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Spinosad and nucleopolyhedrovirus mixtures for control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in maize

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Abstract

Spinosad (Dow AgroSciences) is a neurotoxin mixture produced during fermentation of a soil actinomycete that has high activity towards Lepidoptera. Diet surface contamination bioassays were performed with *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) and Spinosad alone and in mixtures. The interaction of SfMPNV + Spinosad mixtures in *S. frugiperda* larvae was generally independent or slightly antagonistic in nature, although weak synergism was detected in mixtures containing 3 ppm Spinosad + 20 or 70 occlusion bodies/mm^2 diet of SfMNPV. Mean time to death was not a reliable indicator of mortality over time in larvae exposed to SfMNPV–Spinosad mixtures because Spinosad killed larvae quickly whereas virus mortality occurred at a much lower rate. Therefore, threshold tolerance analysis was employed to generate time–response curves that showed two clear phases; an initial response to Spinosad until ~100 h followed by virus-induced mortality at 120–250 h post-contamination. A field trial was performed to assess the degree of pest control achieved by SfMNPV–Spinosad mixtures applied to maize. Recovery of *S. frugiperda* larvae was significantly reduced in all treatments compared to recovery from control plots. The mixture of SfMNPV with 3 ppm Spinosad resulted in ca. 90% *S. frugiperda* control, which was 12.5–32% greater than for plots treated with SfMNPV alone. The impact of low concentrations of Spinosad on non-target arthropods present in the maize crop was evaluated in a field trial. Application of 3 ppm Spinosad had very little effect on the abundance of insect natural enemies present on maize plants, whereas application of the product label recommended rate of 200 ppm Spinosad had effects similar to those observed following application of chlorpyrifos. The use of low concentrations of Spinosad merits further study as a means of controlling lepidopteran pests either alone or in combination with other entomopathogens.

Keywords: NPV–Spinosad interaction; Baculovirus; Formulation; Fall armyworm; Non-target impact

1. Introduction

The formulation of entomopathogens can greatly affect their efficiency as biological insecticides (Burges and Jones, 1998). Specifically, formulation can influence the stability of the pathogen in storage and the efficiency of the application to the crop. Moreover, certain formulation adjuvants can enhance the activity of the pathogen and improve environmental persistence (Jones et al., 1997). One way to increase the activity of the pathogen is to mix it with small quantities of synergistic substances such as optical brighteners (Shapiro and Dougherty, 1994), inorganic acids (Cisneros et al., 2002b; Shapiro and Bell, 1982) or sublethal concentrations of chemical insecticides (Peters and Coaker, 1993). However, the interaction between a pathogen and other compounds may also be antagonistic due to decreased feeding or a change of gut pH (Chancey et al., 1973; Fuxa, 1979; Pingel and Lewis, 1999) or each entity may act independently, leading to additive mortality (Koppenhöfer and Kaya, 2000; McVay et al., 1977).

Spinosad (Dow AgroSciences) is a mixture of spinosyns A and D produced during fermentation of the soil actinomycete *Saccharopolyspora spinosa* Mertz and Yao (Sparks et al., 1998). Spinosad is a neurotoxin with a novel mode of action involving the nicotinic acetylcholine receptor and probably GABA receptors as well

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(Salgado, 1997, 1998). Exposure causes a cessation of feeding followed, some 24 h later, by paralysis and death. Spinosad is primarily a stomach poison with some contact activity and is particularly toxic to Lepidoptera and Diptera. However, toxicity tests indicate that Spinosad has virtually no toxicity to birds and mammals and relatively low toxicity to certain insect natural enemies (Bret et al., 1997), although a number of insect predators and parasitoids appear to be susceptible to Spinosad intoxication (Cisneros et al., 2002a; Elzen et al., 2000; Tillman and Mulrooney, 2000). Spinosad is classified by the US Environmental Protection Agency as an environmentally and toxicologically reduced risk material (Saunders and Bret, 1997).

Larvae of the fall armyworm *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) are the principal pests of maize production in Mesoamerica. Infestation levels over 55% can cause a 15–73% reduction in crop yield (Hruska and Gould, 1997). The crop damage caused by *S. frugiperda* larvae is highly apparent and growers often apply synthetic insecticides in spray and granular formulations to control the pest. However, the incorrect use of chemical insecticides by resource-poor rural growers results in a high prevalence of chronic pesticide poisoning in farm workers from southern Mexico and Nicaragua (Hunt et al., 1999; McConnell and Hruska, 1993).

Given the need for safe, sustainable, and economical pest control for Mesoamerican maize farmers, we have been evaluating the multinucleocapsid nucleopolyhedrovirus of *S. frugiperda* (SfMNPV) as a biological insecticide. Spray application between 1.2×10^{12} and 6×10^{12} viral occlusion bodies (OBs)/ha in water results in approximately 40% infection of *S. frugiperda* larvae collected at 2 days post-application and reared in the laboratory until death or pupation (Martínez et al., 2000). Natural parasitism typically contributes an additional 20% mortality giving an overall prevalence of around 60%.

Formulation may improve this degree of control in two different ways. First, the use of feeding stimulants may increase consumption of virus inoculum by the target pest. Granular phagostimulant formulations based on nixtamalized maize flour have recently been shown to significantly increase control of lepidopteran pests using *Bacillus thuringiensis* Berliner (Tamez-Guerra et al., 1998, 2000) and SfMNPV (Castillejos et al., 2002). Second, the efficacy of virus treatments may be increased by the incorporation of substances, such as optical brighteners, that enhance the activity of the virus (Hamm, 1999) or insecticidal substances that cause complimentary mortality, resulting in improved pest control (Morris et al., 1974).

The objectives of the present study were to characterize the interaction between SfMNPV and very low concentrations of Spinosad and to determine the feasibility of using SfMNPV–Spinosad mixtures for control of *S. frugiperda* in maize. For this, we performed laboratory bioassays of virus and Spinosad alone and in mixtures. We then performed a field trial to assess the degree of pest control achieved by SfMNPV–Spinosad mixtures. Finally, we evaluated the possible impact of low concentration Spinosad applications on non-target arthropods present in the maize crop.

