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Rapid communication

Combined effect of biopesticides on the digestive enzymatic profiles of *Cnaphalocrocis medinalis* (Guenée) (the rice leaffolder) (Insecta: Lepidoptera: Pyralidae)

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Abstract

Plant extracts, especially botanical insecticides, are currently studied more and more because of the possibility of their use in plant protection. Many of the natural plant compounds and organic compounds used in the control of insect pests are known to affect digestive enzymes. When fed a diet of rice leaves treated with botanical insecticides and bacterial toxins in bioassays, activities of the digestive enzymes protease, amylase, and lipase in the rice leaffolder larvae are affected. Digestive enzyme activities were affected by botanical insecticides and bacterial toxins individually and in combination. When combined, the effect was more severe at low concentration. There were statistically significant differences ($P \leqslant 0.05$) in enzyme activities in combined and individual treatments. The combination of *Btk* and botanical insecticides caused a two-fold decrease in enzyme activity even at reduced concentration. Clear dose–response relationships were established with respect to enzyme activity. A synergistic effect of botanical insecticides and bacterial toxins was found when combined in low doses. These effects are most pronounced in early instars.

Keywords: Neem; *Vitex*; *Bt*; Biopesticides; *Cnaphalocrocis medinalis*; Toxicity; Enzyme; Protease; Amylase; Lipase

1. Introduction

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 nontarget organisms (Abudulai et al., 2001). Botanical insecticides and microbial pesticides are highly effective, safe, and ecologically acceptable (Weinzierl and Henn, 1991; Senthil Nathan et al., 2005b, c; Senthil Nathan and Kalaivani, 2005).

The common trend in the past two decades toward reducing reliance on synthetic insecticides for control of insect pests in agriculture, forestry, and human health has renewed worldwide interest in *Bacillus thuringiensis* Berliner (*Bt*) as an environmentally desirable alternative (Gill et al., 1992). In recent years, an increasing number of reports on the development of resistance to *Bt* in agriculture have been published (Tabashnik, 1994); the lethal dose of *Bt* is also instar dependent and the susceptibility of mature larvae is very low (Tabashnik and Carrière, 2004). The combination of *Bt* with a baculovirus has been tested (Navon, 1993), but studies of combinations with other biopesticides are necessary and desirable.

The Indian neem tree, *Azadirachta indica* A. Juss. (Meliaceae), has been found to be a promising source of natural pesticides and several constitutions (or

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preparations) of its leaves and seed show marked insect control potential (see review by Schmutterer (1990)). Neem seed kernel extract (NSKE) suppresses the feeding, growth, and reproduction of insects (Ascher et al., 1984; Mordue and Blackwell, 1993; Smirle et al., 1996; Shafeek et al., 2004; Senthil Nathan et al., 2005b, c). Due to their relative selectivity, neem products can be recommended for many integrated pest management programs (Schmutterer, 1990). *Vitex negundo* L. (Verbenaceae) is an important aromatic and medicinal plant with pesticidal properties, and it is used predominantly for its pest control properties (Kirthikar and Basu, 1981). Due to observed antifeedant and growth regulation effects (Senthil Nathan et al., 2005b, c), it is apparent that botanical insecticides affect insect physiology in many different ways and that other modes of action, especially effects on enzyme activities, are still to be discovered. In this paper we describe experiments that examine potential effects of botanical insecticides and bacterial toxin on activities of digestive enzymes in the rice leaffolder *Cnaphalocrocis medinalis* (Guenée) (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, Tamilnadu, India, and Paddy Breeding Station, Tamilnadu 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 (L:D) photoperiod and 85% relative humidity. Rice plants were grown in earthenware pots, 18 cm tall with a 20-cm-diameter top; each pot held 15 plants and gave 62 tillers. The pots were placed in about 10 cm of water in a metal tray in the greenhouse (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.

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 mixture (Multidec drops; Ashok Pharmaceuticals, Chennai-24, India) to enhance oviposition. After 2 days, the potted plants were removed from the oviposition cage. Leaf portions containing eggs were clipped and placed on moist filter paper in Petri dishes. These eggs were used to establish the culture of *C. medinalis*.

2.2. Preparation of Bacillus thuringiensis Kurstaki (Btk)

Delfin WG, which contains *B. thuringiensis kurstaki* (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 3 μg/mL.

