# REPLICATION OF A *MAMESTRA BRASSICAE* NUCLEAR POLYHEDROSIS VIRUS IN A NEWLY ESTABLISHED *MAMESTRA BRASSICAE* CELL LINE

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**Summary.** – A continuous cell line, designated MbL-3, was newly established from minced neonate larvae of the cabbage armyworm, *Mamestra brassicae.* This new cell line is heteroploid, containing triploid cells predominantly at the frequency of about 50%. Doubling time of the cell population is 33 hrs. The Akutsu isolate of a *Mamestra brassicae* nuclear polyhedrosis virus (MbNPV) was examined for replication in 16 continuous cell lines, including the cell line MbL-3, from seven lepidoptera species: *Mamestra brassicae, Pseudaletia separata, Spodoptera exigua, Spodoptera frugiperda, Spodoptera littoralis, Spodoptera litura* and *Plutella xylostella*. Of these cell lines, only the MbL-3 cells supported a high replication of the virus. Four other *Mamestra* cell lines and a *P. separata* cell line showed a low virus-susceptibility, while the other cell lines were not permissive for the virus infection. Maximum infection rate of MbL -3 cells to the Akutsu isolate was 23.4%. The growth kinetics of this virus isolate in MbL-3 cells showed that the virus was released from infected cells 24 hrs p.i. and reached a maximal titer 96 hrs p.i. The number of polyhedral inclusion bodies (PIBs) reached a maximum of $10^{7.5}$ PIBs/ml 120 hrs p.i.

**Key words:** nuclear polyhedrosis virus; *Mamestra brassicae* cell line

## Introduction

Nuclear polyhedrosis viruses (NPVs) are the representative of invertebrate viruses with supercoiled double-stranded DNA genomes of 80 to 230 kbp, belonging to the subfamily *Eubaculovirinae* of the family *Baculoviridae* (Francki *et al.*, 1991). NPVs have been found in many insect species belonging to the orders *Lepidoptera, Hymenoptera, Coleoptera, Diptera, Orthoptera, Trichoptera* and *Crustacea* (Martignoni and Iwai, 1981). They are considered to be the ecologically sound agents for control of insect pests in agriculture and forestry because they are highly specific for target pests and non-hazardous for non-target organisms and the environment (Adams and McClintock, 1991). Moreover, they have recently been employed as expression vectors for many foreign genes (Tanada and Kaya, 1993).

Since continuous insect cell lines were first established by Grace (1962) from a lepidopteran insect *Antheraea eucalypti,* many insect cell lines have been described and the number of studies on the *in vitro* replication of insect-pathogenic viruses have dramatically increased (Granados and Federici, 1986; Tanada and Kaya, 1993).

The cabbage armyworm *Mamestra brassicae* is a common pest of cole crops, and it attacks also rice, pear, onion and tomato. MbNPV has been reported to be one of the key factors suppressing the population of this insect in the field (Vlak and Gröner, 1980). Recently, *in vivo* studies on the host range, pathogenicity and biochemical characteristics of MbNPV have been extensively conducted (Vlak

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**Abbreviations:** FBS = foetal bovine serum; MbNPV = *Mamestra brassicae* nuclear polyhedrosis virus; MOI = multiplicity of infection; NPV = nuclear polyhedrosis virus; p.i. = post infection; PIB = polyhedral inclusion body; SDS = sodium dodecyl sulfate; SE = standard error

and Gröner, 1980; Brown *et al.*, 1981; Wiegers and Vlak, 1984). To our knowledge, however, there have been only a few reports dealing with the *in vitro* replication of MbNPV (Miltenburger and David, 1976; Evance *et al.*, 1981).

In the present paper, the establishment of a cell line from neonate larvae of *M. brassicae* and the successful replication of MbNPV in this newly established cell line are described.

## Materials and Methods

*Insect and cell lines.* A laboratory-maintained culture of *M. brassicae* was kindly provided by Dr. C. Goto, National Agriculture Research Center, Tsukuba, Japan. The larvae were reared on artificial diet (Okada, 1990; H. Hayashi, personal communication) at 27 °C under a photoperiod of 16L-8D. The origin and source of 16 insect cell lines used are listed in Table 1. The cells were grown in the IPL-41 medium (Dougherty *et al.*, 1981) supplemented with 10% heat-inactivated (60 °C, 30 mins) foetal bovine serum (FBS) (Whittaker M.A. Products, Inc., Maryland) at 27 °C, and subcultured at 4- to 5-day intervals.