2. Materials and methods

2.1. Bioassays

To determine the activity of an SfMNPV isolate previously characterized by Escribano et al. (1999), bioassays were performed based on the technique described by Del Rincón-Castro and Ibarra (1997). All laboratory procedures were performed at 25 ± 1 °C, 75–85% RH, and 12 h:12 h L:D photoperiod. Occlusion bodies (OBs) were produced in fourth-instar *S. frugiperda* larvae individually maintained in 25 ml plastic cups containing a semi-synthetic diet based on soya and maize without formaldehyde (modified from Mihm, 1984). Virus-killed larvae were triturated in 0.1% (w/v) sodium dodecyl sulfate (SDS) and centrifuged at 90*g* for 5 min. The supernatant was centrifuged at 3000*g* for 10 min and pelleted OBs were resuspended in sterile distilled water, counted using a bacterial counting chamber, and stored at 4 °C for 24 h prior to use. Sterile plastic petri dishes (9 cm diameter) were half-filled with semi-synthetic diet. The diet was allowed to solidify and was then contaminated with one of the following five concentrations of OBs: 10, 50, 100, 250, and 500 OBs/mm^2 diet surface. OBs were suspended in a volume of 250 μl of 0.1% (vol/vol) Triton X-100 solution.

A rectangular plastic grid 70×54 mm divided into 12 squares with an internal area of 15×15 mm was pressed into the diet to form 12 identical compartments into each of which was placed a second-instar *S. frugiperda* larva taken from the laboratory culture maintained at El Colegio de la Frontera Sur (ECOSUR), Tapachula, Mexico. The grid was covered with a thin glass slide and the lid of the petri dish. Forty-eight larvae were used for each concentration. A similar number of control larvae were placed in petri dishes containing 0.1% Triton X-100 and diet alone. Larvae were checked twice daily for mortality until 19 days post-contamination by which time survivors had pupated. Viral deaths were confirmed by examination of Giemsa-stained smears of insect cadavers. The OB bioassay was performed three times.

The above bioassay procedure was repeated using a commercial Spinosad preparation (Tracer, Dow Agrosciences). For tests involving Spinosad, the following seven concentrations of active ingredient (a.i.) were employed: 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 parts per

million (ppm) a.i. in a solution of 0.1% Triton X-100. Forty-eight larvae were treated with each concentration. Control larvae were exposed to diet with 0.1% Triton X-100 alone. Evaluation of larval mortality commenced at 18 h post-contamination and continued twice daily thereafter until survivors had pupated. The Spinosad bioassay was performed four times.

Virus-induced mortality data were subjected to probit regression analysis using the PCProbit program (CINVESTAV-IPN, Mexico). Due to a moderate degree of overdispersion, Spinosad mortality data were analyzed in GLIM (Numerical Algorithms Group, 1993), with a binomial error structure specified. GLIM presents the results of such analyses in terms of χ^2 statistics (Crawley, 1993). Overdispersion was corrected following Williams' procedure using the GLIM macro described by Collett (1991). The results of scaled analyses are presented as F statistics with the scale parameter indicated. In all cases, the behavior of models was checked by examination of the distribution of residuals and fitted values using the model checking macro present in the program. The mean time to death was calculated using GLIM with a normal error distribution; individuals that did not succumb to virus infection were excluded from the analysis (Farrar and Ridgway, 1998).

2.2. Interaction NPV–Spinosad

To determine the nature of the interaction between nucleopolyhedrovirus and Spinosad, a bioassay was performed as described above using mixtures of SfMNPV (5, 20, and 70 OBs/mm^2 diet) and Spinosad (0.05, 0.5, and 3 ppm a.i.) in a solution of 0.1% Triton X-100. Results of the previous bioassays indicated that when treated individually, these concentrations were expected to cause approximately 18–50% infection by virus and 13–50% mortality by Spinosad. As before, 48 larvae were contaminated with each substance alone or in combination (NPV + Spinosad). Evaluation of larval mortality commenced at 18 h post-contamination and continued twice daily thereafter until survivors had pupated. Control larvae ($N = 162$) were not exposed to virus or Spinosad. The experiment was performed four times.

To determine the nature of the interaction between SfMNPV and Spinosad when bioassayed as mixtures, we performed two types of analysis. First, the expected mortalities were calculated following the formula described by Finney (1964) assuming independent action, in which percentage expected mortality $E = [O_{\mathrm{NPV}} + O_{\mathrm{Spin}}(1 - O_{\mathrm{NPV}})] \times 100$, where O_{NPV} is the proportional mortality produced by NPV alone and O_{Spin} is the proportional mortality produced by Spinosad alone. The difference between observed and expected mortalities was then analyzed by the log likelihood ratio test (G test) adjusted using Williams' correction, following the procedures described by Sokal and Rohlf (1981). Adjusted G values approximate to the χ^2 distribution for large sample sizes such as that used in this study.

Second, we applied the threshold tolerance analysis procedures described by Preisler et al. (1999) for the analysis of mortality over time for bioassays with mixtures. In threshold tolerance models, each individual in a population is assumed to have a particular tolerance to the toxicant or pathogen. The individual responds (dies) if and when the concentration or dose exceeds the threshold tolerance of that individual. The probability of response (p_i) by time t_i of insects from the replicate j simultaneously administered a virus mixed with an insecticide can be generalized as

$$p_i = 1 - \exp[-\exp(\alpha + \beta\tau_I)], \tag{1}$$

where α and β are the parameters of the linear predictor of the generalized linear model and τ_I is a non-parametric transformation of the time points t_{ij} generated by fitting a smoothing routine (*loess*) to the observed mortality over time, within the generalized additive fitting function (*gam*) described by Hastie (1992), using the S-Plus statistical package (Statistical Sciences, 1993). Estimates for α and β were generated using the binomial maximum likelihood function (2) where s_{ij} is the number of insects alive at time point t_{ij}, and d_{ij} is the number of insects that died in the interval $(\tau_{ij}, \tau_{i+1,j})$ since the previous observation and $q_{ij} = [p_{(I+1)j} + p_{ij}]/(1 - p_{ij})$, which is the conditional probability of an insect responding during each interval between observations (Preisler et al., 1999)

$$\prod_{j=1}^{J} \prod_{i=1}^{I_i} q_{ij}^{dij} (1 - q_{ij})^{sij - dij}. \tag{2}$$

In the case of the treatments involving 3 ppm Spinosad and/or SfMNPV, the maximum likelihood function did not converge. Response curves were therefore fitted using the complimentary log–log output from *gam*. Confidence limits were established using deleted jackknife procedures based on 10 randomly generated data subsets each with 12 insects deleted from each of the four replicates, as described by Efron and Tibshirani (1993). It was not possible to compare lethal time (LT_{50}) values of different treatments because, in many cases, mortality did not reach 50%. Mean time to death was therefore analyzed in GLIM with normal errors (Hernández-Crespo et al., 1999). The usual model checking procedures were performed.

