Available online at www.sciencedirect.com
ScienceDirect
Biological Control 42 (2007) 216–225
Biological Control
www.elsevier.com/locate/ybcon

Abundance and genetic structure of nucleopolyhedrovirus populations in greenhouse substrate reservoirs

Rosa Murillo [a], Delia Muñoz [a], M. Carmen Ruíz-Portero [b], M. Dolores Alcázar [b], José E. Belda [b], Trevor Williams [a], Primitivo Caballero [a,*]

[a] *Departamento de Producción Agraria, Universidad Pública de Navarra, Pamplona 31006, Spain*
[b] *Departamento de Biología Aplicada, Universidad de Almería, Almería 04120, Spain*

Received 10 January 2007; accepted 23 April 2007
Available online 29 April 2007

Abstract

The abundance and genetic structure of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) occlusion body (OB) populations in greenhouse substrates in southern Spain was determined during an 18 month period over an area of 426 km^2. To detect the presence of OBs, substrate samples were incorporated into a semi-synthetic diet and fed to first instar *S. exigua*. SeMNPV OBs were detected in 34% of substrate samples ($N = 267$). The prevalence of substrates positive for OBs did not differ significantly between samples from different crops or from four different geographical zones. Seasonally, Spring and Summer samples had significantly greater OB concentrations than samples taken in Autumn and Winter, concurrent with changes in the host insect population. Restriction fragment length polymorphism analysis revealed at least nine different variants. Substrate pH was identified as a major factor influencing the abundance and genetic composition of the soil OB population. Certain variants were more prevalent in substrates of more alkaline pH suggesting genotype-specific differences in survival outside the host. The prevalence of mixed variant infections was inversely related to an increasing substrate pH. Greenhouse substrates of southern Spain represent an abundant and diverse reservoir for the survival of SeMNPV. Studies are required to determine the degree to which agropractices that conserve soil OB populations would favor long-term microbial control of lepidopteran pests in greenhouse crops.

Keywords: Greenhouse crops; Nucleopolyhedrovirus; Occlusion body population; Genetic structure; Soil reservoir

1. Introduction

The persistence of nucleopolyhedrovirus (*Baculoviridae*) occlusion bodies (OBs) outside the host insect is determined by numerous habitat-dependent biotic and abiotic factors. On plant surfaces, a key habitat for OB survival, persistence is negatively correlated with irradiation by solar ultraviolet light, high pH, and temperature (Jones et al., 1993; Young, 2001), and OBs may lose their insecticidal properties within a few hours. In contrast, soil represents a stable long-term reservoir for OBs in the environment (Jaques, 1964; Olofsson, 1988; Thompson et al., 1981). OB survival in soil is believed to decline with increasing pH as the integrity of the OB matrix is compromised by alkaline conditions (Jaques, 1974; McLeod et al., 1982). High temperatures and the presence of microbial agents can also reduce the persistence of OBs in soil (Peng et al., 1999; Young, 2001). However, as OBs and infected corpses are washed off plants by rainfall, soil represents the final destination for the vast majority of the OB population. The importance of soil in host-NPV population dynamics depends on the rate of transport of OBs, by rainsplash or crawling arthropods, back onto plant surfaces where they can be transmitted to susceptible foliar-feeding insects (Fuxa and Richter, 2001; Hochberg, 1989).

The beet armyworm, *Spodoptera exigua* (Hübner), is a major lepidopteran pest infesting horticultural crops

* Corresponding author. Fax: +34 948 169 732.
E-mail address: pcm92@unavarra.es (P. Caballero).

1049-9644/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2007.04.015

worldwide. In large extensions of greenhouses (~40,000 ha) in Almeria, southern Spain, *S. exigua* larvae cause severe crop losses, requiring repeated pesticide applications from May to October each year. Natural populations of *S. exigua* infesting wild plants and outdoor crops in southern Spain suffer annual epizootics of NPV disease (Caballero et al., 1992a), that probably initiate from OBs transported from the soil reservoir (Fuxa, 2004). However, NPV epizootics in greenhouse crops tend to occur only when larval populations reach high densities because chemical control measures have failed to suppress the pest. Outbreaks of pesticide-resistant strains of *S. exigua* can be fairly common in Almería (Lasa et al., 2007a) and they usually result in devastating damage to the affected crop. Consequently, there exists a strong demand for a bioinsecticide, based on *S. exigua* multiple nucleopolyhedrovirus (SeMNPV), that is currently being developed for use in greenhouses of this region (Lasa et al., 2007b).

Genotypic diversity in isolates from SeMNPV-killed larvae has been observed repeatedly in many parts of the world (Caballero et al., 1992b; Gelernter and Federici, 1986; Hara et al., 1995; Kolodny-Hirsch et al., 1993; Kondo et al., 1994; Muñoz et al., 1998, 1999; Murillo et al., 2001; Vlak et al., 1981). In contrast, genetic diversity in soil OB populations and the factors that influence the abundance of OB populations in the substrate of commercial greenhouses remain unknown.

This study aimed to (i) quantify the abundance of SeMNPV OBs in the substrates of greenhouses in Almeria, Spain, (ii) determine the genotypic diversity of the soil OB populations and, (iii) examine the relationship between the abundance and genetic structure of the SeMNPV population and a selection of temporal, agronomic and physicochemical factors in greenhouse substrates. The results of these studies have clear implications for risk assessment of SeMNPV insecticides applied to greenhouse crops and provide valuable ecological information for the biopesticide registration process.

2. Materials and methods

2.1. Insects and virus

A laboratory colony of *S. exigua*, with no history of exposure to pesticides, was maintained on a wheatgerm semi-synthetic diet (Greene et al., 1976), under controlled conditions (26 ± 2 °C, $70 \pm 5\%$ R.H., and photoperiod 16 h:8 h L:D) in the Universidad Pública de Navarra (Pamplona, Spain). The SeMNPV strain SP2 (SeMNPV-SP2) was originally isolated from *S. exigua* cadavers collected during an epizootic in greenhouses at El Ejido (Almería, Spain) in 1990 (Caballero et al., 1992b). SeMNPV-SP2 comprises at least four different genotypes (Muñoz et al., 1999). Occlusion bodies were produced by feeding healthy fourth instar *S. exigua* with virus-contaminated diet. OBs were extracted from virus-killed larvae by homogenizing them in 0.1% (w/v) sodium dodecyl sulfate (SDS) and filtering through cheesecloth. OBs were centrifuged at 3000*g* for 10 min, washed with 0.1% NaCl (w/v) and centrifuged again. The resulting pellet containing the OBs was resuspended in bidistilled water and the OB concentration was determined using an improved Neubauer hemocytomer (Hawksley, Lancing, UK) under phase-contrast microscopy. Counted suspensions of OBs were stored at 4 °C until use.

