Research Article

Received: 23 October 2017 Revised: 21 December 2017 Accepted article published: 3 January 2018 Published online in Wiley Online Library: 23 February 2018

(wileyonlinelibrary.com) DOI 10.1002/ps.4846

Remarkably efficient production of a highly insecticidal Chrysodeixis chalcites nucleopolyhedrovirus (ChchNPV) isolate in its homologous host

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

Abstract

BACKGROUND: A Chrysodeixis chalcites nucleopolyhedrovirus from the Canary Islands (ChchNPV-TF1) has proved to be effective for control of *Chrysodeixis chalcites* on banana crops. Commercialization of this virus as a bioinsecticide requires an efficient production system.

RESULTS: The sixth instar (L_6) was the most suitable for virus production, producing 1.80×10^{11} occlusion bodies (OB)/larva and showed a lower prevalence of cannibalism (5.4%) than fourth (L_4) or fifth (L_5) instars. Inoculation of L_6 at 24 h post molting produced six times more OB (5.72×10^{11} OB/larva) than recently molted L_6 larvae (1.00×10^{11} OB/larva). No significant differences were recorded in mean time to death (165–175 h) or OB production per larva (3.75×10^{11} to 5.97×10^{11}) or per mg larval weight (1.30×10^{11} to 2.11×10^{9}), in larvae inoculated with a range of inoculum concentrations (LC_{50}–LC_{90}). Groups of infected L_6 larvae reared at a density of 150 larvae/container produced a greater total number of OBs (8.07×10^{13} OB/container) than lower densities (25, 50 and 100 OB/container), and a similar number to containers with 200 inoculated larvae (8.43×10^{13} OB/container).

CONCLUSION: The processes described here allow efficient production of sufficient OBs to treat ~ 40 ha of banana crops using the insects from a single container.

Keywords: *Chrysodeixis chalcites*; Alphabaculovirus; ChchNPV; *in vivo* virus production; biological insecticide; cannibalism

1 INTRODUCTION

The tomato looper, *Chrysodeixis chalcites* (Esper) (Lepidoptera: Noctuidae) is considered a major lepidopteran pest in many parts of Europe, Africa and the Middle East.[1–4] This pest causes serious damage to banana crops in the Canary Islands,[3–5] and to horticultural greenhouse crops in Spain and other parts of Europe.[4] Previous studies revealed that an isolate of C. chalcites nucleopolyhedrovirus from southern Tenerife (Canary Islands), named ChchNPV-TF1, was a highly effective biological insecticide for control of this pest.[4,7–9] The pathogenicity and virulence of this strain is comparable with that of the most pathogenic and virulent baculoviruses currently commercialized as the basis for insecticidal products.[10–13]

Commercial production of baculovirus insecticides requires an efficient and economically viable occlusion body (OB) production system. As obligate pathogens, viruses require living hosts for replication, and this can be achieved only in host larvae (*in vivo*) or in cell culture (*in vitro*). The expenses associated with both these systems can make it difficult to produce virus-based insecticides at competitive costs.[14,15] In addition, *in vitro* system techniques, currently used in recombinant baculovirus expression systems, vaccine development and gene therapy,[16] face important technical constraints. For instance, cell culture production can lead to a variety of mutations in baculovirus genomes that can reduce the biological activity of insect viruses,[17–20] which together with the high cost of cell culture media, has hindered the development of suitable reactor-based production systems.[14,21]

At present, the only viable option for large-scale production of baculoviruses involves using the host insect as a biofactory, and so all the baculovirus products used against different insect pests worldwide are currently produced using *in vivo* production systems.[14,22–24] Each baculovirus requires a specific combination of conditions to maximize its production. Although most baculoviruses present a narrow host range, others have a wider host range and can be produced in several host species.[14]

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

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

b *Instituto de Ecología AC, Xalapa, Mexico*

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

In addition, efficient *in vivo* production systems also require optimization of diverse biological variables, particularly the larval stage at inoculation and inoculum concentration that strongly influence the total yield of OBs. These two parameters need to be precisely determined for each virus–host system to maximize the trade-off between speed of kill, larval biomass and OB production.[24,25] Larval rearing density during the period that follows inoculation is also influential in the growth and survival of infected insects.[23] In general, when cannibalism is not an issue of concern, larvae tend to be reared at the highest densities possible to reduce handling times, while keeping larval stress to a minimum to avoid reductions in host weight gain. However, cannibalism may be an issue when rearing late instar larvae gregariously, and this can greatly reduce survival and overall yields of OBs.[26]

In this study our aim was to evaluate the influence of inoculum concentration, growth stage at inoculation and rearing density on the quantities of ChchNPV-TF1 OBs produced in *C. chalcites* larvae from the same geographical region. Our findings should prove valuable for the commercial production of ChchNPV-based insecticides for control of this pest in banana and horticultural crops.

2 EXPERIMENTAL METHODS

2.1 Insect source, rearing and virus strain

Larvae of *C. chalcites* were obtained from a laboratory culture established in 2007 at the Universidad Pública de Navarra (Spain), using insects collected from banana crops in southern Tenerife, and maintained at the Instituto Canario de Investigaciones Agrarias (ICIA), Tenerife (Spain). This colony was refreshed periodically with insects collected from the same island. Larvae were reared at 25 ± 1 °C, 70 ± 5% humidity, and a photoperiod of 16: 8 h (light: dark), on a semi-synthetic diet described by Greene *et al.*[27] Adults were fed 30% w/v diluted honey. The ChchNPV-TF1 strain was originally isolated from a single larva collected during a natural epizootic in a banana crop in southern Tenerife, Canary Islands (Spain).[7] ChchNPV-TF1 was characterized and selected from among other strains due to its high pathogenicity, measured by concentration–mortality metrics, and rapid speed of kill. ChchNPV-TF1 OBs were amplified in a single passage in fifth instar *C. chalcites* larvae. OBs from virus-killed larvae were extracted, filtered through muslin, washed twice with 0.1% SDS and once with 0.1 M NaCl and finally resuspended in distilled water. Purified OBs were stored at 4 °C. All experimental procedures described in the following sections were performed at 25 ± 1 °C and 70 ± 5% humidity.

