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Genetic and phenotypic variability in *Spodoptera exigua* nucleopolyhedrovirus isolates from greenhouse soils in southern Spain

Rosa Murillo, Sonia Elvira, Delia Muñoz, Trevor Williams, Primitivo Caballero *

Departamento de Producción Agraria, Universidad Pública de Navarra, Pamplona 31006, Spain

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Abstract

Genotypic and phenotypic variation of SeMNPV was examined in seven isolates of SeMNPV originating from occlusion body (OB) populations in the soil of greenhouses in Spain. Semi-quantitative PCR indicated that some of the isolates were composed of a single dominant genotype, whereas other isolates were composed of two or three genotypes in equal proportions. The coexistence of genotypes could be explained by trade-offs among the three phenotypic traits analyzed, namely pathogenicity (LD_{50}), speed of kill (mean time to death), and OB yields, so that increases in one trait were accompanied by decreases in another. Mixed genotype isolates tended to behave differently to single genotype isolates. Two of the genotypic mixtures were significantly more pathogenic (lower LD_{50} values) for *Spodoptera exigua* (Hübner) larvae than the single genotypes that they comprised. OB yield/insect was greater for single genotype compared to mixed genotype isolates, despite genotype-specific differences in mean times to death. Total OB production/insect was positively correlated with time to death. Two out of three of the mixed genotype isolates had lower OB yield/mg insect weight at death compared to single genotype isolates. Each genotypic combination appeared to interact to produce a unique phenotype. This suggests the existence of trade-offs between traits leading to the coexistence of distinct genotypes and genotypic mixtures with similar transmissibility.

Keywords: *Spodoptera exigua*; Nucleopolyhedrovirus; Soil isolates; Genetic variability; Phenotypic variability

1. Introduction

Baculoviruses are pathogens of numerous forest and agricultural pest insect species, particularly the larval stages of Lepidoptera. Their high degree of pathogenicity and host specificity permit their use as biological insecticides (Moscardi, 1999). However, successful pest control with these products has not always been achieved for a number of reasons. The lack of effective control observed following trials against certain pests has been attributed to the use of slow-acting virus strains or isolates with low pathogenicity (Ribeiro et al., 1997; Williams et al., 1999). To overcome these problems, it is necessary to identify and select highly insecticidal strains (those that combine high pathogenicity and fast speed of kill) appropriate for inundative control targeted at a particular host. This is likely to be possible for numerous host-crop combinations since, based on the diversity of insect species, the interspecific diversity of baculoviruses is expected to be enormous (Miller, 1997). However, what is probably more interesting for baculovirus insecticide development is the diversity existing within populations of a particular baculovirus species (Van Regenmortel, 2000). Restriction endonuclease profiles of field-collected isolates invariably reveal a high degree of genotypic diversity occurring between different isolates (Cooper et al., 2003; Getting and McCarthy, 1982; Hodgson et al., 2001), while in vitro and in vivo cloning of such isolates demonstrates the presence of a wide array of genotypically distinct variants within each isolate (Knell and Summers, 1981; Lee and Miller, 1978; Maeda et al., 1990; Smith and Crook, 1988). This genotypic variation often

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

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translates into phenotypic differences in pathogenicity (50% lethal dose, LD_{50}), virulence (speed of kill), and productivity (number of progeny virus particles) (Cory and Myers, 2003; Muñoz and Caballero, 2001). In this study, we refer to pathogenicity as the capacity of the virus to enter the host, establish infection, reproduce, and cause death, whereas virulence is taken to mean the time elapsing between initial infection and death of host (Thomas and Elkinton, 2004).

Genotypic and phenotypic diversities have been widely reported between and within different geographical isolates of the multiple nucleopolyhedrovirus of *Spodoptera exigua* (SeMNPV). This virus is of particular interest for the bioinsecticide industry for two reasons. First, it displays a very low LD_{50} value and a fast speed of kill, and the host range is restricted to *S. exigua* alone (Smits, 1987; Smits and Vlak, 1988). Second, *S. exigua* is a major pest of agricultural crops in tropical and subtropical regions and has often developed resistance to chemical insecticides (Brewer and Trumble, 1989, 1994; Brewer et al., 1990; Chaufaux and Ferron, 1986; Mascarenhas et al., 1998).

In the region of Almería (Spain), insecticide resistant populations of *S. exigua* (Hübner) inflict important economic losses each year because of a lack of effective chemical or biological control agents. Analysis of natural SeMNPV populations in the soils of Almería revealed the existence of between isolate and within-isolate genotypic diversity in 160 SeMNPV isolates (R. Murillo, unpublished data). Four single genotype isolates and three mixed genotype isolates were selected for detailed study because they contain the most abundant genotypes present in the soil SeMNPV population. Natural NPV isolates have been obtained usually from virus-killed larvae and very few studies have been reported on the biological activity of soil derived isolates. However, soil populations of viral occlusion bodies (OB) constitute an important source of inoculum from which disease outbreaks have been initiated in susceptible larvae (Hochberg, 1989; Young and Yearian, 1986).

