Discover Viruses

RESEARCH Open Access

Selection of *Anticarsia gemmatalis* nucleopolyhedrovirus variants for the in vitro production of a biopesticide

Andrea Farias de Almeida[1], Márcia Regina da Silva Pedrini[2], Anderson Fernandes de Brito[3], Bergmann Morais Ribeiro[4], Márcio Martinello Sanches[5], William Sihler[6] and Marlinda Lobo de Souza[6*]

*Correspondence:
Marlinda Lobo de Souza
marlinda.souza@embrapa.br

Full list of author information is available at the end of the article

Abstract

The Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV) has been used as a biopesticide to control the "soybean caterpillar" in Brazil. Production of baculoviruses has been performed in vivo through the infection of larvae under laboratory conditions. The in vitro production has been limited by the occurrence of mutations and deletions in viral isolates following serial passages in cell culture. This study aimed to select productive and genetically stable AgMNPV isolates suitable for in vitro production. Initially six variants with high polyhedra production in cell culture (Many Polyhedra - MP) were selected through plaque assay purification. The molecular characterization of isolates was performed by DNA restriction analysis and protein synthesis analysis. The two most productive isolates after serial passages were further analyzed in cell suspension cultures, using IPLB-SF21 cells adapted to Sf900II medium. Occlusion body (OB) production was monitored daily to evaluate potential for in vitro biopesticide manufacturing. The MP variants did not present molecular alterations compared to the type-isolate AgMNPV-2D. Among the variants, the AgMNPV-MP5 isolate showed the highest productivity yielding 5.3×10^8 OBs/mL and approximately 300 OBs/cell after eight days of infection. These results indicate AgMNPV-MP5 as a possible candidate for biopesticide production.

Keywords AgMNPV, Baculovirus, Biological control, Cell culture, Many polyhedra variants

1 Introduction

Baculoviruses are invertebrate viruses from the family Baculoviridae, which were previously divided into two genera [1]: Nucleopolyhedrovirus (NPV) and Granulovirus (GV). The current classification subdivides the family into four genera: *Alphabaculovirus* (lepidopteran-specific NPVs), *Betabaculovirus* (lepidopteran-specific GVs), *Gammabaculovirus* (hymenopteran-specific NPVs) and *Deltabaculovirus* (dipteran-specific NPVs) [2]. They contain circular double-stranded DNA and are characterized by enveloped rod-shaped nucleocapsids found inside a protein matrix, forming the occlusion bodies (OB).

This structure has a diameter between 0.5 and 15 μm that allows the viability of infective particles outside their natural host. The other viral phenotype is the budded virus (BV) responsible for cell to cell infection inside the host [3].

The use of the baculovirus Anticarsia gemmatalis nucleopolyhedrovirus (AgMNPV), (*Alphabaculovirus angemmatalis*) in Brazil represented the largest viral biopesticide pest control program in the country and one of the largest in the world [4]. In 2003, more than two million of hectares of soybean crops were treated with AgMNPV to control the soybean caterpillar *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Erebidae). However, in subsequent years, the area treated exclusively with AgMNPV declined sharply to approximately 300,000 hectares. This reduction was primarily due to the emergence of other pest species affecting soybean crops, which led to an increased demand for broad-spectrum chemical pesticides to manage multiple pests simultaneously [5].

Initially, in the eighties, the production of AgMNPV was carried out using natural populations of *A. gemmatalis* in the field. The infected caterpillars were then taken to the laboratory for virus purification and formulation [4]. This type of production required intense manual labor and depended on the natural occurrence of the caterpillar in soybean plantations for a long period of time. Currently the commercial production of this virus is done by multiplying of the viruses in larvae (in vivo), requiring a laborious process, using insects raised with artificial diet and demanding a specific infrastructure.

An alternative to increase the availability of an insect virus-based biopesticide is its large-scale production in cell cultures (in vitro). The AgMNPV production in insect cell culture has been studied in different laboratories [6, 7, 8, 9]. The in vitro production of baculoviruses offers a number of advantages: specific cell lines can be selected, preserved and stored in cryogenic media for future use in viral multiplication [3], and baculoviruses isolates (genetic, seasonal or geographic) with better infectivity in specific cell lines can also be selected. However, the development of in vitro baculovirus production has been limited because of the accumulation of genomic alterations caused by the serial passage of the virus in cell cultures [10]. The generation of FP (Few Polyhedra) mutants and the formation of defective interfering (DI) viruses are the main genetic alterations resulting from the passage effect. Both become predominant compared to the parental virus and drastically reduce polyhedra production [11, 12, 13].

Besides the FP phenotype, many polyhedra (MP) variants have also been isolated during virus serial passage in cell culture. These MP variants have been considered an interesting alternative for the development of in vitro production of baculoviruses. MP variants are morphologically similar to wild type virus, capable of forming multiple occlusion bodies in the cell nucleus. The selection of stable virus with a MP phenotype, isolated during serial passage in cell culture have been described for the baculoviruses Lymantria dispar multiple nucleopolyhedrovirus - LdMNPV [14, 15] and Helicoverpa armigera single nucleopolyhedrovirus - HaSNPV [16].

The selection of stable genetic isolates that display the best in vitro production of viral biopesticides is important for broadening the scale of baculovirus production. Several viral clones isolated by plaque assay were selected from a natural isolate of AgMNPV collected from infected caterpillars in Londrina, Brazil in 1979 (AgMNPV-79) [17]. Genomic variants of this virus showed varied virulence in cell culture and also variations in DNA restriction profile [18]. The AgMNPV-2D clone [19] has been considered the

prototype of AgMNPV and is widely used in the molecular biology studies of this virus. The complete genome sequence of the AgMNPV-2D virus has been determined [20].

In this work, we used six MP variants from AgMNPV-2D. The variants were assessed for OB production and stability after seven consecutives passages in cell culture. The two most productive variants (AgMNPV-MP2 and AgMNPV-MP5) were compared to AgMNPV-2D (wild type) in suspension cell culture aiming future scale-up for bioreactor production.

2 Materials and methods

2.1 Cell static culture

Trichoplusia ni Hübner, 1802 (Lepidoptera: Noctuidae) cells, BTI-Tn-5B1-4 [21], known as "High five" cells and *Spodoptera frugiperda* Smith & Abbot, 1797 (Lepidoptera: Noctuidae) IPLB-SF21, American Type Culture Collection, MD) [22] were grown as monolayers in TMNFH medium supplemented with 10% fetal bovine serum, at 27°C. The cells were seeded at a density of 1×10^6 per 60 mm^2 well.

2.2 Cell suspension culture

Insect cells (IPLB-SF21, American Type Culture Collection, MD) [22] were adapted to culture medium SF900II SFM (Gibco, Invitrogen®) supplemented with 1% (v/v) of antibiotic/antimicotic (Invitrogen®) and used in all the viral replication experiments. These cells were kept in 125 mL Erlenmeyer flasks (Schott®, Germany) with 50 mL working volume using an orbital shaker (Tecnal® – TE340, Brazil) with agitation of 120 rpm and controlled temperature of 28 °C. Cell subcultivations were carried out every 3 days to maintain the cells in the exponential cell growth phase.