2.2 Effect of larval instar on OB production, cannibalism and mean time to death

Groups of 50 recently molted (2–8 h previously) *C. chalcites* larvae of each of the fourth, fifth and sixth instars (L_4, L_5 and L_6), were starved for 12 h and inoculated with ChchNPV-TF1 OBs using the droplet feeding method.[28] Inoculated larvae were placed individually in 30 ml plastic cups containing semi-synthetic diet, covered with a ventilated plastic cap and incubated until they died or pupated. A single concentration producing ∼ 90% larval mortality (LC_{90}) was used to inoculate each instar: 5.56×10^6, 5.00×10^7 and 9.02×10^8 OB/ml for L_4, L_5 and L_6, respectively.[8] As controls, identical numbers of larvae were inoculated with food dye and sucrose solution (10% sucrose and 0.001% Fluorella Blue dye), without OBs. This procedure was performed on three occasions using different batches of insects.

Larvae were checked every 8 h until death. When observed to be moribund (∼ 8 h prior to death), larvae were weighed individually, and placed individually in microcentrifuge tubes until death. However, L_4 larvae were too small to be weighed with consistent precision. Therefore, we could not obtain the value of OBs/mg larval weight in this instar. After death, all virus-killed larvae (at least 40 for each larval stage and replicate) were frozen and stored at −20 °C until required for OB titration. For this, cadavers were thawed, homogenized in 0.1% SDS, filtered through muslin and duplicate samples of OBs for each insect were counted in a Neubauer chamber. The influence of instar on the mean time to death (MTD) was analyzed by recording larval mortality at 8-h intervals.

To estimate cannibalism, the L_4, L_5 and L_6 stages were inoculated each with their corresponding LC_{90} concentration of OBs using the droplet feeding technique. Larvae that ingested the inoculum in 10 min were placed in groups of 50 in rectangular plastic containers of 13.5 cm (length) × 10.5 cm (width) × 8.5 cm (height) and covered with a ventilated plastic lid. A piece of semi-synthetic diet of 10 × 5 × 1 cm was placed in each container on top of a plastic grid. Groups of 50 larvae of each instar were inoculated with food dye and sucrose solution as negative controls. Numbers of larvae that reached the pupal stage, cannibalized larvae (that were partially or totally consumed), and virus-killed larvae were recorded daily. The experiment was performed on nine occasions using different batches of insects.

2.3 Effect of inoculation time on OB production and MTD

Groups of 25 L_6 insects were inoculated with 9.02×10^8 OB/ml, which was expected to result in 90% mortality,[8] using the droplet feeding method. Larvae were inoculated at one of two different physiological times during the L_6 stage: (1) recently molted (1–8 h post molting) and (2) 24 h after molting had occurred (24-h-old L_6). Inoculated larvae were placed individually in 30 ml plastic cups with artificial diet and incubated until death or pupation. Mortality was recorded at 8-h intervals to estimate MTD. Groups of 25 larvae of each physiological age were inoculated with food dye and sucrose solution without OBs as negative controls. The experiment was performed on three occasions using different batches of insects. Larvae were weighed individually when they were moribund (∼ 8 h prior to death), at which time they were placed individually in microcentrifuge tubes. Following death, larvae were homogenized in 0.1% SDS, OBs were extracted and filtered through muslin and duplicate samples were counted in a Neubauer counting chamber.

2.4 Effect of inoculum concentration on MTD and OB production

Five groups of 25 larvae (24-h-old L_6), were inoculated with a range of 2.5-fold dilutions: 9.02×10^8, 3.56×10^8, 1.41×10^8, 5.57×10^7 and 2.20×10^7 OB/ml, previously calculated to kill between 90% and 50% of larvae in a sample.[8] Each group of larvae was placed in one of the rectangular plastic containers with diet, as described previously. As controls, identical numbers of larvae were inoculated with food dye and sucrose solution, without OBs. To determine the influence of inoculum concentration on the MTD, larval mortality was recorded at 8-h intervals. Larvae were weighed when they were visibly moribund (∼ 8 h prior to death), and incubated individually in microcentrifuge tubes until death, whereupon OBs were extracted and duplicate samples from each larva were counted using a counting chamber. The experiment was performed on six occasions using different batches of insects.

2.5 Effect of larval rearing density on cannibalism rates and OB production

A batch of 600 *C. chalcites* larvae (24-h-old L_6) were orally inoculated with the LC_{90} (9.02×10^8 OB/ml) using the droplet-feeding method and distributed in groups of 1, 25, 50, 100, 150 and 200 in the rectangular plastic containers with diet, as mentioned previously. Identical numbers of larvae were inoculated with food dye and sucrose solution, without OBs, as negative controls. Each container was checked daily for 8 days post inoculation. The numbers of pupae and cannibalized larvae (disappeared or partially devoured) were noted and diseased larvae were collected and weighed immediately prior to death. Following death, virus-killed larvae were stored at −20 °C until required for titration, whereupon they were thawed, homogenized in 0.1% SDS, filtered through muslin and duplicate samples of OBs were counted in a Neubauer chamber. The experiment was performed 10 times using different batches of insects.