*Establishment of MbL-3 cell line.* A method developed by Hara *et al.* (1993) was employed to establish the new cell line. Eggs of *M. brassicae* were surface-sterilized with 70% ethanol for 30 secs and with 0.1% $HgCl_2$ for 10 mins, and then rinsed three times with sterile distilled water. Hatched neonate larvae were minced with scissors, and the fragments were transferred to 25 $cm^2$ tissue culture flask (Falcon, 3813) and incubated at 27 °C in IPL-41 medium containing 10% heat-inactivated FBS.

*Cell growth kinetics.* Cells were seeded at a density of 2.0 x $10^5$/ml in 25 $cm^2$ flasks and incubated at 27 °C. Cell counts were made every 24 hrs for 10 days.

*Karyotype analysis* was conducted as described previously by Hara *et al.* (1993).

*Virus and in vitro inoculation.* The Akutsu isolate of MbNPV was provided as infected dead larvae by Dr. M. Okada, Chugoku National Agricultural Experiment Station, Fukuyama, Japan. The dead larvae were homogenized in 1 mol/l NaCl and the resulting suspension was filtrated through several layers of cheesecloth. PIBs were washed in 1 mol/l NaCl and centrifuged at 3,000 rpm for 10 mins at 4 °C.

Third- to fourth-instar *M. brassicae* larvae were fed on artificial diet surface-contaminated with 4.0 x $10^6$ PIBs/larva. Five days after inoculation, haemolymph from several living larvae was pooled and centrifuged at 8,000 rpm for 10 mins. The supernatant, containing extracellular virus "infected haemolymph" was stored at -80 °C until use. For virus inoculation *in vitro*, 0.1 ml of the infected haemolymph diluted 1:100 in IPL-41 medium was incubated with 0.4 ml of cells in exponential phase in a multiwell tissue culture plate (Falcon, 3047) at 27 °C with a cell density of 6 x $10^5$/ml. Glutathione (0.6 mg/ml), for prevention of melanization, and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml) were added. Wells were examined daily for 10 days for the presence of PIBs by phase-contrast microscopy. Infection rate was calculated 5 days p.i. in 1,000 cells on the basis of the polyhedral formation.

*Kinetics of virus growth.* 0.4 ml of the cells in exponential phase were seeded per well in a multiwell tissue culture plate (Falcon, 3047) at a density of 6.0 x $10^5$ cells/ml in IPL-41 medium supplemented with 10% heat-inactivated FBS, antibiotics and glutathione. The Akutsu isolate of MbNPV was inoculated to cells at a multiplicity of infection (MOI) of 0.05 $TCID_{50}$/cell. Virus adsorption was allowed to proceed by centrifugation at 1,600 rpm for 1 hr at 27 °C. Subsequently, cells were washed once with IPL-41 medium and resuspended in fresh medium (0.5 ml/well). At various intervals, a portion of the culture (plate) was removed and centrifuged at 3,000 rpm for 10 mins. The supernatant was stored at -80 °C until virus titration. The virus titer was determined by the technique described by Hara *et al.* (1994). $TCID_{50}$ values were calculated in a standard way.

For quantification of PIBs, infected cell cultures were harvested at various intervals, and centrifuged at 3,000 rpm for 10 mins. The pellet was suspended in IPL-41 medium containing 1% SDS. The mixture was then incubated at 27 °C for 30 mins, followed by three independent PIB counts in a haemocytometer.