2.3 Virus

The AgMNPV-2D viral isolate was obtained from Dr. James Maruniak (University of Florida, USA). This virus is stored in the Embrapa/Cenargen Invertebrate Virus Collection under the number BRM 004998.

Fourth instar *A. gemmatalis* larvae were *per os* infected with AgMNPV-2D by placing them on a diet containing surface-applied polyhedra (10^6 OB per diet surface). The hemolymph was collected on the 4th day post-infection (d.p.i.), by larvae bleed and cysteine was added at 10 mM. Hemolymph was then passed through a syringe filter (0.45 µm) for sterilization. This solution was used as inoculum (passage 0) for three individual infections on Tn5B1-4 cells plated at a density of 2×10^6 cells/flask in T25 cell culture flasks. At the end of the incubation period (1 h, 27 °C), the inoculums were removed. The cells were then rinsed with serum free TNMFH medium and maintained in complete medium at 27 °C. This infection constituted the first passage (P1) of virus in cell culture. The viral variants were purified by plaque assay. Serial dilutions of virus containing solutions were used to infect insect cells for 1 h and after that, the virus solution was removed and an agarose overlay was added to the infected cells in order to restrict viral spread, preventing indiscriminate infection through the liquid growth medium. After 5–7 d.p.i., live cells were stained with a dye (neutral red) and dead infected cells appeared as non-stained group of infected cells known as a plaque. Cytopathic effects were observed under phase contrast microscopy at 400X. The AgMNPV MP variants obtained by plaque assay were selected based on the presence of various polyhedra in

cell nucleus and high BV titers. They were named AgMNPV MP1, MP2, MP3, MP4, MP5 and MP6, respectively.

2.4 Restriction endonuclease analysis of viral DNA

Budded viruses from passage P1 in BTI-Tn-5B1-4 cells were purified in a 25% sucrose cushion by ultracentrifugation in a Sorvall SW28 rotor at 24,000 rpm, for 75 min at 4 °C [23]. For DNA extraction the pellet was suspended in 500 µL of disruption buffer (10 mM Tris, pH 7.6; 10 mM EDTA, pH 8.0; 0.25% SDS), containing 500 $\mu g.mL^{-1}$ of Proteinase K, and the sample was incubated overnight at 37 °C. DNA was extracted with phenol: chloroform and then digested with *Hin*dIII and *Pst*I enzymes, according to the manufacture (Promega®) at 37 °C for 3 h. The digested DNA samples were loaded into a 0.8% agarose gel, containing ethidium bromide (0.3 µg/mL) and electrophoresed at 40 V.

2.5 Protein synthesis analysis of infected cells

BTI-Tn-5B1-4 cells were seeded at a density of 1×10^6 per 60 mm^2 dish and incubated overnight at 27 °C. Subsequently, the cells were inoculated with the AgMNPV-2D single infection and MP variants (multiplicity of infection - MOI = 10); The mock-infected control cells received the same treatment, except that the inoculum did not contain any virus. After viral adsorption for 1 h, cells were washed and incubated in complete culture medium at 27 °C for 5 days. The samples were disrupted and submitted to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Invitrogen®). To visualize bands, the gel was treated with staining solution of coomassie brilliant blue R 250 (Sigma-Aldrich®), methanol/etanol and acetic acid according to [24].

2.6 Serial passage of BV

The two most productive AgMNPV MP variants and AgMNPV-2D were selected to assess the stability of virus through seven serial passages in BTI-Tn-5B1-4 seeded at a density of 2×10^6 cells in T25 cell culture flasks. Aliquots of supernatant from each flask were collected at 72 h post-infection (h.p.i.). The total number of polyhedra per cell was determined based on the number of cells possessing polyhedra and number of occlusion bodies per mL (OB/mL). The last one (polyhedra density) was calculated after lysis of infected cells with 1% sodium dodecyl sulfate at room temperature for 1 h. Infected cells at 72 h.p.i. were pelleted by centrifugation at 3000 rpm in a microcentrifuge for 5 min. The supernatant containing budded virus (1 mL) was used as a viral inoculum (P1) for a new passage, at a density of 2×10^6 cells in T25 cell culture flask. In the same way, subsequent passages always used 1 mL of the previous supernatant. Triplicate flasks of 2×10^6 infected cells were done for every passage. Typical cells of each serial passage were observed using a phase contrast microscope (Olympus® CX40, Japan).

2.7 Virus multiplication in cell suspension culture

The two most productive AgMNPV MP variants and the AgMNPV-2D isolate cultivation was performed through infection of SF21 cells. Healthy SF21 cells (95% viability) were infected with BVs of AgMNPV isolates. These infections were performed with time of infection (TOI) of 5.0×10^5 viable cells/mL. The viral inoculum used in all the experiments had a MOI of 2.0 for all the isolates. Samples were removed daily for

counting infected and non-infected cells, as well as for counting the occlusion bodies (OBs) formed during the infection process.

2.8 Cell concentration and viability in suspension culture

Cell concentration was estimated using a phase contrast microscope (Olympus® CX40, Japan) and a Neubauer hemocytometer (Bright-Line Hemocytometer, Sigma®). Counting in triplicate was performed in each sample, with 200 cells counted on both sides of the hemocytometer [25]. The concentration of viable cells and viability was determined by cell counting, using the exclusion technique and trypan blue staining (Sigma-Aldrich®) at a final concentration of 0.1% (v/v) [26].

2.9 Occlusion body (OB) concentration

To release and count the OBs produced by the infected cells, a volume of 1% (p/v) of sodium dodecyl sulfate (SDS) was added to 0.5 mL of infected cell suspension. The mixture was incubated at 28 °C for at least one hour to dissolve the cell membranes and release the OBs. The solution containing OBs was diluted appropriately with water and counted in a hemocytometer with the aid of a light microscope (Olympus CX40, Japan) (800 × magnification). Each sample was counted in triplicate, and volumetric (OBs/mL) and specific (OB/cell) OB production was estimated.

2.10 Viral titer ($TCLD_{50}$) and multiplicity of infection (MOI)

For viral titer ($TCLD_{50}$ – 50% of tissue concentration lethal dose) determination, the cell viability MTT assay was performed [27]. Because a baculovirus is a lytic virus, that is, causes cell lysis during the infection process, it leads to decreased cell growth that can be detected by using MTT, a reagent that quantifies the viable cells present in the cultivation [28]. The multiplicity of infection (MOI), therefore, was calculated by the ratio between viral titer and the number of infected cells.