2.6 Statistical analysis

The means of final larval weights, OB production and OBs/mg larval weight values of each replicate were calculated and subjected to univariate analysis of variance (ANOVA), when data were normally distributed. OB production values across larval rearing densities were normalized by square root transformation ($\sqrt{x}$) prior to ANOVA. Comparisons of larval weights and OB production at different ages (following molting and 24 h later) were performed by *t*-test. The correlation between OB production and larval weight was examined by Pearson's coefficient. MTD values recorded at different larval stages, inoculation times and inoculum concentrations were determined by fitting Weibull survival models using the Generalized Linear Interactive Modeling (GLIM) program.[29] Finally, the average prevalence of pupation, cannibalized larvae (i.e., larvae that disappeared or were partially devoured) and virus-killed larvae in each replicate were all normally distributed and were subjected to ANOVA and Tukey test. All analyses, except Weibull survival analysis, were performed using SPSS v. 23 (IBM SPSS Statistics, Softonic, Barcelona, Spain).

3 RESULTS

3.1 Effect of larval instar on OB production, cannibalism and MTD

No virus-induced mortality was recorded in the control group. The yield of OBs from each larva was significantly affected by instar at inoculation ($F_{2,6} = 187.4$, $P < 0.001$), which increased from $8.87 \times 10^9 \pm 4.23 \times 10^8$ OB/larva in L_4 to $1.80 \times 10^{11} \pm 1.03 \times 10^{10}$ OB/larva in L_6 (Fig. 1A). Production was positively correlated with larval weight across the three instars tested (Pearson's $r = 0.982$, $P < 0.001$). The L_4, L_5 and L_6 stages differed significantly in MTD values ($F_{2,6} = 145.90$, $P < 0.001$), with MTD ± SE values of 140 ± 1, 152 ± 1 and 167 ± 1 h, respectively.

The prevalence of cannibalism, defined as larvae that disappeared or were partially devoured, differed significantly with instar, being lowest in L_6 (5.3%), highest in L_4 (18%) and intermediate in L_5 ($F_{2,24} = 13.7$, $P < 0.001$) (Fig. 1B). Larvae that were not victims of cannibalism either succumbed to polyhedrosis disease (79–86%), the prevalence of which did not differ significantly among instars ($F_{2,24} = 2.4$, $P = 0.109$), or pupated (2.8–8.4%), which was significantly more prevalent in L_6 than in L_4 ($F_{2,24} = 5.3$, $P = 0.012$) (Fig. 1B).

According to these results, although L_6 larvae took longest to die, this instar yielded greater numbers of OBs and showed a lower prevalence of cannibalism than the other instars, and so L_6 was selected as the most appropriate stage for use in the subsequent studies.

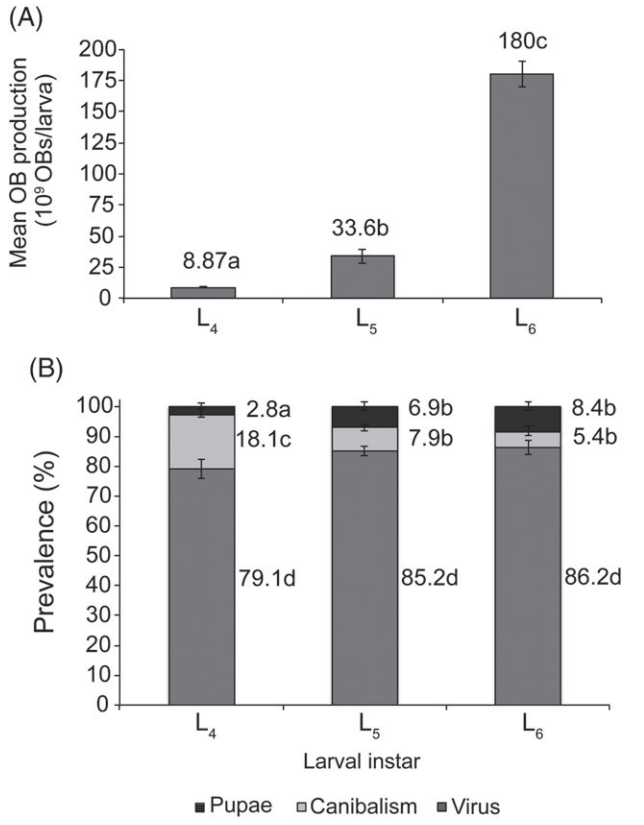

Figure 1. A) Mean OB production of ChchNPV-TF1 in L_4, L_5, and L_6 *C. chalcites* larvae. Values above bars indicate average OB production. Different letters accompanying values indicate significant differences between them (Tukey, $P < 0.05$). B). Percentages of larvae reaching the pupal stage, larval cannibalism and virus-killed larvae in *C. chalcites* L_4, L_5 and L_6 instars inoculated with their corresponding LC_{90} of OBs.

3.2 Effect of inoculation time on OB production and MTD

No virus-induced mortality was registered in the control group. At 24 h after molting, L_6 insects weighed 1.4 times more than newly molted L_6 ($t = 13.8$; d.f. = 5; $P < 0.001$), with average (± SE) live weights of 315 ± 41 and 230 ± 36 mg, respectively. However, this increase in larval weight resulted in a 5.7-fold increase in the average number of OBs produced per larva ($t = 3.156$; d.f. = 5; $P = 0.025$) (Fig. 2A). Similarly, the efficiency of OB production (OB/mg larval weight) increased by 4.2-fold in 24-h-old L_6 larvae compared with recently molted insects ($t = 3.573$; d.f. = 5 $P = 0.016$) (Fig. 2B). Mean OB production per larva was positively correlated with larval weight (Pearson's $r = 0.981$, $P = 0.001$). Finally, MTD values did not differ significantly, with a MTD value of 168 h for L_6 larvae and 176 h for 24-h-old L_6 larvae.

