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Why do Spodoptera exigua multiple nucleopolyhedrovirus occlusion bodies lose insecticidal activity on amaranth (*Amaranthus hypocondriacus* L.)?

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ABSTRACT

Amaranth (*Amaranthus hypocondriacus* L.) is a pseudocereal that is widely consumed in Mexico and other tropical regions, but which is attacked by *Spodoptera exigua* (Hübner) larvae. Products based on Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) can be highly effective insecticides, but when applied to amaranth viral occlusion bodies (OBs) lost 50% of their activity in a 6 h period. A series of experiments was performed to examine the role of solar radiation, leaf oxidative enzymes, leaf pH and insect feeding behavior, all of which failed to explain the reduced prevalence of virus infection in *S. exigua* larvae on amaranth. The concentration of the flavonoid rutin on amaranth leaves was > 200-fold higher than on another preferred host plant, sweet pepper (*Capsicum annuum*). However, in laboratory assays, rutin generally resulted in an increased prevalence of infection when administered in mixtures with semi-synthetic diet and OBs. We conclude that the cause of OB inactivation on amaranth remains elusive, although understanding how this will influence its efficacy as a biological insecticide requires field studies.

1. Introduction

Amaranth, *Amaranthus hypocondriacus* L., is a pseudo-cereal widely grown in Mexico since before the Prehispanic period. Increasing interest in its nutritional properties (Bressani, 2003; Perales-Sanchez et al. 2014) and its ability to grow under a range of climatic conditions in Mexico, have resulted in an expansion in amaranth production to approximately 7.3 million ha and a national production of 27.5 million tonnes in 2017 (SIAP 2017). Compared to popular cereals such as maize, wheat and rice, amaranth has a balanced amino acid profile rich in lysine, tryptophan and methionine, and a high total protein content (Bressani et al., 1987; Ravindran et al., 1996). Moreover, amaranth can be important nutritional source for people with celiac disease, diabetes or hypercholesterolemia (Bressani, 2003; Lucero-López et al., 2011).

Larvae of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) are one of the most important pests of amaranth crops in the central region of Mexico (Aragón et al., 1997; Pérez-Torres et al., 2011). Control of this pest is an issue of concern for organic growers as few effective control measures are presently available. Biological control using products based on Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) can be as effective as chemical insecticides in some crops (Kolodny Hisrch et al., 1993), and where high levels of pesticide resistance exist in pest populations (Lasa et al., 2007a). This virus has been registered for its use in Mexico in tomato, chile pepper and eggplant under the name of Spod-X® (FMC Agroquímica de Mexico, Zapopan), although its use could be expanded to include other crops such as amaranth.

The efficacy of virus-based insecticides depends on numerous factors related to the insect pest, the virus pathogen, the food plant, the environment and their interactions (Ment et al., 2018; Cory and Deschodt, 2018). For example, susceptibility to infection decreases as larvae grow so that applications of OBs to crops are usually targeted at highly susceptible early instars (Briese, 1986). Similarly, knowledge of the feeding behavior of insect pests on different crops can be used to target OB applications with greater efficiency (Smits et al., 1987; Kolodny-Hirsch et al., 1997). The crop can also have a major influence on the persistence of OBs on plant surfaces. Examples of this include the presence of phylloplane compounds that favor OB adhesion to leaf surfaces or that reduce OB viability, or plant structures that can shelter OBs from harmful ultra-violet solar radiation (reviewed by Williams, 2018).

The present study aimed to evaluate the persistence of SeMNPV OBs as a biological insecticide for control of *S. exigua* in amaranth crops. First, we determined the persistence of OBs on amaranth foliage under

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field conditions and then investigated a series of factors (sunlight, insect feeding behavior, pH and leaf surface chemistry) that may be responsible for the low levels of OB persistence that we initially observed. These studies were performed with reference to a preferred host, sweet pepper (*Capsicum annuum* L.), for which OB persistence and efficacy studies had been performed previously (Lasa et al., 2007a,b).

2. Materials and methods

2.1. Insect colony, virus and plants

Larvae of *S. exigua* were obtained from a laboratory colony started in 2012 in the Instituto de Ecología AC (INECOL), Xalapa, Mexico. First to third instars were reared in groups using semi-synthetic diet adapted from Hoffman's tobacco hornworm diet (Hunter-Fujita et al. 1998) under controlled conditions of 25 ± 2 °C, 70 ± 10% relative humidity and 14 h:10 h light–dark photoperiod. Fourth instars were individualized in 30 ml plastic cups with the same diet and reared until pupation. Adults were placed in nylon mesh cages, fed *ad libitum* on a 10% (wt./vol.) sucrose solution and allowed to oviposit on paper towels.

The SeMNPV virus strain used in experiments was kindly supplied by P. Támez-Guerra (Universidad Autónoma de Nuevo León, Mexico) and was identical in terms of restriction profiles to that of SeMNPV-US2, which is the principal active ingredient of the biological insecticide Spod-X (Certis USA LLC, Columbia, MD). Viral occlusion bodies (OBs) were produced by inoculating fourth instars using the droplet feeding technique (Hughes and Wood 1981). Larvae that consumed inoculum within 10 mins were subsequently reared on diet until death. To extract OBs, virus-killed larvae were triturated, suspended in distilled water and insect debris was removed by filtration through an 80 μm mesh. OBs were counted using a Neubauer improved chamber (Hawksley, Lancing, United Kingdom), adjusted to a concentration of $1x10^9$ OBs/ml and stored at 4 °C prior to use.

Plants used in the experiments were amaranth and sweet pepper (*C. annuum* var. annuum). Amaranth plants were obtained from seedlings 20 cm tall grown in an organic plant nursery in Puebla, Mexico. Sweet pepper plants were grown from seeds (Happy Flower Mexicana SA de CV, Iztapalapa, Mexico). Both species were maintained in a mixture of compost, soil and volcanic pumice (2:1:1), in black plastic bags (20 × 20 × 25 cm), in a greenhouse outside the INECOL laboratory.

