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IN VITRO STUDIES ON THE VIRULENCE OF *SPODOPTERA LITURA* BACULOVIRUS

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Baculoviruses have been used as biopesticides against economic pests in agriculture, forestry and landscapes. *In vitro* production of baculoviruses has often been considered especially because of the ease of large-scale propagation. In this study, *in vitro* production was investigated by the infectivity of two genera of baculoviruses, Nucleopolyhedrosis virus (NPV) and Granulovirus (GV) to cultured lepidopteran cells. Production of baculoviruses depends on the ability of these cells to replicate in an optimal condition. *Spodoptera litura* baculovirus isolated locally was investigated for its virulence to two cell lines by inoculating with both forms of the virus, occlusion-body derived virions (PDV) and budded virus (BV), from SpltNPV and SpltGV. Efforts to develop cell cultures from the local *Spodoptera litura* for use in replicating these local baculoviruses was, however, unsuccessful due to cell deterioration or microbial contamination. A cell line from *Spodoptera frugiperda* (Sf9) was susceptible to budded virus obtained directly from the insect, but not to viral progeny. While these results suggest Sf9 cells have potential for replicating these baculoviruses, the *Spodoptera litura* cell line, TUAT-Spli-221 appeared not to be susceptible.

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VIRULASI VIRUS BACULO *SPODOPTERA LITURA* DIDALAM KULTUR TISU SERANGGA

Oleh

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Pengerusi: **Profesor Norani Abdul Samad, Ph.D.**

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Virus baculo telah digunakan sebagai racun terhadap serangga perosak utama di dalam sektor pertanian, perhutanan dan lanskap. Pembuatan virus baculo secara kultur semakin diberi perhatian terutamanya kemudahan di dalam pembuatan secara pukal. Di dalam kajian, pembuatan secara kultur telah dilakukan kepada dua jenis virus baculo, iaitu virus Nucleopolihedrosis (NPV) dan virus Granulosis (GV), untuk melihat kemampuan virus-virus tersebut menjangkiti sel kultur lepidoptera. Pembuatan virus baculo ini bergantung kepada kemampuan sel untuk mereplikasi virus pada kadar yang optimum. Virus baculo *Spodoptera litura* tempatan telah dikaji tahap jangkitannya terhadap dua jenis sel kultur dengan menginokulasikan terhadap dua jenis virus, iaitu virus virion and virus budded dari SpltNPV dan SpltGV. Kemampuan untuk menghasilkan sel kultur daripada *Spodoptera litura* bagi mereplikasikan virus baculo di atas tidak berhasil kerana deteriorasi sel atau pencemaran mikrob. Sel Sf9 dapat dijangkiti virus budded yang didapati daripada larva, tetapi sel tersebut tidak dapat dijangkiti virus progeni. Ujikaji ini menunjukkan sel Sf9 mempunyai potensi untuk mereplikasi virus baculo di atas, tetapi sebaliknya bagi sel *Spodoptera litura*, TUAT-Spli-221.

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LIST OF ABBREVATIONS

Ac	- *Autographa californica*
BDSA	- N-Benzyldimethylamine
Bm	- *Bombyx mori*
BV	- budded virus
Cp	- *Cydia pomonella*
DDSA	- Dodecenyl Succinic Anhydride
DIPs	- defective interfering particles
DMSO	- Dimethylsulfide
ECV	- extracellular virus
EF	- enhancing factor
EGT	- ecdysteroid glucosyltransferase
FBS	- Fetal bovine serum
FP	- few polyhedra
GV	- Granulovirus
Hz	- *Helicoverpa zea*
IPM	- Integrated pest management
Ld	- *Lymantaria dispar*
M&M	- Mitsuhashi and Maramorosch
MNA	- Methyl Nadic Anhydride
MNPVs	- multiple nuleopolyhedroviruses
MOI	- multiplicity of infection
NOV	- nonoccluded virus
NPV	- Nucleopolyhedrosis virus
OBs	- occlusion bodies
ODV	- occlusion-body derived virus
OV	- occluded virus
p.i.	- post infection
PDV	- polyhedral-derived virions
PIBs	- polyhedra inclusion bodies
PTM	- Potato tuber moth
REN	- restriction endonuclease
RIKEN	- Physical and chemical research
SDS	- sodium dodecyl sulphate
Se	- *Spodoptera exigua*
SNPVs	- singly nucleopolyhedrosis virus
Spli	- *Spodoptera littoralis*
Splt	- *Spodoptera litura*
SyF	- synergistic factor