3.3 Effect of inoculum concentration on MTD and OB production

Virus-induced mortality was not registered in the control group. Inoculum concentration did not significantly affect the final weight ($F_{4,25} = 0.905$, $P = 0.476$), or the MTD of infected larvae. Diseased insects died with mean body weights between 290 ± 35 and 358 ± 48 mg and at between 165 and 175 h post infection,

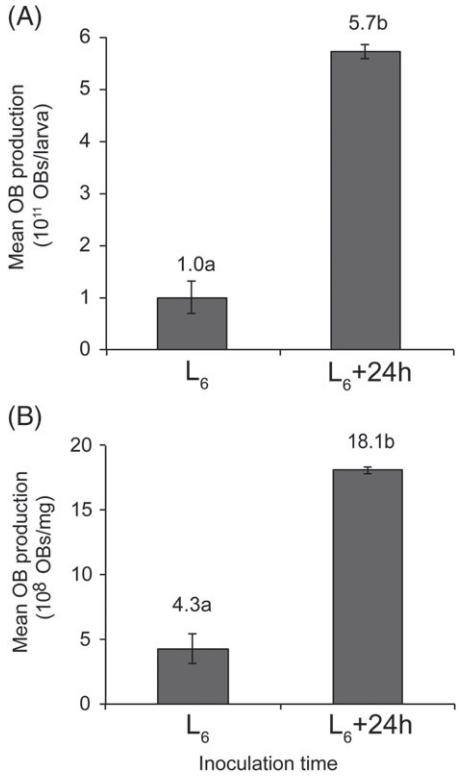

Figure 2. A) Mean OB production per larva of ChchNPV-TF1 (OBs/larva) and B) mean OB production per mg of larva of ChchNPV-TF1 (OBs/mg) in *C. chalcites* L_6, newly molted (L_6) or 24 h after molt to L_6 had occurred ($L_6 + 24$). Values above bars indicate mean OB production. Different letters accompanying values indicate significant differences between these values (Tukeyt-test, $P < 0.05$).

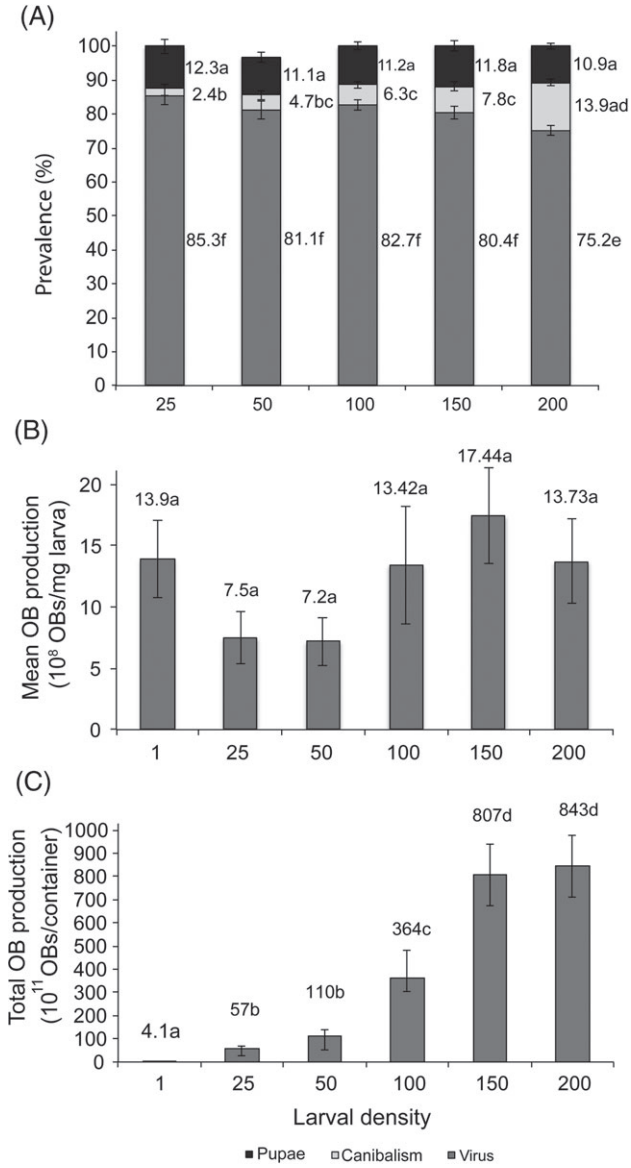

Figure 3. A) Percentages of larvae reaching the pupal stage, larval cannibalism and virus-killed larvae reared at larval densities: 1, 25, 50, 100, 150 and 200 in rectangular plastic containers. Twenty-four h old L_6 *C. chalcites* larvae were inoculated with the ChchNPV-TF1 LC_{90} (9.02×10^8 OBs/ml). B) Mean OB production per mg of larva (OBs/mg) and C) total OB production per container in 24-h-old L_6 *C. chalcites* larvae in rectangular plastic containers at larval densities: 1, 25, 50, 100, 150 and 200. Values above bars indicate mean OB production. Same letters accompanying values indicate no significant differences between them (Tukey, $P > 0.05$).

depending on inoculum concentration. Similarly, no significant differences were observed in mean OB production per larva ($F_{4,25} = 0.359$, $P = 0.835$), ranging from $3.75 \times 10^{11} \pm 1.19 \times 10^{11}$ to $5.97 \times 10^{11} \pm 1.79 \times 10^{11}$ OB/ml. Also, OB production/mg larval weight varied from $1.30 \times 10^9 \pm 4.63 \times 10^8$ to $2.11 \times 10^9 \pm 6.66 \times 10^8$ OB/mg larva but did not differ significantly among the inoculum concentrations tested ($F_{4,25} = 0.408$, $P = 0.801$). However, the highest inoculum concentration (9.08×10^8 OB/ml) resulted in the highest percentage of virus-killed larvae (97%) and therefore the highest overall OB yield (7.17×10^{12} OBs per group of 25 inoculated larvae).