2.2. Greenhouse sampling

The soil substrate of Almería greenhouses are commonly formed by a clay-loam soil base, covered by a 5–10 cm deep layer of finely crushed white volcanic porous substrate with a grain diameter ranging between 1 and 3 mm. Between these layers, manure is incorporated as an organic fertilizer. The layers tend to become mixed over time as old crops are uprooted and new ones are planted directly in the substrate. Soils were sampled during an 18 month period between July 2002 and December 2003. Samples were taken over an area of 426 km^2, in which 40,000 ha of greenhouses are located around the city of Almería, southern Spain. For sampling, this area was divided into four geographical zones based on climate, agronomic characteristics, and structural features (Fig. 1). Samples were collected from 20 randomly chosen greenhouses each month ($N = 360$), that usually involved different numbers of samples from all four geographical zones. All greenhouses were producing crops using standard insect pest control measures, principally based on the application of synthetic insecticides. Each greenhouse was sampled only once. Samples were taken by scraping away the upper 5 cm of substrate at depths of 5–10 cm. Each sample comprised a mixture of subsamples from three points combined into a total of 500 g substrate from each greenhouse: one taken under a row of crops and two taken from randomly selected points between crop rows. Samples were classified taking into account the date, crop, and geographical zone from which they were collected. These samples were immediately transported to the Universidad Pública de Navarra and stored at 4 °C for 2–8 weeks prior to analysis, during which time the infectivity of OBs did not decrease significantly (R. Murillo, unpublished data). For each sample, the substrate pH value was measured using a digital pH meter probe (Crison Instruments, Alella, Spain) immersed in 25 g substrate + 100 ml bidistilled water.

To determine the prevalence of natural NPV infection in the *S. exigua* population, one hundred larvae were collected during monthly substrate sampling from those greenhouses with spontaneous *S. exigua* infestations between July and December 2003. The larvae were reared individually on semi-synthetic diet in the laboratory, under the controlled conditions described above, until death or pupation. Virus-induced mortality was confirmed by observing the viral occlusion bodies (OBs) under phase-contrast microscopy. The cadavers of NPV-killed larvae were stored individually at −20 °C for analysis.

A

B

Zone	Name	Mean annual temperature (°C) (min –max)	Range of substrate pH	Geographical position	Main crops	General age of structures	Magnitude of *S. exigua* infestations
1	Adra-El Ejido	19.4 (8.7 -30.5)	7.7-8.7	36°41´N 2°47´W	Mainly sweet pepper	Recent	Very severe, very common
2	Roquetas-La Mojonera	18.1 (9.6 -2 8.3)	7.6-8.4	36°45´N 2°36´W	Sweet pepper, tomato	Recent	Severe, common
3	La Cañada	21.6 (7.9 -3 5.4)	7.2-8.4	37°03´N 2°23´W	Tomato, melon	Recent	Common
4	Níjar	20.5 (6.9 -3 8.5)	7.7-9.1	36°58´N 2°12´W	Tomato, melon	Older	Occasional

Fig. 1. Location and geographical zones in area of sampling (A) and agronomic characteristics and range of substrate pH values observed in each zone (B).

2.3. Substrate bioassay

To determine the sensitivity of the substrate bioassay, a substrate sample (pH 8.1) originating from an Almerian greenhouse was selected at random and processed as described by Richards and Christian (1999). Substrate was air dried at 20 °C, passed through a 1 mm gauge sieve and autoclaved. One of five different concentrations of SeMNPV-SP2 OBs was added to a 10 g sample of sterilized substrate, air dried overnight and then incorporated into 100 ml of semi-synthetic diet at a temperature of 50 °C. This resulted in substrate-diet mixtures containing 7.4, 22.2, 66.6, 200, and 600 OB/ml of diet (equivalent to 74–6000 OB/g substrate sample). The substrate-diet mixture was agitated thoroughly using a hand-held kitchen blender for 1 min. The mixture was then poured into clean plastic trays (14 cm diameter) to form a layer ~1 cm thick, and allowed to solidify. When solid, the mixture was cut into 25 small pieces of ~5 mm diameter and placed individually in the lid of 1.5 ml Eppendorf tubes. Tubes had been pierced with a hot needle to allow ventilation. Mid-first instars of *S. exigua* from the laboratory colony were individually placed on the diet pieces and the tubes were sealed. A group of 25 larvae were placed on sterile substrate-diet mixture as controls. Insects were maintained at 26 ± 2 °C in darkness. Virus-induced mortality was recorded at 7 days post-inoculation by which time all infected larvae had died (Muñoz et al., 1999). The procedure was performed three times.

Once the sensitivity of the bioassay had been determined, substrate samples originating from Almería were air dried, sieved but not autoclaved, and incorporated into insect diet at the rate of 10 g substrate/100 ml diet, as described above. Groups of 50 first instar *S. exigua* were individually fed with diet mixed with one of the substrate samples, as describe above. Additional groups of larvae were individually fed with diet mixed with sterile substrate as controls. Larvae were held at 26 ± 2 °C in darkness and virus-induced mortality was determined at 7 days post-treatment, and confirmed by microscope observation of OBs. Seventy-eight substrate samples were excluded from the study since the larvae suffered a high incidence of non-specific mortality (>10%), possibly due to pesticide contamination resulting in a total of 282 substrate samples.

2.4. Identification of genotypic variants

All the larvae that died from NPV disease during the bioassays corresponding to the first eight months of

sampling ($N = 56$), and 10 more larvae per month from the rest of the sampling period ($N = 100$), were collected for genotypic analysis. OBs from individual virus-killed larvae were purified and counted as described above. Greenhouse-collected larvae died of infection in late instars and produced large quantities of OBs that did not require additional amplification whereas larvae from substrate bioassays often died in early instars and produced small quantities of OBs. The OBs from every individual larva that died in bioassays were amplified by incorporating OBs into a mixture of 10% w/v sucrose, 0.001% (w/v) Fluorella blue at a concentration of 78 OB/μl (predicted to cause 50% mortality based on previous studies) and fed to 20 fourth instar *S. exigua*. After inoculation, these insects were reared on virus-free diet at constant temperature (25 ± 2 °C) and darkness until death or pupation. Cadavers were collected individually and stored at −20 °C until used.

