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Evaluation of seven viral isolates as potential biocontrol agents against *Pseudoplusia includens* (Lepidoptera: Noctuidae) caterpillars

Talita M. Alexandre [a,b], Zilda Maria A. Ribeiro [c], Saluana R. Craveiro [c,d], Fabiane Cunha [a,b], Ines Cristina B. Fonseca [e], Flávio Moscardi [e], Maria Elita B. Castro [c,*]

[a] *Universidade Federal do Paraná, UFPR, Curitiba-PR, Brazil*
[b] *Embrapa Soja, 86001-970 Londrina-PR, Brazil*
[c] *Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica W5 Norte Final, 70770-917 Brasília-DF, Brazil*
[d] *Universidade de Brasilia, 70910-900 Brasilia-DF, Brazil*
[e] *Universidade Estadual de Londrina, 86051-990 Londrina-PR, Brazil*

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ABSTRACT

The caterpillar *Pseudoplusia includens* (Walker, 1857) (Lepidoptera, Noctuidae), known as soybean looper, is a pest that has recently assumed greater importance in soybean in Brazil. Isolates of nucleopolyhedroviruses (NPVs) of this pest have been identified from cotton in Guatemala and soybean farms in Brazil, providing an interesting perspective of potential use of viral insecticide against the insect in lieu to chemical insecticides. With the objective to contribute to the characterization studies of this virus, morphological and molecular analyses and biological activity were carried out with seven *P. includens* viral isolates (I-A to I-G). Electron microscopy of viral samples, purified from macerated infected larvae, showed particles with typical morphology of the *Baculoviridae* family, genus *Alphabaculovirus* (*Nucleopolyhedrovirus* – NPV) presenting virions with only a single nucleocapsid per envelope (SNPV) occluded in a protein matrix, forming occlusion bodies (OB). This virus was then classified as *P. includens single nucleopolyhedrovirus* (PsinSNPV). OB particles analyzed in SDS–polyacrylamide gel showed an intense band corresponding in size to NPV polyhedrin protein. DNA restriction profiles of the PsinSNPV isolates showed differences in the fragment size and number suggesting the existence of genotypic variants, except between I-E and I-F profiles that were similar. Among the isolates tested for infectivity against *P. includens*, I-A, I-E and I-F were the most virulent. Survival times (ST_{50}) varied according to viral concentration, with significant differences among isolates for the three higher concentrations.

1. Introduction

The soybean looper, *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae; Plusiinae), is a species with distribution restricted to the western hemisphere, occurring from northern United States of America (USA) to southern South America (Alford and Hammond, 1982). It may feed on a large number of host plants, including crops of economic importance such as soybean, cotton, beans, tobacco, sunflower and various vegetables (Bueno et al., 2007). In Brazil, this insect has become a key pest of soybean in the last five years, being controlled mainly by applications of broad spectrum chemical insecticides (Bueno et al., 2007), usually requiring much higher dosages of insecticides than the major soybean defoliating noctuid and a concurrent species, the velvetbean caterpillar, *Anticarsia gemmmatalis* (Hübner) (Morales et al., 1995). In this context, use of a viral insecticide based on the PsinSNPV to control *P. includens* would be important to overcome problems related to reduction of natural enemies and possible selection of resistance to chemical insecticides in populations of the insect related to current control practices (Bueno et al., 2007).

Baculoviruses are a large virus group belonging to the family *Baculoviridae* that infect different insect orders, mainly Lepidoptera (Theilmann et al., 2005). Presently, it is subdivided into four genera, the *Alphabaculovirus* (lepidopteran-specific nucleopolyhedrovirus, NPV – Groups I and II), *Betabaculovirus* (lepidopteran-specific granulovirus), *Gammabaculovirus* (hymenopteran-specific NPV), and *Deltabaculovirus* (dipteran-specific NPV) (Jehle et al., 2006; www.ictvonline.org). Baculoviruses have large and covalently closed circular genomes of double-stranded DNA (range from 80 to 180 kbp), which are packaged singly or multiply in an enveloped, rod-shaped virion (Friesen and Miller, 2001; Theilmann et al., 2005). Two morphologically distinct forms of infectious particles are generated in the baculovirus cycle, the occlusion derived virus (ODVs), comprising enveloped virions embedded within a crystalline matrix of protein (polyhedrin for NPVs and granulin

* Corresponding author. Fax: +55 61 3448 4673.
E-mail address: elita@cenargen.embrapa.br (Maria Elita B. Castro).

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for GVs), and budded virus (BVs), consisting of a single virion enveloped by a plasma membrane. Due to their specificity and high virulence to a number of insect pest species, they have been used worldwide to control lepidopteran pests in many crops (Gröner, 1986; Moscardi, 1999; Moscardi et al., 2002). The *A. gemmatalis multiple nucleopolyhedrovirus* (AgMNPV) is one of the most successful and recognized examples of virus use by farmers (Moscardi, 1999, 2007), being employed in soybean in Brazil, Argentina, Paraguay and Mexico.

A number of studies based on the DNA restriction patterns have been used to investigate variability among NPV isolates obtained from geographically separate populations of a given host species and to distinguish NPVs isolated from different insects (Gettig and McCarthy, 1982; Caballero et al., 1992; Tanada and Kaya, 1993; Boughton et al., 1999; Richards et al., 1999; Takatsuka et al., 2003; Cooper et al., 2003; Graham et al., 2004; Cory et al., 2005; Martins et al., 2005; Figueiredo et al., 2009).

