Research Article

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Insecticidal efficacy and persistence of a co-occluded binary mixture of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) variants in protected and field-grown tomato crops on the Iberian Peninsula

Maite Arrizubieta,[a] Oihane Simón,[a] Luis M Torres-Vila,[b] Elisabete Figueiredo,[c] Javier Mendiola,[b] Antonio Mexia,[c] Primitivo Caballero[a,d] and Trevor Williams[e*]

Abstract

BACKGROUND: A binary co-occluded mixture (HearSP1B:LB6) of *Helicoverpa armigera* single nucleopolyhedrovirus (HearNPV) variants was previously found to be highly pathogenic under laboratory conditions. The insecticidal efficacy and persistence of this mixture were determined in greenhouse and field-grown tomato crops in Spain and Portugal.

RESULTS: Concentrations of 10^9–10^{11} occlusion bodies (OBs) L^{-1} of HearSP1B:LB6 resulted in 89–100% mortality of larvae on treated tomato plants in growth chambers. In protected tomato crops, application of 10^{10} OBs L^{-1} of HearSP1B:LB6 was as effective as *Bacillus thuringiensis* (*Bt*) and spinosad in reducing the percentage of damaged fruits, and resulted in higher larval mortality than the *Bt* treatment. In open-field tomato crops, virus treatments were as effective in reducing the percentage of damaged fruit as spinosad, *Bt* and chlorpyrifos treatments. The persistence of the insecticides on tomato plants was negatively correlated with solar radiation in both field and greenhouse settings. Residual insecticidal activity of OBs on protected tomato crops at 6 days post-application was 55 and 35% higher than that of *Bt* and spinosad respectively. On field-grown tomato, OB persistence was significantly lower than with spinosad or chlorpyrifos.

CONCLUSION: The efficacy and persistence of HearSP1B:LB6 OBs were comparable with those of commercial insecticides in both field and greenhouse tomato crops. Future studies should focus on reducing application rates to determine insecticidal efficacy at lower OB concentrations.

Keywords: alphabaculovirus; crop protection; residual activity; *Bacillus thuringiensis*; spinosad

1 INTRODUCTION

Spain is the world's fourth largest producer of tomatoes, with an average of 4.3 million t $year^{-1}$, whereas Portugal produces an additional 1.1 million t $year^{-1}$.[1,2] The total area of production comprises approximately 60 000 and 17 000 ha in Spain and Portugal respectively, with 25% (Spain) and 60% (Portugal) of the production in greenhouses and the remainder as field crops.[1,2]

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), also known as the tomato fruitworm, is one of the most important pests of tomato.[3] Females mainly lay eggs in the flowering period, although larvae may attack any phenological stage of the plant and are especially likely to damage the fruit. Therefore, the pest's preference for flowers and fruits, in addition to its polyphagy, high mobility and fecundity, makes it a major pest.[4] Quality control in the tomato processing industry set the damage limit to 2–5% of harvested tomatoes, but if larvae are present, this limit is reduced to 0–2%.[3,5]

Control of this pest is usually achieved by applying chemical insecticides, especially organophosphates, the carbamate

* *Correspondence to: Trevor Williams, Instituto de Ecología AC, AP 63, Xalapa, Veracruz 91070, Mexico. E-mail: trevor.inecol@gmail.com*

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

b *Servicio de Sanidad Vegetal, Consejería de Agricultura DRMAyE, Gobierno de Extremadura, Mérida, Spain*

c *Centro de Engenharia dos Biossistemas, Instituto Superior de Agronomia, Universidade de Lisboa, Lisbon, Portugal*

d *Departamento de Producción Agraria, Universidad Pública de Navarra, Pamplona, Spain*

e *Instituto de Ecología AC, Xalapa, Veracruz, Mexico*

methomyl or pyrethroids.[3,6] However, excessive dependence on chemicals has led to a variety of problems, such as increased production costs due to multiple insecticide applications, development of marked insecticide resistance in the pest population, elimination of beneficial insects and the presence of pesticide residues that may restrict the commercialisation of tomato crops from this region.[7–9] Frequent use of insecticides also leads to a need for continual monitoring and analysis of residues in tomato fruits and tomato-based products.[10] These constraints have motivated the search for alternative control methods, including the use of biological insecticides.[11,12]

HearNPV is an alphabaculovirus (Baculoviridae) that has been used as a biological insecticide for control of this pest on cotton, soybean, pigeon pea, maize and tomato in several parts of the world.[13] The virus comprises a single nucleocapsid containing a single genome within each virion, with dozens of virions occluded into each viral occlusion body (OB). The virus is specific to certain species of *Heliothis* and *Helicoverpa*,[14] but shows the highest pathogenicity, in terms of dose–mortality metrics, to larvae of *H. armigera*.[15] As in other alphabaculoviruses,[16–19] natural isolates of the single nucleocapsid morphotype of this virus, known as HearSNPV, comprise heterogeneous mixtures of genotypic variants that contribute to the transmission and survival of the pathogen in the host population.[20,21]

In a previous study, a novel binary mixture of HearSNPV variants, named HearSP1B:LB6, had high insecticidal activity against *H. armigera* larvae under laboratory conditions.[22] This unique combination of variants was produced by isolating individual variants *in vitro*, mixing and co-occluding them in different proportions and testing the pathogenicity of the resulting OBs, using insect bioassay concentration–mortality metrics.[22] The HearSP1B:LB6 mixture comprised equal proportions of the most pathogenic variant present in a field isolate of HearSNPV from Badajoz (Spain), named HearSP1, and the fastest-killing variant, HearLB6, from an isolate collected from dead larvae near Seville (Spain). These variants were mixed and co-occluded into OBs using a procedure that had been successfully employed for co-occlusion of mixtures of other alphabaculovirus variants.[23] The HearSP1B:LB6 co-occluded mixture was 1.7–2.8-fold more pathogenic than any of the genotypes present in the HearSP1 or HearLB6 populations.[22] The presence of variant mixtures within individual OBs was confirmed using end-point dilution and qPCR techniques.[22] Additionally, a production system for HearSP1B:LB6 OBs was developed, aimed at efficient production of this binary mixture (Arrizubieta M, unpublished).

Registration of bioinsecticidal products requires field trials to be performed under typical crop production conditions to demonstrate the efficacy of the product for pest control. The efficacy of biological insecticides can vary in laboratory, greenhouse and open-field conditions.[24–27] Furthermore, insect populations often differ in their susceptibility to a particular virus strain present in a virus-based insecticide, as local virus genotypes tend to be more pathogenic to local pest populations than genotypes from geographically distant regions.[15,19,28,29]

The efficacy of a virus-based insecticide is also strongly influenced by its persistence on the surface of the crop plant, as with greater persistence the probability of the pest consuming a lethal dose of OBs over time also increases.[30] Solar ultraviolet (UV) radiation is the main factor affecting the persistence of OBs deposited on plant surfaces.[31] However, the incidence of UV radiation can vary greatly from region to region, with crop phenology and with growing conditions. For example, exposure to UV radiation is greatly reduced in greenhouse-grown crops compared with those grown in an open field, as the greenhouse's plastic structure filters much of the incident UV.[32]

As a contribution to the registration of the binary variant mixture as the active ingredient of an insecticidal product, the present study aimed to determine the efficacy of HearSP1B:LB6 OBs for the control of *H. armigera* and the persistence of OBs on tomato crops under both protected and open-field conditions. The performance of OBs was compared with that of commercial products commonly used for the control of this pest on tomato crops.