3.4 Effect of larval density on OB production

No virus-induced mortality was recorded in the control group. The different larval densities tested (1, 25, 50, 100, 150 and 200) did not significantly influence mean (± SE) larval weight immediately prior to death (317 ± 23, 354 ± 30, 318 ± 14, 295 ± 19, 303 ± 13 and 327 ± 21 mg, respectively) ($F_{5,54} = 1.003$; $P = 0.425$). The prevalence of larvae that reached the pupal stage varied between $11 \pm 2\%$ and $12 \pm 2\%$, and did not differ significantly among densities ($F_{4,40} = 0.150$; $P = 0.962$). By contrast, cannibalism increased significantly with rearing density from $2.4 \pm 1\%$ at 25 larvae/container to $14 \pm 1\%$ at the density of 200 larvae/container ($F_{4,40} = 13.69$; $P < 0.001$) (Fig. 3A). By contrast, the prevalence of virus deaths decreased significantly with rearing density from $85 \pm 2\%$ in the 25 larvae density to $75 \pm 1\%$ in the 200 larvae density ($F_{4,40} = 3.22$; $P = 0.022$).

Mean OB production varied from $7.19 \times 10^8 \pm 1.97 \times 10^8$ to $1.74 \times 10^9 \pm 3.87 \times 10^8$ OB/mg larval weight but did not differ significantly among larval densities ($F_{5,54} = 1.427$; $P = 0.229$) (Fig. 3B). However, the total OB production per container varied significantly with rearing density ($F_{5,54} = 27.216$; $P < 0.001$) (Fig. 3C). Of the gregarious rearing treatments, the containers with 200 or 150 larvae were the most productive, with $8.43 \times 10^{13} \pm 1.87 \times 10^{13}$ and $8.07 \times 10^{13} \pm 1.86 \times 10^{13}$ OB/container, respectively, whereas the density of 25 or 50 larvae/container were the least productive treatments (with $5.72 \times 10^{12} \pm 1.33 \times 10^{12}$ and $1.10 \times 10^{13} \pm 2.74 \times 10^{12}$ OBs, respectively), and the 100 larvae treatment was intermediate. By contrast, individual larvae reared

alone produced $4.13 \times 10^{11} \pm 8.15 \times 10^{10}$ OBs (Fig. 3C), similar to the value for L_6 insects inoculated at 24 h post molting reported in the previous experiment.

4 DISCUSSION

We describe an efficient procedure for the production of large quantities of ChchNPV-TF1 OBs using the homologous host reared on semi-synthetic diet alone or gregariously. Specifically, the key variables of instar, timing of inoculation, inoculum concentration and post-inoculation rearing density were examined systematically for their effects on OB yields.

ChchNPV-TF1 OB production in L_6 *C. chalcites* was 20- and 5-fold higher than in L_4 and L_5, respectively. Larval weight gain has been observed to result in exponential increases in OB yields in several other host–baculovirus systems.[14,24,30–32] As observed in the present study, this is due to the positive relationship between insect weight at death and OB production, which is dependent on the weight of the larvae at the moment of inoculation and the increase in larval weight during infection.[33,34] As such, the final larval instar is often selected for OB production,[26] or the penultimate instar if the final instar is resistant to infection.[34–36] Other authors have made use of the relationship between larval body weight and OB production by applying juvenile hormone analogues (JHA) to increase larval weight gain[32,37] or generate supernumerary host instars in order to increase OB yields per insect.[38]

Another important consideration for the choice of host instar for OB production is cannibalism. This is a frequent behavior in many species of Lepidoptera during the larval stage and is stage-dependent in some species, with later instars often showing an increasing tendency to devour conspecifics.[26,39] Additionally, nucleopolyhedrovirus-infected larvae often show lower mobility and sluggish responses, making them more likely to be victims of conspecific predation than healthy insects.[40,41] However, this was not the case in *C. chalcites*, as insects in the L_6 stage were less prone to cannibalistic behavior than those in the L_5 or L_4 stage. A similar tendency was observed in *Spodoptera exigua* when JHA-treated larvae were compared with untreated conspecifics.[42] In both species, casual observations indicated that L_6 were less agile and apparently less prone to engage in aggressive interactions that earlier instars.

In the present study, the moment of inoculation was also highly influential in OB production. When inoculated as 24-h-old L_6 larvae, OB production increased 6-fold compared with the yield that resulted from the inoculation of recently molted conspecifics of the same instar. This effect appears to involve a combination of increased weight at inoculation and increased replication efficiency in 24-h-old infected larvae, in which the mean production of OB/mg larval weight was over 4-fold higher compared with recently molted L_6 larvae.

The inoculum OB concentration influences overall OB production by affecting speed of kill and the proportion of insects that succumb to lethal polyhedrosis disease.[24–26] High inoculum concentrations usually reduce the duration of the infection, as larvae die in earlier developmental stages and produce fewer OBs, because infected larvae have less time to develop and the virus has less time to replicate.[22,43] By contrast, if the inoculum concentration is low, fewer insects acquire a lethal infection. In the present study, inoculum concentrations ranging from the LC_{90} to LC_{50} resulted in similar speed of kill values (MTD), which allowed infected insects to reach a similar growth stage with similar yields of OB/larva. Nonetheless, the highest inoculum concentration resulted in the greatest proportion of virus-killed larvae and the greatest overall number of OBs produced from each batch of inoculated insects. These results are consistent with those of other baculovirus OB production systems, such as *Spodoptera exigua*–SeMNPV,[35,42,44] *Spodoptera exempta*–SpexMNPV,[45] *Spodoptera littoralis*–SpliNPV,[46] *Anticarsia gemmatalis*–AgMNPV[47] and *Helicoverpa armigera*–HearNPV.[20,26] Each of these viruses was used to inoculate their homologous host species and the highest production of OBs was obtained using quantities of inoculum close to the 90 or 95% lethal concentrations.

