Research Article

Received: 6 May 2013 Revised: 17 July 2013 Accepted article published: 26 July 2013 Published online in Wiley Online Library: 22 August 2013

(wileyonlinelibrary.com) DOI 10.1002/ps.3617

Stage-specific insecticidal characteristics of a nucleopolyhedrovirus isolate from *Chrysodeixis chalcites* enhanced by optical brighteners

Alexandra Bernal,[a] Oihane Simón,[a] Trevor Williams[b] and Primitivo Caballero[a,c*]

Abstract

BACKGROUND: *Chrysodeixis chalcites* is a major noctuid pest of banana crops in the Canary Islands. The stage-specific susceptibility of this pest to *C. chalcites* single nucleopolyhedrovirus (ChchSNPV-TF1) was determined, as well as the effect of selected optical brighteners as enhancers of primary infection.

RESULTS: Susceptibility to ChchSNPV-TF1 occlusion bodies (OBs) decreased as larval stage increased; second instars (L_2) were 10 000-fold more susceptible than sixth instars (L_6). Virus speed of kill was 42 h faster in L_2 than in L_6. OB production increased in late instars; L_6 larvae produced 23-fold more OBs than L_4. Addition of 10 mg mL^{-1} Tinopal enhanced OB pathogenicity by 4.43- to 397-fold depending on instar, whereas 10 μL mL^{-1} Leucophor resulted in potentiation of OB pathogenicity from 1.46- to 143-fold. Mean time to death decreased by 14 to 26 h when larvae consumed OBs in mixtures with 10 mg mL^{-1} Tinopal, or 10 μL mL^{-1} Leucophor, although in these treatments OB yields were reduced by up to 8.5-fold (Tinopal) or up to 3.8-fold (Leucophor).

CONCLUSION: These results have clear applications for the use of ChchSNPV-TF1 as a biological insecticide in control programs against *C. chalcites* in the Canary Islands.

Supporting information may be found in the online version of this article.

Keywords: *Chrysodeixis chalcites*; nucleopolyhedrovirus; insecticidal activity; optical brighteners; pest management

1 INTRODUCTION

The golden twin spot tomato looper, *Chrysodeixis chalcites* (Esper) (Lepidoptera: Noctuidae), is an important polyphagous pest of crops including tobacco, tomato, cotton, crucifers, legumes, maize, soybean, potato, artichoke, cauliflower and ornamental crops.[1,2] In Spain, this pest has been responsible for major losses (> 30% of total production) in banana crops in the Canary Islands,[3] and in vegetable crops over a large area of greenhouses in Almeria, southern Spain.[4] Chemical-based control measures against this pest currently require multiple applications of insecticides that tend to increase production costs and can hamper the commercialization of products that may contain pesticide residues,[5] hence the need to assess alternative methods to control this pest.

Baculoviruses are promising control agents for a number of lepidopteran pests due to their favorable insecticidal properties, host specificity and outstanding safety record.[6] A number of baculoviruses are currently produced on a commercial scale and applied to large areas of crops, such as the nucleopolyhedrovirus of *Anticarsia gemmatalis* (AgMNPV) in Brazil.[7] Because of its high pathogenicity and virulence, a singly encapsidated strain of *C. chalcites* nucleopolyhedrovirus (ChchSNPV, family Baculoviridae, genus *Alphabaculovirus*) isolated from a single larva collected from banana crops in southern Tenerife (Canary Islands, Spain), called ChchSNPV-TF1, was selected from among other ChchSNPV strains from the Canary Islands and other regions including Almería or The Netherlands (Bernal A, unpublished). The pathogenicity and virulence of this strain is comparable with that of the most pathogenic and virulent baculoviruses currently commercialized as bioinsecticide products.[7,8]

To determine the likely efficacy of a virus pathogen as the basis for a bioinsecticide product, the susceptibility of the different pest instars to the virus strain should be determined. Host stage can

* *Correspondence to: Primitivo. Caballero, Dpto. Producción Agraria, Universidad Pública de Navarra, Campus Arrosadía s/n, 31006, Pamplona, Navarra, Spain. E-mail: pcm92@unavarra.es*

a *Instituto de Agrobiotecnología, CSIC-Gobierno de Navarra, Mutilva Baja, Spain*

b *Instituto de Ecología A.C., Xalapa, Veracruz, Mexico*

c *Dpto. Producción Agraria, Universidad Pública de Navarra, Pamplona, Spain*

affect the characteristics of the concentration–mortality response and survival time of virus infected hosts.[9,10] A mixture of larval stages of *C. chalcites* is likely to be present at any one time in the field due to overlapping pest generations, making the determination of instar-related pathogenicity and virulence of practical importance for optimal timing of virus insecticide applications.

Certain components of the formulation may increase the insecticidal activity of the pathogen. Optical brighteners can enhance insect susceptibility to virus infection by disrupting the peritrophic membrane[11,12] or by inhibiting the sloughing of infected midgut cells.[13] Previous studies have demonstrated that these compounds increase OB pathogenicity in laboratory bioassays[14,15] or when applied to crops.[16]

In the present study, the susceptibility of different *C. chalcites* instars to an NPV isolated from *C. chalcites* larvae, ChchSNPV-TF1, alone and in mixtures with selected optical brighteners, and the influence of these compounds on speed of kill and OB production characteristics, were determined. The results of these studies provide valuable information for the development of this virus as a biological control agent against *C. chalcites* in the Canary Islands.

2 MATERIAL AND METHODS

2.1 Insect source and viruses

Chrysodeixis chalcites larvae were obtained from a laboratory colony at the Universidad Pública de Navarra, Spain, that was established with pupae received from the Instituto Canario de Investigaciones Agrarias (ICIA), Tenerife, Spain, in 2007, and refreshed periodically with pupae from the Canary Islands. Larvae were reared at $25 \pm 1\ ^{\circ}C$, $70 \pm 5\%$ humidity, with a 16/8 h light/dark photoperiod, on a semisynthetic diet described by Greene *et al.*[17] Adults were fed with 300 mg mL^{-1} honey solution.