2.2. Experiment 1. Persistence of OBs on amaranth leaves

This test was performed in a field in Xalapa, Veracruz (19°30′39″N; 96°56′41″W) on a sunny day in August 2014. Amaranth plants were 80–110 cm in height, with a minimum of 20 true leaves. Four randomly selected plants were each sprayed with 8 ml of one of the following suspensions: i) $1x10^9$ OBs/liter or ii) $5x10^8$ OBs/liter. OB treatments included 0.05% (vol./vol.) Tween 80 (Sigma Aldrich, Mexico) as wetting agent. Each plant was considered as one replicate. Treatments were applied using a 500 ml capacity hand sprayer (Valigra SA de CV, Mexico). A similar group of four plants were sprayed with water and 0.05% Tween 80 as control. Treatments were applied at 7.00 am, shortly after sunrise. Three leaves were randomly collected from each plant immediately after spraying and before deposits had fully dried (0 h). Similar three leaf samples were also taken at 3, 6, 12 and 30 h post-application. Each group of three leaves was placed in a 250 ml ventilated plastic container and 20 larvae in the fourth instar, which had molted between 8 and 24 h previously, were released. Larvae were allowed to feed on leaves for 5 h at 25 ± 1 °C and were then placed individually in 30 ml plastic cups with semi-synthetic diet. Insects were reared at 25 ± 1 °C for 9 days, when virus-induced mortality was recorded. In cases of doubt, virus deaths were confirmed by direct observation of OBs in Geimsa-stained smears of insect cadaver tissues (Lacey and Kaya, 2007). During the experiment, incident solar radiation in the visible spectrum was measured at the upper 10 cm of the plants, at 30 min intervals, using a portable light meter (YK-10LX, LT Lutron, Taipei, Taiwan).

2.3. Experiment 2. Persistence of OBs on amaranth under sunlight or dark conditions

To determine whether the loss of insecticidal activity of SeMNPV OBs observed in the first experiment was mainly due to exposure to solar radiation, a second experiment was performed in September 2014 to compare the persistence of SeMNPV OBs on plants maintained under natural sunlight or dark conditions. Two groups of four randomly selected amaranth plants, similar to those used in the previous experiment, were sprayed at 7.00 am with 8 ml/plant of $1x10^9$ OBs/liter + 0.05% Tween 80 as described in the first experiment. Four plants were also sprayed with water and 0.05% Tween 80 as control. Immediately after spraying, as soon as residues had dried, one group of four plants was transferred to a dark room at 26 ± 1 °C, whereas the other group of treated plants and the control plants remained in the field. Three leaves were randomly collected from each plant in each treatment at 1, 6 and 12 h post-application. Following the procedures described in the first experiment, each group of three leaves was placed in a 250-ml plastic container with 20 fourth instars that were allowed to feed on leaves during 5 h. Larvae were then placed individually in 30 ml plastic cups with semi-synthetic diet and reared at 25 ± 1 °C for 9 days after which virus-induced mortality was recorded. Visible solar radiation in the field was measured at half hour intervals as described in the previous experiment.

2.4. Experiment 3. OB persistence on amaranth and sweet pepper in dark conditions

To determine whether the loss of insecticidal activity of OBs on amaranth was due to an intrinsic property of the plant, OB persistence was compared on amaranth and sweet pepper, for which previous persistence studies had been performed under greenhouse conditions (Lasa et al., 2007a). In July 2015, amaranth plants of 80–90 cm height with 18 true leaves and pepper plants of 40–50 cm height with 12 true leaves were randomly assigned to treatment and control groups. Four plants of each species were each sprayed with 7.5 ml of $1x10^9$ OBs/liter suspension + 0.05% Tween 80, as described in the first experiment. A similar group of four plants was sprayed with water and 0.05% Tween 80 as control. Plants were sprayed at 8.30 am and 40 min later, when residues had dried, plants were placed in a dark room. Two randomly selected leaves from each plant were taken at 1, 6 and 12 h post-application and placed together in a 250 ml plastic container with 20 larvae that had molted to the second instar, in the previous 8–24 h period. Larvae were allowed to feed on leaves during 5 h and were then individualized in wells of 12-well cell-culture plates with semi-synthetic diet. Insects were incubated at 25 ± 1 °C for 9 days after which virus-induced mortality was recorded.

The pH of the spray residue on amaranth and sweet pepper leaves was measured 5 min after spraying, when spray deposits were still moist. For this, the pH of the upper leaf surfaces of the upper 10 cm section of leaves on plants was measured using plastic pH indicator strips (Hydrion Spectral 0–14, Micro Essential Laboratory Inc., New York). Two measurements of two different leaves of four different plants (n = 8) were performed for each plant species.

To examine the physical condition of OBs on sweet pepper and amaranth leaves, 5x5 mm leaf samples were cut, from leaves that had been sprayed with OB suspension and 0.05% Tween 80 at 4–6 h previously. Samples were mounted on aluminum sample holders with double adhesive carbon conductive tape and photographed at x10,000 using a scanning electron microscope (FEI 250 Quanta FEG) under low-vacuum conditions. Leaf samples were not dehydrated or coated prior to observation.

2.5. Experiment 4. Feeding of S. exigua larvae on amaranth and sweet pepper

The area of leaf surface consumed by *S. exigua* larvae on amaranth and sweet pepper was compared to determine whether differences in feeding behavior could explain the lower mortality observed in insects that fed on OB-treated amaranth plants in the previous experiment. Leaf discs were obtained from young fully-expanded leaves of the upper canopy of plants, similar of those described in experiment 3. Leaf discs (19.6 mm diam., 301 mm^2) were cut from leaves of amaranth and sweet pepper taken from the upper 10 cm section of plants. Discs were cut using a cork borer and avoiding the midrib and large lateral veins. Each disc was placed on a moist filter paper disk in the center of a 90 mm Petri dish. A single *S. exigua* third instar that had molted during a 16 h starvation period prior to the test, was released inside each Petri dish and allowed to feed on the leaf disc during a 24 h period. Petri dishes were incubated in darkness at 24 ± 1 °C and 90–95% humidity. After 24 h, each disc was photographed, and the consumed leaf surface area was quantified using the Image J program (Rasband, 2016). The same procedure was also performed to quantify feeding behavior in fourth instars. Undamaged leaf discs were used as reference discs in this study. The test was performed using a total of 81 (amaranth) or 85 larvae (sweet pepper) in the third instar and 89 (amaranth) or 87 larvae (sweet pepper) in the fourth instar. Finally the average weight of the leaf discs for each plant species was obtained by individually weighing 30 leaf discs within an hour of removal from the leaf.