2.3. Preparation of neem seed kernel (NSKE) and Vitex negundo leaf extracts (VNLE)

Neem seed kernel and *V. negundo* were collected from forests in the Marudamalai hills, Bharathiar University, Coimbatore, Tamil nadu, India. Fifty-gram seed kernels of *A. indica* (from three trees) and *V. negundo* leaves (from five plants) 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 the stock solution, the required concentrations 0.01%, 0.25%, 0.5%, 1%, and 2% were prepared using water. The combined extracts were prepared by mixing equal volumes of NSKE and VNLE (1:1, v/v).

2.4. Bioassay and treatments

Bioassays were performed with second to fifth instars of *C. medinalis* using the concentrations of 0.25%, 0.5%, 1%, and 2% of NSKE and VNLE, and 0.5, 1, 2 and 3 μg/mL of *Btk*. Control leaves were treated with distilled water. A minimum of 20 larvae/concentration were used for all the experiments and the experiments were replicated five times (totally, $n = 100$). The effective concentration (EC_{50}) was calculated using Probit analysis (Finney, 1971).

Fresh rice leaves (*Oryza sativa* L) were sprayed with different concentrations of *Btk*, NSKE, and VNLE and allowed to air dry. Control leaves were treated with distilled water alone. Second to fifth instar larvae were starved for 4 h and then fed leaves treated with the different concentrations of *Btk*, NSKE, or VNLE. The uneaten leaves were removed every 24 h, and the larvae were fed fresh treated leaves. A minimum of 20 larvae/concentration were used in each experiment and all experiments were replicated five times (totally, $n = 100$).

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 anaesthetized with $5 \times 5\text{-mm}^2$ cotton pads soaked in ether and the

entire digestive tract was dissected out in ice-cold insect Ringer's solution. The Malpigihian 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. Protease activity

The enzyme assays were carried out as described by Snell and Snell (1949). The reaction mixture of 1 mL of substrate (50 ppm bovine serum albumin), 1 mL of gut tissue extract and 0.1 mL solution of $MgSO_4$ was incubated at 37 °C, pH 11.7, for 1 h. The control was made in the same way but 1 mL of heat-treated extract, was added. The reaction was terminated by adding 1 mL of 50% trichloroacetic acid. The differences in absorbance were measured at 600 nm in a spectrophotometer (Jasco (Japan) UV/VIS Spectrophotometer Mode lV-570 Model).

2.7. Amylase activity

Amylase activity was determined based on the method of Bernfield (1955) as described by Ishaaya and Swirski (1970) employing 3,5-dinitrosalicylic acid reagent. The reaction mixture consisted of 2 mL of 2% freshly prepared starch solution, 1 mL of 0.01 M phosphate buffer (pH 7.2), and 0.25 mL of enzyme extract. After incubating for 60 min at 37 °C, the enzyme activity was terminated by adding 0.4 mL of 3,5-dinitro salicylic acid reagent. The reaction mixture was maintained at 100 °C for 5 min. Absorbance of the sample was measured in optical density (OD) units at 550 nm against a blank in which the enzyme extract was replaced with deinonized water. The amylolytic activity was expressed in terms of the weight of the reducing sugars (glucose) produced by the enzyme action per unit weight of gut, per unit time, using glucose as the standard.

2.8. Lipase activity

The enzyme assays were carried out as described by Cherry and Crandall (1932). One milliliter of gut tissue extract (the control tube was placed in a boiling water bath for 15 min to destroy the enzyme activity and then cooled), 0.5 mL of phosphate buffer solution (pH 8.0), and 2 mL of olive oil (Riviera max. acidity 1%) emulsion were added, shaken well and incubated at 37 °C. After 24 h, 3 mL of 95% alcohol and two drops of 2% phenolphthalein indicator were added to each tube (control and experimental), the tubes were titrated separately with 0.05 N NaOH solution using a Hamilton microburette, and the end point of titration was marked by the appearance of permanent pink color.

2.9. Statistical analysis

The effective concentration was calculated using Probit analysis (Finney, 1971), and values were expressed as means of five replicates with standard errors. Data from enzyme activity were subjected to analysis of variance (ANOVA of arcsine square root transformed percentages). Differences between the treatments were determined 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 toxins affected the digestive enzymatic profiles of *C. medinalis* at several doses. Tables 1–3 and Figs. 1–3 demonstrate the efficacy and insecticidal activity of botanical insecticides and bacterial toxins against digestive enzyme activities of the rice leaffolder. Neem is unlikely to significantly enhance the activity of *Bt* toxin when applied topically. EC_{50} values of *Btk*, NSKE, and VNLE against rice leaffolder are shown in Fig. 3. NSKE was the most potent in all experiments with the lowest EC_{50} (0.7%, 1.1%, and 1.5% third to fifth instars, respectively).

