

In vitro infectivity of *Spodoptera frugiperda multiple nucleopolyhedrovirus* to different insect cell lines

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Abstract – The objective of this work was to evaluate the response of an in vitro host range to *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV), a pathogenic virus to the fall armyworm (*Spodoptera frugiperda*, Lepidoptera: Noctuidae), for the further development of a biopesticide based on cell culture systems. The cell lines from *Bombyx mori* (BM-5), *Lymantria dispar* (IPLB-LD-625Y), *Trichoplusia ni* (BTI-Tn-5B1-4), *Anticarsia gemmatalis* (UFL-AG-286), and *S. frugiperda* (IPLB-SF-21AE and Sf9) were tested for their susceptibility to a highly-virulent Brazilian isolate of SfMNPV. The cytopathic effects induced by the virus, the production of viral particles, and the synthesis of viral polypeptides were examined and compared. Both *S. frugiperda* cell lines showed hypertrophy of cell nuclei and production of many polyhedra. The SDS-Page of radiolabeled proteins showed that the cell protein synthesis was shutoff, while an intense band of about 30 kDa, recognized as polyhedrin, was synthesized. The other cell lines did not show polyhedra production, although some of them underwent morphological changes and protein synthesis shutdown in response to virus infection. The SF-21 and Sf9 cell lines are recommended for further in vitro production of SfMNPV.

Index terms: *Baculovirus*, biological control, cell culture, fall armyworm, in vitro production, SfMNPV.

Infectividade in vitro de *Spodoptera frugiperda multiple nucleopolyhedrovirus* a diferentes linhagens celulares de insetos

Resumo – O objetivo deste trabalho foi avaliar uma gama de hospedeiros, in vitro, quanto à resposta a *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV), um vírus patogênico à lagarta-do-cartucho (*Spodoptera frugiperda*; Lepidoptera: Noctuidae), para o posterior desenvolvimento de um biopesticida baseado em sistema de cultura de células. As linhagens celulares de *Bombyx mori* (BM-5), *Lymantria dispar* (IPLB-LD-625Y), *Trichoplusia ni* (BTI-Tn-5B1-4), *Anticarsia gemmatalis* (UFL-AG-286) e *S. frugiperda* (IPLB-SF-21AE e Sf9) foram testadas quanto a sua suscetibilidade a um isolado brasileiro de SfMNPV altamente virulento. Os efeitos citopáticos induzidos pelo vírus, a produção de partículas virais e a síntese de polipeptídeos virais foram examinados e comparados. Ambas as células de *S. frugiperda* apresentaram hipertrofia dos núcleos celulares e produção de muitos poliedros. O SDS-Page de proteínas com radiomarcagem mostrou que as proteínas celulares apresentaram inibição de síntese, enquanto uma intensa banda de cerca de 30 kDa, reconhecida como poliedrina, era sintetizada. As outras linhagens celulares não apresentaram produção de poliedros, apesar de algumas terem apresentado alterações morfológicas e inibição de síntese proteica em resposta à infecção viral. As linhagens celulares SF-21 e Sf9 são recomendadas para a posterior produção in vitro de SfMNPV.

Termos para indexação: *Baculovirus*, controle biológico, cultura de células, lagarta-do-cartucho, produção in vitro, SfMNPV.

Introduction

Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV), genus *Alphabaculovirus*, family *Baculoviridae*, is pathogenic to the fall armyworm (*Spodoptera frugiperda*; Lepidoptera: Noctuidae) and has a great potential to be used as a

biocontrol agent. The insect is a severe pest in most countries, and it is native to the tropical regions of the Western Hemisphere, from the United States to Argentina (Farias et al., 2014).

In a screening with 22 baculovirus isolates from different maize producing regions in Brazil, an isolate

named as SfMNPV-19 showed the lowest LC_{50} and the highest mortality rate at lower concentrations, leading to intense disruption of the host integument (Barreto et al., 2005). In contrast to other reported SfMNPV isolates, however, an isolate named SfMNPV-6nd did not cause liquefaction to larvae, a very important feature for virus pesticides production. The virulence of both isolates was compared by Vieira et al. (2012), who reported that the LC_{50} of SfMNPV-6nd is not significantly different from that of isolate 19, although it took considerable longer periods to kill *S. frugiperda* larvae. In fact, in preliminary experiments conducted by our team, the isolate 19 also showed better results of infection than the isolate 6nd, in the available cell lines.