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Lepidopteran larvae of the common cutworm, *Spodoptera litura* are a major pest of vegetable crops and young forest trees in Malaysia (Sajap, 1995a). Most notably, this insect is a serious pest of tobacco plants (*Nicotiana tabacum*) as well as maize, tomato, groundnuts, legumes and many other economically important crops. Tobacco cultivation in this country is a profitable enterprise since the crop has an excellent domestic and export market potential (Yunus, 1975). Since the caterpillars cause leaf damage, they lessen leaf collecting and cause spoilage that affects the quality of tobacco leaves, thereby causing high revenue loss. All stages of larvae devour these leaves and a larva usually needs two tobacco leaves to complete development (Yahya, 1985).

Because of the economic losses caused by this pest, insecticides are commonly used in controlling the pest in Malaysia, especially methamidophos, acephate and permethrin (Yahya and Abdul Karim, 1989). However, these insects gradually develop resistance to most of the common commercial pesticides. To reduce excessive use of chemical insecticides, as well as herbicide and fertilisers, integrated pest management (IPM) programmes have been introduced. IPM

approaches ensure greater profit for farmers, less pollution to the environment and reduced health hazards to farmers.

Baculoviruses infect many economically important insect pests (Yunus and Ho, 1980) and so are potential candidates for biological control (Muhammer, 1996). Because of the devastating effects that they can have on natural populations of insects, they are an obvious choice for agricultural insect pest control. Hence, baculoviruses formulations can be used to control insect pest in vegetables, fruits, forests and crop plantations.

Baculoviruses have only been isolated from invertebrates and are the causative agent of fatal diseases in insects (Van Oers and Vlak, 1997). They are highly virulent to specific insect species, and are not pathogenic to vertebrates or plants. These features make them useful in biological control of insect pests for reducing pest populations. Most baculoviruses are host specific that is; each is capable of infecting only the species from which it was isolated although some have been shown capable of infecting several host species.

To date, baculoviruses-based biopesticides have only been produced in larvae, which is tedious and time consuming. Insect tissue and cell culture has a great potential for studying many aspects of virus-host relationships. In addition to their use for studying baculoviruses, tissue and cell cultures are promising for viral

production as biopesticides. To accomplish this, *in vitro* host ranges of the viruses need to be established as well as the productivity of various cell lines.

Cell lines of *S.litura* have been established in several Asian countries, including China (Shih *et al.*, 1997), Japan (Mitsuhashi, 1995), and India (Pant *et al.*, 1998). Although, several cell lines have been established, very few have been shown to replicate virus. Not all baculoviruses isolated from infected larvae can be replicated in established cell lines and it is very rare for replication of granulosis virus (GV) *in vitro* (Winstanley and Crook, 1993). *S. litura* baculoviruses have been isolated in China, Japan, and India (Mitsuhashi, 1995). *S. litura* nuclear polyhedrosis virus (SpltNPV) replication has been reported in cell lines, but there is no published report of successful cultivation of SpltGVs replication. Both NPVs and GVs from *S. litura* have been isolated in Malaysia and may have potential as a biopesticide (Sajap *et al.*, 2000).

Baculoviruses of the same species isolated from different geographical isolates may have different virulence to particular cell lines. Kislev (1986) showed that NPVs isolated in different areas give different levels of infection, ranging from 90% of cells infected to less than 3%. Cells from the same species were usually susceptible to infection and possibly even within the same genus.