A single nucleopolyhedrovirus of *P. includens* (PsinSNPV) obtained from the insect on cotton in Guatemala has been studied under laboratory and field conditions, regarding aspects as dosage and temperature response (Livingston et al., 1980; Kunimi et al., 1997; Ali and Young, 1991), effect of viral enhancers (fluorescent brighteners) (Zou and Young, 1996; Young, 2001), field persistence, control efficacy, effect on post larval stages and transmission (Mcleod et al., 1982; Young and Yearian, 1982). This isolate was also used for initial laboratory trials in Brazil (Morales and Moscardi, 1993a; Morales et al., 1993). Most recently, some other isolates have been obtained from the insect in soybean in Brazil. Due to the changing status of *P. includens* as a pest of importance on this crop, there have been initiatives to evaluate the potential of PsinSNPV as a biological insecticide. It is important to select the best isolates to use against *P. includens*. Therefore, we initiated a study of the morphology, genetics, and biological activity of several PsinSNPV isolates.

2. Materials and methods

2.1. Insects and viral isolates

Infected *P. includens* larvae from different localities in South and Central Brazil were collected in soybean farms (Table 1), brought to Embrapa Soja, Londrina, Paraná state, and prepared for an initial examination under a phase-contrast microscope. From those larvae, seven viral isolates were used for morphological analysis by electron microscopy, genomic analyses with restriction endonucleases and for virulence and survival time evaluations through bioassays.

2.2. Purification of viral occlusion bodies (OBs)

Occlusion bodies (OBs) from *P. includens* larvae infected with each of the seven PsinSNPV isolates were purified and then used as stocks for electron microscopic observations, SDS–polyacrylamide gel electrophoresis, DNA extraction and bioassays. Infected larvae were macerated in homogenization buffer (1% ascorbic acid; 2% sodium dodecyl sulphate – SDS; 0.01 M Tris–HCl, pH 7.8; and 0.001 M EDTA), filtered through six layers of gauze and was first given a low speed spin at 4000*g* for 5 min to remove the larval debris

Table 1
Viral isolates obtained from infected *Pseudoplusia includens* larvae.

Isolates	Host insect	Collection date	Collection local	Institution	Crop
Psin-IA (I-A)	*Pseudoplusia includens*	1972	Guatemala/Central America	University of Arkansas/USA	Cotton
Psin-IB (I-B)	*Pseudoplusia includens*	January 2006	Londrina, PR	Embrapa Soja	Soybean
Psin-IC (I-C)	*Pseudoplusia includens*	January 2006	Maringá, PR	Embrapa Soja	Soybean
Psin-ID (I-D)	*Pseudoplusia includens*	February 2006	Iguaraçu, PR	Embrapa Soja	Soybean
Psin-IE (I-E)	*Pseudoplusia includens*	February 2007	Iguaraçu, PR	Embrapa Soja	Soybean
Psin-IF (I-F)	*Pseudoplusia includens*	February 2008	Dourados, MS	Soybean farmer' field	Soybean
Psin-IG (I-G)	*Pseudoplusia includens*	February 2008	Sertanópolis, PR	Soybean farmer' field	Soybean

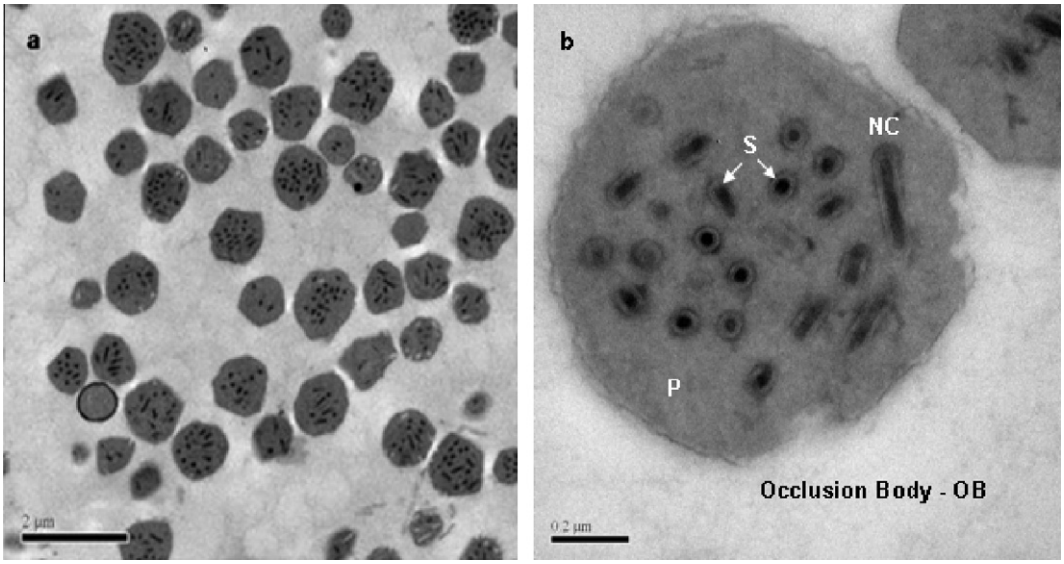

Fig. 1. Transmission electron micrographs of *Pseudoplusia includens* NPV occlusion bodies (OB) showing virions containing single (S) nucleocapsids (NC) embedded in the crystalline matrix of polyhedrin protein (P). This polyhedral morphology was found in all isolates examined. Scale bars: (a) 2 µm; (b) 0.2 µm.

and then centrifuged at 12,000*g* for 15 min to pellet the occlusion bodies. The pellets were suspended in TE buffer (0.01 M Tris–HCl, pH 7.8 and 0.001 M EDTA) and centrifuged at 12,000*g* for 15 min. The OB-containing pellets were again suspended in TE buffer with 0.1% SDS and 0.1 M NaCl and then collected by centrifugation. The final OB suspensions were prepared in Milli-Q H_2O and stored at −20 °C.

