Available online at www.sciencedirect.com
ScienceDirect
Biological Control 40 (2007) 89–96
Biological Control
www.elsevier.com/locate/ybcon

Efficacy of optical brightener formulations of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) as a biological insecticide in greenhouses in southern Spain

Rodrigo Lasa [a], Carmen Ruiz-Portero [b], María D. Alcázar [c], José E. Belda [b], Primitivo Caballero [a], Trevor Williams [a,*]

[a] *Departamento de Producción Agraria, Universidad Pública de Navarra, 31006 Pamplona, Spain*
[b] *Departamento de Biología Aplicada, EPS, Universidad de Almería, 04120 Almería, Spain*
[c] *Unidad de Entomología, Laboratorio de Sanidad Vegetal, 04745 La Mojonera, Almería, Spain*

Received 21 April 2006; accepted 28 June 2006
Available online 12 July 2006

Abstract

The efficacy of optical brightener formulations of a native Spanish isolate of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) was determined for control of *S. exigua* on greenhouse grown sweet pepper (*Capsicum annuum*) in Almeria, Spain. In laboratory bioassays involving diet surface contamination, the 50% lethal concentration in fourth instars was reduced 25-fold and 5731-fold, compared to SeMNPV occlusion bodies (OBs) alone, or in mixtures with 0.1% and 1% of the stilbene-derived optical brightener leucophor AP, respectively. The efficacy of spray applications of 1×10^{12} OBs/Ha SeMNPV alone, or in mixtures with 0.1% leucophor AP, was tested in a greenhouse planted with sweet pepper. The prevalence of virus infection in larvae collected at intervals post-application and reared on artificial diet in the laboratory was very high (62–97% mortality). Compared to the treatment involving SeMNPV OBs alone, OBs applied in mixtures with leucophor AP resulted in a significant increase in the prevalence of infection in larvae collected at 2 days post-application, but not in insects collected subsequently. In contrast, a chemical insecticide treatment, lufenuron, performed poorly (<45% mortality). Persistence of OBs on leaf surfaces was examined by subjecting leaf samples to laboratory bioassay in second instar *S. exigua*. The reduction in OB activity over an 8 day period was greater in leaves sampled from the upper crop canopy compared to those from the lower part of the plant but persistence was not improved in the presence of leucophor AP. The plastic greenhouse structure reduced the intensity of incident UV-B (280–315 nm) readings by ~90% compared to external readings. Applications of leucophor AP did not adversely affect the growth of sweet pepper plants over a 14-day period. We conclude that SeMNPV should be adopted as a biological insecticide in greenhouses of this region.

Keywords: Baculovirus; Efficacy; Formulation; Greenhouse crops; Nucleopolyhedrovirus; Persistence; Sweet pepper

1. Introduction

Larvae of the beet armyworm, *Spodoptera exigua* (Hübner), cause serious damage in greenhouse crops in many parts of the world. The greenhouses of Almeria, in southern Spain cover some 28,000 ha and are the largest producers of protected crops in Europe. Horticultural production in this region involves an expenditure of over 6 million euros/year (US$7 million) on insecticides for control of *S. exigua*. The intensive use of chemicals for the control of this pest has resulted in high levels of resistance to virtually all commercial insecticides in many parts of the world, including Almeria (Belda, 1994; Brewer and Trumble, 1994; Mascarenhas et al., 1998; Moulton et al., 2002; Smagghe et al., 1997, 2003; Wang et al., 2006). Heavy use of insecticides also leads to a need for continual monitoring of pesticide residues in greenhouse produce (Garrido et al., 2004). Finally,

* Corresponding author. Fax: +34 948 169 732.
E-mail address: trevor.williams@unavarra.es (T. Williams).

1049-9644/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2006.06.015

the lack of effective biological agents for control of *S. exigua* means that broad-spectrum chemical insecticide use, targeted at *S. exigua* populations, represents a barrier to the effective deployment of natural enemies for the control of other important insect pests (whitefly, aphids, thrips, etc.) in greenhouses of the region. An effective means for the biological control of *S. exigua* is urgently required.

The use of *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) as a biological insecticide against *S. exigua* larvae has proved to be valuable in covered crops in northern Europe (Bianchi et al., 2001; Smits et al., 1987). This virus was first registered in the United States under the name Spod-X® and was thereafter registered in The Netherlands and other countries (Kolodny-Hirsch et al., 1997; Smits and Vlak, 1994). However, the insecticidal activity of Spod-X is adversely influenced by the presence of defective mutant genotypes (Muñoz et al., 1998), and laboratory studies indicate that Spanish populations of *S. exigua* are significantly more susceptible to a native Spanish isolate (named SeMNPV-SP2) than they are to Spod-X (Belda et al., 2000).

The insecticidal properties of baculoviruses can be improved by the use of formulations that include stilbene-derived optical brighteners. These compounds can increase susceptibility to NPV infection by disrupting the peritrophic membrane (Okuno et al., 2003; Wang and Granados, 2000) or inhibiting sloughing (Washburn et al., 1998) or virus-induced apoptosis of insect midgut cells (Dougherty et al., 2006). Laboratory studies on mixtures of SeMNPV and various optical brighteners have demonstrated increased pathogenicity, *sensu* Thomas and Elkinton (2004), compared to SeMNPV alone, especially in late instars (Hamm and Chandler, 1996; Murillo et al., 2003; Shapiro, 2000; Shapiro and Argauer, 2001), but field testing of such mixtures has not be reported. However, previous studies have indicated that the cost of these adjuvants (Martínez et al., 2000) and their influence on the growth of crops (Goulson et al., 2003) may give cause for concern.

Following application to plant surfaces, baculovirus occlusion bodies (OBs) are rapidly inactivated by solar ultraviolet (UV) radiation, particularly in the UV-B range of 280–320 nm (Killick, 1990; Morris, 1971). Stilbene optical brighteners can significantly improve OB persistence by absorbing UV radiation and re-emitting the energy as visible blue light (Dougherty et al., 1996; Shapiro, 1992; Shapiro and Farrar, 2003). However, in glasshouses, virus inactivation proceeds less rapidly than under field conditions due to filtering of UV radiation by the glass structure (Smits et al., 1987). The majority of greenhouses in Almeria are constructed of plastic sheets that also filter part of the UV spectrum. In addition, a whitewash treatment of the plastic applied during the summer months is likely to reduce further the intensity of UV radiation incident upon crop leaf surfaces.