Spodoptera frugiperda is a very important pest in South America, causing significant damage to several different crops. It is the most important Lepidoptera pest for maize in Brazil, with damages to yield reaching up to 52% (Figueiredo et al., 2006). The use of chemical pesticides and of genetically modified maize to control the pest have been raising environmental concerns and generating resistant biotypes (Farias et al., 2014).

Large-scale production of the SfMNPV baculovirus is still obtained in vivo by infecting healthy larvae. Therefore, the liquefaction of the larvae integument is a disadvantage to this process. Moreover, the large-scale production of baculovirus is also hindered by the cannibalistic behavior of the fall armyworm. This requires larval individualization, which is labor-intensive, increases the risk of contamination, and increases the costs of biopesticide production (Valicente et al., 2013). Therefore, in vitro production has a great potential to overcome these difficulties (Reid et al., 2014).

Cell cultures of insects are an interesting means to select systems that support viral replication (Castro et al., 2006; Almeida et al., 2010; Huynh et al., 2015). These cell systems are useful in studies of virus-host interactions, but the present technical limitations should be solved for their need in large-scale production of biopesticides, such as the accumulation of “few polyhedra mutants” or “defective interfering particles” in the cell culture (Moscardi et al., 2011; Reid et al., 2014). Other application of similar importance for cell culture is the synthesis of recombinant proteins by genetic engineering (Granados et al., 2007). In this sense, the Sf9 (*S. frugiperda*) and “High-Five” (*Trichoplusia ni*) cell lines have been studied

extensively as to their ability to grow in suspension cultures using serum-free media (Reid et al., 2014).

Selecting productive cell lines is an essential initial step for the establishment of SfMNPV in vitro production. Previous work with other SfMNPV isolate 18, which is less virulent than the isolate 19, was done exclusively with *S. frugiperda* cell lines in suspension culture (Almeida et al., 2010). However, it is well known that baculovirus species can infect cell lines originated from other Lepidoptera species. In the case of *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV), the polyhedra production in some cell lines obtained with different Lepidoptera was similar to that obtained with original host cell line, or even better (Castro et al., 2006). The response of insect cells to baculovirus infection involves apoptosis and protein synthesis shutdown (Du & Thiem, 1997). Several baculoviruses overcome cell defense mechanisms by expressing anti-apoptotic genes, as well as host-range factors that prevent global protein synthesis shutdown.

Because the production of baculovirus in cell culture is a system independent of the insect, features such as liquefaction are not pertinent. Most important in this context is the quality of the polyhedra formed as for the maintenance of their ultrastructure and virulence. In addition, it is important to select cell lineages that have desirable traits for in vitro culture. Studies on the subject have shown that baculoviruses establish unique interactions with different cell lines, resulting in various types of productive or nonproductive infections.

The objective of this study was to evaluate the response of six different lepidopteran cell lines to SfMNPV-19.

Materials and Methods

Six lepidopteran cell lines were assayed: *Trichoplusia ni* BTI-Tn-5B1-4 (Granados et al., 1994), also known as “High Five cells”; *Anticarsia gemmatalis* UFL-AG-286 (Sieburth & Maruniak, 1988); *Spodoptera frugiperda* IPLB-SF-21AE (Vaughn et al., 1977) and Sf9 (Granados et al., 2007); *Lymantria dispar* IPLB-LD-652Y (Goodwin et al., 1978); and *Bombyx mori* BM-5 (Grace, 1967). The cells were maintained at 27°C in 10% heat-inactivated bovine fetal serum supplemented medium. The culture medium used was TNMFH (Grace’s insect medium supplemented with

lactalbumin hydrolysate and yeastolate), except for the UFL-AG-286 cells, which were cultivated in TC-100 (Gibco-BRL, Grand Island, NY, USA).

The cells were infected with the virus isolate SfMNPV-19 (Barreto et al., 2005). Tissue culture plates (60 mm²) were seeded with 1x10⁶ cells and allowed to attach overnight (12–14 hours). The cultures were inoculated with SfMNPV-19 at a multiplicity of infection (MOI) of 10. Mock-infected cells did not contain any virus. After viral adsorption for 1 hour, cells were washed and incubated in complete medium at 27°C.

