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Development and characterization of a continuous cell line, AFKM-On-H, from hemocytes of the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera, Pyralidae)

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Abstract The corn borer, *Ostrinia nubilalis*, is a very important pest in different countries, and the in vitro system of the insect could be a useful tool for isolation and characterization of the pathogens and physiological responses of the insect. In this context, a cell line was derived from the hemocytes of the European corn borer and was named AFKM-On-H for, respectively, *O. nubilalis*, Armand Frappier, King Mongkut Institutes, and Hemocytes. This cell line was initiated and maintained in Ex-Cell 400 medium supplemented with 10% heat-inactivated fetal bovine serum. The cells, mostly spherical in shape, not firmly attached to the plastic culture flasks, were passaged up to 200 times by repeated gentle pipetting of the cells. The doubling times at the 80th and 125th passages at 28°C and at the 122th and 169th passages at 25°C were 40, 29, 35, and 34 h, respectively. The AFKM-On-H cell line was further characterized by the morphology, karyotype, random amplified polymorphic DNA analysis, and isozyme profiles. Susceptibility of the cell line to cytoplasmic polyhedrosis viruses (CPV) *Euxoa scandens* (EsCPV), *Dendrolimus punctatus* (DpCPV), and *Choristoneura fumiferana* (CfCPV); nuclear polyhedrosis viruses [*Autographa californica* (AcMNPV) wild type and recombinant, *Antherea yammamai* (AnyaNPV)]; and *Chilo* iridescent virus was demonstrated. Relative sensitivities of the cell line to *Bacillus thuringiensis* and *Metarhizium anisopliae* toxins and effects of the molting hormone 20-hydroxyecdysone on this new hemocyte cell line were characterized.

Keywords Hemocyte cell line · European corn borer · Molting hormone · Polymerase chain reaction · Baculovirus · *Chilo* iridescent virus · Cytoplasmic polyhedrosis virus · *Bacillus thuringiensis* · Destruxins

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Introduction

Numerous different insect cell lines have been developed with the primary aim of producing Baculovirus pesticides (Weiss and Vaughn 1986; Goodman and McIntosh 1994; Murhammer 1996) and recombinant proteins (Davis et al. 1993; Granados et al. 1994; Palomares and Ramírez 1998; Palomares et al. 2003). However, insect cell lines from various origins also have wide application ranges, such as the isolation of viruses from field-collected insects and the separation of a specific virus strain from a mixture (Belloncik et al. 1996). New insect cell lines also have their importance in conducting virus replication and in cell–pathogen interaction studies, as well as in screening, testing, and identifying in vitro different bioproducts with ecdysone-like activity (Sohi et al. 1995; Trisyono et al.

2000). In this context, we were interested to develop an in vitro cell system from the European corn borer, polyphagous, an important major pest of corn in many European and North American countries (Farinos et al. 2004; Linacre and Thompson 2004), including the Quebec province, where this insect causes primarily important damages to corn (Hudon and LeRoux 1986) and, in the fall season, pepper plantations.

Four cell lines have already been reported from this insect. The UMC-OnE cell line was obtained from embryonic tissues and demonstrated to be responsive to 20-hydroxyecdysone (20 HE) and some ecdysone agonists (Trisyono et al. 2000). Another cell line from pupal ovaries of this insect was reported to be susceptible to microsporidian infection (Kurtti from Trisyono et al. 2000). Later on, two continuous cell lines (BCIRL/AMCY-OnFB-GES-1 and BCIRL/AMCY-OnFB-GES-3) were developed from larval fat body of *Ostrinia nubilalis* (Goodman et al. 2001a). BCIRL/AMCY-OnFB-GES-1 cell line supported (Goodman et al. 2001b), poorly, the replication of *Autographa californica* (AcNPV). We report in this paper on the development and characterization of an additional cell line from the hemocyte of the European corn borer and its reaction to insect viruses, microbial toxins, and the molting hormone 20 HE.

Material and Methods

Cell line establishment. Peppers that were heavily infested with *O. nubilalis* larvae were obtained from Dr. Gérard Mailloux, *Institut de Recherches et de Développement en Agro-alimentaire*, Quebec, department of Agriculture, St Bruno de Montarville, Quebec, 50 km from Montreal. The last instar larvae were then separated and surface sterilized with 70% alcohol. Using a sterile needle, the larva was punctured in one leg and hemolymph of individual larvae was asceptically collected in a flask containing 5 ml of Ex-Cell 400 medium pH 6.2 (JHR Biosciences, Lenexa, KS) supplemented with 10% of fetal bovine serum (FBS) and 1% of antibiotic solution (100 µg/ml penicillin and 100 µg/ml streptomycin). A total of nine flasks were prepared. Some primary cultures were also initiated in Grace medium supplemented with 10% FBS. However, no successful evolution of these primary cultures was obtained using this medium. All the primary cultures were incubated at 28°C.

Twenty-one days after the establishment of the primary cultures, the medium of each flask with floating cells was removed and put in an another flask. Medium was added to the remaining attached monolayer cell flasks. Twenty-five days later, the flasks demonstrating sufficient large amounts of round or fibroblastic attached cells were selected.

Cell subcultures. Old medium was discarded from the flask. Then, using rubber policeman or by repeated gentle pipetting, the attached cells were suspended in new medium and transferred to two new flasks weekly. The use of pancreatin solution to better detach the fibroblastic cells was not satisfactory. One month later, a flask of cells with the best satisfactory cell growth was conserved and further subcultured at 28°C and, recently, at 25°C. Up to 200 passages of this cell line, AFKM-On-H, were done in Excell medium by gentle pipetting of the attached cells every 5–7 days. More recently, the established cell line was adapted to growth in Grace medium supplemented with 10% FBS. The AFKM-On-H cell line is now stored both in liquid nitrogen and at −80°C; these two methods preserve the cells equally well.

Microscopical analysis of cell morphology. The cells were observed using a contrast phase light microscope. Ultrathin sections of cells treated as previously described (Belloncik et al. 1996) were analyzed under an electron microscope.

Cell growth measurement. Cells were seeded in triplicate at 2×10^5 cells per milliliter in T-25-cm^2 flasks. The number of viable cells was evaluated every 48 h for 8 d using a hemacytometer and trypan blue staining. Growth curves of the AFKM-On-H cell line were determined at the 80th and 125th passages at 28°C and at the 122th and 169th passages at 25°C.