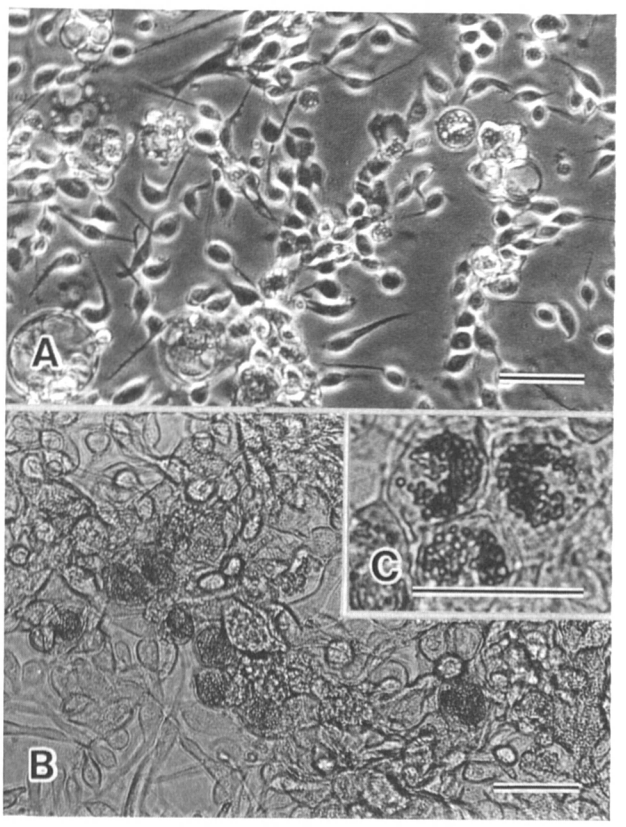


**Fig. 1**
**Phase-contrast microphotographs of MbL-3 cells newly established from neonate larvae of the cabbage armyworm *Mamestra brassicae***
(A) Normal cells (bar = 50 µm). (B) and (C) Cells 5 days p.i. with the Akutsu isolate of MbNPV (bar = 50 µm).

## Results

*Establishment of the MbL-3 cell line*

Eight primary cultures were initially prepared from minced neonate larvae of *M. brassicae*. About 7 days after seeding the culture, spherical cells were released from larval tissue fragments, followed by a slow increase in the number of cells. Two months later, continuous cell growth was observed in only one culture, while other cultures deteriorated at this time. About 100 days later, the flask bottom was fully covered with adherent cells. At the first passage, cells were harvested by use of cell scraper and transferred into a new flask containing fresh medium. Cells were then subcultured at 7- to 10-day intervals. Approximately 200 days later, fibroblast-like cells (Fig. 1A) became predominant and, thereafter, the passage of cells was performed at 4- to 5- day intervals. This established cell line was designated MbL-3.

*Characterization of the MbL-3 cell line*

The population of the Mbl-3 cells was found to be heteroploid and polyploid. Triploid cells accounted for approximately 50% of the population (Fig. 2). The growth curve of the MbL-3 cells is shown in Fig. 3. A lag phase of 2.5 days was followed by an exponential growth phase. The stationary phase was initiated on the day 7. The population doubling time of the MbL-3 cells was 33.6 ± 7.8 hrs. The cell density reached a maximum of $2.2 \times 10^6 \pm 0.2$ cells/ml on the day 8.

*Susceptibility of 16 lepidopteran cell lines to MbNPV*

Table 1 shows the results of experiments with 16 lepidopteran cell lines, including the newly established MbL-3 cell line, inoculated with the Akutsu isolate of MbNPV. Of these, only the MbL-3 cell line was highly susceptible to this virus (Fig. 1B, 1C) with cell infection rate of 23.4 ± 1.7%. Two homologous cell lines, NIAS-MB-25 and NIAS-MB-32, and the *Pseudaletia separata* cell line NIAS-LeSe-11 showed a lower susceptibility to the Akutsu isolate; the infection rate in these cell lines was below 10%. In two other *Mamestra* cell lines, SE-MaBr-1 and NIAS-MaBr-92, only a few cells formed PIBs, showing very low susceptibility to the virus. Other cell lines of five lepidopteran species were not susceptible at all.

*Growth kinetics of MbPNV in MbL-3 cells*

The result of a growth kinetics study of the Akutsu isolate in the MbL-3 cell line is shown in Fig. 4. The extracellular virus began to increase slowly 48 hrs p.i., followed by a logarithmic growth phase. At 96 hrs p.i., the titer reached a maximum of $10^{3.9}$ $TCID_{50}$/ml. The number of PIBs began to increase rapidly at 24 hrs p.i. This was followed by a stationary phase and a maximum ($10^{7.5}$ PIBs/ml) was reached 120 hrs p.i.

