TESIS DE MÁSTER

Vertical transmission efficiency and insecticidal propreties of *Spodoptera exigua* Nucleopolyhedrovirus (SeMNPV) genotypes for their use in biological control

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Curso 2010-11

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Agradecimientos

Me gustaría agradecer la ayuda y el apoyo recibidos a la hora de realizar esta memoria a muchas personas. En Primer lugar a Rosa Murillo y Primitivo Caballero por permitirme realizarla en su departamento, en especial a Rosa por su paciencia al orientarme y guiarme día tras día sin perder la sonrisa.

Al Gobierno Vasco por concederme la beca, ya que gracias a ella me ha sido posible cursar este máster.

A todos los profesores que tanto en el colegio como en la licenciatura como en el máster me han enseñado el gusto por el conocimiento y el significado del esfuerzo.

A los compañeros de laboratorio que me han animado y arropado hasta el final, en especial a Cristina por toda su paciencia y horas invertidas que me han ayudado mucho en la realización del trabajo.

A mis compañeros de máster por lo momentos pasados y por todo lo que me han enseñado.

A Lourdes por abrirme los ojos e introducirme el gusanillo de la biología desde muy temprano.

Y finalmente a mi familia a Koldo por el apoyo recibido, por estar allí todos los días, por creer en mí todo este largo tiempo y acompañarme en todas mis decisiones.

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Abstract

The *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) is a naturally occurring pathogen of *S.exigua* larval populations. Some bioinsecticides based on the SeMNPV have been commercialized in Spain to be used in biological control against this pest in greenhouses of sweet pepper by spray applications. Recent studies have demonstrated that the transmission of the virus from parents to offspring (vertical transmission) is frequent and could be a promising feature in field applications. The aim of this work was to study the convenience of using mixed populations of two SeMNPV genotypes that had been proved to exhibit either interesting insecticidal properties (SeG25) or the capability to be transmitted through host generations (SeAl1). Interestingly, the three mixed populations containing the 25, 50 and 75% of SeG25 improved the pathogenicity (CL_{50}) respect to the SeAl1 genotype. However in terms of virulence (MTD) and productivity (OBs/larva), no differences were found between the genotypes or their mixtures. Finally, the capacity to induce persistent infections of each genotype and their mixtures was evaluated in adult survivors to a sublethal dose of the virus using RT-PCR. Transcripts for the *ie0* early gene were detected in adult survivors to viral challenge with the SeG25 and the three mixed population. Another two viral genes, *DNA-polymerase* and *polyhedrin*, that are expressed in the early and late phases respectively were also tested and no transcripts were detected for these genes, suggesting very low level of transcriptional activity.

Resumen

El nucleopoliedrovirus de *Spodoptera exigua* (SeMNPV) es un patógeno natural de las poblaciones larvarias de *S. exigua* que constituye la base de un bioinsecticida comercializado en España para el control biológico de esta plaga en pimiento. Recientes estudios han demostrado que la transmisión del virus a la descendencia (transmisión vertical) se da con frecuencia y podría ser una característica deseable para su uso en aplicaciones de campo. En el presente trabajo se discute la conveniencia de utilizar una mezcla de dos genotipos SeAl1 (transmisión vertical) y SeG25 (transmisión horizontal) en determinadas proporciones para mejorar las características que cada uno de ellos presenta por separado y así explotar cada una de las vías de transmisión. La patogenicidad (CL_{50}) del genotipo SeG25, y de cualquiera de las mezclas que contienen un 25, 50 o 75 % del mismo, fue más alta que la del aislado SeAl1. Sin embargo, en términos de virulencia (TMM) y productividad (OBs/larva) no se observaron diferencias significativas entre genotipos ni entre sus mezclas. Además se evaluó la capacidad de producir infecciones encubiertas de cada genotipo y sus mezclas sometiendo larvas de *S. exigua* a infecciones subletales del virus. Se encontraron transcritos del virus para el gen temprano *ie0* mediante RT-PCR en los adultos supervivientes a infecciones provocadas por el genotipo SeG25 y todas las mezclas. También se testaron otros dos genes virales que se expresan de manera temprana y tardía en la infección de baculovirus (*DNA-polimerasa* y *polihedrina*) para los que en ningún caso se detectaron transcritos.