2.3. Transmission electron microscopy

For morphological identification of the virus, occlusion bodies (OBs) from seven isolates were processed for transmission electron microscopy (TEM). The purified OBs were fixed by 2.5% glutaraldehyde in 0.02 M sodium cacodylate buffer (pH 7.2) for 2 h, and then post-fixed by 1% osmium tetroxide in the same buffer for 1 h. The fixed samples were dehydrated by immersion in 0.5% uranyl acetate and washed in a graded series of ethanol, and then embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were prepared with a Reichert OMU3 Ultramicrotome (Reichert Supernova Ultramicrotome), stained with 2% uranyl acetate followed by Reynolds lead citrate (Reynolds, 1963), and observed in a JEOL 1011 transmission electron microscope at 80 kV.

2.4. Polyacrylamide gel electrophoresis (SDS–PAGE) of occlusion bodies (OB)

The OBs were mixed individually with equal volumes of 2× sample buffer (125 mM Tris–HCl, pH 6.7; 4% SDS; 30% glycerol; 0.002% bromophenol blue, containing reducing agent 10% 2-β-mercaptoethanol). After boiling for 5 min, the samples were loaded onto the 16% polyacrylamide gel containing SDS as described by Laemmli (1970). OB samples of the seven PsinSNPV isolates, along with a sample of known AgMNPV-2D polyhedrin protein (33-kDa) for comparison of the polyhedrin size, were loaded on the same denaturing gel, which was afterwards stained with Coomassie brilliant blue.

2.5. Purification of DNA from OBs

DNA of all the seven isolates was purified from OBs for a comparative restriction endonuclease analysis, and the procedures used were based on standard method (O'Reilly et al.,1992). Virions were released by dissolving 1×10^9 OB/ml in an alkaline solution (0.1 M Na_2CO_3, 0.17 M NaCl, 0.01 M EDTA, pH 10.9) at 37 °C for 30 min. DNA was extracted from alkali-liberated virions by overnight incubation at 37 °C with 1% SDS and 0.5 mg/ml proteinase K (final concentration), followed by extractions with phenol:chloroform:isoamylalcohol (25:24:1). In order to precipitate the DNA, a 0.1 volume of 3 M sodium acetate, pH 5.2 and 2 volumes of absolute ethanol were added to the aqueous phase and then incubated at −20 °C, overnight. The precipitated DNA was recovered by centrifugation in the microfuge at 14,000*g* for 15 min, and washed with 70% (v/v) ethanol. The dried DNA was then suspended in sterile TE buffer (pH 8.0) and stored at 4 °C until use. DNA concentration was estimated by comparison to known concentrations of standard DNA (λ DNA).

2.6. Viral DNA cleavage with restriction endonucleases (REN)

Viral DNA (1–2 μg) was cleaved with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I under the conditions recommended by the manufacturers (Gibco BRL or Sigma) individually. The generated DNA fragments were analyzed by 1% agarose gel electrophoresis carried out at 40 V, overnight (14–16 h), using 1× TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA, pH 8.0). The 1 kb plus DNA ladder (Gibco BRL) was used as molecular size marker. The gel was stained with 0.5 μg/ml ethidium bromide, visualized, and photographed with an Eagle Eye II still video system (Stratagene).

2.7. Bioassays: determination of lethal concentration (LC_{50}) and survival time (ST_{50})

In order to investigate the pathogenic potential of isolates from diseased *P. includens* larvae, bioassays were carried out using insects obtained from an existing *P. includens* colony. Third-instar *P. includens* larvae (40 larvae/viral concentration) were infected with the different isolates by incorporation of each virus into an artificial diet (Greene et al., 1976), according to procedures of Morales and Moscardi (1993b), at five viral concentrations (625, 2500, 10,000, 40,000, 160,000 occlusion bodies – OB/ml of diet) per isolate. The larvae were placed in 50-ml plastic vials (2 larvae/vial) containing the treated diet and incubated in environmental chambers at 26 ± 2 °C, relative humidity of 70 ± 10%, and photoperiod of 12L:12D. Larvae fed untreated diet were used as controls. Mortality was checked daily and dead larvae were frozen at −20 °C. Mortal-

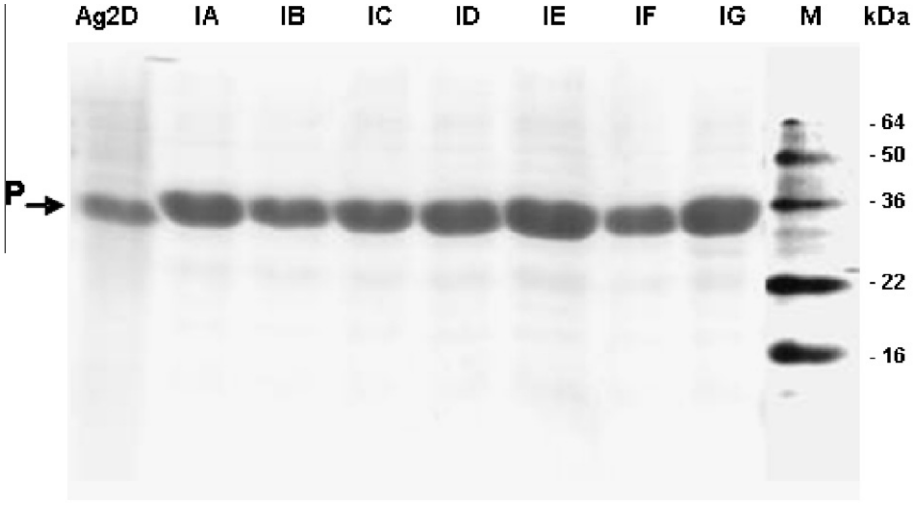

Fig. 2. Analysis of *Pseudoplusia includens* SNPV structural proteins. Polyacrylamide gel (SDS–PAGE 15%) stained with *Coomassie Blue* (A). At left, AgMNPV polyhedra with its known 33 kDa polyhedrin. At right, molecular weights are expressed in kilodaltons.

ity data were submitted to Probit analysis (Finney, 1971), using POLO-PLUS program (Russell et al., 1977) to estimate the median lethal concentration (LC_{50}), as well as associated parameters (95% fiducial limit, slope, χ^2) for all isolates. The median survival times (ST_{50}) for the isolates were estimated using the Kaplan–Meyer Product-Limit estimator method in JMP SAS (2008), and were compared using log-rank tests (Kabfleisch and Prentice, 1980).