In vitro infectivity was evaluated by phase contrast, electron microscopy analysis, and by the kinetics of protein synthesis. At postinfection (p.i) periods of 0, 24, 48, 72, and 96 hours, cells were observed for cytopathic effects by phase-contrast microscopy. Cells were collected at 96-hour p.i for electron microscopy analysis. Infected and mock-infected cells were centrifuged at 3.000 g for 3 min, washed with PBS, pH 6.2, and centrifuged again. Sedimented cells were resuspended in a buffer solution of 0.1mol L⁻¹ sodium cacodylate and 2.5% glutaraldehyde, and then transferred to microcentrifuge tubes for incubation at 4°C. Fixed samples were treated with 0.05 mol L⁻¹ sodium cacodylate, and then fixed with 2% osmium tetroxide. After a new wash using sodium cacodylate buffer, samples were dehydrated in ethanol at 30, 70, 90, and 100%, and subjected to a mixture of ethanol:Spurr resin at the rates of 3:1, 2:1, 1:1, ending with 5 mL of pure Spurr. The blocks were mounted in Spurr and kept at 37°C, for 72 hours. The specimens were then cut in the ultramicrotome with a diamond knife. Meshes containing the samples were stained with 2% uranyl acetate. Samples were photographed in a transmission electron microscope Jeol 1011 (Jeol, Peabody, MA, USA) (Sample et al., 2007).

The [³⁵S] methionine protein labeling of the infected cells was done based on the protocol described by Sample et al. (2007). Cells were seeded at a the density of 1x10⁶ per 60 mm² dish and incubated overnight at 27°C to allow their attachment. Subsequently, the cells were inoculated with the virus (SfMNPV-19) at an MOI of 10. The mock-infected control cells received the same treatment, except for the inoculum without virus. After viral adsorption for 1 hour, cells were washed and incubated in complete culture medium at 27°C. The medium was replaced by phosphate

buffered saline (PBS, pH 6.2) at postinfection periods of 24, 48, and 72 hours, and the incubation continued for 30 min (starvation period). The medium was removed, and 50 µCi of [³⁵S] methionine in 0.6 mL of PBS was added for 1-hour pulse. Mock-infected cells were labeled at the time 0, after 1-hour of virus adsorption. After labeling, the cells were transferred to a microfuge tube, pelleted by centrifugation for 60 s, rinsed with PBS, and frozen at -20°C. The samples were disrupted and subjected to SDS–polyacrylamide gel electrophoresis (SDS–Page). To visualize the bands, the gel was treated with a fluorographic reagent Amplify (Amersham Biosciences, Little Chalfont, United Kingdom) for 30 min, dried, and exposed to a Kodak X-Omat AR film (Kodak, Rochester, NY, USA) for 5 days, at -70°C. The autoradiogram was analyzed for the presence of labeled proteins.

In order to verify the presence of apoptosis in some cell lines, a test was conducted to observe DNA fragmentation. The apoptosis tests were performed in cells without evidence of polyhedra presence. The cell lines Tn-5B1-4, AG-286, BM-5, and LD-625Y were seeded at 1x10⁶ density per 60 mm² dish, and incubated overnight at 27°C to allow their attachment. Subsequently, the cells were inoculated with the virus SfMNPV-19 at an MOI of 10. The mock-infected control cells received the same treatment, except for the inoculum without virus. At 96-hour p.i., the cells were suspended in 0.1 mL PBS, and total DNA was isolated with the DNeasy blood & tissue kit (Qiagen, Hilden, Germany). The DNA was electrophoretically separated in 1.0% agarose gel stained with ethidium bromide.

Results and Discussion

Morphological analysis by phase-contrast microscopy (Figure 1) showed that infected IPLB-SF-21AE and Sf9 cells lead to successful viral replication with many polyhedra formation. *Bombyx mori* cells (BM-5) seemed to change their morphology from round-refractive to “groundnut-shape”, although without polyhedra production. *Anticarsia gemmatalis* (UFL-AG-286) cells showed slight alterations, such as a small increase in size in comparison to the control. There were no morphological changes in *Trichoplusia ni* (BTI-Tn-5B1-4) and *Lymantria dispar* (IPLB-LD-625Y) cells. Although the IPLB-SF-21AE showed