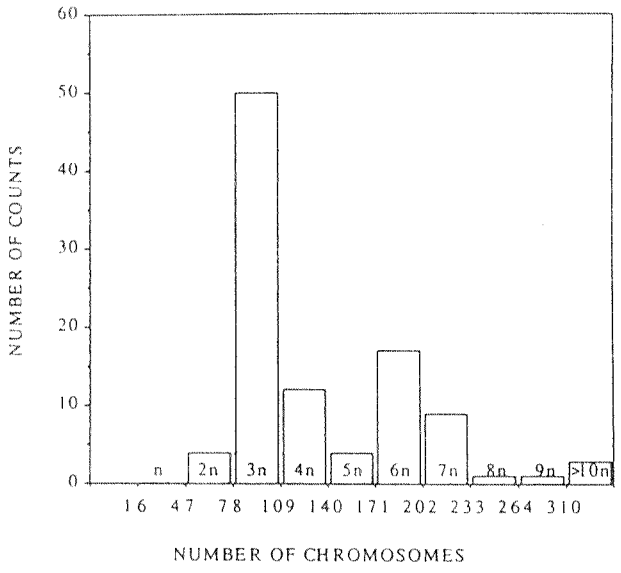


**Fig. 2**
**Histogram of the number of chromosomes of the *Mamestra brassicae* cell line MbL-3 at the passage 39**

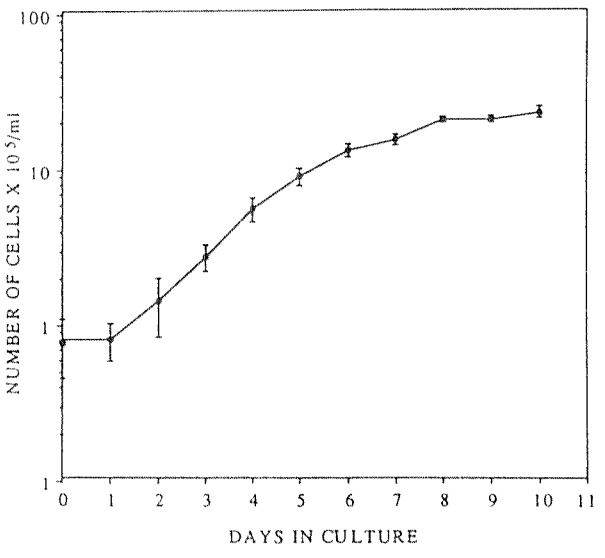


**Fig. 3**
**Growth curve of the *Mamestra brassicae* cell line MbL-3 at the passage 46**
Vertical lines represent SE.

**Table 1. Susceptibility of insect cell lines to the Akutsu isolate of *Mamestra brassicae* NPV**

| Insect species | Cell line | Primary explant | Source | Susceptibility to MbNPV[f] |
|---|---|---|---|---|
| *Mamestra brassicae* | MbL-3 | Neonate larvae | This study | +++ |
| | SES-MaBr-1 | Fat body | J.Mitsuhashi[a],1991 | + |
| | NIAS-MB-25 | Pupal ovary | J.Mitsuhashi,1991 | ++ |
| | NIAS-MB-32 | Pupal ovary | J.Mitsuhashi,1983 | ++ |
| | NIAS-MaBr-92 | Hemocyte | J.Mitsuhashi,1991 | + |
| *Pseudaletia separata* | NIAS-LeSE-11 | Fat body | J.Mitsuhashi,1991 | ++ |
| *Spodoptera exigua* | Se3FH | Neonate larvae | K.Hara[b],1993 | – |
| | Se4FH | Neonate larvae | K.Hara,1993 | – |
| | Se5FH | Neonate larvae | K.Hara,1993 | – |
| | Se6FHA | Neonate larvae | K.Hara,1993 | – |
| | Se6FHB | Neonate larvae | K.Hara,1993 | – |
| *Spodoptera frugiperda* | SF9 | Pupal ovary | T.Hara[c], 1991 | – |
| | SF21AEII | Pupal ovary | J.Vaughn[d], 1979 | – |
| *Spodoptera littoralis* | CLS79 | Pupal ovary | J.Vaughn, 1979 | – |
| *Spodoptera litura* | TUAT-SpLi-221 | Pupal ovary | J.Mitsuhashi,1990 | – |
| *Plutella xylostella* | PXL/C | Neonate larvae | T,Sato[e], 1990 | – |

[a]Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan.
[b]Institute of Biological Control, Kyushu University, Fukuoka, Japan.
[c]Institute of Genetic Resources, Kyushu University, Fukuoka, Japan.
[d]Department of Agriculture, Beltsville, Maryland, USA.
[e]National Institute of Sericultural and Entomological Science, Ibaraki, Japan.
[f]Infection rate: more than 20% (+++), less than 10% (++), less than 1% (+); PIBs not formed (–).