The ChchSNPV-TF1 strain used in this study was isolated from a single infected *C. chalcites* larva during a viral epizootic in banana crops in the Canary Islands (Bernal A, unpublished). OBs used in bioassays, were amplified in a single passage through fourth instars (L_4) of *C. chalcites*. For this, overnight starved larvae that had molted in the previous 12 h, were inoculated orally with an OB suspension (10^6 OBs mL^{-1}) and reared until death. OBs from virus-killed larvae were extracted and filtered through cheesecloth. OBs were washed twice with 1 mg mL^{-1} SDS and once with 0.1 mol L^{-1} NaCl and finally resuspended in double-distilled water. OB suspensions were quantified using an improved Neubauer hemocytometer (Hawksley, Lancing, UK) under phase contrast microscopy at × 400 and stored at $4\ ^{\circ}C$.

2.2 Virus identification

The identity of the virus was determined by sequencing amplicons generated using degenerate oligonucleotide primers.[18] DNA was extracted from purified OBs by releasing virions from 100 µL OB suspension (10^9 OBs mL^{-1}) by treatment with 100 µL 0.5 mol L^{-1} sodium carbonate, 50 µL 10% (w/v) SDS in a final volume of 500 µL and incubating for 10 min at $60\ ^{\circ}C$. Undissolved OBs and other debris were removed by low-speed centrifugation ($3800 \times g$, 5 min). The supernatant containing the virions was treated with 25 µL proteinase K (20 mg mL^{-1}) for 1 h at $50\ ^{\circ}C$. Viral DNA was extracted with saturated phenol–chloroform, subjected to alcohol precipitation and resuspended in 0.1× TE buffer (Tris-EDTA, pH 8). DNA concentration was estimated at A_{260}. PCR was performed followed standard procedures using the degenerate oligonucleotides for *polyhedrin*, *lef-8* and *lef-9* genes[18] and a High Fidelity Taq DNA Polymerase (Prime Star HS DNA polymerase, Takara, Japan). PCR amplifications were purified using a QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and purified products were cloned into pGEM-T Easy vector (Promega, Fitchburg, WI, USA). Nucleotide sequences were determined in an ABI PRISM 377 automated DNA sequencer (Sistemas Genómicos S.A., Valencia, Spain), employing standard M13 and M13 reverse primers. Finally, a BLAST search was performed using the NBCI database.

2.3 Susceptibility of *C. chalcites* instars to ChchSNPV-TF1 OBs

Bioassays were carried out on L_2, L_3, L_4, L_5 and L_6 instars of *C. chalcites* to determine instar-specific responses to ChchSNPV-TF1 OBs. The mean lethal concentration (LC_{50}), mean time to death (MTD) and OB production (OBs $larva^{-1}$) were determined following *per os* inoculation, carried out using the droplet-feeding method.[19]

Pre-molt *C. chalcites* larvae were starved for 8–12 h at $25 \pm 1\ ^{\circ}C$, visually checked to have molted to the correct instars, and then allowed to drink from an aqueous suspension containing 100 mg mL^{-1} sucrose, 0.01 mg mL^{-1} Fluorella blue and OBs at one of five different concentrations. These were 160, 800, 4×10^3, 2×10^4 and 1×10^5 OBs mL^{-1} for L_2; 320, 1.6×10^3, 8×10^3, 4×10^4 and 2×10^5 OBs mL^{-1} for L_3; 3.2×10^3, 1.6×10^4, 8×10^4, 4×10^5 and 2×10^6 OBs mL^{-1} for L_4, 3.2×10^4, 1.6×10^5, 8×10^5, 4×10^6 and 2×10^7 OBs mL^{-1} for L_5; 3.2×10^5, 1.6×10^6, 8×10^6, 4×10^7 and 2×10^8 OBs mL^{-1} for L_6. For all instars OB concentrations were obtained using fivefold dilution series from the highest to the lowest concentrations. These concentration ranges were previously determined to kill between 95 and 5% of the experimental insects in each instar. Larvae that ingested the suspension within 10 min were transferred to individual wells of a 25-well tissue culture plate with a semisynthetic diet plug. Bioassays with 25 larvae per virus concentration and 25 larvae as negative controls were performed three times. Larvae were reared at $25 \pm 1\ ^{\circ}C$, and larval mortality was recorded every 12 h until the insects had either died or pupated. Virus-induced mortality was subjected to logit analysis using the POLO-PC program.[20] Relative potencies were calculated as the ratio of effective concentrations relative to L_2 instars.[21]

Time mortality results were subjected to Weibull survival analysis using the Generalized Linear Interactive Modeling program GLIM 4.[22] OB concentrations used for the time mortality analysis were those that resulted in ~90% larval mortality namely: 5.13×10^4, 2.36×10^5, 5.56×10^6, 5.00×10^7 and 9.02×10^8 OBs mL^{-1} for L_2, L_3, L_4, L_5 and L_6, respectively. Bioassays with 25 larvae per treatment and 25 larvae as negative controls were performed three times. The time mortality distribution among the different instars was analyzed graphically. Larval mortality was recorded at 8 h intervals until the insects had either died or pupated. Only individuals that died from polyhedrosis disease, confirmed by the microscopic observation of OBs, were included in the analyses.

OB production was determined in L_4, L_5 and L_6. Larvae were inoculated with the OB concentration that resulted in ~90% larval mortality, as described in the time mortality study. Groups of 25 larvae were inoculated for each treatment and the whole study was performed three times. All the larvae that died of virus disease (minimum 50 larvae per virus treatment) were collected and stored at -20 °C until used for OB counting. For this, each larva was thawed, homogenized in 1 mL distilled water, and the number of OBs per larva was determined by counting in triplicate. The average values of the OB counts from each replicate were analyzed by ANOVA using the SPSS v12 program.