Rearing density effects on larval development and body weight have been examined in some species of Lepidoptera. For example, *Mamestra brassicae* larvae reared at high densities developed faster, had a smaller body size and were more susceptible to disease than conspecifics reared at lower densities.[48] The larval densities tested here had no significant influence on larval body weight, but did affect cannibalistic behavior. Cannibalism in *C. chalcites* tended to be lower than reported for other lepidopteran species, such as *H. armigera*,[49] *S. exigua*[37] and *S. frugiperda*,[39] so that gregarious rearing was feasible in the ChchNPV-TF1 OB production system. However, as the prevalence of cannibalism increased with increasing rearing density, the overall OB yield at the highest density (200 larvae/container) was similar to the overall yield observed at the density of 150 larvae/container. Because the density of 150 larvae/container requires the inoculation of 25% fewer larvae to set up each container, compared with the 200 larvae/container treatment, the density of 150 larvae/container was selected as the most appropriate post-inoculation rearing density.

The OB production process developed here resulted in remarkable yields, which as far as we are aware, are among the highest observed for any lepidopteran nucleopolyhedrovirus pathosystem reported to date. With 1.81×10^9 OB/mg larval weight or 5.7×10^{11} OB/larva, the productivity of ChchNPV-TF1 in *C. chalcites* was higher than the productivity reported for other nucleopolyhedroviruses in permissive hosts which range between 3×10^6 and 6×10^6 OB/mg or 1×10^9 and 5×10^9 OBs/larva in nucleopolyhedroviruses in commercial production.[14,25,50] However, because instar at the moment of infection has a marked influence on total yield, OB/mg larval weight is a more representative measure of virus productivity. Accordingly, ChchNPV-TF1 productivity per mg of larval weight is 53-fold higher than the *H. armigera*–HearNPV system (3.42×10^7 OB/mg),[30] 129-fold higher than the *A. gemmatalis*–AgMNPV system (1.38×10^7 OB/mg),[47] 138-fold higher than the JHA-modified *S. exigua*–SeMNPV system (1.31×10^7 OB/mg),[35] or 240-fold higher than the *Trichoplusia ni*–AcMNPV system (7.5×10^6 OB/mg).[51] At 5.7×10^{11} OB/larva the production of ChchNPV-TF1 represents an~ 100-fold higher OB yield than any of the previously mentioned nucleopolyhedrovirus pathosystems. Indeed, the productivity of ChchNPV-TF1 was closer to that of granuloviruses, many of which produce more OBs than nucleopolyhedroviruses.[14] The high OB yields suggest a remarkably efficient use of host cellular resources for OB production by the ChchNPV-TF1 virus.

The productivity ratio (PR) may be a more valuable parameter to measure productivity in production systems, as PR values indicate the relationship between the amount of inoculum required to infect a larva and the number of OBs produced following the death of each infected larva.[52] However, we were unable to calculate the PR for ChchNPV-TF1 as we did not quantify the number of

inoculum OBs ingested by the different instars. To date, the highest PR value reported for a nucleopolyhedrovirus is that of SeMNPV in its homologous host (1.2×10^6),[14] although how this compares with the ChchNPV-TF1 system remains to be determined. Nevertheless, the inoculation of 24-h-old L_6 larvae with the corresponding LC_{90} inoculum concentration, followed by rearing at a density of 150 larvae/container resulted in the production of 8.07×10^{13} OBs of the ChchNPV-TF1 isolate.

Recent studies have demonstrated highly efficient control of *C. chalcites* infestations on banana crops following applications of ~ 1×10^9 OB/l, equivalent to 2×10^{12} OB/ha, as application volumes of ~ 2000 l/ha are often required for effective coverage of banana plants.[4] Application of ChchNPV-TF1 OBs proved to be as or more effective for crop protection, as commercial insecticides based on indoxacarb or *Bacillus thuringiensis* var. *kurstaki*.[4,9] Given this, a single container of 150 virus-killed *C. chalcites* larvae would, theoretically, provide sufficient OBs to treat ~ 40 ha of banana crops. Even if a fraction of the OBs were lost during processing and formulation of an insecticidal product, the productivity of this production system is outstanding compared with that of other nucleopolyhedroviruses, in which tens or hundreds of larvae have to be infected, reared and harvested to provide sufficient OBs for a single hectare treatment.[12,53,54] We conclude that the efficient production of ChchNPV-TF1 OBs involves inoculation of 24-h-old L_6 insects with an inoculum of 9.02×10^8 OB/ml (LC_{90}) followed by rearing at a density of 150 larvae/container. The *C. chalcites*-ChchNPV-TF1 pathosystem represents a remarkable opportunity for the production of a biological insecticide for control of this pest in greenhouse and field crops.

ACKNOWLEDGEMENTS

We thank Noelia Gorría and Itxaso Ibáñez (UPNA) for technical assistance and insect rearing, and Gabriel Mercado (INECOL) for providing logistical support to TW. This study received financial support from the Instituto Nacional de Investigaciones Agrarias (Project RTA2010-00016-C02-02 and RTA2013-00114-C02-02) and the Programa Nacional Español de I+D+I (Projects AGL2008-05456-CO3-01 and AGL2011-30352-CO2-01).