3. Results and discussion

3.1. Morphological description of the Pseudoplusia includens SNPV isolates

Samples of virus suspensions from seven uncharacterized PsinSNPV isolates were examined first by phase contrast microscopy

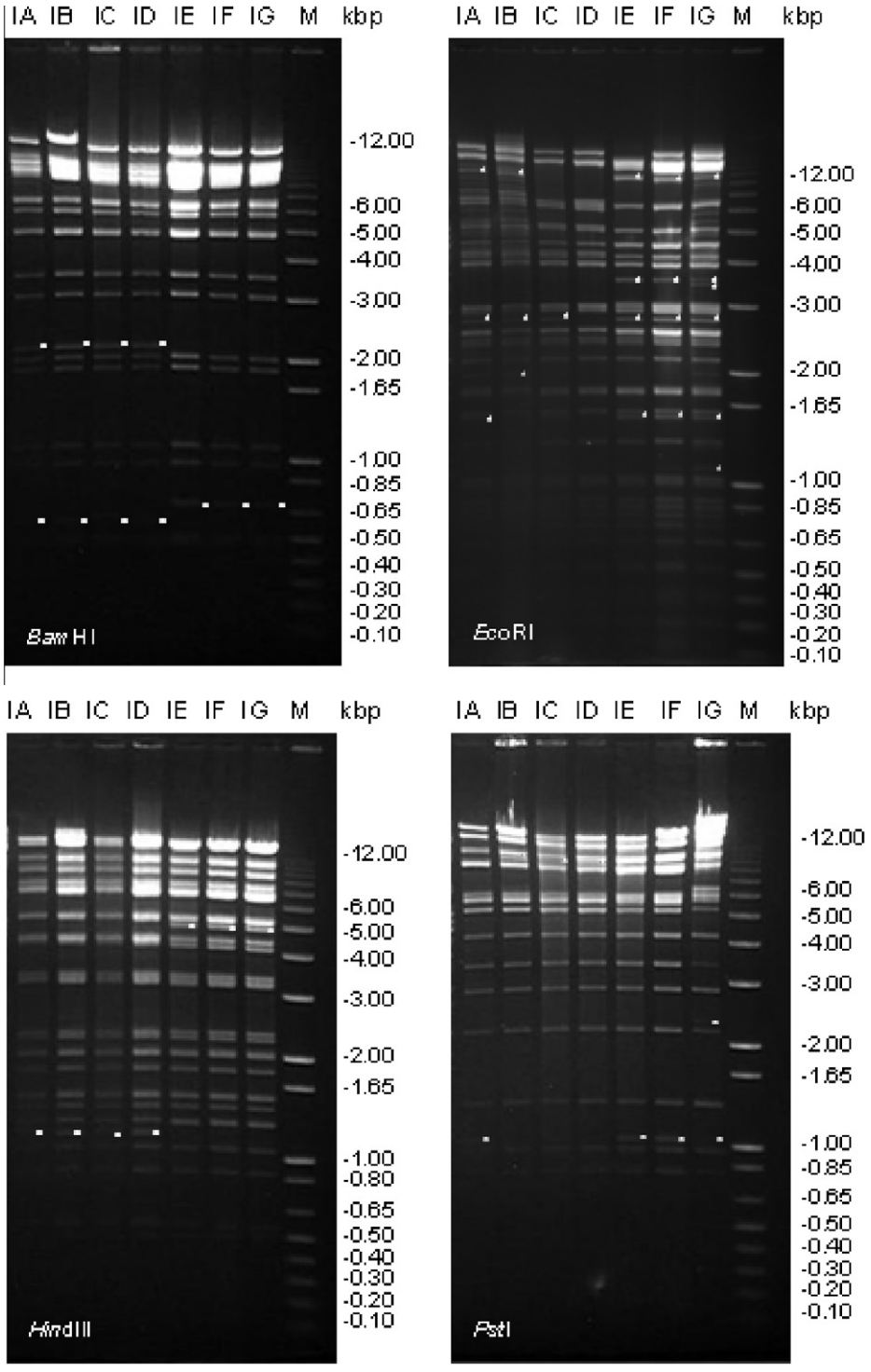

Fig. 3. Comparison of four DNA restriction profile patterns among PsinSNPV isolates (I-A, I-B, I-C, I-D, I-E, I-F and I-G). DNAs were digested with *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I; restriction fragments were separated by electrophoresis in 1.0% agarose gel. Each novel band is marked by an asterisk. The presence of submolar bands suggests that these isolates can be a mixture of more than one strain of the virus. Molecular marker (M): 1 kb plus DNA Ladder (Invitrogen).