The present study aimed to evaluate the efficacy of optical brightener formulations in greenhouse conditions of Almeria and to examine OB persistence and possible plant growth effects associated with the use of these adjuvants. For this, we selected the native Spanish SeMNPV-SP2 isolate and the optical brightener leucophor AP which is an inexpensive stilbene derivative that had been identified as a potent synergist of nucleopolyhedrovirus in a previous study (Martínez et al., 2003).

2. Materials and methods

2.1. Insect colony, virus strain and optical brightener

Spodoptera exigua larvae were obtained from a laboratory colony maintained at 25 ± 2 °C temperature, 70 ± 5 °C humidity and 16:8 light dark photoperiod in the Universidad Pública de Navarra, Spain. The wild type SeMNPV-SP2 used in this study, was isolated from infected *S. exigua* larvae during a viral epizootic in vegetable greenhouses in El Ejido, southern Spain (Caballero et al., 1992). This isolate comprises a mixture of at least four genotypes (Muñoz et al., 1999). SeMNPV was produced in fifth instar *S. exigua* larvae orally inoculated and reared on artificial diet until death. Virus-killed larvae were triturated and occlusion bodies (OBs) were purified as described previously (Muñoz et al., 1998). The OBs were suspended in distilled water, counted using a Neubauer improved chamber (Hawksley, Lancing, United Kingdom) and stored at 4 °C prior to use. The optical brightener leucophor AP (CAS Registry No. 68444-86-0), an anionic disulfonated stilbene brightener derivative with a molecular weight of 970.5 (Croma, Guipuzcoa, Spain), was obtained as a liquid and diluted to 0.1% and 1% (vol/vol) using distilled water for laboratory studies or tap water for greenhouse spray applications.

2.2. Laboratory activity of SeMNPV + leucophor AP mixtures

To quantify SeMNPV potentiation by two different concentrations of leucophor AP, laboratory bioassays were performed using a diet surface contamination technique described by Cisneros et al. (2002). A sterile plastic lid (102 × 102 × 4 mm) was filled with artificial diet to form a 2 mm thick layer. When solidified, a 1.0 ml volume of virus suspension was spread evenly using a hard loop over the diet surface (11,000 mm^2). Each bioassay involved five concentrations of virus estimated to result in 10–90% larval mortality. A plastic grid 100 × 100 mm divided into 25 squares, each with an internal area of 20 × 20 mm, was pressed into the diet to form 25 identical compartments, into each of which was placed a fourth instar *S. exigua*. The plastic grid was then covered with a filter paper and another lid. Larvae were held at 25 ± 1 °C, 70% RH in the dark and checked for mortality after 7 days. The bioassay was performed three times using 25 larvae per concentration. Both untreated controls and optical brightener control larvae (leucophor AP only) were treated identically. All treatments included 0.05% (vol/vol) wetter-sticker (Agral®, Syngenta Agro SA, Madrid, Spain) to reduce clumping of OBs. Results were subjected to logit regression with the

Generalized Linear Interactive Modeling (GLIM) program with a binomial error distribution specified (Numerical Algorithms Group, 1993).

2.3. Efficacy of formulations in greenhouse conditions

Experiments on the efficacy of optical brightener formulations of SeMNPV were conducted on sweet pepper (*Capsicum annuum* L.; Solanaceae) in greenhouses in Almeria during October 2003. The experiment involved four treatments: (i) control without virus, (ii) 1×10^{12} OBs/ha SeMNPV alone, (iii) 1×10^{12} OBs/ha SeMNPV + 1% leucophor AP, (iv) chemical insecticide at the label recommended rate of 0.05% lufenuron (Match 5 EC®, Syngenta). Each plot comprised 10 plants of 1.2 m height and planted at 1 m intervals with a 1 m space between rows, giving each plot an area of 10 m^2. Six replicate plots were marked for each treatment in a fully randomized design. A natural *S. exigua* larval infestation was augmented by releasing 20 laboratory-reared *S. exigua* second instars per plant. All applications were made using a compressed-air hand sprayer (Matabi 7®, Antzuola, Guipuzcoa, Spain) and all applications included 0.05% Agral wetter-sticker. All plots were treated with 750–800 ml of each spray treatment to run off, which is equivalent to the standard volume of spray application used in greenhouse crops in the region (500–1000 l/ha).

Between 12 and 25 *S. exigua* larvae were randomly collected by hand from plants in each plot at time point 0 (before application), and at 2, 5 and 8 days after application, and reared in the laboratory in 25 ml plastic cups with artificial diet at 25 °C until death or pupation. Virus mortality was registered daily. Percentage mortality was calculated for each treatment, normalized by arcsine transformation and subjected to repeat measures analysis of variance (ANOVA) in SPSS ver.12.0 (SPSS, Chicago, IL). The characteristics of the variance–covariance matrix were examined for this and all subsequent multivariate analyses by applying Mauchly's sphericity test (Stevens, 2001). The significance of treatment effects at each sample time were determined by within-subject pairwise comparisons among the estimated marginal means with Bonferroni correction.

2.4. Persistence of OBs on leaf surfaces

To evaluate the influence of optical brightener on the persistence of SeMNPV OBs on sweet pepper foliage, leaves were selected at random from the upper or lower halves of the plant at 1 h after application, and at 2, 5 and 8 days after spray application. Each sample involved six leaves randomly collected from each half of the plant among the 10 plants in each plot. Six replicate plots of each treatment were evaluated. These leaves were placed in labeled polythene bags, immediately frozen and stored at −20 °C until use. Apical leaves that appeared and expanded following spray application were not sampled to avoid errors from analyzing foliage that had not been treated with OBs. Similarly, leaves that were obviously contaminated by infected corpses of larvae were also rejected for sampling.