2.6. Experiment 5. Is the OB-inactivating factor(s) on amaranth leaves thermolabile?

The possible involvement of enzymes or other thermolabile substances in OB inactivation on amaranth was evaluated. For this a 6 g wet weight sample of amaranth leaves collected from the upper 10 cm of plants was mixed with 20 ml of distilled water and crushed in a ceramic mortar. The resulting homogenate was filtered through a 0.8 mm metal gauze and samples of 1 ml of the resulting aqueous extract were placed in 1.5 ml Eppendorf tubes. Samples were randomly assigned to one of two temperature treatments: (i) 24 °C (ambient laboratory temperature) for 15 min or (ii) 100 °C during 10 min using a calibrated laboratory heating block. Heated samples were allowed to cool for 5 mins. OB suspension was then assigned to one of the following treatments: i) OB suspension mixed with unheated amaranth extract to a final concentration of $5x10^5$ OBs/ml, ii) an identical concentration of OBs mixed with heat-treated amaranth extract, iii) an identical concentration of OB suspension mixed with 0.001% fluorella blue food coloring (positive control) and iv) water with fluorella blue (without OBs) as a negative control. OB suspensions in heated or unheated amaranth extracts were incubated for 24 h in dark conditions at 24 ± 1 °C and used in droplet feeding assays (Hughes et al., 1986). Identical assays were performed using OB samples that had been mixed with amaranth extracts 5–10 min prior to the bioassay (named 0 h samples). Droplet feeding bioassays were performed using groups of 30 *S. exigua* larvae that had molted to the third instar during a 12 h overnight starvation period. These larvae were allowed to drink OB suspension or control solution during a 15 min period. Larvae that drank the experimental inocula were identified by the blue (fluorella blue) or green (amaranth extract) color of their intestine. Inoculated larvae were allowed to walk over pieces of absorbent paper towel for a few seconds to reduce external contamination by OB suspensions before being placed individually in 50 ml plastic cups with a piece of semi-synthetic diet and incubated in darkness at 25 ± 1 °C. Virus-induced mortality was evaluated 7 days later. Two replicates were performed in consecutive weeks; as the results were nearly identical and additional replicates were not performed.

2.7. Experiment 6. Identification and quantification of phenolic compounds on sweet pepper and amaranth leaf surfaces.

A sample of five leaves of sweet pepper and amaranth were collected from three different greenhouse-grown plants i.e., three replicates of each species. The surface area of each leaf was determined using Image J software. Each group of five leaves was immersed in 10 ml analytical grade methanol (Sigma-Aldrich, HPLC grade) for 20 s. The methanol was then evaporated under reduced pressure in a rotary evaporator (Büchi RII) and the yielded extract was re-dissolved in 1 ml methanol (Sigma, LCMS grade) with 0.1% formic acid (Sigma, LCMS grade), filtered through a 0.2 µm PTFE filter placed in a 1.5 ml sample vial and stored at -20 °C prior to analysis.

Samples were analyzed using a 1290 Infinity Agilent ultrahigh resolution liquid chromatograph (UPLC) coupled to a 6460 Agilent triple quadrupole mass spectrometer (MS-QqQ). The mobile phases were water with MS grade 0.1% formic acid and acetonitrile with 0.1% formic acid. The column was an Agilent, Zorbax SB-C18, 2.1x50 mm, 1.8 µm with a flow of 0.1 ml/min and an injection volume of 1 µl operating at a temperature of 40 ± 0.8 °C. Chromatographic separation, identification and quantitation of individual phenolic compounds were performed using a dynamic multiple reaction monitoring acquisition method (dMRM) as described previously (Jiménez-Fernández et al., 2018) (Supplemental Information, Section II).

2.8. Experiment 7. Effect of rutin on OB activity

To evaluate the effect of rutin on SeMNPV OB activity against *S. exigua* larvae two treatments were performed: i) a 3 g sample of semi-synthetic diet was mixed with 14.5 mg rutin (Sigma-Aldrich, St. Louis, MO) dissolved in 100 µl methanol, ii) 3 g of diet mixed with 100 µl methanol. In this way rutin was present at a concentration of ~4.8 mg rutin/g of diet, a similar concentration to that previously determined on amaranth leaves in experiment 5. Four pieces of diet of ~130 mg (2x2x3 mm) from each treatment were placed in a Petri dish and groups of 30 *S. exigua* third instars were allowed to feed during 3 h at 24 ± 1 °C. After this period, 5 µl of SeMNPV OB suspension at one of three concentrations (5×10^5, 1×10^6 and 5×10^6 OBs/ml) were applied to the pieces of diet (equivalent to 2500, 5000 or 25,000 OBs/piece of diet). Larvae were then allowed to feed on OB-treated diet pieces for 6 h and after that, 24 larvae were randomly collected, individualized and incubated with virus free diet in a 24-well culture cell plate at 26 ± 1 °C. Virus-induced mortality was recorded at 7 days post-inoculation. During all tests, a similar group of larvae were fed methanol-treated diet or and methanol + rutin treated diet (without OB treatments) as controls. The entire experiment was performed on three occasions (3 replicates).