1. Background and objectives

Insects can be affected by a wide range of pathogens, producing chronic lethal infections that importantly impact on host population dynamics (Anderson and May, 1981; Kalmakoff and Crawford, 1982). Baculoviruses are one of the most studied groups of insect pathogens, primarily because of their good qualities to be used as insect pest control agents (Moscardi, 1999). They are arthropod-specific DNA viruses and therefore safe to human and environmental friendly. The occlusion bodies (OBs) produced by baculoviruses, make them able to persist outside the host for considerable periods if they are protected from ultraviolet irradiation. OBs are thought to be the main mechanism of baculovirus transmission and persistence. Insect-infecting baculoviruses have been reported worldwide from over 600 host species, mainly from the order Lepidoptera (van Regenmortel *et al*., 2000), including agricultural and forest pests (Fuxa, 1991; Moscardi, 1999). The genus alphabaculovirus gathered those baculovirus infectious for lepidopteran species, formally known as Nucleopolyhedrovirus (NPV).
Larval infestations of the *Spodoptera exigua* in greenhouses crops such as sweet pepper are very frequent in Almeria and had been controlled by weekly chemical applications for decades. Moreover, the intensive use of chemicals insecticide for the control of this pest has resulted in high levels of resistance (Mascarenhas *et al*., 1998; Moulton *et al*., 1999, 2002; Smagghe *et al*., 1997, 2003; Torres Vila *et al*., 1998). The *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) has been deeply studied for its promising insecticidal properties such as high pathogenicity and virulence (Caballero *et al*., 2010). As a result, a bioinsecticide based on SeMNPV autochthonous strains is being producing with the trade name of VIREX® (Biocolor) and commercialized by the local growers association COEXPAL (Asociación de Cosecheros y Exportadores de Almeria). In total three SeMNPV-based products are registered in Spain to be used as an alternative method to chemical control in Almerian greenhouses against *S. exigua* larvae (Spod-X: Certis U.S.A., Spexit: Andermatt Biocontrol). This is especially interesting because of the full compatibility of the virus with natural enemies used for simultaneous pests and insect pollinators, a great handicap to grow crops under biological conditions.

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Baculovirus are well known as horizontally transmitted pathogens. Horizontal transmission (HT) occurs when NPV-infected larvae die and a massive number of new OBs are liberated over the environment. Susceptible hosts of the same generation became infected when they ingest OBs with the food. Although this is the best known route of transmission of the NPVs, recent studies have shown that the virus transmission from parents to offspring (vertical transmission, VT), is not only possible (Kukan, 1999) but frequent (Cabodevilla *et al.*, 2011; Vilaplana *et al.*, 2010: Kouassi *et al.*, 2009; Kem *et al.*, 1011). Vertical transmission implies the virus passing from covertly infected adults to the offspring (Goulson and Cory, 1995; Rothman and Myers, 2000) and involved still unknown mechanisms that lead the virus to be maintained in the host cell (Hughes *et al.,* 1997; Burden *et al.,* 2002). Nevertheless, two scenarios have been reported for NPVs producing infections in asymptomatic insect: persistent infections when the virus showed transcriptional activity as a low level, opposed to latent infection when the virus is essentially dormant (Burden *et al.,* 2003)

The vertical transmission (VT) and the prevalence of the covert infections produced by SeMNPV in the *S. exigua* population of Almerian greenhouses have been addressed in recent studies (Cabodevilla *et al*, 2011a, 2011b). Interestingly, the 16% of adults captured directly in the field harbored viral transcripts indicating a high prevalence of covert infections in field populations (Cabodevilla *et al*, 2011a). Studies on the genotypic and phenotypic variation of the SeMNPV genotypes associated either to vertical or horizontal transmission route revealed that some genotypes appear to be better adapted to one of both routes according to differential biological properties. Overall horizontally transmitted (HT) genotypes were more pathogenic and virulent than vertically transmitted (VT) genotypes, while VT genotypes induced higher levels of covert infections on adults when the insect were challenge as larvae with sublethal viral doses. Namely, the VT genotype SeAl1 was able to establish covert infections by inoculation with sublethal doses and could be transmitted through up to 5 generations affecting almost all individuals. The HT genotype SeG25, originally isolated from soil samples, showed better pathogenicity qualities (CL_{50}), virulence (MTD) and productivity of polyhedra (Cabodevilla *et al.,* 2011a).

The aim of this work was to study the convenience of using different mixtures of both genotypes, SeG25 and SeAl1, in certain proportions for improving the insecticidal features that each one has separately against *S. exigua* larvae. So far, isolates has been

characterized traditionally in our laboratory taking into account three biological features: pathogenicity, virulence and OB productivity. In this study, in addition to those characteristic, we assessed the capability of genotypes to persist in the host population for long periods through examination of covert infections in adults.

2. Materials and methods

2.1. Insects and viruses.

The *Spodoptera exigua* larvae used in this study were selected from a virus-free laboratory culture originally obtained from Andermatt Biocontrol AG (Grossdietwil, Switzerland) and maintained in continuous rearing in the insectary facilities of the Universidad Pública de Navarra at a constant temperature (25 ± 1 ºC) and 16h: 8 ligth: dark cycle on semisynthetic diet (Elvira *et al.,* 2010).

