Effect of Biopesticides Applied Separately or Together on Nutritional Indices of the Rice Leaffolder *Cnaphalocrocis medinalis*

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Laboratory assays were done to evaluate the effect of neem seed kernel extract (*Azadirachta indica* A. Juss), *Vitex negundo* L. (Lamiales: Verbenaceae) leaf extract, and *Bacillus thuringiensis* (Berliner), applied separately or together, on nutritional indices of the rice leaffolder *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Pyralidae). All three biopesticides suppressed feeding and larval growth and low concentrations affected the larval performance. The combined effect of the three biopesticides resulted in a considerable decrease in nutritional indices, indicating strong deterrence. Dose response relationships were established with respect to frass production and larval growth. The efficiency of conversion of ingested and digested food was considerably reduced.
KEY WORDS: Biopesticides; *Bt*; neem; *Vitex*; *Cnaphalocrocis medinalis;* nutrition; physiology.

INTRODUCTION

The rice leaffolder *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Pyralidae) is a major insect pest of rice (*Oryza sativa* L.) and has become a major threat to the rice population in several Asian countries (tropical and subtropical Asia) (18-20). During the last decade the pest has posed an increasingly important threat to rice production (7). Of the eight species of rice leaffolder, the most widespread and important one is *C. medinalis.* It is the only leaffolder species that survives on weeds in rice fields between rice crops (7). 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 insecticide, and lethal effects on non-target organisms (11).

The Indian neem tree, *Azadirachta indica* A. Juss (Meliaceae), has been found to be a promising source of natural pesticides, given that several constituents of its leaves and seed show marked insect control potential. The neem seed kernel extract (NSKE) is known to suppress the feeding, growth and reproduction of insects due to its relative selectivity. Neem products can be recommended for many programs on integrated pest management (1,6,16). *Vitex negundo* L. (Verbenaceae) showed a promising pesticidal activity against insects and is used predominant for its pest control properties (4,17,18). The screening of phytochemical effects at tritrophic levels is useful for the evaluation of the compatibility of *Bacillus thuringiensis (Bt)* with allelochemicals used in crop breeding programs for developing natural resistance to insects, and the selection of plant allelochemicals that

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synergize *Bt* could be used in *Bt* formulations for controlling insect pests (20). Formulation additives may enhance the activity of *Bt* and could increase both host range and biological activity of the toxin. Investigations have tested the property of chemical compounds and have shown that various plant allelochemicals do increase *B. thuringiensis* activity (10,13,18).

From the reviewed literature, this research attempts to study the combined action of *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*), NSKE and *V. negundo* leaf extract (VNLE) on food selection, digestion and absorption in relation to control strategy of *C. medinalis*.

MATERIALS AND METHODS

Laboratory mass culture of *C. medinalis* *Cnaphalocrocis medinalis* larvae were collected from the paddy fields in and around Coimbatore district, Tamilnadu, India, and the Paddy Breeding Station, Tamilnadu Agricultural University, Coimbatore. Larvae were reared in a greenhouse on potted rice plants covered with mesh sleeves at 27±2°C; 10L:14D; 85% r.h. Rice plants were grown in 18-cm-tall earthenware pots with a 20-cm-diam 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 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 *Btk* Delfin WG (Sandoz (India) Limited, Mumbai, India), which contains *Btk*, serotype 3a, 3b (85%) and dispersing agents (15%) with a potency of min 53,000 SU/mg, 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^{-1}.

Preparation of NSKE and of *V. negundo* leaf extract Neem seed kernel and *V. negundo* were collected from forests in the Marudamalai hills, Bharathiar University, Coimbatore. Fifty g of seed kernels of *A. indica* and *V. negundo* leaves 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, several concentrations ranging from 0.10% to 3.0% were prepared using water. The combined extracts were prepared by mixing equal volumes of NSKE and VNLE (1:1, v/v) (18).

