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ORIGINAL PAPER

Can mixtures of horizontally and vertically transmitted nucleopolyhedrovirus genotypes be effective for biological control of *Spodoptera exigua*?

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Abstract Previous studies identified distinct genotypes of *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) that were associated with horizontal transmission (named HT-SeG25) or vertical transmission (named VT-SeAl1) in the host insect, *S. exigua* (Lepidoptera: Noctuidae). We examined the use of mixtures of occlusion bodies (OBs) of these genotypes as the basis for a virus preparation that could provide immediate pest control and establish a persistent sublethal infection in the survivors of an OB application for transgenerational pest suppression. Mixtures of HT-SeG25 + VT-SeAl1 comprising 25:75 or 75:25 % of each genotype, respectively, resulted in improved OB pathogenicity in terms of concentration-mortality metrics compared to OBs of VT-SeAl1 alone or similar values compared to OBs of the HT-SeG25 genotype alone. In contrast, no significant differences were observed in speed of kill or mean OB production per larva. Laboratory and greenhouse trials revealed that the prevalence of sublethal infection in adults that survived OB treatments in the larval stage increased with the proportion of VT-SeAl1 present in the inoculum, as determined by qPCR. Greenhouse trials indicated that the 75 % VT-SeAl1 + 25 % HT-SeG25 mixture was as effective as methoxyfenozide in preventing pest damage to pepper fruits. The potential contribution of vertically transmitted genotypes to transgenerational suppression of pest populations is discussed.

Keywords SeMNPV · Genotypes · Intergenerational transmission · Persistent infection · Laboratory and field trials · Insecticide efficacy

Key message

- Distinct genotypes of SeMNPV are associated with horizontal or vertical routes of transmission.
- Virus occlusion bodies involving a mixture of 75 % of vertically transmitted +25 % of horizontally transmitted genotypes were as effective as methoxyfenozide for protection against *Spodoptera exigua* feeding damage to pepper fruits.
- Novel combinations of horizontally and vertically transmitted genotypes in an alphabaculovirus-based insecticide could provide immediate pest control and contribute to transgenerational control of *S. exigua* larval populations.

Introduction

Natural populations of baculoviruses tend to be highly diverse, particularly in the case of the lepidopteran nucleopolyhedroviruses in the genus *Alphabaculovirus* (Erlandson 2009). Alphabaculovirus diversity can vary across geographical regions (Cory and Myers 2003; Ogembo et al. 2007; Williams et al. 2011), or more locally (Bernal et al. 2013a; Murillo et al. 2007), and even within isolates

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obtained from individual diseased insects (Cory et al. 2005; Hodgson et al. 2001; Redman et al. 2010). The diversity of alphabaculovirus populations is transmitted and maintained because the emergent phenotypic traits that arise from genetic diversity favor the survival of the pathogen (Simón et al. 2012a, b). Similarly, the value of retaining genetic diversity (Cory and Franklin 2012), or modifying genetic diversity present in virus-based biological control products (Arrizubieta et al. 2015; Bernal et al. 2013b), is becoming increasingly recognized. Recent studies involving the cloning of genotypes from highly pathogenic natural isolates, followed by the characterization of novel genotype mixtures that have been co-occluded within virus occlusion bodies (OBs), have resulted in the identification of unique combinations with marked improvements in OB pathogenicity or speed of kill characteristics (Arrizubieta et al. 2015; Bernal et al. 2013b).

Natural insect populations frequently harbor covert baculovirus infections that are not lethal and which do not result in the characteristic signs and symptoms of lethal polyhedrosis disease (Burand et al. 2011; Kemp et al. 2011; Kouassi et al. 2009). These insects can develop to adulthood, reproduce, and transmit the infection vertically to their offspring (Burden et al. 2003; Vilaplana et al. 2010; Virto et al. 2014). As such, vertical transmission has been suggested as a pathogen survival strategy when opportunities for horizontal transmission are highly restricted (Cory and Myers 2003). However, both transmission routes may coexist despite apparently opposing strategies of pathogen virulence and host survival. Specifically, horizontal transmission is favored following massive systemic virus replication and death of the host, whereas vertical transmission requires low levels of virus replication that permit the survival and efficient reproduction of the insect host (Cory 2015).

The *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) is a highly pathogenic host-specific alphabaculovirus that has been used as the basis of biological insecticides for control of the Beet armyworm, *S. exigua* (Lepidoptera: Noctuidae) (Jiang et al. 2004; Kolodny-Hirsch et al. 1997; Smits and Vlak 1988, 1994; Sun 2015). A SeMNPV-based insecticide has been developed for use on large extensions (>40,000 Ha) of greenhouse grown horticultural crops in Almería, southern Spain (Lasa et al. 2007). Field-caught adults of *S. exigua* collected in this region harbored persistent SeMNPV infections that were transmitted to their offspring, some of which subsequently died of polyhedrosis disease during laboratory rearing (Cabodevilla et al. 2011a). Sampling and analysis of distinct genotypes isolated from Almería (Spain) revealed marked differences in their insecticidal phenotypes and their tendency to produce covert, sublethal infections in their hosts. These genotypes were associated with different routes of transmission (Cabodevilla et al. 2011a). Genotypes isolated from soil samples were assumed to originate from larvae that had died of polyhedrosis disease and would likely be transmitted horizontally (HT). In contrast, genotypes isolated from the laboratory-reared progeny of field-caught moths were assumed to have been transmitted vertically (VT). In general, VT genotypes were capable of producing a high prevalence of persistent infection in adults that survived an inoculum challenge during the larval stage. HT genotypes tended to have higher OB pathogenicity and faster speed of kill compared to VT genotypes (Cabodevilla et al. 2011a).

Following the application of baculovirus-based insecticides a portion of the pest population often survives to adulthood and may reproduce and lay eggs on the same, or a nearby crop. During this period, the original inoculum applied to the crop is rapidly inactivated by solar ultraviolet radiation and diluted by rainfall and the growth of the plants, leaving little inoculum available to infect and control the following generation of pest larvae (Ignoffo 1992; Sun et al. 2004). Given this scenario, and based on previous observations that genotype interactions can produce unexpected phenotypic characteristics in genotype mixtures, we performed the present study using mixtures of OBs of different genotypes to explore interactions between genotypes that employ differing strategies of transmission. Specifically, we examined the possibility of using mixtures of genotypes to provide immediate pest control by HT genotypes combined with transgenerational control through VT genotypes, which could reduce the need for repeated insecticide applications against the target pest. For this, three mixtures of SeMNPV OBs, comprising different proportions of VT and HT genotypes, were evaluated for their insecticidal properties in laboratory studies and subsequently for their efficacy as crop protection agents in field trials.