Two isolates of the SeMNPV involved in either vertical (SeAl1) or horizontal (SeG25) transmission were selected for its good features to preferentially exploit one of those transmission routes. The SeAl1 was isolated from the larval progeny of adults collected from greenhouses of Almeria (Spain, 2007), in which fatal infections by NPV were spontaneously developed. Thus the SeAl1, was vertically transmitted (VT) from parents to offspring (Cabodevilla, 2008). The SeG25, was obtained from OBs present in the greenhouses soil-substrate environment (Murillo *et al.,* 2007), and therefore considered to be horizontally transmitted (HT). Three laboratory artificial populations were performed by mixing OBs from SeAl1 or SeG25 single genotypes isolates at three relative proportions: 75% of SeAl1+ 25% of SeG25 (named 75:25), 50% of SeAl1 + 50% of SeG25 (named 50:50) and 25% of SeAl1 + 75 % of SeG25 (named 25:75).

2.2. Characterization of insecticidal properties

OBs from the single genotypes SeAl1 and SeG25, and the OB-mixed populations 75:25, 50:50, and 25:75 were compare in terms of pathogenicity (Medium Lethal Concentration, CL_{50}), virulence (Mean Time to Death, MTD) and OB productivity (OB/Larva).

a. Determination of OB concentration-mortality response

The pathogenicity of the virus was determined by bioassay following an adaptation of the Hughes and Wood method (1981) describe by Muñoz *et al.,* (2001). Groups of 24 newly molted *S. exigua* second-instar (L_2) larvae were starved for 8 to 12 h and then allowed to drink from an aqueous suspension containing 50% (w/v), the food dye Fluorella blue, sterile water, and one of the following OBs/ml concentrations: 3×10^3, 9×10^3, 2.7×10^4, 8.1×10^4, and 2.45×10^5 . These concentrations were previously

probed to produce a range of mortality between 10 and 90% in previous studies. Larvae that ingested the suspension within 10 minutes were individualized in 24 well tissue culture plate provided with semisynthetic diet and reared at 26 ± 2°C. Three independent replicates of the bioassays containing 24 larvae per virus concentration and 24 mock-infected larvae as controls were performed. Larval mortality was recorded at six days post-infection. Virus induced mortality data were subjected to logit analysis using the GLIM 4 program (Generalized Linear Interactive Modeling, Numercial Algorithms Group, Oxford, UK) with a binomial structure specified (Crawley, 1993). Model behaviour was checked by examination of the distribution of residuals and fitted values.

b. Determination of OB production and virulence

Five groups of 24 fourth instars (L4) larvae were droplet-infected with 5×10^7 OB/ml of the virus population, previously estimated to result in near to 90% of larval mortality (Murillo *et al.,* 2003). 24 larvae were mock-infected and used as controls. The bioassay was performed three times. Larvae that did not respond to tactile stimuli were recorded as dead. Mortality data were recorded every eight hours for five days. The results of individuals that died due to NPV infection by different isolates were subjected to Weibull survival analysis in GLIM. The validity of the Weibull model was determined by comparing fitted values with Kaplan–Meier survival function estimated values (Crawley, 1993).

Larvae that died of NPV infection were harvested and stored at -20ºC until used for OB counting. Ten NPV cadavers were randomly selected from each virus population and larvae were homogenized in 1 ml of distilled water. Virus production was estimated by counting triplicate samples of diluted OB suspension using a Neubauer hemocytometer under a phase-contrast microscope. OB production data were analyzed by Krustal Wallis and Mann Whitney U no parametric statistic tests using the SPSS v12 program, since dataset did not satisfy model checking procedures.

2.3. Covert infections induced by sublethal dose

Covertly infected insect lines were set up by sublethally dosing larvae with the SeAl1 and SeG25 genotypes and the three OB-mixed populations previously characterized. Pre-molt *S. exigua* fourth instars were selected and starved overnight.

Once they molted 48 L_4 larvae were inoculated by the droplet feeding method as describe above using an OB suspension containing 9×10^3 OB/ml of each virus treatment or alternatively water for mock-infected controls. This concentration equated to a dose of 29.7 OBs /larva as fourth instars larvae ingested an average volume of 3.3 µl (Smits *et al.*, 1987). This OB amount had previously produced a 50% of larval mortality for fourth instars larvae in similar conditions (Cabodevilla*, et al.,* 2010a). Inoculated larvae were reared through to pupation at 26 ± 2 ºC and mortality was recorded daily. The experiment was performed independently three times. Adult survivors were frozen at -80ºC to be subjected to viral RNA extraction and transcripts detection.