2.3. Field trial: NPV–Spinosad mixtures

A field trial was performed in a maize field close to the village of Mazatán, Chiapas, Mexico (14°52′44″N, 92°27′45″W) at approximately 20 m altitude, during the month of July, 2000. During this period, the weather was hot (daily range 23–36 °C) with regular rainfall in

the afternoons (~300 mm/month). Insecticides had not been applied to the crop, prior to the experiment. Maize plants were planted at a density of approximately 40,000 plants/ha and were 50–60 cm tall at the start of the trial. Plants were divided into 40 experimental blocks of 6×6 m with a barrier of 5 m of maize plants between blocks. Plants within blocks were manually infested each with 4 second-instar *S. frugiperda* larvae from the laboratory culture. Twenty-four h later, each of the blocks was randomly assigned to one of the following treatments: (i) control water spray, (ii) Spinosad (Tracer) applied at the product label recommended concentration of 200 ppm (equivalent to 60 g a.i./ha), (iii) Spinosad applied at 3 ppm (equivalent to 0.9 g a.i./ha), (iv) 1.2×10^{12} OBs/ha SfMNPV, and (v) 1.2×10^{12} OBs/ha SfMNPV + 3 ppm Spinosad. Applications were made in a volume of 8.6 liters/treatment (equivalent to 300 liters/ha) using a manual knapsack sprayer fitted with a cone nozzle, with 0.02% (vol/vol) Agral Plus (Zeneca) as wetter-sticker. There were eight replicate blocks assigned to each treatment.

At 2, 5, and 10 days post-application, 20 randomly selected plants from each block were cut, placed in plastic bags, and transported to the laboratory, where living *S. frugiperda* larvae were transferred to individual plastic cups containing semi-synthetic diet and reared through to pupation as described previously (Williams et al., 1999). The number of larvae that died of virus infection or parasitoid emergence was noted.

The number of larvae recovered from experimental plants was subjected to ANOVA in GLIM for each sample point. The prevalence of infection by virus and the emergence of parasitoids were analyzed using binomial error structures. Small degrees of overdispersion were taken into account by scaling the error distribution, as described above.

2.4. Effect of Spinosad on non-target arthropods

A field trial was performed to determine the possible impact of low concentrations of Spinosad on non-target arthropods. The experimental site was a maize field adjacent to the experimental site described above. Maize plants were planted at a density of approximately 30,000 plants/ha and were 80–100 cm tall at the start of the trial. Part of the field (0.3 ha) was divided into blocks 6×6 m with 5 m of maize planted between blocks. Seven randomly selected blocks were assigned to each one of the following treatments: (i) water control, (ii) chlorpyrifos (Lorsban 480 EM, Dow AgroSciences) at the recommended rate of 0.75 liter/ha, (iii) Spinosad applied at 200 ppm (60 g a.i./ha), and (iv) Spinosad applied at 3 ppm (0.9 g a.i./ha). As in the previous experiment, applications were made using a knapsack sprayer in a volume of 300 liters/ha with 0.02% Agral Plus included as a wetter-sticker.

At 1, 3, and 7 days post-application, the number of arthropods present on 15 randomly selected plants in each block was checked and recorded. Sampled plants were never re-sampled. Insects were classified post hoc into eight groups based on ecological and taxonomic relationships. Natural enemy groups were earwigs (*Doru taeniatum* Dorhn), *Orius* spp., predatory beetles, spiders, and other predators, which included syrphid larvae, *Chrysoperla* spp., *Solenopsis* spp., etc. Groups of other insects were classified as lepidopteran larvae, staphylinid (*Tachyporus* sp.) and nitidulid (*Carpophilus* sp.) beetles that were abundant on maize plants, or other insects, which included thrips, aphid colonies, phytophagous bugs, etc.

The number of arthropods observed on maize plants was subjected to repeat measures MANOVA using SAS (SAS Institute, 1992) with the eight groups of natural enemies and other insects described above as dependent variables. The significance of treatment effects at each time point and multiple comparisons between treatments were interpreted in terms of F statistics generated from Pillai's Trace (Winer, 1971). To compare treatment effects on a particular arthropod group within a sample, univariate ANOVA or Kruskal–Walis non-parametric analyses was employed (depending on the data distributions of each arthropod group), followed by the LSD procedure for means separation. A Bonferroni correction was applied giving a critical value of $\alpha = 0.0083$, instead of the conventional $\alpha = 0.05$, to minimize the risk of type I errors (Sokal and Rohlf, 1981).

3. Results

3.1. Bioassay

The LC_{50} value for SfMNPV was calculated at 70.3 OBs/mm^2 of diet surface (range of 95% C.L.: 53.0–91.6 OBs/mm^2). The probit regression equation was $y = 0.81 \log(x) + 3.50 \quad (\chi^2 = 4.66,\ \mathrm{df} = 3,\ P > 0.05)$. Larvae died 5–12 days post-inoculation as reported previously for this isolate (Cisneros et al., 2002b; Martínez et al., 2000). There were no viral deaths in the controls. The LC_{50} calculated for Spinosad was 2.98 ppm (range of 95% C.L.: 2.25–4.06 ppm). The logit regression equation was $y = 0.49 \log_e(x) - 1.47$ (in terms of the odds ratio $\log_e[p/q]$, scale parameter = 1.034 following Williams' correction for overdispersion). There was a significant negative relationship between mean time to death and $\log_e$ Spinosad concentration ($F_{(1,365)} = 27.9,\ P < 0.001$) ranging from 47.6 ± 7.8 h at 0.1 ppm to 23.5 ± 1.2 h at 10 ppm (although evaluation of mortality commenced at 18 h post-contamination, by which time a considerable number of larvae had died at the highest concentrations).