Materials and methods

Insects and viruses

The *Spodoptera exigua* insects used in the study were obtained from a virus-free laboratory culture started using pupae obtained from Andermatt Biocontrol AG (Grossdietwil, Switzerland) and were maintained in the insectary facilities of the Universidad Pública de Navarra, Pamplona, Spain. Insects were reared on semisynthetic diet (Elvira et al. 2010) at 25 ± 1 °C, 50 ± 5 % relative humidity and 16:8 h light: dark photoperiod. Two SeMNPV genotypes were used to produce OB mixtures. The SeMNPV-A11 genotype (abbreviated to VT-SeA11) was isolated from the offspring of field-caught adults, which died spontaneously

when reared individually on semisynthetic diet. As these larvae had never been intentionally exposed to virus, the infection was assumed to pass from parents to offspring (Cabodevilla et al. 2011a). The SeMNPV-G25 genotype (abbreviated to HT-SeG25) was originally isolated from soils in the horticultural area of Almeria, Spain (Murillo et al. 2007). Both genomes of both genotypes have been sequenced recently and examined at the genomic level (Theze et al. 2014). OBs of each genotype were produced by inoculating fourth instar *S. exigua* larvae with a concentration of 5×10^7 OBs/ml of each genotype, using the droplet-feeding method (Hughes and Wood 1981). Larvae that drank the suspension within 10 min were reared individually on semisynthetic diet until death. The identity of viral progeny was verified by comparison of *Bgl*II restriction endonuclease profiles from newly produced OBs with that derived from inoculum OBs (Fig. 1). Virus-killed cadavers were homogenized in sterile distilled water and filtered through muslin. The resulting OB suspensions were counted in triplicate using a Neubauer hemocytometer under a phase-contrast microscope.

To produce experimental inocula, OBs suspensions were prepared for each genotype in equal concentrations and mixed in one of the following three genotypic proportions: 75 % VT-SeAl1 + 25 % HT-SeG25 (named 75:25), 50 % VT-SeAl1 + 50 % of HT-SeG25 (named 50:50), and 25 % of VT-SeAl1 + 75 % HT-SeG25 (named 25:75).

Insecticidal properties of mixtures of OBs

To examine the influence of possible interactions between genotypes on the insecticidal properties of each mixture, compared to those of the individual component genotypes (VT-SeAl1, HT-SeG25), bioassays were performed to determine inoculum pathogenicity, speed of kill, OB production and the prevalence of sublethal infection in surviving insects. Pathogenicity was estimated in terms of concentration-mortality response using the droplet-feeding method. For this, groups of 24 first instar larvae were allowed to molt overnight without food and then, as second instars, were allowed to drink one of the following five OB concentrations which were the same for each of the virus treatments: 2.45×10^5, 8.1×10^4, 2.7×10^4, 9×10^3, and 3×10^3 OBs/ml in 10 % sucrose solution with 0.001 % Fluorella blue food color. Another group of 24 larvae was mocked-infected as a control. Larvae that ingested the suspension within 10 min were individually transferred to 24-compartment plates (Corning, New York, USA), in which each well contained a piece of semisynthetic diet and reared at 25 ± 2 °C and 50 ± 5 % RH in darkness. Treated larvae were monitored for 7 days post-

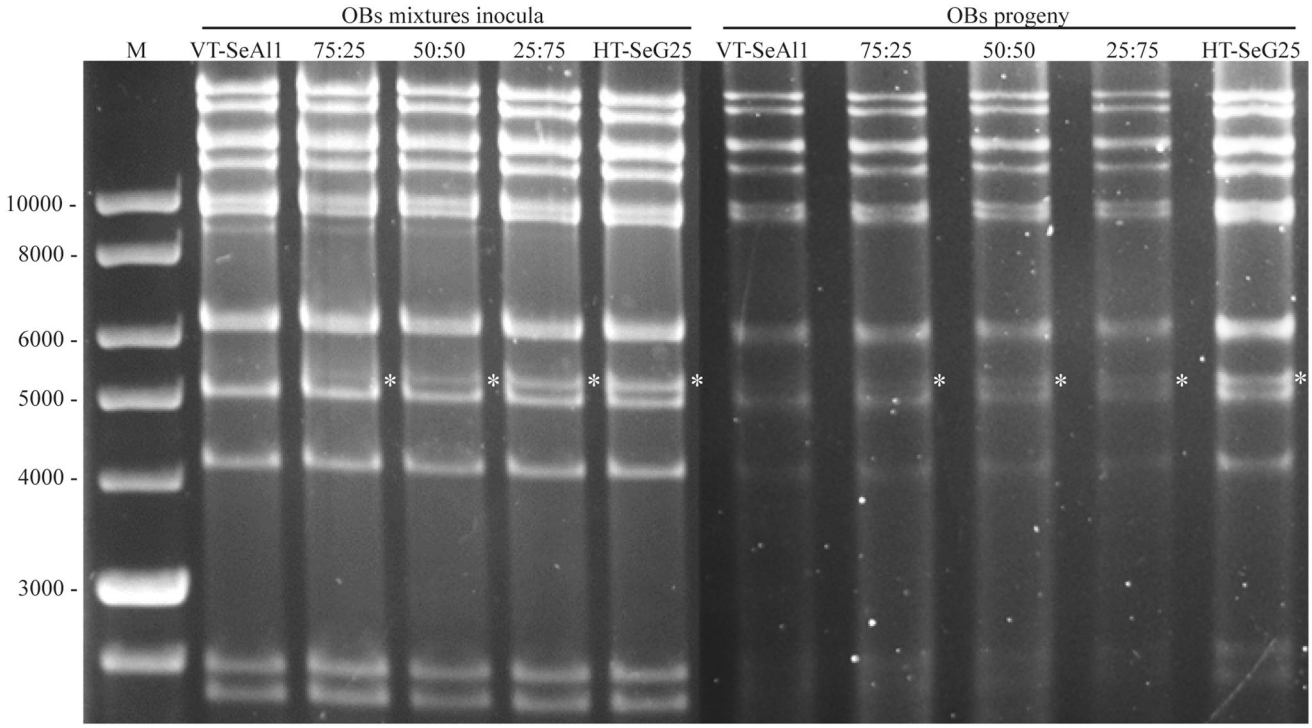


Fig. 1 Genomic DNA digested with *Bgl*II from OBs used as inoculum and OBs progeny from larvae dosed with VT-SeAl1, 75:25, 50:50, 25:75, and HT-SeG25 treatments at bioassay. *Asterisks* indicate the marker fragment characteristic for HT-SeG25 genotype. A 1-Kb DNA ladder (Bioline) was used as a molecular size marker (M)

