Effects of Spinosad and Neem on the Efficacy of a Nucleopolyhedrovirus on Pickleworm Larvae[1]

D. Michael Jackson,[1] Martin Shapiro,[2] and B. Merle Shepard[2]

J. Agric. Urban Entomol. 30: 28–37 (2014)

ABSTRACT Formulations of neem (Neemix® 4.5) and spinosad (SpinTor® 2SC) were tested for their effects when mixed with the multicapsid nucleopolyhedrovirus virus (AgMNPV) from the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), for control of pickleworm larvae, *Diaphania nitidalis* (Stoll) (Lepidoptera: Pyralidae). In these experiments, neem had no additive effects in combination with AgMNPV. However, combinations of AgMNPV with spinosad as low as 1.0×10^{-6} of the original 22.8% suspension showed added efficacy against pickleworm larvae. These biorational insecticides are compatible with nucleopolyhedrovirues and may be useful in management of this primary pest of cucurbits.

KEY WORDS *Diaphania nitidalis*, AgMNPV, Neemix, azadirachtin, Spintor, spinosyn

Pickleworm, *Diaphania nitidalis* (Stoll) (Lepidoptera: Pyralidae), is a major pest of Cucurbitaceae (York 1992, Capinera 2001, 2008). Larvae are especially difficult to control because they typically feed within the fruits of various cucurbits, such as cucumber, *Cucumis sativus* L.; cantaloupe, *Cucumis melo* L.; pumpkin, *Cucurbita pepo* L.; and squash, *Cucurbita* spp. (Dupree et al. 1955, Reid & Cuthbert 1956, Elsey 1981, Capinera 2005). Pickling cucumbers are especially vulnerable because damaged fruits are unmarketable (Hughes et al. 1983, Zehnder et al. 1996). This pest can be one of the most important production problems for cucurbit growers in the eastern United States (Canerday & Dilbeck 1968, Gianessi & Williams 2011). Because of the low threshold for injury by pickleworm larvae (Hughes et al. 1983), cucurbit crops are often sprayed on a schedule. Thus, there is a need for biologically related pest management tools, including entomopathogens, for this important vegetable pest.

The baculoviruses (nucleopolyhedroviruses and granuloviruses) (Baculoviridae) are by far the most important group of the entomopathogenic viruses for insect pest management (Evans 2000). Baculoviruses are generally regarded as safe and effective bio-insecticides against a wide range of lepidopteran pests (Moscardi 1999, Lacey et al. 2001). Nucleopolyhedroviruses (NPV) are promising biological control agents for lepidopterous pests because of their specificity and safety (Burges & Jones 1986), but use of entomopathogenic viruses for vegetable pest management has been hampered by their relatively high cost, slow activity,

[1] Accepted for publication 1 July 2014.
[2] Corresponding Author, USDA, ARS, U.S. Vegetable Laboratory, 2700 Savannah Highway, Charleston South Carolina USA. E-mail: mike.jackson@ars.usda.gov
[3] Coastal Research and Education Center, Clemson University, Charleston South Carolina USA.

and susceptibility to UV degradation (Moscardi 1999). No naturally occurring baculoviuses have been reported from field-collected *D. nitidalis*; however, Jackson et al. (2008, 2009) showed that second instar pickleworms are susceptible to baculoviruses isolated from some other lepidopteran species. A multiply embedded nucleopolyhedrovirus (AgMNPV) from the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) (Carner & Turnipseed 1977), and a multiply embedded nucleopolyhedrovirus (AcMNPV) from the alfalfa looper, *Autographa californica* (Speyer) (Lepidoptera: Noctuidae) (Vail et al. 1972), were the most efficacious NPVs against second-instar pickleworms (Jackson et al. 2008). Five of six stilbene fluorescent (optical) brighteners significantly increased efficacy of AgMNPV against pickleworm larvae (Jackson et al. 2008). In addition, recent research has investigated the use of plant extracts as feeding stimulants, virus enhancers, and sunlight protectants to increase efficacy and persistence of NPV formulations against lepidopteran pests (Shapiro et al. 2007a,b, 2008, 2009a,b, 2012, Jackson et al. 2009, El Salamouny et al. 2009a,b).