3.2. Interaction NPV–Spinosad

Viral concentrations of 5, 20, and 70 OBs/mm^2 caused 21.5–60.8% mortality of *S. frugiperda* larvae in agreement with the mortality predicted from the results of the previous bioassay (Table 1). Similarly, Spinosad concentrations of 0.05, 0.5, and 3 ppm a.i. caused 23.5–67.4% mortality. *G* tests applied to observed mortality and expected mortality data from NPV–Spinosad mixtures containing 0.05 or 0.5 ppm a.i. indicated that observed mortality was statistically similar to expected mortality (an independent effect) or was slightly less than the expected value (an antagonistic effect). In contrast, mixtures containing 3 ppm Spinosad resulted in independent mortality at 5 OBs/mm^2 and a greater than expected mortality at 20 and 70 OBs/mm^2 (a weak synergistic effect) (Table 1). No mortality occurred in control larvae.

The OB concentration did not significantly affect the mean time to death either alone or when mixed with Spinosad ($F_{(2,44)} = 1.38,\ P = 0.26$). In contrast, increasing the concentration of Spinosad caused a highly significant decrease in the mean time to death of larvae exposed to Spinosad alone or in mixtures with SfMNPV ($F_{(3,44)} = 42.6,\ P < 0.001$). However, mean time to death was not a reliable indicator of mortality over time in larvae exposed to SfMNPV–Spinosad mixtures because Spinosad killed larvae quickly whereas virus mortality occurred at a much lower rate. This pattern was clearly revealed by threshold tolerance analysis of the cumulative response over time of insects exposed to SfMNPV and Spinosad alone or in mixtures (Figs. 1A–O). Mortality of larvae inoculated with virus alone commenced at approximately 120 h post-inoculation (5 days), whereas the response to Spinosad was much faster (<48 h), especially at the concentration of 3 ppm (Fig. 1F), although at the lowest concentration of Spinosad (0.05 ppm) insect mortality was noticeably delayed (Fig. 1D). As a result of these differences, many of the time–response curves generated by virus–Spinosad mixtures show two clear phases; an initial response to Spinosad until ~100 h, followed by virus-induced mortality beginning at 120 h post-contamination and increasing gradually until 250–400 h post-contamination (Figs. 1G–O).

3.3. Field trial: SfMNPV–Spinosad mixtures

Recovery of larvae was significantly reduced in all treatments compared to recovery from control plants at 2 days ($F_{(3,28)} = 15.7,\ P < 0.001$), 5 days ($F_{(3,28)} = 13.9,\ P < 0.001$), and 10 days post-application ($F_{(3,28)} = 5.24,\ P = 0.006$) (Figs. 2A–C). Application of 200 ppm Spinosad reduced total larval recovery to a single larva at 2 days post-application, increasing to a total of six larvae at 10 days post-application. Because of very low recovery, this treatment was not included in the subsequent analyses. Application of 3 ppm Spinosad, with or without virus, reduced larval recovery to approximately one-quarter of the recovery from control plants.

Percentage virus infection varied non-significantly from 30% to 44% in larvae collected at 2 days post-application and reared in the laboratory for the SfMNPV + Spinosad treatment and the SfMNPV alone treatment, respectively ($F_{(1,14)} = 1.26,\ P = 0.28$, scale parameter = 1.56). The prevalence of virus infection fell

Table 1
Interaction of nucleopolyhedrovirus (SfMNPV) and Spinosad in second-instar *Spodoptera frugiperda*

Conc. NPV (OBs/mm^2)	Conc. Spinosad (ppm)	Number dead	Number tested	Percentage of mortality observed	Percentage of mortality expected	G_{Adj}[a]	P[b]	Interaction[c]
0	0.0	0	162	0.0	—			
5	0.0	38	177	21.5	—			
20	0.0	74	189	39.2	—			
70	0.0	107	176	60.8	—			
0	0.05	44	187	23.5	—			
5	0.05	71	190	37.4	39.9	0.21	N.S.	Indep.
20	0.05	73	187	39.0	53.5	7.84	**	Antag.
70	0.05	111	186	59.7	70.0	4.33	*	Antag.
0	0.5	59	189	31.2	—			
5	0.5	91	191	47.6	46.0	0.10	N.S.	Indep.
20	0.5	99	191	51.8	58.2	1.56	N.S.	Indep.
70	0.5	114	190	60.0	72.5	6.65	**	Antag.
0	3.0	124	184	67.4	—			
5	3.0	150	189	79.4	74.5	1.26	N.S.	Indep.
20	3.0	176	192	91.7	80.2	10.67	**	Synerg.
70	3.0	186	191	97.4	87.2	15.10	***	Synerg.

[a] Adjusted *G* test values (χ^2 distribution).
[b] Probability $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, N.S. means $P > 0.05$.
[c] SfMNPV–Spinosad interaction classified as antagonistic, independent or synergistic.