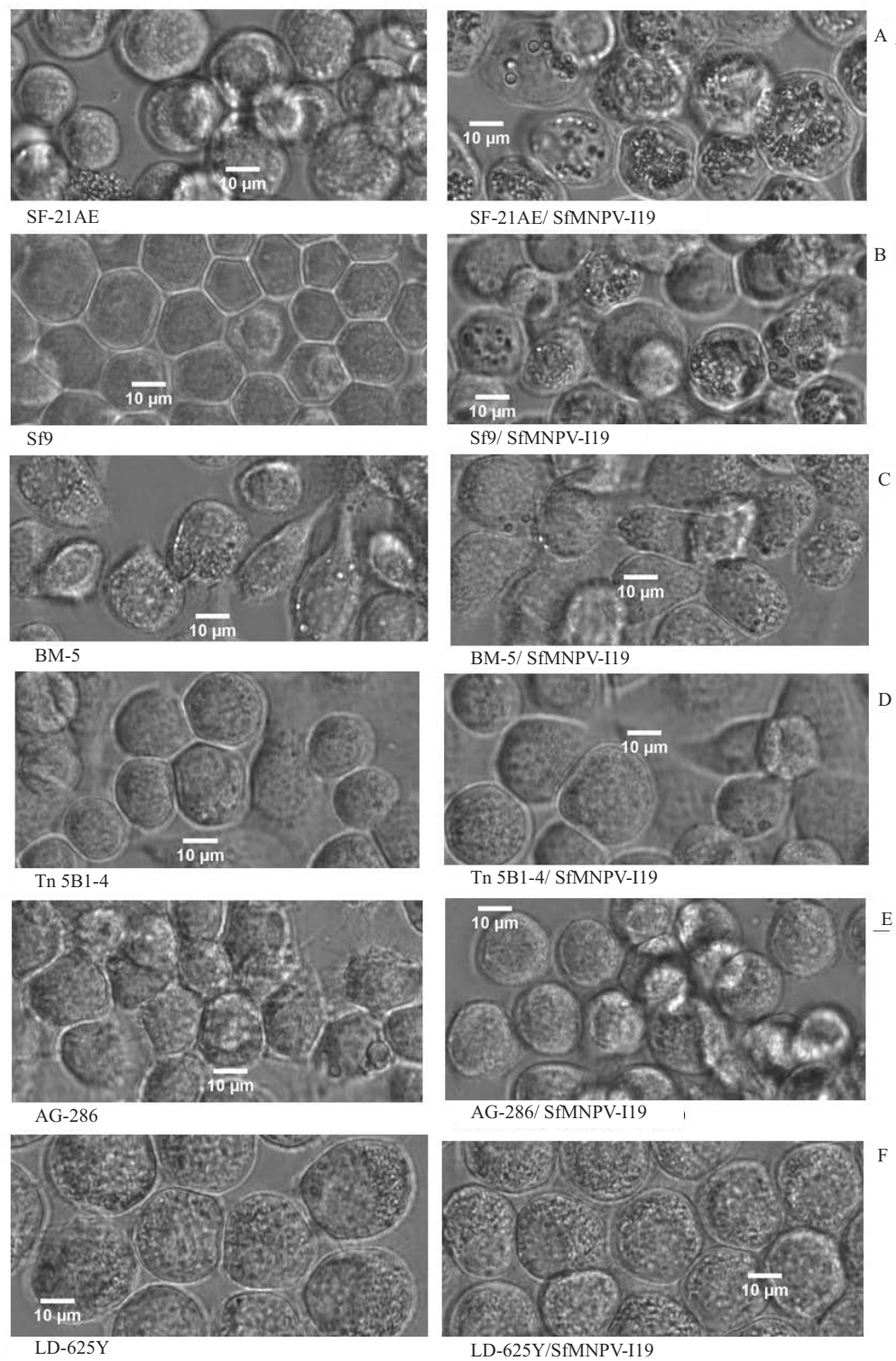


Figure 1. Phase-contrast micrographs of mock-infected cells (left), and cells infected with SfMNPV-19 96-hour p.i. (right) (40X) *Spodoptera frugiperda* IPLB-SF-21AE (A) and Sf9 (B); *Bombyx mori* BM-5 (C), *Trichoplusia ni* BTI-Tn 5B1-4 (D); *Anticarsia gemmatalis* UFL-AG-286 (E); and *Lymantria dispar* IPLB-LD-625Y (F).

more polyhedra than Sf9, at 72-hour p.i., the presence of polyhedra became similar in both cells at 96-hour p.i.