Extracts from seeds of the neem tree, *Azadirachta indica* A. Juss. (Meliaceae), have been evaluated extensively for control of lepidopterous larvae. Neem and its active ingredient azadirachtin have antifeedant, growth regulatory, sterilant, and insecticidal properties (Schmutterer 1990, Mordue [Luntz] & Nisbet 2000). However, results with neem on many lepidopterous pests have been variable (Schmutterer 1990, Isman 2006), and in field trials, neem products have not always been efficacious against pickleworm larvae (Bavaresco 2007).

Spinosad, another bioinsecticide, is produced via fermentation from a naturally occurring soil Actinobacteria, *Saccharopolyspora spinosa* Mertz & Yao (Actinomycetales: Pseudonocardiaceae) (Mertz & Yao 1990). The insecticidal components of spinosad are spinosyn A and spinosyn D, which are typically applied as a mixture (Thompson et al. 2000, Anonymous 2013b). Spinosad is effective against a range of arthropod pests and is especially effective against Lepidoptera (Sparks et al. 1999). Spinosad has a low mammalian toxicology (Cleveland et al. 2001), and it has reduced activity against many beneficial insects (Thompson et al. 2000, Sparks et al. 2001, Méndez et al. 2002). Spinosad has been classified as a bioinsecticide (Copping & Menn 2000), and it has been certified for use for organic agriculture (Racke 2007). Spinosad and neem products are labeled for pickleworm control (Anonymous 2013a,b), and these materials have been recommended widely for organic production (Bradley 2007, Jackman 2008, Layton 2013, Zehnder 2013), home use (Anonymous 2013c, Layton 2013), and regular commercial production of cucurbits (Olson et al. 2010, Webb & Olson 2013).

Neem, or its active component, azadirachtin, has been evaluated as an enhancer of nucleopolyhedroviruses against several lepidopteran pests, including gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) (Shapiro et al. 1994, Cook et al. 1996), *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Murugan et al. 1999, Kumar et al. 2008, Wakil et al. 2012), *Spodoptera litura* F. (Lepidoptera: Noctuidae), *S. frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) (Senthil Nathan & Kalaivani 2005, 2006, Zamora-Avilés et al. 2013), and the cabbage butterfly, *Pieris brassicae* (L.) (Lepidoptera: Pieridae) (Bhandari et al. 2009). In these studies, NPV efficacy was generally increased in an additive fashion, indicating compatibility between the insecticidal properties of these viruses and neem (Shapiro et al. 1994, Senthil Nathan & Kalaivani 2005, Kumar et al. 2008). For example, Shapiro et al. (1994) reported that neem seed extract resulted in faster virus-caused mortality of gypsy moth larvae. Kumar et al. (2008) reported an additive interaction between azadirachtin and the NPV for *H. armigera*.

Spinosad also can increase the efficacy of NPVs against lepidopterous larvae. For example, Khattab (2007) reported increased efficacy of SpilNPV against the cotton leaf worm, *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae), in mixtures with spinosad. Méndez et al. (2002) found up to a 32% increase in control of *S. frugiperda* in field trials with maize using mixtures of SfMNPV and spinosad. However, the interaction between spinosad and an NPV can be complex and not necessarily additive (Méndez et al. 2002, Hesketh et al. 2009). The objective of the research described herein was to evaluate the efficacies of neem and spinosad in combination with the entomopathogenic virus AgMNPV against second instar pickleworms.