Bioassay and treatments Bioassays were performed with fourth and 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^{-1} of *Btk*. Control leaves were treated with distilled water. A minimum of 30 larvae per concentration were used for each experiment; the experiments were replicated five times. Larval weight /mortality was recorded after 7 days at 28°C and 16L:8D photoperiod and the effective concentration (EC_{50}) was calculated using probit analysis (2).

Fresh rice leaves were sprayed with different concentrations of *Btk*, NSKE and VNLE and air-dried. Control leaves were treated with distilled water alone. Fourth and fifth instars were starved for 4 h and then fed with leaves treated with various 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 per concentration were used for each experiment; the experiments were replicated five times.

Feeding deterrency index A feeding deterrency index was estimated by using a leaf cut choice–test (5,7). In a 15-cm-diam petri dish lined with a moist filter paper disc, 3-cm-long leaf cuts from TN 1 rice plants were treated on each side with various concentrations of *Btk* (5 to 20 μg ml^{-1}), NSKE and VNLE (1% to 3%). Control leaf cuts were treated with distilled water alone. The leaf cuts were dried at room temperature and then fourth and fifth instar *C. medinalis* which had been starved for 4 h were introduced into each arena containing one treated and one untreated leaf disc in alternate positions on moist filter paper. Experiments were carried out with two larvae in a petri dish in five replicates. Consumption was recorded using a digitizing leaf area meter (Model LI-3000, Li-cor) after 24 h. The index of feeding deterrency was calculated as (C-T)/ (C+T) X 100, where 'C' is the consumption of the control leaf cut and 'T' of the treated leaf cut.

Quantitative food utilization efficiency measures A gravimetric technique was used to determine weight gain, food consumption and feces produced. All weights were measured using a monopan balance accurate to 0.1 mg. The newly molted first to fifth instar larvae were starved for 4 h. After measuring the initial weight of the larvae, they were individually introduced into separate containers. The larvae (20 larvae per concentration, five replicates) were allowed to feed for a period of 24 h on weighed quantities of *Btk*-, NSLE- and VNLE-treated and untreated TN 1 rice leaves. Larvae were again weighed. The difference in weight of the larvae gave the fresh weight gained during the study period. Sample larvae were weighed, oven-dried (48 h at 60°C) and then reweighed to establish a percentage dry weight of the experimental larvae. The leaves remaining at the end of each day were oven-dried and reweighed to establish a percentage dry weight conversion value to allow for the estimation of diet dry weight. The quantity of food ingested was estimated by subtracting the diet (dry weight) remaining at the end of each experiment from the total dry weight of diet provided. Feces were collected daily and weighed, then oven-dried and reweighed to estimate the dry weight of excreta. The experiment was continued for 4 days and observations were recorded every 24 h.

Consumption, growth rates and post-ingestive food utilization efficiencies (all based on dry weight) were calculated in the traditional manner (17,23,25). Consumption index (CI) = E/TA; Relative growth rate (RGR) = P/TA; Approximate digestibility (AD) = 100(E-F)/E; Efficiency of conversion of ingested food (ECI) = 100 P/E; Efficiency of conversion of digested food (ECD) = 100 P/(E-F), where A = mean dry weight of insect during T, E = dry weight of food consumed, F = dry weight of feces produced, P = dry weight gain of insect and T = duration of experimental period.

Statistical analysis Data from feeding deterrence and nutritional indices were analyzed with ANOVA of arcsine transformed percentages followed by Tukey's multiple range test ($P \leq 0.05$) (14). In Tukey's test we used descending order: the highest values differing from the averages as detected by statistical testing are marked with the letter 'a', the next lower with 'b', etc. (24).