2.11 Transmission electron microscopy (TEM)

Infected cells at 72 h.p.i. were suspended and centrifuged at 3,000 × *g*/3 min. The supernatant was discarded and the cell pellet was washed with PBS buffer, pH 6.2. Initially, the samples were fixed in solution containing 0.1 M sodium cacodylate and 2.5% glutaraldehyde and kept at 4 °C. They were then treated with 0.05 M sodium cacodylate, followed by addition of 1% osmium tetroxide. After a new washing in sodium cacodylate, the samples were dehydrated in ethanol and embedded in Spurr`s resin. The samples were sectioned in an ultramicrotome and assembled in copper coated grids. Finally, the grids were contrasted with 3% uranyl acetate for 20 min and analyzed in a Transmission Electron Microscope Jeol® 1011 at 80 kV.

2.12 Scanning electron microscopy (SEM)

The size of AgMNPV-2D and MP variants were studied by scanning electron microscopy. Infected cells at 96 h.p.i. were suspended and centrifuged at 3,000 × *g*/3 min. The supernatant was discarded and the cell pellet was washed with PBS buffer, pH 6.2. Initially, the samples were fixed in solution containing 0.1 M sodium cacodylate and 2.5% glutaraldehyde and kept at 4 °C. They were then treated with 0.05 M sodium cacodylate, followed by addition of 1% osmium tetroxide. After a new washing in sodium cacodylate,

the samples were dehydrated in ethanol. Then the samples were dried by the critical point method using CO_2 in a Baltec® CPD 030 device, coated with 25 nm of gold in a Balzers MED 010 device and observed under a Zeiss® model DSM 962 scanning electron microscope. At least 20 polyhedra per sample were measured and the data were submitted to statistical test ANOVA and the means compared using Tukey-Kramer test ($p < 0.05$).

3 Results

3.1 Selection of AgMNPV MP variants

All AgMNPV MP variants selected presented high polyhedra production in the cell nuclei (Fig. 1), and the infection patterns were consistent through the serial passages. The AgMNPV MP variants did not present deletions or the presence of submolar bands in the DNA restriction profile compared to AgMNPV-2D (Fig. 2). The protein synthesis profiles of infected cells were similar in the MP variants and AgMNPV-2D and different from the profile of non-infected cells (mock-infected control). The presence of 30KDa band in the infected cells was recognized as the viral polyhedrin, which was absent in the mock-infected cells (Fig. 3). The AgMNPV MP2 and MP5 presented the highest OB production among the variants (6.8×10^7 and 6.2×10^7 OB/mL respectively), corresponding to approximately 200 OBs/cell and were selected for serial passages studies. They were assessed for seven consecutives passages. Although the number of OB had a decrease, especially after the fourth passage, the number of OBs/cell remained relative stable in the MP variants (around 80 OBs/cell) compared to the isolate-type AgMNPV-2D (30–40 OBs/cell). The presence of few polyhedra (FP) phenotype in the MP variants infected cells was not observed. On the other hand, a large amount of FP cells was observed after the AgMNPV-2D serial passages.

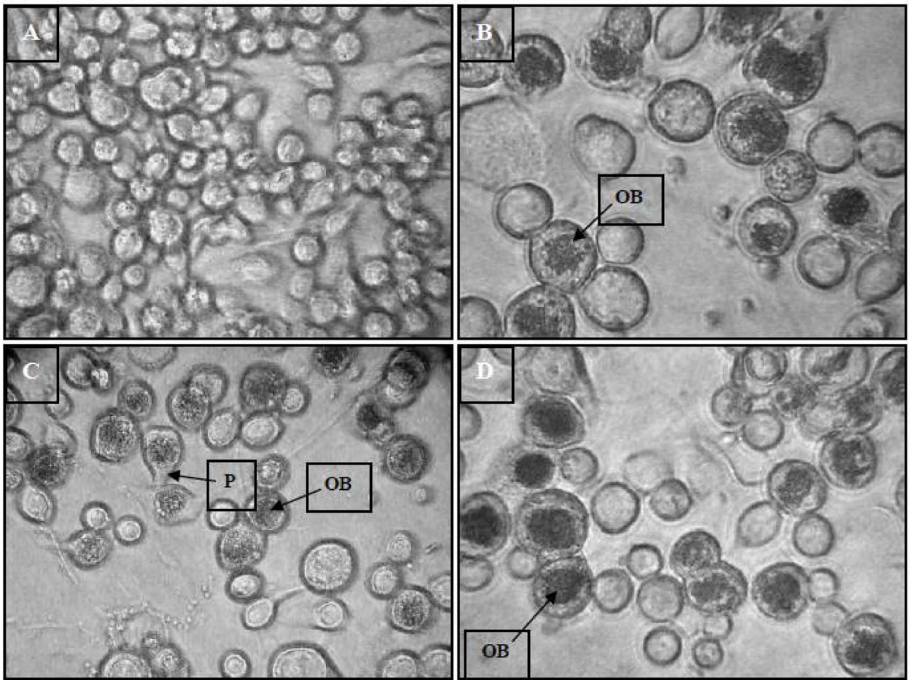

Fig. 1 AgMNPV variants in BTI-Tn-5B1-4 cells cultivated in static culture. **A** mock-infected control cells. **B** AgMNPV-2D (Passage1). **C** AgMNPV-MP2 (Passage2). **D** AgMNPV-MP5 (Passage2). The arrows indicate occlusion bodies accumulation in the cell nucleus (OB) and cell protrusion (P)