Materials and Methods

Pickleworm colony. Pickleworms used for bioassay studies were reared at the USDA, ARS, U.S. Vegetable Laboratory (USVL) in Charleston, SC, by methods modified from those of Elsey et al. (1984). Modifications included using a multi-purpose Lepidoptera diet (BioServe Product# F9772; wheat germ, soy flour base) (BioServe, Frenchtown, NJ), the elimination of plastic louvers in the rearing crispers, and layered instead of crumpled paper towels for pupation sites (Jackson et al. 1998). Larvae for bioassays were fed on the multi-purpose diet until the second instar when they were used for bioassays.

AgMNPV isolate. A Brazilian isolate of AgMNPV (G. R. Carner, Clemson University, Clemson, SC) came from diseased larvae collected in southern Brazil (Carner & Turnipseed 1977) and was produced in laboratory-reared *A. gemmatalis* by standard techniques (Fuxa & Richter 1999, Jackson et al. 2008, 2009). The formulation of AgMNPV for all tests was applied at a concentration of 1×10^7 viral occlusion bodies (OB) per dish (9-cm diam. × 1.5 cm deep) in one mL of distilled water.

Bioassays. Bioassays of pickleworm larvae were modified from published procedures (Shapiro & Farrar 2003) and were similar to the techniques we have used previously (Jackson et al. 2008, 2009). Bioassays were conducted using new 9–cm diameter sterile plastic Petri dishes that were discarded after one use. One mL of virus suspension, biorational insecticide (dilutions of neem or spinosad), or a mixture of AgMNPV and a biorational insecticide was pipetted onto the surface of the same multi-purpose diet (about 0.6 cm thick) used for colony rearing and allowed to air-dry in the laboratory. Sterile distilled water was pipetted onto the surface of the diet in the control treatments. Ten second instars of pickleworms from the laboratory colony were gently placed on the surface of the diet using soft forceps or a small brush. For each replication, there were five plates per treatment, and an equal number of untreated diet controls. Larvae were allowed to feed at ambient laboratory temperatures (22–24°C). Plates were examined several times over a two-week period, and the numbers of dead larvae, live larvae, prepupae, and pupae were recorded. Experiments were replicated three times (over time) with five dishes per treatment per replication. Thus, a total of 150 larvae were bioassayed for each treatment for each experiment.

For the first experiment, seven concentrations of neem (Neemix® 4.5, Certis USA, Columbia, MD) (Anonymous 2013a) and an untreated control were evaluated. Concentrations tested were the original suspension (OS) (4.5% azadirachtin), and dilutions of 0.1 [1×10^{-1}], 0.01 [1×10^{-2}], 0.001 [1×10^{-3}], 0.0001 [1×10^{-4}], 0.00001 [1×10^{-5}], 0.000001 [1×10^{-6}], and an untreated control (distilled water).

For the second experiment, thirteen treatments were evaluated. They were AgMNPV (1×10^{7} OB), neem concentrations of 0.01 [1×10^{-2}], 0.001 [1×10^{-3}], 0.0001 [1×10^{-4}], 0.00001 [1×10^{-5}], 0.000001 [1×10^{-6}] with and without the addition of the AgMNPV formulation, 1% Blankophor P167 with and without AgMNPV, and an untreated control (distilled water). Blankophor P167 is a diamino stilbene disulfonic acid derivative and serves as a fluorescent (optical) brightener that enhances the efficacy of AgMNPV against pickleworm larvae by protecting against ultraviolet radiation (Jackson et al. 2008).

For the third experiment, eight concentrations of spinosad (SpinTor® 2SC, Dow AgroSciences, Indianapolis, IN) (Anonymous 2013b) and an untreated control were evaluated. Spinosad concentrations tested were the original suspension (OS) (22.8% spinosad, a mixture of spinosyn A and spinosyn D), and dilutions of 0.01 [1×10^{-2}], 0.001 [1×10^{-3}], 0.0001 [1×10^{-4}], 0.0002 [2×10^{-5}], 0.00001 [1×10^{-5}], 0.00002 [2×10^{-6}], 0.000001 [1×10^{-6}], and an untreated control (distilled water).