2.4 Selection of optical brighteners

The degree of enhancement of OB activity by optical brighteners depends on the host–pathogen system, the chemical composition of the optical brightener and the instar and concentration used.[15,23] To select the most effective optical brightener, preliminary tests were performed on L_2 and L_4 *C. chalcites* using a total of eight optical brighteners from three different chemical groups (Table 1). All compounds were dissolved in double-distilled water at a concentration of 10 mg mL^{-1} for powder brighteners (Tinopal UNPA-GX and Tinopal UNPA-GX free acid) or 10 μL mL^{-1} for liquid brighteners (Blankophor CLE, Leucophor AP, Leucophor SAC, Leucophor UO, Blankophor ER, Hostalux SN). A single OB concentration that corresponded to the LC_{50} for each instar: 1.45×10^3 and 1.95×10^5 OBs mL^{-1} for L_2 and L_4, respectively, was used to inoculate larvae by the droplet-feeding method. Bioassays with 25 larvae per treatment and 25 larvae as negative controls were performed three times. Larvae were reared at 25 ± 1 °C, and larval mortality was recorded every 12 h until the insects had either died or pupated. The results were analyzed by fitting generalized linear models with a binomial error structure specified in GLIM 4.[22]

2.5 Effects of Tinopal UNPA-GX and Leucophor UO on the insecticidal properties of ChchSNPV-TF1 OBs

Based on the results of preliminary tests, Leucophor UO and Tinopal UNPA-GX were selected to determine their effects on the insecticidal activity of ChchSNPV-TF1 OBs against the different instars of *C. chalcites*. Two different concentrations were tested; 1 and 10 mg mL^{-1} for Tinopal UNPA-GX or 1 and 10 μL mL^{-1} for Leucophor UO. Bioassays were performed on L_2, L_3, L_4, L_5 and L_6 using the droplet-feeding method. Batches of 25 larvae of each instar were starved for 8 to 12 h at 25 ± 1 °C and then allowed to drink from an aqueous suspension containing 100 mg mL^{-1} sucrose, 0.01 mg mL^{-1} Fluorella blue, optical brighteners at two different concentrations and OBs. An identical number of larvae were fed with identical solutions without OBs, as controls. For viral treatments alone, the concentrations for each instar were those used in susceptibility assays, whereas for the OB suspensions that included optical brighteners, the concentration ranges used were based on the following fivefold dilution series: 16, 80, 400, 2×10^3 and 1×10^4 OBs mL^{-1} for L_2; 32, 160, 800, 4×10^3 and 2×10^4 OBs mL^{-1} for L_3; 320, 1.6×10^3, $8x10^3$, 4×10^4 and 2×10^5 OBs mL^{-1} for L_4; 3.2×10^3, 1.6×10^4, 8×10^4, 4×10^5 and 2×10^6 OBs mL^{-1} for both L_5 and L_6. Each bioassay was performed three times. Larvae were reared at 25 ± 1 °C and larval mortality was recorded every 12 h until the insects had either died or pupated. Virus-induced mortality was subjected to logit analysis using the POLO-PC program.[20]

Time mortality, data subjected to Weibull survival analysis, was only performed for viral treatments including OBs and 10 mg mL^{-1} Tinopal UNPA-GX or 10 μL mL^{-1} Leucophor UO. The OB concentrations used for the time mortality analysis were those that resulted in ~90% larval mortality, that were the same concentrations described in the susceptibility assay for OB alone treatments. For OB suspensions containing 10 mg mL^{-1} Tinopal UNPA-GX the OB concentrations used were 7.50×10^3, 1.24×10^4, 9.58×10^4, 5.11×10^5 and 1.54×10^6 OBs mL^{-1}, for L_2, L_3, L_4, L_5 and L_6, respectively. In mixtures with 10 μL mL^{-1} Leucophor UO the concentrations that produced ~90% mortality were 3.32×10^4, 1.07×10^5, 5.67×10^5, 9.61×10^5 and 7.66×10^6 OBs mL^{-1}, for L_2, L_3, L_4, L_5 and L_6, respectively. Larval mortality was recorded every 8 h and only individuals that died from polyhedrosis disease, confirmed by the microscopic observation of OBs, were included in the analyses. Bioassays with 25 larvae per treatment and 25 larvae as negative controls were performed three times.

Finally, OB production in the three treatments; OBs alone, OBs in mixtures with 10 mg mL^{-1} Tinopal UNPA-GX and OBs in mixtures with 10 μL mL^{-1} Leucophor UO, was determined in L_4, L_5 and L_6 that died in the previous speed of kill assay. All the larvae that died of virus disease (minimum 20 larvae per virus treatment) were collected and stored at -20 °C until used for OB counting. Each larva was thawed, homogenized in 1 mL distilled water and the number of OBs larvae^{-1} was determined by counting in triplicate. The experiment was performed three times. The average OB counts from each replicate were normalized by logarithmic transformation prior to ANOVA using the SPSS v12 program.

3 RESULTS

3.1 The virus used in this study belongs to ChchNPV species

Sequence analysis revealed that the ChchSNPV-TF1 isolate from a *C. chalcites* larva in Tenerife is a variant of *Chrysodeixis chalcites* single nucleopolyhedrovirus (ChchSNPV). The amplified sequences had the highest identity with the ChchSNPV isolate from The Netherlands (Genbank accession number AY864330.1) (Fig. S1). The amplified *polyhedrin* gene sequence was 541 bp long with 99% identity (533/541 nt identical) to the Dutch ChchNPV isolate (Fig. S1A) and 98% identity with *Trichoplusia ni* NPV (range 532/541 nt identical). Amplification of the *lef-8* gene resulted in a product of 714 bp, with > 99% sequence identity with ChchNPV (722/725 nucleotides identical) (Fig. S1B) and 87% with TnSNPV (628/725

Table 1. Chemical composition of eight optical brightener from three different chemical groups

Chemical group: product	Chemical composition (% active component)	Supplier
Stilbene acid derivatives:		
Blankophor CLE	$C_{30}H_{20}N_6Na_2O_6S_2$ (91%)	Clariant, Barcelona, Spain
Leucophor AP	$C_{40}H_{42}N_{12}O_{10}S_{2.2}Na$ (90%)	Clariant, Barcelona, Spain
Leucophor SAC	$C_{48}H_{42}O_{24}S_6$ (90%)	Clariant, Barcelona, Spain
Leucophor UO	$C_{48}H_{42}O_{24}S_4$ (90%)	Clariant, Barcelona, Spain
Tinopal UNPA-GX	$C_{40}H_{44}N_{12}O_{10}S_2$ (90%)	Sigma Chemical Co, St. Louis, MO, USA
Tinopal UNPA-GX free acid	$C_{40}H_{42}N_{12}O_{10}S_2Na_2$ (90%)	Sigma Chemical Co, St. Louis, MO, USA
Styryl-benzenic derivative:		
Blankophor ER	$C_{24}H_{16}N_2$ (91%)	Clariant, Barcelona, Spain
Pyrazoline derivative:		
Hostalux SN	$C_{21}H_{26}ClN_3O_3S$ (90%)	Clariant, Barcelona, Spain

nucleotides identical). Finally, the PCR fragment for *lef-9* was 293 bp long with > 99% sequence identity with ChchSNPV (295/296 nucleotides identical) (Fig. S1C) and 92% with TnSNPV (272/296 nucleotides identical). These results confirmed that the TF1 isolate from the Canary Islands was indeed a variant of ChchSNPV.