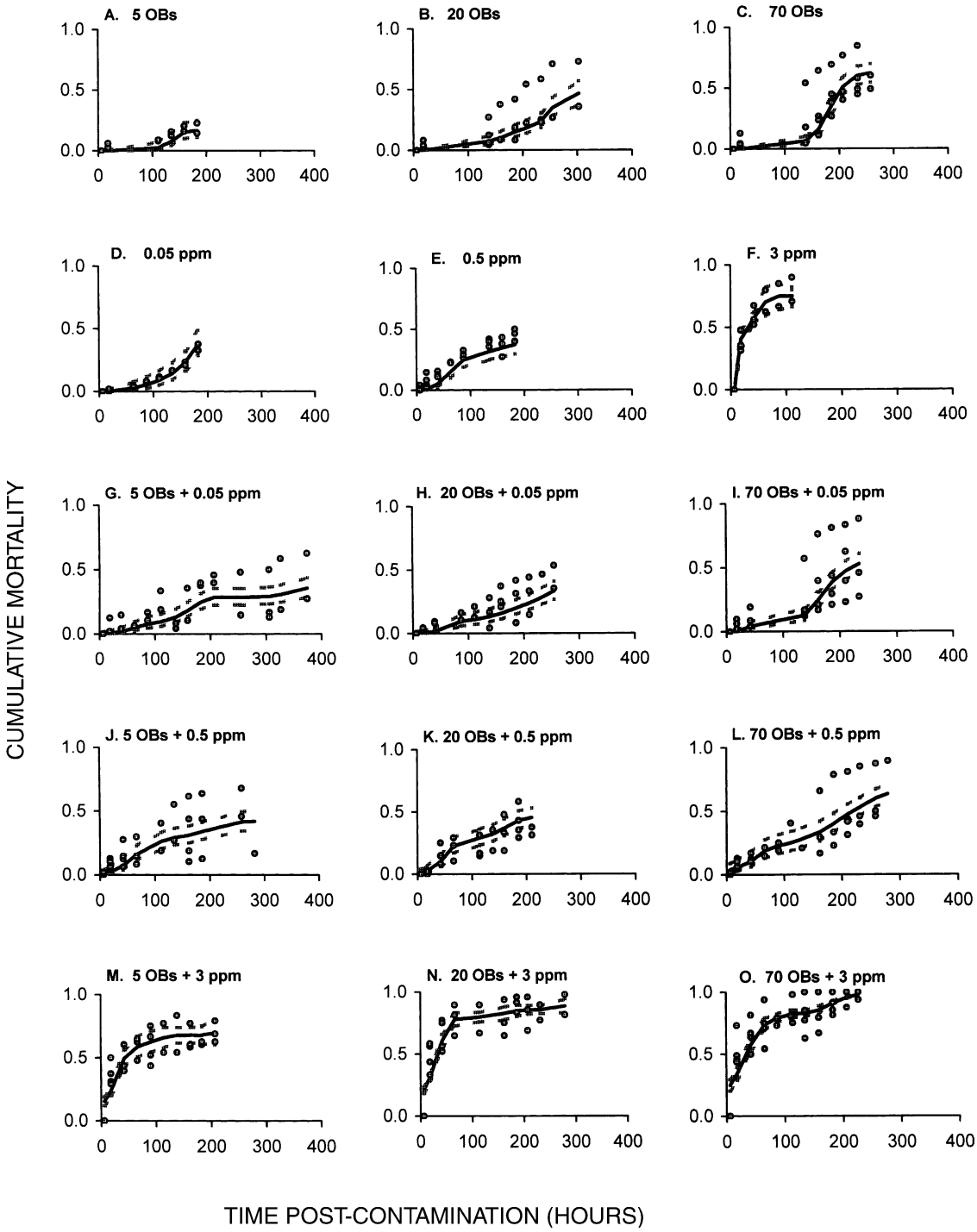

Fig. 1. Cumulative mortality of *Spodoptera frugiperda* larvae contaminated in the second instar with (A) 5, (B) 20, and (C) 70 OBs/mm^2 SfMNPV or (D) 0.05, (E) 0.5, and (F) 3 ppm Spinosad alone or in mixtures (G)–(O). Continuous line indicates threshold tolerance analysis fitted values, dashed line indicates 95% confidence interval. To achieve suitable estimates, in certain cases, it was necessary to exclude replicates from this analysis (indicated by the number of datapoints shown on each graph).

markedly in larvae from the virus alone treatment and was just 3% in larvae collected at 10 days post-application and reared in the laboratory (Fig. 2C). In contrast, the prevalence of virus infection in larvae from the SfMNPV + Spinosad treatment fell only slightly, reaching 24% in larvae collected at 10 days post-application and reared in the laboratory ($\chi^2 = 6.0$, df $= 1$, $P = 0.014$). No viral infections were detected in larvae collected from plots that had not been treated with OBs.

Only two species of parasitoid emerged from field-collected larvae. The solitary braconid egg-larval endoparasitoid *Chelonus insularis* Cresson accounted for 48%

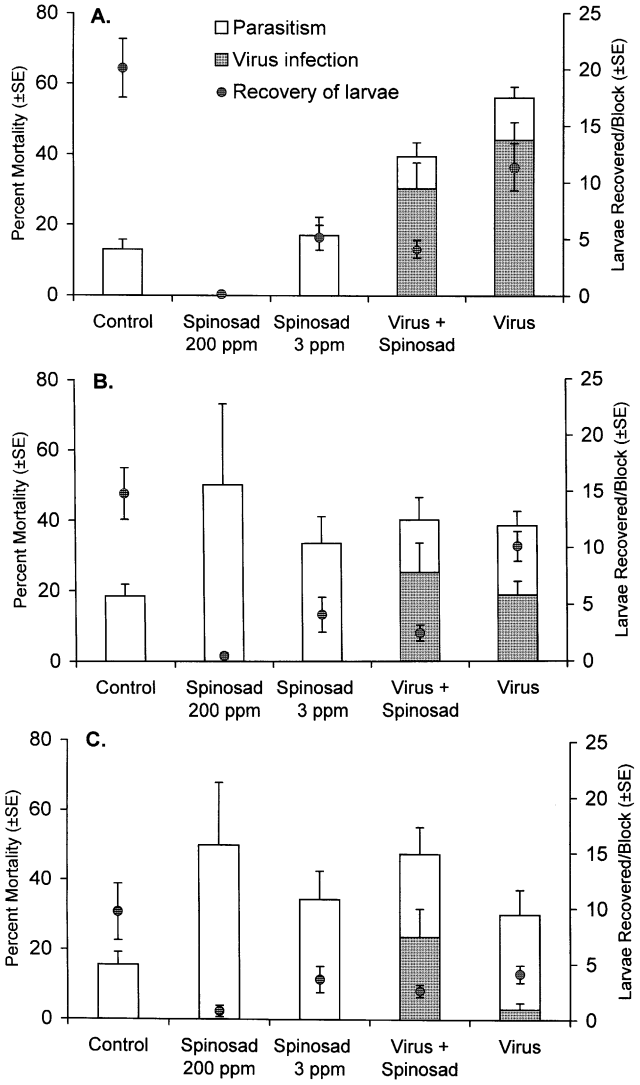

Fig. 2. Effect of application of Spinosad at 200 ppm and 3 ppm, 1.2×10^{12} OB/ha of SfMNPV, and the mixture of 1.2×10^{12} OB/ha SfMNPV + 3 ppm Spinosad on the recovery of *Spodoptera frugiperda* larvae (dots) from experimental plants at (A) 2 days, (B) 5 days, and (C) 10 days post-application. Larvae were reared in the laboratory to determine the prevalence of infection by virus (gray columns) and the emergence of parasitoids (white columns). Control plants were treated with water + wetter-sticker alone.

