Bulletin of Insectology ***61*** *(2): 299-302, 2008*
ISSN 1721-8861

Effects of nucleopolyhedrovirus based product on *Spodoptera littoralis*

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Abstract

Nucleopolyhedroviruses (NPV) are considered among the most promising microbiological control agents for *Spodoptera littoralis* (Boisduval) (Lepidoptera Noctuidae). In a first laboratory experiment, a new SlNPV-based formulation was compared with a conventional Bt toxin product in order to evaluate its potential role as bioinsecticide for the short-term control of *S. littoralis*. Both formulations were tested on second instar larvae at the recommended dosages for treatment of vegetable crops. Four days after the treatments, Bt toxins caused higher mortality and significantly reduced leaf damages; on the contrary no significant differences were recorded between SlNPV and control treatments. Further investigations were carried out to assess the impact of SlNPV on *S. littoralis* over time. The mean lethal time (LT50) for second instar larvae treated with SlNPV was 7.32 days and it was significantly shorter than LT50 in control group.

Key words: *Spodoptera littoralis*, mean lethal time, SlNPV, baculovirus, *Bacillus thuringiensis*.

Introduction

The Egyptian cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera Noctuidae) is an important and widespread pest. Owing its polyphagy this species causes economical yield losses on several crops (Carter, 1984). Considerable damages are recorded regularly on cotton, spinach, alfalfa, pepper, eggplant, tomato, lettuce, bean, strawberry, and also some ornamental crops are attacked. *S. littoralis* has been recorded throughout Africa, the Middle East and in Mediterranean basin (Pineda *et al*., 2007). In Italy, most of damages are limited to the southern regions (Sannino, 2003).

Common control strategies for *S. littoralis* rely on chemical sprays, which are detrimental to entomophagous, pollinators, and other non-target insects. Moreover, intensive uses of board-spectrum insecticides have selected high levels of resistance in several leafworm populations (Ishaaya *et al.*, 1995; Miles and Lysandrou, 2002; Smagghe *et al.*, 1999). Therefore alternative control techniques need to be evaluated.

Delta-endotoxins derived from *Bacillus thuringiensis* Berliner (Cry proteins) are by far the commonest active ingredients of commercial microbial insecticides (Lacey *et al.*, 2001). Cry 1C toxins are considered as the most effective on larvae of *S. littoralis* whereas Cry 1A toxins are only marginally active (Escriche *et al.*, 1998).

Baculoviridae are a large family of entomopathogenic viruses whose double-stranded circular DNA is contained within enveloped, rod-shaped virions that infect many invertebrates, particularly Lepidoptera species (Moscardi, 1999). Baculoviruses show both high host specificity and intense virulence to susceptible insects. Moreover, virions are protected against environmental inactivating agents by the polyhedral occlusion bodies (OBs) and they can retain infectivity for several years (Groner, 1987). For these reasons baculoviruses are considered among the most promising insect microbial control agents. Several studies have demonstrated that different isolates of *S. littoralis* nucleopolyhedrovirus (SlNPV) can cause high mortality in larval populations of this pest (Jones *et al.*, 1994; Maeda *et al.*, 1990; Toprak *et al.*, 2006; 2007). The major restrictive factor for the application of SlNPV and other entomopathogenic viruses is the relatively long time between the infection and the death of the insects (van Beek and Hughes, 1998).

The present paper reports information from two different laboratory experiments. In 2006, a research was carried out with the aim to compare the short-time efficacy of a new microbial insecticide containing SlNPV with a commercial formulation of Bt toxins already registered for *S. littoralis*. Both mortality and leaf damage reduction were considered. The second experiment was performed in 2007 in order to evaluate the effect over time of the experimental formulation of SlNPV.

Materials and methods

Insect rearing

S. littoralis individuals were reared on bean (*Phaseolus vulgaris* L.) in Plexiglas cages kept in climatic chamber (25 ± 1 °C, 65 ± 10% RH, and a 16L:8D photoperiod) at Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA) of University of Bologna, Italy. The rearing was established in 2005 from larvae collected on spinach (*Spinacia oleracea* L.) in Latina province, central Italy. Before the beginning of the assays, the rearing was replenished with individuals taken from the same areas.