REFERENCES

1 van Oers MM, Herniou EA, Usmany M, Messelink GJ and Vlak JM, Identification and characterization of a DNA photolyase-containing baculovirus from *Chrysodeixis chalcites*. *Virology* **330**:460–470 (2004).
2 Shepard BM, Hammig MD, Carner GL, Ooi PAG and Smith JP, Implementing integrated pest management in developing and developed countries, in *Integrated Pest Management: Dissemination and Impact*, ed. by Peshin R and Dhawan AK. Springer, Dordrecht, pp. 275–305 (2009).
3 Del Pino M, Carnero A, Cabello T and Hernández-Suárez E, La lagarta o bicho camello, *Chrysodeixis chalcites* (Esper, 1789), una plaga emergente en los cultivos de platanera de Canarias. *Phytoma* **225**:21–24 (2011).
4 Fuentes G, Hernández-Suárez E, Simón O, Williams T and Caballero P, Chrysodeixis chalcites nucelopolyhedrovirus (ChchNPV): natural occurrence and efficacy as a biological insecticide on young banana plants in greenhouse and open-field conditions on the Canary Islands. *PLoS ONE* **12**:e0181384 (2017).
5 Domínguez E, López-Cepero J and Nogueroles C, Identificación y control de plagas y enfermedades, in *Calidad y sostenibilidad en el cultivo de la platanera en Canarias*, ed. by Nogueroles C. ASPROCAN, Tenerife, pp. 145–170 (2012).
6 Cabello T and Belda J, Noctuidos plaga (Lepidoptera: Noctuidae) en cultivos hortícolas en invernaderos, in *Sanidad Vegetal en la Horticultura Protegida*, ed. by Moreno R. Consejería de Agricultura y Pesca, Seville, pp. 179–211 (1994).
7 Bernal A, Carnero A, Hernández-Suárez E, Williams T, Caballero P and Simón O, A native variant of Chrysodeixis chalcites nucleopolyhedrovirus: the basis for a promising bioinsecticide for control of *C. chalcites* in Canary Islands' banana crops. *Biol Control* **67**:101–110 (2013).
8 Bernal A, Williams T, Caballero P and Simón O, Stage-specific insecticidal characteristics of a nucleopolyhedrovirus isolate from *Chrysodeixis chalcites* enhanced by optical brightener. *Pest Manag Sci* **70**:798–804 (2014).
9 Simón O, Bernal A, Williams T, Carnero A, Hernández-Suárez E, Muñoz D *et al.*, Efficacy of an alphabaculovirus-based biological insecticide for control of *Chrysodeixis chalcites* (Lepidoptera: Noctuidae) on tomato and banana crops. *Pest Manag Sci* **71**:1623–1630 (2015).
10 Haase S, Sciocco-Cap A and Romanowski V, Baculovirus insecticides in Latin America: historical overview, current status and future perspectives. *Viruses* **7**:2230–2267 (2015).
11 Knox C, Moore SD, Luke GA and Hill MP, Baculovirus-based strategies for the management of insect pests: a focus on development and application in South Africa. *Biocontrol Sci Technol* **25**:1–20 (2015).
12 Moscardi F, Assessment of the application of baculoviruses for the control of Lepidoptera. *Annu Rev Entomol* **44**:257–289 (1999).
13 Sun X, History and current status of development and use of viral insecticides in China. *Viruses* **7**:306-319 (2015).
14 Grzywacz D and Moore S, Production, formulation and bioassay of baculoviruses for pest control, in *Microbial Control of Insects and Mite Pests from Theory to Practice*, ed. by Lacey LA. Academic Press, San Diego, pp. 109–124 (2017).
15 Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M and Goettel MS, Insect pathogens as biological control agents: back to the future. *J Invertebr Pathol* **132**:1–41 (2015).
16 van Oers MM, Opportunities and challenges for the baculovirus expression system. *J Invertebr Pathol* **107**:3–15 (2011).
17 Dai X, Hajós JP, Joosten NN, van Oers MM, Ijkel WFJ, Zuidema D *et al.*, Isolation of a *Spodoptera exigua* baculovirus recombinant with a 10.6 kbp genome deletion that retains biological activity. *J Gen Virol* **81**:2545–2554 (2000).
18 Pijlman GP, van den Born E, Martens DE and Vlak JM, *Autographa californica* baculoviruses with large genomic deletions are rapidly generated in infected insect cells. *Virology* **283**:132–138 (2001).
19 Pijlman GP, Vrij J, van den End FJ, Vlak JM and Martens DE, Evaluation of baculovirus expression vectors with enhanced stability in continuous cascaded insect-cell bioreactors. *Biotechnol Bioeng* **87**:743–753 (2004).
20 Sun X, Sun X, Bai B, Werf WVD, Vlak JM and Hu Z, Production of polyhedral inclusion bodies from *Helicoverpa armigera* larvae infected with wild type and recombinant HaSNPV. *Biocontr Sci Technol* **15**:353–366 (2005).
21 Claus JD, Gioria VV, Miceloud GA and Visnovsky G, Production of insecticidal baculoviruses in insect cell cultures: potential and limitations, in *Insecticides – Basic and Other Applications*. InTech, Rijeca, pp. 127–152 (2012).
22 Kumar CM, Sathiah N and Rabindra RJ, Optimizing the time of harvest of nucleopolyhedrovirus infected *Spodoptera litura* (Fabricius) larvae under *in vivo* production systems. *Curr Sci* **88**:1682–1684 (2005).
23 Rabindra RJ, Sathiah N, Jayanth KP and Gupta RK, Commercial scale production and formulation of nucleopolyhedrovirus of *Helicoverpa armigera* and *Spodoptera litura*, in *Recent Advantages in Integrated Pest Management*, ed. by Singh A, Bombawale N, Trivedi TP. National Centre for Integrated Pest Management (NCIPM), New Delhi, pp. 32–39 (2003).
24 Reid S, Chan L and van Oers M, Production of entomopathogenic viruses, in *Mass Production of Beneficial Organisms*, ed. by Morales-Ramos JA, Rojas MG, Shapiro-Ilan DI. Elsevier, Amsterdam, pp. 437–484 (2014).
25 Grzywacz D, Moore D and Rabindra RJ, Mass production of entomopathogens in less industrialized countries, in *Mass Production of Beneficial Organisms*, ed. by Morales-Ramos JA, Rojas MG, Shapiro-Ilan DI. Elsevier, Amsterdam, pp. 519–553 (2014).
26 Arrizubieta M, Simón O, Williams T and Caballero P, Determinant factors in the production of a co-occluded binary mixture of *Helicoverpa armigera* alphabaculovirus (HearNPV) genotypes with desirable insecticidal characteristics. *PLos One* **11**:e0164486 (2016).
27 Greene GL, Leppla NC and Dickerson WA, Velvetbean caterpillar: a rearing procedure and artificial medium. *J Econ Entomol* **69**:487–488 (1976).