The density of viable OBs on leaf samples was estimated by bioassay. Batches of frozen leaves (6 leaves/sample) were triturated in a blender and 10 g (wet weight) were mixed with 40 g of artificial diet containing 0.1% leucophor AP. All bioassays included leucophor AP in the mixture to avoid the possible variation of activity caused by traces of the optical brightener residue that could be present on leucophor treated foliage. The mixture was agitated and spread in a Petri dish, allowed to solidify and fed to groups of 30 second instar *S. exigua*. Virus mortality was recorded 1 week after larvae had been transferred to the diet.

The relationship between the prevalence of mortality observed in the bioassay and the density of viable OBs on leaf surfaces was determined by calibration of the bioassay. A calibration curve was obtained by bioassay of 10 g samples of homogenized leaves, mixed with one of four different quantities of OBs (5×10^4, 5×10^5, 5×10^6, and 5×10^7 OBs), and stirred thoroughly into artificial diet containing 0.1% leucophor AP. Leaves for the calibration curve had been collected in untreated areas of the greenhouse and frozen at the same time as the experimental leaf samples. The relationship between foliar wet weight and surface area was established by weighing and measuring the surface of 20 groups of four randomly selected leaves. This was achieved using the image analysis program (WINDIAS, Delta-T devices Ltd, Cambridge, UK). The density of OBs on foliage could then be estimated by comparing the percentage mortality of virus treatments, in larvae that consumed diet + experimental leaf samples with the resulting calibration curve. Mortalities of larvae from each replicate were corrected for control mortality by applying Abbott's (1925) formula. Estimated OBs density were normalized by $\log_e$ transformation and subjected to repeat measures ANOVA in SPSS ver.12.0.

2.5. Optical brightener effects on plant growth

Four different concentrations of leucophor AP were evaluated to determine the effect of this product on plant growth. Sweet pepper plants were planted in plots of 1×1 m in an 800 m^2 greenhouse in the experimental center of "La Mojonera", Almeria, Spain. Spray applications were performed in July 2004. Leucophor AP was diluted in sterile water to obtain concentrations of 0.1%, 1.0% and 5.0% (vol/vol). A control treatment, with water alone, was used as the reference treatment. All applications included 0.05% Agral wetter-sticker. Thirty plants were individually sprayed with each concentration of leucophor AP using a compressed-air hand sprayer (Matabi 7®, Antzuola, Guipuzcoa, Spain) to run-off. Plant height, plant dry weight and the number of leaves per plant were determined at 14 days after spraying. Two plants were lost during the experiment. Results were subjected to multivariante ANOVA using SPSS ver.12.0.

2.6. Greenhouse UV measurements

The intensity of UV-B radiation was determined in the range 280–315 nm using a portable spectroradiometer (model IL1400A, International light Inc., Newburyport, MA) fitted with a solar blind vacuum photodiode detector (SEL240/SPS300/T/W) from the same manufacturer. Readings were taken at randomly selected points outside and inside commercial greenhouses of the Almeria region. All measurements were performed between 12 and 20 September 2005, on sunny, cloudless days. The following measurements were taken:

(a) Daily variation in solar UV-B radiation

The intensity of solar UV-B was measured simultaneously every hour from 1000 to 1800 h in the exterior and interior of five different commercial greenhouses to estimate the pattern of changes in UV-B intensity during the day. Internal greenhouse measures were taken at ground level in the central corridor of each greenhouse. External measurements were made at ground level at a distance of 3–4 m from the greenhouse door.

(b) Variation in UV-B intensity between greenhouses

UV-B radiation values were measured inside five different greenhouses in the experimental center "Las Palmerillas" in Almeria. Another 15 values were registered outside the greenhouses in direct sunlight at the same time. The greenhouses were unplanted at the time of measurement and differed in orientation and structure. Four of the five greenhouses were covered with a lifespan plastic film and the other covered with a polymethacrylate structure and a shade cloth. Measurements were taken between 1330 and 1430 h, corresponding to the maximum observed daily UV-B intensity. Spectroradiometer readings between greenhouses were subjected to ANOVA in SPSS ver.12.0.

(c) Variation in UV-B in different parts of the sweet pepper crop

Incident UV-B was measured for 20 randomly selected sweet pepper plants (1.6 m height) at four randomly selected points: (a) at the top of the plant canopy, (b) in the central part of the upper crop canopy, (c) in the central part of the lower crop canopy and (d) at soil level in the corridor between plants. Measurements were performed in four different commercial greenhouses between 1330 and 1400 h. Results were subjected to non-parametric Kruskal–Wallis test.

3. Results

3.1. Laboratory activity of SeMNPV + leucophor AP mixtures

No mortality was observed in untreated controls or in insects treated with optical brightener alone. LC_{50} values were reduced from 57.3 OBs/mm^2 diet surface for insects treated with SeMNPV alone to 2.29 and 0.01 OBs/mm^2 in the presence of 0.1% and 1% leucophor AP, respectively (Table 1). Concentration-mortality regression lines for SeMNPV alone and SeMNPV + leucophor AP could not be fitted in parallel, therefore, relative potency was calculated as the ratio of LC_{50} values (Robertson and Priesler, 1992). The potency of SeMNPV in fourth instars was increased 25 times and 5731 times in mixtures containing 0.1% and 1% leucophor AP, respectively.