Differences in protease, amylase, and lipase activities in the gut in the control and treated third instar larvae are shown in Table 1. Treatment with botanical insecticides and bacterial toxins significantly decreased the activity of the digestive enzymes in individual and combined treatments. The maximal suppression of digestive enzyme activity was obtained by combination of botanical insecticides and bacterial toxins at 1 μg/mL *Btk*, 0.1% NSKE, and 0.1% VNLE in all larval instars. Similarly, there was significant reduction in the activities of protease (maximum of 82%), amylase (maximum of 90%), and lipase (maximum of 92%) in the combination treatment. Digestive enzyme activity was considerably decreased when the insects were fed on leaves treated with both *Btk* and botanical insecticides, compared to control treatment. Digestive enzyme activities significantly decreased with increasing concentrations of *Btk*, NSKE, and VNLE in all larval instars tested (Tables 1–3, Figs. 1–3).

There were statistically significant differences ($P \leqslant 0.05$) in enzyme activities in individual and combined treatments (Tables 1–3, Figs. 1–3). Our data demonstrate that suppression of the digestive enzyme activity is among the symptoms of toxicity that were observed following exposure to these bioinsecticides. Insects fed with 1 μg/mL *Btk*, 0.1% NSKE, and 0.1%

Table 1
Digestive enzyme activities ($\times 10^4$/mg/min) of third instar larvae of *C. medinalis* after treatment with *Btk,* NSKE, and VNLE

Treatments	Protease	Amylase	Lipase
Control	12.05 ± 0.91^{a}	3.64 ± 0.03^{a}	0.84 ± 0.06^{a}
Btk (μg/mL)			
1	10.56 ± 0.95^{ab}	3.06 ± 0.03^{b}	0.61 ± 0.04^{b}
2	9.21 ± 0.86^{b}	2.79 ± 0.03^{a}	0.47 ± 0.03^{c}
3	8.10 ± 0.71^{b}	2.11 ± 0.02^{ab}	0.35 ± 0.02^{c}
NSKE (%)			
0.1	10.21 ± 0.91^{ab}	2.96 ± 0.03^{a}	0.54 ± 0.04^{b}
0.25	8.89 ± 0.72^{b}	2.21 ± 0.01^{ab}	0.41 ± 0.02^{c}
0.5	7.47 ± 0.70^{bc}	1.75 ± 0.01^{b}	0.29 ± 0.01^{d}
VNLE (%)			
0.1	10.41 ± 0.95^{b}	3.02 ± 0.04^{a}	0.58 ± 0.03^{b}
0.25	9.05 ± 0.81^{b}	2.44 ± 0.02^{ab}	0.45 ± 0.02^{c}
0.5	7.93 ± 0.68^{bc}	1.98 ± 0.01^{b}	0.32 ± 0.02^{c}
Btk(μg/mL) + NSKE (%)			
1.0 + 0.10	9.45 ± 0.78^{b}	2.54 ± 0.02^{ab}	0.45 ± 0.02^{c}
2.0 + 0.25	6.32 ± 0.52^{c}	1.23 ± 0.02^{b}	0.23 ± 0.01^{d}
Btk(μg/mL) + VNLE (%)			
1.0 + 0.10	9.76 ± 0.59^{b}	2.75 ± 0.02^{ab}	0.48 ± 0.02^{c}
2.0 + 0.25	6.84 ± 0.52^{c}	1.59 ± 0.02^{b}	0.27 ± 0.03^{d}
NSKE (%) + VNLE (%)			
0.10 + 0.10	9.05 ± 0.71^{b}	2.39 ± 0.02^{ab}	0.42 ± 0.03^{c}
0.25 + 0.25	5.98 ± 0.52^{c}	1.05 ± 0.10^{b}	0.16 ± 0.007^{e}
Btk (μg/mL) + NSKE (%) + VNLE (%)			
1.0 + 0.10 + 0.10	2.11 ± 0.12^{d}	0.37 ± 0.01^{c}	0.07 ± 0.003^{f}

Means within columns followed by the same letter are not significantly different (Tukey's test, $P \leqslant ;0.05$).