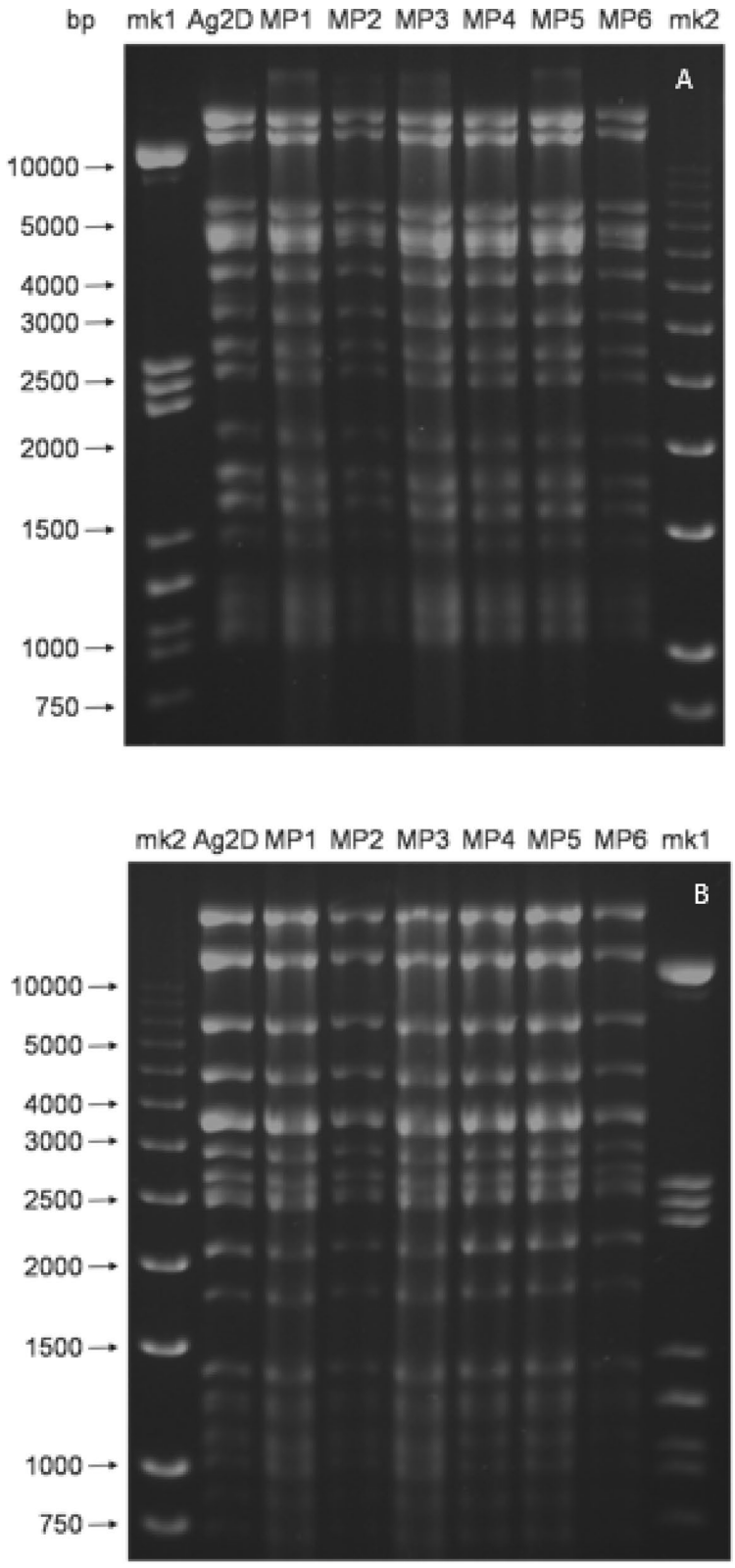

Fig. 2 Restriction endonuclease analysis of AgMNPV-2D and MP variants (MP1 to MP6). Viral genomic DNA was digested with *Hind*III (**A**) and *Pst*I (**B**). The fragments were separated by 0.8% agarose gel electrophoresis, and the gel was stained with ethidium bromide. Markers in base pairs: (mk1) DNA lambda/*Pst I* (mk2) 1 Kb DNA Ladder

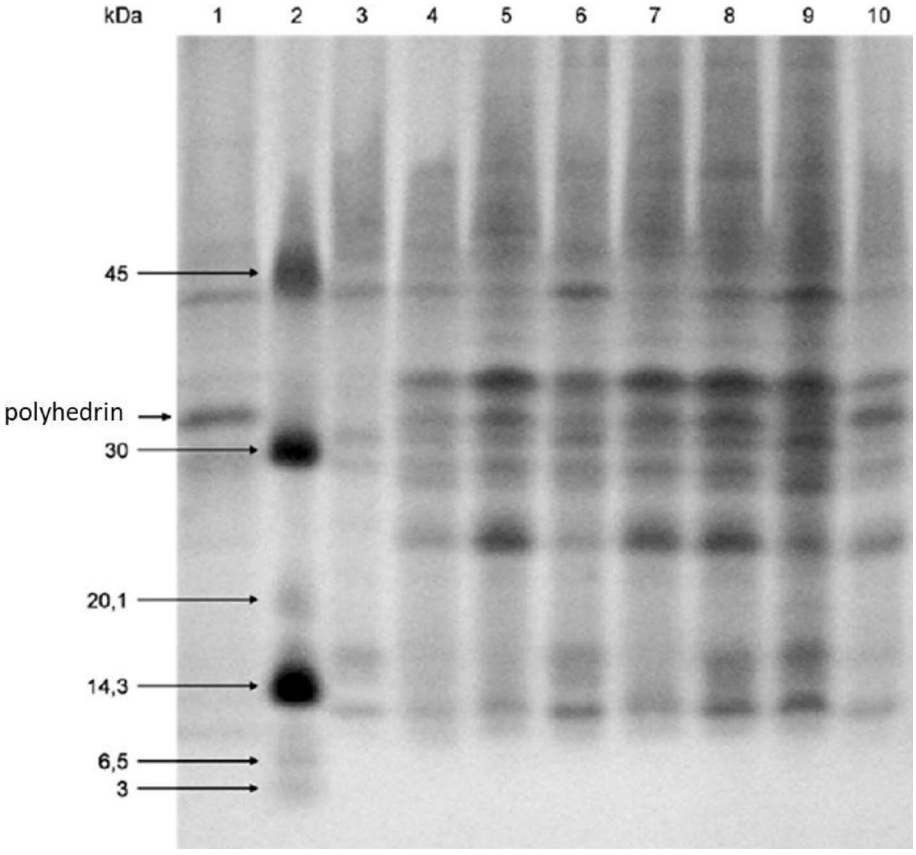

Fig. 3 BTI-Tn-5B1-4 cell protein profile in SDS-PAGE 15%. (1) positive control: AgMNPV-2D semi-purified OBs. (2) Low range rainbow molecular weight marker. (3) Mock-infected control cells. (4) AgMNPV-2D infected cells. (5–10) AgMNVPV variants infected cells: MP1, MP2, MP3, MP4, MP5, MP6

3.2 Cell viability during virus infection in suspension culture

The AgMNPV MP2 and MP5 variants were selected for cell suspension culture assay. The viability of SF21 cells non-infected and infected with the AgMNPV-2D, AgMNPV-MP2 and AgMNPV-MP5 isolates was analyzed. Only mock-infected cells showed increased cell viability along the five days of the experiment. All infected cells showed growth similar to the non-infected cells up to the second day of infection. SF21 cells infected with AgMNPV-2D, AgMNPV-MP2 and AgMNPV-MP5 showed maximum cell concentration of 1.4 ± 0.07, 2.0 ± 0.10 and $0.52 \pm 0.26 \times 10^6$ viable cells/mL, respectively (Fig. 4). On the 3rd day p.i. AgMNPV-2D and AgMNPV-MP2 infected cells stopped growing. On the other hand, AgMNPV-MP5 stopped growing on the 2nd d.p.i. indicating a faster replication of this isolate compared with the other viruses tested.

3.3 Occlusion body production in cell suspension culture

Mean OB production was $(1.3 \pm 0.21) \times 10^8$ OB/mL after 8 d.p.i. for the AgMNPV-2D isolate and $(1.9 \pm 0.30) \times 10^8$ OB/mL for the AgMNPV-MP2 isolate, showing similar OB production curves. The mean OB production for the AgMNPV-MP5 isolate was higher than the other to viruses, reaching $5.3 \pm 0.85 \times 10^8$ OB/mL (Fig. 5).

