BIOCONTROL SCIENCE AND TECHNOLOGY
2020, VOL. 30, NO. 6, 602–608
https://doi.org/10.1080/09583157.2020.1738344

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SHORT COMMUNICATION

Vertical dispersal of nucleopolyhedrovirus occlusion bodies in soil by the earthworm *Amynthas gracilus*: a field-based estimation

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ABSTRACT

The soil is the most important environmental reservoir for nucleopolyhedrovirus occlusion bodies (OBs). We employed a diet + soil bioassay technique to demonstrate that the earthworm *Amynthas gracilus* was capable of vertical transport of Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) occlusion bodies (OBs) under field conditions. Incubation of OBs with earthworms in sand did not adversely affect the prevalence of virus-induced mortality, but variation in insect mortality was markedly increased, possibly due to changes in the particle size distribution following earthworm processing of OB-contaminated sand. We conclude that earthworms are likely to interact frequently with soil OB populations.

ARTICLE HISTORY
Received 11 December 2019
Accepted 29 February 2020

KEYWORDS
Spodoptera frugiperda; baculovirus; persistence in soil; earthworm-mediated movement; bioassay

Nucleopolyhedroviruses (*Alphabaculovirus*: Baculoviridae) are lethal pathogens of lepidopteran larvae that can be highly effective biological insecticides (Lacey et al., 2015). The soil is the principal environmental reservoir of nucleopolyhedrovirus occlusion bodies (OBs) (Fuxa & Richter, 1996). Viral OBs can remain in the soil for extended periods before they are transported back on to plants by wind currents and rain-splash (Fuxa & Richter, 2001). They are subsequently transmitted when susceptible larvae consume OB-contaminated plant tissues.

Previously, we demonstrated that the earthworm *Eisenia fetida* (Haplotaxida: Lumbricidae) was capable of transporting OBs vertically in an artificial soil held between glass plates under controlled laboratory conditions (Infante-Rodríguez et al., 2016). The intestine of *E. fetida* was found to be slightly acidic and did not inactivate OBs during passage through the gut. In the present study, we examined the capacity of a different species, *Amynthas gracilus* (Haplotaxida: Megascolecidae), a cosmopolitan earthworm that is widespread in Mexico, to transport nucleopolyhedrovirus OBs in a natural, characterised soil, under field conditions.

For this, we initially calibrated a soil-diet incorporation bioassay for the detection of OBs in soil (Murillo et al., 2006; Richards & Christian, 1999). Samples of 10 g (dry wt.)

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Supplemental data for this article can be accessed at https://doi.org/10.1080/09583157.2020.1738344

of a characterised clay soil (supplemental material, Table S1) were obtained from the grounds of the Instituto de Ecología AC, Xalapa, Mexico, air-dried, sieved through a 1 mm mesh sieve and were thoroughly mixed with 1 ml of serially-diluted OB suspension (containing 5×10^3–5×10^8 OBs) of a Nicaraguan isolate of Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV). Each OB-contaminated soil sample was then mixed with 50 g of semi-synthetic diet (supplemental material, Table S2) to form a homogeneous paste that was divided equally among the wells of a 24-well tissue culture plate. A single *S. frugiperda* larva that had molted to the second instar within the previous 6–24 h, was placed in each well and allowed to feed on the soil-diet mixture for 48 h, after which the mixture was replaced with clean virus-free diet. Control larvae were treated identically but consumed an OB-free soil-diet mixture. Larvae were incubated at 25 ± 0.5°C in darkness and the number of virus-killed larvae was recorded five days later (7 days post-inoculation). Death by polyhedrosis was confirmed by microscopic observation of OBs in Giemsa-stained smears of larval tissues. The bioassay was performed on four occasions (replicates) using different batches of insects. The results were subjected to logit regression in GLIM4 with a quasibinomial error structure specified to account for minor overdispersion.