Table 2
Digestive enzyme activities ($\times 10^4$/mg/min) of fourth instar larvae of *C. medinalis* after treatment with *Btk,* NSKE, and VNLE

Treatments	Protease	Amylase	Lipase
Control	15.91 ± 1.21^{a}	6.23 ± 0.42^{a}	1.22 ± 0.10^{a}
Btk (μg/mL)			
1	14.62 ± 1.20^{a}	5.71 ± 0.38^{a}	1.09 ± 0.09^{a}
2	13.06 ± 1.00^{ab}	4.37 ± 0.35^{b}	0.91 ± 0.07^{b}
3	11.94 ± 0.98^{b}	3.19 ± 0.26^{b}	0.78 ± 0.05^{c}
NSKE (%)			
0.1	13.94 ± 1.20^{a}	5.05 ± 0.42^{a}	0.98 ± 0.08^{a}
0.25	12.21 ± 1.00^{b}	3.94 ± 0.29^{b}	0.76 ± 0.04^{c}
0.5	10.52 ± 1.00^{c}	2.51 ± 0.19^{bc}	0.59 ± 0.03^{d}
VNLE (%)			
0.1	14.15 ± 1.42^{a}	5.47 ± 0.41^{a}	1.02 ± 0.90^{a}
0.25	12.71 ± 1.10^{b}	4.19 ± 0.38^{b}	0.85 ± 0.07^{b}
0.5	10.98 ± 0.98^{c}	2.96 ± 0.15^{bc}	0.64 ± 0.04^{c}
Btk(μg/mL) + NSKE (%)			
1.0 + 0.10	11.21 ± 0.95^{c}	3.54 ± 0.29^{b}	0.89 ± 0.07^{c}
2.0 + 0.25	8.79 ± 0.62^{d}	1.96 ± 0.10^{c}	0.43 ± 0.03^{d}
Btk(μg/mL) + VNLE (%)			
1.0 + 0.10	11.95 ± 0.79^{b}	3.98 ± 0.25^{b}	0.96 ± 0.07^{ab}
2.0 + 0.25	9.21 ± 0.62^{d}	2.15 ± 0.18^{c}	0.55 ± 0.03^{cd}
NSKE (%) + VNLE (%)			
0.10 + 0.10	10.27 ± 0.92^{c}	3.12 ± 0.21^{b}	0.81 ± 0.05^{b}
0.25 + 0.25	7.11 ± 0.53^{de}	1.45 ± 0.12^{c}	0.36 ± 0.01^{e}
Btk (μg/mL) + NSKE (%) + VNLE (%)			
1.0 + 0.10 + 0.10	4.21 ± 0.25^{f}	0.64 ± 0.04^{d}	0.15 ± 0.003^{f}

Means within columns followed by the same letter are not significantly different (Tukey's test, $P \leqslant 0.05$).

VNLE showed maximum reduction in protease (74% in fourth instar and 72% in fifth instar), amylase (90% in fourth instar and 83% in fifth instar), and lipase (88% in fourth instar and 86% in fifth instar), compared with controls (Table 3, Figs. 3 and 4). The combined treatment of *Btk*, NSKE, and VNLE affected digestive enzyme activities to a greater extent. The enzyme activities of the third to fifth instar larvae were decreased considerably more after treatment with the combination of *Btk*, NSKE, and VNLE in a dose-dependent manner.

4. Discussion

Crude botanical insecticides have been used for centuries and were well known in tribal and traditional cultures (see review by Schmutterer (1990)). Naturally occurring biopesticides seem a logical choice for investigation. Several studies have shown that feeding is necessary for the stimulation of enzyme activities (Sibley, 1981; Broadway and Duffey, 1988). Exposure of *C. medinalis* larvae to sublethal doses of *Btk* in the laboratory reduced digestive enzyme activity (Senthil Nathan, 2000). Higher enzyme activity in the midgut of control insects is most probably due to consumption and utilization of large quantities of food. Imbalance in enzyme–substrate complex and inhibition of peristaltic movement of the gut (Hori, 1969) might have inhibited the enzyme activity in the treated insects. Chapman (1985) 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 condition of the rice leaffolder and reflects the absorption, digestion, and positive transport of nutrients in the midgut. *Btk* caused 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 fluid. The

Table 3
Digestive enzyme activities ($\times 10^4$/mg/min) of fifth instar larvae of *C. medinalis* after treatment with *Btk,* NSKE, and VNLE