inoculation for signs of infection and virus-induced mortality. The bioassay was performed on three occasions. Virus-induced mortality results were subjected to logit analysis using the GLIM 4 program (Generalized Linear Interactive Modeling, Numerical Algorithms Group, Oxford, UK) with a binomial error structure specified (Crawley 1993). The interaction between VT-SeAl1 OBs and HT-SeG25 OBs was evaluated using the formula of Tabashnik (1992):

$$\mathrm{LC}_{50}(R_{\mathrm{VT-SeAl1}} : R_{\mathrm{HT-SeG25}}) = \left(\frac{R_{\mathrm{VT-SeAl1}}}{\mathrm{LC}_{50}(\mathrm{VT-SeAl1})} + \frac{R_{\mathrm{HT-SeG25}}}{\mathrm{LC}_{50}(\mathrm{HT-SeG25})}\right)^{-1},$$

where $R_{\mathrm{VT\text{-}SeAl1}}$ and $R_{\mathrm{HT\text{-}SeG25}}$ are the relative proportions of each genotype in the mixture and LC_{50}(VT-SeAl1) and LC_{50}(HT-SeG25) indicate the 50 % lethal concentration values of each genotype alone. Synergistic or antagonistic interactions between genotypes would result in a change in the effectiveness of the mixture, resulting in a lower or higher LC_{50} value than predicted by Tabashnik's formula, respectively.

To determine speed of kill and OB production, groups of larvae were treated with a single concentration of OBs that resulted in ~90 % mortality based on previous studies (Cabodevilla et al. 2011a). For this, groups of 24 recently molted fourth instar larvae were allowed to drink a suspension of 5×10^7 OBs/ml for each treatment: 75:25, 50:50, 25:75, or OBs of each single genotype alone, as described above. A group of 24 larvae was treated identically using a solution without OBs as a control. Three replicates of the experiment were performed. Virus mortality was registered at 8 h intervals for 5 days (Cabodevilla et al. 2011a). Time-mortality data were subjected to Weibull survival analysis in GLIM. The validity of the Weibull model was determined by comparing fitted values with Kaplan–Meier survival function estimated values (Crawley 1993).

Virus-killed larvae were individually collected and stored at −20 °C until used for OB counting. To quantify the number of OBs in each insect, 10 virus-killed cadavers were randomly selected from each treatment, thawed, and individually homogenized in 1 ml of distilled water. OB production was estimated by counting triplicate samples of diluted OB suspension using a Neubauer hemocytometer under a phase-contrast microscope. OB production values could not be normalized by transformation and were analyzed by Kruskal–Wallis and Mann–Whitney U tests in SPSS v19.0.

Covert infection in survivors of an inoculum challenge

The prevalence of sublethal infection in the adult survivors of larvae that consumed OB inoculum in the fourth instar was quantified by quantitative PCR (qPCR). For this, groups of 48 recently molted larvae were allowed to drink a suspension of 9×10^3 OBs/ml of one of five treatments: 75:25, 50:50, 25:75, and the VT-SeAl1 or HT-SeG25 genotype OBs alone. This OB concentration was previously estimated to result in ~50 % mortality in fourth instar larvae (Cabodevilla et al. 2011a). Another group of 48 larvae were treated with sucrose solution and Fluorella blue as mock-infected controls. Inoculated larvae were placed individually in 15 ml plastic cups with semisynthetic diet and reared at 25 ± 2 °C and 50 ± 5 % RH in darkness and checked daily until death or adult emergence. Within 24-h post-emergence, adult survivors were frozen at −80 °C prior to qPCR analysis. The whole experiment was independently performed three times.

The prevalence of sublethal infection in adult survivors was quantified by qPCR as described previously (Virto et al. 2013). For this, total DNA was extracted from the abdomen of adults using Master Pure complete DNA purification kit (Epicentre Biotechnologies) and was diluted in 20 µl sterile Milli-Q water. Control samples and blank extraction samples containing water alone were processed in parallel to detect cross-contamination during the extraction. qPCR was performed using SYBR green fluorescence in 96-well reaction plates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Specific primers were designed to target a conserved sequence in the *DNA polymerase* gene of VT-SeAl1 and HT-SeG25 genotypes (Theze et al. 2014). The qPCR reactions were performed in a total volume of 10 µl, containing 5 µl of SYBR green, 0.4 µl of both forward and reverse primers (10 µM), and 1 µl of DNA template. The protocol conditions involved an initial denaturation at 95 °C for 30 s, followed by 45 amplification cycles of 95 °C for 5 s, 60 °C for 30 s, and a dissociation stage of 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s, as described previously (Virto et al. 2013). The critical Cq value for detection of sublethal infections was previously established as 33 cycles, so Cq values higher than this value were classified as negative samples. All qPCR procedures were performed using filter pipette tips in a qPCR hood.

Virus mortality was subjected to analysis of variance (ANOVA) in SPSS v19.0. The prevalence of covert infection in adult survivors was analyzed by fitting generalized linear models in GLIM 4 with a binomial error distribution specified and virus treatment as a factor. The percentages of infected insects were normally distributed and were subjected to *t* test for pairwise comparison. Mean viral load in each covertly infected adult insect was estimated, and values were subjected to analysis of variance (ANOVA) using SPSS v19.0.

Efficacy of single genotype OBs on pepper plants

The effects of genotypes and OB application rate on larval mortality were evaluated in a small greenhouse trial in May 2012. Groups of nine sweet pepper plants (*Capsicum annuum* var. Melchor), 1.5 m in height, planted in plastic tubs were taken to each of 12 small experimental greenhouses (each 10 m^2) in the Instituto de Formación e Investigación Agraria y Pesquera (IFAPA), Almería, Spain. A piece of paper containing ~200 *S. exigua* eggs from the laboratory colony was stapled to the underside of a medium-sized leaf half way up each plant. Plants were observed daily until larvae reached the second instar. OBs of the single genotypes VT-SeAl1 and HT-SeG25 at one of three concentrations (2×10^7, 1×10^8 and 5×10^8 OBs/l), and a water control were applied to four groups of three plants. One group of plants from each virus concentration treatment was placed in each of four greenhouses (each greenhouse representing a replicate). Similarly, groups of three plants were treated with water as a control and placed in four control treatment greenhouses. All applications were made to run-off in a volume of 125 ml/plant with 0.15 % (v/v) of the wetter-sticker Agral 90 (Syngenta Agro S.A., Madrid, Spain), using a 2 l capacity compressed-air hand sprayer (Pamex, Promopastor SL, Valencia, Spain). At 4 days post-application, 25 larvae per plant were collected, placed individually in 25 ml plastic cups with semisynthetic diet in laboratory conditions, and checked daily for virus mortality until death or pupation. The percentage of virus mortality in each repetition (plot) of each treatment was calculated and subjected to ANOVA using SPSS v19.0. Percentage values did not require transformation prior to ANOVA. Mean separation was performed by Tukey post hoc test ($P \leq 0.05$).