The present study aimed to determine the genotypic composition of a selection of SeMNPV isolates collected from greenhouse soils in Almería, compare their insecticidal activity in terms of median lethal dose (LD_{50}), speed of kill in terms of mean time to death (MTD), and productivity, as the number of OBs yielded upon larval death. This information will be of great value in understanding the differences in characteristics that determine the transmissibility of distinct genotypes, alone and in mixtures, in agroecosystems.

2. Materials and methods

2.1. Insects and viruses

Larvae of *S. exigua* were obtained from a laboratory colony maintained at constant temperature (25 ± 2 °C), photoperiod (16:8 h, light/darkness), and relative humidity (75%), and reared on a wheatgerm-based semi-synthetic diet (Greene et al., 1976).

Table 1
Classification of soil isolates according to their genotypic composition (single or mixed) and name of the genotypes they comprise

Soil isolates	Classification[a]	Genotypic composition[b]
SeSP03-17	Single	Se-G24
SeSP03-18	Single	Se-G25
SeSP02-13	Single	Se-G26
SeSP03-14	Single	Se-G27
SeSP02-10	Mixed	Se-G25 + Se-G26
SeSP02-34	Mixed	Se-G24 + Se-G25 + Se-G26
SeSP02-83	Mixed	Se-G24 + Se-G26

[a] Classification determined by visualization of viral DNA with restriction endonucleases (R. Murillo, unpublished data).
[b] Genotypic composition determined by PCR (this paper).

Seven viral isolates were selected from a collection of soil samples taken from greenhouses of Almería (Spain) during 2002 and 2003 (R. Murillo, unpublished data). To obtain these soil isolates, the bioassay method developed by Richards and Christian (1999) was adapted as follows: 25 g of air-dried and sieved soil was incorporated into 100 ml of semi-synthetic diet and offered to first instar *S. exigua*. Each NPV-killed larva was considered as a single isolate. Restriction endonuclease analysis revealed that these isolates (named SeSP03-17, SeSP03-18, SeSP02-13, SeSP03-14, SeSP02-10, SeSP02-34, and SeSP02-83) could be distinguished by restriction fragment length polymorphisms (RFLP) (Table 1). A natural isolate, SeSP90-01, obtained from virus-killed *S. exigua* larvae collected in 1990 in greenhouses of Almería was used as a reference isolate. This isolate was previously shown to consist of a mixture of genotypes (Caballero et al., 1992). All isolates were produced by feeding healthy fourth instar *S. exigua* with virus-contaminated diet. Virus OBs were purified according to Muñoz et al. (1997) and stored at 4 °C for up to 2 weeks until use. OB concentrations were determined using an improved Neubauer hemocytometer (Hawksley, Lancing, UK) under phase-contrast microscopy.

2.2. Estimates of the relative composition of SeMNPV isolates

Genotypic composition of the isolates was determined by PCR. The relative proportion of the different genotypes present within mixed genotype isolates (SeSP02-10, SeSP02-34, and SeSP02-83) was assessed by semi-quantitative PCR. The number of PCR cycles (15, 20, 25 or 30 cycles) and the use of equal amounts of template DNA were subjected to preliminary testing in all samples to ensure reliable and repeatable results. As controls, different experimental preparations were constructed in the laboratory by mixing the corresponding OB suspensions at ratios of 1:1, 3:1, and 1:3. These preparations were named P1 (consisting of SeSP03-18 and SeSP02-13 mixed

in a ratio of 1:1), P2 (SeSP03-17, SeSP03-18, and SeSP02-13 mixed in a ratio of 1:1:1), P3 (SeSP03-17 and SeSP02-13 mixed in a ratio of 1:1), P4 (SeSP03-17 and SePS02-13 in a ratio of 3:1), and P5 (SeSP03-17 and SePS02-13 in a ratio of 1:3) (Table 1). Mixtures P1, P2, and P3 were designed to reflect the composition of the most commonly observed mixed genotype isolates from the soil and they were therefore employed as reference preparations. To obtain similar quantities of template DNA (0.5 ng), genomic DNA was treated with *Sma*I to open the closed circular genome, loaded onto a 0.6% agarose gel, electrophoresed, stained with ethidium bromide, and visualized under UV light. Images were captured and band intensity was quantified using Quantity One V.4.2.2 software (Bio-Rad, Hercules, USA). Fragment intensity readings were compared with known concentrations of λ-*Hin*dIII fragments.