Karyotype analysis. Chromosome numbers were determined for the cell line using a modified Schneider's protocol (Schneider 1973). Cells in the logarithmic growth phase were incubated for 24 h in a culture medium containing 50 µg/ml of colchicine (Sigma, St. Louis, MO). The cell pellet obtained from the treated culture flask was washed with distilled water and then allowed to swell in it for 10 min. After centrifugation, the cells were fixed with glacial acetic acid and methanol (1:3). After the last centrifugation, they were stained with orcein (2% in a 30% acetic acid solution in distilled water) for 24 h. A drop of stained cell suspension was put on a microscope slide and covered with a lamella, which was pressed to spread the chromosomes. The number of chromosomes was recorded and the percentage of cells of each polyploidy observed was calculated from at least 30 metaphase cell spreads.

Characterization using random amplified polymorphic DNA markers. *Ostrinia nubilalis* larvae tested were obtained from the Research Station, Agriculture Canada, St Jean d'Iberville. Cell lines from *O. nubilalis* (AFKM-On-H), *Mamestra brassicae* (Mabr) (Inoue and Mitsuhashi 1985) from the National Institute of Agrobiological sciences, Tsukuba, Japan; *Bombyx mori* (BmN4) (Maeda

1989) from Dr. Hajime Mori, Kyoto Institute of Technology, Japan; and *Aedes aegypti* (Singh), from Dr. Gérard Devauchelle, Station de pathologie comparée, Ales, France, were used. Extraction, purification, and quantitation of DNA from *O. nubilalis* larvae and cell cultures were performed according to previously described methods (Léry et al. 2003); PCR amplification in 25-µl reaction mixtures containing 20 ng of genomic DNA proceeded as reported above. The following random primers were used: 5 AATCGGGCTG 3′, 5′ CCGCATCTAC 3′, and 5′ GTTTCGCTCC 3′. After fractionation using 1.5% agarose gel electrophoresis, amplification products were characterized according to size, and band patterns from individual larvae and cell cultures were compared by using the similarity coefficient S_{ab} as defined by Nei and Li (1979), where $S_{ab}=2N_{ab}/(Na+N_b)$, Na and N_b = number of bands on the gel for the samples a and b, respectively, and N_{ab} = number of common bands on the gel for the two samples.

Isozyme analysis. For isozyme analysis, we compared AFKM-On-H with *Spodoptera frugiperda* cell clonal isolate Sf9 (Vaughn et al. 1977), *B. mori* BmN4 (Maeda 1989) and *Trichoplusia ni* TN-368 (Hink 1970) cell lines. Sf9, BmN4, and TN-368 were from cell collection of Dr. S. Imanishi, National Institute of Agrobiological Sciences, Tsukuba, Japan. Using an AuthentiKit procedure (Corning, Walpole, MA), the isozymes detected were isocitric dehydrogenase, phosphogluco mutase, phosphogluco isomerase (PGI), and glucose6-phosphate dehydrogenase.

Cell susceptibility to viruses. The following viruses were used: (1) Nonoccluded virus suspension of *Galleria mellonella* (GmMNPV), *A. californica* (AcMNPV), *Helicoverpa armigera* (HaNPV), *Spodoptera exigua* (SpeiNPV), *B. mori* (BmNPV), and *Antherea yamamai* (AnyaNPV) nucleopolyhedrosis viruses; (2) genetic recombinant virus (AcLuci9.4) of *Ac*MNPV carrying a Luciferase gene; (3) occluded virions released by alkaline treatment of polyhedra of *Euxoa scandens* (EsCPV), *B. mori* (BmCPV), *Dendrolimus punctatus* (DpCPV), and *Choristoneura fumiferana* (CfCPV) cytoplasmic polyhedrosis viruses (CPVs); (4) *Chilo* iridescent virus (CIV) suspension; and (5) Densonucleosis virus produced in *G. melonella* larvae.

Briefly, the cells, except for those infected with virus 2, were seeded in flasks at a concentration of 2×10^5/ml. After cell attachment overnight, the medium was removed and replaced for 1 h by 1 ml of virus suspension at approximately 1 MOI. Then, 4 ml of culture medium was added, and the cells were incubated at 28°C and observed daily for one to several wk for cytopathic effects and/or viral polyhedra formation. Additional electron microscopy was performed for viral replication confirmation when needed.

As for *Ac*MNPV (AcLuci9.4) carrying a luciferase gene (virus 2), cells in 150 µl of medium suspension were seeded in a 96-well plate at a final concentration of 30,000 cells/well, and 25 µl of virus suspension was then added. After an incubation at 25°C for 3 and 6 d, the medium was removed, 100 µl of lysis buffer was added to each well for 30 min, and the relative luminescent units (RLU) were counted in the cell lysate using a luminometer (Tomita et al. 1995).

Effects of the molting hormone 20 HE. Cells were seeded at a concentration of 2×10^5/ml and cultured at 25°C in Ex-Cell 400 medium supplemented with 10% FBS. 20 HE was added to the culture medium at final concentrations of 0.046, 0.46, 2.78, and 5.57 µg/ml of culture medium. The treated cells were observed daily with a phase contrast microscope, and their viability was evaluated after 168 h of 20 HE treatment.

Response of AFKM-On-H cell line to Bacillus thuringiensis toxins. Four commercial formulations were tested: (1) Thuricide (var, *Kurstaki*) from Thermo Ecotek (Ontario, Canada) containing cry1Aa, cry1Ab, cry1Ac, and cry2Aa toxins (Schnepf et al. 1998); (2) Trident (var. *tenebrionis*) from Sandoz Agro Canada containing cryIIIA and cryIIIBb toxins (Wraight and Ramos 2005); (3) Teknar-HPD (var. *israelensis*) from Zoecon Canada (Mississauga, Canada) containing cry4A, cry4B, cry10A, cry11A, and cyt1A (Schnepf et al. 1998); and (4) blank formulation (graciously provided by Zoecon Canada) containing the inert ingredients of the Teknar minus the bacterial products.

Evaluation tests of cell response to Bt toxin were performed in 96-well plates (Falcon, Lincoln Park, NJ). In summary, the toxins of each formulation were dissolved, treated, and used in the toxicity tests as described previously and without modification (Charpentier et al. 1995, 2002). The cell lysis was monitored under microscope for 24 h postcontact of the toxin solution on the cell and the tissue culture toxic dose 50% ($TCTD_{50}$) was calculated with the Kärber formula (Kärber 1931)

Response of AFKM-On-H cell line to destruxins, mycotoxins of Metarhizium anisopliae. A crude extract of destruxins (Vey and Quiot 1989; Brousseau et al. 1996) was dissolved in acetone first and completed by water for a final concentration of 200 µg/ml (stock solution) and then filtered on 0.45-µm millipore paper and tested on cells in a 96-well plate. One hundred fifty microliters of cell suspension (35,000 cells per milliliter) was added in each of the 96 wells of the plate and incubated overnight at 28°C. The following day, 50 µl of each serial twofold dilution of the toxin (from 1/2,000) was added to the wells (eight wells/toxin dilution and eight wells for a 1/2,000

acetone concentration control). The plates were then incubated at 28°C for 72 h; the cytotoxic effect was then recorded under microscope, and the $TCTD_{50}$ was calculated as for *B. thuringiensis* toxin.