2.4. Total RNA extraction

Total RNA was extracted from adults that survived to a virus infection and were frozen at -80ºC immediately after emergence using TRIZOL reagent (Invitrogen) following the manufacturer's instructions. The abdomens were dissected with sterilized toothpicks on a plate and homogenized in 300 µl of TRIZOL reagent (50-100 mg of tissue). Then 100µl chloroform was added to separate two phases by centrifugation at 12,000 × g for 15 min at 4ºC. The aqueous phase was transferred to a fresh tube and the RNA precipitated using 125 µl of isopropanol and centrifuged at 12,000 × g for 10 min at 4ºC. Pelleted RNA was washed with 500 µl of 75% ethanol and resuspended in 50µl diethylpyrocarbonate (DEPC) water. All equipment and reagents were previously sterilized and treated with DEPC to remove RNases. RNA samples were treated with DNAse to avoid DNA contamination. Blank extraction samples containing only water were processed in parallel to detect cross-contamination during the extraction.

2.5. Detection of persistent infections of SeMNPV

To detect the virus presence in asymptomatic insects, specific viral genes activity was study using the Reverse Transcritase Polymerase Chain Reaction (RT-PCR). As transcripts for certain genes might not be found due to the lack of activity in covert infections conditions, we select essential genes for viral multiplication that are known to be active at different stages of the cascade expression of a NPV infectious cycle (Rhormann, 2011). Therefore the *ie*-0, *DNA-polymerase* and *polyhedrine* genes were targeted representing the very early, early and late stages respectively. *DNA-*

polymerase and *polyhedrine* primers used in this study were designed and amplification conditions optimized by Virto (2011). *Ie0* PCR amplification conditions were 35 cycles at a annealing temperature of 54ºC . Primers features are described in Table 1

Table1. Nucleotide name, orientation and sequence of the oligonucleotides used in the study. The Primers location and amplicon size is referred to the SeMNPV-US1 sequence (GenBank database, accession number AF169823)

Oligonucleotide name	Target gene	Orientation	Sequence (5'-3')	Amplicon size	Location (nt)
SednapolF	DNA - polymerase	Forward	ATGACTTCTTCGTCGTCGTC	300	90226-90245
SednapolR		Reverse	TAGCACGTCGTGTTAGCGTG		90545-90526
SepolhF	Polihedrin	Forward	ATGTATACTCGCTACAGCTA	300	1-20
SepolhR		Reverse	TGTCTTCCATGAAACGCGTC		319-300
Seie0F	Seie0	Forward	CTATAGCTCGACGCTCGGTG	491	131937-131959
Sie0R		Reverse	ATCGTCTTCGATACCGCGAG		132447-132428

RT-PCR was performed in two successive steps. First, cDNA synthesis was performed from 4 µl of total insect RNA previously treated with DNAse, using the ImProm-II reverse transcriptase (Promega) and one of the reverse primer (SedNApolR, SepolhR or Seie0R, Table 1) according to the manufacturer's instructions. An aliquot of the reaction (1/4) was then subjected to PCR amplification with *Taq* DNA polymerase (Bioline) and the two internal primers for the corresponding gene according (table 1). PCR reaction mixture contained 2.5 µl of NH_4, 0.25 µl of dNTP mix (10 mM), 1.25 µl of $MgCl_2$ (15 mM), 0.5 µl of each primer (10 pmol/µl) and 0.2 µl *Taq* DNA polymerase (Bioline). The first PCR amplification was carried out in a total reaction volume of 25 µl containing 5 µl of DNA template using the following conditions: initial denaturation of 2 min at 94ºC; 28 cycles of 1 min at 94ºC, 30 s at 52ºC, 50 s at 72ºC; and final extension of 7 min at 72ºC DNA fragments were visualized in a UV transiluminator,

photographed and examined using the molecular analyst program GeneSnap (from SynGen ver.7.05.02, Synoptics Ltd. 1993-2008). Negative controls, containing all the PCR reagents and either blank extraction or sterile distilled water, were included.

3. Results

3.1. Pathogenicity

OB concentration-mortality response was compared between the single genotypes SeAl1, and SeG25 and the mixed genotypic populations 75:27, 50:50, and 25:75 in terms of LC_{50}. Overall mortality increased significantly with OB concentration (χ^2 = 415.3; df=1; P< 0.05) (Table 2). Since the interaction between virus treatments and log_e(virus concentration) was not significant (χ^2 = 2.08; df=4, P> 0.05) the mortality response for all five treatments were fitted with a common slope (Table 2). No evidences of overdispersion were observed in datasets, and no mortality by virus infection was registered in mock-infected control larvae. Virus treatment differed significantly in their pathogenicity (χ^2 = 23.89; df=4; P< 0.05) when the minimum model was fitted. According to LC_{50} values the single genotypes SeAl1 was the least pathogenic, being 3.10, 2.45, and 2.36 times less pathogenic than SeG25 and the mixed populations 25:75 and 75:27 respectively (Table 2). All three mixed genotype populations induced similar mortality response to larvae between them and also in comparison to the SeG25 genotype. Thus, the addition of the SeG25 at any proportion to the SeAl1 genotypes induced similar behavior than the single genotype alone. Potencies were calculated as the ratio of effective concentrations relative to the SeAl1 isolate.