Genomic diversity for the Spanish SeMNPV isolates has been localized to a region, the *hr*1 (Muñoz et al., 1999), and although we cannot exclude the possibility that other variants are present that do not vary in this region, detailed REN analysis studies indicate that they must exist at a very low frequency. Amplification of *hr*1 enabled identification of the different SeMNPV isolates from Almería (R. Murillo, unpublished data). The primers used (5′-ctttgtcatcgtcacctacg-3′ and 5′-gagatcatc atcgatgaaatc-3′) were designed to hybridize to flanking fragments of the homologous region *hr*1, that has been reported to be a region with a high capacity of discrimination between SeMNPV genotypes (Muñoz et al., 1999). PCR products were loaded in 1.5% agarose gel and subjected to electrophoresis. The relative proportion of each genotype was estimated by densitometric analysis, as described above. Intensity readings were performed three times for each PCR product (amplicon) and the mean values were compared along a series of 15, 20, 25, and 30 amplification cycles.

2.3. Determination of dose–mortality and dose–time

The pathogenicity of SeSP02-13, SeSP03-14, SeSP03-17, SeSP03-18, SeSP02-10, SeSP02-34, SeSP02-83, and SeSP90-01 was determined by the droplet-feeding method (Hughes and Wood, 1981). Newly molted second instars were starved for 16–20 h and allowed to drink from an aqueous suspension containing OBs, 10% sucrose, and 0.001% (w/v) Fluorella Blue. Six viral doses containing 1, 3, 9, 27, 81, and 243 OBs/larva of each viral inoculum were prepared to produce mortalities between 5 and 95%. The different suspensions and an OB-free solution were supplied to seven batches of larvae, each containing 30 individuals, for each virus treatment. Larvae that ingested droplets within 10 min were individually transferred to 24-well tissue-culture plates containing diet and reared at 25 ± 2 °C. Mortality was recorded every 12 h for 7 days. Bioassays were performed independently on four occasions.

2.4. Determination of OB yield and time to kill

The number of OBs produced in each infected insect at the moment of death was determined by inoculating fourth instar *S. exigua* with a single concentration (6453 OB/ml) of each isolate which was previously estimated to result in ~90% mortality (Murillo et al., 2003). Starved newly molted larvae were individually weighed and allowed to drink from aqueous suspensions containing OBs, 10% sucrose, and 0.001% (w/v) Fluorella Blue. Fifteen inoculated larvae were transferred individually to diet and maintained at 25 ± 2 °C until death or pupation. Larvae with clear symptoms of advanced NPV infection were transferred individually to vials. Larvae were monitored every 8 h for 5 days. Larvae that did not respond to tactile stimuli were recorded as dead. Approximately, 12 h after death, cadavers were weighed and frozen at −20 °C. Ten NPV-killed larvae were randomly selected from each treatment (isolate) for OB titration. Cadavers were homogenized individually in 1 ml of bidistilled water, filtered through two layers of cheesecloth, and appropriately diluted for OB quantification. The number of OBs from each infected larva was estimated by counting triplicate samples of the diluted suspensions using an hemocytometer in a phase-contrast microscope. The experiment was performed six times.

DNA from fourth instar *S. exigua* inoculated with mixed genotype isolates was processed by semi-quantitative PCR. Only amplified inocula showing identical REN profiles to those of the original inocula were considered for productivity analysis. The rest (10%), which may have contained contaminants of some kind, were not.

2.5. Statistical analysis

Dose–mortality data were subjected to logit analysis using the GLIM program (Generalized Linear Interactive Modeling, Numerical Algorithms Group, Oxford, UK), with a binomial error structure specified (Crawley, 1993). Model behavior was checked by examination of the distribution of residual and fitted values. Time–mortality results of individuals that died due to NPV infection by different isolates were subjected to Weibull analysis (Aitikin et al., 1989). The validity of the Weibull model was determined using the Kaplan macro, present in the GLIM program. Individuals that did not die from virus infection were excluded from the analysis (Farrar and Ridgway, 1998). Mean OB yield/insect was calculated for each repetition and $\log_e$-transformed prior to analysis of variance with normal error structure. The initial weight of larvae and speed of kill were included as covariables in the model. Linear regression of total OB yields from individual insects against time to death for all isolates tested was performed following $\log_e$ transformation. Datasets that did not satisfy model checking procedures were subjected to non-parametric tests (Kruskall–Wallis and Mann–Whitney) using SPSS v.12 (SPSS, Chicago, IL).

3. Results

3.1. Identification of genotypes present in virus isolates

Restriction endonuclease analysis of the SeMNPV carried out in a previous study indicated that isolates SeSP02-13, SeSP03-14, SeSP03-17, and SeSP03-18 consisted of a single dominant genotype, whereas isolates SeSP02-10, SeSP02-34, and SeSP02-83 comprised two or three genotypes (Table 1).

According to the nomenclature system used in our laboratory, the name given to a particular genotype includes the first letter of the generic and specific name of the insect host (Se for *S. exigua*) followed by the letter G (Genotype) and an Arabic number which indicates the order in which they have been identified. For instance, the isolate SeSP03-17 consisted in a single genotype that were identified and characterized under the name Se-G24 (R. Murillo, unpublished data), although none of the genotypes mentioned in this study were subjected to purification by cloning. The following three distinct RFLP-*Bgl*II genotypes found were subsequently named Se-G25, Se-G26, and Se-G27 (obtained from isolates SeSP03-18, SeSP02-13, and SeSP03-14, respectively).