2 EXPERIMENTAL METHODS

2.1 Virus and insects

The co-occluded genotypic mixture used in the present study HearSNPV-SP1B + HearSNPV-LB6, abbreviated here to HearSP1B:LB6, was characterised previously in terms of concentration–mortality response, speed of kill and OB production.[22] All virus preparations were propagated in fifth-instar *H. armigera* larvae by the droplet feeding method.[33] The wild-type HearSNPV-SP1 isolate (HearSP1)[24,34] was included as a reference treatment in open-field trials.

The *H. armigera* colony used for artificial infestations in greenhouse and persistence assays was established with pupae received from the Centre for Ecology and Hydrology (CEH) (Oxford, UK) and maintained in the Universidad Pública de Navarra (Pamplona, Spain) at 25 ± 2 °C, 70–80% relative humidity and a 16:8 h L:D photoperiod on a semi-synthetic diet.[35]

2.2 Determining the optimal OB concentration

A preliminary assay was performed in a growth chamber under laboratory conditions with the aim of selecting the optimal HearSP1B:LB6 OB concentration to be used in greenhouse and open-field trials described below. Groups of three tomato plants of ∼1.2 m height were sprayed with 10^9, 10^{10} and 10^{11} OBs L^{-1}, supplemented with 0.2% (v/v) of a commercial wetter-sticker based on nonylphenoxy polyethoxy ethanol (Agral®; Syngenta Agro, Madrid, Spain), using a total of 60 mL for each plant. Plants sprayed with just water and wetter-sticker were included as negative controls. When plants were completely dry (∼1 h), three tomato leaves from each plant, each comprising five primary leaflets, were cut at the base of the petiole, and the cut end was placed in 50 mL glass cups containing Hoagland nutritive solution.[36] Leaves were then placed individually in a 2 L glass container. Each container was artificially infested with ≈ 150 second-instar *H. armigera* larvae from the laboratory colony, covered with muslin and maintained at 25 ± 2 °C, 70–80% relative humidity and a 16:8 h L:D photoperiod during a week. Groups of 20 living larvae were collected at random from each container at three different intervals, following 1, 3 and 5 days of exposure. Moribund larvae or those that had fallen off experimental plants were not collected. The collected larvae were individualised in 30 mL plastic cups containing artificial diet and incubated under the same conditions until death or pupation. Larval mortality was recorded daily. The experiment was performed 3 times. Results were subjected to repeated-measures analysis of variance (ANOVA) and Tukey's test ($P < 0.05$) for homogeneous groups using SPSS v.21 (IBM SPSS Statistics; SPSS, Chicago, IL).

2.3 Greenhouse trials

Greenhouse trials were performed in 2011 in an experimental greenhouse of 18 m length × 16 m width, with a total area of

288 m^2, located at the Instituto Superior de Agronomia (Lisbon, Portugal). Cropping practices such as bed formation, drip irrigation, application of fertilisers, transplanting, maintenance and manual weeding were performed following established procedures in the studied area. Plug seedlings were transplanted with a ball of peat at the 3–5-true-leaf stage, in pairs of rows separated by 20 cm between each row, and 50 cm distance between row pairs. Plants were spaced at 40 cm intervals along the rows, resulting in a density of ∼38 000 plants ha^{-1}. Greenhouse plastic was treated with a chalk-based shade product (Spraychalk; Mardenkro, Baarle-Nassau, The Netherlands), water dilution 1:8, as is usual in the region for summer protected crops.

The experiment involved four treatments: (i) HearSP1B:LB6 OBs applied at a concentration of 10^{10} OBs L^{-1} (following the results of the growth chamber study described in Section 2.2), equivalent to 10^{13} OBs ha^{-1}; (ii) Turex® 50 WP [50% *Bacillus thuringiensis* (*Bt*) (w/w), 25 000 IU mg^{-1}, from Biosani, Portugal; applied at 1 kg ha^{-1}]; (iii) Spintor® [SC, 48% spinosad (w/v) from Dow AgroSciences, Seville, Spain; applied at 250 mL ha^{-1}]; (iv) control treatment (water). Turex and Spintor were applied at the product label recommended rates. All treatments included 0.2% Agral® wetter-sticker and were applied using 18 L hand-operated knapsack sprayers fitted with a cone nozzle.

The entire greenhouse was divided into 16 plots, according to a 4 × 4 Latin square design, with four replicates per treatment. Experimental plots comprised 7.5 m long sections of two double rows (7.5 m^2) comprising 28 plants (22 border plants and six central plants). Each plot was artificially infested with 112 larvae by placing two *H. armigera* second instars from the laboratory colony on each of the two youngest clusters of tomato fruits on each of the 28 plants. Larvae were allowed to feed on plants for 1 day. After that, each plot was sprayed with 750 mL of each insecticide treatment, equivalent to an application volume of 1000 L ha^{-1}. All treatments were applied between 18:00 and 20:00 h. The entire trial was performed twice, on 19 July and 13 September. The efficacy of HearSP1B:LB6 OBs was evaluated by estimating the number of surviving larvae and the number of fruit feeding injuries present on the six central plants of each plot at 10 days after application. The numbers of living larvae and fruit feeding injuries involving direct feeding damage caused by *H. armigera* were measured by direct counting. Results were subjected to repeated-measures ANOVA and Tukey's test ($P < 0.05$) for homogeneous groups using SPSS software. The correlation between fruit feeding damage and larval mortality was determined using the Pearson coefficient, as both variables were normally distributed.

2.4 Open-field trials

Open-field trials were conducted in 2012 at CICYTEX Research Centre (Finca La Orden, Badajoz, Spain). Soil was prepared according to usual cropping practices in the study area: ploughing, harrowing, bed formation, drip irrigation and transplanting. Tomato seedlings were transplanted with a ball of peat at the 3–5-true-leaf stage in single rows spaced at intervals of 1.5 m on 1.0 m wide beds. Plants were spaced 25–26 cm apart along the rows, resulting in a density of ∼26 000 plants ha^{-1}. Cultural practices, including the use of herbicide, fungicide, fertiliser, irrigation, crop maintenance and manual weeding, were performed according to usual procedures. Plots were inspected daily for minor pests; liquid sulfur was applied on four occasions to control mites. Experimental plots were arranged in a randomised plot design, with four replicate plots per treatment. The whole trial consisted of 48 experimental plots. Each plot was 4 m long by one row wide (6 m^2), comprising 15–16 plants. Plots were separated from each other by a buffer row of untreated plants. Plots at the edges of the experimental area were surrounded by additional rows of untreated plants to reduce edge effects. Two pheromone traps were placed at the edge of the experimental area and inspected twice weekly to acquire information on likely infestation by *H. armigera*.