3.2. Efficacy of formulations in greenhouse conditions

A total of 754 *S. exigua* larvae were recovered from greenhouse plots, all of which were third, fourth and fifth instars (Fig. 1). Average virus mortality of control larvae varied from 2.7% and 9.9% depending on time point, indicating the natural presence of SeMNPV in the insect population. Mortality in larvae collected from virus and

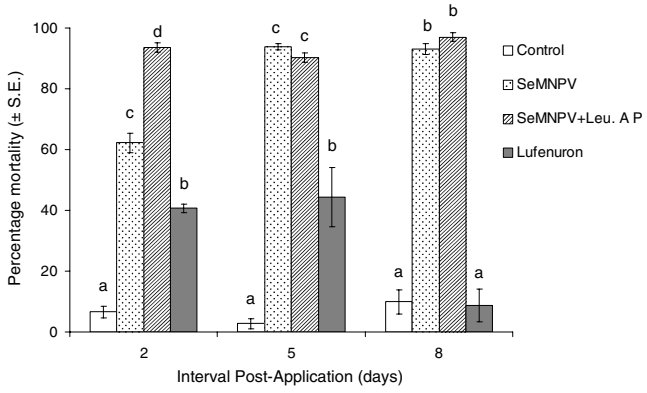

Fig. 1. Prevalence of virus mortality of *Spodoptera exigua* larvae collected from greenhouse sweet pepper plants at 2, 5 and 8 days after application and reared in the laboratory until death or pupation. Mortality of insects recovered from plants treated with lufenuron was not due to virus infection. Average (±SE) sample size in each collection of larvae was 75.0 ± 11.4 insects per treatment. Bars labeled with identical letters were not significantly different for comparisons between treatments within each sample (repeated measures ANOVA with intra-subject pairwise comparison of estimated marginal means, $P > 0.05$ Bonferroni correction).

Table 1
Logit regression analysis of concentration-mortality response of fourth instar *Spodoptera exigua* inoculated with SeMNPV alone and with leucophor AP by the diet surface contamination method

Treatment	Slope (±SE)	LC_{50} (OBs/mm^2)	Range of 95% CI	Relative potency[a]
SeMNPV	0.589 ± 0.08	57.31	43.92–76.59	1
SeMNPV + 0.1% Leucophor AP	0.948 ± 0.09	2.29	1.73–3.03	25.02
SeMNPV + 1% Leucophor AP	0.561 ± 0.12	0.01	0.004–0.019	5731

[a] Calculated as the ratio of LC_{50} values compared to SeMNPV OBs alone.

chemical treatments differed significantly from that of the control ($F_{3,19} = 54.1$, $P < 0.001$). Formulation of SeMNPV with 1% leucophor AP resulted in a significant increase in the prevalence of mortality in larvae collected at 2 days post-application and reared in the laboratory until death, compared to SeMNPV alone, whereas at 5 or 8 days post-application, SeMNPV alone, or in formulation with optical brightener, both resulted in ~90% mortality in insects collected and reared in the laboratory until death (treatment * time interaction $F_{6,38} = 3.09$, $P = 0.014$). In contrast, the insect growth regulator lufenuron performed poorly. This chemical insecticide applied at the recommended rate resulted in less than 45% mortality in insects reared in the laboratory, highlighting the problem of pest resistance to commercially available pesticides in this region.

3.3. Persistence of OBs on leaf surfaces

Virus mortality in control larvae fed mixtures of untreated leaves + diet varied from 0.1% to 11.2%, indicating a low presence of naturally occurring OBs. A logit regression ($y = 0.8130x - 12.07$) of larval mortality on the density of viable OBs (OBs/mm^2 leaf surface) was performed based on a total area (upper + lower leaf surfaces) of 82,500 mm^2 for the 10 g leaf samples used to calibrate the assay. The proportion of virus mortality observed in insects that fed on foliage + diet mixtures was inserted into the regression equation to estimate the number of viable OBs present on foliage samples, and thus, their density on a given area of foliage. The density of viable OBs immediately after spraying was estimated to be approximately 180–200 OBs/mm^2 and did not differ initially according to leaf location or formulation (Fig. 2A and B). Mortalities observed in larvae that fed on leaves sampled at 5 days post-application were equivalent to about 34 OBs/mm^2 in the upper crop canopy (Fig. 2A) and equivalent to 122 OBs/mm^2 in the lower crop canopy (Fig. 2B). The reduction in activity over time was greater in leaves sampled from the upper crop canopy compared to those from the lower part of the plant ($F_{1,20} = 7.26$, $P < 0.014$). Formulation of SeMNPV with optical brightener did not increase virus persistence compared with the virus alone ($F_{1,20} = 0.021$, $P = 0.885$). Eight days after application a small increase in the density of viable OBs was observed in upper canopy foliage that was probably due to contamination of leaves by OBs from infected cadavers that died on upper parts of the plant.

3.4. Optical brightener effects on plant growth

Mean (±SE) plant height varied from 30.5 ± 0.8 cm for plants treated with 1% leucophor AP to 32.6 ± 1.1 cm for plants treated with 0.1% leucophor AP. Mean plant biomass (dry weight) varied from 41.6 ± 3.4 g for plants treated with leucophor AP to 47.8 ± 3.6 g in the control plants. Mean number of leaves per plant varied from 37.5 ± 1.9 for

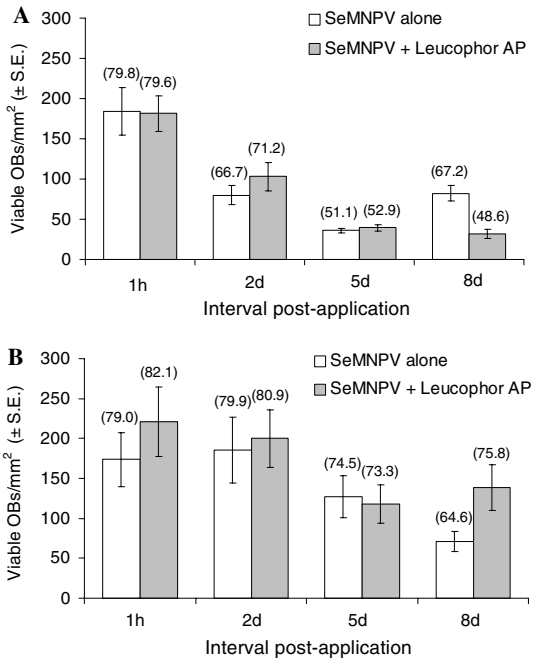

Fig. 2. Variation of the density of viable occlusion bodies (OBs) on leaf surfaces over time in leaves sampled from (A) upper crop canopy and (B) lower crop canopy. OB densities were estimated by a calibrated laboratory bioassay. Numbers in parenthesis above the bars indicate the percentage of virus mortality observed in each bioassay.

plants treated with 1% leucophor AP to 46.2 ± 3.3 for control plants. The application of different concentrations of leucophor AP (0.1%, 1.0% and 5.0%) on the foliar surface did not significantly affect sweet pepper plant height, biomass or number of leaves, measured at 14 days post-application, compared to control plants (MANOVA, Pillai's trace $F_{9,342} = 1.419$, $P = 0.18$).