Viral DNA was obtained by dissolving the OB matrix in an alkaline solution (0.3 M Na_2CO_3, 0.5 M NaCl, 0.03 M EDTA; pH 10.5), incubating the virions with proteinase K, followed by phenol/chloroform extraction and alcohol precipitation (Muñoz et al., 1997). The concentration of DNA was estimated by agarose gel electrophoresis. Approximately 2 μg of viral DNA was incubated with 10 units of restriction enzymes PstI and BglII for 4–12 h. Reactions were stopped by addition of loading buffer (0.25% w/v bromophenol blue, and 40% w/v sucrose in water), loaded in 0.7% agarose gels with TAE buffer (40 mM Tris–Acetate; 1 mM EDTA), and electrophoresed at 20–40 V for 6–12 h. Ethidium bromide stained gels were photographed on a UV transilluminator using a Bio-Rad Gel-doc and software (Bio-Rad, Madrid, Spain).

2.5. Data analysis

Concentration-mortality data from the substrate bioassay calibration study were subjected to logit analysis by generalized linear modeling (Numerical Algorithms Group, 1993) with a binomial error distribution. The frequencies of substrate samples found to contain OBs and the frequencies of different variants were compared for crop type, geographical zone and season by log likelihood ratio test (G test) (Sokal and Rohlf, 1981). Seasonal effects were compared by grouping results from samples taken in Spring (March, April, May), Summer (June, July, August), Autumn (September, October, November) and Winter (December, January, February). The prevalence of virus-induced mortality in substrate bioassays was compared against the variables of season and type of infections (single or multiple variant) by subjecting them to non-parametric Kruskal–Wallis and Mann–Whitney tests. Median values rather than means have been reported for analyses involving non-parametric statistics. The relationship between substrate pH and observed mortality in the bioassays was determined by linear regression.

3. Results

3.1. Calibration of substrate bioassay

The concentration-mortality response was estimated from the logit regression odds ratio $\ln(p/q) = 1.046$ [± 0.114] ln(OB concentration) −8.269 [± 0.843], where values in square parentheses indicate SE of slope and intercept estimates. The LC_{50} value of the SeMNPV-SP2 in sterilized substrate that had been inoculated with OBs was estimated at 2.72×10^3 OBs/g of substrate (range of 95% confidence intervals: 2.01×10^3–3.72×10^3) for mid-first instar *S. exigua* reared on a 10% (w/v) substrate-diet mixture. No virus or non-specific mortality was registered in the control insects. There was no evidence of overdispersion in the mortality data. The lowest threshold for OB detection in greenhouse substrate using this bioassay was defined by the death of 1/75 larvae that, according to the logit regression, would have consumed an estimated concentration of 43 OBs/g of substrate.

3.2. Presence of OBs in substrate samples and greenhouse-collected larvae

Of a total of 282 substrate samples collected and analyzed over an 18 month period, SeMNPV OBs were detected in 34.4% (in which at least one larva died of NPV infection). The spatial distribution of OBs was homogeneous, with no significant differences found in the frequency of OB positive samples from different zones ($G = 1.19$, df = 3, $P = 0.755$) or different crops ($G = 11.3$, df = 6, $P = 0.124$) (Fig. 2). OBs were also frequently detected in substrate samples from uncultivated areas outside of greenhouses (shown as "uncultivated" in Fig. 2). The frequency of OB positive samples did not differ according to season ($G = 5.07$, df = 7, $P = 0.166$). Substrate samples in which no OBs were detected were excluded from the remainder of the analyses.

The prevalence of OBs, reflected in the proportion of NPV-killed larvae per sample, varied markedly over the

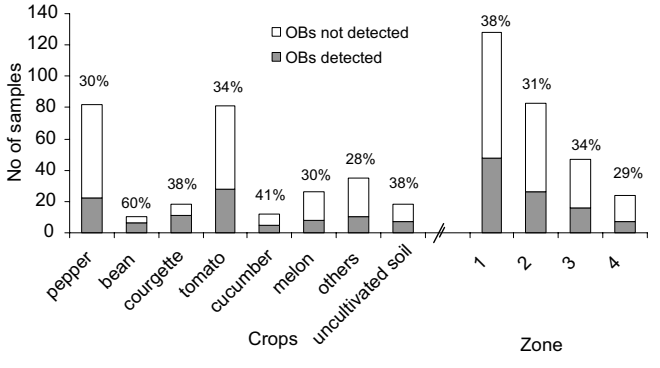

Fig. 2. Abundance of samples in which OBs were detected and not detected in substrate collected from different crops, uncultivated substrate, and four designated geographical zones (shown in Fig. 1). The percentage of samples with OBs is indicated above each column.

sampling period, from a minimum of <1% in December 2002 to a maximum of 80% in July and September 2002 (data not shown). Seasonally, Spring and Summer samples showed significantly greater mortalities (median 10.5%) than those from Autumn and Winter (median mortalities 4.2% and 2.1%, respectively) (Fig. 3A) (Kruskal–Wallis $H = 35.5$, df = 3, $P < 0.001$). The prevalence of natural virus-induced mortality in larvae collected from infested greenhouse crops was consistently low and never exceeded 3.5% in any sample from any month.

3.3. Relationship of substrate pH to virus-induced mortality

Substrate pH values ranged from 7.3 to 9.5 in all samples tested. The average pH value varied significantly for samples collected from different zones (Kruskal Wallis $H = 16.1$, df = 3, $P = 0.001$) with the most alkaline substrates in zones 3 and 4 (Fig. 3B). Substrate pH also varied seasonally ($F_{3,156} = 44.6$, $P < 0.001$), with the highest values observed in samples taken in the Autumn (Fig. 3C). The prevalence of virus-induced mortality in substrate bioassays was negatively correlated with substrate pH for all samples across seasons and zones (Mortality = −0.19 substrate pH + 1.75, $R^2 = 0.235$, $F_{1,158} = 48.5$, $P < 0.001$) (Fig. 3D). Samples in which no mortality was registered were excluded from the analysis (mean [± SE] pH of OB negative substrates was 8.35 ± 0.03).