The experiment involved six treatments: (i) HearSP1B:LB6 OBs applied at a concentration of 10^{10} OBs L^{-1} (equivalent to 10^{13} OBs ha^{-1}, following the results of the growth chamber study described in Section 2.2); (ii) HearSP1 OBs applied at a concentration of 10^{10} OBs L^{-1}, equivalent to 10^{13} OBs ha^{-1}; (iii) Turex® [50% *Bt* (w/w) from Certis, Spain; applied at 2 kg ha^{-1}]; (iv) Spintor® [SC, 48% spinosad (w/v) from Dow AgroSciences; applied at 250 mL ha^{-1}]; (v) Dursban® [75% chlorpyrifos (w/w) from Dow AgroSciences; applied at 1.25 kg ha^{-1}]; (vi) control treatment (water). All commercial insecticides were applied at product label recommended rates for tomato. All treatments included 0.2% Agral® wetter-sticker and were applied using 18 L hand-operated knapsack sprayers with a cone nozzle. All treatments were applied between 18:00 and 20:00 h. Treatments were applied in a volume of 600 mL in each plot, equivalent to an application volume of 1000 L ha^{-1}.

Treatments were applied either 3 or 5 times, depending on how many times economic threshold levels were reached or surpassed during the course of the trial. In all, four plots from each treatment received three applications (24 plots in total) and four plots from each treatment received five applications (24 plots in total). The first treatment was applied when 3% of fruits showed characteristic *H. armigera* feeding damage, which is the action threshold in integrated pest management (IPM) programmes against *H. armigera* in tomato crops in this region.[3,37] Thereafter, applications were performed every 10–13 days, which represents the usual time interval between treatments against *H. armigera* on processing tomato in this region. The first application was performed on 11 June, and the second on 21 June; in both cases all fruits were green. The third, fourth and fifth treatments were applied on 3, 16 and 26 July, which coincided with the presence of ∼5, 50 and 65% of red fruit development respectively.

Plots were inspected for *H. armigera* damage twice weekly from early fruit set to 1 week before harvesting. The percentage of larval damage was estimated in each plot every 3–4 days by examining 100 randomly chosen fruits, which included green fruits larger than 2.5 cm in diameter, as well as pink and red fruits of all sizes. Insect feeding damage was classified as recent or old (scarred injuries). Data were grouped by fortnights (first fortnight: 1–15 June; second fortnight: 16–30 June; third fortnight: 1–15 July; fourth fortnight: 16–31 July). Percentages were normalised by arcsine transformation prior to analysis, followed by repeated-measures analysis of variance (ANOVA) and Tukey's test ($P < 0.05$). Within-subject pairwise comparisons were used to determine the time effects on fruit damage among the estimated marginal means with Bonferroni correction ($P < 0.05$).

Following established criteria, plots were harvested when the 80% red fruit stage was reached. All tomato fruits in the central 1.5 m^2 of each plot were manually picked on a single occasion to simulate mechanical harvesting. Fruits were individually inspected, weighed and classified into one of five groups: unmarked green fruits, damaged green fruits, unmarked red fruits, scarred red fruits and rotten red fruits. Scarred red fruits were marketable fruits, according to quality standards required by the processing industry, in which larval damage was superficial and well healed, whereas rotten red fruits were unmarketable fruits in

which larval perforations were recent, unhealed, deep and usually rotting.

For analysis, fruit production was expressed in t ha^{-1}, estimated from the harvested weight of tomatoes (kg m^{-2}) in each plot. The total number of fruits, fruit weight, percentage of healthy fruits and total yield per hectare were subjected to two-way factorial ANOVAs to examine the effects of insecticide, the number of sprays applied and their interaction. Percentage values were normalised by arcsine transformation prior to analysis. Mean separation was performed by Tukey's test ($P < 0.05$). All analyses were performed using Systat (2000)[38] statistical software.

2.5 Comparison of OB and insecticide persistence

The estimated UV radiation during the greenhouse trials was provided by the Instituto Geofisico Dom Luis Meteorological Station (Instituto Português do Mar e da Atmosfera, IPMA), while the data in open-field trials in Badajoz were collected by the Badajoz Meterological Station (Agencia Estatal de Meteorología, AEMET).

During greenhouse trials, three terminal leaflets were randomly collected from 15 leaves located in the upper half of the central plants of each plot. These samples were taken at 1, 60, 132 and 204 h post-application. In field-grown tomato crops, three terminal leaflets were collected from 30 leaves from the upper half of 15 treated plants at 1, 60, 156 and 228 h post-application. The three leaflets from each leaf were pooled, placed in labelled polythene bags, immediately frozen and stored at −20 °C until use. Therefore, greenhouse trials had four replicate plots per treatment, for each collection time, while open-field trials involved eight replicate plots per treatment. The concentrations of viable OBs and each commercial insecticide on leaflet samples were estimated by bioassay. For this, frozen leaflets were triturated, and a 2 g sample (wet weight) of each leaflet sample was thoroughly mixed with 8 g of artificial diet. The resulting mixture was divided equally among five wells of a 24-well plate. A single second-instar larva was placed in each well and incubated at 25 ± 2 °C, 70–80% relative humidity and 16:8 h L:D photoperiod. Therefore, each replicate in greenhouses was assayed using 75 larvae (five larvae were used for each of the 15 pooled leaflet samples), whereas in open-field trials each replicate was tested using 150 larvae (five larvae for each of the 30 pooled leaflet samples). Larval mortality was recorded daily for 7 days.

The relationship between the prevalence of mortality observed in the bioassay and the insecticide concentration on leaf surfaces was determined by prior calibration of the bioassay technique. For this, 10 g of homogenised leaves collected prior to the application of insecticide treatments and 40 g of artificial diet were mixed with one of the five different concentrations of insecticides: 10^5, 10^6, 10^7, 10^8 and 10^9 OBs of HearSP1B:LB6 or HearSP1 L^{-1} diet; 1.6, 3.2, 8.0, 20 and 40 mg of Turex L^{-1} diet (equivalent to 0.8–20 mg AI L^{-1} *Bt*); 0.016, 0.625, 0.25, 1.0 and 4.0 µL L^{-1} of Spintor (equivalent to 0.0077–1.9 mg AI L^{-1} spinosad); 0.32, 1.6, 8.0, 40 and 200 mg L^{-1} for Dursban (equivalent to 0.24–150 mg AI L^{-1} chlorpyrifos). For each insecticide–diet combination, the resulting mixture was divided equally among the 48 wells of two 24-well plates. A single second-instar larva was placed in each well and incubated at 25 ± 2 °C, 70–80% relative humidity and 16:8 h L:D photoperiod during a week. As a negative control, a 10 g sample of untreated homogenised leaves was mixed with 40 g of artificial diet and included in the bioassay. The entire calibration procedure was performed in triplicate. Logit regressions of larval mortality on the logarithm of insecticide concentration were computed (Table 1), and the quantities of the different insecticides per gram of leaf material were estimated by comparing the percentage mortality of larvae that consumed diet + leaf sample mixtures with the corresponding calibration curve for each insecticide. The logarithm of residual insecticide estimated concentrations was subjected to repeated-measures analysis of variance (ANOVA) using SPSS software. The significance of time effects on insecticidal persistence was determined by within-subject pairwise comparisons among the estimated marginal means with Bonferroni correction ($P < 0.05$). In addition, the Pearson coefficient was calculated in order to determine the correlation between insecticidal persistence and incident UV radiation, as both variables were normally distributed.