Results

Cell line establishment. Nine flasks of primary culture were successfully obtained from hemocytes of *O. nubilalis*, each flask from one individual larva, using Ex-Cell 400 medium supplemented with 10% FBS. Attempts to use Grace medium were not successful. No contamination or melanization was encountered, and the hemocytes attached to the culture flask bottom quickly in the first few hours. Two types of cells were equally predominant: fibroblast-like cells and spherical cells. The numbers of both increased during the first 3 wk, with a predominance later on of slightly attached or floating spherical cells. From the 25th day, cell subcultures were attempted.

Subcultures. Successful subcultures of the primary cultures were obtained by passaging the spherical cells. Attempts to subculture the strongly attached fibroblast-like cells either by pipeting, using rubber policeman, or pancreatine treatment were unsuccessful. Only the flasks with a high density of growing spherical cells gave a viable subculture. We thereafter selected one of the most rapid-growing cell flasks, which was further long-term passaged. The early subcultures were made at an interval of 10 to 14 d when the cell monolayer was almost confluent and a high number of spherical cells was predominant. Later on, the interval of subcultures decreased to less than 1 wk. Up to 200 passages of the cell line named AFKM-On-H were made in Ex-Cell 400 medium supplemented with 10% FBS. After the 125th passage, some of the cells were transferred to Grace medium plus 10% FBS. Interestingly, this adaptation to a different medium did not affect the cell growth. The AFKM-On-H cell line is now stored either in liquid nitrogen or in a freezer at −80°C; these two methods were proved to be equally effective to conserve the viability of this cell line.

Microscopical analysis of cell morphology. Light microscopical observations of AFKM-On-H cells showed the predominance of the spherical cells (Fig. 1). The ratio of spherical/fibroblast-like cells varies slightly with the age of the subculture. However, more than 90% of the cells are of the spherical type. Electron microscope observations (Fig. 2) demonstrated normal ultrastructure of the cells and the absence of any bacterial, viral, and microsporidial contamination.

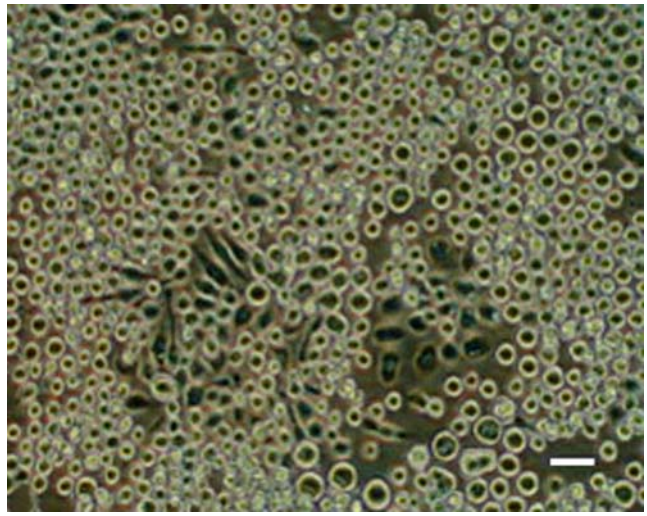

Figure 1. Light microscopy. Phase-contrast photographs of a monolayer of AFKM-On-H cell line. *Bar*: 30 μm.

Cell growth measurement. The cell growth evaluation and the cell doubling time of AFKM-On-H are given in Fig. 3. Population doubling times were approximately 40 and 29 h at the 80th and 125th passages at 28°C and 36 and 34 h at the 122th and 169th passages when maintained at 25°C.

Karyotype analysis. The results obtained demonstrate that the AFKM-On-H cell line at the 170th passage is 55% haploid with an average of 18 chromosomes per cell, 27% diploid with an average of 32 chromosomes per cell, and 18% tetraploid with an average of 58 chromosomes per cell.

Characterization using random amplified polymorphic DNA markers. Amplification of DNA from *O. nubilalis* larvae and cell line with three primers yielded a total of 55 scorable bands, mostly 0.2–1.5 kb in length. Some of the data with primer 5′ AATCGGGCTG 3′ are shown in Fig. 4. On average, one observed about 12 bands for each DNA sample and primer being used. None of these bands could be qualified as a universal marker present in all *O. nubilalis* samples, but 28 out of the 55 reported bands were found at frequencies of 75% or higher. Most of the remaining bands appeared at much lower frequencies, some even being observed in a single DNA sample. The distribution of the similarity coefficient S_{ab} (defined in the "Material and Methods" section) was quite heterogenous, with averages between 0.7 and 0.85, according to the primer. At least with primer CCGCATCTAC, one could not always distinguish on the basis of individual S_{ab} values alone between samples from *O. nubilalis* and samples from other insect species. However, when computing an average S_{ab} for each *O. nubilalis* sample, one observes that all intraspecific comparisons, including the cell line under investigation, cluster

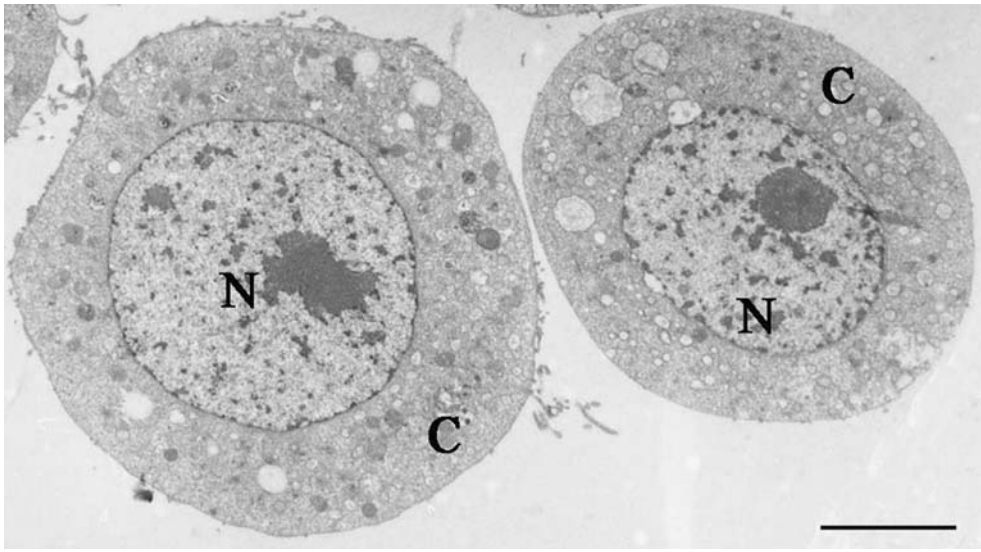

Figure 2. Electromicrograph showing the ultrastructure of AFKM-On-H cell line. *N* nucleus, *C* cytoplasm. *Bar* 5 μm.

above a 0.7 S_{ab} value, opposite to a figure below 0.4 when one performs interspecific comparisons (Table 1).