3.4. Genetic diversity in substrate OB populations

Six distinct variants showing a single dominant genotype were distinguished by BglII restriction fragment length polymorphism (RFLP) of viral DNA isolated from 177 NPV-killed larvae (Fig. 4). Of these insects, 21 were field-collected larvae that died in the laboratory from natural NPV infections and 156 were infected cadavers from the substrate bioassays. These genotypes were named Se-G24, Se-G25, Se-G26, Se-G27, Se-G28, and Se-G29. The Se-G24, Se-G25, and Se-G26 *Bgl*II profiles were identical to those previously reported for SeMNPV-SP2 (Muñoz et al., 1999) whereas Se-G27, Se-G28, and Se-G29 have not been previously reported. Muñoz et al. (1999) also described an additional variant called SP2D, cloned from the wild-type isolate, but we did not detect this variant in the present study. Compared to Se-G24, Se-G27 presented a characteristic BglII fragment of around 31 kb, while the Se-G24 fragments BglII-C and BglII-E were absent in Se-G27. Se-G28 showed a marker fragment of ~9 kb with respect to Se-G24 and the absence of Se-G24 BglII-K and BglII-L of 4.8 and 4.2 kb, respectively (Fig. 4). The Se-G29 BglII profile differed from that of Se-G24 in the size of the BglII-K fragment, which at ~4.5 kb, is the smallest observed so far for the SeMNPV-SP2 strain (Fig. 4). All six isolates were detected in larvae from the substrate bioassays, whereas only two were isolated from greenhouse-collected larvae, in which 65% of profiles corresponded to that of Se-G25 and 35% corresponded to Se-G24.

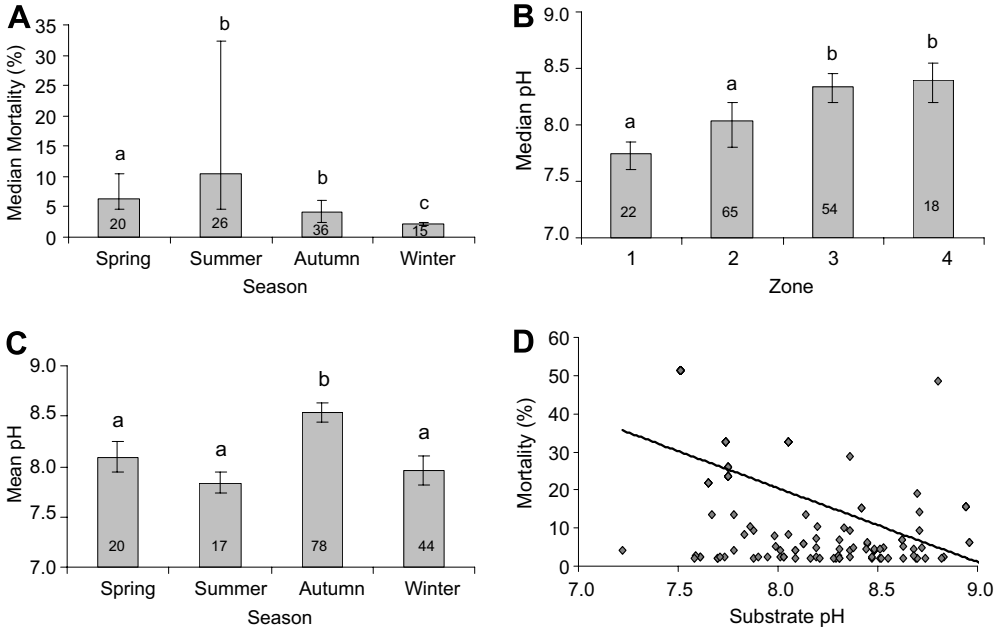

Fig. 3. Prevalence of SeMNPV-induced mortality by season observed at bioassay against *S. exigua* first instars (A). Mean or median values of substrate pH of samples collected in four geographical zones (B) and over seasons (C). Regression of prevalence of virus induced mortality in bioassayed substrate samples against substrate pH (Mortality (%) = −19.41 (substrate pH) + 175.78) (D). Columns headed by different letters indicate significant differences between medians by Mann–Whitney test ($P < 0.05$) or between means by *t*-test ($P < 0.05$). Error bars are the 25 and 75 percentiles for the medians (A and B) and 95% confidence limits for the means (C). Numbers inside columns indicate sample sizes.

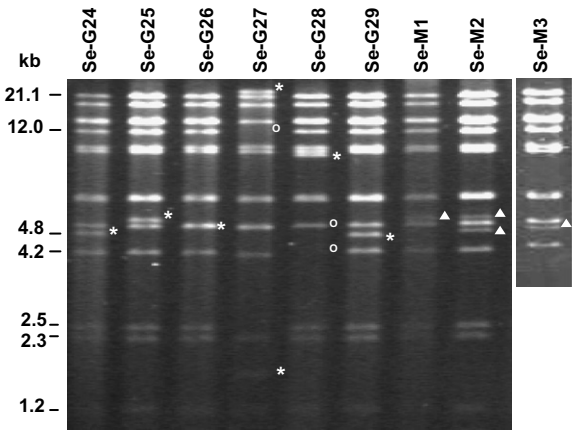

Fig. 4. Restriction endonuclease profiles with *Bgl*II of viral DNA extracted from isolates with single (Se-G24, Se-G25, Se-G26, Se-G27, Se-G28, and Se-G29) or co-dominant genotypes (Se-M1, Se-M2, and Se-M3). An asterisk indicates polymorphic fragments, the position of fragments absent with respect to isolate Se-G24 are indicated by a circle and the submolar fragments are indicated by a triangle. The molecular sizes in kb of some of the fragments are shown to the left of the figures.

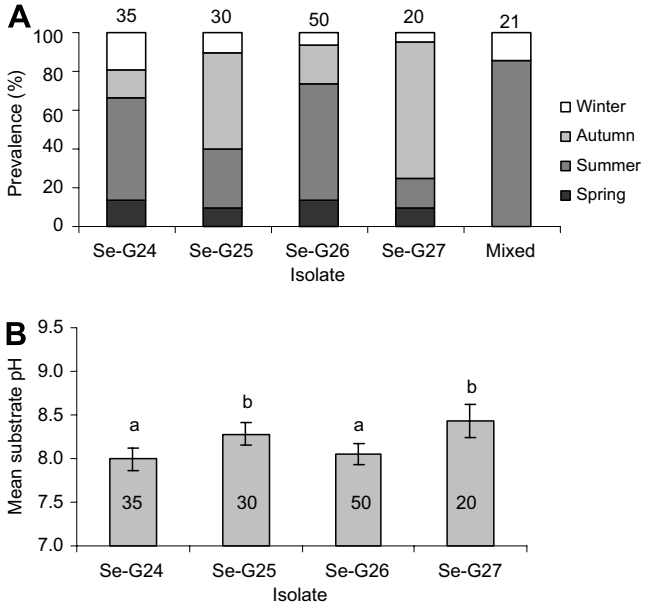

Fig. 5. Frequency of the single dominant variants (Se-G24, Se-G25, Se-G26, and Se-G27), and mixtures of two co-dominant variants (mixed) isolated from each zone (A) Numbers above each bar indicate total number of samples. Mean substrate pH of samples from which single dominant variants were isolated (B). Error bars are 95% confidence limits. Columns headed by different letters were significantly different by *t*-test ($P \leqslant 0.05$).