Table 1. Logit regressions of larval mortality on the concentration of insecticides per gram of leaves, used to calibrate the persistence bioassay

	Treatment	Logit regression	log (units)
Greenhouse	HearSP1B:LB6 OBs	$y = 28.30x - 53.85$	OBs
	Bt	$y = 43.37x + 127.44$	mg
	Spintor	$y = 55.00x + 223.22$	µL
Open field	HearSP1B:LB6 OBs	$y = 24.28x - 49.73$	OBs
	HearSP1 OBs	$y = 23.30x - 45.70$	OBs
	Bt	$y = 41.32x + 122.38$	mg
	Spintor	$y = 55.72x + 224.40$	µL
	Chlorpyrifos	$y = 62.20x + 178.91$	mg

3 RESULTS

3.1 Determination of OB concentration

No virus mortality was registered in control larvae reared following exposure to control plants, indicating the absence of natural or accidental contamination of experimental plants, and that the *H. armigera* colony insects used to infest the tomato plants were healthy. The percentages of larval mortality due to polyhedrosis disease in insects that fed on plants treated with 10^9 OBs L^{-1} were 88.9, 96.7 and 88.0% in larvae collected at 1, 3 and 5 days after virus application respectively. These percentages increased to 100% at every collection time in plants treated with 10^{10} and 10^{11} OBs L^{-1} (Fig. 1). No significant differences were observed in the prevalence of virus-induced mortality among the different concentrations tested ($F_{2,6} = 4.96$, $P > 0.05$). As 10^{10} OBs L^{-1} was the lowest concentration that provided 100% mortality of experimental insects on tomato plants, this concentration was selected for use in greenhouse and open-field trials.

3.2 Greenhouse trials

In the first trial, the number of released larvae that disappeared from plants, i.e. the reduction in the initial infestation,[39] was taken as an indicator of total larval mortality, regardless of whether it was specifically related to the treatments applied. At 10 days post-application, significantly fewer larvae were present in plots treated with HearSP1B:LB6 OBs or commercial insecticides (93.2–97.9% mortality) than in control plots (75.0%) ($F_{3,12} = 48.6$, $P < 0.05$) (Fig. 2A). HearSP1B:LB6 OBs (97.9%) and spinosad (97.9%) treatments resulted in significantly higher larval mortality than *Bt* (93.2%) (Tukey's test, $P < 0.05$) (Fig. 2A). Larval mortality was inversely correlated with fruit damage (Pearson's $r = -0.92$); plants treated with HearSP1B:LB6 OBs or either of the commercial insecticides had significantly fewer damaged fruits

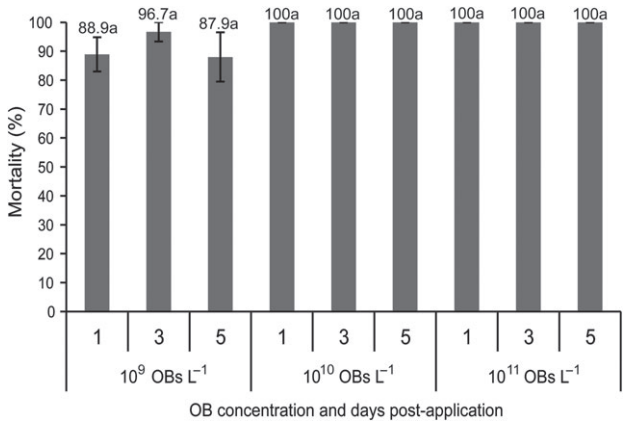

Figure 1. Percentage of virus-induced mortality in second-instar *H. armigera* larvae that fed on tomato leaves treated under laboratory conditions for 1, 3 and 5 days after the application of 10^9, 10^{10} and 10^{11} HearSP1B:LB6 OBs L^{-1}. Bars labelled with the same letters did not differ significantly (repeated-measures ANOVA followed by Tukey's test at $P < 0.05$, see text). Vertical lines indicate the standard error.

(12.1–17.3%) than control plants (25.3%) ($F_{3,12} = 9.9$, $P < 0.05$) (Fig. 2A). However, the different treatments resulted in similar percentages of damaged fruits, with 12.1% of damaged fruit in plots treated with HearSP1B:LB6 OBs, 16.3% in *Bt*-treated plots and 17.3% in spinosad-treated plots (Tukey's test, $P > 0.05$) (Fig. 2A).

The results of the second trial were very similar to those of the first trial. Larval mortality at 10 days post-application was significantly higher in plots treated with the different insecticides (90.6–100%) than in control plots (72.9%) ($F_{3,12} = 20.3$, $P < 0.05$) (Fig. 2B). Plots treated with HearSP1B:LB6 OBs (100%) resulted in significantly higher larval mortality than *Bt* (90.6%) (Tukey's test, $P < 0.05$), whereas spinosad resulted in an intermediate prevalence of mortality (97.9%) (Tukey's test, $P > 0.05$) (Fig. 2B). Larval mortality was inversely correlated with fruit damage (Pearson's $r = -0.94$); plots treated with viral OBs or commercial insecticides had significantly fewer damaged fruits (11.9–15.5%) than control plots (28.9%) ($F_{3,12} = 7.3$, $P < 0.05$). No significant differences were observed in the percentages of damaged fruits among the insecticide-treated plots (15.4, 15.5 and 11.9% for HearSP1B:LB6, *Bt* and spinosad respectively) (Tukey's test, $P > 0.05$) (Fig. 2B).

3.3 Open-field trial

No significant differences were detected between plots sprayed 3 or 5 times for any of the variables studied ($F_{1,33} < 3.12$, $P > 0.09$ in all cases), or in the interaction insecticide × number of applications ($F_{5,33} < 2.36$, $P > 0.07$ in all cases). Therefore, the results for plots with different numbers of applications (three or five) were pooled for all subsequent analyses.

Fruit damage (recent and scarred) showed a clear seasonal pattern (Fig. 3). Significant differences between 14 day intervals (fortnights) ($F_{3,69} > 76.91$, $P < 0.001$ in all cases), insecticides ($F_{5,69} > 15.92$, $P < 0.001$ in all cases) and also in the fortnight × insecticide interaction ($F_{15,69} = 5.24$, $P < 0.001$) were recorded, so that each 14 day period was considered separately in the following one-way ANOVAs.

In the first fortnight, no differences were observed in the percentage of damaged fruits, which was always lower than 1% in all insecticide treatments ($F_{5,15} = 0.72$, $P = 0.62$) (Fig. 3A). However, in the second and third fortnights (Figs 3B and C), control plots presented more damaged fruits, either scarred or recent