TABLE 1. Effective concentrations (EC_{50}) of *Bacillus thuringensis* subsp. *kurstaki* (*Btk*), neem seed kernel extract (NSKE) and *Vitex negundo* leaf extract (VNLE) against fourth and fifth instar larvae of *Cnaphalocrocis medinalis*

Treatment	Larval instar	
	IV	V
Btk (μg ml^{-1})	2.2±0.3*	3.1±0.4*
NSKE (%)	1.0±0.2	1.6±0.3
VNLE (%)	1.6±0.3	2.1±0.3

*Values are means of five replicates ± S.E.

RESULTS

Antifeedant effects of *Btk*, NSKE and VNLE Figure 1 shows the feeding deterrency index of fourth instar *C. medinalis* after treatment with *Btk*, NSKE and VNLE. It can be seen that the incorporation of neem and *Vitex* in the diet (rice leaves) significantly reduced the growth of *C. medinalis* larvae in a concentration- and dose-dependent manner. This clearly indicates that the combination produced a strong effect even at lateral larval stages. The EC_{50} values of *Btk*, NSKE and VNLE against rice leaffolder are shown in Table 1. NSKE was most potent in all experiments with the least EC_{50} (1.6% and 2.1% fourth and fifth instars, respectively).

Effect of *Btk*, NSKE and VNLE on nutritional indices of *C. medinalis* The nutritional indices of fourth instar *C. medinalis* larvae after treatment with *Btk*, NSKE and VNLE are provided in Table 2. NSKE and VNLE treatment had a detrimental effect upon *C. medinalis* larval growth and development, and NSKE proved to be the most detrimental to the larvae at the concentrations tested. Larvae fed the lower dose moulted into progressive instars. However, between the end of the fourth instar and the beginning of the fifth instar, the spinning behavior was completely stopped, feeding reduced and the larvae regurgitated semisolid sticky substances to build up a tunnel using the debris in which the pupa is formed. A decrease in the consumption index, relative growth rate, and efficiency of conversion of ingested and digested food was noticed after treatment with *Btk*, NSKE and VNLE (Table 2). The combined treatment of *Btk*, NSKE and VNLE affected the nutritional indices to an even greater extent. The approximate digestibility was slightly increased but was not significant (Table 2). A similar trend was observed in the nutritional indices of fifth instars after treatment with *Btk*, NSKE and VNLE. The concentration-dependent reduction in feeding relative to controls was directly related to the reduction in food consumption (Table 3). The nutritional parameters of the fifth instar larvae were decreased, with a pronounced effect following treatment with the combination of *Btk*, NSKE and VNLE in a dose-dependent manner. This result clearly indicates that neem and *Vitex* belong to the category of unacceptable host plants for pest insects. *Btk*, NSKE and VNLE did not severely affect the fifth instar. In addition to significant decreases in ECI and ECD, treatments with *Btk*, NSKE and VNLE also produced significant decreases in relative consumption rate and relative growth rate. NSKE and VNLE in combination with *Btk* was the most potent growth inhibitor of the test treatments. This disruption of the normal feeding physiology of *C. medinalis* by *Btk* with NSKE and VNLE was elicited at a lower concentration for larvae.

TABLE 2. Nutritional indices of fourth instar larvae of *Cnaphalocrocis medinalis* after treatment with *Bacillus thuringensis* subsp. *kurstaki* (*Btk*), neem seed kernel extract (NSKE) and *Vitex negundo* leaf extract (VNLE)