3.5. Genotypic structure of substrate OB populations

The most common substrate variant was Se-G26 (32%) followed by Se-G24, Se-G25, and Se-G27 that were observed with frequencies of 22, 19, and 12%, respectively. These four variants were detected from substrate samples collected throughout the 18 month sampling period and in all four zones, whereas Se-G28 and Se-G29 were detected only once and twice in zones 1 and 2, respectively. The remaining 15% of profiles showed the presence of submolar fragments, indicating that these isolates comprised a mixture of at least two variants. Profiles showing mixtures of Se-G26 and Se-G25 (5% of total restriction profiles), of Se-G24 and Se-G25 (5%), and of Se-G24 and Se-G26 (4%) (Fig. 4, lanes M1–M3) were the most frequently observed mixtures, representing 90% (21/24) of the observed variant mixtures. The frequency of different variants in substrate samples did not differ significantly across zones ($G = 20$, df $= 12$, $P = 0.067$), but differed seasonally; the frequency of Se-G24, Se-G26 and mixtures was significantly higher in the Summer samples, while that of Se-G25 and Se-G27 was higher in the Autumn ($G = 48$, df $= 12$, $P < 0.001$) (Fig. 5A). Variants Se-G25 and Se-G27 were isolated from substrates with a significantly higher average pH (mean 8.3 ± 0.10) than isolates Se-G24 and Se-G26 (mean 8.0 ± 0.07) ($F_{3,132} = 5.2$; $P < 0.001$) (Fig. 5B). Genotypes Se-G28 and Se-G29 were isolated at extremely low frequencies and were not included in these analyses.

Mixed variant infections were observed in substrates with significantly lower median pH values than single genotype infections (Fig. 6A) (Mann–Whitney $U = 358$, $P < 0.001$). Mixed genotype infections were more likely to be observed in samples with a high prevalence of mortality, indicating a high density of OBs, in which median mortality was more than double that seen in samples causing only single variant infections (Mann–Whitney $U = 963$, $P < 0.05$) (Fig. 6B).

4. Discussion

Nucleopolyhedrovirus OBs bind very strongly to soil particles, especially to the clay component of soil (Christian et al., 2006). Previous attempts to quantify the presence of OBs in soil have employed desorbant chemicals and ultrasonification to dislodge OBs prior to quantification (Ebling and Holmes, 2002; England et al., 2001; Evans et al., 1980). The diet incorporation bioassay technique developed by Richards and Christian (1999), overcomes this problem by delivering soil bound OBs directly to the alkaline insect midgut to initiate lethal infection. Diet-incorporation bioassay proved a sensitive technique to detect and quantify SeMNPV OBs in the substrates of greenhouses in Almería, Spain ($LC_{50} = 2.72 \times 10^3$ OBs/g of substrate; $LC_{10} = 322$ OBs/g of substrate). This level of sensitivity was very similar to that reported for the detection of *Helicoverpa armigera* NPV ($LC_{50} = 2.15 \times 10^3$ OBs/g of substrate) in Australian substrates (Richards and Christian, 1999). The diet-incorporation bioassay was more sensitive than a PCR based technique reported by England et al. (2001) that detected 1.5×10^4 and 4.2×10^4 OBs/g of detritus and forest litter, respectively. It was also more sensitive than a complex procedure involving lyophilization, OB extraction and PCR amplification of baculovirus DNA described by Ebling and Holmes (2002), with a sensitivity

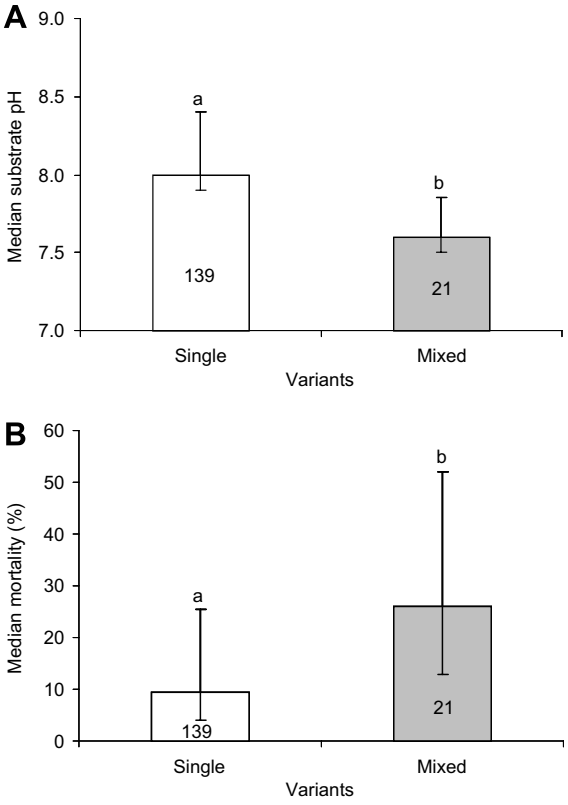

Fig. 6. Median substrate pH (A) and percentage of insect mortality (B) observed in substrate samples from which single and mixed genotype SeMNPV variants were isolated. Columns headed by different letters indicate significant differences by Mann–Whitney test ($P < 0.01$). Error bars are the 25 and 75 percentiles. Numbers within columns indicate sample sizes.

that varied from 2×10^2 to 10^3 OBs/g of lyophilized samples from different soil horizons.

The OBs of SeMNPV were found to be abundant across different zones of the Almeria region and in greenhouses growing different crops, all of which are attacked by *S. exigua*. The corpses of virus-infected insects invariably contain between 10^6 and 10^9 OBs, whereas the density of OBs in the soil was considerably lower than this; we observed between ~1% and 80% mortality in substrate bioassays representing approximately 40–10,000 OBs/g of substrate. Previous studies have reported that OBs persist in the soil despite the unstable conditions of seasonal crop habitats. Fuxa and Richter (1999) reported that OBs of *Anticarsia gemmatalis* MNPV (AgMNPV) persisted in the soil after a single viral release, even in plots in which crops were rotated. Agricultural operations such as disking, harrowing, planting and cultivating did not reduce the quantity of AgMNPV OBs in soils collected from soybean crops (Fuxa and Richter, 1996). However, factors such as harsh weather, and elevated pH values or high temperatures rapidly degrade OBs in the soil. The air temperature inside greenhouses during the Summer months may exceed 40 °C but sunlight and rainfall effects on soil OB populations are likely to be minimal as the plastic structure greatly reduces UV penetration (Kittas and Baille, 1998; Lasa et al., 2007b), and almost all irrigation is subterranean. In addition, the incidence of OBs in samples from non-cultivated substrates was comparable to that of cultivated substrate, indicating that OBs can persist over extended periods in the absence of the insect host and the host plant. As such, the substrate is likely to represent an important habitat for OB survival.