The specific OB production for each isolate is shown in Fig. 6. The AgMNPV-MP2 isolate presented the OB/cell ratio similar to the AgMNPV-2D, with an average 72.37 ± 11.58 OB/cell and 75.71 ± 12.11 OB/cell, respectively. However, the AgMNPV-MP5 isolate,

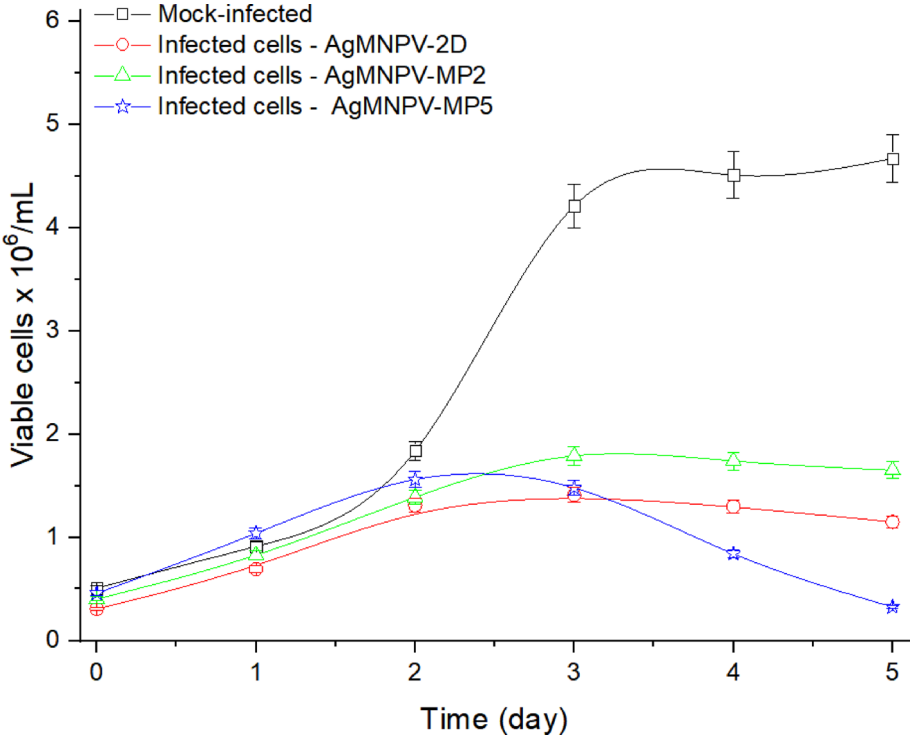

Fig. 4 Growth curves of non-infected and infected SF-21 cells in suspension culture with the different AgMNPV variants and AgMNPV-2D. Samples were collected for cell-counting expressed as viable cells × 10^6 /mL. Vertical bars indicate the standard error

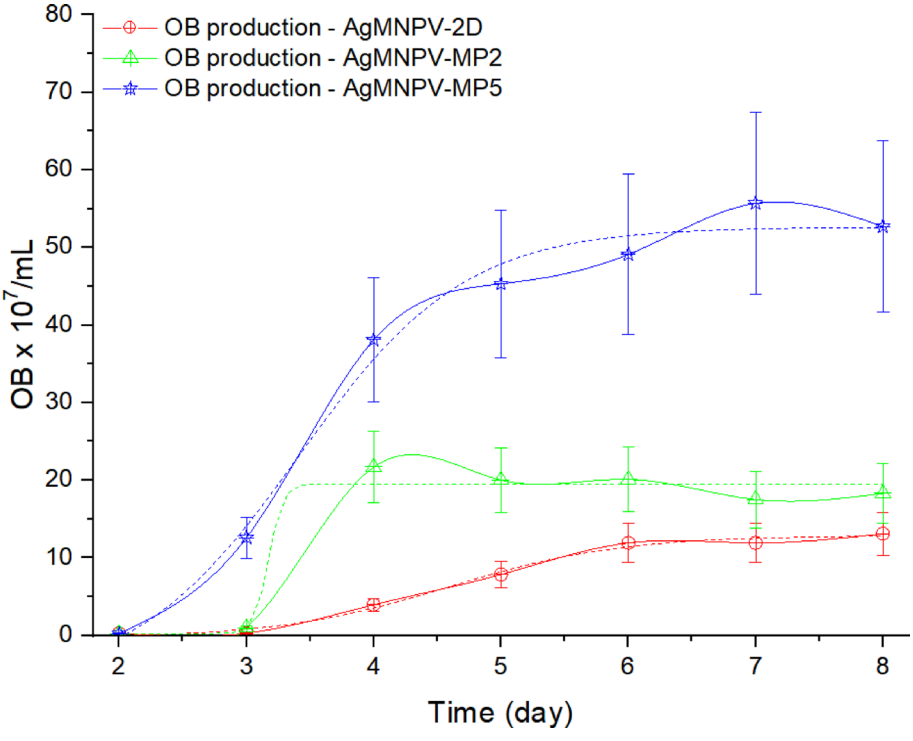

Fig. 5 Occlusion bodies (OB) production of different AgMNPV variants and AgMNPV-2D in SF-21 cells. Samples were collected for OB-counting expressed as OB × 10^7 /mL. Vertical bars indicate the standard error

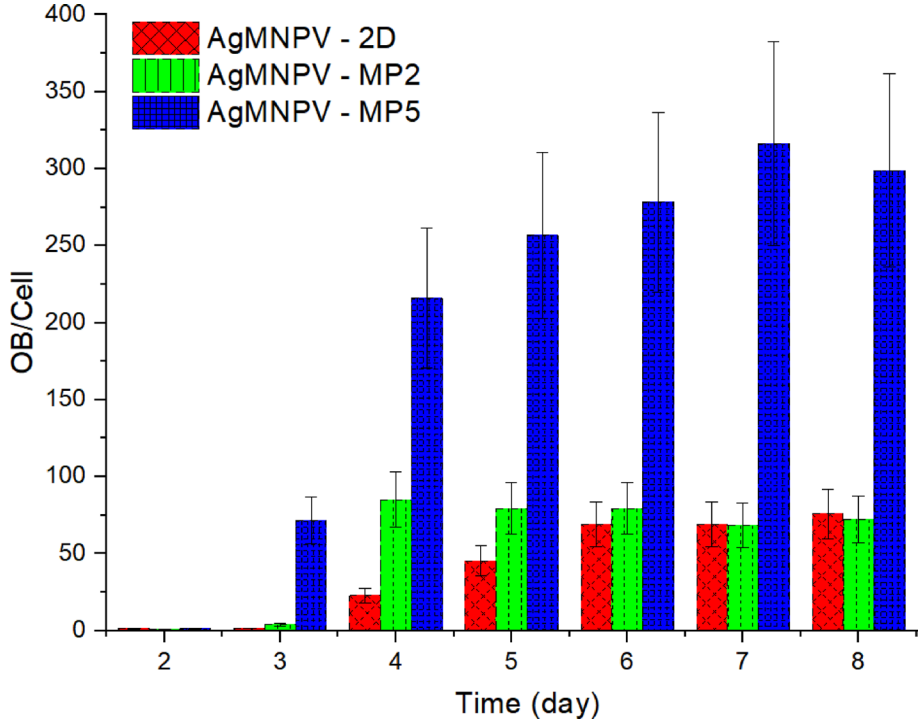

Fig. 6 Specific OB production of different AgMNPV variants and AgMNPV-2D in SF-21 cells. Samples were collected for OB-counting expressed as OB/cell. Vertical bars indicate the standard error