for the presence of occlusion bodies (OBs) and then by transmission electron microscopy (TEM). TEM micrographs of *P. includens* SNPV OBs revealed the presence of several (up to 21) virions containing a single nucleocapsid packaged within a single viral envelope (Fig. 1). The OBs exhibit pleomorphic shapes with sizes ranging from 0.5 to 1.3 μm in diameter, most of them around 1 μm. This size is at the lower end of the size range (0.15–15 μm) reported for NPVs (Theilmann et al., 2005). Single-nucleocapsid NPVs isolated from other lepidopteran insects have shown similar sizes, with OB diameters ranging from 0.5 to 2.0 μm, such as *Clanis bilineata* SNPV (Wang et al., 2008), *Ectropis obliqua* SNPV (Ma et al., 2006), *Adoxophyes honmai* NPV (Ishii et al., 2003), *Thysanoplusia orichalcea* SNPV (Cheng and Carner, 2000), and other SNPV cited by Sudhakar and Mathavan (1999). The polyhedral structure of the PsinSNPV isolates observed by TEM was confirmed by detection of PsinSNPV polyhedra in SDS–PAGE gels. All isolates analyzed in this work showed a major protein species corresponding in molecular mass to the polyhedrins, as reported for other NPVs (Fig. 2).

3.2. Comparative analyses of the viral DNA restriction patterns

The genome DNA of each of the seven PsinSNPV isolates was cleaved with the enzymes *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I to compare restriction profiles generated among them (Fig. 3). The analyses were done by comparison of additional fragments present in relation to number of fragments common to all isolates generated by each cleavage. Some differences were observed among the restriction patterns of the viral isolates analyzed, particularly for digestion with *Eco*RI that revealed an even greater range of variation in terms of number of detectable DNA fragments (from 26 to 32 fragments). Besides 26 *Eco*RI fragments common in all isolates, there were three extra fragments of about 10, 2.7 and1.6 kbp (or 1.8) for I-A and I-B isolates, respectively; a 2.7 kbp fragment for I-C; four fragments of 10, 3.7, 2.7 and 1.6 kbp for I-E and I-F; and six fragments of 10, 3.7, 3.6, 2.7, 1.6, and 1.2 kbp for I-G isolate. In all other REN profiles the variation was only of one or two extra fragments. The combination resulting of the DNA analyses with the four restriction enzymes showed that from the seven isolates only two, I-E and I-F, exhibited the same number and mobility of fragments in the gels, demonstrating thus that these isolates might have similar genomes.

Wild-type baculovirus isolates are frequently found to be genetically heterogeneous and this is typically indicated by the presence of submolar bands in the DNA restriction profiles (McIntosh et al., 1987; Smith and Crook, 1988a, 1988b). Therefore, the heterogeneity observed among the isolates here analyzed could be related with those bands present in submolar quantities on the gels. On the other hand, when our REN fragment gels were compared to other published REN fragment gels, since we have not yet PsinSNPV genome sequence data, the REN patterns of the PsinSNPV isolates were distinct from those other already known SNPV, such as *Trichoplusia ni* SNPV (Bilimoria, 1983; Davis and Wood, 1996; Erlandson et al., 2007), *Buzura suppressaria* SNPV (Liu et al., 1993), *Orgyia antique* SNPV (Richards et al., 1999), *Orgya pseudotsugata* SNPV (Leisy et al., 1986), *Orgyia ericae* SNPV (Yang et al., 2006), *Helicoverpa* (*Heliothis*) *zea* SNPV (Granados et al., 1981; McIntosh and Ignoffo, 1983), *Heliothis armigera* SNPV (Sudhakar and Mathavan, 1999), *T. orichalcea* SNPV (Cheng and Carner, 2000), *A. honmai* SNPV (Ishii et al., 2003; Takahashi et al., 2008). However, this comparison was not exhaustive and we still do not know whether the virus described here is a new virus or a variant of a previously described virus.

3.3. Median lethal concentration (LC_{50}) and survival time (ST_{50})

The χ^2 values related to the different tested isolates LC_{50} were non-significant, indicating that the data were homogeneous for all isolates and fit the probit analysis model utilized (Finney, 1971). The I-A isolate (from Guatemala) resulted in the lowest LC_{50}, but did not statistically differ from I-E (from Iguaraçu, PR, Brazil, season 2006/07) and I-F (from Dourados, MS, Brazil). These three isolates were the most virulent against *P. includens* (Table 2). However, I-F also did not differ from I-D and I-E. On the other hand, I-E did not differ from I-B, I-D, and I-F. The analysis related to the median survival time (ST_{50}) revealed that ST_{50} did not follow the same pattern of the LC_{50} in the comparisons among PsinSNPV isolates (Table 3). The lowest viral concentration tested (650 OB/ml of diet) was excluded from this analysis as it caused less than 50% mortality in infected insects. In general, ST_{50} decreased with increase in viral concentration. At 2500 OB/ml of diet, there were no significant differences among isolates. At 100,000 OB/ml of diet, the isolate I-A resulted in the lowest ST_{50}, but did not differ from I-D and I-F. At 40,000 OB/ml of diet, lowest ST_{50} values were observed for I-B and I-G, which were significantly different from I-C to I-D, but did not differ from the other isolates. At 160,000 OB/ml of diet I-D resulted in the highest ST_{50}, not significantly differing from I-C, but differing from the other isolates.

Genotypic variants of the same baculovirus, a common event in field population, have been reported to differ in their DNA restriction profiles and also in their biological activities (Caballero et al., 1992; Takatsuka et al., 2003; Cory and Myers, 2003; Cory et al., 2005). In the present work, PsinSNPV isolates from different localities were analyzed and compared essentially based on their DNA restriction patterns, virulence, and survival time, since all isolates were similar in their general morphology. DNA cleavage using four restriction enzymes generated profiles exhibiting variation in their genomes with evidence of submolar bands in most of the profiles. This suggests that some of these isolates could in fact be a mixture of more than one strain of the virus. According to Cory et al. (2005), small genomic changes may result in significant modifications in the biological activity of baculoviruses. However, our biological activity data are quite preliminary since the isolates here evaluated were originally obtained from field-collected *P. includens* larvae and then multiplied in laboratory assays. Therefore, further sequencing studies, besides being helpful in the identification and characterization of the isolates, might help elucidate if the variable genomic regions detected here are related to LC_{50} and ST_{50} differences observed among the isolates evaluated.