Treatment	CI (mg/mg/day)	RGR (mg/mg/day)	AD (%)	ECI (%)	ECD (%)
Control	0.492±0.036a[z]	0.159±0.011a	54.21±4.12bc	32.40±1.78a	59.78±4.12a
Btk (μg ml^{-1})					
1.0	0.361±0.031b	0.098±0.006b	55.09±4.51b	27.41±1.52a	49.76±4.0ab
2.0	0.324±0.028c	0.082±0.005b	55.98±4.46b	25.31±1.42b	45.23±4.23b
3.0	0.278±0.021cd	0.066±0.005c	57.71±4.81ab	23.78±1.32b	35.64±3.75c
NSKE (%)					
0.10	0.304±0.025c	0.080±0.007b	56.27±4.01b	26.39±1.78ab	48.36±3.96b
0.25	0.263±0.020d	0.060±0.007c	57.09±4.05ab	22.99±1.32b	40.27±3.85d
0.50	0.220±0.019d	0.046±0.003d	59.30±4.31a	21.13±1.16b	35.64±2.83e
VNLE (%)					
0.10	0.321±0.022c	0.086±0.009b	55.98±4.12b	27.07±1.75a	48.36±4.23b
0.25	0.294±0.021c	0.073±0.006b	56.43±4.18b	24.94±1.62b	44.21±4.12b
0.50	0.243±0.020d	0.054±0.005c	58.71±4.51a	22.39±1.05b	38.14±4.0c
Btk (μg ml^{-1}) + NSKE (%)					
1.0+0.10	0.187±0.018de	0.043±0.003d	57.46±4.28ab	22.40±1.79b	40.74±3.75bc
2.0+0.25	0.093±0.007f	0.010±0.001e	59.94±4.71a	10.99±0.59c	18.34±1.76d
Btk (μg ml^{-1}) + VNLE (%)					
1.0+0.10	0.198±0.015d	0.047±0.004cd	56.98±4.52b	24.23±1.18b	42.54±3.75bc
2.0+0.25	0.096±0.007f	0.012±0.001e	59.01±4.75a	13.19±0.75c	22.36±1.42d
NSKE (%) + VNLE (%)					
0.10+0.10	0.165±0.014e	0.036±0.003d	57.84±4.00a	22.15±1.0b	38.31±2.96c
0.25+0.25	0.082±0.007f	0.008±0.0006e	60.05±4.57a	9.86±0.56d	16.43±1.02d
Btk (μg ml^{-1}) + NSKE (%) + VNLE (%)					
1.0+0.10+0.10	0.035±0.002g	0.003±0.0001f	64.31±5.01a	6.59±0.41e	10.26±0.68a

CI, consumption index.
RGR, relative growth rate.
AD, approximate digestibility.
ECI, efficiency of conversion of ingested food.
ECD, efficiency of conversion of digested food.
[z]Within columns, means (±S.E.) followed by a common letter do not differ significantly (Tukey's test, $P \leq 0.05$).

DISCUSSION

Recently, there has been research on plant extracts and essential oils as alternatives to the broad use of conventional pesticides. The toxic action mechanisms of tertranorterpeniods have not been uncovered and are still under investigation (15). However, the onset of toxic signs is usually rapid. Our present study and data describe the onset of toxic actions of *Btk*, NSKE and VNLE on rice leaffolder. Murugan *et al.* (12) and Koul and Isman (9) have shown that decrease in relative consumption rate can occur independently of extrinsic chemosensory effects. From this study, it appears that NSKE and VNLE have both antifeedant and growth inhibitory activity on *C. medinalis*.

The reduction in dietary utilization suggests that reduction in growth may result from both behavioral and physiological (post-ingestive) effects (9). A significant correlation between deterrency and the toxicity of injected secondary plant compounds in locusts has already been reported (8). This conclusion is corroborated by the results of direct bioassays for feeding deterrency, and the leaf-cut-choice tests (where substantial feeding inhibition on treated leaf disc was observed). The addition of *Btk*, at concentrations ranging from

TABLE 3. Nutritional indices of fifth instar larvae of *Cnaphalocrocis medinalis* after treatment with *Bacillus thuringensis* subsp. *kurstaki* (*Btk*), neem seed kernel extract (NSKE) and *Vitex negundo* leaf extract (VNLE)