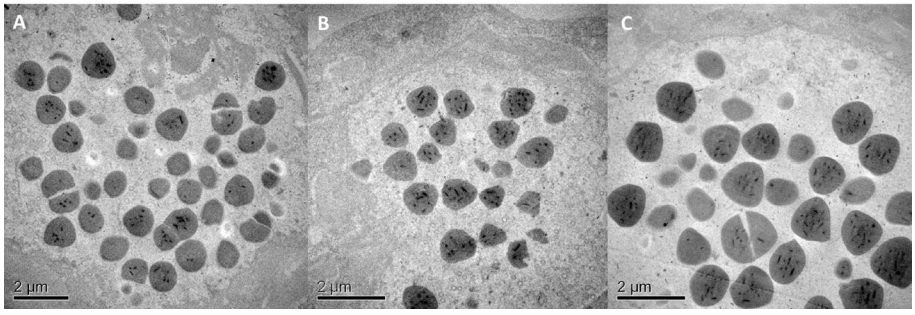

Fig. 7 Transmission electron micrographs of SF21 cells infected with AgMNPV- 2D **A** AgMNPV-MP2 **B** and AgMNPV-MP5 **C**, at 72 h.p.i

despite undergoing the same purification process as AgMNPV-MP2, obtained the best OB/cell ratio, reaching 297.73 ± 47.64 OB/cell, and was considered the best isolate for OB production after 8 d.p.i., using the SF21 line cultivated in SF900II SFM medium, in suspension.

The ultrastructural analysis of the two many polyhedra variants (MP2 and MP5) and AgMNPV-2D by Transmission Electron Microscopy (TEM), is shown in Fig. 7. No morphological differences were observed among the AgMNPV MP variants and the parental virus (AgMNPV-2D). The cells showed the same baculovirus typical effects infection such as cell rounding, nuclear hypertrophy, virogenic stroma formation, virus assembly and polyhedra production. Likewise, no significant difference was found in the polyhedral size among AgMNPV-2D (2.09 µm), AgMNPV-MP2 (2.03 µm) and AgMNPV-MP5 (2.18 µm) isolates ($F = 5.46$, $p < 0.0001$) after Scanning Electron Microscope (SEM) examination.

4 Discussion

The selection of stable genetic isolates is important for the in vitro cultivation of baculoviruses, mainly in relation to occlusion body production, which is the insect infective form of the virus. In this work we did not find molecular alterations by viral DNA restriction analysis, in the MP variants compared to AgMNPV-2D; more importantly, FP phenotypes in AgMNPV-MP2 and MP5 infected cells were not observed after seven consecutive passages in suspension cell culture, whereas many AgMNPV-2D infected cells with FP mutants were observed. The baculovirus AgMNPV, represented by their different genetic isolates (AgMNPV-2D, AgMNPV-MP2 and AgMNPV-MP5), was also analyzed for its OB production capacity, using suspension cultivation of *S. frugiperda* cells (SF21). Of the isolates studied, AgMNPV-MP5 demonstrated the best capacity to produce occlusion bodies.

Accumulation of Few polyhedra mutants (FP) after AgMNPV 2D passages in static cell culture was previously reported by our group [13]. Ultrastructural analysis showed typical signs of FP mutant formation such as decrease in the number of polyhedral per cell, polyhedra aberrant morphology and low numbers of virions occluded in the protein matrix. Restriction endonuclease analysis of the viral DNA revealed that lower and higher passages had similar profiles demonstrating that there were no large insertions or deletions in their genomes and indicating the generation of FP mutants instead of defective interfering viruses.

Slavicek et al. [14] showed that isolates of the baculovirus LdMNPV produced different concentrations of virus OB when used in the *Lymantria dispar* Linnaeus, 1758 (Lepidoptera: Erebidae) 652Y cell line. Pedrini et al. [16] analyzed genetic isolates of the Helicoverpa armigera single nucleopolyhedrovirus (HaSNPV) multiplied during serial passage in *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) cells, selecting mutants (ppC19) that produced five times more occlusion bodies than typical FP mutants (FP8AS), which normally produce fewer occlusion bodies than the wild-type HaSNPV virus. Ferreira et al. [29] observed genetic differences between two AgMNPV clones (Ag79-01 and AgL-16) obtained from the same wild-isolate, a Brazilian field isolate (AgMNPV-79). They were cultivated in static culture and no cytopathic difference was noted; these clones also presented differences of virulence in larvae infection compared to AgMNPV-2D.

The infectivity of the virus prototype AgMNPV-2D in seven different cell lines was investigated using static cultures [9]. The cells lines BTI-Tn-5B1-4, from *T. ni*, and UFL-AG-286, from *A. gemmatalis*, were highly productive in terms of production of polyhedra and budded virus. Almost 100% of Tn-5B1-4 and AG-286 cells showed formation of polyhedra by 96 h p.i. At this same period, 70% of the IPLB SF21 cells, from *S. frugiperda*, produced polyhedra particles. However, TN-368 cells, from *T. ni*, and Sf9 cells, derived from IPLB-Sf21 cell line, presented low infection rates. Moreover, a cell line of *L. dispar* (Ld652Y) showed no susceptibility to the virus while a cell line of *Bombyx mori* Linnaeus, 1758 (Lepidoptera: Bombycidae) (BM 5) resulted in apoptosis.

The in vitro production of AgMNPV using *S. frugiperda* cells (Sf9 cell line) in a stirred tank bioreactor was analyzed by Rodas et al. [8]. OBs production in infected cultures performed in bioreactor with SF900II medium (MOI 1), was lower in terms of OBs/cell as compared to that observed in shaker bottles. At higher passages the loss of virulence was accompanied by a loss in budded virus titer, a decreased number of PIBs produced

and an altered DNA restriction pattern, indicating the synthesis of defective interference particles (DIPs).

In the present study we used SF21 cells in suspension cultivation and obtained a mean of 73.48 OB/cell for the AgMNPV-MP2 isolate and 297.73 OB/cell for the AgMNPV-MP5 isolate, demonstrating better capacity to produce this genetic isolate. Besides, no morphological difference was found between the two many polyhedra variants and the parental virus (AgMNPV-2D).

In vitro production of the isolate AgMNPV-MP5 was very significant because it presents a high specific production of OBs, only seen in few in vitro production systems. For example, the baculovirus Helicoverpa armigera SNPV cultured on *H. zea* cells produced 222 OB/Cell [30] and S. frugiperda MNPV cultivated in *S. frugiperda* cells produced an average of 400 OB/cell in lab scale [31] and 300 OB/cell in bioreactor [32], respectively.