2.9. Statistical analysis

Virus-induced mortality results were analyzed by fitting generalized linear models (GLMs) with a binomial error structure with treatment and time as fixed factors (Numerical Algorithms Group, 1993). The results of GLMs are given as χ^2 statistics. Minor overdispersion in the mortality results was taken into account by scaling the error distribution where necessary. The results of scaled analyses are given as F statistics. Mean leaf disc weights were compared by *t*-test, but leaf disc area consumed for amaranth and sweet pepper were not normally distributed and were compared within instars by Kolmogorov–Smirnov two-sample test using SPSS v.17 (SPSS Inc., Chicago).

3. Results

3.1. Experiment 1. Persistence of OBs on amaranth leaves

Virus-induced mortality of larvae that consumed leaf samples taken

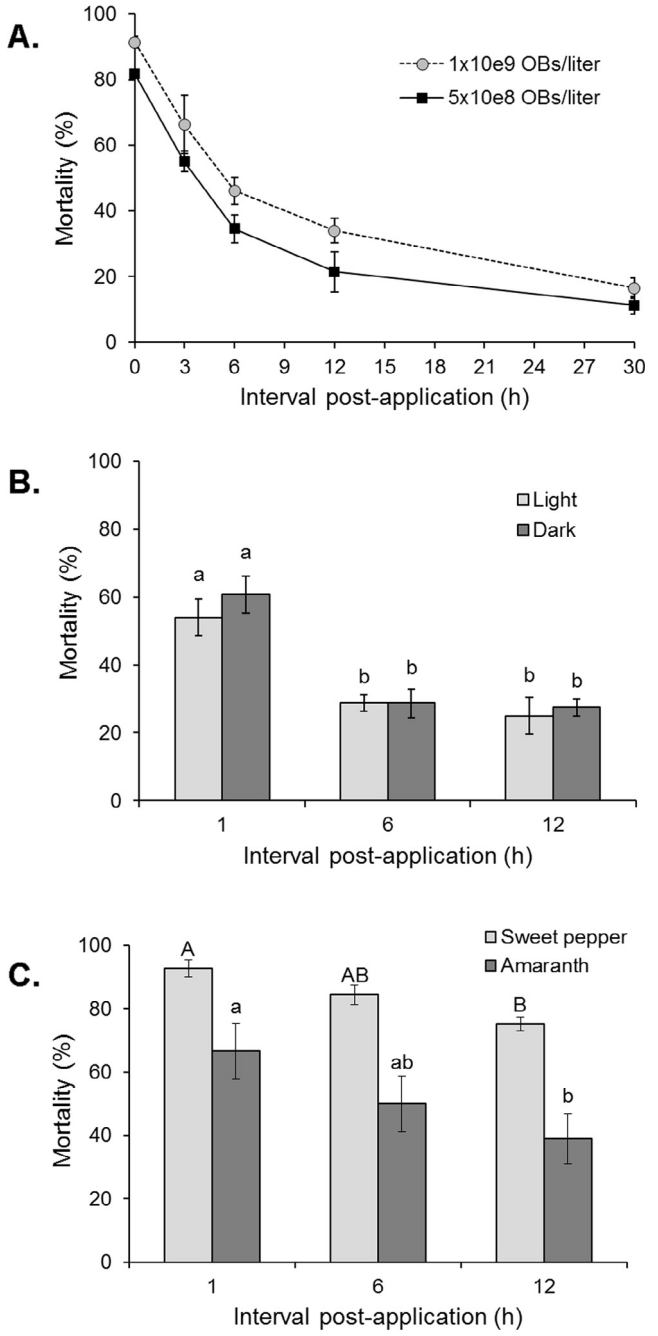

Fig. 1. Mean (± SE) percentage of virus-induced mortality of *Spodoptera exigua* fourth instars that consumed: (A) leaves collected at different intervals after the application of two concentrations of SeMNPV occlusion bodies (OBs) on potted amaranth plants under field conditions, (B) leaves from OB-treated amaranth plants that were held under field (light) or dark laboratory conditions, (C) leaves from OB-treated sweet pepper and amaranth plants under field conditions. In all cases larvae that consumed leaves were reared individually on semi-synthetic diet until death or pupation. In all cases vertical bars indicate SE. Columns headed by identical letters (for comparisons among upper or lower case letters) did not differ significantly (GLM, $P > 0.05$).

immediately following application of OBs was 82 and 91% for the $5x10^8$ and $1x10^9$ OBs/l treatments, respectively. A significantly higher prevalence of virus-induced mortality was observed in the higher concentration treatment ($F_{1,39} = 4.2$, $P < 0.05$, scale parameter = 2.05), although in both treatments, virus-induced mortality fell significantly in the samples taken at 6, 12 and 30 h post-application ($F_{1,38} = 96.35$, $P < 0.001$, scale parameter = 2.05) (Fig. 1A). No virus-induced mortality was observed in larvae that consumed leaves from control plants. The intensity of visible solar radiation at the moment of OB applications was < 100 lx (lumens/m^2) but increased to a peak of over 80,000 lx at 7.5 hrs post-application and again the following day at 29 h post-application (Supplemental Information, Section IA)

3.2. Experiment 2. Persistence of OBs on amaranth under sunlight or dark conditions

No significant differences were observed in virus-induced mortality in larvae that fed on the leaves of OB-treated plants maintained under light or dark conditions ($\chi^2 = 0.407$, df = 1, $P = 0.48$). However, in both light and dark treatments the prevalence of virus-induced mortality declined significantly over time ($\chi^2 = 32.9$, df = 1, $P < 0.001$) (Fig. 1B). The day of the experiment was cloudier than that of the first experiment, but was still bright with over 25,000 lx in the middle of the day (Supplemental Information, Section IB). As OBs on amaranth leaves in the dark rapidly lost infectivity, it was clear that solar radiation was not responsible for the rapid reduction in OB activity on amaranth leaves. No mortality was observed in control amaranth leaves treated with water and Tween 80.