Treatments	Protease	Amylase	Lipase
Control	18.93±1.50[a]	8.90±0.56[a]	2.50±0.21[a]
Btk (μg/mL)			
1	17.41±1.61[a]	7.94±0.49[a]	2.23±0.19[a]
2	15.21±1.41[b]	6.21±0.38[b]	1.98±0.16[a]
3	13.46±1.23[b]	5.04±0.42[b]	1.65±0.12[a]
NSKE (%)			
0.1	16.18±1.42[a]	6.92±0.58[ab]	2.10±0.18[a]
0.25	14.57±1.31[ab]	5.67±0.42[b]	1.42±0.12[a]
0.5	12.11±0.98[bc]	4.10±0.38[bc]	1.27±0.10[ab]
VNLE (%)			
0.1	16.42±1.42[a]	7.12±0.61[ab]	2.20±0.18[a]
0.25	14.94±1.26[b]	6.06±0.48[b]	1.81±0.16[a]
0.5	13.07±1.15[b]	4.93±0.35[c]	1.53±0.14[a]
Btk(μg/mL) +NSKE (%)			
1.0+0.10	14.21±1.23[b]	5.21±0.41[b]	1.97±0.15[a]
2.0+0.25	10.05±0.95[d]	3.40±0.28[cd]	0.81±0.07[b]
Btk(μg/mL)+VNLE (%)			
1.0+0.10	14.74±1.29[b]	5.98±0.36[b]	2.05±0.19[a]
2.0+0.25	11.25±0.95[cd]	3.51±0.25[cd]	1.10±0.10[b]
NSKE (%)+VNLE (%)			
0.10+0.10	13.78±1.15[b]	4.71±0.21[c]	1.75±0.18[ab]
0.25+0.25	9.00±0.70[d]	3.21±0.19[d]	0.69±0.05[bc]
Btk (μg/mL)+ NSKE (%)+VNLE (%)			
1.0+0.10+0.10	5.23±0.35[e]	1.54±0.10[f]	0.36±0.04[d]

Means within columns followed by the same letter are not significantly different (Tukey's test, $P \leqslant 0.05$).

damage to the midgut caused a decrease in digestive enzyme activities (Eguchi et al., 1972; Mathavan et al., 1989; Smirle et al., 1996; Senthil Nathan et al., 2005a).

Decreased levels of digestive enzymes at higher concentration of *Btk*, NSKE, and VNLE suggest reduced phosphorous liberation for energy metabolism, and decreased rate of metabolism, decreased rate of transport of metabolites and may be due to the direct effects of *Btk*, NSKE, and VNLE on enzyme regulation. In the present study, after NSKE and VNLE treatment, the biochemical parameters and enzymatic profiles were markedly affected. It is evident that exposure to botanical insecticides in larval diet has significant effects on several enzyme activities 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 larval exposure to neem (Smirle et al., 1996; Senthil Nathan, 2000). Active principles present in the neem and *Vitex* (azadirachtin, vitexin, etc.,) are responsible for such effects. In conclusion, neem and *Vitex* had significant effects on larval *C. medinalis*, and they act synergistically with *Bt* toxin, causing reduction of digestive enzyme activity. However, Johnson et al. (1990) made an exhaustive study of protease activity in the midguts of larvae of susceptible and resistant strains of *Plodia interpunctella* Hubner and the results indicated that resistance was not due to obvious changes in larval midgut protease activity. A recent study by Oppert et al.