Although the in vitro baculovirus production is still economically less viable than in vivo production [33], the advances achieved in this and other recent studies [32] can contribute to change this perspective, especially in a possible scenario with intensive and skilled labor required for larvae rearing and problems with microbial contamination. It was necessary 40 tons of AgMNPV infected *A. gemmatalis* larvae to treat one million ha of crops, when this biopesticide was widely sprayed in Brazil [34] highlighting the challenges for in vivo production.

The genetic stability of OB is a problem for baculovirus in vitro production that our strategy helped to overcome. Another problem regarding baculovirus in vitro production is the loss of pathogenicty and virulence of produced OB. Some studies with bioassays reported the low mortality of larvae treated with cell culture derived AgMNPV, especially after serial passages [8, 35], while in other studies, the AgMNPV OB obtained from cell culture presented high pathogenicity and virulence [29, 36]. Therefore, is essential to test the efficiency of AgMNPV MPs for larvae control prior to large-scale in vitro production.

5 Conclusions

An important strategy to optimize the baculovirus in vitro production is the selection of many polyhedra (MP) variants. These variants are more stable and form many polyhedra in the cell nucleus even after consecutive passages in cell culture. Comparison of two MP variants, with the same morphology as the type virus, showed that AgMNPV-MP5 genetic isolate had the best occlusion body (OB) production capacity in suspension culture of SF21 cells, reaching a mean productivity of 297 OB/cell and 5.3×10^8 OB/mL. These results indicate that many polyhedra variants derived from the same virus can have different capacities to produce occlusion bodies when subjected to the same infection process. Besides, these MP viruses could be used in vitro for the production of baculoviruses as an alternative for the in vivo production.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s44370-025-00034-9.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
The authors thank to the Rosana Falcão (EMBRAPA) for the Scanning electron microscopy assistence and Dr Marcos Rodrigues de Faria (EMBRAPA) for the statistical analysis.

Author contributions
Conceptualization: AFA, MRSP, MLS; Methodology: AFA, MRSP, MLS; WS; AFB, Formal analysis and investigation: AFA, MLS, WS, BMR, AFB; Writing - original draft preparation: AFA, MRSP, MLS, MMS; Writing - review and editing: All authors; Funding acquisition: AFA, MRSP, MLS; Resources: MRSP, MLS, BMR; Supervision: MRSP, BMR, MLS.

Funding
This project received funds from Embrapa (grant number 02.10.01.003.00.00 to MLS), Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (grant number 304223/2021-2 to BMR), Fundação de apoio à pesquisa do Distrito Federal -FAPDF (grant number 193–00001749/2022-31 to BMR) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES - doctoral fellowship of AFA.

Data availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable. This work was developed using insect cell cultures and insect viruses (baculovirus). This group of viruses are non-pathogenic for humans, vertebrates and plants.

Consent for publication
All authors agreed to publish the study.

Competing interests
Marcio Martinello Sanches declares he is an Editorial Board Member of Discover Viruses and confirms that he was not involved in the handling or decision-making of its own submission.

Author details
[1]Departamento de Biotecnologia, Centro de Biotecnologia, Universidade Federal da Paraíba, Via Ipê Amarelo S/N, Campus I, João Pessoa CEP 58051- 900, SP, Brasil
[2]Departamento de Engenharia de Alimentos, Universidade Federal do Rio Grande do Norte, Av. Senador Salgado Filho 3000, Lagoa Nova, Natal CEP 59.072-970, RN, Brasil
[3]Instituto Todos pela Saúde (ITpS), Av. Paulista, 1.938 – 16/andar, São Paulo CEP 01310-942, SP, Brasil
[4]Instituto de Ciências Biológicas, Departamento de Biologia Celular, Universidade de Brasília, Prédio K, Brasília CEP 70910-900, DF, Brasil
[5]Laboratório de Fitopatologia, Embrapa Gado de Corte, Campo Grande CEP 79106-550, Brasil
[6]Embrapa Recursos Genéticos e Biotecnologia, Laboratório de Virologia de Insetos, Parque Estação Biológica, Brasília CEP 70770-917, DF, Brasil

Received: 30 May 2025 / Accepted: 16 October 2025
Published online: 23 October 2025

References

1. Theilmann DA, Blissard GW, Bonning B, Jehle J, O´Reilly DR, Rohrmann GF, Thiem S, Vlak JM. (2005) Family Baculoviridae. *In*: Virus taxonomy: Eight report of international committee on taxonomy of viruses. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editor 177–185.
2. Harrison RL, Herniou EA, Jehle JA, Theilmann DA, Burand JP, Becnel JJ, Krell PJ, Van Oers M, Mowery JD, Bauchan GR, ICTV Report Consortium. ICTV virus taxonomy profile: Baculoviridae. J Gen Virol. 2019;99:1185–6. https://doi.org/10.1099/jgv.0.001107.
3. King LA, Possee RD. (1992) The baculovirus expression system: a laboratory guide New York: Chapman & Hall, xiv:229.
4. Moscardi F. Assessment of the application of baculoviruses for control of lepidoptera. Annu Rev Entomol. 1999;44:257–89. https://doi.org/10.1146/annurev.ento.44.1.257.
5. Sosa-Gómez DR. Microbial control of soybean pest insects and mites. In: Lacey LA, editor. Microbial control of insect and mite pests: from theory to practice. Amsterdam: Elsevier; 2017. pp. 199–208.
6. Claus JD, Remondetto GE, Guerrero SA, Demonte AM, Murguía M, Marcipar AJ. *Anticarsia gemmatalis* nuclear polyhedrosis virus replication in serum free and serum-reduced insect cell cultures. J Biotechnol. 1993;31:1–15. https://doi.org/10.1016/0168-1656(93)90133-8.
7. Castro MEB, Souza ML, Araujo S, Bilimoria SL. Replication of *Anticarsia gemmatalis* nuclear polyhedrosis virus in four lepidopteran cell lines. J Invertebr Pathol. 1997;69:40–5. https://doi.org/10.1006/jipa.1996.4624.
8. Rodas VM, Marques FH, Honda MT, Soares DM, Jorge SAC, Antoniazzi MM, Medugno C, Castro MEB, Ribeiro BM, Souza ML, Tonso A, Pereira CA. Cell culture derived AgMNPV bioinsecticide: biological constraints and bioprocess issues. Cytotechnology. 2005;48:27–39. https://doi.org/10.1007/s10616-005-3175-7.
9. Castro MEB, Ribeiro ZMA, Souza ML. Infectivity of *Anticarsia gemmatalis nucleopolyhedrovirus* to different insect cell lines: Morphology, viral production, and protein synthesis. Biol Control. 2006;36:299–304. https://doi.org/10.1016/j.biocontrol.2005.10.002.