Lethal and sublethal infection by single genotype OBs and mixtures of OBs in greenhouse trials

Greenhouse trials were performed on sweet pepper plants (*Capsicum annuum* var. Melchor), planted in experimental greenhouses (110 m^2) during October 2012 and October 2013. The crop was cultivated following standard practices used in commercial greenhouses of this region, including predator releases of *Amblyseius swirskii*, *Nesidiocoris tenuis*, and *Orius laevigatus* for biological control of whiteflies and thrips. Four blocks of 110 m^2, comprising five rows with 20 plants per row, were planted at a distance of 1.1 m between rows. Each block was divided into five experimental plots that contained 20 plants: one plot in the center of the block and the other four plots at each corner of the block. The three central plants in each plot were selected for insect release and treatment applications to avoid border effects and to minimize cross-contamination. Five treatments were applied at a concentration of 1×10^8 OBs/l: (i) VT-SeAl1 OBs alone, (ii) HT-SeG25 OBs alone, (iii) the OB mixture 75:25, (iv) the OB mixture 25:75, and (v) water as a control. These OB mixtures were selected because they had proved to be more pathogenic than VT-SeAl1 OBs alone in the laboratory bioassays. Four replicates (one per block) were performed using a randomized design. Artificial infestation, OB application, insect collection, and analyses on the prevalence of lethal polyhedrosis disease in larvae and sublethal infection in adult survivors were performed as described above (see "Covert infection in survivors of an inoculum challenge" and "Efficacy of single genotype OBs on pepper plants" sections).

Susceptibility of the progeny of treated insects to OBs

Pupae that had been collected and survived the greenhouse trials in 2013 (from the treatments involving VT-SeAl1, 75:25, 25:75, HT-SeG25 OBs, and the water control) were classified and sorted according to treatment of origin, placed in groups of 10 males and 10 females in paper bags, and allowed to mate in the laboratory. Egg masses from these insects were allowed to hatch and reared on semisynthetic diet. Egg masses were not subjected to surface decontamination treatment. Groups of 24 first instar larvae were allowed to molt overnight without food and then, as second instars, were allowed to drink OB suspensions at one of the concentrations and following the procedures described in "Insecticidal properties of mixtures of OBs" section. In all cases, larvae were treated with 25:75 OBs as inoculum. Three independent replicates were performed. The concentration–mortality relationship was estimated by logit regression ("Insecticidal properties of mixtures of OBs" section). This treatment was selected based on the results obtained in the bioassays described in "Insecticidal properties of mixtures of OBs" section, which showed that the 25:75 mixture was as pathogenic as HT-SeG25 and produced a similar prevalence of covert infection as VT-SeAl1.

Influence of single and mixed OB treatments on pest feeding damage

Insect feeding damage following the application of OB and insecticide treatments was examined in greenhouse trials performed in September 2014. For this, four greenhouse blocks of 110 m^2 were planted with sweet pepper and each divided into 10 experimental plots, representing two replicates in each greenhouse, giving a total of eight replicates per treatment. Three central plants per plot were artificially infested by stapling a piece of paper containing

~200 *S. exigua* eggs on one leaf of each plant, as described in "Efficacy of single genotype OBs on pepper plants" section. Five treatments were applied to each group of central plants at 5×10^8 OBs/l: (i) VT-SeAl1 OBs alone, (ii) HT-SeG25 OBs alone, (iii) 75:25 OB mixture, (iv) the insect growth regulator methoxyfenozide (Runner® 24 %, Bayer Crop Science), applied at the label recommended rate of 0.05 % (w/v), and v) water control. Treatments were distributed in a randomized design. Feeding injury was estimated on leaves and fruits at 8 days post-application. In order to estimate foliar feeding damage, nine leaves per plant were sampled by randomly selecting and collecting three leaves from three branches of each plant at three different heights in the upper (110–140 cm), middle (60–100 cm), and lower (10–50 cm) sections of each plant. Four categories were used to classified samples: (a) 0–25 %, (b) 26–50 %, (c) 51–75 %, and (d) 76–100 % of foliar area consumed. All fruits of the treated plants were collected and examined for signs of *S. exigua* feeding damage (rotting, perforation or scars) and classified as damaged or undamaged fruit. The frequencies of foliar feeding damage were compared across treatments by log likelihood ratio test (*G*-test). The proportion of damaged fruit was subjected to ANOVA followed by Tukey's post hoc test ($P \leq 0.05$) (SPSS v19.0).

Results

Insecticidal properties of the horizontally and vertically transmitted genotypes and their mixtures

Virus mortality was not observed in control insects. OB pathogenicity significantly differed across virus treatments ($\chi^2 = 23.89$; df = 4; $P < 0.001$). The virus treatment × OB concentration interaction was not significant ($\chi^2 = 2.08$; df = 4; $P > 0.72$) so that regressions could be fitted with a common slope (Table 1). In terms of OB potency, VT-SeAl1 genotype OBs were 3.1, 2.5, and 2.4-fold less pathogenic than HT-SeG25, 25:75, and 75:25 OB mixtures, respectively, (Table 1). The three mixed-genotype OB preparations and HT-SeG25 OBs alone had similar OB potencies (*t* test; $P > 0.05$). To evaluate genotypic interactions, the expected LC_{50} values were estimated for each mixture of OBs, assuming that each genotype's OBs acted in an independent (additive) manner (Tabashnik 1992). A positive interaction was detected for the 75:25 mixture, given that the upper 95 % confidence interval of the LC_{50} was lower than the expected LC_{50} value (Table 1).

The speed of kill for fourth instar larvae did not differ significantly between virus treatments (*t* test; $P > 0.05$) (Table 1). The mean (±SE) time to death varied from 85.0 ± 0.2 to 86.1 ± 0.2 h for insects infected with HT-SeG25, VT-SeAl1 genotypes, and their mixtures. Similarly, median OB production did not differ significantly between virus treatments (Kruskal–Wallis: $\chi^2 = 1.925$; df = 4; $P = 0.75$), ranging from 4.9×10^8 OBs/larva to 6.8×10^8 OBs/larva for the HT-SeG25 variant and the mixture 25:75, respectively, (Table 1).