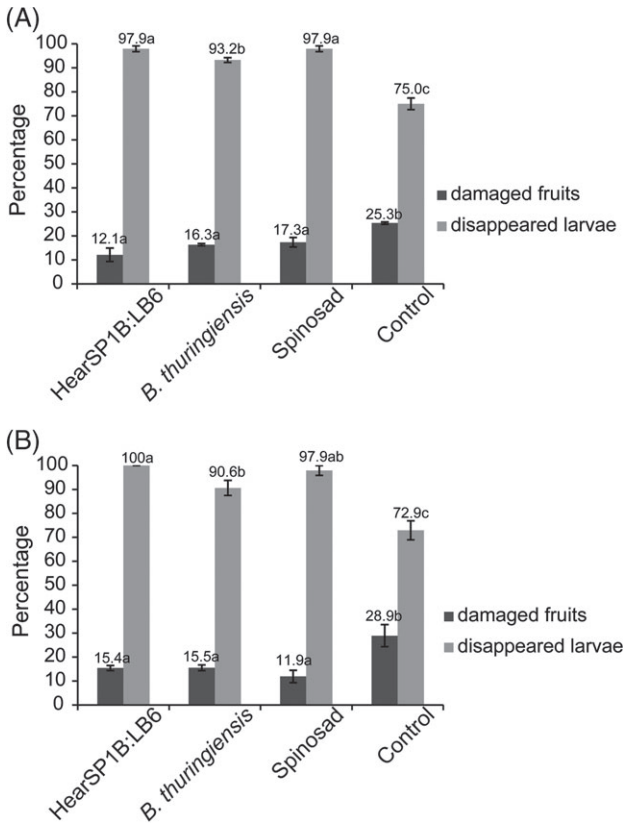

Figure 2. Percentage reduction in infestation (dead and disappeared larvae) and percentage damaged fruits on protected tomato plants at 10 days after the application of HearSP1B: LB6 OBs, *Bt* and spinosad: (A) first trial; (B) second trial. Bars labelled with the same letters did not differ significantly (repeated-measures ANOVA followed by Tukey's test at $P < 0.05$, see text). Vertical lines indicate the standard error.

(11.2–21.2%), than plots treated with the different insecticides (3.7–7.2%) (second fortnight: $F_{5,15} = 18.68$, $P < 0.001$; third fortnight: $F_{5,15} = 54.76$, $P < 0.001$). Finally, in the fourth fortnight, the percentages of scarred fruits differed significantly among insecticides ($F_{5,15} = 44.28$, $P < 0.001$), with significantly lower values in insecticide-treated plots (2.1–4.7%) than in control plots (14.7%), whereas recent damage was very low (<0.4%) and similar among all treatments ($F_{5,15} = 1.00$, $P = 0.45$) (Fig. 3D).

The percentages of fruits with recent damage were higher in the second fortnight (1.3–3.4%) compared with the first (0.3–0.9%), third (0.2–0.7%) and fourth fortnights (0.05–0.2%) in plots treated with the different insecticides ($F_{3,9} > 41.2$, $P < 0.001$ in all cases). In the control treatment, the percentage of recently damaged fruits was higher in the second (5.6%) and third (3.6%) fortnights than in the first (0.5%) or fourth (0.4%) fortnights ($F_{3,9} = 12.7$, $P < 0.002$). Lower percentages of scarred fruits were observed in the first fortnight (0.1% in all cases), compared with the second, third and fourth fortnights, in plots treated with the different insecticides (3.8–6.2%, 2.7–3.8%, 2.4–6.6%, 3.6–4.4% and 2.1–3.3% for HearSP1B:LB6, HearSP1, *Bt*, spinosad and chlorpyrifos respectively) ($F_{3,9} > 9.1$, $P < 0.05$ in all cases). In control plots the percentages of scarred fruits increased significantly over time until the third fortnight (0.1, 6.6 and 17.6% for the first, second and third fortnights respectively) and was 14.7% in the fourth fortnight ($F_{3,9} = 69.7$, $P < 0.001$). As a result, similar quantities of green undamaged fruits were harvested in all insecticide treatments (8.5–12.3 t ha^{-1}) ($F_{5,39} = 0.70$, $P = 0.63$)

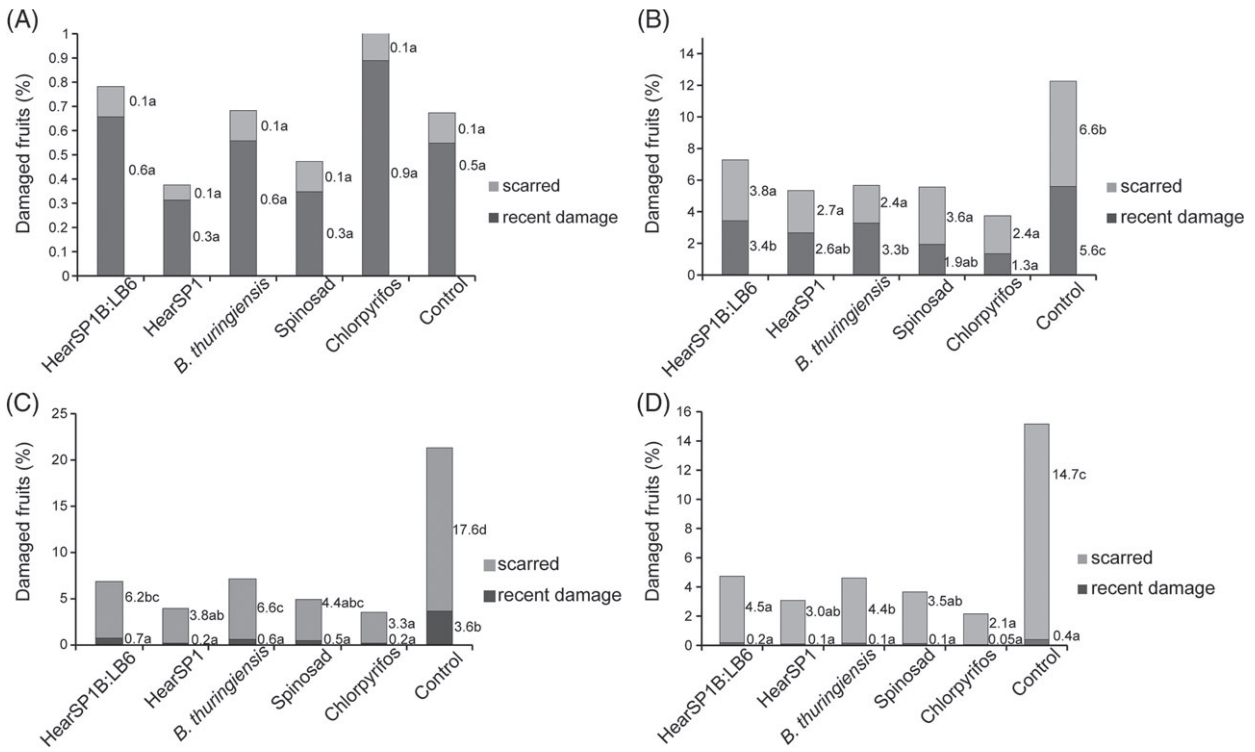

Figure 3. Percentages of damaged fruits, either scarred or recent, in field-grown tomato crops after the application of HearSP1B:LB6 OBs, HearSP1 OBs, *Bt*, spinosad and chlorpyrifos, and their seasonal progression through the four fortnights of the months of June and July: (A) first fortnight; (B) second fortnight; (C) third fortnight; (D) fourth fortnight. Bars labelled with the same letters did not differ significantly (ANOVA followed by Tukey's test at $P < 0.05$, see text).