10. Krell PJ. Passage effect of virus infection in insect cells. Cytotechnology. 1996;20:125–37. https://doi.org/10.1007/BF00350393.
11. Gooijer CD, Van Lier FLJ, Van den End EJ, Vlak JM, Tramper J. A model for baculovirus production with continuous insect cell cultures. Appl Microbiol Biotechnol. 1989;30:497–501.
12. Kumar S, Miller LK. Effects of serial passage of *Autagrapha Californica* nuclear polyhedrosis virus in cell culture. Virus Res. 1987;7:335–49. https://doi.org/10.1016/0168-1702(87)90047-5.
13. Rezende SHMS, Castro MEB, Souza ML. Accumulation of few-polyhedra mutants upon serial passage of *Anticarsia gemmatalis* multiple nucleopolyhedrovirus in cell culture. J Invertebr Pathol. 2009;100:153–9. https://doi.org/10.1016/j.jip.2008.12.002.
14. Slavicek JM, Mercer MJ, Kelly ME, Hayes-Plazolles N. Isolation of a baculovirus variant that Exibts enhanced polyhedra production stability during serial passage in cell culture. J Invertebr Pathol. 1996;67:153–60. https://doi.org/10.1006/jipa.1996.0023.
15. Slavicek MJ, Hayes-Plazolles N, Kelly ME. Identification of a *Lymantria dispar* nucleopolyhedrovirus isolate that does not accumulate few-polyhedra mutants during extended serial passage in cell culture. Biol Control. 2001;22:159–68. https://doi.org/10.1006/bcon.2001.0969.
16. Pedrini MRS, Nielsen LK, Reid S, Chan LCL. Properties of a unique mutant of *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus that exhibits a partial many polyhedral and few polyhedral phenotype on extended serial passaging in suspension cell cultures. Vitro Cell Dev Biol -Anim. 2005;41:289–97. https://doi.org/10.1290/0503019R.1.
17. Maruniak JE, Johnson D, Gowan D. (1986) AgNPV replication in cell cultures and physical map of its genomic variants. J Cell Biochem (supplement) 10 C, 49.
18. Maruniak JE, Maruniak AG, Souza ML, Zanotto PMA, Moscardi F. Physical maps and virulence of *Anticarsia gemmatalis* nucleopolyhedrovirus genomic variants. Arch Virol. 1999;144:1991–2006. https://doi.org/10.1007/s007050050720.
19. Johnson DW, Maruniak JE. Physical map of *Anticarsia gemmatalis* nuclear polyhedrosis virus (AgMNPV-2) DNA. J Gen Virol. 1989;70:1877–83. https://doi.org/10.1099/0022-1317-70-7-1877.
20. Oliveira JVC, Wolff JLC, Garcia-Maruniak A, Ribeiro BM, Castro MEB, Souza ML, Moscardi F, Maruniak JE, Zanotto PMA. Genome of the most widely used viral biopesticide: *Anticarsia gemmatalis* multiple nucleopolyhedrovirus. J Gen Virol. 2006;87:3233–50. https://doi.org/10.1099/vir.0.82161-0.
21. Granados RR, Guoxun L, Derksen ACG, McKenna KA. A new insect cell line from *Trichoplusia Ni* (BTI-Tn-5B1-4) susceptible to *Trichoplusia Ni* single enveloped nuclear polyhedrosis virus. J Invertebr Pathol. 1994;64:260–6. https://doi.org/10.1016/S0022-2011(94)90400-6.
22. Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P. The establishment of two cell lines from the insect spodoptera Frugiperda (Lepidoptera:Noctuidae). Vitro. 1977;13:213–7. https://doi.org/10.1007/BF02615077.
23. O'Reilly DR, Miller LK, Luckow VA. Baculovirus expression vectors: A laboratory manual. New York: Cold Spring Harbor Laboratory; 1992.
24. Gallagher SR. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). Curr Protoc Essent Lab Tech. 2008;00:731–7325. https://doi.org/10.1002/9780470089941.et0703s00.
25. Nielsen LK, Smyth GK, Greenfield PF. Hemocytometer cell count distributions: implications of non-poisson behaviour. Biotechnol Prog. 1991;7:560–3. https://doi.org/10.1021/bp00012a600.
26. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2015;111(A3B1–A3B3). https://doi.org/10.1002/0471142735.ima03bs111.
27. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–63. https://doi.org/10.1016/0022-1759(83)90303-4.
28. Mena JA, Ramírez OT, Palomares LA. Titration of Non-Occluded baculovirus using a cell viability assay. Biotechniques. 2003;34:260–4. https://doi.org/10.2144/03342bm05.
29. Ferreira BC, Melo FL, Silva AMR, Sanches MM, Moscardi F, Ribeiro BM, Souza ML. Biological and molecular characterization of two *Anticarsia gemmatalis multiple nucleopolyhedrovirus* clones exhibiting contrasting virulence. J Invertebr Pathol. 2019;164:23–31. https://doi.org/10.1016/j.jip.2019.03.008.
30. Lua LH, Pedrini MRS, Reid S, Robertson A, Tribe DE. Phenotypic and genotypic analysis of *Helicoverpa armigera* nucleopolyhedrovirus serially passaged in cell culture. J Gen Virol. 2002;83:945–55. https://doi.org/10.1099/0022-1317-83-4-945.
31. Almeida AF, Macedo GR, Chan LCL, Pedrini MRS. Kinetic analysis of *in vitro* production of wild-type *Spodoptera Frugiperda* nucleopolyhedrovirus. Braz Arch Biol Technol. 2010;52:285–91. https://doi.org/10.1590/S1516-89132010000200006.
32. Klafke K, Sanches MM, Sihler W, Souza ML, Tonso A. Bioreactor production process of spodoptera Frugiperda multiple nucleopolyhedrovirus biopesticide. Pathogens. 2023;12:1001. https://doi.org/10.3390/pathogens12081001.
33. Martínez-Balerdi M, Caballero J, Aguirre E, Caballero P, Beperet I. Baculoviruses as microbial pesticides: Potential, Challenges, and market overview. Viruses. 2025;17:917. https://doi.org/10.3390/v17070917.
34. Moscardi F. A nucleopolyhedrovirus for control of the Velvetbean caterpillar in Brazilian soybeans. In: Vincent C, Goettel MS, Lazarovits G, editors. Biological control: A global perspective: case studies from around the world. Wallingford, UK: CAB International; 2007. pp. 344–52.
35. Sanches MM, Guimarães GC, Sihler W, Souza ML. Successful co-infection of two different baculovirus species in the same cell line reveals a potential strategy for large in vitro production. Braz J Microbiol. 2021;52:1835–43. https://doi.org/10.1007/s42770-021-00622-z.
36. Sanches MM, Sihler W, Silva CEP, Guimaraes GC, Benito NP, Sosa-Gomez DR, Souza ML. Characterization of a Chrysodeixis includens nucleopolyhedrovirus isolate from Brazilian Cerrado and assessment of its co-infection with Anticarsia gemmatalis multiple nucleopolyhedrovirus. Braz Arch Biol Technol (Online). 2019. https://doi.org/10.1590/1678-4324-2019180688

Publisher's note