3.5. Greenhouse UV measurements

The highest intensity of incident UV-B radiation (75–85 μW/cm^2) outside greenhouses was observed between 1300 and 1400 h each day that readings were taken (Fig. 3A). The intensity of UV-B radiation inside greenhouses during the same period was reduced by 75–95% of that of the external incident UV-B, with values in the range of 4–20 μW/cm^2 due to the filtering effect of the plastic sheeting.

A survey of five different greenhouse structures indicated that greenhouses differed significantly in UV-B transmission ($F_{4,70} = 366$, $P < 0.001$) with average (±SE) values in greenhouses constructed of polyethylene plastic films between 7 ± 0.24 and 17.8 ± 0.33 μW/cm^2, compared to 3.4 ± 0.33 μW/cm^2 in the greenhouse constructed of polymethacrylate (exterior UV-B intensity while readings were taken was 76.75 ± 3.81 μW/cm^2).

Significant differences were also observed in UV-B intensity in different parts of the crop (Kruskal-Wallis $H = 27.85$, df = 3, $P < 0.001$), despite significant differences between greenhouses (Kruskal-Wallis $H = 16.98$, df = 3, $P = 0.001$).

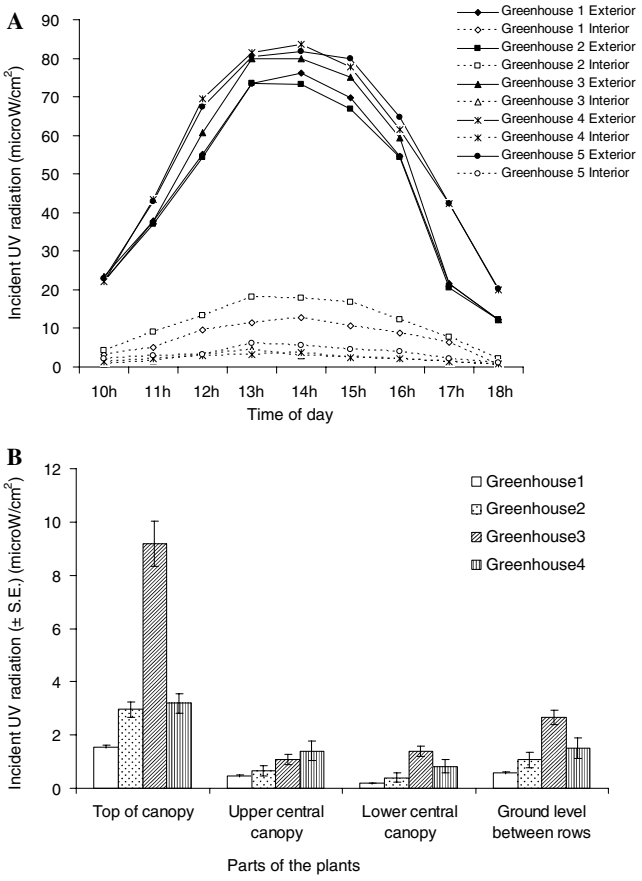

Fig. 3. Measurement of intensity of incident UV-B radiation. (A) Daily variation of incident UV-B radiation inside and outside greenhouses; (B) Intensity of UV radiation measurements in different part plants of sweet pepper crops in four different greenhouses in Almeria measured at 1330–1400 h.

Measurements taken in the upper crop canopy were always higher than observed in other parts of the plants (Fig. 3B). Summer whitewash treatment reduced UV-B penetration compared to the unwhitewashed greenhouses that were used to measure daily variation in UV (Fig. 3A) and the influence of plastic verses polymethacrylate structure.

4. Discussion

The present study aimed to determine the efficacy of SeMNPV alone and in mixtures with an optical brightener for control of *S. exigua* larvae in greenhouse conditions. The laboratory LC_{50} of SeMNPV in fourth instar *S. exigua* was reduced by 25-fold and 5731-fold when present in mixtures with 0.1% and 1% leucophor AP, respectively. A positive relationship between brightener concentration and the potentiation of nucelopolyhedrovirus pathogenicity has been established previously (Martínez et al., 2003; Shapiro and Argauer, 1997; Thorpe et al., 1999; Zou and Young, 1996). The degree of optical brightener potentiation also tends to be greater in late instars that are normally more resistant to baculovirus infection than their younger counterparts (Hamm, 1999). These results are consistent with those reported previously for *S. exigua* inoculated with SeMNPV in mixtures with Tinopal LPW (Hamm and Chandler, 1996; Murillo et al., 2003) or Blankophor HRS (Shapiro and Argauer, 2001), wherein LC_{50} values were reduced by between 144- and 876-fold depending on the brightener, host instar and method of inoculation.

Application of 1×10^{12} OBs/ha SeMNPV in greenhouses resulted in excellent control of *S. exigua* larvae, exceeding that reported in preliminary greenhouse trials in Spanish sweet pepper crops (Belda et al., 2000). Similar rates of application have proved to be highly effective for *S. exigua* control on tomato (Smits et al., 1987) and ornamentals (Young and McNew, 1994), whereas lower rates of application (3×10^{11} OBs/ha) have proved to be very efficient in chrysanthemum (Bianchi et al., 2000b) and other horticultural crops (Kolodny-Hirsch et al., 1997).