For the fourth experiment, twelve treatments were evaluated. They were AgMNPV (10^{7} OB), spinosad concentrations of 0.00002 [2×10^{-5}], 0.00001 [1×10^{-5}], 0.000002 [2×10^{-6}], 0.000001 [1×10^{-6}] with and without the addition of the AgMNPV formulation, Blankophor P167 (1%) with and without AgMNPV, and an untreated control (distilled water).

Data analysis. Data from larval bioassays were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of the SAS system, version 9.1 (SAS 2009). When treatment effects were significant at the 5% level, means were separated using Tukey's Honestly Significant Difference Procedure (SAS 2009). The LD_{50} for neem and spinosad was determined by probit regression analysis (SAS 2009). Chi-square goodness-of-fit tests of observed and expected values (Steel & Torrie 1960) were conducted to determine whether there were additive or synergistic effects of neem or spinosad with AgMNPV.

Results and Discussion

Neem treatments alone at 1×10^{-4}, 1×10^{-5}, and 1×10^{-6} of the original suspension (OS) caused little mortality of pickleworm larvae (Table 1). However, significant mortality occurred at the 10^{-3} OS rate of neem-alone, and at the higher concentrations (OS and dilutions of 1×10^{-1} and 1×10^{-2} OS) there was 100% mortality of larvae tested (Table 1). Probit analysis indicated that the LD_{50} for neem-alone was 5.3×10^{-4} and 5.6×10^{-4} OS for experiments 1 and 2, respectively (Table 1). When neem was mixed with AgMNPV, there were no additive effects on pickleworm mortality at any rate (Table 1). Neem levels below 1×10^{-4} OS were ineffective when tested alone, and these levels also did not enhance AgMNPV efficacy. Conversely, the addition of AgMNPV to neem at 1×10^{-3} OS did not increase mortality above the 1×10^{-3} OS treatment alone (Table 1).

Table 1. Effect of neem and AgMNPV on mortality of second instar pickleworms in laboratory bioassays.

Treatment	Percent mortality	
	Exp. 1	Exp. 2
Neem original suspension [OS][a]	100.0 a[b]	----
Neem 1×10^{-1} OS	100.0 a	----
Neem 1×10^{-2} OS	100.0 a	100.0 a[b]
AgMNPV plus Blankophor P167 (1%)	----	71.0 ab
Neem 1×10^{-3} OS	69.0 b	62.0 b
AgMNPV plus neem 1×10^{-3} OS	----	63.5 b
AgMNPV plus neem 1×10^{-4} OS	----	37.0 c
AgMNPV alone	----	35.0 c
AgMNPV plus neem 1×10^{-5} OS	----	31.0 c
AgMNPV plus neem 1×10^{-6} OS	----	28.0 c
Blankophor P167 alone (1%)	----	0.0 d
Neem 1×10^{-4} OS	0.0 c	0.0 d
Neem 1×10^{-5} OS	0.0 c	0.0 d
Neem 1×10^{-6} OS	0.0 c	0.0 d
Untreated control	0.0 c	0.0 d

[a]Original neem concentration ca. 4.5%.
[b]Means (±SE) in the same column followed by a common letter are not significantly different ($P = 0.05$, Tukey's Honestly Significant Difference Procedure) (SAS 2009).

For experiment 3, spinosad-alone treatments as low as 1×10^{-6} OS caused larval mortality that differed significantly from the untreated control (Table 2). However, for experiment 4, significant mortality was not achieved until spinosad was applied at the 1×10^{-5} OS rate. Probit analysis indicated that the LD_{50} for spinosad-alone was 8.6×10^{-6} and 1.3×10^{-5} OS for experiments 3 and 4, respectively. Chi Square analysis indicated that there were significant additive effects when AgMNPV was mixed with spinosad. The AgMNPV-alone treatment caused 32% mortality, and spinosad-alone at the 1×10^{-5}, 2×10^{-6}, and 1×10^{-6} OS levels caused mortalities of 24%, 16%, and 10%, respectively (Table 2). However mixtures of AgMNPV with 1×10^{-5}, 2×10^{-6}, and 1×10^{-6} OS spinosad increased larval mortality additively to 78%, 52%, and 44%, respectively.