The 50% lethal concentration (LC_{50}) of OBs was estimated at 1.4×10^6 OBs/g soil (95% C.I.: 6.0×10^5–3.5×10^6) (Figure 1(A)). The regression slope (±SE) was 0.8027 ± 0.0774 (scale factor 1.93). Virus-killed *S. frugiperda* larvae were observed at a low prevalence (0%–10% mortality) following ingestion of concentrations as low as 500 OBs/g soil (Figure 1(A)). No control larvae died of lethal polyhedrosis disease. These findings are similar to the estimated LC_{50} value (2.7×10^6 OBs/g soil) of the soil bioassay performed previously using an artificial soil and a different colony of *S. frugiperda* (Infante-Rodríguez et al., 2016). Infante-Rodríguez et al. (2016) exposed larvae to OB-contaminated soil for a 4-day period, rather than the 2-day period that we used, although the resulting LC_{50} values were similar, indicating that the 2-day period was sufficient for the acquisition of a lethal infection in a fraction of the experimental insects.

As it was extremely difficult to collect and bioassay earthworm faeces to determine their effect on OB pathogenicity, we performed an experiment to determine the effect on OB integrity and insecticidal activity following incubation of OBs on a neutral substrate with earthworms. For this, 2×10^9 SfMNPV OBs were thoroughly mixed with samples of 140 g of moist sand (19.7% moisture) collected from a local river. The sand had been washed overnight with tap water, rinsed with distilled water and dried in an oven at 50°C prior to use. The OB-contaminated sand was placed in a 250 ml capacity plastic tub (11 cm diameter × 4 cm height) with a ventilated lid together with two *A. gracilus* adults (mean live wt. 1.21 ± 0.07 g) that were placed on the sand surface. An identical OB-contaminated sand sample was placed in a separate tub without earthworms. Another tub containing sand without OBs was used as the control. All tubs were incubated at 20°C in darkness in a bioclimatic chamber for 7 days, after which a 0.5 g sand sample was taken from each tub for scanning electron microscope (SEM) examination, and a 10 g sample of sand from each tub was taken for determination of the insecticidal activity of OBs by the soil incorporation bioassay. The experiment comprised six replicates. Percentage mortality variances differed among treatments, so that treatments were compared by Mann–Whitney U test. For SEM observation sand samples were air-dried, mounted on an aluminium stub, coated with gold/palladium (Quorum Q150R, USA), and observed and photographed in a SEM at 5 kV (FEI Quanta 250 FEG, Thermo Fisher Scientific, USA).