3.2 Susceptibility of *C. chalcites* instars to ChchSNPV-TF1 OBs

Susceptibility to infection decreased with increasing larval instar. LC_{50} values were 1.45×10^3 OBs mL^{-1} for L_2, 1.48×10^4 OBs mL^{-1} for L_3, 1.95×10^5 OBs mL^{-1} for L_4, 1.80×10^6 OBs mL^{-1} for L_5 and 2.20×10^7 OBs mL^{-1} for L_6 (Table 2).

Speed of kill decreased with increasing larval instar (Weibull hazard function: $\alpha = 7.0698$). MTD values for L_2, L_3, L_4, L_5 and L_6 instars were 126, 137, 142, 150 and 168 h, respectively (Table 2). However, MTD values for L_3, L_4 and L_5 did not differ significantly from one another.

Mean OB production values also increased significantly with larval instar ($F_{(2,6)} = 226.553$, $P < 0.001$), with an average of 4.83×10^9 OBs larva^{-1} in L_4, 2.27×10^{10} OBs larva^{-1} in L_5 and 1.10×10^{11} OBs larva^{-1} in L_6 (Table 2).

3.3 Tinopal UNPA-GX and Leucophor UO were selected as the most effective enhancers of OB pathogenicity

Inoculation of L_2 and L_4 *C. chalcites* with OBs alone resulted in 38 and 39% mortality, respectively (Table 3). Among the different optical brighteners tested, only three resulted in a significant increase in the insecticidal activity of ChchSNPV-TF1 OBs against L_2 ($\chi^2 = 9.266$; d.f. = 8; $P < 0.001$). Inoculation of OBs in mixtures with Tinopal UNPA-GX, Tinopal UNPA-GX (free acid), and Leucophor UO resulted in 64, 62 and 59% mortality, respectively (Table 3). In L_4, all optical brighteners significantly enhanced OB activity with mortalities of 51–97%, with the exception of Blankophor ER. The most active optical brighteners in L_4 were the same as those that enhanced OB activity in L_2: Tinopal UNPA-GX (97% mortality), Tinopal UNPA-GX (free acid) (96% mortality) and Leucophor UO (89% mortality). Because Tinopal UNPA-GX and Tinopal UNPA-GX free acid were different forms of the same compound and produced the same enhancement, Tinopal UNPA-GX was selected with Leucophor UO (a cheaper compound) to study the potentiation effect on *C. chalcites* at two different concentrations; 1 and 10 mg mL^{-1} for Tinopal UNPA-GX or 1 and 10 μL mL^{-1} for Leucophor UO.

3.4 Tinopal UNPA-GX and Leucophor UO in mixtures with ChchSNPV-TF1 OBs increased OB pathogenicity and speed of kill but reduced OB production

The addition of 1 mg mL^{-1} Tinopal UNPA-GX or 1 μL mL^{-1} Leucophor UO to OB suspensions enhanced OB pathogenicity in all instars except L_2. By contrast, mixtures of OBs and optical brighteners at 10 mg mL^{-1} for Tinopal UNPA-GX or 10 μL mL^{-1} for Leucophor UO enhanced OB pathogenicity in all instars tested. Mixtures of OBs and 10 mg mL^{-1} Tinopal UNPA-GX enhanced OB pathogenicity from 4.43- to 397-fold for L_2 to L_6 instars, respectively, whereas 10 μL mL^{-1} Leucophor UO had a lower potentiation effect, from 1.46- to 143-fold for L_2 to L_6 instars, respectively. The potentiation effect was of a greater magnitude in the three later instars, L_4, L_5 and L_6 (Table 4).

MTD values were reduced in mixtures of OB and either of the optical brighteners. Specifically, OBs in mixtures with 10 mg mL^{-1} Tinopal UNPA-GX resulted in a reduction of 19–26 h for L_2 to L_6, respectively. Similarly OBs in mixtures with 10 μL mL^{-1} Leucophor UO resulted in a reduction of 14–16 h for L_2 to L_6, respectively, compared with that of the OBs alone (Weibull hazard function $\alpha = 6.4883$) (Fig. 1A).

The addition of 10 mg mL^{-1} Tinopal UNPA-GX or 10 μL mL^{-1} Leucophor UO significantly reduced the mean number of OBs per larva compared with OBs alone ($F_{(2,6)} = 134.966$, $P < 0.001$; $F_{(2,6)} = 221.249$, $P < 0.001$; and $F_{(2,6)} = 106.897$, $P < 0.001$ for L_4, L_5 and L_6, respectively). Mean OB yield was reduced by 1.95- to 8.45-fold for L_4 and L_6, respectively, with the addition of Tinopal UNPA-GX, whereas Leucophor UO reduced the total OB production 1.38- to 3.79-fold for L_4 to L_6, respectively, compared with OBs alone treatments. Tinopal UNPA-GX, which reduced MTD values more drastically, resulted in lower OB yields than Leucophor UO (ANOVA, Tukey $P < 0.001$) (Fig. 1B). The decrease in OB production was correlated with the increased speed of kill of the virus when inoculated in mixtures with optical brighteners.