Isozyme analysis. Taken together, the isozyme patterns for AFKM-On-H cells were able to help in distinguishing this cell line from the others examined and used in our laboratories. Sf-9 cells generated patterns different from that of the corn borer cell line in all isozymes except for PGI. On the other hand, the Bm-N4 patterns differed from those of AFKM-On-H only in the case of PGI. Lastly, TN-368 was quite similar to AFKM-On-H except for the slight difference in the PGI pattern (Fig. 5).

Cell susceptibility to viruses. AFKM-On-H cell line was found to be nonpermissive to GmMNPV, BmNPV, SpexNPV, HaNPV, BmCPV, and *G. melonella* densonucleosis virus. On the other hand, based on the production of polyhedra, the AFKM-On-H cell line was demonstrated to be susceptible to infection by AcMNPV, AnyaNPV, EsCPV, CfCPV, and DpCPV. Furthermore, expressions of luciferase in this cell line recorded at 3 and 6 d postinfection were 4×10^8 and 2.25×10^8 RLU, respectively, confirming the replication of AcNPV. As for comparison of level of expression, infection of Sf9 cell line with the NPV recombinant resulted in a luciferase expression of 5.5×10^8 and 1×10^8 RLU at 3 and 6 d, respectively (Fig. 6).

Strong cytopathic effects such as modification of the morphology, such as elongation budding and hypertrophy of the cells (Fig. 7), characterized the contact of AFKM-On-H with CIV. Several ultrathin sections demonstrated internalization of virus particles in cytoplasm of cells without, however, a typical CIV replication.

Effects of the molting hormone 20 HE. Cell clumping and filamentous cytoplasmic elongations were observed after the 20 HE application (Fig. 8). The reaction of the cells to the molting hormone depended largely on the doses.

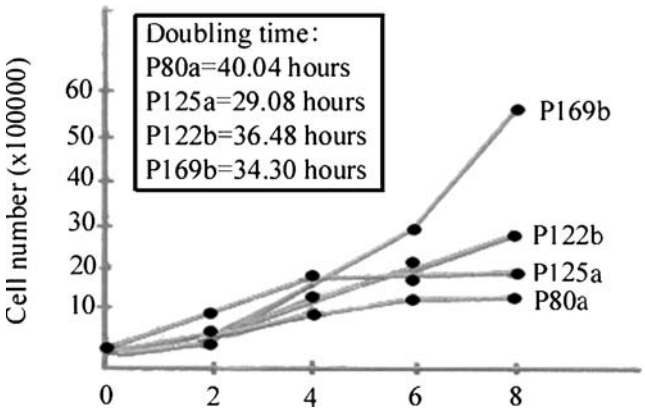

Figure 3. Growth curves of AFKM-On-H cell line at passages 80 (P80), 125 (P125), 122 (P122), and 169 (P169) maintained at 28°C (*P80a* and *P125a*) and 25°C (*P122b* and *169b*).

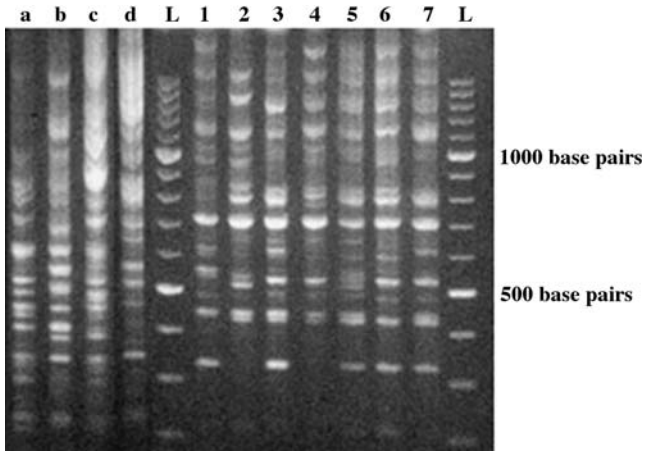

Figure 4. Random amplified polymorphic DNA profile of *a B. mori* (BmN4), *b Mamestra brassicae* (Mabr), *c Aedes aegypti* (Ae), *d* AFKM-On-H cells and *O. nubilalis* individual larva (*1–7*) using the primer A-04 (5′ AATCGGGCTG 3′). *L* DNA ladder, a multiple of 100 base pairs.

Table 1. Average of individual S_{ab} values coefficient

	Specimens				
	O. nubilalis larva	Cell cultures			
	n=7	AFKM-ON-H	Ae	Mabr	BmN4
Average S_{ab}[a]	0.788	0.783	0.263	0.414	0.320
Min S_{ab}	0.723	0.718	0.209	0.366	0.281
Max S_{ab}	0.833	0.842	0.347	0.472	0.371

[a] S_{ab} as defined by Nei and Li (1979), where $S_{ab}=2N_{ab}/(Na+N_b)$; Na and N_b = number of bands on the gel for the samples a and b, respectively, and N_{ab} = number of common bands on the gel for the 2 samples