28 Hughes PR and Wood HA, A synchronous *per oral* technique for the bioassay of insect viruses. *J Invertebr Pathol* **37**:154–159 (1981).
29 Crawley MJ, *GLIM for Ecologists*. Blackwell Science, Oxford (1993).
30 Kalia V, Chaudhari S and Gujar GT, Optimization of production of nucleopolyhedrovirus of *Helicoverpa armigera* throughout larval stages. *Phytoparasitica* **29**:23–28 (2001).
31 Monobrullah MD and Nagata M, Optimization of *Spodoptera litura* (Fab.) nucleopolyhedrovirus production in homologous host larvae. *Int J Trop Insect Sci* **20**:157–165 (2000).
32 Shieh TR, Industrial production of viral pesticides. *Adv Virus Res* **36**:315–343 (1989).
33 Hodgson DJ, Vanbergen AJ, Watt AD, Hails RS and Cory JS, Phenotypic variation between naturally co-existing genotypes of a Lepidoptera baculovirus. *Evol Ecol Res* **3**:687–701 (2001).
34 Shapiro M, *In vivo* production of baculoviruses, in *The Biology of Baculoviruses*, ed. by Granados RR, Federici BA. CRC Press, Boca Raton, pp. 31–62 (1986).
35 Lasa R, Caballero P and Williams T, Juvenile hormone analogs greatly increase the production of a nucleopolyhedrovirus. *Biol Control* **41**:389–396 (2007).
36 Shapiro M, Bell RA and Owens CD, *In vivo* mass production of gypsy moth nucleopolyhedrosis virus, in *The Gypsy Moth: Research Toward Integrated Pest Management*, ed. by Doane CC, MacManus ML, U.S. Department of Agriculture, Washington, DC, pp. 633–655 (1981).
37 Glen DM and Payne CC, Production and field evaluation of codling moth granulosis virus control of *Cydia pomonella* in the United Kingdom. *Ann Appl Biol* **104**:87–88 (1984).
38 Lasa R, Pagola I, Ibañez I, Belda JE, Williams T and Caballero P, Efficacy of *Spodoptera exigua* multiple nucleopolyhedrovirus as a biological insecticide for beet armyworm control in greenhouses of southern Spain. *Biocontr Sci Technol* **17**:221–232 (2007).
39 Chapman JW, Williams T, Escribano A, Caballero P, Cave R and Goulson D, Age-related cannibalism and horizontal transmission of a nuclear polyhedrosis virus in larval *Spodoptera frugiperda*. *Ecol Entomol* **24**:268–275 (1999).
40 Boots M, Cannibalism and the stage-dependent transmission of a viral pathogen of the Indian meal moth, *Plodia interpuctella*. *Ecol Entomol* **23**:118–122 (1998).
41 Polis GA, The evolution and dynamics of intraspecific predation. *Annu Rev Ecol Syst* **12**:225–251 (1981).
42 Elvira S, Williams T and Caballero P, Juvenile hormone analog technology: effects on larval cannibalism and the production of *Spodoptera exigua* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. *J Econ Entomol* **103**:577–582 (2010).
43 Cory JS and Myers JH, Adaptation in an insect host–pathogen interaction. *Ecol Lett* **7**:632–639 (2004).
44 Elvira S, Gorría N, Muñoz D, Williams T and Caballero P, A simplified low-cost diet for rearing *Spodoptera exigua* (Lepidoptera: Noctuidae) and its effect on *S. exigua* nucleopolyhedrovirus production. *J Econ Entomol* **103**:17–24 (2010).
45 Cherry AJ, Parnell MA, Grzywacz D and Jones KA, The optimization of *in vivo* nuclear polyhedrosis virus production in *Spodoptera exempta* (Walker) and *Spodoptera exigua* (Hübner). *J Invertebr Pathol* **70**:50–58 (1997).
46 Grzywacz D, McKinley DJ, Moawad G and Cherry A, The *in vivo* production of *Spodoptera littoralis* nuclear polyhedrosis virus. *J Virol Method* **71**:115–122 (1998).
47 Moscardi F, Leite LG and Zamataro E, Production of nuclear polyhedrosis virus of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae): effect of virus dosage, host density and age. *An Soc Entomol Bras* **26**:121–132 (1997).
48 Goulson D and Cory JS, Responses of *Mamestra brassicae* (Lepidoptera: Noctuidae) to crowding: interactions with disease resistance, color phase and growth. *Oecologia* **104**:416–423 (1995).
49 Kakimoto T, Fujisaki K and Miyatake T, Egg laying preference, larval dispersion, and cannibalism in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Ann Entomol Soc Am* **96**:793–798 (2003).
50 Cherry AJ, Parnell MA, Grzywacz D and Jones KA, The optimization of *in vivo* nuclear polyhedrosis virs production in *Spodoptera exempta* (Walker) and *Spodoptera exigua* (Hübner). *J Invertebr Pathol* **70**:50–58 (1997).
51 Cory JS, Clarke EE, Brown ML, Hails RS and O'Reilly DR, Microparasite manipulation of an insect: the influence of the *egt* gene on the interaction between a baculovirus and its lepidopteran host. *Funct Ecol* **18**:443–450 (2004).
52 Hunter-Fujita FR, Entwistle PF, Evans HF and Crook NE, *Insect Viruses and Pest Management*. Wiley, Chichester.
53 Behle RW and Popham HJR, Laboratory and field evaluations of the efficacy of a fast-killing baculovirus isolate from *Spodoptera frugiperda*. *J Invertebr Pathol* **109**:194–200 (2012).
54 Williams T, Goulson D, Caballero P, Cisneros J, Martínez AM, Chapman JW *et al.*, Evaluation of a baculovirus bioinsecticide for small-scale maize growers in Latin America. *Biol Control* **14**:67–75 (1999).