Table 2. Logit regression analysis of virus induced mortality in second instar S. exigua inoculated with single genotype or mixed genotype SeMNPV isolates.

Isolate	LC_{50} (× 10^4) OBs/ml	Range of 95 % C.I (x104)	Intercept ± SE	Potency	P
SeAl1	5.98	4.43-8.08	-7.908 ± 0.36	1	-
75:25	2.55	1.82-3.59	-7.369 ± 0.36	2.36	0.027
50:50	3.45	2.48-4.85	7.585 ± 0.36	1.73	0.093
25:75	2.45	1.75-3.42	7.337 ± 0.36	2.45	0.023
SeG25	1.95	1.40-2.74	7.176 ± 0.36	3.10	0.01

Log regression of number of responding insect against log_e (virus dose) given in terms of loge odds ratio: loge (p/q) = a + bx. Regression was fitted in GLIM with a common slope of 0.72± 0.033 (SE) for all virus genotype. 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 SeAl1 isolate.

3.2. Virulence and OB production

The MTD (±SE) speed of kill in fourth instars did not significantly vary between virus treatments from a minimum of 85.00 ±0.23 h to a maximum of 86.14 ± 0.23 h for insects infected with SeG25 and SeAl1 respectively (χ^2 = 211.5; df=1; P> 0.05). (Weibull hazard function α=12.71) (Figure 1). Similarly, median OB production did not show differences between virus treatments (Kruskall Wallis: χ^2=1.925 fd=4, p>0.05) with a minimum of 2.57×10^9 ($2.56\ 10^8$ - $2.36\ 10^8$) for the SeG25 isolate to a maximum of 2.80×10^9 (3.35×10^8 - 3.02×10^8) for the mixture 25:75 (Figure 2).

These results suggest that vertically and horizontally transmitted genotypes share similar capability for production of OBs and speed of kill rate at the conditions used in this study.

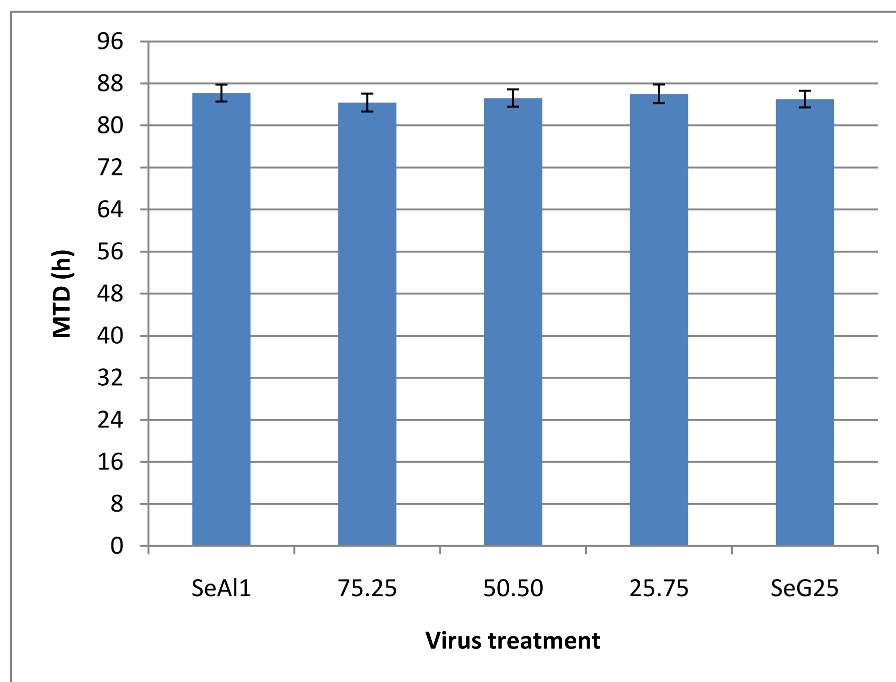

Figure 1. Speed of kill for single genotype (SeAl1 and SeG25) and OB- mixed genotypic populations (75:25, 50:50, 25:75) after inoculation with 5×10^7 OB/ml of each virus treatment in L_4 larvae.