This was confirmed by semi-quantitative PCR amplification of a hypervariable region from isolates SeSP03-17, SeSP03-18, SeSP02-13, and SeSP03-14 which resulted in a single amplicon for each isolate, the sizes of which corresponded to those of the genotypes Se-G24, Se-G25, Se-G26, and Se-G27, respectively (R. Murillo, unpublished data). Isolates SeSP02-10, and SeSP02-83 yielded two amplicons each, corresponding to genotypes Se-G25 + Se-G26 and Se-G24 + Se-G26, respectively (Fig. 1). Similarly, SeSP02-34 yielded three amplicons corresponding to genotypes Se-G24, Se-G25, and Se-G26. Densitometric quantification of these amplicons revealed that the different genotypes were present at statistically equal proportions within each one of the three mixed genotype isolates (Fig. 1).

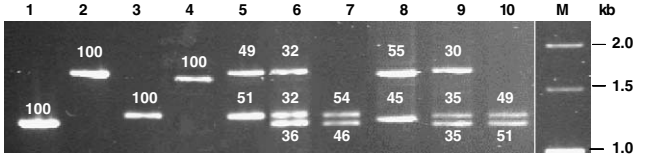

Fig. 1. Electrophoresis of amplicons obtained by semi-quantitative PCR from the single genotype isolates SeSP03-17 (lane 1), SeSP03-18 (lane 2), SeSP02-13 (lane 3), and SeSP03-14 (lane 4); laboratory artificial population P1 consisting in a mixture of the SeSP03-18 and SeSP02-13 isolates at the relative proportion of 1:1 (lane 5); P2 of the SeSP03-17, SeSP03-18, and SeSP02-13 isolates at 1:1:1 (lane 6); and P3 of the SeSP03-17 and SeSP02-13 isolates at 1:1 (lane 7); and the mixed genotype isolates SeSP02-10 (lane 8), SeSP02-34 (lane 9), and SeSP02-83 (lane 10). The proportion of each genotype is shown on top of or under the corresponding amplicons. A commercial molecular size marker, Bioron (Biolabs), is shown on the right most lane and its corresponding fragment sizes in kilobasepair are indicated.

The semi-quantitative PCR conditions were adjusted using experimental virus preparations: P1 (1:1 SeSP03-18/SeSP02-13), P2 (1:1:1 SeSP03-17/SeSP03-18: SeSP02-13), and P3 (1:1 SeSP03-17/SeSP02-13) (Fig. 1). Additional tests using P4 (SeSP03-17 and SePS02-13 in a ratio of 3:1) and P5 (SeSP03-17 and SePS02-13 in a ratio of 1:3) confirmed that the PCR assay was not significantly affected by the ratio of genotypes present in genotypic mixtures (data not shown). Amplicon intensities exactly reflected the genotypic composition when 20 amplification cycles were performed (Student's t test; $P > 0.05$) (Fig. 1). We observed that 15 cycles did not produce visible amplification products and that 25 and 30 cycles produced significantly different mean intensity readings for each amplicon (Student's t test; $P < 0.05$). Therefore, PCR were stopped after 20 cycles, during the mid-logarithmic phase. However, we cannot eliminate the possibility that additional genotypes were present at an abundance below the PCR threshold of detection.

3.2. Pathogenicity of single and mixed genotype SeMNPV isolates

Laboratory bioassays in second instar *S. exigua* indicated that the mixed genotype isolates SeSP02-34 and SeSP02-83 were the most pathogenic isolates tested, with LD_{50} values statistically similar to that of the reference isolate SeSP290-01 (Table 2). These values were 1.5–2.5 times lower than those of the single genotype isolates SeSP03-17, -18, and SeSP02-13, with which they share the genotypes Se-G24, Se-G25, and Se-G26 (Tables 1 and 2). In contrast, the mixed genotype isolate SeSP02-10, which contained genotypes Se-G25 and Se-G26, was as pathogenic as the single genotype isolates SeSP03-18 and SeSP02-13, comprising genotypes Se-G25 and Se-G26, respectively (Tables 1 and 2). For all virus treatments, mortality increased with increasing dose ($\chi^2_1 = 1216$, $P < 0.005$). Since the interaction between isolates and $\log_e$[virus dose] was not significant, dose–mortality responses for all eight isolates were fitted with a common slope ($\chi^2_7 = 7.10$, $P = 0.418$) (Table 2). No evidence of overdispersion was observed in datasets, and no mortality by virus infection was registered in mock-infected control insects. Model simplification indicated that isolates could be assigned a posteriori to one of the following four groups based on the dose–response phenotype: (i) SeSP03-17 (least pathogenic isolate), (ii) SeSP02-13, (iii) SeSP03-18, SeSP03-14, and SeSP02-10, and (iv) SeSP290-01, SeSP02-34, and SeSP02-83 (most pathogenic isolates).