3.3. Experiment 3. OB persistence on amaranth and sweet pepper in dark conditions

Virus-induced larval mortality was significantly lower when larvae ingested OB-treated amaranth than OB-treated sweet pepper ($F_{1,23} = 46.9$, $P < 0.001$, scale parameter = 1.55). For both plant species, the prevalence of mortality decreased significantly over time ($F_{1,22} = 15.3$, $P < 0.001$, scale parameter = 1.55) (Fig. 1C). The mortality of *S. exigua* larvae at all time points was approximately 30% lower for larvae fed on amaranth compared to values observed in the sweet pepper treatment. No mortality was observed in control amaranth leaves treated with water and Tween 80. Measurements of pH of leaf surfaces revealed that both amaranth and sweet pepper leaves had a pH of 6–7 in all cases. To check on the physical integrity of OBs we examined the upper surface of sweet pepper and amaranth leaves by low vacuum scanning electron microscopy (Fig. 2). OBs were intact and appeared to be randomly distributed over the surface of leaves of both species. The width of OBs varied between 1.3 and 1.8 μm under low vacuum conditions which did not involve prior dehydration of samples.

3.4. Experiment 4. Feeding of S. exigua larvae on amaranth and sweet pepper

The mean (± SE) wet weight of leaf discs (284 mm^2) was similar for amaranth (48.7 ± 1.4 mg) and sweet pepper (49.0 ± 1.1 mg) ($t = 0.146$; df = 58; $P = 0.885$). However, the leaf surface area consumed by *S. exigua* larvae in a 24 h period was 6.2-fold higher for amaranth than for sweet pepper in third instar insects (K-S two sample test, $P < 0.001$) and 3.4-fold higher in fourth instars (K-S two sample test, $P < 0.001$) (Fig. 3A,B).

3.5. Experiment 5. Is the OB-inactivating factor(s) on amaranth leaves thermolabile?

The prevalence of virus-induced mortality of larvae that consumed control OBs with fluorella blue was 96.7% and 91.7% following 0 h and 24 h of incubation, respectively. The mortality of larvae that consumed OBs that had been incubated for 24 h with heated and unheated amaranth extracts did not differ according to treatment ($\chi^2 = 0.862$, df = 2, $P > 0.05$) or over time (0 h sample *vs.* 24 h sample) ($\chi^2 = 3.35$, df = 1, $P > 0.05$). The prevalence of virus mortality at the first time point varied between 92 and 97% whereas at 24 h post-treatment virus-induced mortality was 89–92% depending on treatment. The fact that OBs were not inactivated in the unheated leaf extract treatment

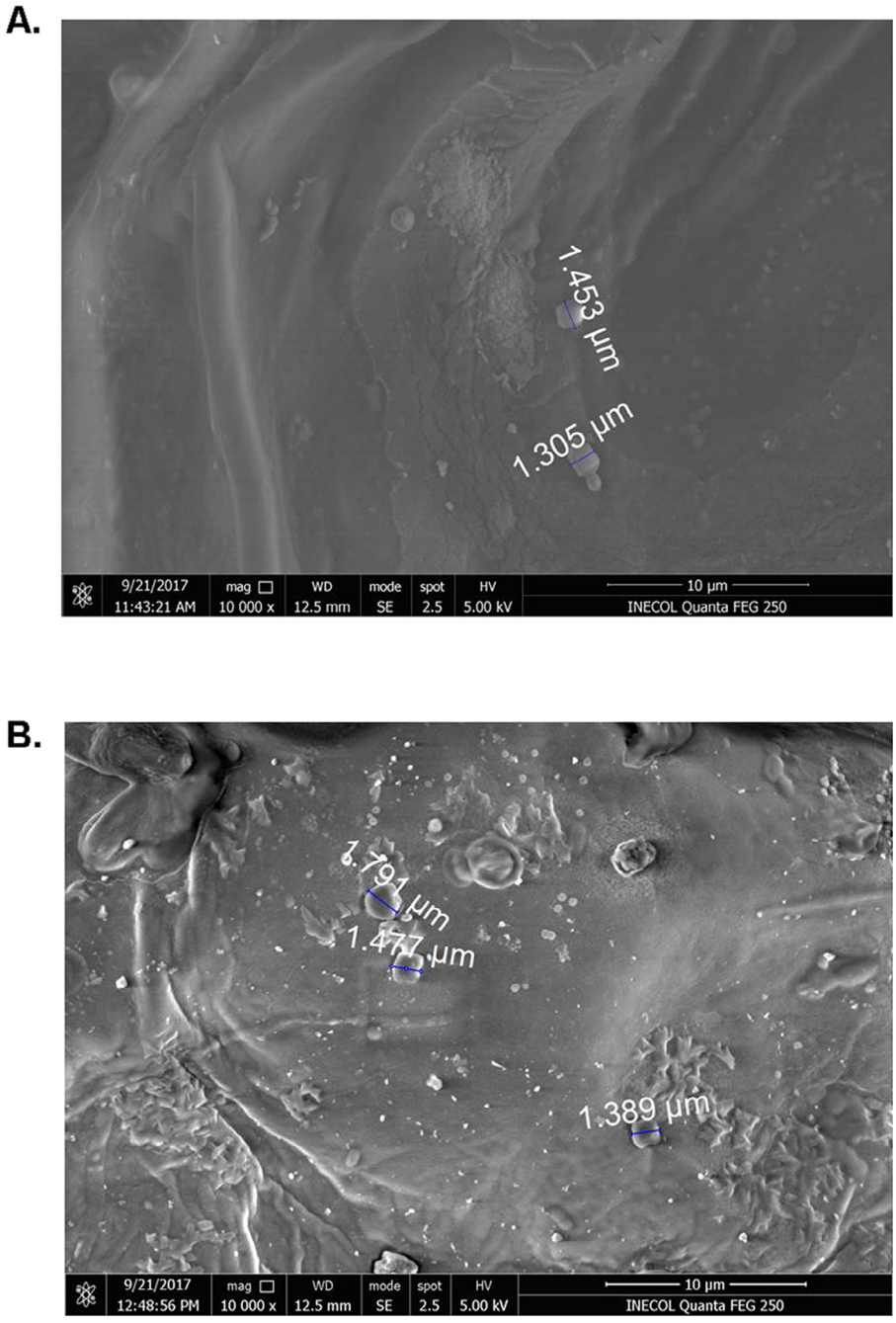

Fig. 2. Low vacuum scanning electronmicrographs of intact occlusion bodies (OBs) on the upper surface of (A) amaranth and (B) sweet pepper leaves that had been treated 24 h previously with OB suspension. Values indicate side-to-side width of OBs.

suggests that the extraction process likely deactivated the OB-inactivating factor.