Ultrastructural analysis of the two infected *S. frugiperda* cell lines showed, as expected, cell nuclei hypertrophy and production of many polyhedra at 96-hour p.i. (Figures 2 A and B). Other typical baculovirus induced effects, such as the formation of virogenic stroma, nucleocapsids, and virions were also observed during the first days postinfection. None of the other cell lines showed virus particles inside the cell nuclei after viral incubation (Figures 2 C, D, E, and F).

However, *Lymantria dispar* and *Trichoplusia ni* cells showed several vesicles and vacuoles in the cytoplasm.

Both IPLB-SF-21AE and Sf9 cells, infected with SfMNPV and labeled with [³⁵S] methionine, showed that polypeptide synthesis patterns in the beginning of the infection differ from that observed in the control cells (Figures 3 A and B). In fact, some viral proteins that initially seemed to comigrate with the host proteins were progressively more evident in the cycle of infection, along with the disappearance of host cell protein synthesis. At late times, post-infection (from 48 to 72-hour p.i.), an intense band of about 30 kDa, was

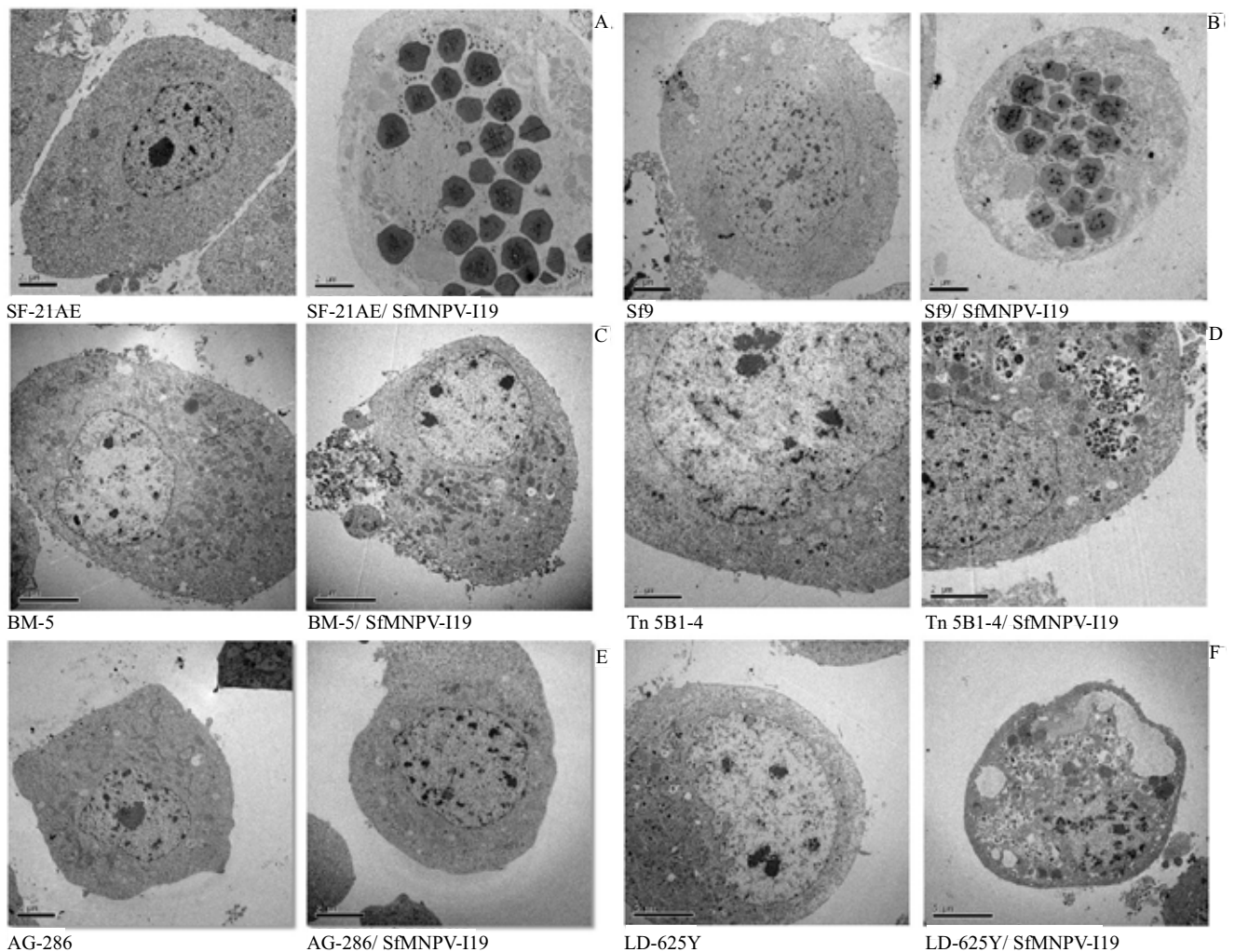


Figure 2. Electron micrographs of mock-infected cells (left) and cells infected with SfMNPV-19 at 96-hour p.i. (right). *Spodoptera frugiperda* IPLB-SF-21AE (A) and Sf9 (B); *Bombyx mori* BM-5 (C), *Trichoplusia ni* BTI-Tn 5B1-4 (D); *Anticarsia gemmatalis* UFL-AG-286 (E); and *Lymantria dispar* IPLB-LD-625Y (F).