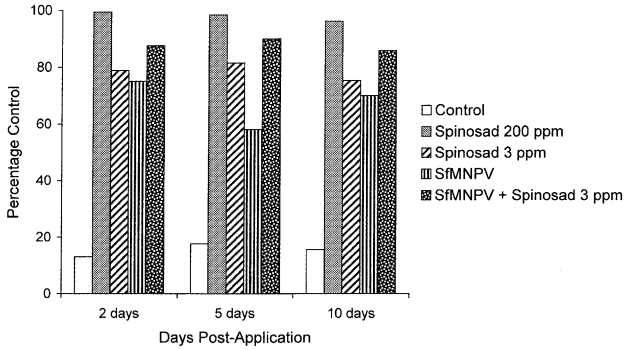

Fig. 3. Percentage control of *Spodoptera frugiperda* larvae at 2, 5, and 10 days post-application following application of Spinosad (3 and 200 ppm), or 1.2×10^{12} OB/ha SfMNPV, or the mixture of 3 ppm Spinosad + 1.2×10^{12} OB/ha SfMNPV. Control plants were treated with water + wetter-sticker alone.

of parasitism, whereas the gregarious eulophid larval ectoparasitoid *Euplectrus comstockii* Howard accounted for 52% of observed parasitism. Percentage parasitoid emergence in larvae collected from control plots was similar at all sample points (13–16% at 2 days and 10 days post-application, respectively). Application of 3 ppm Spinosad and/or SfMNPV did not significantly affect the prevalence of parasitoid emergence from larvae collected at 2 days ($F_{(3,28)} = 0.12$, $P = 0.94$, scale parameter = 1.37), 5 days ($\chi^2 = 1.13$, df = 3, $P = 0.77$) or 10 days post-application ($F_{(3,28)} = 0.81$, $P = 0.50$, scale parameter = 1.36). The apparently high prevalence of parasitism in larvae collected from plots treated with 200 ppm Spinosad was an artifact of very low sample sizes (Figs. 2A–C).

Using these results, the percentage of *S. frugiperda* control could be calculated as $[1 - (c - y - v - p)/c] \times 100$, where c is the number of larvae recovered from control plots, y is the number of larvae recovered from treated plots, v and p are the number of larvae that died from virus infection or parasitism in the laboratory, respectively. Pest control in control plots was due to parasitism alone (Fig. 3). The product label recommended application of 200 ppm Spinosad gave virtually 100% control during the experimental period whereas percentage control in 3 ppm Spinosad-treated plots varied between 75 and 82%. The mixture of SfMNPV with 3 ppm Spinosad resulted in approximately 90% *S. frugiperda* control, which was 12.5–32% greater than control for SfMNPV alone (Fig. 3). Clearly, these estimates do not take into account differences in the prevalence of predation in experimental plots, but they do serve to highlight the combination of differences in larval recovery and post-collection mortality between treatments.

3.4. Impact of Spinosad on non-target arthropods

The abundance of natural enemies and other insects on maize plants was markedly reduced by application of Spinosad or chlorpyrifos compared to control plants ($F_{(24,201)} = 5.02$, $P < 0.001$) (Figs. 4A–C). The abundance of arthropods did not change significantly between samples taken at 1 day and 3 days post-application ($F_{(8,65)} = 1.85$, $P = 0.08$) but increased significantly between the observations at 3 days and 7 days post-application ($F_{8,65} = 5.38$, $P < 0.001$), presumably due to movement of arthropods into insecticide-treated plots from neighboring untreated maize plants. The interaction of sample point with treatment was not significant ($F_{(48,420)} = 1.09$, $P = 0.33$).