Microbial products tested

Turex (ScamBiosistem, Modena, Italy), a commercial product containing endotoxins derived from *B. thuringiensis* subsp. *kurstaki* and *aizawai* (25.000 ITU/mg), was used as positive control. The SlNPV formulation, which is still not registered in Italy, was kindly supplied

by IntrachemBio (Bergamo, Italy).

Assays were conducted using the following dosages that were derived from label recommendations for the treatment of vegetable corps: 3 g/l (1.5 Kg/ha) for Turex; 0.5 ml/l = $5 \cdot 10^7$ OB/m^2 (250 ml/ha) for SlNPV formulation.

Experiment 1: Bt toxins vs SlNPV assays

The experiment was carried out using five replicates for each treatment (SlNPV, Bt toxins, and control). For each replicate a jar with two bean plants was arranged. The plants were treated with a pump sprayer until leaf dripping and allowed to dry at room temperature. The control plants were sprayed with distilled water. Plants were isolated form pot soil by an aluminium film and a cylindrical Plexiglas cage (Ø = 9 cm; h = 27 cm) was placed over the plants. Five second-instar larvae of *S. littoralis* were introduced in each cage and the upper side of the cage was closed with fine gauze to allow air circulation.

The experiment was carried out in the Laboratory for the Biological Agriculture of Centro Agricoltura Ambiente "Giorgio Nicoli" (Crevalcore, Bologna) in a climatic chamber at: 25 ± 0.1 °C diurnal temperature and 22 ± 0.1 °C nocturnal temperature, 60 ± 2% RH, under a light dark photoperiod of 14 L:10 D.

On the fourth day after starting the experiment mortality of larvae and damage on leaves were recorded. Post-cotyledon leaves (two per each plant and thus four in each replicate) were analyzed in order to estimate leaf damage. The extent of leaf blade eaten by the larvae was assessed as percentage on the total leaf area using the program Image J 1.37v (Wayne Rasband, National Institues of Health, USA).

Experiment 2: SlNPV lethal time assay

The time response of *S. littoralis* to viral formulation was assessed on second-instar larvae that were continuously fed on fresh leaves excised from bean plants. Both SlNPV and control plants were treated as described above. Each replicate consisted of 20 larvae reared from the same egg mass. Ten of them were fed by SlNPV treated leaves, the others were used as control. In total, 10 replicate were set-up for each treatment. Larvae were individually held in a Petri dish to avoid cannibalism. A disc of filter paper was placed in each dish and regularly moistened to maintain the humidity. Mortality was checked daily for 14 days after starting the experiment; at each examination the leaf was replaced. The same parameters of climatic chambers reported for the previous experiment were used.

Data analysis

The mortality of *S. littoralis* larvae was analyzed by one-way ANOVA. In experiment 1, Tukey's test was used for multiple comparison of means ($p < 0.05$).

Owing to heteroscedasticy of the variances, leaf damage data were analyzed by Kruskal-Wallis non-parametric ANOVA followed by Dunn's test for multiple comparisons of means ($p < 0.05$).

Kaplan-Meier Estimators were used on pooled data (regardless of replicate) to calculate mean lethal times and 95% confidence intervals. A non-parametric test was used since survivors were present in both groups, and alive larvae were considered as censored data. The log-linear rank test was used to identify significant differences ($p < 0.05$) between SlNPV group and control group.

The trend of mortality, corrected by Abbott's formula (Abbott, 1925), as function of days post treatment was analyzed by the curvilinear regression: $y = A / (1 + B \cdot \rho^x)$ (Snedecor and Cochran, 1980).

Results

Bt toxins vs SlNPV assay

Four days after treatment the mortality was 88% for Bt, 28% for SlNPV, and 24% for control (table 1). ANOVA showed significant differences among treatments [$p = 0.0022$; $F(2, 15) = 10.62$]. The percentage of mortality in Bt was significantly higher ($p < 0.001$) than in other treatments. On the contrary, the mortality of SlNPV did not show statistically significant differences in comparison to the mortality of control.