Capacity to induce covert infections of the horizontally and vertically transmitted genotypes and their mixtures

All OB treatments including single genotypes (VT-SeAl1 and HT-SeG25) and three mixed-genotype OB preparations were used to inoculate fourth instar larvae. Mean (±S.E.) virus-induced mortality was similar among treatments ($F = 1.72$; df = 4, 10; $P = 0.22$), ranging from 35.4 ± 11.5 % in the 75:25 treatment to 64.6 ± 3.4 % in the VT-SeAl1 treatment (Fig. 2a). Virus mortality was not present in control larvae. *Bgl*II restriction profiles confirmed the identity of viruses causing lethal infections and the presence of genotype-specific marker fragments characteristic for HT-SeG25 and VT-SeAl1 genotypes (Fig. 1).

The prevalence of sublethal infection in the adult survivors of an OB inoculum treatment in the larval stage was determined by qPCR (Fig. 3a). The prevalence of sublethal infection differed significantly with OB treatment ($F = 20.80$; df = 5, 16; $P < 0.001$). Sublethal infection in adults was most prevalent in the survivors of VT-SeAl1 OB inoculum (90 % sublethal infection) and lowest in the HT-SeG25 OB treatment (51 % sublethal infection). Survivors of the OB mixtures had an intermediate prevalence of sublethal infection (72–87 %). Unexpectedly, 20 % of the adults from the control group tested positive for covert infection, suggesting a low level of covert infection in the insect colony.

Viral load was quantified in adults tested positive. Insects infected with VT-SeAl1 genotype harbored the highest viral load (mean = $4.54 \times 10^{-1} \pm 3.89 \times 10^{-2}$ pg viral DNA/insect), although no significant differences were observed between treatments ($F = 0.20$; df = 5, 8; $P = 0.95$). The estimated viral DNA load in mock-infected adults was $3.88 \times 10^{-1} \pm 8.35 \times 10^{-2}$ pg viral DNA/insect. Adults infected with the OB mixed populations 75:25, 50:50, 25:75, and the HT-SeG25 genotype were estimated to harbor $3.37 \times 10^{-1} \pm 5.14 \times 10^{-3}$, $4.19 \times 10^{-1} \pm 1.38 \times 10^{-1}$, $4.53 \times 10^{-1} \pm 8.11 \times 10^{-2}$, and $4.24 \times 10^{-1} \pm 5.57 \times 10^{-4}$ pg viral DNA/insect, respectively.

Greenhouse trial

At 4 days post-application, between 276 and 295 larvae were collected from each virus treatment and were checked daily for virus mortality. Larval mortality increased

Table 1 Mean lethal concentration (LC_{50}), confidence intervals (95 %), relative potency, expected LC_{50} using the method of Tabashnik, mean time to death (MTD), and median OB yield of the VT-SeAl1, 75:25, 50:50, 25:75, and HT-SeG25 treatments in second instar *S. exigua* larvae

Treatment	LC_{50} ($\times 10^4$) (OBs/ml)	95 % confidence interval ($\times 10^4$)		Relative potency	Expected LC_{50} ($\times 10^4$) (OBs/ml)	MTD (h)	95 % confidence interval		Median OB yield ($\times 10^8$ OBs/larva)	Interquartile range ($\times 10^8$ OBs/larva)
		Low	High				Low	High		
VT-SeAl1	5.98 a	4.43	8.08	1	–	86.1 a	84.6	87.8	5.8 a	5.4
75:25	2.55 b	1.82	3.59	2.4	3.94	84.3 a	82.6	86.1	5.0 a	5.4
50:50	3.45 a,b	2.48	4.85	1.7	2.94	85.2 a	83.6	86.9	5.6 a	5.1
25:75	2.45 b	1.75	3.42	2.5	2.34	86.0 a	84.3	87.8	6.8 a	5.9
HT-SeG25	1.95 b	1.40	2.74	3.1	–	85.0 a	83.4	86.6	4.9 a	5.0

Logit regressions were fitted with a common slope of 0.72 ± 0.033 (±SE) for all virus treatments. Relative potencies were calculated as the ratio of effective concentrations relative to the VT-SeAl1 genotype. Mean time to death (MTD) values were estimated by Weibull survival analysis (Weibull hazard function $\alpha = 12.71$). Median OB production was analyzed by Kruskal–Wallis test. Values followed by the same letters did not differ significantly for comparisons among treatments within each column ($P > 0.05$)

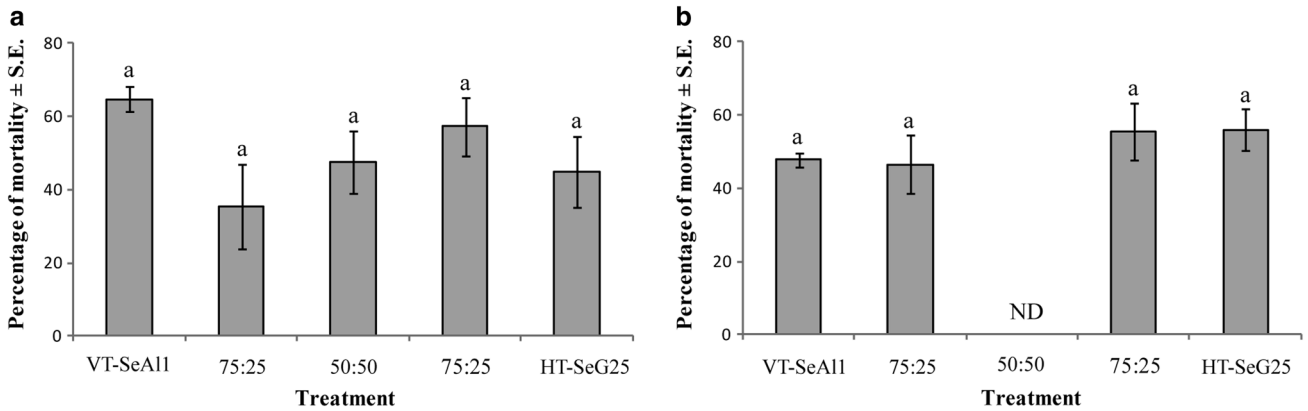


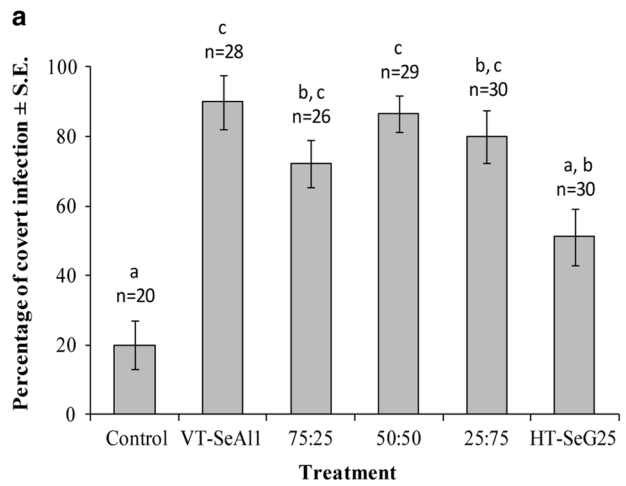
Fig. 2 Mean percentage of viral mortality in *S. exigua* larvae in the covert infection bioassays performed in **a** laboratory and **b** greenhouse conditions. Columns labeled with *identical letters* did not differ significantly (ANOVA). *ND* no data