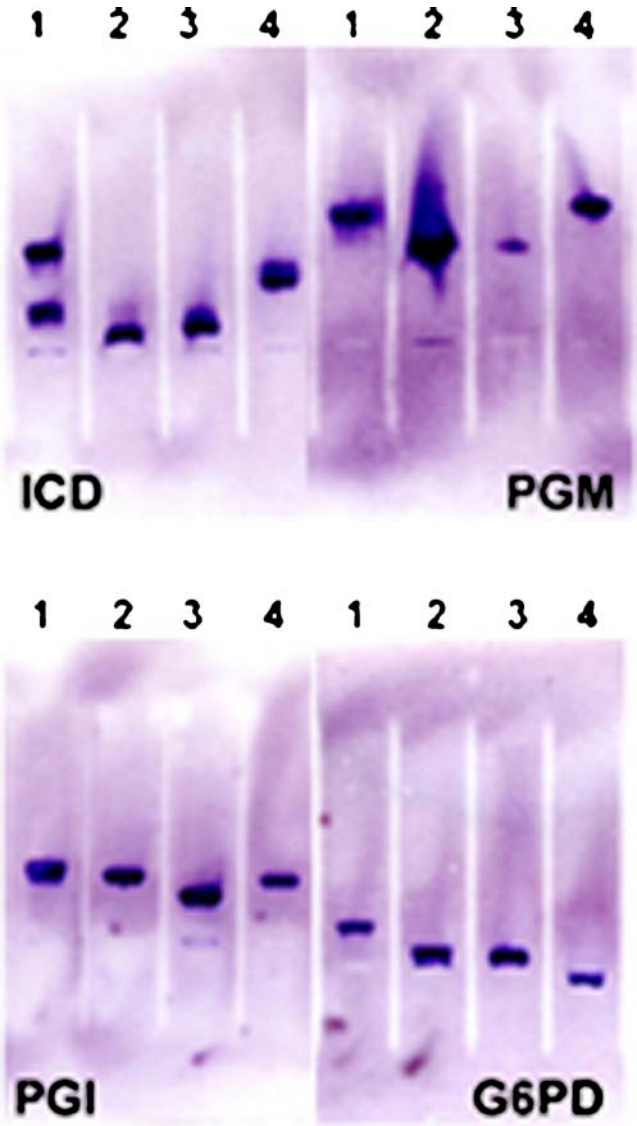

Figure 5. Isozyme pattern of AFKM-On-H cells in comparison with Sf9, TN-368, and Bm-N4 cell lines. *Lane 1* Bm-N4. *Lane 2* AFKM-On-H. *Lane 3* TN-368. *Lane 4* Sf-9. The gels show bands of isocitric dehydrogenase, phosphogluco mutase, PGI, and glucose6-phosphate dehydrogenase isozymes.

Preliminary results showed that doses of 0.046 and 0.46 μg/ml of culture medium of 20 HE stimulated the proliferation of AFKM-On-H cells. However, a strong inhibition (40 and 60%) of the cell growth was obtained after the cell treatment with 2.79 μg and 5.57 μg/ml, respectively, of culture medium of this product. Further quantified results will be presented in a further paper.

Response of AFKM-On-h cell line to B. thuringiensis toxins and destruxins, mycotoxins of M. anisopliae. $TCTD_{50}$ of these toxins on AFKM-On-H cell line in comparison to the reactions of other cell lines from *S. frugiperda* (Sf9), the Colorado potato beetle (LDA1, LDA2, LDL1, LDE1) (Charpentier et al. 2002), and *H. armigera* (KMITL-HA-1) (Charoensak 2003) are stated in Table 2. The results obtained demonstrated the sensitivity to the solubilized crystal toxins from commercial preparations of *B. thuringiensis* of the new hemocyte *O. nubilalis* cell line. The Btk in the thuricide was, as expected, the most effective, and the Bti surprisingly gave some toxic effect. This cell line also demonstrated a very high sensitivity to *M. anisopliae* mycotoxins.

Discussion

Several tissues and cells, undifferentiated or differentiated, are used for establishment of continuous insect cell lines. The most common sources of cell lines are the embryonic, ovarian, and fat tissues. Among the cell lines developed, those derived from hemocytes are in few numbers. However, these cells are the most convenient to collect, and cultures derived from hemocytes are far less heterogeneous than those from embryonic or ovarian tissues are. Special attention must be given, however, if the cells produce melanin, which did not cause problems during the establishment of the primary culture of *O. nubilalis* hemocytes.

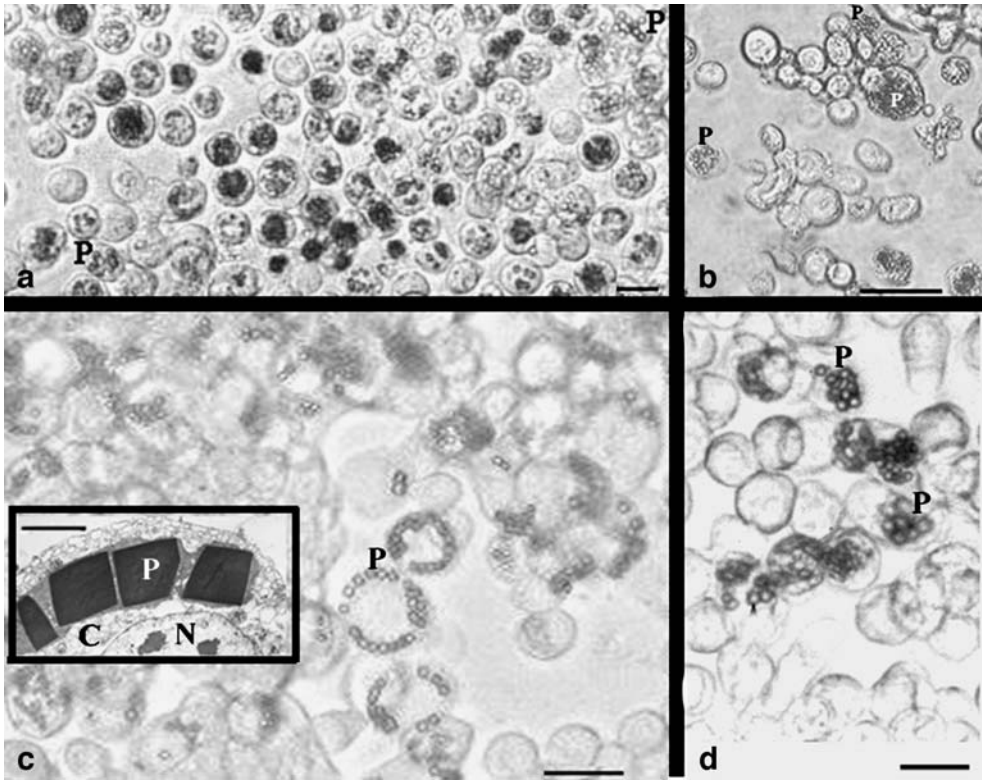

Figure 6. Light microscope observations of AFKM-On-H cells treated with virus. *P* viral polyhedra. *Bar*: 20 μm. *a* AcNPV. *b* AnyaNPV. *c* EsCPV. *Inset*: ultrathin section of infected cell. *N* nucleus. *C* cytoplasm. *Bar*: 5 μm. *d* DpCPV.

Depending on the insect, the cell, and tissue origins, a variable length of time is necessary from the primary culture establishment and the first successful subculture. This "dormant" stage could be several wk, as long as several mo, or more than a yr. In this regard, AFKM-On-H could be considered as very fast in terms of cell line establishment. Furthermore, according to the doubling time evaluated, AFKM-On-H could be characterized as a fast-growing cell line as well. The growth of this cell line is slightly faster than that of the one developed by Trisyono et al. (2000) from embryonic tissues of the same insect species. Interestingly, the cells incubated at 25°C demonstrated, as expected, slower growth than those maintained at 28°C, but on the other hand, the cell line maintained at lower temperature was more productive in terms of number of total viable cells after 8 d of culture.