4 DISCUSSION

Strategies for the biological control of *C. chalcites* in banana crops have been poorly explored to date. The control of *C. chalcites* currently presents a number of difficulties due to the low number of plant protection products authorized for this crop, the difficulty in the correct application of these compounds and an absence of commercial biological control products.[3] In the present study, larval susceptibility to ChchSNPV-TF1 OBs was determined across different instars and the effect of OB and

Table 2. LC_{50} values, relative potencies, mean time to death (MTD) and mean OB yield of ChchSNPV-TF1 in different instars of *C. chalcites*

Instar	LC_{50} (OB mL^{-1})	Relative potency	Fid. lim. (95%) Low	Fid. lim. (95%) High	MTD (h)	Fid. lim (95%) Low	Fid. lim (95%) High	Mean OB yield ($\times 10^9$ OBs larva^{-1})	Fid. lim. (95%) Low	Fid. lim. (95%) High
L_2	1.45×10^3	1	–	–	126a	123	132	–	–	–
L_3	1.48×10^4	0.098	0.06	0.18	137ab	129	142	–	–	–
L_4	1.95×10^5	0.007	0.004	0.014	142b	134	147	4.83a	3.60	6.05
L_5	1.80×10^6	0.001	0.0009	0.002	150bc	140	154	22.74b	10.54	34.93
L_6	2.20×10^7	0.0001	0.00009	0.0002	168c	153	170	110.38c	60.22	160.54

Logit regressions were fitted in POLO Plus.[20] A test for non-parallelism was not significant for all larval stages ($\chi^2 = 9.12$; d.f. = 4; $P = 0.058$) which allowed regressions to be fitted in parallel with a common slope of 0.891 ± 0.095. Relative potencies were calculated as the ratio of effective concentrations relative to L_2 instars.[21] MTD values were estimated by Weibull survival analysis (hazard function $\alpha = 7.0698$).[22] The mean OB production was analyzed by ANOVA using the SPSS v12 program, ($F_{(2,\ 6)} = 226.5$, $P < 0.001$; values followed by identical letters did not differ significantly, Tukey test $P > 0.05$).

Table 3. Mortality percentage in L_2 and L_4 *C. chalcites* instars following treatment with ChchSNPV-TF1 OBs alone or in mixtures with 10 mg mL^{-1} of powered optical brighteners or 10 μL mL^{-1} of liquid optical brighteners

Treatment	L_2 mortality (%)	*P*	L_4 mortality (%)	*P*
ChchSNPV-TF1 OBs alone	38	–	39	–
ChchSNPV-TF1 + Blankophor ER	38	> 0.05	51	> 0.05
ChchSNPV-TF1 + Tinopal UNPA-GX	64	< 0.05	97	< 0.001
ChchSNPV-TF1 + Tinopal UNPA-GX (free acid)	62	< 0.01	96	< 0.001
ChchSNPV-TF1 + Leucophor SAC	36	> 0.05	79	< 0.01
ChchSNPV-TF1 + Leucophor UO	59	< 0.05	89	< 0.001
ChchSNPV-TF1 Hostalux SN	43	> 0.05	83	< 0.001
ChchSNPV-TF1 + Leucophor AP	39	> 0.05	79	< 0.001
ChchSNPV-TF1 + Blankophor CLE	36	> 0.05	67	< 0.05

OB concentrations used for L_2 and L_4 instars were 1.45×10^3 and 1.95×10^5 OB mL^{-1}, respectively. The percentage of mortality of OBs alone was compared with that observed in mixtures with optical brightener in each instar. The results were analyzed using a generalized linear model with binomial error specified. No evidence of overdispersion was observed in the results (GLM).

Table 4. LC_{50} values, relative potencies and slope of different treatments; virus alone (TF1) and virus with Tinopal UNPA-GX sodium salt (TF1 + Tinopal) and Leucophor UO (TF1 + UO) among the different *C. chalcites* instars

Instar	Treatment	LC_{50} (OBsmL^{-1})	Relative potency	Fiducial limits (95%)		Slope	Standard error
				Low	High		
L_2	TF1 alone	1.45×10^3	1.00	–	–	0.83	0.09
	TF1 + 1 mg mL^{-1} Tinopal	2.71×10^3	0.54	0.26	1.12	0.74	0.09
	TF1 + 1 μL mL^{-1} UO	4.26×10^3	0.34	0.16	0.72	0.79	0.09
	TF1 + 10 mg mL^{-1} Tinopal	3.27×10^2	4.43	2.36	8.34	0.94	0.09
	TF1 + 10 μL mL^{-1} UO	9.95×10^2	1.46	0.76	2.80	0.84	0.09
L_3	TF1 alone	1.48×10^4	1.00	–	–	1.07	0.09
	TF1 + 1 mg mL^{-1} Tinopal	2.53×10^3	5.85	3.30	10.38	0.80	0.09
	TF1 + 1 μL mL^{-1} UO	6.82×10^3	2.17	1.16	4.07	.83	0.09
	TF1 + 10 mg mL^{-1} Tinopal	6.39×10^2	23.19	13.75	39.12	0.99	0.10
	TF1 + 10 μL mL^{-1} UO	2.48×10^3	5.96	3.35	10.66	0.78	0.08
L_4	TF1 alone	1.95×10^5	1.00	–	–	0.88	0.09
	TF1 + 1 mg mL^{-1} Tinopal	2.65×10^4	7.35	9.66	14.96	0.56	0.08
	TF1 + 1 μL mL^{-1} UO	5.04×10^4	3.87	4.27	9.89	0.60	0.08
	TF1 + 10 mg mL^{-1} Tinopal	2.98×10^3	65.43	52.58	110.27	0.85	0.09
	TF1 + 10 μL mL^{-1} UO	9.72×10^3	20.08	10.71	45.40	0.73	0.08
	TF1 alone	1.80×10^6	1.00	–	–	0.89	0.09
L_5	TF1 + 1 mg mL^{-1} Tinopal	1.29×10^5	13.98	8.12	24.08	0.94	0.09
	TF1 + 1 μL mL^{-1} UO	2.70×10^5	6.67	3.64	9.22	0.79	0.08
	TF1 + 10 mg mL^{-1} Tinopal	2.55×10^4	70.41	60.75	121.66	0.98	0.09
	TF1 + 10 μL mL^{-1} UO	4.47×10^4	40.21	26.39	50.12	0.96	0.09
	TF1 alone	2.20×10^7	1.00	–	–	0.80	0.08
L_6	TF1 + 1 mg mL^{-1} Tinopal	2.38×10^5	89.16	71.06	93.59	0.63	0.08
	TF1 + 1 μL mL^{-1} UO	3.61×10^5	59.84	26.87	68.88	0.60	0.08
	TF1 + 10 mg mL^{-1} Tinopal	5.36×10^4	397.13	283.32	750.54	0.70	0.08
	TF1 + 10 μL mL^{-1} UO	1.49×10^5	142.56	111.71	210.13	0.62	0.08

Logit regressions were fitted in POLO Plus.[20] Relative potencies were calculated as the ratio of effective concentrations relative to ChchSNPV-TF1 OBs alone.

optical brightener mixtures on the insecticide properties of this virus were determined.