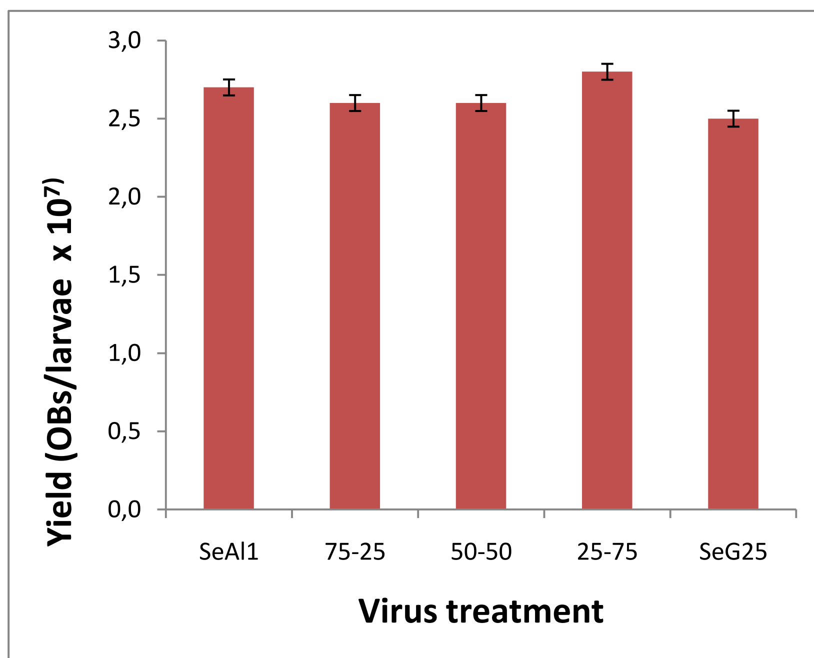

Figure 2. OB production of single genotype (SeAl1 and SeG25) and OB-mixed genotypic populations (75:25, 50:50, 25:75) after inoculation with 5×10^7 OB/ml of each virus treatment in L_4 larvae.

3.3. Induction of covert infections

L_4 instar larvae infected with 9×10^3 OB/ml of single genotypes (SeAl1 and SeG25) and OB-mixed genotypic populations (75:25, 50:50, 25:75) did not show the baculovirus typical symptoms such as liquefaction of cadavers upon death in all cases. After obervations of the cadavers under phase contrast microscopy we recognized two different infectious agents responsible of the mortality (Table 3).

Table 3. Larval Mortality percentage due to bacterial or viral infection and number of survivor insects after inoculation with 9×10^3 OB/ml of different virus treatment.

Virus treatment	% Mortality [1]		Number of Survival insect
	Bacteria	NPV	
SeAl1	63	26	9
75: 25	63	24	5
50:50	64	32	5
25:75	61	29	8
SeG25	61	29	8
Control	76	2	15

[1] N= 75

NPV induced mortality was lower than expected and did not exceed 29 % at any case. Unexpectedly, high rates of mortality due to bacteria were registered in all virus treatment with a range of 76 to 63%. Larvae that were not challenged with virus (control) were also found to be affected by bacterial infections. Therefore the number of insects that reached adults stage was extremely low. Survivors to both infections were disposed to be analyzed by RT-PCR.

3.4. RT-PCR results

Viral transcripts were detected in total RNA extracted from adults survivors to virus treatment with the genotype SeG25 and the mixed populations 75:25, 50:50 and 25:75 for the *ie0* gene in different proportions (table 4), suggesting a persistent infection was present. Neither amplification for *DNA-polymerase* not *polyhedrin* genes were obtained at any treatment (Figure 3b, and 3c). RNA from adults treated with water (table 3, fig. 3, lane 6) considered as controls for detections of cross-infections or environment contaminations did no amplified for any gene, confirming the insect used in this experiment were free of virus and no cross-contaminations occur during the bioassay. Also 10 individuals directly introduced in our laboratory from the insectary facilities were probed negative for viral transcripts by the same means (data not shown).

Table 4. Percentages of positive adults for transcripts of corresponding genes *ie0*, *DNA-polymerase* and *polyhedrin* survivors to a virus challenge with genotypes SeAl1, SeG25 and the OBs mixed populations (75:25, 50:50, 25:75), and no treated control.

Virus treatment	***ie0***	***DNA-polymerase***	***polyhedrin***	N
SeAl1	0	0	0	2
75:25	0	0	0	1
50:50	100	0	0	3
25:75	100	0	0	2
SeG25	50	0	0	6
Control	0	0	0	6

The electrophoresed PCR products were found to show some non-specific fragments that are very common when total RNA as a template is used (Murillo, com. pers). We only considered as positive amplifications those bands showing the expected amplicon size, namely 491, 300 and 300 bp for *ie0, DNA-polymerase and polyhedrin* genes respectively.