Treatment	CI (mg/mg/day)	RGR (mg/mg/day)	AD (%)	ECI (%)	ECD (%)
Control	0.625±0.051a	0.314±0.029a	66.43±5.16b	50.30±4.65a	75.72±5.96a
Btk (μg ml^{-1})					
1.0	0.603±0.045a	0.275±0.025a	67.47±5.51b	45.77±4.09a	67.84±5.62b
2.0	0.571±0.043ab	0.237±0.021b	67.98±5.50b	41.63±3.72ab	61.24±5.56b
3.0	0.504±0.040b	0.210±0.019b	71.63±5.90a	41.82±3.25ab	58.39±5.21b
NSKE (%)					
0.10	0.561±0.043ab	0.211±0.019b	68.29±5.75ab	37.73±2.98b	55.20±5.10b
0.25	0.411±0.036c	0.119±0.014d	69.54±5.79ab	29.71±2.54c	41.95±4.25d
0.50	0.359±0.033c	0.102±0.011d	73.69±6.07a	28.42±2.32c	38.57±4.79d
VNLE (%)					
0.10	0.575±0.040ab	0.255±0.020ab	67.93±6.00b	44.39±3.25a	65.31±5.21ab
0.25	0.421±0.037c	0.164±0.018c	68.09±6.21b	38.97±3.00b	57.24±4.56b
0.50	0.378±0.031c	0.141±0.011c	72.02±6.51a	35.02±3.25b	48.63±4.81c
Btk (μg ml^{-1}) + NSKE (%)					
1.0+0.10	0.394±0.029c	0.132±0.012c	69.54±5.79ab	33.51±2.60b	48.19±3.75c
2.0+0.25	0.161±0.018f	0.025±0.002e	74.98±5.98a	15.06±1.31d	21.43±1.70ef
Btk (μg ml^{-1}) + VNLE (%)					
1.0+0.10	0.432±0.029c	0.154±0.014c	69.27±6.01ab	35.87±2.01b	51.77±4.12bc
2.0+0.25	0.193±0.016c	0.037±0.003b	74.52±6.25a	19.59±0.95d	26.29±1.90e
NSKE (%) + VNLE (%)					
0.10+0.10	0.321±0.021d	0.103±0.010d	70.52±6.54ab	32.31±2.00b	45.82±3.60cd
0.25+0.25	0.129±0.012g	0.024±0.003e	75.39±6.52a	14.07±0.75d	18.67±1.02f
Btk (μg ml^{-1}) + NSKE(%) + VNLE (%)					
1.0+0.10+0.10	0.067±0.009h	0.006±0.0004f	79.24±6.29a	8.21±0.68e	12.93±0.72g

CI, consumption index.
RGR, relative growth rate.
AD, approximate digestibility.
ECI, efficiency of conversion of ingested food.
ECD, efficiency of conversion of digested food.
[z]Within columns, means (±S.E.) followed by a common letter do not differ significantly (Tukey's test, $P \leq 0.05$).

0.1 to 3 μg ml^{-1}, to plant extracts had adverse effects upon the growth and development of *C. medinalis*. The consumption of *Btk* with plant extracts resulted in retarded growth and affected the nutritional physiology of the larvae. When combined with plant extracts, *Btk* increased the larval mortality percentage and reduced time to kill when compared with treatments containing only *Btk*. The addition of *Btk* to plant extracts caused a significant mortality and overall inhibition due to avoidance of the treated diet (3).

The results also showed that relative consumption and growth rates were significantly lower among fourth instars restricted to a diet containing *Btk* plus plant extracts. Furthermore, utilization efficiencies for larvae exposed to *Btk* plus NSKE were significantly reduced. The consumption of plant extract and *Btk* resulted in retarded growth and development of the larvae, similar to the results obtained using whole neem extracts (22). The addition of plant extract to *Btk* increased the larval mortality percentage due to biologically active compounds in these plant extracts and may enhance the *Btk* activity (21,22).