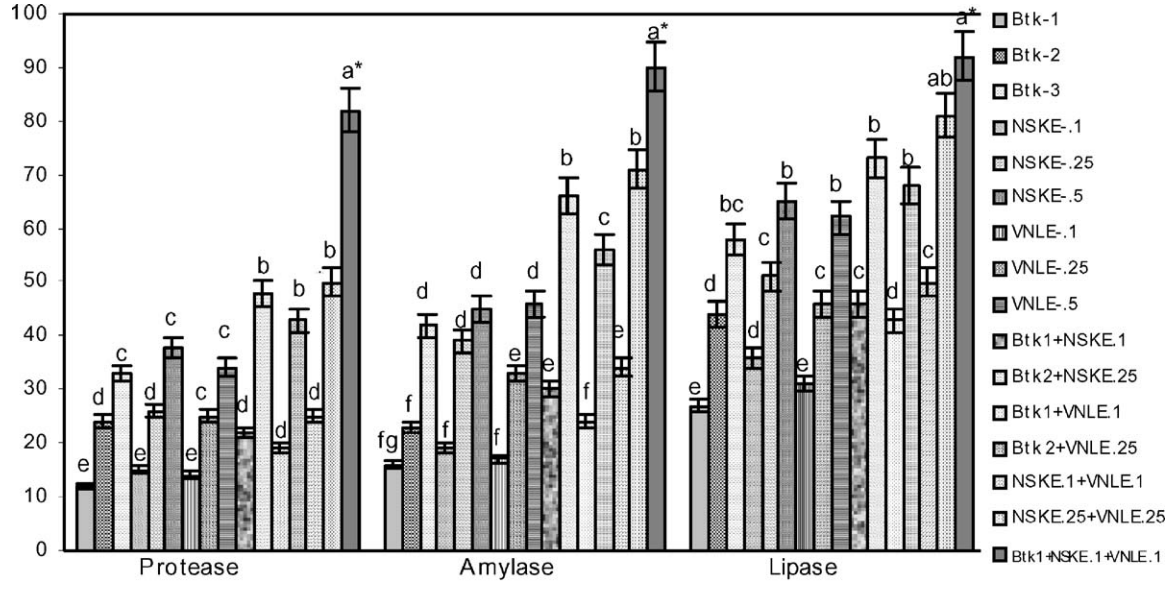

Fig. 1. Percentage reductions of digestive enzyme activities in third instar larvae of *C. medinalis* after treatment with *Btk,* NSKE, and VNLE. *Within bar (individual enzyme), means followed by the same letter do not differ significantly (Tukey's test, $P \leqslant 0.05$) Treatments: *Btk*, μg/mL; NSKE and VNLE, %.

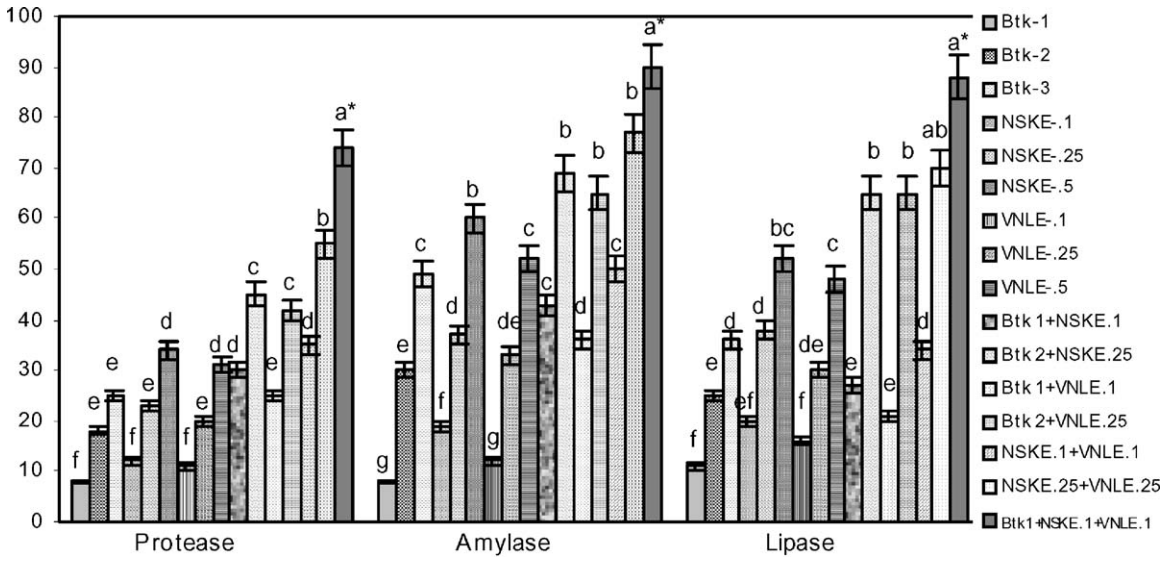

Fig. 2. Percentage reductions of digestive enzyme activities in fourth instar larvae of *C. medinalis* after treatment with *Btk,* NSKE and VNLE. *Within bar (individual enzyme), means followed by the same letter do not differ significantly (Tukey's test, $P \leqslant 0.05$) Treatments: *Btk*, μg/mL; NSKE and VNLE, %.