3.6. Experiment 6. Identification and quantification of phenolic compounds on sweet pepper and amaranth leaf surfaces.

Two main phenolic compounds were detected in amaranth and sweet pepper leaves: rutin (quercetin-3-O-rutinoside) and kampferol-3-O-glucoside (Fig. 4A-C). The mean (± SD) concentration of rutin on the surface of amaranth leaves was 78.45 ± 11.34 µg/cm^2 compared to the estimated presence on sweet pepper leaves (< 0.5 µg/cm^2). The estimated concentration of kaempferol-3-O-glucoside on amaranth was 0.92 ± 1.08 µg/cm^2 whereas this compound was below detectable levels on sweet pepper leaves.

3.7. Experiment 7. Effect of rutin on OB activity

Virus-induced mortality increased significantly with OB dose (χ^2 = 115.6, df = 2, P < 0.001) (Fig. 5). Virus-induced mortality was significantly higher in the presence of rutin in the low (2500 OBs) and intermediate (5000 OBs) treatments and was similar with or without rutin at the highest dose (25000 OBs) (χ^2 = 13.07, df = 1, P < 0.001). No mortality was observed in larvae that fed methanol-treated diet or and methanol + rutin treated diets without OBs and used as controls.

4. Discussion

Application of SeMNPV OBs to amaranth crops resulted in a rapid loss of OB activity, measured as virus-induced larval mortality in leaf

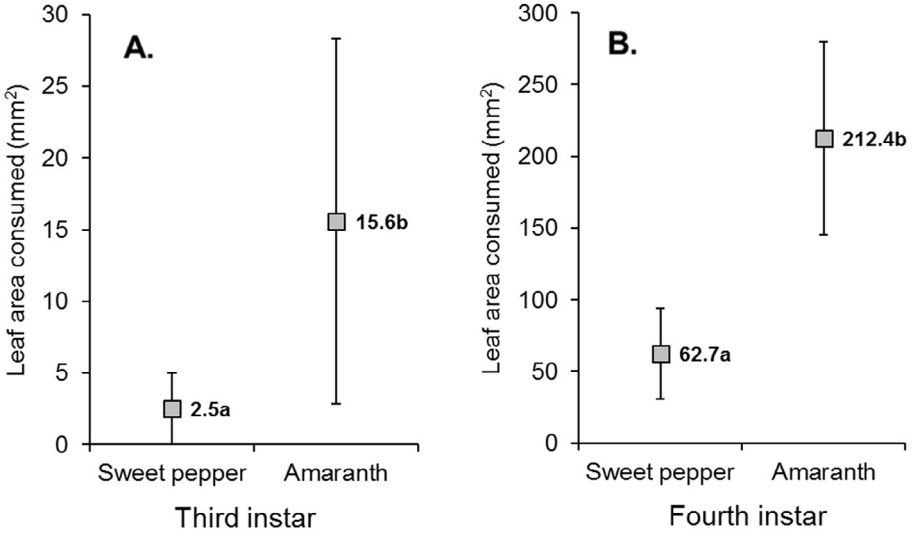

Fig. 3. Mean leaf area consumed by each *Spodoptera exigua* larva of (A) third and (B) fourth instar exposed to leaf discs of sweet pepper or amaranth during 24 h. Values next to points indicate mean value. Vertical bars indicate SD. Means followed by the same letter did not differ significantly (KS two sample test, $P < 0.05$).

bioassays. Several studies have focused on improving SeMNPV OB persistence on crop foliage as this virus is used as the basis for biological insecticides in many countries (Hunter-Fujita et al., 1998). Previous studies have reported a 50% loss in OB activity in a period of approximately 2–7 days depending on crop and exposure to sunlight (upper or lower canopy leaves) (Kolodny Hisrch et al., 1993; Bianchi et al., 2002; Lasa et al., 2007a; Shapiro et al., 2012). This is markedly longer than the ~ 6 h period required for 50% loss of activity that we