significantly with OB concentration ($F = 41.76$; df = 2, 21; $P < 0.001$), but did not differ significantly between genotypes ($F = 0.183$; df = 1, 22; $P = 0.673$) (Fig. 4). Mortality ranged from 28.8 ± 3.4 to 19.8 ± 3.8 % at the lowest OB concentration (2×10^7 OBs/l) to between 80.3 ± 3.6 and 80.7 ± 4.6 % at the highest concentration (5×10^8 OBs/l) for the VT-SeAl1 and HT-SeG25 treatments, respectively. Lethal polyhedrosis disease was not observed in control larvae.

Prevalence of covert infection in insects recovered from field trials

Adult insects that survived the treatments of 1×10^8 OBs/l in greenhouse trials during the larval stage were tested for sublethal infection by qPCR. The percentage of larvae that succumbed to the infection was similar among treatments ($F = 0.62$; df = 3, 12; $P = 0.61$) and ranged from 46.4 ± 8.0 % to 56 ± 5.7 % (Fig. 2b). Lethal polyhedrosis disease was not observed in control larvae. The prevalence of sublethal infection in adults differed significantly with OB treatment ($F = 18.8$; df = 4, 19; $P < 0.001$). The prevalence of sublethal infection in adult survivors increased with the proportion of VT-SeAl1 genotype present in the mixture (Fig. 3b) and was highest for insects treated with the VT-SeAl1 genotype OBs alone (76 %) and lowest in the insects that survived HT-SeG25 genotype OBs (45 %), with intermediate values in the OB mixtures. Sublethal infection was detected in 31 % of control insects, probably due to the presence of inapparent sublethal or latent infection in the laboratory insect colony.

The estimates of DNA quantities in positive adults did not differ significantly between treatments ($F = 0.21$; df = 4, 12; $P = 0.92$) and ranged from $1.54 \times 10^{-1} \pm 1.54 \times 10^{-2}$ pg viral DNA/insect (HT-SeG25 genotype) to $4.19 \times 10^{-1} \pm 9.32 \times 10^{-2}$ pg viral DNA/insect (VT-SeAl1 genotype). A mean value of $2.34 \times 10^{-1} \pm 4.46 \times 10^{-2}$ pg viral DNA/insect was measured in control insects.



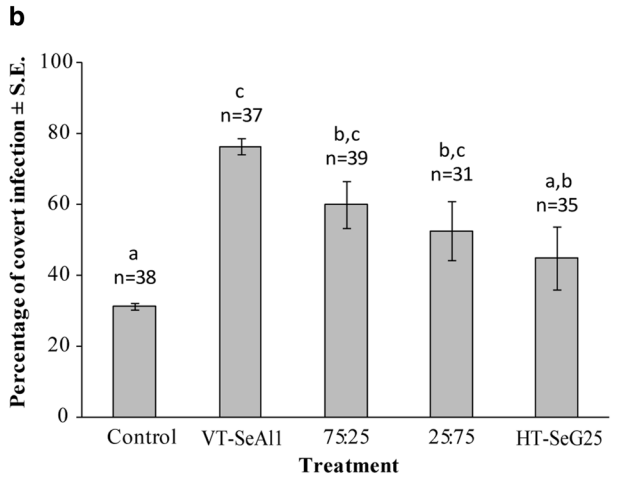


Fig. 3 Mean percentage of adults that tested positive for *DNA polymerase* gene amplification (qPCR), after sublethal inoculation with single genotypes VT-SeAl1, HT-SeG25, and their OB mixtures (75:25, 50:50, and 25:75) in **a** laboratory and **b** field conditions. Numbers above the columns indicate the number of individuals tested (*n*). Columns labeled with *identical letters* did not differ significantly (*t* test, $P < 0.05$)

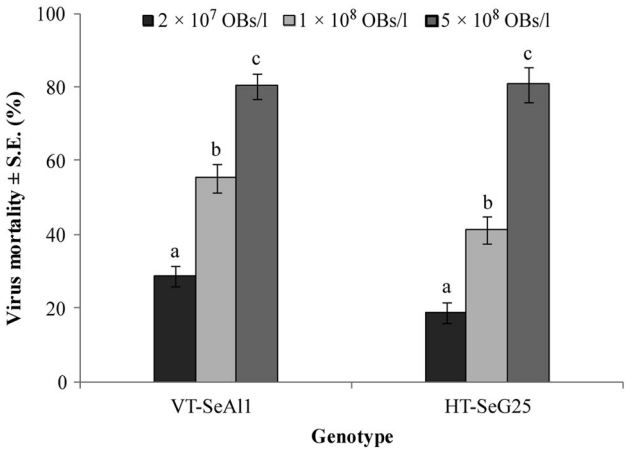


Fig. 4 Mean percentage of virus mortality of *S. exigua* larvae collected at 4 days post-application using three dosages: 2×10^7, 1×10^8, and 5×10^8 OBs/l and two genotypes: VT-SeAl1 and HT-SeG25. Insects were reared in the laboratory until death or pupation. Columns labeled with *different letters* indicate significant differences for comparisons of application rate within each genotype treatment (Tukey, $P < 0.05$)

Susceptibility of the offspring (F_1) of field-treated insects to superinfection

The concentration-mortality response of progeny (F_1) from field-treated individuals that survived treatment with OBs of VT-SeAl1, 75:25, 25:75, HT-SeG25, and controls, was determined following inoculation of second instars with the 25:75 OB mixture. Overall mortality increased significantly with OB concentration ($\chi^2 = 735$; df = 1; $P < 0.001$), whereas no significant interaction of OB concentration × virus treatment was detected ($\chi^2 = 0.17$; df = 4; $P = 0.99$). No effect of field treatment on parental adults was observed, since insect from all treatments were equally susceptible to disease ($\chi^2 = 6.13$; df = 4; $P = 0.18$) (Table 2). No overt infections were observed in mocked-infected larvae.