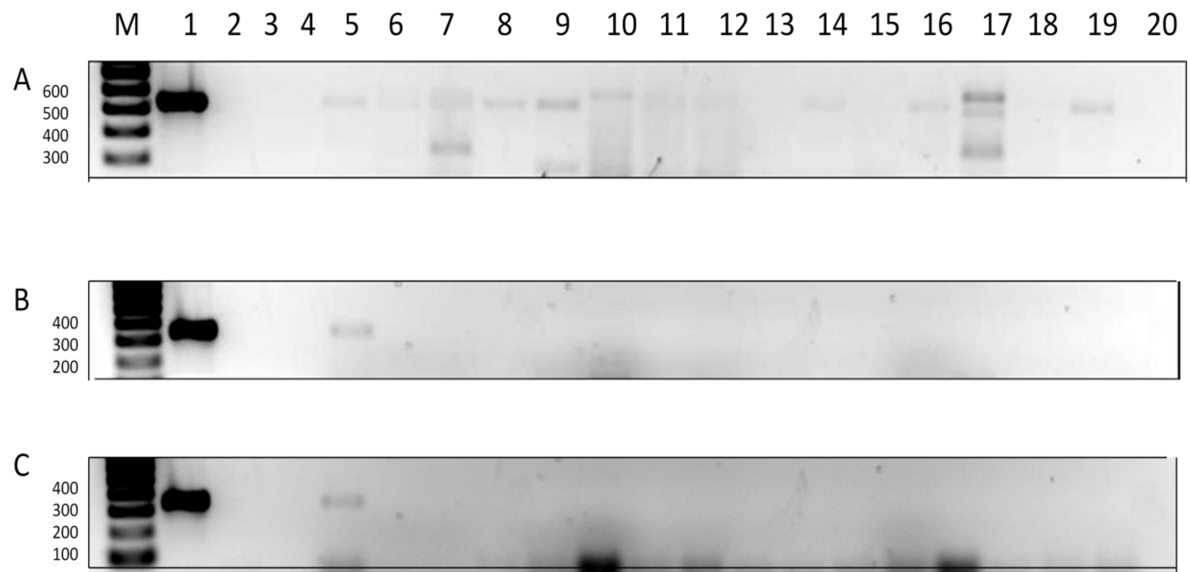

Figure 3. Detection of SeMNPV *ie0* (A), *polyhedrin* (B) *and DNA-polymerase*(C) gene expression using RT-PCR. Marker (M), PCR + (lane2), PCR- (lane3), RT- (lane 4), RT+ (lane 5), RNA from adults survivors to a virus challenged with treatments: Control.(lane 6), SeAl1 (lane 7, 8), , 75:25 (lane 9), 50:50 (lane 10, 11, 12), 25:75.(lane 13, 14), SeG25 (lane 15, 16, 17, 18, 19,

Discussion

The aim of this study was to determine whether a mixture of the genotypes SeAl1 and SeG25 showing certain good insecticidal properties improved the features that each one has separately. SeAl1 occurs naturally in field populations as a vertically transmitted genotype (Cabodevilla *et al.,* 2011a) and was capable to be transmitted through up to five 5 generations affecting virtually all individuals (Cabodevilla *et al.,* 2011b). SeG25 was obtained from soil-substrate and has been demonstrated to have high pahogenicity and virulence, as long as to produce large numbers of OB upon larval death. Despite both genotypes are capable to be vertically or horizontally transmitted (Cabodevilla *et al*., 2011b) we hypothesize that each genotype could have been specialized to better exploit one transmission route as a result of a long-term adaptation based on their biological features.

So far, to define the insecticidal properties of an NPV isolate, three parameters were take into account: the pathogenicity as the viral dose needed to kill the 50% of a treated population, the virulence as the time elapsed between inoculation and death for the 50% of a treated population, and the OBs production as the amount of virus particles obtained upon larval death. A number of studies have been conducted to determine the best candidate for a biopesticides development based on those phenotypic characteristics for different NPV species, including *S. exigua* (Murillo *et al*., 2006, Simon *et al*., 2004; Figueiredo *et al.,* 2009; Barrera *et al*., 2011, Jakubowska, *et al*., 2005).

In this study we tested the single genotypes SeAl1 and SeG25, and three OBs mixed populations for their biological properties. In line with previous studies the SeG25 showed 3.1 times more pathogenic than SeAl1 (Cabodevilla *et al*., 2011a). Especially relevant was the behavior of OBs mixed populations. The three OBs-mixed populations showed similar LC_{50} to the SeG25, despite the quantity of this genotype in the mixture. Thus, as low as 25% of SeG25 in the OBs mixture was enough to improve significantly the pathogenicity respect to the SeAl1 genotype alone.