Table 2. Mean ± SE yield (t ha^{-1}) of green fruits, either damaged or undamaged, and red fruits, either rotten, scarred or undamaged, in open-field tomato crops after the application of HearSP1B:LB6, HearSP1, *Bt*, spinosad and chlorpyrifos[a]

	Green fruits		Red fruits		
	Undamaged	Damaged	Undamaged	Scarred	Rotten
HearSP1B:LB6	10.4 ± 2.0 a	0.4 ± 0.1 a	153.7 ± 9.6 a	5.2 ± 0.5 ab	7.7 ± 0.9 ab
HearSP1	9.1 ± 2.1 a	0.3 ± 0.1 a	144.8 ± 3.4 a	4.3 ± 0.5 ab	8.9 ± 1.3 b
Bt	8.5 ± 1.3 a	0.3 ± 0.1 a	142.6 ± 9.6 ab	6.0 ± 1.0 b	8.3 ± 0.8 b
Spinosad	8.5 ± 1.1 a	0.1 ± 0.05 a	165.6 ± 14.1 a	3.0 ± 0.5 a	7.2 ± 1.0 ab
Chlorpyrifos	12.3 ± 2.6 a	0.3 ± 0.1 a	153.5 ± 7.4 a	3.0 ± 0.4 a	5.0 ± 0.6 a
Control	11.3 ± 2.0 a	1.2 ± 0.4 b	120.8 ± 6.2 b	18.1 ± 1.7 c	14.9 ± 1.3 c

[a] Values followed by the same letters within the same column did not differ significantly (ANOVA followed by Tukey's test at $P < 0.05$).

(Table 2). In contrast, yields of red undamaged fruits differed significantly among insecticide treatments ($F_{5,39} = 2.78$, $P = 0.03$), with higher yields in insecticide-treated plots (142.6–165.6 t ha^{-1}) than in control plots (120.8 t ha^{-1}) (Table 2). Similarly, lower quantities of either green damaged fruits (0.1–0.4 t ha^{-1}, representing 1.1–3.2% of harvested fruits) ($F_{5,39} = 4.95$, $P < 0.002$), scarred red fruits (3.0–6.0 t ha^{-1}, representing 2.0–4.2% of harvested fruits) ($F_{5,39} = 42.55$, $P < 0.001$) or rotten red fruits (5.0–8.9 t ha^{-1}, representing 4.3–7.6% of harvested fruits) ($F_{5,39} = 10.15$, $P < 0.001$) were collected in insecticide-treated plots compared with control plots: 1.2, 18.1 and 14.9 t ha^{-1} for green, scarred and rotten fruits respectively, equivalent to 9.7, 14.7 and 13.9% of harvested fruits respectively (Table 2).

The prevalence of scarred fruits was higher in the *Bt*-treated plots (6.0 t ha^{-1}, representing 4.2% of harvested fruits) compared with spinosad or chlorpyrifos treatments (3.0 t ha^{-1}, representing 2–3% of harvested fruits), whereas HearSP1B:LB6 and HearSP1 OB applications resulted in intermediate values of scarred fruits (5.2 and 4.3 t ha^{-1}, representing 3.8 and 3.5% of harvested fruits respectively) (Tukey's test, $P < 0.05$) (Table 2). Application of chlorpyrifos resulted in significantly fewer rotten fruits (5.0 t ha^{-1}, representing 4.3% of harvested fruits) compared with HearSP1 OB (8.9 t ha^{-1}, representing 7.6% of harvested fruits) or *Bt* (8.3 t ha^{-1}, representing 7.2% of harvested fruits) treatments, whereas HearSP1B:LB6 OB (7.7 t ha^{-1}, representing 6.6% of harvested fruits) and spinosad (7.2 t ha^{-1}, representing 5.2% of harvested fruits) treatments resulted in intermediate values (Tukey's test, $P < 0.05$) (Table 2).

3.4 Insecticide persistence

The average accumulated dose of UV radiation inside the greenhouse during the sampling period was 4134, 10 294 and 16 585 J m^{-2} at 3, 6 and 9 days after treatment respectively (Fig. 4),

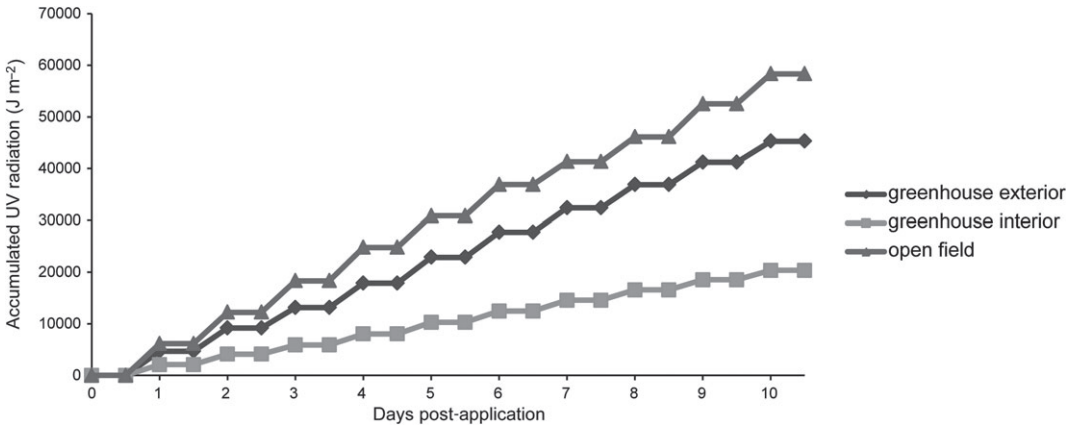

Figure 4. Accumulated ultraviolet (UV) radiation dose (J m^{-2}) during the field trials in protected crops (greenhouse exterior and greenhouse interior) and open-field tomato crops. UV radiation inside the greenhouse (greenhouse interior) was ~45% of the UV radiation outside the greenhouse (greenhouse exterior).

taking into account that the plastic structure and whitewash treatment intercepted ~55% of incident UV radiation, which allowed pollinators to function within the greenhouse (Fig. 4).

The activity of all insecticides on plants decreased significantly over time ($F_{3,9} = 18.9$, $P < 0.05$ for HearSP1B:LB6; $F_{3,9} = 170.5$, $P < 0.05$ for *Bt*; $F_{3,9} = 8.3$, $P < 0.05$ for spinosad) and was negatively correlated with the accumulated dose of UV radiation (Pearson's $r = -0.89$, -0.99 and -0.99 for HearSP1B:LB6 OBs, *Bt* and spinosad respectively). Insecticide concentrations on plant surfaces at 1 h after spraying were estimated to be $2.73 \times 10^5 \pm 8.44 \times 10^2$ HearSP1B:LB6 OBs g^{-1} leaf material (wet weight), 0.201 ± 0.016 mg g^{-1} of *Bt* and 0.0056 ± 0.0002 µL g^{-1} of Spintor, which were taken to represent the initial (100%) value of insecticidal residues. At 3 and 6 days after application, the residual activity of HearSP1B:LB6 OBs on leaves was similar to the activity observed at 1 h after application (94.0–87.7% residual activity), but decreased significantly at 9 days post-application to 13.3% of initial activity (Fig. 5A). The *Bt* and spinosad treatments showed similar patterns of persistence, with 73.6 and 80.5% of initial activity remaining at 3 days post-application respectively, falling to 7.2 and 15.1% at 9 days post-application respectively (Figs 5B and C).