Larvae were markedly less susceptible to infection with increasing instars as has been reported for other species of Lepidoptera.[24,25] This stage-related resistance to infection increases steadily with larval body weight in some species.[26] In many cases, the physiological basis for this process remains uncertain. Larvae are able to rid themselves of primary infection by sloughing off infected gut cells during the molt.[13,27] The peritrophic membrane (PM), is also a key barrier against infection by baculoviruses.[12,28,29] Larvae can resist infection by increasing the thickness of the PM, indicating that the PM is not only a passive physical barrier but can also be remodeled in response to gut infection.[28,30] Susceptibility to viral infection decreased through successive instars as the PM became progressively less permeable.[31]

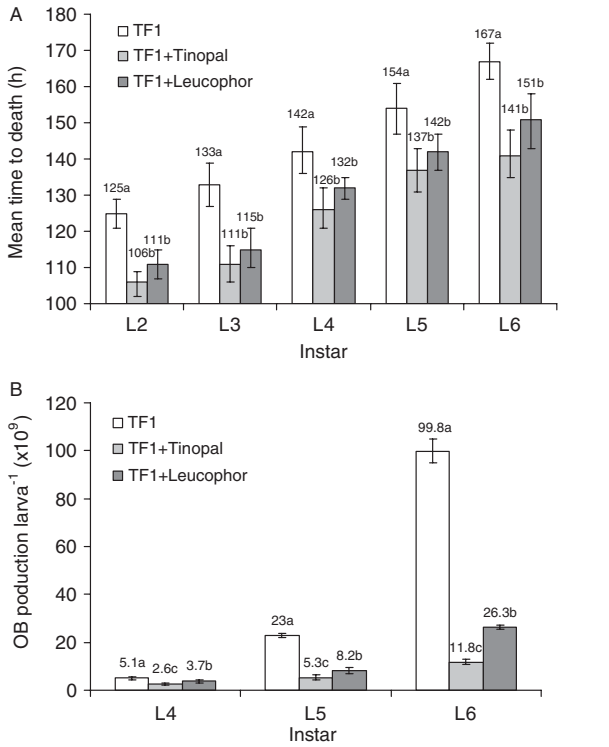

Figure 1. (A) Mean time to death of ChchSNPV-TF1 OBs alone (TF1), or in mixtures with 10 mg mL^{-1} Tinopal UNPA-GX (TF1 + Tinopal) or 10 μL mL^{-1} Leucophor UO (TF1 + UO) across *C. chalcites* instars. Values were estimated by Weibull analysis and are indicated above the bars. Values followed by identical letters did not differ significantly for treatment comparisons within each instar (*t*-test, $P > 0.05$). (B) Mean OB yield obtained after infection of *C. chalcites* larvae inoculated with ChchSNPV-TF1 OBs alone (TF1), or in mixtures with 10 mg mL^{-1} Tinopal UNPA-GX (TF1 + Tinopal) or 10 μL mL^{-1} Leucophor UO (TF1 + UO) in all *C. chalcites* instars. Values above the bars indicate means. Values followed by identical letters did not differ significantly (ANOVA, Tukey $P > 0.05$).

Under field conditions, effective crop protection is favored when all pest instars can be controlled following a single application of a viral insecticide, because in natural populations, overlap of larval generations may be common. Owing to the lower susceptibility of late instars, that are the principal cause of feeding damage in crops, the search for enhancer substances that improve OB insecticidal activity has attracted interest.[14] Certain substances, including optical brighteners, granulovirus enhancins, plant extracts and chitin-synthesis inhibitors can be effective at increasing the insecticidal efficacy of OBs.[12,32–34] Optical brighteners were first identified as viral protectants against inactivation by UV radiation.[35,36] Apart from their UV-protective activity, optical brighteners enhance OB potency and also allow the virus to replicate normally in semi-permissive species, and in resistant insect biotypes.[14,37,38] The efficacy of optical brighteners appears to involve a combination of different effects in the intestinal tract. Brighteners inhibit the sloughing of infected midgut cells[13] and inhibit the apoptotic response of midgut cells,[39] both of which increase the probability of establishment of primary infection in the midgut. Those compounds also inhibit chitin synthesis and dramatically increase PM porosity which facilitates movement of occlusion derived virions from the gut lumen to epithelial cells.[12,30]

Laboratory bioassays in L_2-L_6 confirmed previous observations that optical brighteners can enhance the potency of OBs and reduced larval survival time and OB production. Tinopal UNPA-GX and Leucophor UO were selected for detailed testing because both brighteners were effective in increasing OB potency in preliminary bioassays as observed in previous studies using other alphabaculoviruses.[11,15,16] Leucophor UO is chemically related to Tinopal UNPA-GX and was included for its lower cost. The degree of enhancement of OB potency observed in the present study in L_6 instars treated with Tinopal UNPA-GX was 397-fold, which is of similar magnitude to the 583-fold increase in potency reported in *S. exigua* L_5 treated with mixtures of *S. exigua* nucleopolyhedrovirus OBs and Tinopal UNPA-GX,[40] or the 360-fold increase in potency in *Lymantria dispar* L_2 treated with mixtures of nucleopolyhedrovirus (LdMNPV) and Tinopal LPW.[15] Wang and Granados[12] observed that treatment of *Trichoplusia ni* L_5 inoculated with 10 mg mL^{-1} Calcofluor resulted in complete disruption of PM formation in 2 h suggesting that optical brighteners require only a short time in the gut of the host insect to produce enhancing activity. However, this effect is quickly reversed, after 2 h of feeding on fresh diet a fully developed peritrophic membrane could be observed. However, following application of a brightener in the field, larvae will be feeding continuously on contaminated foliage, so the addition of optical brighteners to baculovirus formulations would likely prove very effective in potentiation of OB activity under field conditions. Formulation of OBs with brighteners could be particularly valuable in situations where it is necessary to control different larval stages simultaneously following application of a virus-based insecticide.