The microsporidia are highly infectious and are an important regulation factor of corn borer in nature, as well as in the laboratory (Belloncik et al. 1988). This insect collected from the field is commonly infected by micro-

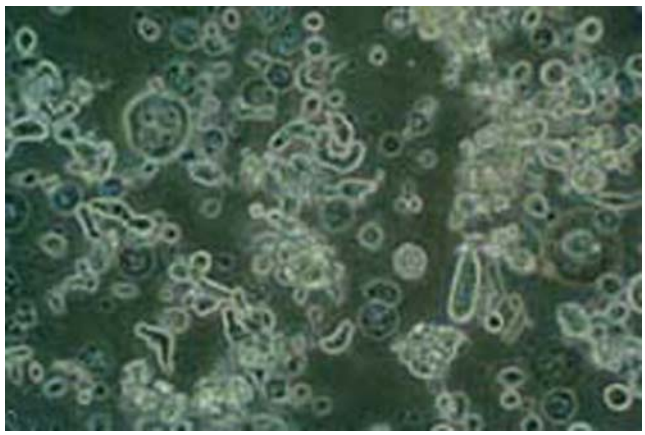

Figure 7. Monolayer of AFKM-On-H cells observed 24 h posttreatment with CIV suspension. Cytotoxic effects: elongation, budding, and hypertrophy of cells. *Bar*: 20 μm.

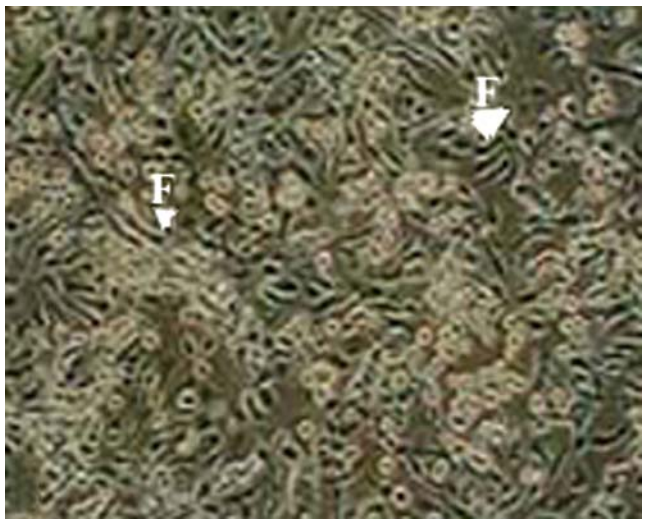

Figure 8. Effect of 20 HE 0.046 μg/ml of culture medium on AFKM-On-H cell line. Cytoplasmic cell filamentous elongations (*F*). *Bar*: 20 μm.

Table 2. Cytotoxicity ($TCTD_{50}$) of toxins from *B. thuringiensis* and *M. anisopliae*

Formulation (μg/ml)	AFKM-On-H	Sf9	KMITL-HA-1[a]	LDA1[b]	LDA2[b]	LDL1[b]	LDE1[b]
Thuricide (Btk)	1.45[c]	1.45	2.99	20.56	14.20	7.97	18.03
Teknar-HPD (Bti)	5.49	5.65	13.06	1.12	0.47	0.47	1.43
Trident (Btt)	26.03	25.31	26.75	4.93	20.50	4.98	7.07
Blank[d]	16.63	16.40	15.26	27.89	10.60	17.57	20.03
Destruxin	0.0049	0.0046	0.2870	0.1700	0.2300	0.5200	0.4600

[a] From Charoensak (2003)
[b] From Charpentier et al. (2002)
[c] Toxin (μg/ml)
[d] The formulation "blank" graciously provided by Zoecon Canada contains all the inert elements of Teknar minus the bacterial products

sporidia. This pathogen was proved to persist in different insect species by a transovarial transmission mechanism. This could lead to the development of persistently infected cell lines (Petchawaran, personal communication) if the primary cells are derived from these infected insects. As for the AFKM-On-H cell line, despite undergoing more than 170 passages, no microsporidial or other microbial infections were noted after extensive light and electron microscopical observations. Therefore, this healthy new cell line can be safely used. This cell line will be further used for the replication study of microsporidia and other pathogens.

No virus was yet isolated from field-collected or reared *O. nubilalis* insects. However, infection in the laboratory of the corn borer larvae with nuclear polyhedrosis viruses, such as Ac NPV (Lewis and Johnson, 1982; Belloncik et al. 1988) and CIV (Belloncik et al. 1988), was demonstrated. The newly hemocyte cell line was demonstrated to be susceptible to several virus infections and, therefore, extend the in vitro infection range of NPVs from *A. californica* and *A. yamamai* and CPVs from *E. scandens*, *D. punctatus* and *C. fumiferana*. Furthermore, the *O. nubilalis* cell line was demonstrated to be nonsusceptible to GmMNPV, which could be helpful to discriminate, if necessary, between Ac and GmMNPV infection.

Comparison of luciferase expression levels in Sf9 and AFKM-On-H cell extracts at 3 and 6 d postinfection shows a slight difference between *O. nubilalis* and Sf9 cell line reactions to AcNPV replication. The results tend to indicate that the Sf9 begins virus production slightly earlier. At 6 d, the infection in Sf9 was at a maximum level, and therefore, fewer cells were available for luciferase extraction. This could explain lower luciferase expression at 6 d in Sf9 than in the *O. nubilalis* cell line. However, statistical analysis, not performed in our studies, could clarify and confirm, or not confirm, these interpretations.

CPV infections, strictly restricted to the midgut of the insect larvae, are very common in nature (Belloncik and Mori 1998). However, as for another species of Pyralidae, *G. melonella*, *O. nubilalis* larvae are not susceptible to CPVs. Extensive attempts to isolate CPV from the corn borer larvae collected from the field, as well as to adapt some CPVs such as *E. scandens* and *B. mori* viruses to this insect reared in laboratory, failed (Belloncik, unpublished results). In this study, successful replication of three CPVs in a cell line derived from hemocytes of *O. nubilalis* corroborates, therefore, the assumption (Belloncik 1996) that cells in an insect demonstrate different susceptibility to CPVs than insect cells cultivated in vitro. A cell line can replicate CPVs irrespective of their origin (i.e., their original host), as well as of the tissue of origin of the cell line.