The isolate I-A (obtained from Guatemala) was studied in the 1980s by Livingston et al. (1980) and Young and Yearian (1982) regarding larval mortality in laboratory and in the field, as well as other aspects; however, there have not been recent research with the PsinSNPV, specially those related to investigating the bio-

Table 2
Median lethal concentrations (LC_{50}) obtained for *P. includens* third-instar larvae infected with different PsinSNPV isolates.

Isolates[a]	LC_{50}	95% Fiducial limits		Slope ± SE[b]	χ^2
		Lower	Upper		
I-A	2500e	1833	3408	2.07 ± 0.31	0.00 ns[c]
I-B	5800abc	4188	7766	2.00 ± 0.24	2.18 ns
I-C	8713ab	5915	14,485	1.51 ± 0.24	2.08 ns
I-D	4751bcd	3459	6424	1.98 ± 0.24	0.33 ns
I-E	2974cde	1797	4500	1.21 ± 0.17	3.00 ns
I-F	2811de	1855	3984	1.53 ± 0.20	3.38 ns
I-G	9274a	6590	13,526	1.61 ± 0.21	0.03 ns

[a] A – from Guatemala; B – from Londrina-PR; C – from Maringá-PR; D – from Iguaraçu-PR season 2005/2006, E – from Iguaraçu-PR season 2006/2007; F – from Dourados-MS; and G– from Sertanópolis-PR. Different letters after LC_{50} values for each isolate are significantly different ($P = 0.05$).
[b] SE, standard error.
[c] Non-significant chi-square (χ^2) values.

Table 3
Median survival times (ST_{50}) and associated parameters for *P. includens* third-instar larvae infected with different PsinSNPV isolates.

Concentration (OB/ml of diet)	Isolates	ST_{50} (d.p.i.)[a]	95% Confidence interval		SE	df	χ^2	*P*
			CI low	CI high				
2500	I-A	8a	8	12	0.44	6	10.37	0.11
	I-B	12a	10	–	0.45			
	I-C	12a	11	12	0.37			
	I-D	10a	9	12	0.43			
	I-E	10a	8	11	0.31			
	I-F	10a	8	12	0.40			
	I-G	12a	10	12	0.34			
10,000	I-A	7b	–	–	0.22	6	45.87	0.001
	I-B	8a	8	9	0.32			
	I-C	8a	8	9	0.27			
	I-D	8ab	7	8	0.32			
	I-E	8a	–	–	0.29			
	I-F	8ab	7	8	0.29			
	I-G	8a	8	10	0.36			
40,000	I-A	7ab	6	7	0.12	6	32.53	0.00
	I-B	6b	–	–	0.17			
	I-C	7a	7	8	0.14			
	I-D	7a	–	–	0.14			
	I-E	6ab	6	8	0.23			
	I-F	6ab	6	7	0.17			
	I-G	6b	–	–	0.15			
160,000	I-A	6b	–	–	0.10	6	64.52	0.00
	I-B	6b	–	–	0.12			
	I-C	7ab	6	7	0.17			
	I-D	7a	–	–	0.10			
	I-E	6b	–	–	0.12			
	I-F	6b	–	–	0.12			
	I-G	6b	6	6	0.10			

[a] Median survival times were determined using the Kaplan–Meier Product-Limit estimator (Collett, 1994); d.p.i. = days post inoculation. Different letters after ST50 values for each dose are significantly different with $a = 0.05$. Chi-square results are from test of the null hypothesis that times to death were not significantly different among virus treatments at each dose (test of equality over isolates log-rank).

logical activity of different viral isolates and their molecular characterization, as conducted in the present work.

Therefore, differences in virulence due to intraspecific heterogeneity, as found in the present work, may represent an important tool to develop the PsinSNPV as a biological insecticide.

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References

Alford, A.R., Hammond Jr., A.M., 1982. Plusiinae (Lepidoptera: Noctuidae) populations in Louisiana soybean ecosystems as determined with looplure-baited traps [*Pseudoplusia includens*, *Rachiplusia ou*, *Trichoplusia* spp.]. Journal of Economic Entomology 75, 647–650.

Ali, A., Young, S., 1991. Influence of larval age and temperature on effectiveness of a nuclear polyhedrosis virus in the soybean Looper, *Pseudoplusia includens* (Lepidoptera: Noctuidae) on soybean. Biological Control 1, 334–338.

Bilimoria, S.L., 1983. Genomic divergence among the single-capsid nuclear polyhedrosis viruses of plusiine hosts. Virology 127, 15–23.

Boughton, A.J., Harrison, R.L., Lewis, L.C., Bonning, B.C., 1999. Characterization of a nucleopolyhedrovirus from the black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae). Journal of Invertebrate Pathology 74, 289–294.

Bueno, R.C.O.F., Parra, J.R.P., Bueno, A.F., Moscardi, F., Di Oliveira, J.R.G., Camillo, M.F., 2007. Sem Barreira. Cultivar Grandes Culturas 93, 12–15.

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

Cheng, Xiao.-Wen., Carner, G.R., 2000. Characterization of a single-nucleocapsid nucleopolyhedrovirus of *Thysanoplusia orichalcea* L. (Lepidoptera: Noctuidae) from Indonesia. Journal of Invertebrate Pathology 75, 279–287.