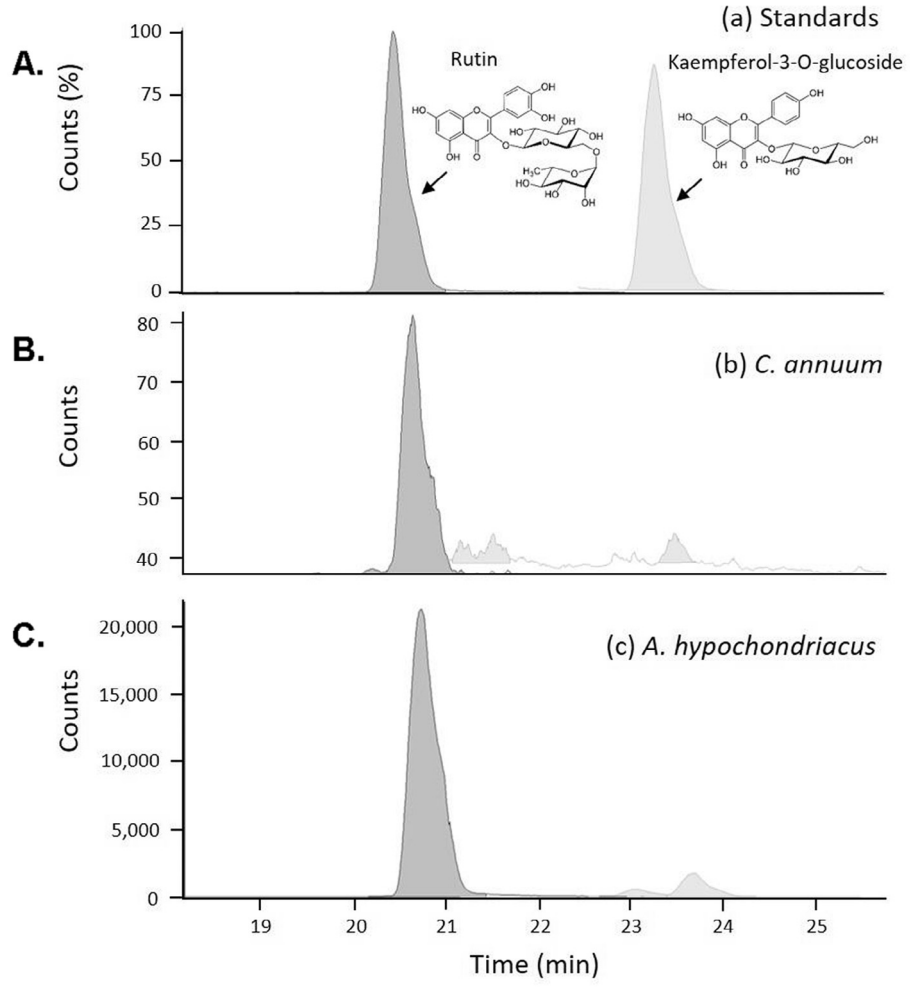

Fig. 4. Representative dMRM chromatogram of identified phenolic compounds: (A) standard products (reference), (B) sweet pepper leaves and (C) amaranth leaves. The y-axis in (A) is percentage, whereas in (B, C) y-axis indicates absolute counts of the compounds in the samples on different scales.

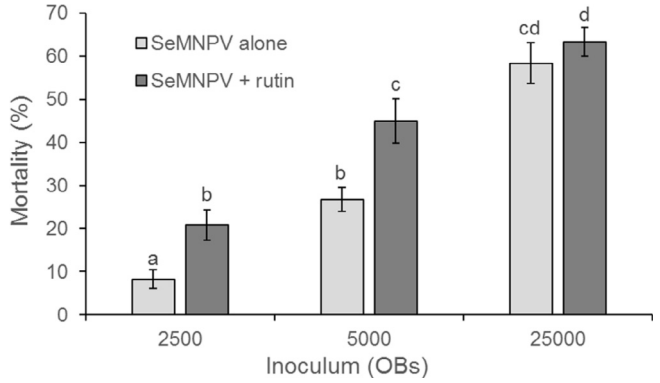

Fig. 5. Mean percentage mortality of *Spodoptera exigua* third instars that consumed semi-synthetic diet treated with one of three doses of SeMNPV OBs alone or SeMNPV OBs mixed with 4.8 mg rutin/g of diet. Vertical bars indicate SE. Columns headed by identical letters did not differ significantly (GLM, $P > 0.05$).

observed on amaranth. Formulation studies have attempted to extend the persistence of OBs on crops through the use of UV-protective substances that reduce the damage caused by solar radiation (Dougherty et al., 1996; McGuire et al., 2001). Radiation protectants were not used in the present study and OBs were applied in a simple aqueous suspension with a dilute wetting agent (Tween 80) to improve the spread and adhesion of spray droplets on leaf surfaces.

To address this issue we initially examined whether solar radiation at the study site was responsible for the loss of OB activity (Shapiro et al. 2002). However, OBs on leaves under light (field) conditions and dark (laboratory) conditions lost activity at a near identical rate over a 12 h period, leading us to reject UV radiation as a cause of OB inactivation on amaranth.

We then performed a direct comparison of OB persistence on amaranth and sweet pepper over a 12 h period. OBs on sweet pepper lost ~10% of the original activity during this period, in line with a published inactivation rate estimate on chrysanthemum (Bianchi et al., 2002). Sweet pepper was selected for this study because our previous experience indicated that SeMNPV is highly effective for *S. exigua* control on this crop (Lasa et al., 2007a). It was clear that OBs applied to amaranth resulted in a lower prevalence of lethal infection compared to sweet pepper plants that were treated with an identical quantity of OB inoculum.

We then questioned whether these results were due to differences in feeding behavior of larvae. However, comparative feeding studies revealed that *S. exigua* third and fourth instars consumed approximately a six-fold and a three-fold larger area when feeding on amaranth compared to sweet pepper leaves, respectively. If the distribution of OBs on leaf surfaces were random, we would expect a 3–6 fold higher dose of OBs consumed by larvae on amaranth and a corresponding increase in the prevalence of virus-induced mortality on amaranth compared to sweet pepper, which was clearly not the case.

We next considered the possibility that amaranth had leaf surface enzymes, surface pH or phylloplane secondary compounds that were responsible for the observed loss of OB activity. Many plants employ physical or chemical defenses against herbivores, pathogens and competitors (Cory and Hoover, 2006). Chemical defenses can be constitutive or induced and usually act directly to reduce herbivore feeding and, in some cases, insect and plant pathogens present on plant surfaces may be inhibited or inactivated (LoPresti, 2016).

Heat inactivation of thermolabile components such as plant oxidative enzymes had no effect on OB activity in mixtures with leaf homogenates of amaranth. Plant peroxidases and polyphenol oxidases produce highly reactive free radicals that can react with phenolic compounds to produce quinones that irreversibly bind to OBs and reduce their solubility in the insect midgut, resulting in a lower prevalence of lethal infection (Felton and Duffey 1990; Hoover et al., 1998).