We detected two main factors influencing the prevalence of virus-induced mortality in substrate: the season and substrate pH. Alkaline pH values observed in the Autumn, probably on account of substrate disinfection treatments applied in greenhouses, resulted in a reduction in virus-induced mortality. Because samples taken inside each greenhouse were pooled and greenhouses were only sampled once, it was not possible to determine the variation of substrate pH over time within individual greenhouses. In early studies, Jaques (1969, 1975) reported that most of the OBs remain bound to soil particles close to the soil surface and OB survival was greater at pHs of 5.1–6.0 than at more alkaline pH values (Jaques, 1985). We cannot exclude the possibility that OBs are inherently less infectious in alkaline substrate, although this seems unlikely given that the pH of the insect midgut, the site of initial infection, is far more alkaline (pH 10–11) than any of the substrate samples that we tested.

Seasonal fluctuations in the prevalence of substrate OB populations coincided with changes in the density of the host insect. *Spodoptera exigua* may complete up to six generations in the greenhouses of southern Spain from May to November with peak population densities between July and September. The abundance of OBs in soil has been demonstrated to increase during natural epizootics in forests (Thompson et al., 1981), soybean crops (Fuxa and Richter, 2001), and soil-dwelling pests of pastures (Crawford and Kalmakoff, 1977), and detailed observations of both forest and pasture insects have demonstrated that virus environmental reservoirs can have a major influence on the population dynamics of these pests (Kalmakoff and Crawford, 1982; Richards et al., 1999).

Greenhouse infestations of *S. exigua* are initiated by the entry of gravid females through holes in the plastic structure or by introducing plants that have been contaminated with eggs or larvae in nursery areas. These insects may have acquired infection by vertical transmission from the parent (Kukan, 1999) and die during the larval stage releasing large quantities of OBs onto plant surfaces that are subsequently translocated to the soil substrate by dew droplets, the run-off of pesticidal sprays and the process of shedding senescent leaves.

The magnitude of the influence of long-lived infective stages of pathogens on host population dynamics depends crucially on the rate of movement between the soil and the plant surfaces (Fuxa and Richter, 2001; Hochberg, 1989). This dispersal represents the transition between a non-transmissible reservoir population and an ephemeral but transmissible OB subpopulation on the host plant.

Different factors may influence the rate of flow of OBs from soil to plant surfaces including air currents, soil type, and rainfall and the interactions of these factors (Fuxa and Richter, 1999, 2001). For example, with a similar density of OBs in soil, the populations of *Spodoptera frugiperda* (J.E. Smith) develop NPV epizootics more readily on grasses than on maize, presumably reflecting more efficient passage of virus back to the host plant (Fuxa, 2004; Fuxa and Geaghan, 1983) or an interaction between virus and host plant chemistry (Hoover et al., 2000). In our case, agronomic practices and the behavior of the host, that often moves between soil surface and host plant during dispersal or after emerging from the pupal stage, are likely to facilitate the movement of OBs from soil to plant surfaces (Hostetter and Bell, 1985), although quantifying OB traslocation in the greenhouse environment requires additional studies.

Six distinct variants showing a single dominant genotype and three types of variant mixtures of SeMNPV were detected within the entire collection of substrate samples analyzed. Some of the single dominant variants (Se-G24, Se-G25 and Se-G26) had BglII profiles identical to those described in a previous study from natural populations of infected larvae in this region (Muñoz et al., 1999) these variants also represented the most frequent genotypes in the substrate OB populations. In addition, Se-G24 and Se-G25 were present in NPV-killed larvae that were field-collected during the present study and their restriction profiles were almost identical to those observed from larvae collected in field crops located 500 km away from Almería (Murillo et al., 2001). However, caution is necessary when interpreting the results of genotype analyses based on RFLP techniques because minority genotypes present at very low concentrations are likely to remain undetected, resulting in an underestimate of the true incidence of mixed infections.

The study of genotypic diversity in substrate OB populations led to the discovery of infrequent genotypes such as Se-G27, Se-G28 and Se-G29 that were not detected in NPV-killed larvae during the entire sampling period. Notably, variant Se-G27 presented identical BglII, PstI and BamHI profiles to the dominant genotype (Se-US2) of a commercial bioinsecticide, Spod-X (Certis, USA) based on an isolate from the United States. This product has been marketed in several countries, including the Netherlands, but has not been registered in Spain. We suspect that Spod-X may have been imported and used by farmers clandestinely to control outbreaks of *S. exigua* that have developed resistance to chemical control measures. Conversations with local phytosanitary officials support such assertions (J.E. Belda, personal observations).

The maintenance of genotypic variation in NPV populations may be promoted by several mechanisms including differential selection, suggesting that particular genotypes perform better under certain ecological conditions (Hodgson et al., 2003), or by a combination of differential host susceptibility and a high degree of spatial structure in the distribution of NPV variants (Cooper et al., 2003). The genetic composition of substrate OB populations in greenhouses of southern Spain is not random, but structured by environmental factors such as pH and season. Certain genotypes (Se-G27 and Se-G25) were more abundant in alkaline substrates and these genotypes became common in the Autumn when substrate pH increased. We assume that this is due to differential loss of genotypes from the OB population. Certain variants may be better able to withstand long periods or more adverse conditions in the substrate than others, possibly due to variation in the size or resilience of OBs. We therefore predict that, in the absence of OBs arriving from external sources, the genetic heterogeneity of OB populations in the substrate is likely to decrease over time, although this requires confirmation from empirical studies.

The results of substrate bioassays may have been influenced by virus genotypic interactions that result in increased pathogenicity, and therefore an increased likelihood of detection in substrates containing OBs with multiple virus genotypes. Such interactions have been reported in *S. frugiperda* MNPV (López-Ferber et al., 2003; Simón et al., 2004, 2005; Simón et al., 2006), and *Panolis flammea* MNPV (Hodgson et al., 2004) and mixtures of certain genotypes may also result in increased pathogenicity, over that of single genotype inocula, in Almerian populations of SeMNPV (Murillo et al., 2006).

These results are clearly pertinent to agroecosystem management and in understanding host-virus population dynamics. These results will also assist in risk assessment applied to natural and genetically modified (GM) baculovirus insecticides as knowledge on survival, population growth and spread can facilitate biosecurity evaluations of environmental release, specifically the likelihood of accumulation of the virus in the substrate reservoir from one growing season to the next, and the likelihood of interactions between NPVs and non-target organisms, such as soil microorganisms and ground-dwelling invertebrates.