Collet, D., 1994. Modelling Survival Data in Medical Research. Chapman and Hall, London.

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

Cory, J.S., Green, B.M., Paul, R.K., Hunter-Fujita, F., 2005. Genotypic and phenotypic diversity of a baculovirus population within an individual insect host. Journal of Invertebrate Pathology 89, 101–111.

Cory, J.S., Myers, J.H., 2003. The ecology and evolution of baculoviruses. Annual Review of Ecology, Evolution, and Systematics 34, 239–272.

Davis, T.R., Wood, H.A., 1996. In *vitro* characterization of a *Trichoplusia ni* single nucleocapsid nuclear polyhedrosis virus. Journal of Invertebrate Pathology 77, 2303–2310.

Erlandson, M., Newhouse, S., Moore, K., Janmaat, A., Myers, J., Theilmann, D., 2007. Characterization of baculovirus isolates from *Trichoplusia ni* populations from vegetable greenhouses. Biological Control 41, 256–263.

Figueiredo, E., Muñzo, D., Murillo, R., Mexia, A., Caballero, P., 2009. Diversity of Iberian nucleopolyhedrovirus wild-type isolates infecting *Helicoverpa armigera* (Lepidoptera: Noctuidae). Biological Control 50, 43–49.

Finney, D.J., 1971. Probit Analysis. Cambridge Univ. Press, London, New York. 333p.

Friesen, P.D., Miller, L.K., 2001. Insect viruses. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Martin, M.A., Lamb, R.A., Roizman, B., Straus, S.E. (Eds.), Fundamental Virology, fourth ed. Lippincott Williams and Wilkins Press, Philadelphia., pp. 443–472.

Gettig, R.R., McCarthy, W.J., 1982. Genotypic variation among wild isolates of *Heliothis* spp. nuclear polyhedrosis viruses from different geographical regions. Virology 117, 245–252.

Graham, R.I., Tyne, W.I., Possee, R.D., Sait, S.M., Hails, R., 2004. Genetically variable nucleopolyhedroviruses isolated from spatially separate populations of the winter moth *Operophtera brumata* (Lepidoptera: Geometridae) in Orkney. Journal of Invertebrate Pathology 87, 29–38.

Granados, R.R., Lawler, K.A., Burand, J.P., 1981. Replication of *Heliothis zea* baculovirus in an insect cell line. Intervirology 16, 71–79.

Greene, G.L., Leppla, N.C., Dicherson, W.A., 1976. Velvetbean caterpillar: a rearing produced and artificial medium. Journal of Economic Entomology 4, 487–488.

Gröner, A., 1986. Specificity and safety of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses: Biological Properties and Molecular Biology, vol. 1. Academic Press, San Diego, Calif., pp. 177–202.

Ishii, T., Nakai, M., Okuno, S., Takatsuka, J., Kunimi, Y., 2003. Characterization of *Adoxophyes honmai* single-nucleocapsid nucleopolyhedrovirus: morphology, structure, and effects on larvae. Journal of Invertebrate Pathology 83, 206–214.

Jehle, J.A., Blissard, G.W., Bonning, B.C., Cory, J.S., Herniou, E.A., Rohrmann, G.F., Theilmann, D.A., Thiem, S.M., Vlak, J.M., 2006. On the classification and nomenclature of baculoviruses: a proposal for revision. Archives of Virology 151, 1257–1266.

SAS, J.M.P., 2008. JMP Statistics and Graphics Guide, Version 7. SAS Institute, Inc., Cary, North Carolina.

Kabfleisch, J.D., Prentice, R.L., 1980. The Statistical Analysis of Time Failure Data. Wiley, New York.

Kunimi, Y., Fuxa, J.R., Richter, A.R., 1997. Survival time and lethal doses for wild and recombination *Autographa californica* nuclear polyhedrosis viruses in different instar of *Pseudoplusia includens*. Biological Control 9, 129–135.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leisy, D., Nesson, M., Pearson, M., Rohrmann, G.F., Beaudreau, G.S., 1986. Location and nucleotide sequence of the *Orgyia pseudotsugata* single nucleocapsid nuclear polyhedrosis virus polyhedrin gene. Journal of General Virology 67, 1073–1079.

Liu, M., Hu, Z., Liang, B., Jim, F., Li, M., Xm, T., 1993. Physical mapping of *Buzura suppressaria* nuclear polyhedrosis virus genome. Archives of Virology 128, 357–362.

Livingston, J.M., Mcleod, P.J., Yearian, W.C., Young, S.Y., 1980. Laboratory and field evaluation of nuclear polyhedrosis virus of the soybean Looper, *Pseudoplusia includens*. Journal Georgia Entomology Society 15, 194–199.

Ma, Xiu-Cui, Xu, Hai-Jun, Tang, Mei-Jun, Xiao, Q., Hong, J., Zhang, Chuan-Xi, 2006. Morphological, phylogenetic and biological characteristics of *Ectropis obliqua* single-nucleocapsid nucleopolyhedrovirus. The Journal of Microbiology 44, 77–82.

Martins, T., Montiel, R., Medeiros, J., Oliveira, L., Simoes, N., 2005. Occurrence and characterization of a nucleopolyhedrovirus from *Spodoptera littoralis* (Lepidoptera: Noctuidae) isolated in the azores. Journal of Invertebrate Pathology 89, 185–192.

Mclntosh, A.H., Ignoffo, C.M., 1983. Restriction endonuclease patterns of three baculoviruses isolated from species of *Heliothis*. Journal of Invertebrate Pathology 41, 27–32.