In contrast with the CPVs, CIV was adapted very easily to *O. nubilalis* and highly replicates in different tissues and cells of larvae such as the fat tissue and hemocytes (Belloncik et al. 1988). However, hemocytes of *O. nubilalis* cultivated in vitro reacted differently to CIV. Clear and strong cytotoxicity as described for other cell lines was noted. This toxicity demonstrated for both insect and vertebrate cell lines is the result of a toxic structural CIV protein and ranges from hypertrophy, to morphological alterations, to cell fusion, to destruction of the cells (Cerutti and Devauchelle 1979, 1980). Observations of ultrathin sections of *O. nubilalis* cell line showed in very few and limited numbers of CIV-treated cell groups of 4–5 virions without clear evidence of complete viral replication. The internalization of virions in some cells as the only replication step could be an explanation of these observations. It was demonstrated by other authors that, in some susceptible insect cells cultivated in vitro, the toxic effect is, if the viral particle concentration is reduced, overcome by a complete viral replication leading to CIV virions synthesis and formation of large intracytoplasmic crystals of virus particles (Kelly and Tinsley 1974; Cerutti et al. 1981; Charpentier et al. 1986). It could be interesting, therefore, to monitor in the AFKM-On-H cell line the cytotoxicity vs. the virus replication according to the virus concentration and to attempt, by cell cloning, to isolate the cells more susceptible to the CIV replication.

As demonstrated for other cell lines (Charpentier et al. 2002; Charoensak 2003), toxins of *B. thuringiensis* induced a strong cytopathic effect. Because BtK is known to be

specific to lepidopterean, the AFKM-On-H cell line was more sensitive to this toxin than to other Bt formulations. Moreover, this cell line was more sensitive than *H. armigera* and equally as sensitive as Sf9. No significant toxicity compared to the blank preparation was noted of trident preparation containing Btt, as noted in case of other insect cell lines. However, as noted for the four Colorado potato beetle (Charpentier et al. 2002) and Sf9 cell lines, a relatively higher sensitivity than expected of AFKM-On-H for the Bti toxin in Tecknar was demonstrated. This effect is most probably related to the association with Bti of CytA (Porter et al. 1993), a nonspecific toxin highly lytic to the cells of different origins.

Several other cell lines has been also demonstrated to be sensitive to the mycotoxins from *M. anisopliae* (Vey and Quiot 1989; Charpentier et al. 2002; Charoensak 2003), but interestingly, the AFKM-On-H cell line, as well as Sf9, demonstrated a very high sensitivity to these mycotoxins from 50 to 100 times more than other cell lines, such as *H. armigera* and *L. decimleneata* cell lines.

The reactions to 20 HE obtained in our experiments using the hemocyte corn borer cell line are different from those described previously on embryonic cell line from the same insect (Trisyono et al. 2000). Using our cell line, depending on the 20 HE dose, cell proliferation or inhibition, as well as filamentous cytoplasmic elongations together with the classical cell clumping effects were obtained and warrant further investigations. In this regard, it is expected that the AFKM-On-H cell line derived from hemocyte of *O. nubilalis* will be an extremely useful tool for screening and identifying the mode of actions of different compounds with ecdysone-like activity at the molecular level. Furthermore, it was demonstrated recently that insect hormones stimulate in vivo and in vitro the transcription of the ie-1 promoter of *B. mori* NPV (Zhou et al. 2002). One important application of the AFKM-On-H cell line will therefore be the use of this hemocyte cell line for the study on the synergetic effects of hormones on insect virus replication.

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References

Belloncik, S. Interactions of cytoplasmic polyhedrosis Viruses with insect cells. Adv Insect Phys. 26: 235–295; 1996.

Belloncik, S.; Arella, M.; Hudon, M.; Martel, P. Biological control program of the European Corn Borer *Ostrinia nubilalis* in the province of Quebec (Canada). Proc. of the 14th International Workshop Group on *Ostrinia nubilalis* meeting (Beijing, China), 103–109; 1988.

Belloncik, S.; Liu, L.; Su, D. M; Arella, M. Characterization and replication of Chinese isolate of *Heliothis armigera*. J. Invertebr. Pathol. 67, 41–47; 1996.

Belloncik, S.; Mori. Cypoviruses. In: Miller, L. K; and L. A. Ball, L. A., eds.: The Insect viruses. Plenum Publishing Corporation. New-York; 1998: 337–369.

Brousseau, C.; Charpentier, G.; Belloncik S. Susceptibility of spruce budworm, *Choristoneura fumiferana* Clemens to destruxins, cyclodepsi-peptide mycotoxyns of *Metarhizium anisopliae*. J. Invertebr. Pathol. 68:180–182; 1996.

Cerutti, M.; Devauchelle, G. Cell fusion induced by an invertebrate virus. Arch. Virol. 61: 149–155; 1979.

Cerutti, M.; Devauchelle, G. Inhibition of macromolecular synthesis in cells infected with an invertebrate virus (iridovirus type 6 or CIV) Arch. Virol. 63: 297–303; 1980.

Cerutti, M.; Guerillon J.; Arella M.; Devauchelle, G. La réplication de l'iridovirus de type 6 (CIV) dans différentes lignées cellulaires. C.R. Acad. Sci. 292: 797–802; 1981.

Charoensak, S. Establishment and some characteristics of cell line derived from embryonic tissue of the american cotton bollworm *Helicoverpa armigera* (Hubner) MSc thesis King Mongkut Institute of Technology, Ladkrabang Bangkok Thailand pp.118; 2003.

Charpentier, G.; Belloncik, S.; Ducros, G.; Fontenille, D.; Tian, L.; and Quiot, J. -M. 1995. Establishment and characterization of three cell lines from *Aedes triseriatus*. J. Med. Entomol. 32: 793–800; 1995.

Charpentier, G.; Belloncik S.; Ducros, G.; Fontenille, D.; Tian, L.; Quiot, J. M. Characterization of cell lines developped from the Colorado potato beetle, *Leptinotarsa decemlineata* say (Coleoptera: Chrysomelidae). In Vitro Cell. Dev. Biol.38A: 73–78; 2002.

Charpentier, G., Bordeleau-Cloutier, P.; Massicotte-Rousseau, Guérillon, J; Devauchelle, G. *Chilo suppressalis* iridescent virus induced antigens in insect cells and tissues: localization and kinetics of appearance. Ann. Inst. Pasteur/ Virol. 137E: 37–49; 1986.

Davis, T. R.; Wickham, T. J.; Mckenna, K. A.; Granados, R. R.; Shuler, M. L.; Wood, H. A. Comparative recombinant protein production of eight insect cell lines. In Vitro Cell. Dev. Biol. Anim., 29A, 388–390; 1993

Farinos, G. P.; De la Poza, P.; Hernandez-Crespo, P.; Ortego, F.; Castanera, P. Resistance monitoring of field populations of the corn borers *Sesamia nonagrioides* and *Ostrinia nubilalis* after 5 years of Bt maize cultivation in Spain. Entomologia Experimentalis et Applicata 110: 23–30; 2004.