identified as polyhedrin, the main structural protein of NPV. The intensity of the radiolabel incorporation detected in this band is consistent with the high levels of polyhedra production observed in the nuclei of these cells (Figures 1 A and B, and Figures 2 A and B). Polyhedrin synthesis in IPLB-SF-21AE and Sf9 reached its maximum at 72-hour p.i.

The infected BTI-Tn-5B1-4, AG-286, BM-5, and IPLB-LD-625Y cells did not produce polyhedrin. However, they showed low levels of cellular protein synthesis after 48-hour p.i., and a complete shutdown of cellular peptide synthesis at 72-hour p.i. This may have occurred due to interactions between viral proteins and cellular translation machinery, as observed in many virus-host interactions (Walsh & Mohr, 2011).

The infected cell lines showed the same DNA pattern, in comparison to the control without fragmentation.

According to our data, only *S. frugiperda* cells were susceptible for the in vitro production of SfMNPV. These cells were previously described as permissive to SfMNPV infection (Simón et al., 2008; Almeida et al., 2010; Beperet et al., 2014). Similar observations occurred with another virus from *Spodoptera* (Yanase et al., 1998). These authors inoculated *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) into eight lepidopteran cell lines, and reported that polyhedra were only produced in the cell line derived from its host (Se031 cells). Morphological changes were also induced by SeMNPV in the “High Five” cells, although no productive infection had occurred