Increased speed of kill among the different larval instars exposed to an OB suspension in mixtures with optical brighteners has been reported in studies on homologous and heterologous alphabaculoviruses,[13,15,38] although other studies have reported no significant differences on speed of kill,[22,40,41] probably as a result of differences in the host-pathogen system under study and the chemical composition of the optical brightener.[11]

The reduction in OB yield observed in larvae inoculated with mixtures of ChchSNPV-TF1 OBs and optical brighteners is consistent with previous studies on SeMNPV[40] and reflects the trade-off between MTD and OB yield.[42] However, this effect is likely to be little consequence in the performance of a virus pesticide for which a high prevalence of lethal infection and rapid death resulting in improved crop protection are the main objectives.

Viral formulations with optical brighteners appear to offer a valuable means of improving the efficacy of ChchSNPV-TF1 as a potential biological insecticide. However, field trials will be required to support these laboratory results and to justify the additional cost of incorporating an optical brightener to the OB formulation.[43] However, the possible negative effects of optical brighteners on pollinators or crop-growth in the field should also be considered[44,45] before employing these substances in virus insecticide formulations on a large scale.

ACKNOWLEDGMENTS

We thank Noelia Gorria and Itxaso Ibáñez (UPNA) for technical assistance. This study received financial support from the Instituto Nacional de Investigaciones Agrarias (Project, RTA2010-00016-C2-02), from the Programa Nacional de España de I + D + i (Project AGL2008-05456-CO3-01/AGR), and from the Gobierno de Navarra (Project IIQ14065:RI1)