Findings suggest that baculoviruses in nature are heterogeneous populations of variant genomes (Brown *et al.*, 1985). In this regard, many variants of

baculoviruses have been isolated, e.g. *Spodoptera frugiperda* MNPV (Maruniak *et al.*, 1984), *Autographa californica* MNPV (Smith and Summers, 1979) and *Pieris rapae* GV (Crook, 1981). Variation in resistance to infection of *Spodoptera littoralis* larvae to *Spodoptera littoralis* NPV has also been documented.

Genetic variant of the same species or genus may result in variation of virulence. One variant may be more pathogenic for a particular pest insect species than others. Cloning strains with best pesticide ability for safety reason are greatly encouraged to avoid any problems encountered for long-term usage.

1.2 Objective

As an approach to these studies, baculoviruses isolated locally GVs and NPVs; were evaluated for their virulence to tissue culture. The objective of this research is to observe whether GVs and NPVs are able to replicate *in vitro*, to obtain permissive cells to these viruses and to evaluate the potential of S. *litura* cell line. To test the susceptibility of insect cells to these baculoviruses, two cell lines were used; one of which was derived from the same species and the other, the same genus, respectively, TUAT-SpLi-221 (*S. litura*) and Sf9 (*S. frugiperda*).

CHAPTER 2

LITERATURE REVIEW

2.1 The Baculoviridae

Baculoviruses are a family of large, enveloped viruses that are pathogenic to arthropods. While found predominantly infecting the order Lepidoptera, i.e. moths and butterflies, they have also been isolated from Hymenoptera, Diptera, Coleoptera, Neuroptera, Trichoptera, and Thysanura as well as the crustacean order Decapoda (shrimp) (Murphy *et al.*, 1995). These viruses have been reported from over 600 species (Blissard and Rohrmann, 1990), and although found in many different species, individual isolates usually have a restricted host range.

Specific baculoviruses are named based on their larval host origin (Murphy *et al.*, 1995). They infect and cause fatal disease in insects, including many pests of crops and forests. Baculoviruses are usually species specific (Sajap *et al.*, 2000). Their specificity and low environmental toxicity make them excellent candidates as biological pesticides in both forest and agricultural applications (Muhammer, 1996). In Brazil, baculoviruses are annually applied to over 1,000,000 ha of soybeans for the control of the velvetbean caterpillar, *Anticarsia gemmatalis* and are also applied to about 100,000 ha of cotton annually in China (Moscardi, 1999).

Several baculovirus pesticides have been registered in North America, Europe and Australia. For example, Gypchek and Spod-X, viral formulations of multiple nucleopolyhedroviruses from the gypsy moth, *Lymantaria dispar* (LdMNPV) and *Spodoptera exigua* (SeMNPV), respectively; GemStar LC, singly-embedded NPV from *Helicoverpa zea* (HzSNPV); and Cyd-X, a granulosis from the codling moth, *Cydia pomonella* (CpGV) have been used to control forest, vegetable, foliage or fruit pests (Possee *et al.*, 1997; Muhammer, 1996).

However, the use of these biological insecticides has only been partially successful because of their limited effectiveness. For example, baculoviruses have a slow rate of killing compared with chemicals, and thus allow the insect pests to continue feeding for many days after treatment. It takes typically from 4 days to 2 weeks for most baculovirus to kill their insect hosts. During the infection period, significant crop damage occurs. In fact, more food is consumed during this time interval by an infected insect than by an uninfected insect (O'Reilly *et al.*, 1991).

Increased feeding of the insect host is a strategy of the virus to maximise its growth and survival rather than evolving to have ideal properties as a pesticide (Miller, 1995). The viral strategy to maximise its progeny production by allowing an infected host to continue feeding is against the purpose of an effective bioinsecticides which is to kill pests as fast as possible. Furthermore, a particular baculovirus has a restricted host range, and so, unlike conventional chemical pesticides, a formulation containing just one virus may not be sufficient when

treating a crop with a complex of pest species. Also, baculovirus pesticides are significantly more expensive than chemical insecticides. Current efforts to genetically engineer baculoviruses may help overcome these deficiencies (Possee *et al.*, 1997).