McIntosh, A.H., Rice, W.C., Ignoffo, C.M., 1987. Genotypic variants in wild-type populations of baculoviruses. In: Maramorosch, K. (Ed.), Biotechnology in Invertebrate Pathology and Cell Culture. Academic Press Inc., San Diego, pp. 305–325.

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

Morales, L., Moscardi, F., 1993a. Virulência para lagartas de *Chrysodeixis includens* (Walker) e *Rachiplusia nu* (Guenée) do virus de *Autographa californica* (Speyer) após passagem seriada em *C. Includens* (Lep.: Noctuidae). Anais da Sociedade Entomológica do Brasil 22, 19–27.

Morales, L., Moscardi, F., 1993b. Comparação entre duas metodologias de bioensaios para vírus entomopatogênicos. Anais da Sociedade Entomológica do Brasil 22, 535–540.

Morales, L., Moscardi, F., Gravena, S., 1993. Potencial do baculovirus de *Autographa californica* (Speyer) no controle de *Chrysodeixis includens* (Walker) e *Anticarsia gemmatalis* Hübner (Lep.: Noctuidae). Pesquisa Agropecuária Brasileira 28, 237–243.

Morales, L., Moscardi, F., Kastelic, J.G., Sosa-Gomez, D.R., Paro, F.R., Soldorio, I.L., 1995. Suscetibilidade de *Anticarsia gemmatalis* Hubner e *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), a *Bacillus thuringiensis* (Berliner). Annais da Sociedade Entomológica do Brasil 24, 593–598.

Moscardi, F., 1999. Assessment of the application of baculoviruses for control of Lepidoptera. Annual Review of Entomology 44, 257–289.

Moscardi, F., 2007. A Nucleopolyhedrovirus for control of the velvetbean caterpillar in Brazilian soybeans. In: Vincent, C., Goettel, M., Lazarovits, G. (Eds.), Biological Control: A Global Perspective. CAB International, Wallingford, pp. 344–352.

Moscardi, F., Morales, L., Santos, B., 2002. The successful use of AgMNPV for the control of velvetbean caterpillar, *Anticarsia gemmatalis*, in soybean in Brazil. In: 35th Annual Meeting of the SIP. Foz do Iguaçu, Brasil, pp. 86–91.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., 1992. Baculovirus Expression Vectors: A Laboratory Manual. W.H. Freeman and Co., Salt Lake City, Utah. pp. 1–347.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. The Journal of Cell Biology 17, 208–212.

Richards, A., Speight, M., Cory, J., 1999. Characterization of a nucleopolyhedrovirus from the vapourer moth *Orgyia antiqua* (Lepidoptera: Lymantriidae). Journal of Invertebrate Pathology 74, 137–142.

Russell, R.M., Robertson, J.L., Savin, N.E., 1977. POLO: A new computer program for probit analysis. Bulletin of the Entomological Society of America 23, 209–213.

Smith, I.R.L., Crook, N.E., 1988a. Physical maps of the genomes of four variants of *Artogeia rapae* granulosis virus. Journal of General Virology 69, 1741–1747.

Smith, I.R.L., Crook, N.E., 1988b. *In vivo* isolation of baculovirus genotypes. Virology 166, 240–244.

Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 26, 31–43.

Sudhakar, S., Mathavan, S., 1999. Electron microscopical studies and restriction analysis of *Helicoverpa armigera* nucleopolyhedrosis virus. Journal of Biosciences 24, 361–370.

Takatsuka, J., Okuno, S., Nakai, M., Kunimi, Y., 2003. Genetic and biological comparisons of ten geographic isolates of a nucleopolyhedrovirus that infects *Spodoptera litura* (Lepidoptera: Noctuidae). Biological Control 26, 32–39.

Takahashi, M., Nakai, M., Nakanishi, K., Sato, T., Hilton, S., Winstanley, D., Kunimi, Y., 2008. Genetic and biological comparisons of four nucleopolyhedrovirus isolates that are infectious to *Adoxophyes honmai* (Lepidoptera: Tortricidae). Biological Control 46, 542–546.

Tanada, Y., Kaya, H., 1993. Insect Pathology. Academic Press, San Diego.

Theilmann, D.A., Blissard, G.W., Bonning, B., Jehle, J.A., O'Reilly, D.R., Rohrmann, G.F., Thiem, S., Vlak, J.M., 2005. Baculoviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy Classification and Nomenclature of Viruses: Eighth Report of the International Committee on the Taxonomy of Viruses. Elsevier, New York, NY, pp. 177–185.

Wang, L., Yi, J., Zhu, S., Li, B., Chen, Y., Shen, W., Wang, W., 2008. Identification of a single-nucleocapsid baculovirus isolated from *Clanis bilineata tsingtauica* (Lepidoptera: Sphingidae). Archives of Virology 153, 1557–1561.

Yang, L.R., Wang, D., Duan, L.Q., Zhang, C.X., 2006. Polyhedrin gene sequence and phylogenetic analysis of a nucleopolyhedrovirus isolated from *Orgyia ericae* Germar. DNA Sequence 17, 215–222.

Young, S.Y., 2001. Enhancement of nucleopolyhedrovirus activity in *Helicoperva zea* (Boddie) and *Pseudoplusia includens* (Walker) larvae with a fluorescent brightener. Journal Entomology Society 36, 162–168.

Young, S.Y., Yearian, W.C., 1982. Nuclear polyhedrosis virus infection of *Pseudoplusia includes* (Lep.: Noctuidae) larvae: effect on post larval stages and transmission. Entomophaga 27, 61–66.

Zou, Y., Young, S., 1996. Use of fluorescent brightener to improve *Pseudoplusia includens* (Lepidoptera: Noctuidae) nuclear polyhedrosis virus activity in the laboratory and field. Journal Economic Entomology 89, 92–96.