We conclude that the substrates of greenhouses of southern Spain harbor an abundant and diverse population of SeMNPV OBs. The density of the substrate OB population fluctuates seasonally following the changes in host abundance. Substrate pH was identified as a major factor influencing both the abundance and genetic composition of the substrate OB population. Indeed, the genetic structure of the substrate OB population is not determined purely by the OB population produced in infected insects, but may represent a distinct subpopulation in which certain variants adopt strategies of long term persistence in the substrate reservoir, whereas others are eliminated by differential survival. Moreover, the adoption of agropractices, such as the use of non-alkaline substrate disinfectants, would conserve OB reservoir populations, and may result in a greater number of infections in subsequent growing seasons, than would been seen following the use of alkaline disinfectants. These intriguing hypotheses are clearly testable under field conditions.

Acknowledgments

We thank Iñaki Pagola and Naiara Lizarraga for laboratory assistance and Noelia Gorria for insect rearing. The study received financial support from CICYT Project AGL2002–04320–C02–01.

References

Caballero, P., Aldebis, H.K., Vargas-Osuna, E., Santiago-Alvarez, C., 1992a. Epizootics caused by a nuclear polyhedrosis virus in populations of *Spodoptera exigua* in southern Spain. Biocontrol Sci. Technol. 2, 35–38.

Caballero, P., Zuidema, D., Santiago-Alvarez, C., Vlak, J.M., 1992b. Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. Biocontrol Sci. Technol. 2, 145–157.

Christian, P.D., Richards, A.R., Williams, T., 2006. Differential adsorption of occluded and nonoccluded insect-pathogenic viruses to soil-forming minerals. Appl. Environ. Microbiol. 72, 4648–4652.

Cooper, D., Cory, J.S., Myers, J.H., 2003. Hierarchical spatial structure of genetically variable nucleopolyhedroviruses infecting cyclic populations of western tent caterpillars. Mol. Ecol. 12, 881–890.

Crawford, A.M., Kalmakoff, J., 1977. A host virus interaction in pasture habitat: *Wiseana* spp. (Lepidoptera: Hepilidae) and its baculovirus. J. Invertebr. Pathol. 29, 81–87.

Ebling, P.M., Holmes, S.B., 2002. A refined method for the detection of baculovirus occlusion bodies in forest terrestrial and aquatic habitats. Pest Manag. Sci. 58, 1216–1222.

England, L.S., Trevors, J.T., Holmes, S.B., 2001. Extraction and detection of baculovirus DNA from lake water, detritus and forest litter. J. Appl. Microbiol. 90, 630–636.

Evans, H.F., Bishop, J.M., Page, E.A., 1980. Methods for the quantitative assessment of nuclear-polyhedrosis virus in soil. J. Invertebr. Pathol. 35, 1–8.

Fuxa, J.R., 2004. Ecology of insect nucleopolyhedroviruses. Agric. Ecosyst. Environ. 103, 27–43.

Fuxa, J.R., Geaghan, J.P., 1983. Multiple-regression analysis of factors affecting prevalence of nuclear polyhedrosis virus in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations. Environ. Entomol. 12, 311–316.

Fuxa, J.R., Richter, A.R., 1996. Effect of agricultural operations and precipitation on vertical distribution of nuclear polyhedrosis virus in soil. Biol. Control 6, 324–329.

Fuxa, J.R., Richter, A.R., 1999. Classical biological control in an ephemeral habitat with *Anticarsia gemmatalis* nucleopolyhedrovirus. Biocontrol 44, 403–419.

Fuxa, J.R., Richter, A.R., 2001. Quantification of soil-to-plant transport of recombinant nucleopolyhedrovirus: effects of soil type and moisture, air currents, and precipitation. Appl. Environ. Microbiol. 67, 5166–5170.

Gelernter, W.D., Federici, B.A., 1986. Isolation, identification and determination of virulence of a nuclear polyhedrosis virus from the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). Environ. Entomol. 15, 240–245.

Greene, G.L., Leppla, N.C., Dickerson, W.A., 1976. Velvetbean caterpillar: a rearing procedure and artificial medium. J. Econ. Entomol. 69, 487–488.

Hara, K., Funakoshi, M., Kawarabata, T., 1995. *In vivo* and *in vitro* characterization of several isolates of *Spodoptera exigua* nuclear polyhedrosis virus. Acta Virol. 39, 215–222.

Hochberg, M.E., 1989. The potential role of pathogens in biological control. Nature 337, 262–265.

Hodgson, D.J., Hitchman, R.B., Vanbergen, A.J., Hails, R.S., Hartley, S.E., Possee, R.D., Watt, A.D., Cory, J.S., 2003. The existence and persistence of genotypic variation in nucleopolyhedrovirus populations. In: Hails, R.S., Beringer, J.E., Godfray, H.C.J. (Eds.), . Ecological Dynamics of Genes. British Ecological Soc. Symp., 15. Blackwell, Oxford, UK, pp. 258–280.

Hodgson, D.J., Hitchman, R.B., Vanbergen, A.J., Hails, R.S., Possee, R.D., Cory, J.S., 2004. Host ecology determines the relative fitness of virus genotypes in mixed-genotype nucleopolyhedrovirus infections. J. Evol. Biol. 17, 1018–1025.

Hoover, K., Washburn, J.O., Volkman, L.E., 2000. Midgut-based resistance of *Heliothis virescens* to baculovirus infection mediated by phytochemicals in cotton. J. Insect Physiol. 46, 999–1007.

Hostetter, D.L., Bell, M.R., 1985. Natural dispersal of baculovirus in the environment. In: Maramorosch, K., Sherman, K.E. (Eds.), . Viral Insecticides for Biological Control. Academic Press, Orlando, FL, pp. 249–284.

Jaques, R.P., 1964. The persistence of a nuclear-polyhedrosis virus in soil. J. Insect Pathol. 6, 251–254.

Jaques, R.P., 1969. Leaching of the nuclear-polyhedrosis virus of *Trichoplusia ni* from soil. J. Invertebr. Pathol. 13, 256–263.

Jaques, R.P., 1974. Occurrence and accumulation of viruses of *Trichoplusia ni* in treated field tests. J. Invertebr. Pathol. 23, 140–152.

Jaques, R.P., 1975. Persistence, accumulation, and denaturation of nuclear polyhedrosis virus and granulosis viruses. In: Vail, P.V. (Ed.), Baculovirus for Insect Pest Control: Safety Consideratons. Am. Soc. Microbiol., Washington, DC, pp. 90–99.