3.3. Dose–time response

Speed of kill was negatively correlated with dose ingested ($F_{1,24} = 89.72$, $P < 0.001$). Speed of kill differed significantly according to isolate ($F_{7,24} = 3.48$, $P = 0.01$) but the interaction between dose and isolate was not significant ($F_{7,24} = 1.81$, $P = 0.131$). Model simplification involving grouping the single genotype or mixed genotype isolates

Table 2
Logit regression analysis of virus induced mortality in second instar *S. exigua* inoculated with single genotype or mixed genotype SeMNPV isolates originating from the soil, compared to a reference isolate (SeSP90-01) originating from virus-killed larvae

Isolate	LD_{50} (OBs/larva)	Intercept ± SE	Relative potency	*P*
Reference isolate				
SeSP90-01	20.8	2.05 ± 0.079	1	—
Single genotype isolates				
SeSP02-13	31.1	2.33 ± 0.081	0.67	0.005
SeSP03-14	29.5	2.29 ± 0.080	0.71	0.013
SeSP03-17	54.3	2.70 ± 0.086	0.39	<0.001
SeSP03-18	28.5	2.27 ± 0.080	0.74	0.025
Mixed genotype isolates				
SeSP02-10	27.7	2.25 ± 0.080	0.75	0.039
SeSP02-34	22.5	2.11 ± 0.078	0.94	0.534
SeSP02-83	23.0	2.12 ± 0.078	0.91	0.456

Log regression of number of responding insect against $\log_e$ (virus dose) given in terms of $\log_e$ odds ratio: $\log_e(p/q) = a + bx$. Regression was fitted in GLIM with a common slope of 0.676 ± 0.022 (SE) for all virus inocula. *P* values were calculated by *t* test of the differences between regressions intercepts compared to that of the wild type. Relative potencies were calculated as the ratio of effective concentrations relative to the SeSP90-01 wild-type isolate.

resulted in a non-significant change in model deviance compared to a model based on eight different isolates ($F_{1,36} = 1.9$, $P = 0.1765$). Therefore, separate regression lines were fitted for single genotype and mixed genotype isolates. It was evident that, for a given dose, insects infected by single genotype isolates died sooner than those infected with mixed genotype isolates (Fig. 2).

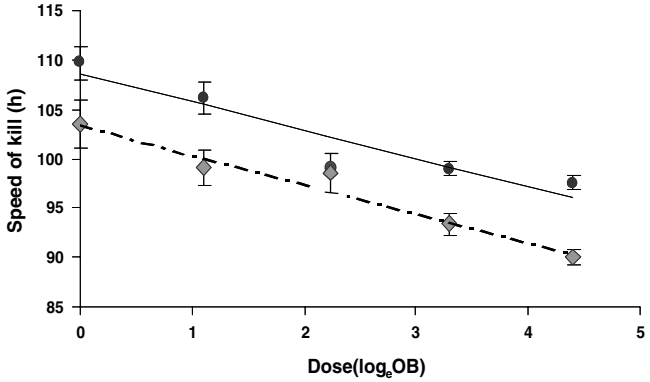

Fig. 2. Relationship between dose and mean speed of kill for SeMNPV single genotype (diamonds) or mixed genotypes (circles) isolates infecting second instar *S. exigua*. Bars indicate standard error. Regression equation for single genotype isolates (dashed line) was speed of kill = 103.1 - 2.78 × ($\log_e$OB) and equation for mixed genotype isolates (solid line) was speed of kill = 108.4 − 2.78 × ($\log_e$OB).

3.4. Time–mortality response

The different isolates killed between 92 and 97% of the treated larvae and no mortality was observed in mock-infected insects. Virus isolates killed larvae infected in the fourth instar in a time range of 72–128 h post-infection (hpi). The instantaneous risk of death increased over time (Weibull shape parameter $\alpha = 14.7$). Comparisons among single genotype isolates revealed that SeSP02-13 and SeSP03-17 both killed larvae significantly faster than SeSP03-14 and SeSP03-18 (Fig. 3). Among the mixed genotype isolates, mean times to death for the SeSP02-10 and SeSP02-34 isolates and the reference isolate SeSP90-01 were intermediate between those of the single genotype isolates, whereas SeSP02-83 had the fastest speed of kill, statistically similar to those of SeSP03-17 and SeSP02-13 (Fig. 3).