Although, several researchers have reported on the enhanced effects of neem (or azadirachtin) on nucleopolyhedroviruses for control of lepidopterous larvae (Shapiro et al. 1994, Cook et al. 1996, Murugan et al. 1999, Kumar et al. 2008, Senthil Nathan & Kalaivani 2005, 2006, Wakil et al. 2012, Zamora-Avilés et al. 2013), a similar result could not be demonstrated in this study. However, results similar to the present study have been reported for experiments with the tobacco budworm, *Heliothis virescens*, using a mixture of azadirachtin and HzSNPV (Koppenhofer & Kaya 2000). In addition, it is known that neem has anti-feedant properties (Schmutterer 1990), which could have affected results in these feeding bioassays. Furthermore, problems with pickleworm control using neem products in the field have been reported (Schmutterer 1990, Bavaresco 2007), and growers

Table 2. Effect of spinosad and AgMNPV on the mortality of second instar pickleworms after three days in laboratory bioassays.

	Percent mortality	
Treatment	Exp. 3	Exp. 4
Spinosad original suspension [OS][a]	100.0 a[b]	----
Spinosad 1×10^{-2} OS	100.0 a	----
Spinosad 1×10^{-3} OS	100.0 a	----
Spinosad 1×10^{-4} OS	94.3 a	100.0 a[b]
AgMNPV plus Blankophor P167 (1%)	----	80.0 a
AgMNPV plus Spinosad 1×10^{-5} OS	----	78.0 a
Spinosad 2×10^{-5} OS	74.8 ab	63.9 ab
AgMNPV plus Spinosad 2×10^{-6} OS	----	52.0 b
AgMNPV plus Spinosad 1×10^{-6} OS	----	44.0 b
AgMNPV alone	----	32.0 bc
Spinosad 1×10^{-5} OS	45.8 bc	24.0 c
Spinosad 2×10^{-6} OS	29.8 cd	16.0 cd
Spinosad 1×10^{-6} OS	22.0 d	10.0 d
Blankophor P167 alone (1%)	----	0.0 d
Untreated control	0.0 e	0.0 d

[a]Original spinosad concentration 22.8%.
[b]Means (±SE) in the same column followed by a common letter are not significantly different ($P = 0.05$, Tukey's Honestly Significant Difference Procedure) (SAS 2009).

are cautioned that neem is not the most efficacious product for pickleworm control.

Conversely, pickleworm control with spinosad has been quite successful. Over the past fifteen years, numerous reports in Arthropod Management Tests (ESA 2013) have reported on the high efficacy of spinosad for control of pickleworm larvae, and this material is widely recommended as a pickleworm management tool (Bradley 2007, Olson et al. 2010, Layton 2013, Webb & Olson 2013, Zehnder 2013). Unlike neem, spinosad increased AgMNPV efficacy in an additive fashion in the present study. Although these results are promising, mixtures of these materials should be evaluated under field conditions to affirm these additive effects.

The AgMNPV tested in these experiments did not originate from pickleworms (Lepidoptera: Pyralidae) but from the velvetbean caterpillar (Lepidoptera: Noctuidae); therefore, it is not surprising that the efficacy of this NPV to pickleworms is quite low (Jackson et al. 2008). However, through the addition of optical brighteners, UV protectants, or bio-insecticides, the efficacy of AgMNPV to pickleworm larvae may be elevated to a more practical level for pest management purposes.

Acknowledgments

We thank Louise Cauthen, Nan Lu, and Jennifer Cook for their technical assistance, and Howard F. Harrison, Jr. and Amnon Levi for reviewing this paper. Technical contribution No. 6287 of the Clemson University Experiment Station.

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