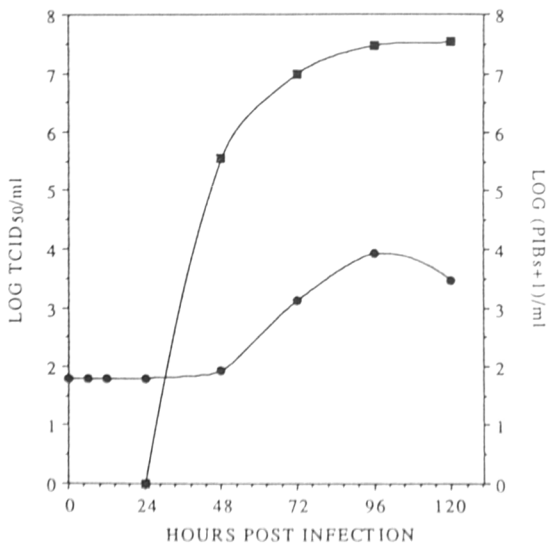


**Fig. 4**
**Replication of the Akutsa isolate of *Mamestra brassicae* NPV in the MbL-3 cell line**
Cells were infected at a MOI of 0.05 $TCID_{50}$/cell. The extracellular virus (●) was monitored by $TCID_{50}$ assay, and the total number of PIBs (■) was assayed after SDS treatment of the cells.

## Discussion

Cell lines with high susceptibility to MbNPV are essential for biological and biochemical characterization of this virus. As yet, however, there have been no reports about the existence of cell lines highly susceptible to MbNPV (Miltenburger and David, 1976; Mitsuhashi, 1977; Mitsuhashi and Shozawa, 1985; Inoue and Mitsuhashi, 1985). In the present study, a MbNPV isolate showed low infectivity for the four existing *Mamestra* cell lines, including NIAS-MB-25 and NIAS-MB-32 cells, and a *P. separata* cell line, but high infectivity for our newly established cell line MbL-3. Our results differ from those previously reported by Mitsuhashi (1977) who claimed that the cell lines NIAS-MB-25 and NIAS-MB-32 were not permissive for MbNPV. Such disagreement may be caused by differences in the virus strains used and/or by changes in virus susceptibility of the cell lines during subcultivation.

The previously established *M. brassicae* cell lines were mainly derived from selected tissues such as ovaries, fat bodies and haemocytes (Miltenburger and David, 1976; Mitsuhashi, 1977; Mitsuhashi and Shozawa, 1985; Inoue and Mitsuhashi, 1985). Recently, Hara *et al.* (1993) have successfully obtained *Spodoptera exigua* cell lines from minced neonate larvae, highly susceptible to *S. exigua* NPV. In this study, we also used minced neonate larvae as the source of primary cultures of *M. brassicae* cells, since it is very likely that the use of highly heterogeneous tissue mixtures may lead to a higher chance of establishing NPV-susceptible cell lines. In fact, this method successfully generated such a cell line in the present study, although the origin of these cells is uncertain. Earlier, Mitsuhashi (1977)

reported that the two previously established *M. brassicae* cell lines (NIAS-MB-25 and -32) consisted mainly of diploid cells but triploid, tetraploid and highly polyploid cells also coexisted. In our new *M. brassicae* cell line MbL-3, the rate of heteroploid cells was above 90% as early as 8 months after initiation of the cell culture; triploid cells were predominant and more highly polyploid cells were also commonly present. This result suggests that the heteroploidy and polyploidy ocurred at the early stage of establishment of this cell line. The growth curve of MbL-3 cells was similar to that of NIAS-MB-32 reported by Mitsuhashi (1977); however, the cell population doubling time (34 hrs) was far shorter than that (48 hrs) of NIAS-MB-32 cells.

One of the most striking aspects of our results is that the MbL-3 cells are highly susceptible to NPVs from the homologous insect species, when compared to the existing lepidopteran cell lines including *M. brassicae* cells. This is the first report on the growth kinetics of the MbNPV in cells from the homologous host. The replication of MbNPV in the Mbl-3 cell line showed three phases: a latent phase, an exponential phase, and a stationary phase similarly to other NPVs (Hara *et al.*, 1994).