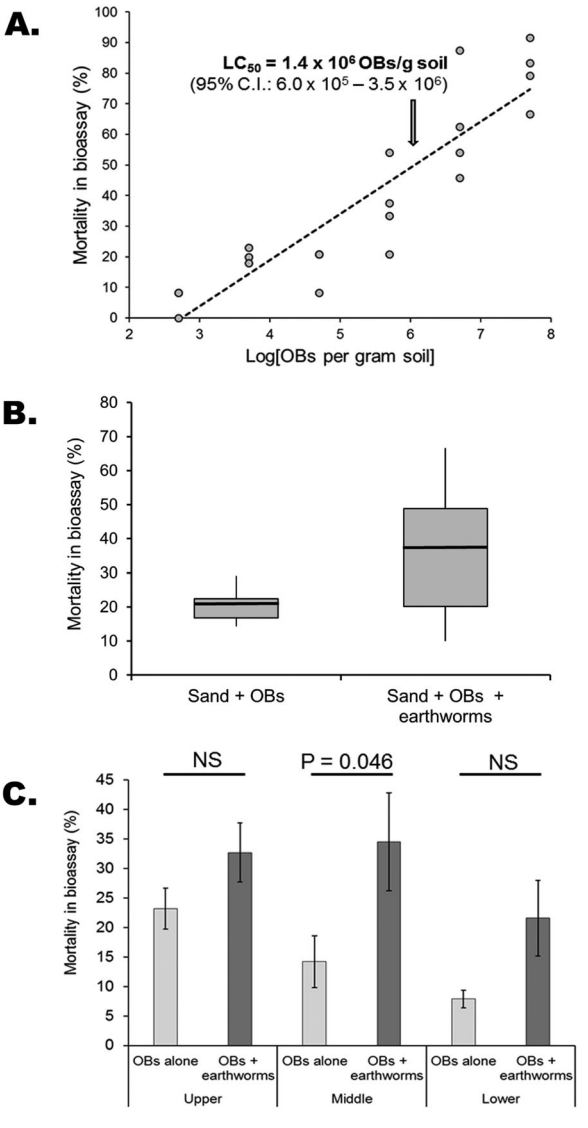

Figure 1. Percentage of mortality of *Spodoptera frugiperda* second instars in soil-diet bioassays (A) Concentration-mortality response of larvae that consumed soil previously contaminated with SfMNPV occlusion bodies (OBs). The 50% lethal concentration was estimated by logit regression. (B) Virus-induced mortality of larvae that consumed diet + sand contaminated with SfMNPV occlusion bodies (OBs) that had been incubated for 7 days alone, or in the presence of earthworms. Horizontal black line indicates median, shaded box indicates interquartile range, vertical bar indicates total range. (C) Mean (± SE) virus-induced mortality of larvae that consumed diet + soil samples taken from upper (0–2 cm depth), middle (6–8 cm depth) and lower (14–16 cm depth) levels of soil in experimental cups, with or without the presence of earthworms. *P* values above pairs of columns indicate a significant difference (ANOVA, Tukey test, $p < 0.05$). NS indicates no significant difference ($p > 0.05$).

It was clear from the photomicrographs that sand particles had numerous OBs adhering to their surface at the beginning of the experiment (supplemental material, Figure S1), and after 7 days incubation, both in the absence and the presence of earthworms (Figure 2

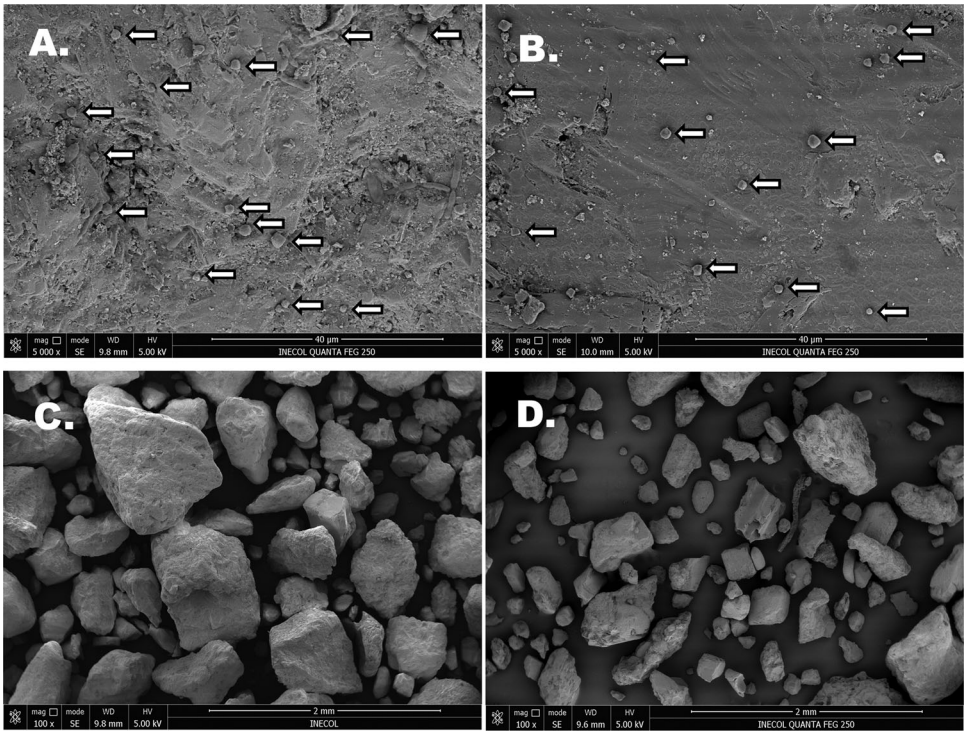

Figure 2. Scanning electron photomicrographs of sand particles contaminated with (A) viral occlusion bodies alone or (B) occlusion bodies that had been incubated with earthworms. Both images were taken at 7 days after the start of the experiment. Arrowheads indicate occlusion bodies adhering to the surface of sand. Sand particles examined (C) prior to the experiment or, (D) after 7 days incubation with earthworms, showed marked changes in the size of particles. Scale bar was 40 µm for A and B, and was 2 mm for C and D.