Compared to SeMNPV alone, application of SeMNPV in mixtures with 1% leucophor AP resulted in increased mortality only in those larvae collected at 2 days post-application and reared in the laboratory until death. This suggests that the optical brightener formulation increased the rate of acquisition of lethal infection compared to larvae that fed on foliage treated with OBs alone. By combining data from various studies involving the application of 5×10^{11} OBs/ha of SeMNPV on chrysanthemum, Smits (1986) estimated that virtually 100% of first to fourth instars acquired a lethal infection within 48 h of application, whereas about 80% of fifth instars became infected during the same period. Since fifth instars are much less sensitive to SeMNPV than early instars (Bianchi et al., 2000a; Smits and Vlak, 1988), applications against populations of *S. exigua* late instars results in poor control (Bianchi et al., 2002). Susceptibility to infection may also change over periods of 24 h or less, depending on the physiological conditions of the larvae (Teakle et al., 1986).

Optical brightener formulations appear to improve the rate of virus acquisition but whether this is reflected in corresponding improvements in the degree of crop defoliation remains to be determined. Evidently, the rate of acquisition of infection in baculovirus formulations with optical brighteners merits further study. In all cases, the prevalence of mortality in larvae from virus treatments was significantly greater than observed with the lufenuron chemical treatment, probably due to pest resistance.

About 90% of solar UV-B radiation is reflected or filtered by the plastic structure of the greenhouse. We therefore expected that the rate of inactivation of OBs applied inside greenhouses would be considerably less than would occur in unprotected field crops. Over 60% of the original virus activity remained on sweet pepper plants at 5 and 8 days after application. Similar results were observed following application of *Trichoplusia ni* nucleopolyhedrovirus in collard plants in glasshouse conditions: 63% of the original virus activity remained after 5 days in comparison with 32% on plants exposed to open field conditions and 83% on plants reared in a dark room (Jaques, 1967).

The persistence of SeMNPV OBs in greenhouse conditions was significantly affected by the intensity of UV radiation that reached each part of the crop (Fig. 3B). Killick and Warden (1991) reported that the shading of lodgepole pine, *Pinus cordata* (Loud.), foliage provided by upper branches decreased the overall UV exposure of virus deposits on many plant surfaces, especially in larger trees. Similarly, our UV-B measurements in sweet pepper revealed that virus persistence was significantly greater on the lower crop canopy than on the upper part of the plant. Other authors have reported differences when comparing virus persistence on the upper and lower leaf surface, as a direct consequence of the intensity of incident UV radiation (Peng et al., 1999; Yearian and Young, 1974; Young and Yearian, 1989). Plastics with selective UV transmission properties may also affect the population densities of other pests including whitefly, aphids and thrips in covered vegetable crops (Costa et al., 2002).

The contribution of other factors to the inactivation of baculoviruses on plants under field conditions appears to be insignificant when compared to sunlight (Young and Yearian, 1986). Few studies have been published on the effect of temperature on the persistence of virus on host plants. Laboratory tests suggest that temperatures of 40 °C or higher for extended periods inactivate baculoviruses. However, high temperatures may also increase the sensitivity of OBs to UV radiation (McLeod et al., 1977). Air temperatures in the greenhouses of Almeria frequently attain 40 °C at midday during the summer months, despite whitewash treatments and abundant ventilation, although we observed good persistence of OBs despite high air temperatures.

In conclusion, laboratory studies indicated that leucophor AP significantly reduced the quantity of OBs needed to cause a lethal infection in fourth instar *S. exigua*. The low cost of leucophor AP (~US$5/kg) may facilitate its use in SeMNPV formulations to improve the control of late instar larvae and increase the rate of acquisition of the infection, with corresponding benefits for the severity of crop damage by this pest on greenhouse crops. The optical brightener had no significant effects on plant growth following single applications at concentrations up to 5%. This agrees with the study of Goulson et al. (2003) who observed 25–40% reductions in the growth of monocots following two applications of optical brightener but no significant effects on the growth of dicotyledonous species. SeMNPV provided excellent control of *S. exigua* infestations in greenhouse sweet pepper crops in Almeria, whereas the insect growth regulator lufenuron performed poorly, probably because of pest resistance. We suggest that SeMNPV should be adopted as a biological insecticide in greenhouses of this region.

Acknowledgments

We thank Noelia Gorría for insect rearing and Jan van der Blom (COEXPHAL) for technical assistance. This study received financial support from MCyT project AGL2002-04320-C02-01 and an FPI fellowship to RL.

References

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267.

Belda, J.E. 1994. Biología, ecología y control de *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) en cultivo de pimiento en invernadero. Unpublished PhD thesis, Universidad de Granada, Spain.

Belda, J.E., Mirasol, E., Escribano, A., Rapallo, S., Caballero, P., 2000. Eficacia de un nucleopoliedrovirus (SeNPV) en el control de *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) en pimiento de invernadero. Bol. San. Veg. Plagas 26, 619–628.

Bianchi, F.J., Snoeijing, I., van der Werf, W., Mans, R.M., Smits, P.H., Vlak, J.M., 2000a. Biological activity of SeMNPV, AcMNPV, and three AcMNPV deletion mutants against *Spodoptera exigua* larvae (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 75, 28–35.

Bianchi, F.J.J.A., Joosten, N.N., Vlak, J.M., van der Werf, W., 2000b. Greenhouse evaluation of dose- and time-mortality relationships of two nucleopolyhedroviruses for the control of beet armyworm, *Spodoptera exigua*, on chrysanthemum. Biol. Control 19, 252–258.

Bianchi, F.J.J.A., Joosten, N.N., Vlak, J.M., van der Werf, W., 2001. The influence of greenhouse chrysanthemum on the interaction between the beet armyworm, *Spodoptera exigua*, and the baculovirus SeMNPV: parameter quantification for a process based simulation model. J. Appl. Entomol. 125, 557–562.

Bianchi, F.J.J.A., Vlak, J.M., van der Werf, W., 2002. Evaluation of the control of beet armyworm, *Spodoptera exigua*, with baculoviruses in greenhouses using a process-based simulation model. Biol. Control 24, 277–284.

Brewer, M.J., Trumble, J.T., 1994. Beet armyworm resistance to fenvalerate and methomyl-resistance variation and insecticide synergism. J. Agr. Entomol. 11, 291–300.