Jaques, R.P., 1985. Stability of viruses in the environment. In: Maramorosch, K., Sherman, K.E. (Eds.), Viral Insecticides for Biological Control. Academic Press, Orlando, FL, pp. 285–359.

Jones, K.A., Moawad, G., Mckinley, D.J., Grzywacz, D., 1993. The effect of natural sunlight on *Spodoptera littoralis* nuclear polyhedrosis virus. Biocontrol Sci. Technol. 3, 189–197.

Kalmakoff, J., Crawford, A.M., 1982. Enzootic virus control of *Wiseana* spp. in the pasture environment. In: Kurstak, E. (Ed.), Microbial and Viral Pesticides. Marcel Dekker, New York, pp. 435–448.

Kittas, C., Baille, A., 1998. Determination of the spectral properties of several greenhouse cover materials and evaluation of specific parameters related to plant response. J. Agric. Eng. 71, 193–202.

Kolodny-Hirsch, D.M., Warkentin, D.L., Alvarado-Rodríguez, B., Kirkland, R., 1993. *Spodoptera exigua* nuclear polyhedrovirus as a candidate viral insecticide for the beet armyworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 86, 314–321.

Kondo, A., Yamamoto, M., Takashi, S., Maeda, S., 1994. Isolation and characterization of nuclear polyhedrosis viruses from the beet armyworm *Spdoptera exigua* (Lepidoptera: Noctuidae) found in Shiga. Japan Entomol. Zool. 86, 314–321.

Kukan, B., 1999. Vertical transmission of nucleopolyhedrovirus in insects. J. Invertebr. Pathol. 74, 103–111.

Lasa, R., Pagola, I., Ibañez, I., Belda, J.E., Williams, T., Caballero, P., 2007a. Efficacy of Spodoptera exigua multiple nucleopolyhedrovirus as a biological insecticide for beet armyworm control in greenhouses of southern Spain. Biocontrol Sci. Technol. 73, 221–232.

Lasa, R., Ruiz-Portero, C., Alcázar, D., Belda, J.E., Caballero, P., Williams, T., 2007b. Efficacy of optical brightener formulations of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) as a biological insecticide in greenhouses in southern Spain. Biol. Control. 40, 89–96.

López-Ferber, M., Simón, O., Williams, T., Caballero, P., 2003. Defective or effective? Mutualistic interactions between virus genotypes. Proc. R. Soc. Ser. B. Biol. Sci. 270, 2249–2255.

McLeod, P.J., Young, S.Y., Yearian, W.C., 1982. Application of a baculovirus of *Pseudoplusia includens* to soybean: efficacy and seasonal persistence. Environ. Entomol. 11, 412–416.

Muñoz, D., Castillejo, J.I., Caballero, P., 1998. Naturally occurring deletion mutants are parasitic genotypes in a wild-type nucleopolyhedrovirus population of *Spodoptera exigua*. Appl. Environ. Microbiol. 64, 4372–4377.

Muñoz, D., Murillo, R., Krell, P.J., Vlak, J.M., Caballero, P., 1999. Four genotypic variants of a *Spodoptera exigua* nucleopolyhedrovirus

(Se-SP2) are distinguishable by a hypervariable genomic region. Virus Res. 59, 61–74.

Muñoz, D., Vlak, J.M., Caballero, P., 1997. *In vivo* recombination between two strains of the genus *Nucleopolyhedrovirus* in its natural host, *Spodoptera exigua*. Appl. Environ. Microbiol. 63, 3025–3031.

Murillo, J., Muñoz, D., Lipa, J.J., Caballero, P., 2001. Biochemical characterization of three nucleopolyhedrovirus isolates of *Spodoptera exigua* and *Mamestra brassicae*. J. Appl. Entomol. 125, 267–270.

Murillo, R., Elvira, S., Muñoz, D., Williams, T., Caballero, P., 2006. Genetic and phenotypic variability in *Spodoptera exigua* nucleopolyhedrovirus isolates from greenhouse soils in southern Spain. Biol. Control. 38, 157–175.

Numerical Algorithms Group, 1993. In: Francis, B., Green, M., Payne, C. (Eds.), The GLIM System: Release 4 Manual. Clarendon Press, Oxford, UK.

Olofsson, E., 1988. Environmental persistence of the nuclear-polyhedrosis virus of the European pine sawfly in relation to epizootics in Swedish pine forests. J. Invertebr. Pathol. 52, 119–129.

Peng, F., Fuxa, J.R., Richter, A.R., Johnson, S.J., 1999. Effect of heat-sensitive agents, soil type, moisture, and leaf surface on persistence of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. Environ. Entomol. 28, 330–338.

Richards, A., Cory, J., Speight, M., Williams, T., 1999. Foraging in a pathogen reservoir can lead to local host population extinction: a case study on a Lepidoptera-virus interaction. Oecologia 118, 29–38.

Richards, A.R., Christian, P.D., 1999. A rapid bioassay screen for quantifying nucleopolyhedroviruses (Baculoviridae) in the environment. J. Virol. Meth. 82, 63–75.

Simón, O., Williams, T., Caballero, P., Lopez-Ferber, M., 2006. Dynamics of deletion genotypes in an experimental insect virus population. Proc. R. Soc. Ser. B. Biol. Sci. 273, 783–790.

Simón, O., Williams, T., Caballero, P., Lopez-Ferber, M., 2005. Physical and partial genetic map of *Spodoptera frugiperda* nucleopolyhedrovirus (SfMNPV) genome. Virus Genes 30, 403–417.

Simón, O., Williams, T., López Ferber, M., Caballero, P., 2004. Genetic structure of a *Spodoptera frugiperda* nucleopolyhedrovirus population: high prevalence of deletion genotypes. Appl. Environ. Microbiol. 70, 5579–5588.

Sokal, R.R., Rohlf, F.J., 1981. Biometry. Freeman, New York.

Thompson, G.C., Scott, D.W., Wickman, B.E., 1981. Long term persistence of the nuclear polyhedrosis virus of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera: Lymantriidae) in forest soil. Environ. Entomol. 10, 254–255.

Vlak, J., Frankenhuyzen, K.V., Peters, D., 1981. Identification of a new nuclear polyhedrosis virus from *Spodoptera exigua*. J. Invertebr. Pathol. 38, 278–297.

Young, S., 2001, Persistence of virus in the environment. In: Baur, M.E., Fuxa, J.R., (Eds.), Factors affecting the survival of entomopathogens. Southern Cooperative Series Bulletin s-301/s-265, (online: http://www.agctr.lsu.edu/s265/default.htm), pp. 35–69.