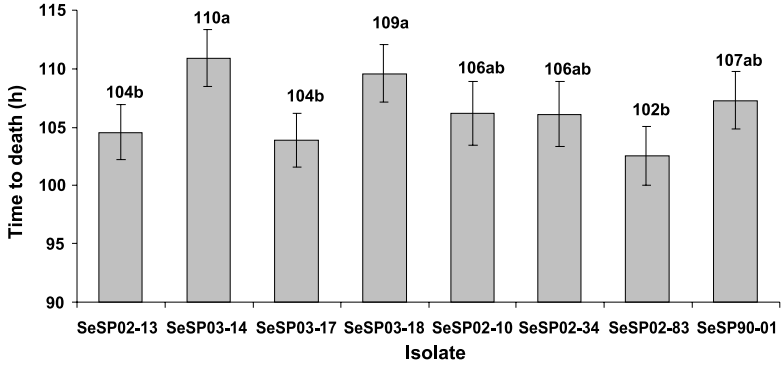

Fig. 3. Mean time to death (MTD) for *S. exigua* inoculated in fourth instar with single genotype (SeSP03-14, SeSP03-17, SeSP03-18, and SeSP02-13) and mixed genotype (SeSP02-10, SeSP02-34, and SeSP02-83) isolates from soil compared to a reference isolate (SeSP90-01). Between 58 and 60 larvae were tested for each treatment. Numbers above columns indicate MTD values averaged over four replicates. Columns headed by different letters are significantly different at $P < 0.01$ (applying a Bonferroni correction for multiple comparisons) by the Weibull analysis.

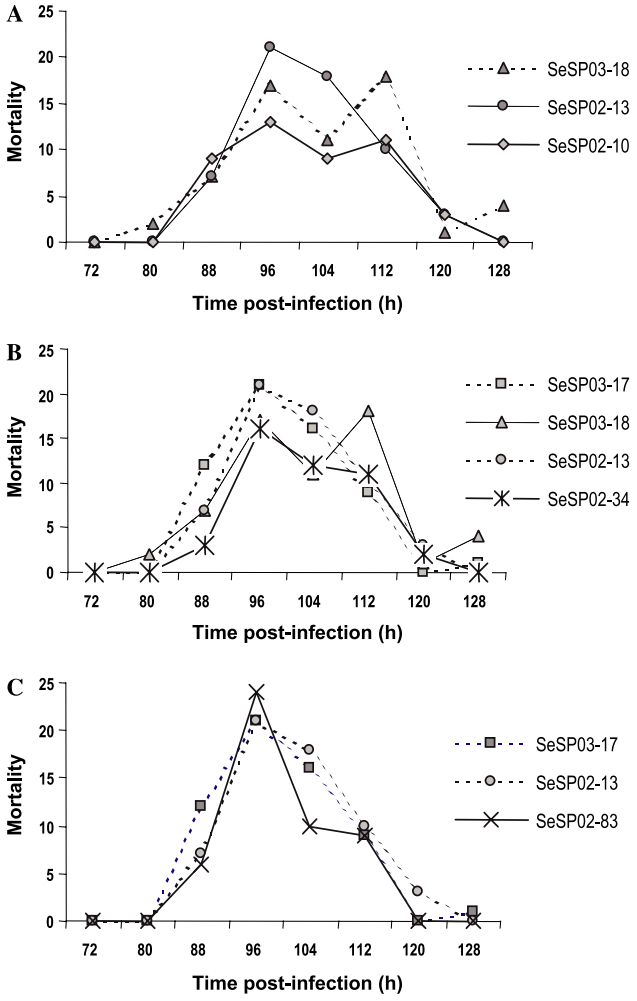

Fig. 4. Mortality of *S. exigua* over time following inoculation in the fourth instar with SeMNPV as single genotype (solid lines) or mixed genotype isolates (dotted lines).

Time–mortality curves of all single genotype isolates showed one or two mortality peaks at 96 and 112 h post-inoculation (Fig. 4A–C). The time–mortality patterns of the mixed genotype isolates (SeSP02-10, SeSP02-34, and SeSP02-83) followed the curves corresponding to the single genotypes present within each of them. For example, SeSP02-10 composed of single genotypes 25 and 26 (isolates SeSP02-13 + SeSP03-18) closely reflected the peaks of mortality observed over time by the single genotype isolates (Fig. 4A). The same patterns were also evident in SeSP02-34 (Fig. 4B) and SeSP02-83 (Fig. 4C).

3.5. Production of progeny OBs

Occlusion body production/larva was not significantly affected by isolate ($F_{7,39} = 2.00$, $P = 0.08$) or the average speed of kill of the isolate considered as a covariable ($F_{1,39} = 1.13$, $P = 0.29$). OB yields per insect differ significantly when isolates were grouped into single genotype isolates and mixed genotype isolates ($F_{1,45} = 12.6$, $P = 0.01$). Larvae killed by single genotype isolates (data pooled for all single genotype isolates) resulted in an average yield of $8.9 \times 10^8 \pm 2.2 \times 10^6$ OB/larva (mean ± SE, $N = 24$), which was significantly more than the average of $6.5 \times 10^8 \pm 2.9 \times 10^6$ OB/larva (mean ± SE, $N = 24$) observed in insects killed by mixed genotype isolates (data pooled for all mixed genotype isolates).