In this study, the potent toxicity that led to high larval mortality exhibited by the extracts could be attributed to the group of toxic biomolecules possessing insecticidal properties

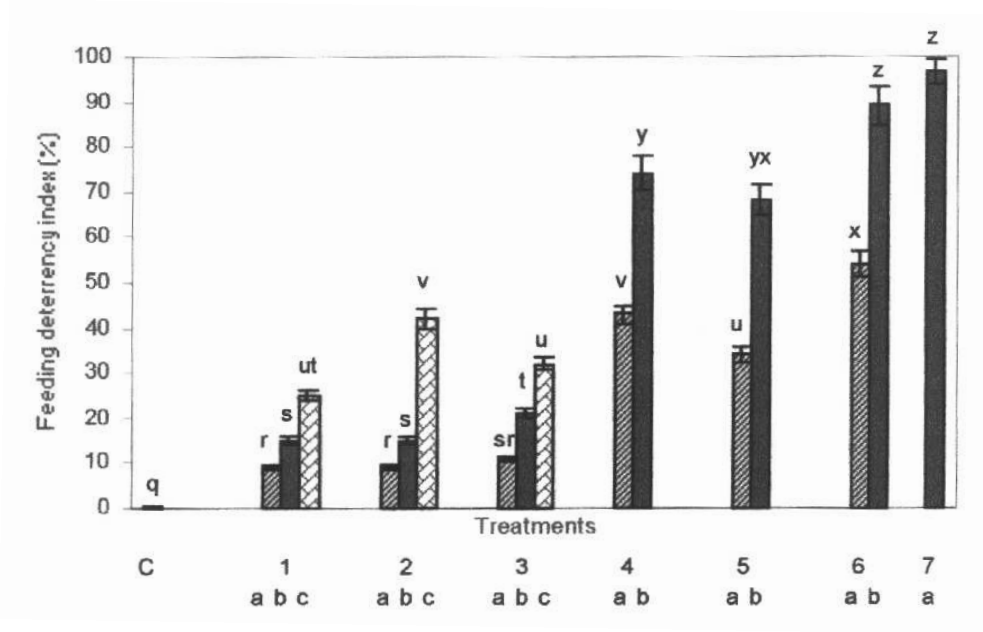

Fig. 1. Feeding deterrency index of fourth instar larvae of *Cnaphalocrocis medinalis* after treatment with *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*), neem seed kernel extract (NSKE) and *Vitex negundo* leaf extract (VNLE). Means (±S.E., indicated by the bars above columns) with a common letter do not differ significantly (Tukey's test, $P \leq 0.05$).

Treatments

C – control

1 – *Btk:* a=5, b=10, c=15 μg ml^{-1}

2 – NSKE: a=1, b=2.5, c=5%

3 – VNLE: a=1, b=2.5, c=5%

4 – *Btk* (μg ml^{-1}) + NSKE (%): a=2.5+0.5, b=5.0+1.0

5 – *Btk* (μg ml^{-1}) + VNLE (%): a=2.5+0.5, b=5.0+1.0

6 – NSKE (%) + VNLE (%): a=0.5+0.5, b=1.0+1.0

7 – *Btk* (μg ml^{-1}) + NSKE (%) + VNLE (%): 1.0+0.25+0.25

present in the plant extracts. From the results of this study, it can be suggested that very low toxicity can be maintained even through a combination of botanical insecticides and a bacterial toxin. These results prove that the application of natural plant products with bacterial toxin, which exert growth inhibition, antifeedant effects and probably some toxic effects on harmful insects, can be a potential control method in plant protection, both for oligophagous and polyphagous insects and for different insect orders. Hence, further investigations should be carried out. The primary effect of these products is to prevent insect feeding and therefore to protect plants from severe effects. The lack of high toxicity to harmful and beneficial organisms is an advantage.

The results of this study also indicate that plant extracts such as NSKE and VNLE enhance the activity of *Btk* and could be an effective alternative to conventional synthetic insecticides for the control of *C. medinalis.* The use of plant extracts as an additive to *Btk* may play a more prominent role in integrated pest management programs in the future.

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