In contrast to the other cases, however, the level of extracellular virus was relatively low, although the degree of PIB production was very high. A similar result was obtained in the *in vivo* experiments: the extracellular virus titer in infectious larval haemolymph was substantially low while numerous PIBs were produced in the same infected larvae (unpublished results). Thus, the Akutsu isolate of MbNPV is a unique in that the major portion of virus population produced is occluded on PIBs, while the minor portion represents the extracellular virus.

Cloning of the *M. brassicae* cell lines highly susceptible to MbNPV as well as the cloning of highly pathogenic virus strains may lead to the better understanding of biological properties of MbNPV *in vitro*.

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## References

Adams JR, McClintock JT (1991): Baculoviridae. In Adams JR, Bonami JR (Eds): *Atlas of Invertebrate Viruses.* CRC Press, Boca Raton, pp. 87–204.

Brown DA, Evans HF, Allen CJ, Kelly DC (1981): Biological and biochemical investigations on five European isolates of *Mamestra brassicae* nuclear polyhedrosis virus. *Arch. Virol.* **69**, 209–217.

Dougherty ED, Weiner RM, Vaughn JL, Reichelderfer CF (1981): Physical factors that affect *in vitro Autographa californica* nuclear polyhedrosis virus infection. *Appl. Environ. Microbiol.* **41**, 1166–1172.

Evance HF, Lomer CJ, Kelly DC (1981): Growth of nuclear polyhedrosis virus in larvae of the cabbage moth, *Mamestra brassicae* L. *Arch. Virol.* **70**, 207–214.

Francki RIB, Fauquet CM, Knudson DL, Brown F (1991): *Classification and Nomenclature of Viruses.* Fifth Report of the International Committee on Taxonomy of Viruses. *Arch. Virol. Suppl.2.* Springer-Verlag, Wien-New York.

Grace TDC (1962): Establishment of four strains of cells from insect tissues grown *in vitro. Nature* **195**, 788–789.

Granados RG, Federici BA (1986): *The Biology of Baculoviruses.* CRC Press, Florida.

Hara K, Funakoshi M, Tsuda K, Kawarabata T (1993): New *Spodoptera exigua* cell lines susceptible to *Spodoptera exigua* nuclear polyhedrosis virus. *In Vitro Cell. Dev. Biol.* **29**, 904–907.

Hara K, Funakoshi M, Tsuda K, Kawarabata T (1994): Susceptibility of lepidopteran cell lines to a *Spodoptera exigua* (Lepidoptera; Noctuidae) nuclear polyhedrosis virus. *App. Entomol. Zool.* **29**, 395–402.

Inoue H, Mitsuhashi J (1985): Further establishment of continuous cell lines from larval fat bodies of the cabbage armyworm, *Mamestra brassicae* (Lepidoptera: Noctuidae). *Appl. Entomol. Zool.* **20**, 496–498.

Martignoni ME, Iwai PJ (1981): A catalogue of viral diseases of insects, mites and ticks. In Burges HD (Ed.): *Microbial Control of Pests and Plant Diseases 1970 – 1980.* Academic Press, London, pp. 897–911.

Miltenburger HG, David P (1976): Nuclear polyhedrosis virus replication in permanent cell lines of the cabbage moth (*Mamestra brassicae* L.). *Naturwissenschaften* **63**, 197–198.

Mitsuhashi J (1977): Establishment and characterization of continuous cell lines from pupal ovaries of the cabbage armyworm, *Mamestra brassicae. Develop. Growth Differ.* **19**, 337–344.

Mitsuhashi J, Shozawa A (1985): Continuous cell lines from larval hemocytes of the cabbage armyworm, *Mamestra brassicae. Develop. Growth Differ.* **27**, 599–606.

Okada M (1990): Present status of the study of pest control by insect viruses. *Agric. Res. Ser. NARC* **18**, 187–293 (in Japanes).

Tanada Y, Kaya HK (1993): *Insect Pathology.* Academic Press, California.

Vlak JM, Gröner A (1980): Identification of two nuclear polyhedrosis viruses from the cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol.* **35**, 269–278.

Wiegers FP, Vlak JM (1984): Physical map of the DNA of a *Mamestra brassicae* nuclear polyhedrosis virus variant isolated from *Spodoptera exigua. J. Gen. Virol.* **65**, 2011–2019.