In terms of speed of kill no similarities were observed in comparison to previous studies conducted with the genotype SeAl1 and SeG25. Here, the peak of the mortality-time response occurred between 85.00 ± 0.23 hours and 86.14 ± 0.23 h for L_4 instar larvae infected for SeG25 and SeAl1 respectively. Cabodevilla *et al.* (2011a) results showed that SeAl1 peak of deaths occurred in 110 ± 2.5 and SeG25 in 100 ± 2.5 h. This

difference can be explained by the variation of temperatures registered during the bioassay between both studies. While Cabodevilla *et al.* (2011a) storage temperature was 25 ± 2ºC, our temperature was 26 ºC ± 2ºC. The influence of the temperature on the larval metabolism has been widely demonstrated (Abbas Ali *et al.*, 1990). Subramanian *et al.*, (2006), reported that the higher temperature the lower time needed to kill the infected host by the virus. Although the differences are not significant, our results show that the HT genotype SeG25 have less OB production and shorter MTD in comparison to the VT genotype SeAl1 following the same trend as previous studies (Cabodevilla *et al.,* 2011a).

Recent findings on SeMNPV covert infections demonstrated that the prevalence of the virus in the host could represent an efficient strategy to persist for long terms in a changeable environment, such as crops systems (Cabodevilla *et al*., 2011c). In order to investigate the capability of the different genotypes and their mixed population to persist in the host we introduce an assay to evaluate the establishment of covert infections in adults as the first steep for the virus to be vertically transmitted to the offspring individuals. Advances in molecular technique development led us to the detection of NPVs in asymptomatic insects. Studies carried out in adults of different lepidopteran virus-host species systems have revealed virus transcriptional activity in asymptomatic individuals (Cabodevilla, 2008; Hudges *et al.,* 1997), while other studies detected both DNA and RNA presence (Burden *et al.,* 2002, 2003; Vilaplana *et al.,* 2010). In our study we wanted to detect the viral RNA that was being transcripted using RT-PCR to determine the level of transcriptional activity of the virus. The *DNA-polymerase*, *polyhedrin* and *ie0* genes were transcribed in different phases of the cascade expression cycle of NPVs (Rhorman *et al.,* 2011). During a lethal infectious cycle of the SeMNPV *ie0* transcripts were found at very early phase, while *DNA-polymerase* and *polyherdin* genes at early and late phases respectively (Simon *et al*., 2004). Our results only showed RT-PCR positives adults for *ie0* gene but not in *DNA-polymerase* and *polyhedrin* genes. The absence of *polyhedrin* transcriptional activity was observed before by Cabodevilla *et al.* (2011a) in a similar experiment using the SeAl1 genotype. The polyhedrin gene function seems to be irrelevant in vertical transmission, as the polyhedrin is not required until the virions were occluded in the OB for the virus release in to the environment (Rhorman *et al.,* 2011). More intriguing is the absence of DNA-polymerase transcripts since this is an essential gene for DNA replication and Cabodevilla *et al.* (2011a) found positive results when adults were tested

for *DNA-polymerase* gene. There might be two possible explanations for that result: i) the low number of samples tested in this study or ii) the viral transcription was in the very early stage of replication when the adults were frozen. Unfortunately, due to the low number of samples available per virus treatment we are not in a position to compare the efficiency of persistent infections establishment between the different OBs mixed populations of SeG25 and SeAl1. In that sense more data will need to be generated to complete the study. All together these findings help us to increase our knowledge on the role of VT genotypes in order to development novel approaches of NPVs applications that explore the possibilities of virus persistence in a multi-generation host during the crop growing season.

Conclusions

1. OB-Mixed populations of the two SeMNPV genotypes SeAl1 and SeG25, containing 25% and 75% of SeG25, improved their pathogenicity respect to the single genotype SeAl1 in 2.3 and 2.4 times respectively.

2. Other two insecticidal properties such us virulence and OBs production showed similar behavior between the single genotypes and the OB-mixed populations, despite difference between the single genotypes SeAl1 and SeG25 have been reported before.

3. SeG25 and the three OB-mixed populations of SeAl1 and SeG25 (72:25, 50:50 and 25:75) were able to produce persistent infections in adults when they were infected as larvae with sublethal doses.

4. Transcripts of the very early gene *ie0* were found in persistently infected adults whilst *DNA-polymerase* and *polyhedrin* genes were not detected in the same samples, suggesting a very low level of viral transcription.

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