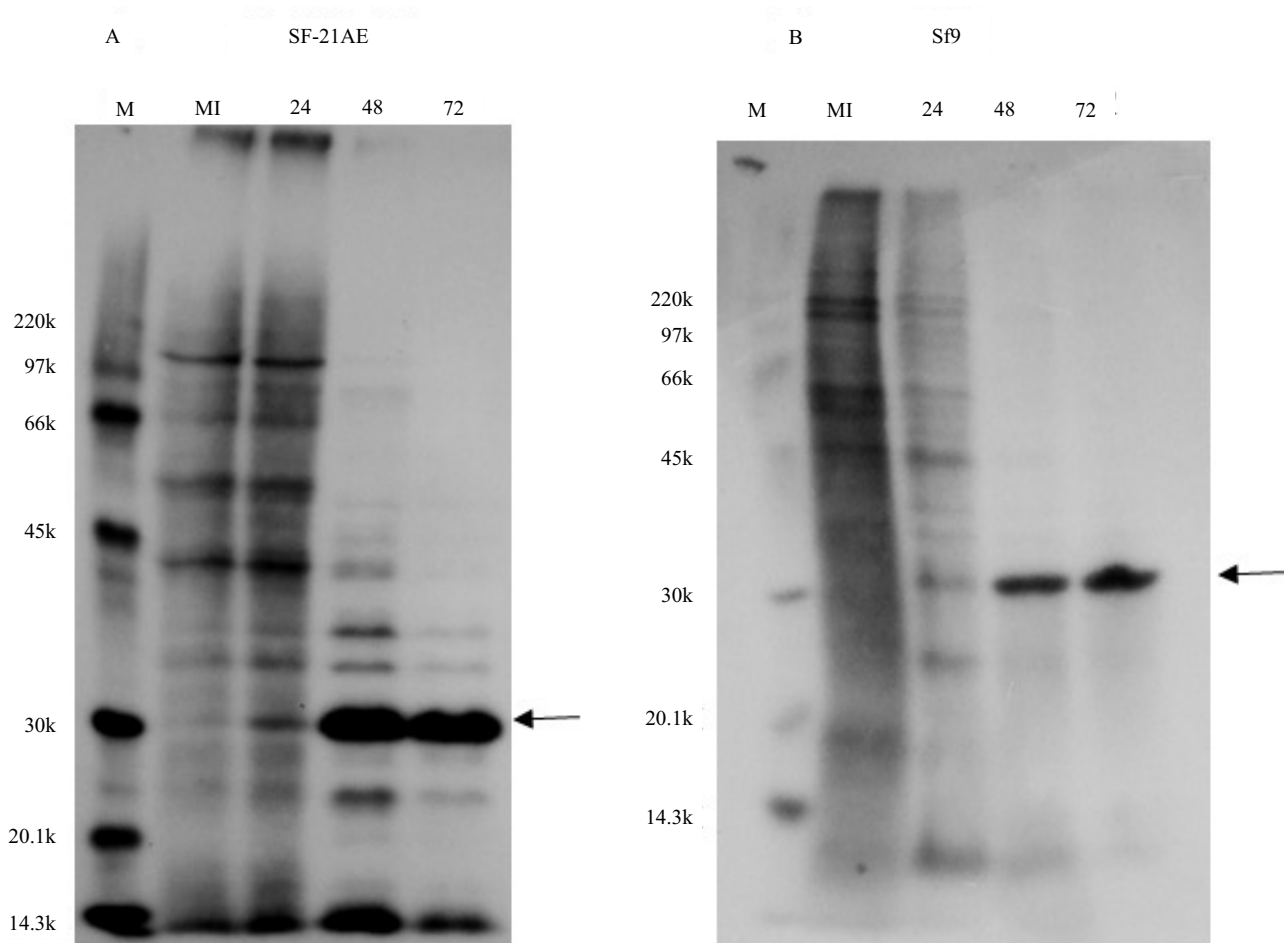


Figure 3. Protein synthesis in SfMNPV-infected insect cells at 24, 48, and 72 hours postinfection. Autoradiogram of a 15% SDS-PAGE from insect cells pulse labeled with [35 S] methionine. Insect cell lines: *Spodoptera frugiperda* IPLB-SF-21AE (A) and Sf9 (B). The arrows indicate the polyhedrin. MI, mock-infected cells; M, molecular mass markers given in kDa.

(Yanase et al., 1998). Nevertheless, Jain & Das (2004) reported that *Spodoptera litura* nucleopolyhedrovirus (SpltNPV), pathogenic to larvae of *Spodoptera litura*, infects the Sf9 cell line, which is derived from another host. Moreover, Ishikawa et al. (2003) showed that LD-625Y cells underwent apoptosis after infection by SeMNPV and SpltNPV.

Inhibition of protein synthesis has been shown to be a common event in cells infected with viruses (Nguyen et al., 2013). The protein shutoff was observed in the interaction between *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and LD-625Y cells (Du & Thiem, 1997), and both apoptosis and the shutdown were suggested as being separate responses of the insect cells to AcMNPV infection. In the present study, we did not observe the occurrence of apoptosis in “High Five”, BM-5, UFL-AG-286, and LD-625Y cells infected with SfMNPV, but there was a protein synthesis shutdown, suggesting a response to the virus infection.

Host cells apoptosis induced by virus infection plays an important role in baculovirus host range (Wu et al., 2016). The mechanism that triggers apoptosis in baculovirus-infected cells relies on an intricate relationship between cellular and viral functions (Ishikawa et al., 2003). For instance, the AcMNPV, a baculovirus with a wide host range, capable to infect more than 25 cell lines, induces apoptosis in *S. litura* and *S. littoralis* cells (Chejanovsky & Gershburg, 1995; Zhang et al., 2002), but can infect the SF-21 and Sf9 cell lines. The product of anti-apoptotic viral gene *p35* was observed as essential for the replication of AcMNPV in the SF-21 cells (Clem et al., 1991). Recently, the anti-apoptotic gene *iap3* of SeMNPV was observed as responsible for the expanded host range for a recombinant virus, including nonpermissive cells to the parental viruses (without *iap3*). This virus was constructed with AcMNPV lacking the *p35* gene and a fragment of SeMNPV (Wu et al., 2016).