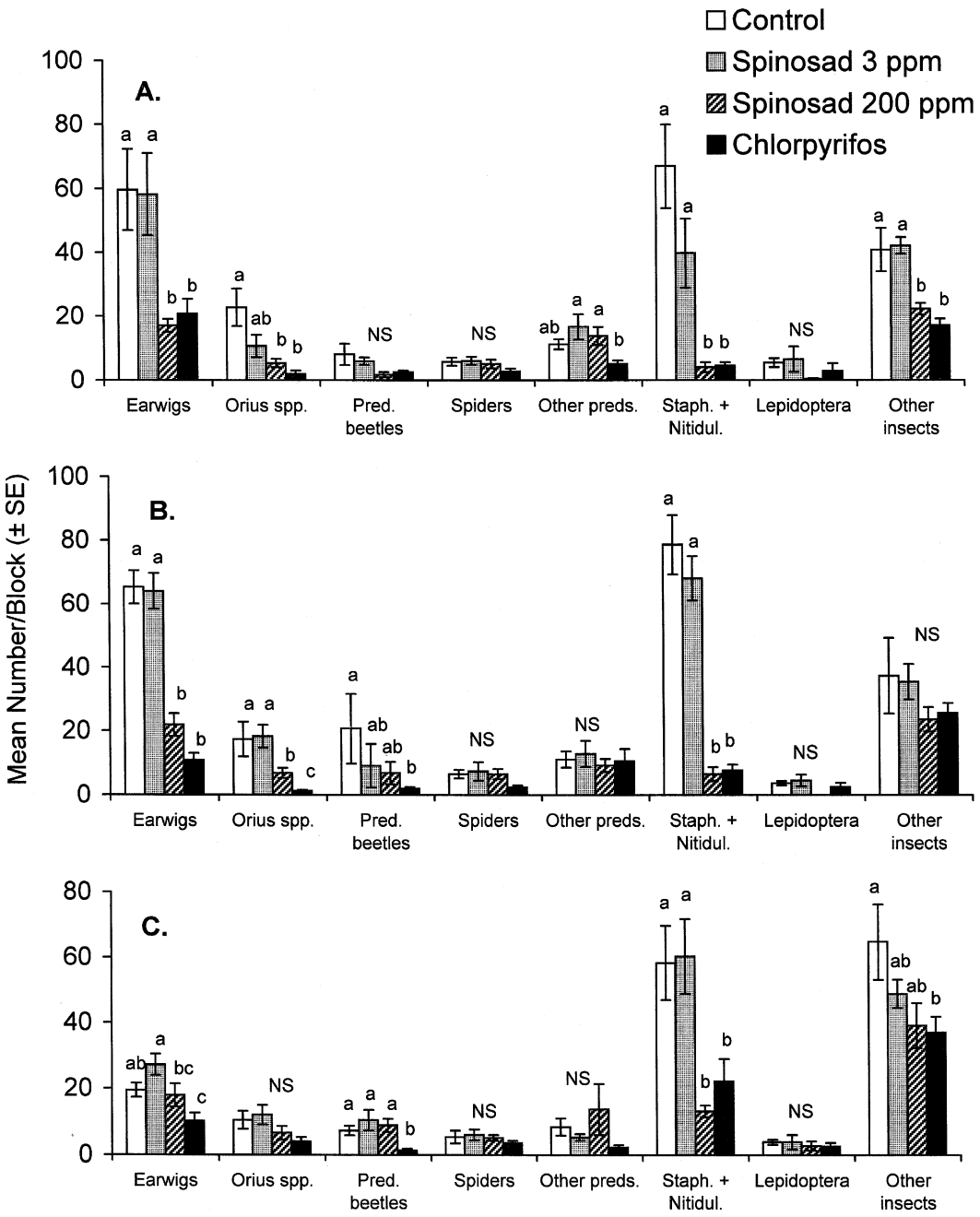

Fig. 4. Effect of application of 3 and 200 ppm Spinosad or 0.75 liter/ha chlorpyrifos on the abundance of eight groups of arthropods on maize plants at (A) 1 day, (B) 3 days, and (C) 7 days post-application. Columns within the same group labeled with the same letter are not significantly different for each sample point separately (ANOVA, LSD $P > 0.05$). Control plants were treated with water + wetter-sticker alone. See text for details of repeated measures analysis. NS means not significant ($P > 0.05$). Treatment differences remained unchanged when the Bonferroni correction was applied ($\alpha = 0.0083$) with the exception of treatment differences in the abundance of *Orius* spp. and other predators which became non-significant at 1 day post-application.

MANOVA procedures indicated that Spinosad applied at 3 ppm affected the abundance of arthropods to a small but significant degree ($F_{(8,65)} = 2.83,\ P = 0.009$), although this effect was not detected by univariate ANOVA applied to the different arthropod groups observed in each sample (Figs. 4A–C). Both Spinosad applied at 200 ppm and chlorpyrifos had a marked effect on the abundance of natural enemies and other insects and this effect persisted for the duration of the study, particularly in the chlorpyrifos treatment (F values given in Table 2). Surprisingly, there was no significant difference between the impact of chlorpyrifos and 200 ppm Spinosad on the abundance of arthropods, although the F value generated by Pillai's Trace was borderline

Table 2
F values generated by Pillai's Trace for comparison of treatments following repeat measures analysis of the effect of application of chlorpyrifos and spinosad at 3 or 200 ppm on the abundance of arthropods observed on maize plants at 1, 3, and 7 days post-application

Treatment	Control[a]	Spinosad 3 ppm	Spinosad 200 ppm
Control[a]	—		
Spinosad 3 ppm	2.82**	—	
Spinosad 200 ppm	16.64***	10.36***	—
Chlorpyrifos	24.86***	18.09***	2.04 (N.S.)

$^{**}P < 0.01$, $^{***}P < 0.001$. N.S., not significant. In all cases, df = 8, 65.
[a] Control plants were treated with water + wetter-sticker alone.

significant ($P = 0.055$) and, in several cases, univariate ANOVA suggested that arthropods were more severely affected by chlorpyrifos than by 200 ppm Spinosad (Table 2, Figs. 4A–C).

The earwig *D. taeniatum*, *Orius* spp., and the staphylinid and nitidulid beetles (*Tachyporus* sp. and *Carpophilus* sp.) were particularly sensitive to 200 ppm Spinosad and chlorpyrifos applications. In contrast, spiders were not significantly affected by the application of Spinosad or chlorpyrifos at any sample time. The abundance of lepidopteran larvae in the experimental plots was low in all samples (Figs. 4A–C).

4. Discussion

A recent survey of biopesticide researchers working in developing countries indicated that formulation was the most important issue in the development of biological insecticides (Harris and Dent, 2000). As spinosyns are produced by fermentation of an actinomycete, Spinosad has been classified as a biopesticide (Copping and Menn, 2000), although it has clearly insecticidal characteristics that differ from the majority of entomopathogen-based biopesticides (Cisneros et al., 2002a; Salgado, 1998). The interaction of Spinosad with entomopathogens has not been previously reported and the possibility of studying SfMNPV–Spinosad combinations was considered to be feasible because Spinosad displays no antifungal, antibacterial or antiviral activity (Bret et al., 1997).

At 70.3 OBs/mm^2 of diet surface (95% C.L.: 53.0–91.6 OBs/mm^2), the LC_{50} value calculated for SfMNPV was similar to the previously published values of 82 and 114 OBs/mm^2 estimated using identical procedures applied to second instar larvae (Cisneros et al., 2002b; Martínez et al., 2000). Likewise, at 2.98 ppm (95% C.L.: 2.25–4.06 ppm), the LC_{50} value calculated for second instar *S. frugiperda* exposed to Spinosad using the diet surface contamination technique was virtually identical to the 3 ppm value (95% C.L.: 1.10–6.60) for spinosyn A reported for *S. frugiperda* larvae of unspecified instar exposed by drench (Bret et al., 1997).

The mortality of larvae treated with SfMNPV mixed with the lowest concentration of Spinosad (0.05 ppm) tended to be less than that expected by independent action, i.e., a degree of antagonism was observed between these entities. In contrast, a weak degree of synergism was observed in the response of larvae exposed to SfMNPV + 3 ppm Spinosad, although the biological explanation for these interactions is currently unknown.