Caballero, P., Zuidema, D., Santiago-Alvarez, C., Vlak, J.M., 1992. Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. Biocontrol Sci. Technol. 2, 145–157.

Cisneros, J., Pérez, J.A., Penagos, D.I., Ruiz, J., Goulson, D., Caballero, P., Cave, R.D., Williams, T., 2002. Formulation of a nucleopolyhedrovirus with boric acid for control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in maize. Biol. Control 23, 87–95.

Costa, H.S., Robb, K.L., Wilen, C.A., 2002. Field trials measuring the effects of ultraviolet-absorbing greenhouse plastic films on insect populations. J. Econ. Entomol. 95, 113–120.

Dougherty, E.M., Guthrie, K.P., Shapiro, M., 1996. Optical brighteners provide baculovirus activity enhancement and UV radiation protection. Biol. Control 7, 71–74.

Dougherty, E.M., Narang, N., Loeb, M., Lynn, D.E., Shapiro, M., 2006. Fluorescent brightener inhibits apoptosis in baculovirus-infected gypsy moth larval midgut cells *in vitro*. Biocontrol Sci. Technol. 16, 157–168.

Garrido, A., Martínez, J.L., Lopéz, T., Cotés, S., Martinez, I., 2004. Monitoring multi-class pesticide residues in fresh fruits and vegetables by liquid chromatography with tandem mass spectrometry. J. Chomatogr. A 1048, 199–206.

Goulson, D., Derwent, L.C., Penagos, D.I., Williams, T., 2003. Effects of optical brighteners included in biopesticide formulations on the growth of crops. Agri. Ecosyst. Environ. 95, 235–240.

Hamm, J.J., 1999. Interactions in entomology: enhanced infectivity of entomopathogenic viruses by fluorescent brighteners. J. Entomol. Sci. 34, 8–16.

Hamm, J.J., Chandler, L.D., 1996. Effects of a nuclear polyhedrosis virus and a fluorescent brightener on colonies of *Spodoptera exigua* (Hübner). J. Entomol. Sci. 31, 355–362.

Jaques, R.P., 1967. Persistence of a nuclear polyhedrosis virus in habitat of host insect *Trichoplusia ni*. I. Polyhedra deposited on foliage. Can. Entomol. 99, 785–794.

Killick, H.J., 1990. Influence of droplet size, solar ultraviolet-light and protectants, and other factors on the efficacy of baculovirus sprays against *Panolis-Flammea* (Schiff) (Lepidoptera: Noctuidae). Crop Protect. 9, 21–28.

Killick, H.J., Warden, S.J., 1991. Ultraviolet penetration of pine trees and insect virus survival. Entomophaga 36, 87–94.

Kolodny-Hirsch, D.M., Sitchawat, T., Jansiri, T., Chenrchaivachirakul, A., Ketunuti, U., 1997. Field evaluation of a commercial formulation of the *Spodoptera exigua* (Lepidoptera: Noctuidae) nuclear polyhedrosis virus for control of beet armyworm on vegetable crops in Thailand. Biocontrol Sci. Technol. 7, 475–488.

Martínez, A.M., Goulson, D., Chapman, J.W., Caballero, P., Cave, R.D., Williams, T., 2000. Is it feasible to use optical brightener technology with a baculovirus bioinsecticide for resource-poor maize farmers in Mesoamerica? Biol. Control 17, 174–181.

Martínez, A.M., Simón, O., Williams, T., Caballero, P., 2003. Effect of optical brighteners on the insecticidal activity of a nucleopolyhedrovirus in three instars of *Spodoptera frugiperda*. Entomol. Exp. Appl. 109, 139–146.

Mascarenhas, V.J., Graves, J.B., Leonard, B.R., Burris, E., 1998. Susceptibility of field populations of beet armyworm (Lepidoptera: Noctuidae) to commercial and experimental insecticides. J. Econ. Entomol. 91, 827–833.

McLeod, P.J., Yearian, W.C., Young, S.Y., 1977. Inactivation of baculovirus *Heliothis* by UV irradiation, dew, and temperature. J. Invertebr. Pathol. 30, 237–241.

Morris, O.N., 1971. Effect of sunlight, ultraviolet and gamma radiations, and temperature on infectivity of a nuclear polyhedrosis virus. J. Invertebr. Pathol. 18, 292–294.

Moulton, J.K., Pepper, D.A., Jansson, R.K., Dennehy, T.J., 2002. Proactive management of beet armyworm (Lepidoptera: Noctuidae) resistance to tebufenozide and methoxyfenozide: baseline monitoring, risk assessment, and isolation of resistance. J. Econ. Entomol. 95, 414–424.

Muñoz, D., Castillejo, J.I., Caballero, P., 1998. Naturally occurring deletion mutants are parasitic genotypes in a wild-type nucleopolyhedrovirus population of *Spodoptera exigua*. Appl. Environ. Microbiol. 64, 4372–4377.

Muñoz, D., Murillo, R., Krell, P.J., Vlak, J.M., Caballero, P., 1999. Four genotypic variants of a *Spodoptera exigua* nucleopolyhedrovirus (Se-SP2) are distinguishable by a hypervariable genomic region. Virus Res. 59, 61–74.

Murillo, R., Lasa, R., Goulson, D., Williams, T., Muñoz, D., Caballero, P., 2003. Effect of Tinopal LPW on the insecticidal properties and genetic stability of the nucleopolyhedrovirus of *Spodoptera exigua* (Lepidoptera: Noctuidae). J. Econ. Entomol. 96, 1668–1674.

Numerical Algorithms Group, 1993. In: Francis, B., Green, M., Payne, C. (Eds.), The GLIM System: Release 4 Manual. Oxford University Press, Oxford, UK.

Okuno, S., Takatsuka, J., Nakai, M., Ototake, S., Masui, A., Kunimi, Y., 2003. Viral-enhancing activity of various stilbene-derived brighteners for a *Spodoptera litura* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. Biol. Control 26, 146–152.