Accordingly, severe apoptotic response has been shown in a study conducted with LD-625Y infected by *Hyphantria cunea* nucleopolyhedrovirus (HycuNPV) with high expression of *iap3* (Ishikawa et al., 2003). These authors suggested that the apoptosis induction in NPV-infected LD-625Y cells is largely dependent on inherent cellular properties, rather than on functions of the NPVs, and indicate that defects in progeny virion production are not merely due to the virus-induced

apoptosis. The data of the present study suggest a defense response of the cells to the baculovirus infection, as well as an anti-apoptotic function mediated by the virus. The genome of SfMNPV-19 is completely sequenced, and the presence of *iap3* was pointed out (Wolff et al., 2008). However, further analysis on gene function is necessary to confirm this hypothesis.

A broader host range could be important for selecting more productive cell lines to be used in large-scale production. The baculovirus AgMNPV has been largely used as a biopesticide in Brazil for controlling *A. gemmatalis* in soybean (Moscardi et al., 2011). In comparison to SfMNPV-19, AgMNPV-2D has a broad capacity of in vitro infection, including cells from other hosts (*S. frugiperda* and *T. ni*). In addition, polyhedra were produced faster at 48-hour p.i. (Castro et al., 2006).

Our study indicates that IPLB-SF-21 cells in static culture are more productive than Sf9 in the beginning of infection with SfMNPV-19. However, at 96-hour p.i., the production of occlusion bodies (OB) became similar in both cell lines. Nevertheless, in a suspension culture infected with another Brazilian isolate (SfMNPV -18), the Sf9 cells seemed to be more productive than SF-21 cells (Almeida et al., 2010). These authors showed that the Sf9 cell-line resulted in better viral production (5.0×10^8 OB mL⁻¹) than the SF-21 cell-line (2.5×10^8 OB mL⁻¹).

The distinct performance between the viral isolates 19 and 18 could be due to the different conditions of the studies. In static cultures, cell growth is limited to the size of the available surface. In the suspension culture, however, the cells develop freely, and growth is limited by other factors (Wu et al., 1998). Moreover, it was not possible to compare the MOI in neither of the studies, since a fixed volume of the virus inoculum (BVs) was used in the suspension culture after the initial release of the caterpillar-produced occlusion bodies (Almeida et al., 2010). Another culture medium was used in the study with the isolate 18 (HyQ SFX-Insect supplemented with 5% bovine fetal serum), as well as a different initial cell density (5×10^5 cell mL⁻¹). Several factors have been shown to affect the volumetric and specific polyhedra production in the Sf9 suspension culture infected with AgMNPV. The initial cell seeding, MOI, and glucose consumption in

infected cells are among these factors (Rodas et al., 2005).

The SfMNPV-6nd has a clear advantage to large-scale biopesticide production *in vivo* because it does not disrupt the larvae integument. However, this characteristic may be unfavorable to control the pest under crop conditions, since it is advantageous for the virus to escape efficiently from the dead larva. Virus polyhedra that remain trapped within the host are less likely to encounter another susceptible individual than virus released via liquefaction (Hawtin et al., 1997). Moreover, the 6nd isolate showed a longer lethal time compared to SfMNPV-19 in bioassays (Vieira et al., 2012).

One of the requirements for a cell line to be considered for *in vitro* production of a baculovirus is its capacity to produce a useful virus, yielding at least 300 OB per cell (Reid et al., 2014). The potential of SF-21 and Sf9 for the production of SfMNPV-19 are similar, according to preliminary studies (5.0×10^8 OB mL⁻¹ and 300 OB per cell). Therefore, the utilization of SfMNPV-19 as a biopesticide may be more interesting when a large-scale *in vitro* production strategy of SfMNPV becomes available.

Conclusions

1. The *Spodoptera frugiperda* SF-21 and Sf9 cell lines are recommended for *in vitro* production of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV), both with similar polyhedra production in static culture.

2. The cell lines BTI-Tn-5B1-4, AG-286, BM-5, and IPLB-LD-625Y are nonpermissive to SfMNPV.

3. The response to SfMNPV varies according to the cell line, and consists in protein synthesis shutoff and distinct morphological changes in the cells.

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