The pattern of insect mortality over time was of particular interest due to the marked differences in the speed of kill of SfMNPV and Spinosad. Standard probit or logit analyses are not applicable to concentration–mortality data from virus–insecticide mixtures, first, because the distribution of binomial responses does not usually follow logistic or Gaussian distributions due to differences in the mode of action and/or interactions between the virus and insecticide and second, because the numbers of responses of individuals from a treated group are related in time (Robertson and Preisler, 1992). Therefore, threshold tolerance analysis was employed to describe the cumulative response of insects to SfMNPV–Spinosad mixtures (McCutchen et al., 1997; Preisler et al., 1999). Virus concentration did not significantly affect time to death presumably because of the restricted range of concentrations used in the experiment. When a broad range of concentrations of virus are used, a negative relationship can be detected between virus concentration and the duration of insect survival (van Beek et al., 2000). The concentration of Spinosad, however, had a clear effect on the speed of kill producing a marked response in less than 48 h followed several days later by virus-induced mortality of those larvae that survived the Spinosad challenge.

The degree of control of *S. frugiperda* larvae was calculated from the reduction in larval recovery from treated plants (presumably due to Spinosad-induced mortality) and the mortality observed in the laboratory due to virus infection and parasitoid emergence. Compared to the virus alone, the degree of pest control was substantially improved by the incorporation of 3 ppm Spinosad into the virus formulation. The use of low concentrations of Spinosad alone may be an efficient means of controlling *S. frugiperda* in maize given that Spinosad is virtually non-toxic to humans and low concentrations of Spinosad had little impact on the abundance of insect natural enemies on maize plants.

At a cost of approximately US$560 per liter in Mexico, Spinosad is a relatively expensive product. The cost of applications involving low concentrations of Spinosad, however, would be just US$1.06 for treatment of 1 ha with 3 ppm Spinosad in 300 liters of water. Somewhat higher concentrations may be necessary to achieve commercially acceptable levels of pest control but preliminary tests indicate that excellent control of *S. frugiperda* is possible with application rates tenfold less than the product label recommendations (P. Tamez-Guerra and T. Williams, unpublished data).

In this study, as in previous studies, insect parasitoids contributed to *S. frugiperda* mortality to an important degree. The braconid egg-larval parasitoid *Chelonus insularis* appears to have a marked impact on *S. frugiperda* populations in Mesoamerica and the southern United States (Andrews, 1988; Ashley et al., 1982; Carrillo-Sánchez, 1993; Wheeler et al., 1989). Larvae infected by SfMNPV are generally unsuitable for reproduction of *C. insularis* as the virus kills the host before the parasitoid can develop and emerge (Escribano et al., 2000). The sensitivity of *C. insularis* to Spinosad has not been determined to date.

The need for detailed studies on the impact of Spinosad on natural enemies in the field has recently been underscored (Cisneros et al., 2002a). Spinosad applied at a concentration of 3 ppm had very little effect on the abundance of natural enemies or other arthropods on maize plants, indicating that the environmental impact of virus formulated with low concentrations of Spinosad is likely to be minimal compared to conventional chemical control measures. In contrast, application of the product label recommended concentration of 200 ppm Spinosad caused a decrease in the abundance of insect natural enemies and other arthropods similar to that observed in plants treated with chlorpyrifos. This was an unexpected result as laboratory bioassays with predators such as *Orius insidiosus* (Say), *Geocoris punctipes* (Say), *Chrysoperla rufilabris* (Burmeister), and the coccinelid *Hippodamia convergens* Guérin-Méneville report contact and/or ingestion LC_{50} values of >200 ppm Spinosad (Elzen, 2001; Schoonover and Larson, 1995).

Clearly, caution is required when making assumptions about pesticide impact on beneficial organisms based on toxicity data generated in laboratory studies (Stark et al., 1995). Contact bioassays of Spinosad at the recommended field rate caused 19–65% mortality in the pteromalid parasitoid *Catolaccus grandis* (Burks) whereas one-quarter of the recommended field rate completely inhibited parasitoid reproduction (Elzen et al., 2000). Moreover, adult braconid and ichneumonid parasitoids appear to be highly susceptible to topical applications of Spinosad whereas sensitivity to residues varies according to parasitoid species (Schneider et al., 2000; Tillman and Mulrooney, 2000). However, field tests involving multiple applications of Spinosad reported very little effect on the abundance of the predators *G. punctipes* and *H. convergens* and the parasitoid *Cotesia marginiventris* (Cresson) in cotton (Tillman and Mulrooney, 2000). In contrast, field tests of spray and phagostimulant formulations of Spinosad indicated that earwigs (*D. taeniatum*) were severely affected by concentrations $\geqslant 160$ ppm Spinosad (Cisneros et al., 2002a). The sensitivity of earwigs to Spinosad was confirmed in the present study. Evidently, the impact of Spinosad on insect natural enemies in the field is an issue that deserves the attention of integrated pest management practitioners.

It is interesting to note that Lepidoptera and spiders were not significantly affected by the application of Spinosad or chlorpyrifos at any sample point. In the case of lepidopteran larvae, this was probably because of their low abundance in the experimental plots. It is usually not necessary to apply insecticides against pest Lepidoptera in maize when natural enemy numbers are high (Castillejos et al., 2001). In the case of spiders (mostly Anyphaenidae and Gnaphosidae), the low impact of insecticide treatments was probably because these spiders almost exclusively inhabited the underside of the leaves closest to the ground and were often observed inside silken tents. As a consequence they probably had little exposure to insecticidal sprays.

In conclusion, the interaction of nucleopolyhedrovirus + Spinosad mixtures in *S. frugiperda* larvae was generally independent in nature, although weak synergism was detected in mixtures containing 3 ppm Spinosad + 20 or 70 OBs SfMNPV. At a cost of just one US dollar/ha, the addition of 3 ppm Spinosad to the virus formulation improved the degree of control of *S. frugiperda* in maize plots by 12–32% compared to application of SfMNPV alone. Application of 3 ppm Spinosad had very little effect on the abundance of insect natural enemies present on maize plants, whereas the application of the product label recommended rate of Spinosad (60 g a.i./ha) had effects similar to those observed following application of chlorpyrifos. The use of low concentrations of Spinosad merits further study as a means of controlling lepidopteran pests either alone or in combination with other entomopathogens.

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