Influence of single and mixed OB treatments on pest feeding damage

Feeding damage was evaluated in leaves and fruits of pepper plants after application of OB treatments at 5×10^8 OBs/l. The highest level of defoliation was registered in control plants (Fig. 5a), which differed significantly from the other treatments ($G = 381$; df = 12; $P < 0.001$). Methoxyfenozide was the most efficient of all the treatments ($G = 47.5$; df = 6; $P < 0.001$), whereas no differences were observed among virus treatments ($G = 7$; df = 4; $P = 0.12$). Methoxyfenozide and virus treatments resulted in significantly lower prevalences of damaged fruits than observed in the control ($F = 15.51$; df = 4, 13; $P < 0.001$); all virus treatments were as effective as the chemical insecticide treatment (Fig. 5b). No virus mortality was observed in larvae recovered from control plots.

Discussion

Combinations of two SeMNPV genotypes that had been proved to exhibit noteworthy insecticidal properties (HT-SeG25), or the capability to be transmitted through host generations (VT-SeAl1), were evaluated for their potential as pest control agents. Interactions between nucleopolyhedrovirus genotypes originating from wild-type isolates

Table 2 Mean lethal concentration (LC_{50}), confidence intervals (95 %), and relative potency of second instar progeny of adults that survived different virus treatments applied in greenhouse conditions

Treatment applied to parental insects	LC_{50} ($\times 10^4$) (OBs/ml)	95 % confidence interval ($\times 10^4$)		Relative potency
		Low	High	
Control	2.58 a	2.01	3.31	1
VT-SeAl1	2.81 a	1.54	5.25	1.0
75:25	3.13 a	2.41	4.07	0.9
25:75	2.20 a	1.65	3.03	0.8
HT-SeG25	2.63 a	2.06	3.37	1.2

The bioassay was performed using the 75:25 OB virus mixture as inoculum

LC_{50} values followed by identical letters do not differ significantly ($P > 0.05$)

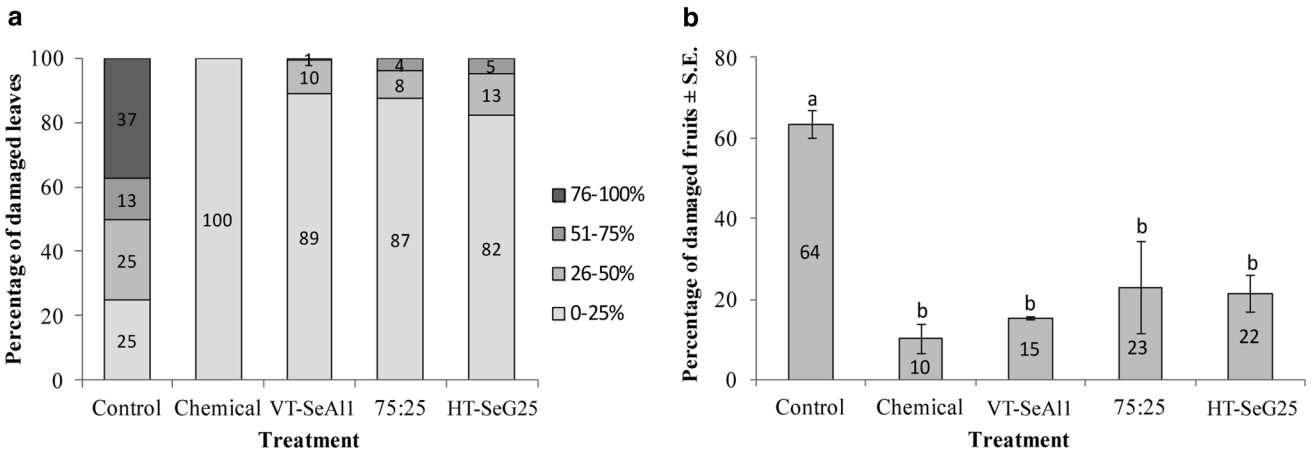


Fig. 5 Pest-inflicted damage on pepper plants at 8 days post-application. **a** Prevalence and severity of damaged leaves in each treatment. Feeding damage by *S. exigua* was classified into one of four categories based on mean percentage of leaf area consumed. **b** Mean percentage of damaged fruits in each treatment. Columns headed by *different letters* differed significantly between treatments (Tukey, $P < 0.05$)

have been identified with important consequences for the phenotype of the virus population, particularly in terms of OB potency, speed of kill and OB production in infected insects (Hodgson et al. 2001; Simón et al. 2006). These traits have a clear influence on the transmission of the pathogen in host populations (Hodgson et al. 2004). However, with notable exceptions (Simón et al. 2013), our ability to predict the emergent properties of genotype interactions within host insects remains extremely limited. For this reason, at present, the phenotypic characteristics of genotypic mixtures can only be determined empirically, which was the rationale of the present study.

The HT-SeG25 and VT-SeAl1 genotypes studied here differ in key phenotypic traits that were clearly related to their respective transmission strategies (Cabodevilla et al. 2011a). In line with previous findings, we observed that HT-SeG25 OBs were threefold more potent than VT-SeAl1 OBs. Genome sequence comparisons identified single-nucleotide polymorphisms in *Se*4 and *Se*5 that implicated these genes in the observed OB phenotype (Serrano et al. 2015; Theze et al. 2014). The presence of the HT-SeG25 genotype at 25 or 75 % of OBs improved the pathogenicity of the inoculum, with the 25 % HT-SeG25 + 75 % VT-SeAl1 mixture exhibiting evidence of mild synergism in OB potency; the explanation for which requires further study.

The prevalence of the VT-SeAl1 genotype in the inocula was associated with an increasing frequency of sublethal infection in adults that survived treatment in the larval stages, both in laboratory and field conditions (Fig. 3). Also, viral loads per insect tended to be higher in adults inoculated with VT-SeAl1 OBs in different proportions compared to those inoculated with the HT-SeG25 genotype alone. Interestingly viral load was previously observed to be positively correlated with the prevalence of vertical transmission in the offspring of sublethally infected adults (Virto et al. 2013). Moreover, evidence of sublethal infection was found in control insects that had originated

from what was believed to be a virus-free colony, although no lethal infections developed in control insects used in laboratory and field experiments. This underlines the pervasive nature of virus infections in natural and laboratory insect populations (Cooper et al. 2003; Kemp et al. 2011; Murillo et al. 2011). Indeed, it may be almost impossible to maintain a lepidopteran colony that is totally free of viruses, albeit at a low prevalence (Possee et al. 2008), as observed in the present study.