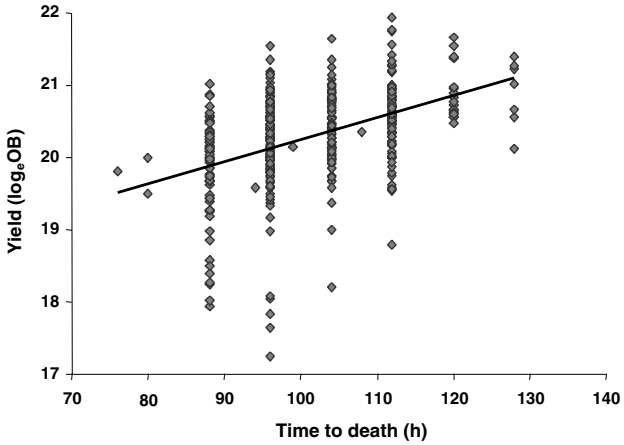

Fig. 5. OB yield and time to death relationship for *S. exigua* inoculated in the fourth instar with SeSP02-13, SeSP03-14, SeSP03-17, SeSP03-18, SeSP02-10, SeSP02-34, SeSP02-83, and SeSP02-01 isolates [regression equation: Yield ($\log_e$ OB) = 17.21 + 0.030 × (time to death in hours)]. Data pooled for all SeMNPV single genotype and mixed genotype isolates.

The yield of OBs per larva was positively correlated with the time elapsed from inoculation to death of each individual ($F_{1,431} = 104$, $P < 0.001$) (Fig. 5). The initial weight of the larva at the moment of inoculation did not significantly influence this outcome ($F_{1,431} = 3.68$, $P = 0.0617$).

The yield of OBs/mg insect weight at death varied significantly between isolates (Kruskall–Wallis: $\chi^2_7 = 16.9$, $P = 0.0181$) (Fig. 6). OB yields of the mixed genotype isolates SeSP02-34 and SeSP02-83 were significantly lower than those of single genotype SeSP03-17, SeSP03-18, and SeSP02-13 isolates. Unexpectedly, the OB yield/mg larval weight was lowest in SeSP03-14, despite the slow speed of kill of this isolate. The value of OB yield/mg larval weight for SeSP02-10 was statistically similar to those of the other six isolates and showed high variation between insects (Fig. 6).

4. Discussion

Comparison of the genotypic composition and phenotypic traits of seven SeMNPV isolates originating from greenhouse soils in Almería, southern Spain, revealed diversity in the genotypic composition and key phenotypic characteristics likely to affect their transmissibility, including pathogenicity, speed of kill, and production of progeny OBs. Soil derived isolates comprised of four single genotype isolates (SeSP03-17, SeSP03-18, SeSP02-13, and SeSP03-14), and three mixed genotype isolates (SeSP02-10, SeSP02-34, and SeSP02-83) each of which consisted of two or three genotypes, present in roughly equal proportions within each of these isolates. The coexistence of

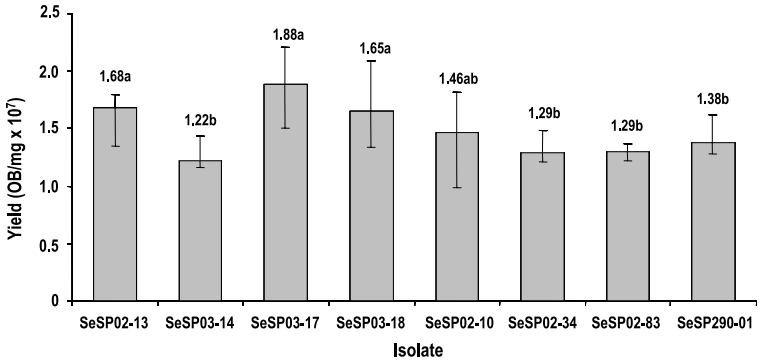

Fig. 6. Median OB yield/mg weight of dead larva obtained from *S. exigua* inoculated in the fourth instar with single genotype SeSP02-13, SeSP03-14, SeSP03-17, SeSP03-18, and mixed genotype SeSP02-10, SeSP02-34, and SeSP02-83 isolates were compared to a reference isolate (SeSP90-01). Between 58 and 60 larvae were tested for each treatment. Bars indicate 25 and 75 percentiles. Columns headed by different letters are significantly different (Mann–Whitney, $P < 0.05$).

different genotypes is a universal phenomenon within natural baculovirus populations (Cory and Myers, 2003), including those of SeMNPV (Caballero et al., 1992; Dai et al., 2000; Hara et al., 1995; Muñoz et al., 1998, 1999).