In open-field crops, no rainfall occurred during the field trial, and the accumulated doses of UV radiation at 3, 7 and 10 days post-treatment were 12 224, 36 885 and 52 517 J m^{-2} respectively (Fig. 4). Residual insecticidal activities on plants decreased significantly over time ($F_{3,21} = 54.3$, $P < 0.001$ for HearSP1B:LB6; $F_{3,21} = 139.4$, $P < 0.001$ for HearSP1; $F_{3,21} = 38.5$, $P < 0.001$ for *Bt*; $F_{3,21} = 12.3$, $P < 0.001$ for spinosad; $F_{3,21} = 37.1$, $P < 0.001$ for chlorpyrifos). Residual insecticidal activity was negatively correlated with accumulated UV radiation (Pearson's $r = -0.92$, -0.96, -0.88, -0.99 and -0.96 for HearSP1B:LB6, HearSP1, *Bt*, spinosad and chlorpyrifos respectively). Similarly to the results observed in protected crops, the concentration of insecticide on leaves at 1 h after treatment was $1.4 \times 10^6 \pm 1.1 \times 10^3$ HearSP1B:LB6 OBs g^{-1}, $1.7 \times 10^6 \pm 4.8 \times 10^2$ HearSP1 OBs g^{-1}, 0.26 ± 0.011 mg g^{-1} of *Bt*, 0.0058 ± 0.0009 µL g^{-1} of Spintor and 0.054 ± 0.0021 mg g^{-1} of chlorpyrifos; these values were taken as the initial (100%) residual activities. Three days after application, the residual activity of HearSNPV OBs had decreased to 40.6 and 62.5% of initial activity for HearSP1B:LB6 and HearSP1 OBs respectively (Figs 5D and E). The activity of HearSNPV OBs continued to decrease to 0.1% of initial activity for HearSP1B:LB6 OBs and 0.05% for HearSP1 OBs at 10 days post-application (Figs 5D and E). In the *Bt* treatment, the residual activity was 33.3% at 3 days post-application, decreasing to 4.9% of initial activity at 10 days post-application (Fig. 5F). The residual activity of spinosad decreased gradually to 21.9% at 10 days post-application (Fig. 5G). Similarly, the activity of chlorpyrifos decreased gradually to 15.6% of initial activity at 10 days post-application (Fig. 5H).

4 DISCUSSION

In the present study, the efficacy and persistence of a binary mixture of HearSP1B and HearLB6 variants co-occluded into OBs[22] were evaluated as the basis for a biological insecticide for control of *H. armigera* on tomato crops grown under greenhouse and open-field conditions in southern Spain and Portugal. Initial growth chamber studies indicated that a concentration of 1×10^{10} HearSP1B:LB6 OBs L^{-1} resulted in 100% mortality of experimental larvae on treated tomato plants. This concentration was estimated to be equivalent to 1×10^{13} OBs ha^{-1} when applied in a spray volume of 1000 L ha^{-1}, which is usual for fruiting tomato plants. Similarly, an unformulated strain of HearSNPV was tested for control of this pest at concentrations of $2.87 \times 10^8 - 1.35 \times 10^{11}$ OBs L^{-1} in greenhouse and field-grown tomatoes and citrus in South Africa, although a rate of 1.15×10^9 OBs L^{-1} (equivalent to 1.15×10^{12} OBs ha^{-1} in an application volume of 1000 L) was selected for further testing and provided excellent pest control.[39] Studies elsewhere have indicated that applications of approximately 1×10^{12} OBs ha^{-1} provides control of this pest in Thailand, India and Botswana on a diversity of crops.[40–42] The high quantities of OBs that we applied, although highly effective as a pest control agent, probably require evaluation at lower concentrations ($10^{11} - 10^{12}$ OBs ha^{-1}) in order to ensure that a virus-based product can be produced at an economically feasible cost.[43] We selected the higher concentration (10^{13} OBs ha^{-1}) rather than the lower one (10^{12} OBs ha^{-1}), given the fear that OBs would be rapidly inactivated owing to the harsh conditions, with high temperatures and intense sunlight, that occur in southern Spain and Portugal during the summer months.

In greenhouses, the HearSP1B:LB6 OB treatment was as effective as spinosad at reducing larval infestation and slightly more effective than the *Bt* treatment. However, the degree of fruit damage was similar among virus, *Bt* and spinosad treatments, which were consistently lower than that of the control. The reduction in infestation (close to 70%) observed in control plots at 10 days after