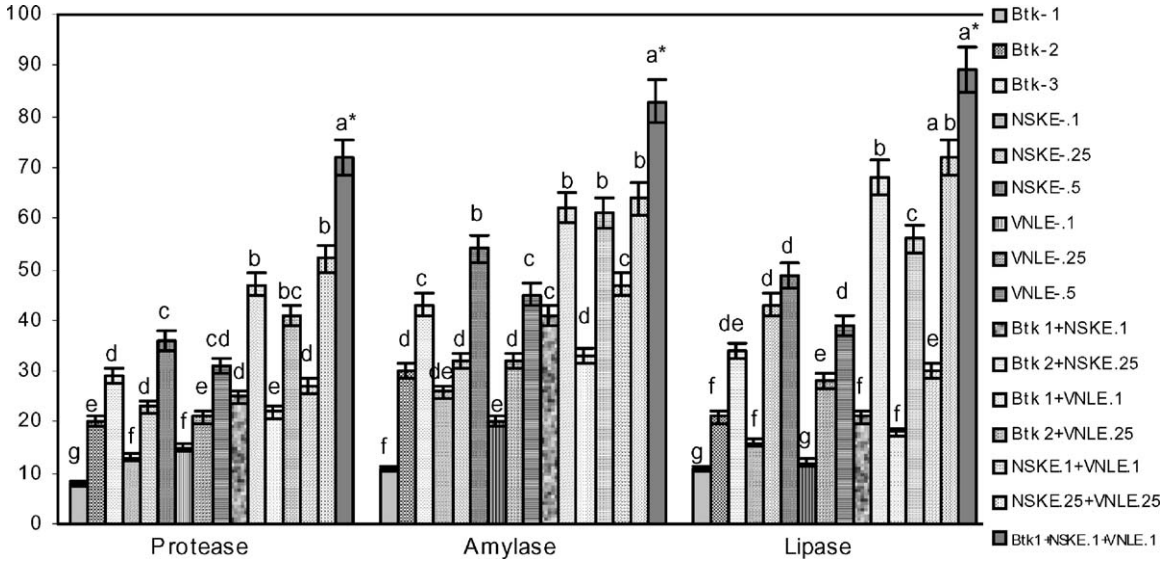

Fig. 3. Percentage reductions of digestive enzyme activities in fifth instar larvae of *C. medinalis* after treatment with *Btk,* NSKE, and VNLE. *Within bar (individual enzyme), means followed by the same letter do not differ significantly (Tukey's test, $P \leqslant 0.05$) Treatments: *Btk*, μg/mL; NSKE and VNLE, %.

(1994) of *P. interpunctella* resistant to *Bt* subsp. *entomodicus* suggests that altered protoxin activation by midgut proteinases is indeed involved in some types of insect resistance to *Bt*. Different proteases can be produced in the insect gut depending on the plant material ingested (Broadway, 1989). Such differences could influences susceptibility through slower activation or faster metabolism of the toxins (Whalon and McGaughey, 1998). Salama et al. (1986) and Ludlum et al. (1991) have reported that aromatic compounds and plant allelochemicals increase *Bt* activity. The results of this study indicate that plant extracts such as NSKE and VNLE enhance the *Btk* activity. The use of plant extracts as additives to *Bt* products may play a more prominent role in integrated pest management programs in the future.

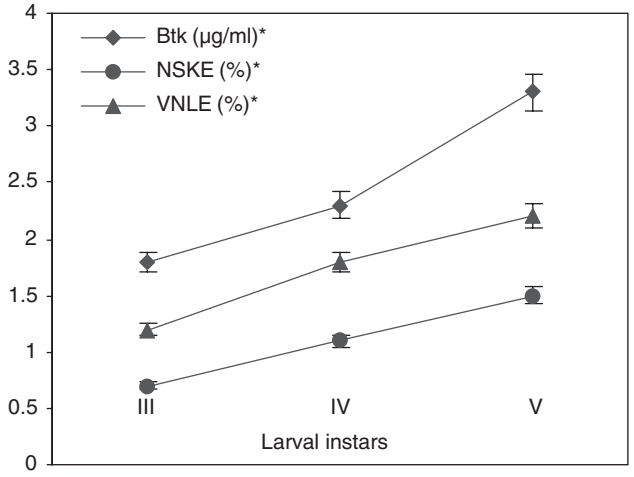

Fig. 4. Effective concentrations (EC_{50}) of *Btk*, NSKE, and VNLE against fourth and fifth instar larvae of *C. medinalis*. *Values are mean of five replicates ± standard error.

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