Most NPV isolates have been obtained from groups of diseased insects collected from the field. Other studies of NPVs have emphasized the importance of analyzing the genotypic composition of isolates from individual insects, rather than groups (Graham et al., 2004; Laitinen et al., 1996; Maeda et al., 1990). The genotypes Se-G24, Se-G25, Se-G26, and Se-G27 represented 22, 19, 32, and 12% of a total of 160 soil isolates subjected to RFLP analysis, respectively. The remaining 15% comprised a mixture of different genotypes indicated by the presence of submolar restriction fragments (R. Murillo, unpublished data). Analysis of the frequency of different genotypes isolated from greenhouse soils has indicated that the soil OB population does not mirror the genotypic composition of the OB population present in infected insects. Instead, soil OBs may represent a distinct subpopulation in which certain variants adopt strategies of long term persistence in the soil reservoir whereas others are eliminated by differential survival, probably in response to their tolerance of soil environmental conditions.

Variation in phenotypic traits may allow different genotypes to coexist, promoting maintenance of genotypic variation within NPV populations (Hodgson et al., 2001). The presence of several SeMNPV genotypes in the soil of greenhouses suggests the existence of trade-offs between phenotypic traits leading to the coexistence of distinct genotypes with a similar fitness. For instance, evidence of within-genotype trade-offs between speed of kill and OB yields have been reported in several NPV–host systems, whereas between-genotype trade-offs have not be examined previously. The most productive genotypes tend to be those that take a long time to kill the host (Burden et al., 2000; Cory and Myers, 2004; Hernández-Crespo et al., 2001; Muñoz et al., 2000; Ribeiro et al., 1997). In this study, OB yield/larvae increased with increasing time to kill, but differences between isolates may be masked by the high variation in this response. Contrary to expectations, we observed one isolate (SeSP02-14) with the slowest speed of kill also had the lowest OB yield/mg larval weight. Isolate SeSP02-14 was the least efficient at converting insect tissue to OBs, and this did not appear to be influenced by host survival time.

The seven soil isolates could be assigned to one of three groups, based on significant differences in their LD_{50} values. A similar magnitude of phenotypic variation in pathogenicity is present in other SeMNPV isolates originating from localized collections made over small geographical areas, or from isolates collected in different countries of the world (Caballero et al., 1992; Hara et al., 1995), indicating that the spatial scale over which isolates are collected does not correlate with increasing variation in phenotypic characteristics.

Mixed genotype isolates tended to behave differently from single genotype isolates. For instance, both pathogenicity and speed of kill at a given dose tended to be higher in mixed genotype compared to single genotype isolates (Table 1, Fig. 2). Interactions among different NPV genotypes during co-infections, may be due to a more efficient exploitation of host resources (Hodgson et al., 2004; Knell, 1996; Simón et al., 2004). However, not all mixed genotype isolates presented the same type of response. Mixed genotype infections produced by SeSP02-34 and SeSP02-83 showed lower LD_{50} and OB yield values than expected from their genotypic composition. For example, the LD_{50} of SeSP02-34, which comprises SeSP03-17, SeSP03-18, and SeSP02-13 in equal proportions, was significantly lower than any of the LD_{50} values corresponding to these isolates. In contrast, SeSP02-10 had a similar LD_{50} value to those of its constituent genotypes present in SeSP03-18 and SeSP02-13. It seems that each genotypic combination interacts to produce a unique phenotype.

The two mixed genotypes SeSP02-34 and SeSP02-83 were the least efficient mixed genotype isolates in converting insect tissue to OBs, but this inefficiency did not influence the survival time of infected insects. The opposite occurs in *Paroreomyza flammea* MNPV; mixed infections

resulted in higher OB yields/insect compared with their corresponding monoclonal genotypes, possibly due to specialization by certain genotypes for particular host resources (Hodgson et al., 2004).

Certain SeMNPV genotypes studied here produced lower yields of OBs but their pathogenicity was higher (indicated by a low LD_{50} value). The number of lethal doses produced by each genotype in each infected host was therefore similar. As a consequence, highly pathogenic mixed genotype infections resulted in low production of OBs per infected host, making their predicted transmissibility similar for single and mixed genotype infections. In a previous study, we observed a clear temporal correlation between periods of high host densities on crops and the abundance of OBs in greenhouse soils (R. Murillo, unpublished data). During such periods, the probability of ingestion of multiple OBs was increased, resulting in an increase in the prevalence of mixed genotype infections.

The results of this study are not simply of academic interest. The soil represents a crucial reservoir for baculoviruses (Fuxa, 2004), and the rate of flow of OBs into and out of this reservoir can have important consequences on the population dynamics of the host (Hochberg, 1991). OBs may move from the soil onto plant surfaces by rainsplash, irrigation, air currents or the movement of invertebrates. Larvae emerging from egg masses placed on the underside of leaves near the soil surface are therefore most likely to become infected by OBs originating from the soil. Future studies should examine the transmissibility of the SeMNPV genotypes to determine whether the phenotypic differences observed in the laboratory are reflected in differences in fitness for certain genotypes, alone and in mixtures, in natural conditions. NPVs have considerable potential as biological insecticides, lending particular significance to an improved understanding of the ecological consequences of using bioinsecticidal products based on genotypically diverse mixtures.

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