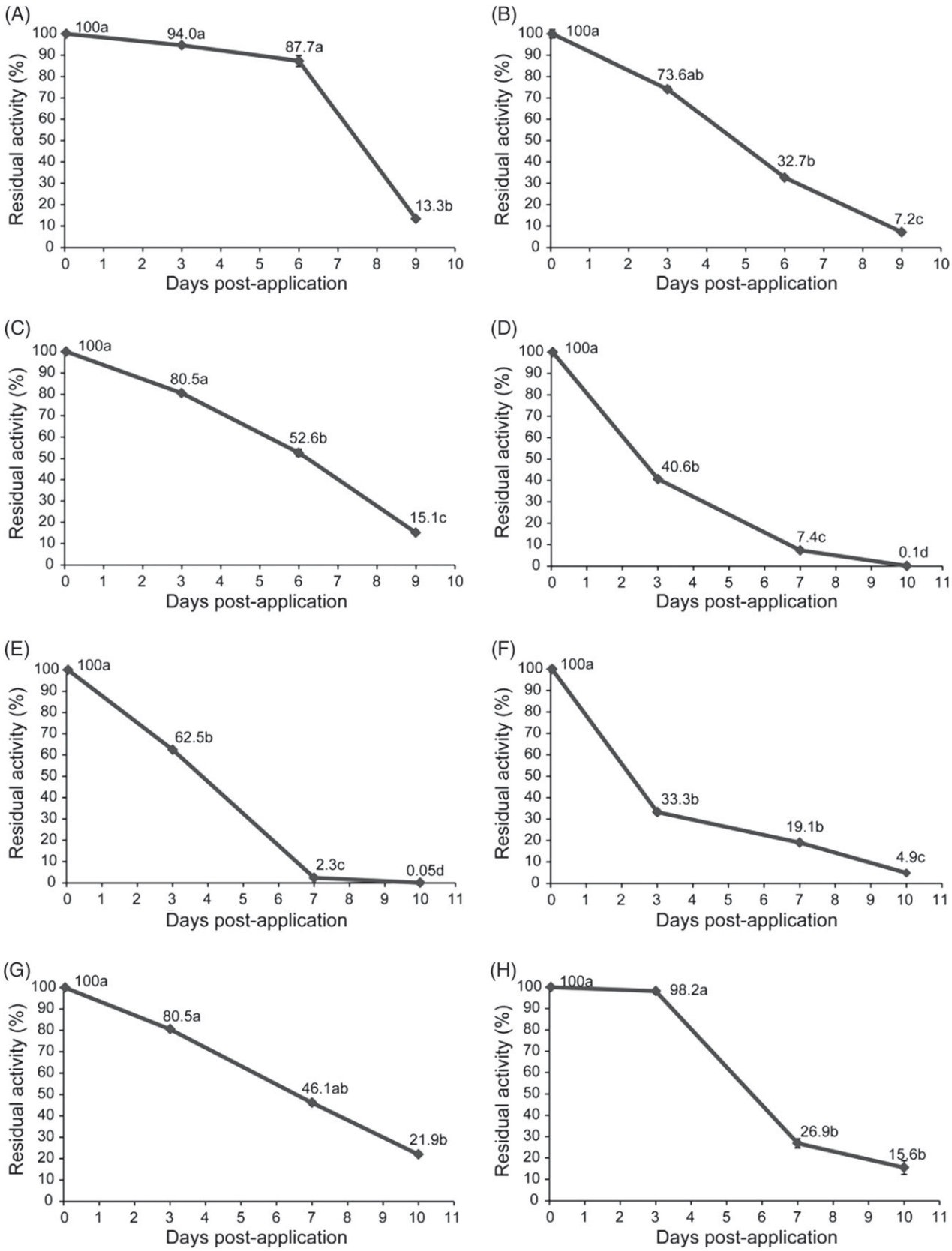

Figure 5. Percentage of insecticide residue on tomato leaves at various intervals after application relative to the quantity of insecticide at 1 h after application: (A) HearSP1B:LB6 OBs, (B) *Bt* and (C) spinosad in protected tomato crops; (D) HearSP1B:LB6 OBs, (E) HearSP1 OBs, (F) *Bt*, (G) spinosad and (H) chlorpyrifos in field-grown tomato crops. Bars labelled with the same letters did not differ significantly (repeated-measures ANOVA with intrasubject pairwise comparison of estimated marginal means, $P > 0.05$, Bonferroni correction, see text). Vertical lines indicate the standard error.

insecticide application might have influenced the results. Previous studies have reported significant reductions in larval infestations after HearSNPV applications, comparable with those of *Bt* or synthetic insecticides, both in protected and field-grown tomato crops.[39,44,45] Moore *et al.*[39] also reported pest mortality exceeding 80% in control plots artificially infested with *H. armigera* eggs 10 days after treatment application, which was attributed to natural mortality and the inability to detect larvae once they had penetrated plants. Fortunately, the fact that the two greenhouse trials produced near-identical results provides strong additional support to the validity of these findings.

The timing of application is an important determinant of insecticide efficacy, as the phenological state plays a crucial role in the association of *H. armigera* with the tomato crop. For instance, before or after flowering, tomato plants are less attractive to ovipositing *H. armigera* females, and mature tomatoes are similarly suboptimal for larval development.[3] Therefore, late insecticide applications, during the tomato maturation period, often have little or no effect in reducing larval damage, because larval densities in the crop are usually low during this phenological stage. This is most likely the reason why the fourth and fifth insecticide applications, which were applied when more than 50% of tomatoes were red, did not improve the degree of crop protection.

The percentage of damaged fruits at harvest was lower in plots treated with HearSNPV OBs (13.6–14.3% of damaged fruits) than in control plots (38.3%), and similar to that observed with the other insecticides tested (8.4–14.3%), which is in agreement with previous studies performed in Australia.[46] As scarred red fruits are the only pest-damaged fruits accepted by processing plants, and given that most green and red rotten fruits are discarded at harvest, the similar yields of marketable tomato fruits (red undamaged fruits) obtained in plots treated with HearSNPV OBs, compared with plots treated with commercial insecticides, indicates that this virus can be a highly effective pest control agent. HearSNPV-based treatments resulted in less than 4% of scarred red fruits, comparable with the figure achieved with the commercial insecticides (2–4.2%) and markedly lower than that of control plots (14%). Field-grown tomato plots treated with HearSNPV OBs in India also proved to be as effective in crop protection as chemical insecticides.[44] Similarly, Cherry *et al.*[47] also observed that chickpea crops treated with different formulations of HearSNPV OBs resulted in yields similar to those of plots treated with *Bt* or chemical insecticides. Interestingly, both the HearSP1B:LB6 and wild-type HearSP1 virus treatments provided similar levels of crop protection in the present study. This may be related to the high rate of application, so that the probability of consuming a lethal dose of OBs was similar for both virus preparations. As laboratory studies indicated that the co-occluded preparation was approximately threefold more pathogenic than the wild-type isolate, the greater insecticidal capacity may only become apparent when lower concentrations of OBs are applied to crops. This is an issue that requires further study in greenhouse and field crop trials.

The persistence of HearSNPV OBs and *Bt* on tomato leaves was markedly higher in the greenhouse than in the open field, whereas the residual persistence of spinosad was similar in the two environments. The persistence of OBs on plant surfaces determines the period during which a lethal dose can be ingested by susceptible insects.[30] Solar radiation is one of the most important factors affecting the persistence of OBs.[48,49] Consequently, inactivation of HearSNPV OBs was slower in protected than in open-field crops, as the plastic structure and whitewash coating are able to filter a large part of incident UV radiation.[32,50] In protected crops, 87% of HearSNPV OBs remained viable at 6 days after treatment, whereas in the open field just 7% of OBs remained viable after 7 days. Similar levels of OB persistence were observed on greenhouse-grown sweet pepper, in which 61% of *Spodoptera exigua* MNPV (SeMNPV) OBs retained their insecticidal activity 6 days post-application.[32] Furthermore, the physicochemical characteristics of the crop can also influence OB degradation, as exudates of some plants can rapidly inactivate OBs. For example, OBs on cotton leaf surfaces were rapidly inactivated by alkaline leaf exudates.[51] Similarly, on chickpea, HearSNPV OBs were degraded almost completely 7 days after treatment,[47] an effect attributed to the isoflavonoids present in leguminous plants.[52] In contrast, tomato is reported to be more favourable for OB persistence on treated foliar surfaces.[53–55] These observations underline the need for crop-specific persistence studies when developing a baculovirus-based bioinsecticide for use on different types of crop.

For the other insecticides, the persistence of spinosad and *Bt* on protected tomato plants was lower than that of HearSNPV OBs. Like the virus, both these insecticides are of natural origin and have gained importance over the past decade as growers have adopted products to be used in integrated pest management programmes that conserve natural enemy populations. The plastic structure of the greenhouse represents an important filter to the passage of UV light,[32,50] so that all the biological-based control measures that we tested probably benefited from partial protection from incident UV in the greenhouse setting. In contrast, in open-field crops, spinosad and chlorpyrifos persisted on tomato foliage for longer periods than HearSNPV OBs. The stability of spinosad, although sensitive to sunlight,[56] might be related to its ability to penetrate leaf tissues by translaminar movement, increasing its persistence under field conditions.[57]

The present study demonstrates that HearSP1B:LB6 OB applications, albeit at a high concentration, were as effective as commercial insecticides for controlling *H. armigera* damage in tomato crops. Moreover, the high persistence of OBs on tomato foliage favours the efficacy of this virus as an insecticide because pest larvae can consume OB-contaminated leaves over several days, increasing the likelihood of consuming a lethal dose. Additional advantages for baculovirus-based insecticides include a minimal safety period before harvest, the absence of xenobiotic residues in food produce and virtually no impact on populations of natural enemies present in greenhouse or open-field crops. Moreover, as baculoviruses are compatible with other pesticides, they may be used in combination with other control agents in IPM programmes in order to manage insecticide resistance.[58]

The results of this study are likely to provide useful efficacy information for the registration of the co-occluded HearSP1B:LB6 variant mixture as a biological insecticide for control of *H. armigera* on tomato crops in this region. Future studies should focus on determining the efficacy of lower OB concentrations at which the differences in the insecticidal characteristics of HearSP1B:LB6 and wild-type HearSP1 OBs may become evident. The use of formulations that improve field persistence and the efficacy of HearSP1B:LB6 OBs and the effective integration of virus-based insecticides with other biorational crop protection products are also issues that deserve additional studies.

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