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 van de Veire M and Degheele D, Toxicity of the insect growth regulator cyromazine on the tomato looper *Chrysodeixis chalcites* (Esper) (Lep: Plusiinae). *Parasitica* **50**:131–133 (1994).
2 van Oers MM, Herniou EA, Usmany M, Messelink GJ and Vlak JM, Identification and characterization of a DNA photolyase-containing baculovirus from *Chrysodeixis chalcites*. *Virology* **330**:460–470 (2004).
3 Del Pino M, Carnero A, Cabello T and Hernández-Suárez E, La lagarta o bicho camello, *Chrysodeixis chalcites* (Esper, 1789), una plaga emergente en los cultivos de platanera de Canarias. *Phytoma* **225**:21–24 (2011).
4 Cabello T and Belda J, Noctuidos plaga (Lepidoptera: Noctuidae) en cultivos hortícolas en invernaderos, in *Sanidad Vegetal en la Horticultura Protegida*, ed. by Moreno R, Consejería de Agricultura y Pesca, Seville, Spain, pp. 179–211 (1994).
5 Perera S and Molina MJ, Plagas y enfermedades en el cultivo ecológico de la platanera, in *El cultivo ecológico de la platanera en Canarias*, ed. by Nogueroles C, Líbano J, Gabinete de Proyectos Agroecológicos S.L., Tenerife, Spain, pp. 77–118 (2007).
6 Szewczyk B, Hoyos-Carvajal L, Paluszek M, Skrzecz I and Souza ML, Baculoviruses re-emerging biopesticides. *Biotechnol Adv* **24**:143–160 (2006).
7 Moscardi F, Assessment of the application of baculoviruses for the control of Lepidoptera. *Annu Rev Entomol* **44**:257–289 (1999).
8 Caballero P, Murillo R, Muñoz D and Williams T, El nucleopoliedrovirus de *Spodoptera exigua* (Lepidoptera; Noctuidae) como bioplaguicida: análisis de avances recientes en España. *Rev Colomb Entomol* **35**:105–115 (2009).
9 Erlandson MA, Genetic variation in field populations of baculoviruses: mechanisms for generating variation and its potential role in baculovirus epizootiology. *Virol Sin* **24**:458–469 (2009).
10 Milks ML, Comparative biology and susceptibility of cabbage looper (Lepidoptera: Noctuidae) lines to a nuclear polyhedrosis virus. *Environ Entomol* **26**:839–848 (1997).
11 Okuno S, Takatsuka J, Nakai M, Ototake S, Masui A and Kunimi Y, Viral enhancing activity of various stilbene-derived brighteners for a *Spodoptera litura* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. *Biol Control* **26**:146–152 (2003).
12 Wang P and Granados RR, Calcofluor disrupts the midgut defense system in insects. *Insect Biochem Mol Biol* **30**:135–143 (2000).
13 Washburn JO, Kirkpatrick BA, Haas-Stapleton E and Volkman LE, Evidence that the stilbene-derived optical brightener M2R enhances *Autographa californica* M nucleopolyhedrovirus infection of *Trichoplusia ni* and *Heliothis virescens* by preventing sloughing of infected midgut epithelial cells. *Biol Control* **11**:58–69 (1998).
14 Shapiro M, Enhancement in activity of homologous and heterologous baculoviruses infectious to beet armyworm (Lepidoptera: Noctuidae) by an optical brightener. *J Econ Entomol* **93**:572–576 (2000).
15 Shapiro M and Shepard BM, Relative efficacies of Congo Red and Tinopal LPW on the activity of the gypsy moth (Lepidoptera: Lymantriidae), nucleopolyhedrovirus and cypovirus. *J Agric Urban Entomol* **25**:233–243 (2008).
16 Lasa R, Pagola I, Ibáñez I, Belda JE, Williams T and Caballero P, Efficacy of a *Spodoptera exigua* multiple nucleopolyhedrovirus as a biological insecticide for beet armyworm control in greenhouses of southern Spain. *Biocontrol Sci Technol* **17**:221–232 (2007).
17 Greene GL, Leppla NC and Dickerson WA, Velvetbean caterpillar: a rearing procedure and artificial medium. *J Econ Entomol* **69**:487–488 (1976).
18 Jehle JA, Lange M, Wang H, Hu Z, Wang Y and Hauschild R, Molecular identification and phylogenetic analysis of baculoviruses from Lepidoptera. *Virology* **346**:180–193 (2006).
19 Hughes PR and Wood HA, A synchronous per oral technique for the bioassay of insect viruses. *J Invertebr Pathol* **37**:154–159 (1981).
20 LeOra Software, *POLO-PC a user's guide to probit or logit analysis*, Berkeley, CA (1987).
21 Robertson JL, Russell RM, Preisler HK and Savin E, *Bioassays with Arthropods*, CRC Press, Boca Raton, FL (2007).
22 Crawley MJ, *GLIM for Ecologists*. Blackwell Science, Oxford (1993).
23 Martínez AM, Simón O, Williams T and Caballero P, Effect of optical brighteners on the insecticidal activity of a nucleopolyhedrovirus in three instars of *Spodoptera frugiperda*. *Entomol Exp Appl* **109**:139–146 (2003).
24 Asser-Kaiser S, Radtke P, El-Salamouny S, Winstanley D and Jehle JA, Baculovirus resistance in codling moth (*Cydia pomonella*) caused by early block virus replication. *Virology* **410**:360–367 (2011).
25 Kouassi LN, Tsuda K, Goto C, Mukawa S, Sakamaki Y and Nakamura M, Biological activity and identification of nucleopolyhedroviruses isolated from *Mythimna separata* and *Spodoptera litura* in Japan. *BioControl* **54**:537–548 (2009).
26 Myers JH, Cory JS, Ericsson JD and Tseng ML, The effect of food limitation on immunity factors and disease resistance in the western tent caterpillar. *Oecologia* **167**:647–655 (2011).
27 Rohrmann GF, Host resistance and susceptibility, in *Baculovirus Molecular Biology* ed. by Rohrmann GF, National Center for Biotechnology Information, Bethesda, MD, pp. 95–109 (2008).
28 Levy SM, Falleiros AMF, Moscardi F and Gregório EA, Susceptibility/resistance of *Anticarsia gemmatalis* larvae to its nucleopolyhedrovirus (AgMNPV): structural study of the peritrophic membrane. *J Invertebr Pathol* **96**:183–186 (2007).
29 Plymale R, Grove MJ, Cox-Foster D, Ostiguy N and Hoover K, Plant-mediated alteration of the peritrophic matrix and baculovirus infection in lepidopteran larvae. *J Insect Physiol* **54**:737–749 (2008).
30 Zhu R, Liu KY, Peng JX, Yang H and Hong HZ, Optical brightener M2R destroys the peritrophic membrane of *Spodoptera exigua* (Lepidoptera: Noctuidae) larvae. *Pest Manag Sci* **63**:296–300 (2007).
31 Lehane MJ, Peritrophic matrix structure and function. *Annu Rev Entomol* **42**:525–550 (1997).
32 Guo HF, Fang JC, Liu BS, Zhong WF and Wan FH, Enhancement of the biological activity of nucleopolyhedrovirus through disruption of the peritrophic matrix of insect larvae by chorflauzuron. *Pest Manag Sci* **63**:68–74 (2007).
33 Shapiro M, Salamouny SE, Sephard BM, Plant extracts as ultraviolet radiation protectants for the beet armyworm (Lepidoptera: Noctuidae) nucleopolyhedrovirus: screening of extracts. *J Agric Urban Entomol* **26**:47–61 (2010).
34 Toprak D, Harris S, Baldwin D, Theilmann D, Gillott C, Hegedus DD, *et al*, Role of enhancin in *Mamestra configurata* nucleopolyhedrovirus virulence: selective degradation of host peritrophic matrix proteins. *J Gen Virol* **93**:744–753 (2012).
35 Dougherty EM, Guthrie KP and Shapiro M, Optical brighteners provide baculovirus activity enhancement and UV radiation protection. *Biol Control* **7**:71–74 (1996).
36 Shapiro M, Use of optical brighteners as radiation protectants for gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. *J Econ Entomol* **85**:1682–1686 (1992).
37 Adams JR, Shepard CA, Shapiro M and Tompkins GJ, Light and electron microscopic investigations on the histopathology of the midgut of gipsy moth larvae infected with LdMNPV plus a fluorescent brightener. *J Invertebr Pathol* **64**:156–159 (1994).
38 Morales L, Moscardi F, Sosa-Gómez DR, Paro FE and Soldorio IL, Fluorescent brighteners improve *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) nucleopolyhedrovirus (AgMNPV) activity on AgMNPV-susceptible and resistant strains of the insect. *Biol Control* **20**:247–253 (2001).
39 Dougherty EM, Narang N, Loeb M, Lynn DE and Shapiro M, Fluorescent brightener inhibits apoptosis in baculovirus-infected gypsy moth larval midgut cells *in vitro*. *Biocontrol Sci Technol* **16**:157–168 (2006).
40 Murillo R, Muñoz D and Caballero P, Host range and biological activity of three *Spodoptera* nucleopolyhedrovirus genotypic variants and the effect of Tinopal LPW on the most active variant. *Int J Pest Manag* **49**:147–153 (2003).
41 Boughton AJ, Lewis LC and Bonning BC, Potential of *Agrotis ipsilon* nucleopolyhedrovirus for suppression of the black cutworm (Lepidoptera: Noctuidae) and effect of an optical brightener on virus efficacy. *J Econ Entomol* **94**:1045–1052 (2001).
42 Cory JS and Myers JH, Adaptation in an insect host-plant pathogen interaction. *Ecol Lett* **7**:632–639 (2004).
43 Martínez AM, Goulson D, Chapman JW, Caballero P, Cave RD and Williams T, Is it feasible to use optical brightener technology with a baculovirus bioinsecticide for resource-poor maize farmers in Mesoamerica?. *Biol Control* **17**:174–181 (2000).
44 Goulson D, Martínez AM, Hughes WOH and Williams T, Effects of optical brighteners used in biopesticide formulations on the behavior of pollinators. *Biol Control* **19**:232–236 (2000).
45 Goulson D, Derwent LC, Penagos DI and Williams T, Effects of optical brighteners included in biopesticide formulations on the growth of crops. *Agricult Ecosys Environ* **95**:235–240 (2003).