Goodman, C. L.; El Sayed G. N.; McIntosh, A. H; Grasela, J. J.; Stiles, B. Establishment and characterization of insect cell lines from 10 lepidopterean species. In Vitro Cell. Dev. Biol.37A: 367–373; 2001a.

Goodman, C. L.; McIntosh, A. H. Production of baculoviruses for insect control using cell culture. In: maramorosh, K.; McIntosh, A. H., eds. Insect biotechnology. Boca Raton, FL: CRC Press: 1994; 33–56.

Goodman, C. L.; McIntosh, A. H.; El Sayed G. N.; Grasela, J. J.; Stiles, B. Production of selected Baculoviruses in newly established lepidopterean cell lines. In Vitro Cell. Dev. Biol.37A: 374–379; 2001b.

Granados, R. R.; Guoxun, L.; Derksen, A. C. G.; Mackenna, K. A. A new cell line from *Trichoplusia ni* (BTI-Tn-5B1-4) susceptible to *Trichoplusia ni* single enveloped nuclear polyhedrosis virus. J. Invert. Pathol., 64: 642–648; 1994.

Hink, W. F. Established cell line from the cabbage looper, *Trichoplusia ni*. Nature, 226: 466–467; 1970.

Hudon M.; LeRoux, E. J. Biology and population dynamics of the European corn borer (*Ostrinia nubilalis*) with special reference to sweet corn in Quebec. I. Systematics, morphology, geographical distribution, host range, economic importance. Phytoprotection 67:39–54; 1986.

Inoue, H.; Mitsuhashi, J. Further establishment of continuous cell lines from larval fat bodies of the Cabbage Armyworm, Mamestra brassicae (Lepidoptera:Noctuidae) Appl.Ent.Zool. 20(4):496–498; 1985.

Kärber, G. Beitrag zur kolliktiven behandlung pharmakologischer reihenversuche Naunyn-Schwiedebergs. Arch. Exp. Pathol. Pharmakol. 162: 680–687; 1931

Kelly, D. C.; Tinsley T. W. Iridescent virus replication: a microscope study of *Aedes aegypti* and *Antherea eucalypti* cells in culture infected with iridescent virus type 2 and 6. Microbios 9: 75–93; 1974.

Léry, X.; LaRue, B.; Cossette, J.; Charpentier, G. Characterization and authentication of insect cell lines using RAPD markers. Insect Biochemistry and Molecular Biology 33: 1035–1041; 2003.

Lewis, L. C.; Johnson, T. B. Efficacy of two nuclear polyhedrosis viruses against *Ostrinia nubilalis* (*Lep: Pyralidae*) in the laboratory and field. *Entomophaga* 27: 33–38; 1982.

Linacre N. A.; Thompson, C .J. Dynamics of insect resistance in Bt-corn. Ecological Modelling 171: 271–278; 2004.

Maeda, S. Gene tranfer vectors of a baculovirus, Bombyx mori nuclear polyhedrosis virus, and their use for expression of foreign genes. J.Mitsuhashi.Boca Raton F, editor:CRC Press.167–181. 1989.

Murhammer, D. W. Production of viral insecticides in cell culture and their use for pest control. Appl. Biochem. Biotechnol. 59: 199–220; 1996.

Nei, M.; Li, W. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269–5273; 1979.

Palomares, L. A.; Joosten, C E.; Hughes, P R.; Granados, R. R.; Shuler, M. L. Novel insect cell line capable of com.plex N-glycosylation and sialylation of recombinant proteins. Biotechnol Prog. Jan–Feb;19:185–92; 2003.

Palomares, L. A.; Ramírez, O. T. Insect cell culture: Recent advances, bioengineering challenges and implications in protein production. In *Advances in Bioprocess Engineering*; Ramírez, O. T., Galindo, E., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands; Vol. II, pp 25–52. 1998.

Porter, A. G., Davidson, E. W., Liu, J. W. Mosquitocidal toxins of bacilli and their genetic manipulation for effective biological control of mosquitoes. Microbiological Reviews 57: 838–861. 1993.

Schneider, I. Karyology of cells in culture. F. Characteristics of insect cells. In: Kruse, P. E. J.; Patterson, M. K. Jr., eds. Tissue culture methods and applications. New York: Academic Press;: 788–790. 1973.

Schnepf, E.; Crickmore, N; Van Rie, J; Lereclus, D; Baum, J; Feitelson, J; Zeigler, D. R; Dean, D. H. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiology and Molecular Biology Reviews 62: 775–806. 1998

Sohi, S. S.; Palli, S. R.; Cook, B. J.; Retnakaran, A. Forest insect cell lines responsive to 20-hydroxyexdysone and two nonsteroidal ecdysone agonists, RH-5849 and RH-5992. J. Insect. Physiol. 41: 457–464; 1995.

Tomita, S.; Kanaya, T.; Kobayashi, J.; Imanishi, S. Isolation of p10 gene from Bombyx mori nuclear polyhedrosis virus and study of its promoter activity in recombinant baculovirus vector system. Cytotechnology 17: 65–70; 1995.

Trisyono, A.; Goodman, C. L.; Grasela, J. J.; McIntosh, A. H.; Chippendale, M.G. Establishment and characterization of an *Ostrinia Nubilalis* cell line and its response to ecdysone agonists. In Vitro Cell. Dev. Biol.36A: 400–404; 2000.

Vaughn, J. L; Goodwin, R. H.; Thomkins, G. J; McCawley, P. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera::noctuidae) In Vitro. 13: 213–217; 1977

Vey, A.; Quiot, J. M. Effet cytotoxique in vitro et chez l'insecte hôte des destruxines toxines cyclodepsipeptidiques produites par le champignon *Metarhizium anisopliae.* Can. J. Microbiology.35: 1000–1008; 1989.

Weiss, S. A.; Vaughn, J. L. Cell culture methods for large-scale propagation of baculoviruses. In: Granados, R. R.; Federici, B. A., ed. The biology of baculoviruses. Vol. II. Boca Rtaon, FL: CRC Press: 63–87; 1986.

Wraight, S. P; Ramos, M. E. Synergistic interaction between *Beauveria bassiana*- and *Bacillus thuringiensis tenebrionis*-based biopesticides applied against field populations of Colorado potato beetle larvae. J. Invert. Pathol. 90: 139–150. 2005.

Zhou, Y.; Xiao, Q.; Zhang, Z.; He, J. Zhang. Foreign insect hormones stimulating the transcription of the *ie-1* promoter of *Bombyx mori* Nuclear polyhedrosis Virus in vivo and in vitro. Biosci. Biotechnol. Biochem. 66: 1488–194; 2002.