(A,B)). None of the OBs appeared to be physically altered following 7 days exposure to earthworms and they appeared to be similarly abundant before and after the incubation period.

Virus-induced mortality of *S. frugiperda* larvae did not differ significantly in larvae that consumed sand + OBs or those that consumed sand + OBs that had been exposed to earthworms (Mann–Whitney $U = 10$, $p = 0.240$) (Figure 1(B)). It was clear that the variation in the treatment with earthworms was markedly higher than the variation without earthworms. We suggest that this may have been due to a change in the particle size distribution in sand with earthworms that was evident by visual inspection and was clearly observed in scanning electron photomicrographs of sand particles prior to the experiment (Figure 2 (C)) and following 7 days incubation with earthworms (Figure 2(D)). This could have facilitated increased ingestion of small particles by *S. frugiperda* larvae in the bioassay. None of the larvae in the control bioassays died from polyhedrosis disease.

To examine the capacity of *A. gracilus* to disperse OBs under field conditions, adult earthworms were collected from a grass-covered lakeside site (19°32'29" N, 96°51'33" W) in the suburbs of the City of Xalapa, Veracruz State, Mexico. Plastic cups of 950 ml capacity were modified by replacing the base with a fine nylon gauze that was held in place by cyanoacrylate glue. Each cup was filled with 460 g dry sieved soil (supplemental

material, Table S1), moistened to near saturation point with 280 ml of distilled water, and placed in the ground in a field containing natural vegetation (mostly grasses, part of an ecological succession experiment) in the grounds of the Instituto de Ecología AC (19° 30'40" N, 96°56'38" W, altitude 1370 m). Each cup was buried up to the lip and two *A. gracilus* adults (mean live wt. 1.36 ± 0.07 g) were placed on the soil surface (surface area 95 cm^2) and allowed to burrow into the soil for 1 h. After this time, 1 ml of OB suspension containing 1×10^9 OBs was applied in drops of ~50 µl over the soil surface using a micropipette. Identical cups were prepared and were treated with OB suspension in the absence of earthworms. Additional cups were treated with a pair of earthworms, but were not inoculated with OB suspension (negative controls). The experiment was performed in September–October 2019, towards the end of the rainy season in this region. Each treatment was replicated seven times.

After 7 days in the field, cups were carefully removed from the ground, placed individually on a plastic tray and transported to a greenhouse for processing. Each cup was placed upside down on a clean sheet of paper to prevent the upper OB-contaminated soil from falling onto the lower layers of soil during sampling. The cup was removed to expose the lowest layer of soil in the cup, at a vertical distance of 16 cm from the surface. A 2 cm soil sample was taken from the lowest section of soil (14–16 cm depth), from the central section of the soil (6–8 cm depth) and from the upper layer (depth 0–2 cm). Each sample was taken using a plastic teaspoon that was never reused. Ten grams of each sample were mixed with 50 g diet and subjected to insect bioassay, as described above. Meteorological data for the experimental period were obtained from a digital weather station located ~150 m from the experimental site: mean (±SE) daytime temperature 24.9 ± 0.5°C, mean nighttime temperature 14.7 ± 0.3°C, mean daily precipitation 10.4 ± 2.8 mm (range: minimum 0.00, maximum 72.4 mm). The percentage of mortality values from bioassays were normally distributed and were subjected to two-way analysis of variance with soil depth and the presence of earthworms as factors. This analysis was performed using the R-based program jamovi (v. 1.0.7).

The presence of earthworms significantly increased the prevalence of mortality in soil bioassays ($F = 11.179$; *d.f.* = 1, 36; $p = 0.002$) (Figure 1(C)). The observed virus-induced mortality decreased significantly with increasing depth of soil ($F = 3.281$; *d.f.* = 2, 36; $p = 0.046$). The mean (±SE) prevalence of mortality was similar in the presence or absence of earthworms in samples taken from the upper (0–2 cm) and lower (14–16 cm) levels, but was significantly increased, from 14.2 ± 4.4% without earthworms to 34.5 ± 8.3% in the presence of earthworms, in samples taken from the middle section (6–8 cm) of the soil in each cup (Figure 1(C)). None of the bioassay larvae died from virus disease in the control treatment (earthworms in the absence of OBs). Overall, nine larvae (0.6%) died from bacterial infection and these insects were excluded from the bioassay results.