Studies on cotton have demonstrated that Helicoverpa armigera nucleopolyhedrovirus (HearNPV) OBs were inactivated within 24 h, even when plants were not exposed to sunlight (Young and Yearian, 1974; Elleman and Entwistle, 1985a). This was attributed to the presence of carbonates and bicarbonates of divalent metal cations (Ca^{2+}, Mg^{2+}) secreted by glandular trichomes resulting in alkaline leaf surfaces (Elleman and Entwistle, 1982). Dew droplets that form on the leaves solubilize these exudates and likely favor the inactivation of OBs (Young et al., 1977). However, OBs on cotton leaves appear physically intact and retain their polyhedral structure (Elleman and Entwistle, 1985a, 1985b). In the case of amaranth, leaf surface pH was neutral or slightly acid, although the pH of spray deposits may have increased as deposits dried (Young et al., 1977). Nonetheless, the acidic to neutral pH characteristics of amaranth leaves would appear to rule out the possibility that leaf alkalinity was responsible for OB inactivation. To check on the physical integrity of SeMNPVs we examined OB-treated sweet pepper and amaranth leaves by low vacuum scanning electron microscopy, but could find no evidence of physical deterioration of OBs such as that often attributed to the presence of alkaline conditions. As the physical characteristics of the leaf surfaces of these plants differed (Fig. 2AB), we could not discount the possibility that OBs adhered poorly to the surface of amaranth leaves and may have fallen off, or been blown off by wind currents. However, this seems unlikely given that OBs on plants held in dark laboratory conditions free from wind currents also experienced a loss of insecticidal activity (Fig. 1B).

Finally, UPLC-MS-QqQ analyses were performed on leaf surface washings. Previous analyses of the composition of secondary metabolites within dried leaves of *Amaranthus* spp. identified the presence of flavonoids, steroids, terpenoids and cardiac glycosides in all species tested, although leaf surface analyses were not performed (Maiyo et al., 2010). Amaranth leaf washings had an approximately two hundred fold higher concentration of the flavonoid rutin than sweet pepper leaves. Rutin comprises the flavonol quercetin and rutinose, a disaccharide sugar. Phenolic compounds such as rutin are often present in plants as antifeedant compounds (Hoffmann-Campo et al., 2006) but may also be phagostimulant, although this effect may be concentration dependent in some species of Lepidoptera (Simmonds 2003). The quantity of rutin present on amaranth leaves (4.8 mg/g wet weight) was over two-fold higher than the concentration reported to inactivate OBs of Helicoverpa zea single nucleopolyhedrovirus on tomato (2.1 μg/g wet weight) in the presence of chlorogenic acid as a minor component (Felton et al., 1987). This led us to examine the influence of rutin on OB infectivity in laboratory bioassays.

Contrary to our expectations, *S. exigua* larvae inoculated with mixtures of rutin and OBs on semi-synthetic diet experienced a higher prevalence of mortality than larvae inoculated with SeMNPV OBs alone, at least at the lower and intermediate inoculum concentrations (2500 and 5000 OBs). This suggests that rutin was not responsible for OB inactivation on amaranth. A minority component, kaempferol-3-O-glucoside was also detected in amaranth leaf surface washings, although at low concentrations and was not tested in mixtures with OBs. Hydrolyzable tannins and isoflavonoids were not detected in analyses performed on amaranth and sweet pepper leaf washings, although these compounds are known to adversely influence OB infectivity in other plant-insect-virus systems (reviewed by Cory and Hoover, 2006). For example, induction of phenolic defenses following jasmonic acid treatment of soya plants also result in decreased NPV infection in *S. frugiperda* larvae, presumably due to the binding of oxidized phenolics to OBs in the midgut (Shikano et al., 2017).

Host plants can also have multiple effects on larval susceptibility to viral pathogens through changes in the immune response (Shikano et al., 2010), or changes in the layered structure and thickness of the peritrophic matrix, which is an important barrier to pathogens present in the insect midgut (Plymale et al., 2008). The host plant also affects

the type and abundance of digestive enzymes that are involved in releasing ODVs from OBs in the insect midgut (reviewed in Shikano 2017). Finally, variation in the rate of nutrient acquisition from different host plants may influence larval growth rate which is correlated with immune response in some species of Lepidoptera (Vogelweith et al., 2013). Consequently, direct comparisons of growth rate, gut physiology and immunological defenses of larvae feeding on amaranth and sweet pepper leaves may provide answers to the differences in the acquisition of infection that we observed on these plants.

Interestingly, a somewhat similar case is that of HearNPV, which has proven to be an excellent biological insecticide for control of this pest on chickpea (*Cicer arietinum*) (Cherry et al., 2000), despite the rapid inactivation of OBs on leaf surfaces. In a detailed study, Stevenson et al. (2010) discovered that the application of OB suspension with a surfactant (0.02% Triton) resulted in a rapid increase in leaf surface isoflavonoids biochanin A and sissotrin, both of which were shown to reduce OB infectivity in bioassays. However, OB inactivation by the isoflavonoids did not fully explain the loss of OB activity observed on sprayed chickpea leaves suggesting that other unknown factors must also be involved.

Producers of organic amaranth in Mexico require effective control measures against *S. exigua* (Pérez-Torres et al., 2011). SeMNPV-based insecticides have proved to be highly effective in a range of crops attacked by this pest and are likely to be effective when used on amaranth, despite the rapid inactivation of OBs. This is because under warm conditions most larvae acquire a lethal infection within a few hours of OB applications, a characteristic that is shared across a range of plant-insect-baculovirus pathosystems in greenhouse and tropical habitats (Lasa et al., 2007b; Del-Angel et al., 2018). Unfortunately, we were unable to identify the components or specific properties of amaranth leaf surfaces that were responsible for the rapid inactivation of SeMNPV OBs. Field tests are required to determine the efficacy of this pathogen as a biological insecticide in organic amaranth crops. Formulation studies should also be conducted to determine whether OB inactivation can be attenuated by microencapsulation, or the addition of buffering or chelation agents (Jones and Burges, 1998) that mitigate the hostile environment of the amaranth phylloplane for nucleopolyhedroviruses.

5. CRediT authorship contribution statement

R. Lasa: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Roles/Writing – original draft; Writing – review & editing. **J.A. Guerreo-Analco**: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization. **J.L. Monribot-Villanueva**: Investigation; Methodology; Validation. **G. Mercado**: Investigation; Methodology; Validation. **T. Williams**: Conceptualization; Data curation; Formal analysis; Roles/Writing – original draft; Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biocontrol.2018.07.014.

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