Peng, F., Fuxa, J.R., Richter, A.R., Johnson, S.J., 1999. Effects of heat-sensitive agents, soil type, moisture, and leaf surface on persistence of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. Environ. Entomol. 28, 330–338.

Robertson, J.L., Priesler, H.K., 1992. Pesticide Bioassay with Arthropods. CRC Press, Boca Raton, FL, USA.

Shapiro, M., 1992. Use of optical brighteners as radiation protectants for Gypsy-moth (Lepidoptera, Lymantriidae) nuclear polyhedrosis-virus. J. Econ. Entomol. 85, 1682–1686.

Shapiro, M., 2000. Enhancement in activity of homologous and heterologous baculoviruses infectious to beet armyworm (Lepidoptera: Noctuidae) by an optical brightener. J. Econ. Entomol. 93, 572–576.

Shapiro, M., Argauer, R., 1997. Components of the stilbene optical brightener Tinopal LPW as enhancers for the gypsy moth (Lepidoptera: Lymantriidae) baculovirus. J. Econ. Entomol. 90, 899–904.

Shapiro, M., Argauer, R., 2001. Relative effectiveness of selected stilbene optical brighteners as enhancers of the beet armyworm (Lepidoptera: Noctuidae) nuclear polyhedrosis virus. J. Econ. Entomol. 94, 339–343.

Shapiro, M., Farrar, R.R., 2003. Fluorescent brighteners affect feeding rates of the corn earworm (Lepidoptera: Noctuidae) and act as enhancers and sunlight protectants for its nucleopolyhedrovirus. J. Entomol. Sci. 38, 286–299.

Smagghe, G., Auda, M., VanLaecke, K., Degheele, D., 1997. Significance of penetration and metabolism on topical toxicity of diflubenzuron in *Spodoptera littoralis* and *Spodoptera exigua*. Entomol. Exp. Appl. 82, 255–260.

Smagghe, G., Pineda, S., Carton, B., Del Estal, P., Budia, F., Vinuela, E., 2003. Toxicity and kinetics of methoxyfenozide in greenhouse-selected *Spodoptera exigua* (Lepidoptera: Noctuidae). Pest Manage. Sci. 59, 1203–1209.

Smits, P.H., 1986. Calculations on the polyhedra intake by beet armyworm larvae feeding in virus sprayed chrysanthemums. In: Samson, R.A., Peters, D., Vlak, J.M. (Eds.), Fundamental and Applied Aspects of Invertebrate Pathology. Veldhoven, Wageningen, Netherlands, pp. 616–619.

Smits, P.H., Vlak, J.M., 1988. Selection of nuclear polyhedrosis viruses as biological control agents of *Spodoptera exigua* (Lepidoptera: Noctuidae). Entomophaga 33, 299–308.

Smits, P.H., Vlak, J., 1994. Registration of the first viral insecticide in the Netherlands: the development of Spod-X based on *Spodoptera exigua* nuclear polyhedrosis virus. Med. Fac. Landbouw. Rijksuniv. Gent. 59/2A, 385–392.

Smits, P.H., van de Vrie, M., Vlak, J.M., 1987. Nuclear polyhedrosis virus for control of *Spodoptera exigua* larvae on glasshouse crops. Entomol. Exp. Appl. 43, 73–80.

Stevens, J., 2001. Applied Multivariate Statistics for the Social Sciences, fourth ed. Lawrence Erlbaum Associates, Inc., Mahway, NJ.

Teakle, R.E., Jensen, J.M., Giles, J.E., 1986. Age-related susceptibility of *Heliothis punctiger* to a commercial formulation of nuclear polyhedrosis-virus. J. Invertebr. Pathol. 47, 82–92.

Thomas, S.R., Elkinton, J.S., 2004. Pathogenicity and virulence. J. Invertebr. Pathol. 85, 146–151.

Thorpe, K.W., Cook, S.P., Webb, R.E., Podgwaite, J.D., Reardon, R.C., 1999. Aerial application of the viral enhancer Blankophor BBH with reduced rates of gypsy moth (Lepidoptera: Lymantriidae) nucleopolyhedrovirus. Biol. Control 16, 209–216.

Wang, P., Granados, R.R., 2000. Calcofluor disrupts the midgut defense system in insects. Insect Biochem. Mol. Biol. 30, 135–143.

Wang, W., Mo, J.C., Cheng, J.A., Zhuang, P.J., Tang, Z.H., 2006. Selection and characterization of spinosad resistance in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). Pestic. Biochem. Physiol. 84, 180–187.

Washburn, J.O., Kirkpatrick, B.A., Haas-Stapleton, E.J., Volkman, L.E., 1998. Evidence that the stilbene-derived optical brightener M2R enhances *Autographa californica* M nucleopolyhedrovirus infection in *Trichoplusia ni* and *Heliothis virescens* by preventing sloughing of infected epithelial cells. Biol. Control 11, 58–69.

Yearian, W.C., Young, S.Y., 1974. Persistence of *Heliothis* nuclear polyhedrosis-virus on cotton plant parts. Environ. Entomol. 3, 1035–1036.

Young, S.Y., McNew, R.W., 1994. Persistence and efficacy of four nuclear polyhedrosis viruses for corn earworm (Lepidoptera: Noctuidae) on heading grain-sorghum. J. Entomol. Sci. 29, 370–380.

Young, S.Y., Yearian, W.C., 1986. Formulation and application of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses: Volume II: Practical Application for Insect Control. CRC Press, Boca Raton, FL, pp. 157–179.

Young, S.Y., Yearian, W.C., 1989. Persistence and movement of nuclear polyhedrosis-virus on soybean plants after death of infected *Anticarsia-gemmatalis* (Lepidoptera: Noctuidae). Environ. Entomol. 18, 811–815.

Zou, Y.Q., Young, S.Y., 1996. Use of a fluorescent brightener to improve *Pseudoplusia includens* (Lepidoptera: Noctuidae) nuclear polyhedrosis virus activity in the laboratory and field. J. Econ. Entomol. 89, 92–96.