The mean leaf damage scores were highest for control, median for SlNPV, and smaller for Bt toxins (table 1). Significant differences among treatments were detected by Kruskal-Wallis test ($p < 0.001$; $H = 31.79$; d.f. = 2). The Dunn's test identified Bt treated leaves as the least damaged. Although the difference was not statistically significant, the SlNPV leaves were less damaged in comparison with the control leaves.

SlNPV lethal time assay

At the fourteenth day a significantly higher mortality was recorded in SlNPV group than in control [$p < 0.001$; $F(3, 16) = 19.60$]. Only 6.00% of larvae survived in virus group whereas 45.92% survived in the control. Two larvae in the control group died because of improper handling; therefore they were not included in the analysis.

Table 1. Effects of treatments on mean percentage of mortality and mean percentage of leaf damages. Mortality and damage were recorded 4 days after the treatment.

Treatment	# replicates	tested larvae	dead larvae at the fourth day	mean percentage of mortality (SD)	mean percentage of leaf damage (SD)
Bt toxins	5	25	22	88.00 (17.89) a[1]	1.03 (1.16) a[2]
SlNPV	5	25	7	28.00 (22.80) b[1]	5.44 (4.11) b[2]
control	5	25	6	24.00 (26.08) b[1]	11.05 (10.46) b[2]

[1] Tukey's test, $p < 0.05$

[2] Dunn's test, $p < 0.05$

Table 2. Kaplan-Meier non-parametric estimates of lethal times for second instar larvae of *S. littoralis*. Larvae assigned to SlNPV group were fed on bean leaves sprayed with $5 \cdot 10^7$ OB/m2 of SlNPV; Control group larvae were fed on bean leaves treated with distilled water.

Treatment	tested larvae	mean lethal time (days)	standard error	95% confidence interval
SlNPV	100	7.32 a[1]	0.38	6.56 – 8.07
control	98	10.67 b[1]	0.42	9.84 – 11.504

[1]Log-rank test, $p < 0.01$

Figure 1 reports cumulative mortality over time of *S. littoralis* larvae. The Kaplan-Meier non-parametric estimates of mean lethal times were 7.32 days for SlNPV group and 10.67 days for control group (table 2). According to the log-rank test the difference between the two treatments is statistically significant.

The logistic function (figure 2), which is expected to describe cumulative mortality over time, fitted the observed data adequately ($r^2 = 0.96$). According to this model a mean mortality of 50% of larvae could be achieved on the ninth day after the treatment with SlNPV.

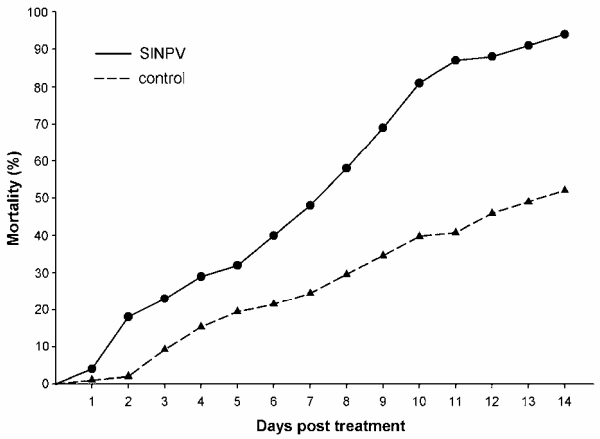

Figure 1. Cumulative time mortality response of second instar larvae of *S. littoralis*. Solid line represents mortality in larvae fed on bean leaves treated with $5 \cdot 10^7$ OB/m^2 of SlNPV; dashed line reports mortality in control group.

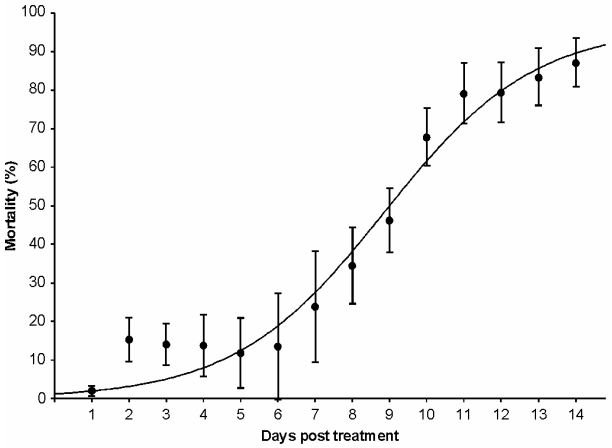

Figure 2. Mean mortality of second instar larvae of *S. littoralis* treated with SlNPV as a function of days after product application. Each value is the mean of ten replicates corrected by Abbott's formula; bars represent the standard errors. Model: $y = A / (1 + B \cdot \rho^x)$, where y = Mortality; x = days post treatment; A = 0.96; B = 60.87; $\rho = 0.61$; $r^2 = 0.96$.