Results from field trials indicated that the establishment of sublethal infection in the survivors of infection was less efficient in the field, compared to observations performed under laboratory conditions. This is likely a result of the heterogeneity in the distribution of OB inocula on host plant surfaces and the feeding behavior of the pest, resulting in increased variability in the dose of OBs ingested by each insect, in addition to possible host plant-related effects on the efficiency of the infection process and subsequent pathogenesis (Hodgson et al. 2002; Raymond et al. 2002). In contrast, under laboratory conditions larvae were inoculated under standardized, highly uniform conditions aimed at minimizing variation within and between batches of virus-treated insects. OB dose ingested and larval stage were factors that were previously reported to affect the acquisition of sublethal infection under controlled conditions (Cabodevilla et al. 2011b).

The strategy for selection of an active ingredient for baculovirus-based insecticides has traditionally focused on the selection of the isolate with the most suitable insecticidal traits (particularly OB pathogenicity and speed of kill), originating from diseased larvae collected in the field during entomopathogen surveys. A change in this paradigm has recently taken place with the development of co-occlusion technology involving the co-occlusion of novel mixtures of genotypes with emergent insecticidal properties (Arrizubieta et al. 2015; Bernal et al. 2013b). The unpredictable nature of genotype interactions led us to examine whether HT and VT genotypes could be combined to provide rapid suppression of the pest following an inundative application of OBs followed by a sustained contribution to pest control by transgenerational mortality of sublethally infected larvae that become lethally diseased, or insects that showed increased susceptibility to OB inoculum on treated crop plants. Both these effects have been observed in laboratory studies (Cabodevilla et al. 2011a, b). The finding of a high prevalence of VT infections in greenhouse experiments indicates that further multigenerational studies are merited to estimate the prevalence of transgenerational mortality in the pest population. In that respect, the concept of transgeneration pathogen-mediated control is akin to the inoculative strategy of biological pest control, that has been proposed for highly transmissible isolates of some pests (Takahashi et al. 2015), and which provides season-long control of *Anticarsia gemmatalis* in soya crops in Brazil (Moscardi 1999). The use of VT isolates in a virus insecticide also opens the way to greater virus dispersal via sublethally infected adults that disperse away from the original site of application to adjacent crops, in which their progeny may die of polyhedrosis disease, thereby providing inoculum for subsequent transmission events (Burden et al. 2003; Vilaplana et al. 2008). However, the relative pest control contributions of horizontal transmission generated through secondary cycling of inocula and vertically transmitted infections that are subsequently activated to lethal disease, have yet to be estimated by quantitative studies.

The transgenerational effects of a pathogen challenge during development have received increasing attention, especially in relation to immune function, fitness costs, and subsequent susceptibility to disease (Tidbury et al. 2011; Wilson and Graham 2015; Zanchi et al. 2011). In this study, we did not observe reactivation in persistently infected offspring derived from adults that survived exposure to virus inocula on host plants. In a previous study developed in similar conditions, up to 20 % of field-collected adults produced offspring that subsequently died from lethal polyhedrosis during rearing under clean laboratory conditions (Cabodevilla et al. 2011a). A different laboratory insect colony found to be sublethally infected during continuous rearing did not succumb to reactivation when studied over five generations (Cabodevilla et al. 2011b), so vertical transmission does not invariably result in lethal disease in a portion of the insect population. Indeed, as demonstrated in the present study, inapparent infection of laboratory colonies of Lepidoptera may be far more common than generally recognized (Hughes et al. 1993; Kouassi et al. 2009; Murillo et al. 2011; Simón et al. 2010). Currently, the underlying mechanism(s) that determine the reactivation of covert into overt lethal infections are poorly understood, although previous studies have identified a number of factors that tend to trigger lethal virus disease. Physiological stress can play a major role in the reactivation of baculovirus infections, which can appear following exposure to chemical compounds (Ilyinykh et al. 2004), nutritionally inadequate diet (Biever and Wilkinson 1978; David and Gardiner 1965), other viruses (Fuxa et al. 1999; Kouassi et al. 2009), and high larval densities during laboratory rearing (Opoku-Debrah et al. 2013), or in natural habitats (Cooper et al. 2003).

As transgenerational virus-induced mortality involves a portion of progeny larvae succumbing to lethal disease during their development, we also examined the susceptibility of the progeny larvae to OB inocula. This involves a combination of responses from the fraction of the population that are sublethally infected and those that are not. In the case of the sublethally infected fraction, exposure to

OB inocula represents an assay of susceptibility to superinfection. Previous studies indicated that the progeny of sublethally infected *S. exigua* adults were 2–3-fold more susceptible to OBs than non-infected insects (Cabodevilla et al. 2011b). In contrast, the bioassays we performed on the progeny of insects exposed to OB inocula applied in greenhouse trials indicated no significant variation in susceptibility to OBs. This may be because the prevalence of sublethal infection registered in the parental insects (30–76 %) was lower than that registered in the laboratory-based study by Cabodevilla et al. (2011b). Alternatively, this may reflect a parental response to nutritional or rearing stresses experienced as larvae in the greenhouse trials, both of which have been reported to reduce the susceptibility of insect progeny to baculovirus infection (Boots and Roberts 2012; Wilson and Graham 2015).

Greenhouse trials indicated that the SeMNPV preparations tested here could provide an excellent level of crop protection, as previously reported in greenhouse trials in southern Spain (Lasa et al. 2007), and elsewhere (Bianchi et al. 2000; Smits et al. 1987). Foliar feeding damage was significantly reduced with respect to water controls, although the insect growth regulator methoxyfenozide provide better foliar protection. However, at high levels of infestation, *S. exigua* can act as a direct pest by feeding on fruits (Lasa et al. 2007). In this sense, the virus treatment was as effective in protecting fruits from larval feeding damage as the chemical insecticide. In addition, NPV-based insecticides exhibit high levels of compatibility with other natural control agents that can be integrated into pest management programs (Jiang et al. 2014).

In summary, mixtures of OBs of horizontally (HT-SeG25) and vertically (VT-SeAl1) transmitted genotypes were as pathogenic as HT-SeG25 OBs alone and also induced covert infection in 60–72 % of adults that survived exposure to OB inocula in the larval stage in both laboratory and greenhouse conditions. The 75:25 mixture proved to be an effective crop protection treatment on greenhouse grown sweet pepper. The inclusion of VT genotypes in combination with highly pathogenic HT genotypes as components of the active ingredient of alphabaculovirus-based insecticides could provide a means of promoting transgenerational pest suppression in agro-ecosystems, although further studies are required to quantify long-term effects on pest suppression.

Author contribution statement

PC conceived and designed research. CV, DN, MMT, and RM conducted experiments. CV contributed new reagents and/or analytical tools. CV, RM, and TW analyzed data. CV, RM, PC, and TW wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

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Conflict of interest Authors C. Virto, T. Williams, D. Navarro, M.M. Tellez, R. Murillo and P. Caballero declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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