For the first time, we demonstrate the vertical transport of nucleopolyhedrovirus OBs by earthworms under field conditions. This observation is relevant because OBs removed from the soil surface will be protected from the harmful effects of ultraviolet radiation. In a previous laboratory study, small quantities of OBs were transported by *E. fetida* to depths of up to 24 cm and no OBs were transported in the absence of earthworms (Infante-Rodríguez et al., 2016). In contrast, in the present study, we detected OBs at depths of 6–8 cm and 14–16 cm in the absence of earthworms, presumably a result of percolation of OBs

from the uppermost soil by the action of rainwater. In the presence of earthworms, however, significantly greater quantities of OBs were detected at intermediate depths, which is usually where earthworms were found to be present when collecting soil samples from experimental cups. Indeed, *A. gracilus* is an epi-endogeic species usually associated with leaf litter and the topsoil layer in the upper ~15 cm of soil (Falco & Coviella, 2016).

Incubation of OB-contaminated sand with earthworms did not adversely affect OB pathogenicity, as indicated by insect bioassay. However, unexpectedly, the median prevalence of infection in samples of sand incubated with earthworms was approximately double that of the sand without earthworms (Figure 1(B)), although this was not statistically significant due to the high variation observed in the bioassays from the treatment with earthworms. We suggest this may have resulted from a shift in the size distribution of sand particles as earthworms ingested individual and aggregates of sand particles which then became disaggregated during passage through the animal's intestine. This effect was clear by visual inspection of sand particles, but was unexpected and was not quantified in the present study. Modification of soil structure is a well-recognized phenomenon in earthworms (Zhang & Schrader, 1993). The overall effect was to produce an increase in the prevalence of fine particles that are likely to have been easier to ingest by *S. frugiperda* larvae in the insect bioassay, resulting in greater heterogeneity in particle size in the earthworm treatment and a corresponding increase in heterogeneity of the larval mortality, compared to insects that fed on diet containing sand + OBs that had not been processed by earthworms. This is an intriguing idea as compared to large or heavy soil particles, OB-contaminated soil particles from earthworm casts might be more readily dispersed back onto crop plants by rain-splash or wind currents in agricultural settings. Indeed, in carefully controlled experiments, artificial precipitation resulted in the highest dispersal of OBs from sandy soil onto cotton plants, whereas air currents were most effective at dispersing OBs from clay soils onto plants, and dispersal from silt soil was intermediate in both cases (Fuxa & Richter, 2001). Nucleopolyhedrovirus OBs also bind strongly to the clay component of soils (Christian et al., 2006). Clearly, the interactions of soil OB populations with soil type, texture and the soil biota are, in general, poorly understood and merit additional study.

Earthworms are a major component of the soil fauna. The density of two earthworms per cup that we used was equivalent to a density of 210 earthworms/m^2, which is within the typical range of earthworm densities in agricultural soils (Edwards & Bohlen, 1996). At these densities, earthworm populations are capable of turning over up to 100 tonnes of soil/ha/yr in temperate regions and considerably more in the tropics (Edwards & Bohlen, 1996). It is clear then that earthworms are likely to interact frequently with OBs present in soil reservoirs due the abundance of these organisms in agricultural and forest ecosystems and the massive quantities of soil that they turn-over on an annual basis.

Acknowledgements

We are very grateful to the following colleagues at the Instituto de Ecología AC: Greta H. Rosas-Saito for electron microscopy, Isabelle Barois Boullard for advice on earthworm biology, Víctor Luna Monterrojo for meteorological data and Adolfo Campos for information on soil characterisation. Daniela P. Arreola A. Flores kindly provided soil characterisation data.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Consejo Nacional de Ciencia y Tecnología: [grant number Ayudante de investigador nivel III student award t].

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