Discussion

Over a short-time period the application of SlNPV was poorly effective on larvae of *S. littoralis*. The conventional Bt formulation caused both higher larval mortality and greater damage reduction. It is well known that Bt toxins kill insects much more rapidly than baculoviruses (van Beek and Hughes, 1998), therefore higher mortality in Bt than in SlNPV four days after treatment was expected. As a consequence of its speed of action, the application of Bt prevented the leaf damage due to *S. littoralis*: approximately only 1% of leaf-blade was eaten in the treated plants. The mean percentages of mortality were very similar in control and SlNPV, on the contrary the mean percentage of leaf damage was approximately twice in the control than in the viral treatment (table 1). Although this difference was not identified as significant by Dunn's test, it could be a consequence of the first symptoms of viral infection that reduced feeding activity of larvae.

54.08% of larvae assigned to the control group died before the end of the experiment and the LT 50 calculated by Kaplan-Meier Estimators in control larvae was only 3.35 days longer than for virus ones. Given that no signs of viral infection could be detected on control larvae, it is likely that such a high mortality could be due to stressing experimental conditions. These conditions could have affected also larvae treated with SlNPV leading to an underestimation of mean lethal time. The logistic regression was performed on mean mortalities corrected by Abbott's formula specifically to take into account the overall high mortality recorded in control larvae. For this reason the estimation of LT 50 in SlNPV treated larvae from the regression was approximately two days longer than the value calculated by Kaplan-Meier Estimators.

Knowledge of the time necessary to kill the target insects is crucial information for the correct timing of pest management programs and several authors performed lethal time analysis on SlNPV isolates (Murillo *et al.*, 2003; Rivkin *et al.*, 2006; Seufi, 2008; Toprak *et al.*, 2006). The LT 50 for second instar larvae of *S. littoralis* ranges from 4.2 to 7.5 days. Different combinations of viral and insect strains and dissimilar conditions of experiments (temperature, humidity, larval stages, artificial diets or host

plants, viral doses, subministration methods, and statistical analyses) were used and comparing data resulting from separate studies is often puzzling. However, results of the present investigation are roughly in agreement with the longest lethal times reported in literature.

Although this assumption needs to be corroborated by open-field studies, it is likely that a mean lethal time greater than seven days is too long to be very useful in preventing economic losses on most of corps. The ineffectiveness in achieving rapid reduction of pest populations is one of the main drawbacks of baculoviruses and one of the reasons why farmers are often averse to use these control agents (Toprak *et al.*, 2007). On the other hand, in crops where economic yield losses are due mainly to the presence of insect fragments in the final products (i.e. spinach and other deep-frozen foods) rather than to direct plant damages, the proper use of SlNPV could actually be useful. Moreover, viruses could persist in the field populations of *S. littoralis* eventually leading to a long-term significant reduction of pest density (Moscardi, 1999). Finally, sprays combination of Bt toxins and SlNPV could be tested in order to increase and enhance the efficacy of microbial products for *S. littoralis* management.

Acknowledgements

We are grateful to E. Ladurner (IntrachemBio) for supplying viral formulation; to M. L. Dindo, L. Depalo and E. Marchetti (DiSTA - Entomologia) for supplying insects. We also thank R. Ferrari (Centro Agricoltura Ambiente "Giorgio Nicoli"), A. Lanzoni and C. Chieco (DiSTA - Entomologia) for technical assistance in lab trials.

The study was funded by Cassa di Risparmio di Bologna Foundation with the assistance of Laboratory for the Biological Agriculture of the Centro Agricoltura Ambiente "Giorgio Nicoli".

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Received June 23, 2008. Accepted September 19, 2008.