In spite of these shortcomings, baculoviruses do have certain advantages over chemical pesticides. For example, chemical pesticides pose a potential health and environmental hazard and can also lead to increased insect resistance, thereby limiting their future use. Integrated pest management (IPM) is becoming a major interest since it involves minimising the use of chemical insecticide and using biological control agents as pest controls in both agricultural and forestry sectors (Sajap, 1995b). Insect viruses such as baculoviruses are suitable for IPM as they have minimum effect on nontarget insects; such as parasitic wasps and honey bees (Muhammer, 1996). Since baculoviruses are species specific, they do not cause lethal effects towards beneficial insects and this characteristic increases its potential as a biological control agent. When applied to crop plantations, baculovirus formulations will not exploit the natural ecology and ecosystem.

Moreover, baculovirus pesticides are of natural base and therefore do not cause health problems in humans or other animals. Most crucially, they sustain continuous effectiveness because susceptible insects do not develop resistance to them as compared to chemical pesticides. Chemical pesticides gradually select resistant insect pests and eliminate susceptible ones. This phenomenon will

progressively increases resistant insect pest populations in the environment and eventually chemical pesticides might become ineffective.

Besides their potential as biopesticides, baculoviruses possess large genomes which make them easy to be manipulated as protein expression system. This is done by inserting foreign or heterologous gene into baculovirus genomes (Pennock, *et al.*, 1984). Baculovirus expression vector systems (BEVS) are preferred for recombinant protein production over both bacterial and mammalian system since insect cells grow at room temperature and do not require carbon dioxide, which simplify the growing and maintenance of cells.

Thus, baculoviruses are being extensively used in research and for medical, pharmaceuticals and veterinary practices (Van Oers and Vlak, 1997). Additionally, baculoviruses are capable of infecting, but not replicating in, a wide range of human and other cell types. This has led to an investigation on baculoviruses as possible gene therapy vectors or as a means of transducing different cell types (Yamao *et al.*, 1999).

2.2 Virion Structure

Baculoviruses contain a double stranded DNA with circular, supercoiled and covalently closed genome varying in size between 100-180 kilobases (Volkman *et al.*, 1995). The genomes are encapsulated in a rod-shaped protein shell or capsid

(Summers and Anderson, 1972). The capsid is 200-400 nm in length and 40-50 nm in width (Harrap, 1972). The DNA is condensed in the capsid with a protein, known as p6.9 protein forming a nucleoprotein structure (Wilson *et al.*, 1987). The combination of the nucleic acid and protein capsid is a nucleocapsid, which is encased in a lipoprotein membrane (envelope) to form virions or virus particles. Baculovirus virions exist in two types, as a single nucleocapsid in an envelope (such as GVs or SNPVs) or many nucleocapsids (multiple) in an envelope (MNPVs).

2.2.1 Genera of Baculoviridae

There are two genera of baculoviruses; the Nucleopolyhedrovirus (NPV) (Rohrmann, 1999) and Granulovirus (GV) (Winstanley and O'Reilly, 1999), both of which have a size of between 1 to 5µm. Nucleopolyhedroviruses have large polyhedral occlusion bodies (OBs) formed by a crystalline matrix composed of polyhedrin protein. This protein primarily functions to protect the virions and viral DNA from environmental decomposition and inactivation by UV light (Blissard and Rohrmann, 1990).

NPVs are divided into two subgenera, single (SNPVs) and multiple (MNPVs) nucleopolyhedroviruses (Murphy *et al.*, 1995). For SNPV, each envelope contains only one virion in the OBs whereas for MNPV, there are multiple nucleocapsids per envelope embedded in the crystalline matrix. The OBs of both subgenera of

NPVs contain multiple virions. The NPV from *Autographa californica* (AcMNPV) is the type species and the most extensively studied. NPVs are able to infect a variety of cells including the midgut, fat body, tracheal, blood cells, hypodermis, Malphigian tubules, muscle sarcolemma, nerve fibre sheaths and pericardial cells (Harrap, 1970).

GVs typically have a single nucleocapsid per envelope and a single virion embedded in an oval occlusion body. However, two or more virions in occlusion bodies have also been reported (Crook and Brown, 1982). OBs of GV are referred as a capsule, which is composed of a protein named granulin. In contrast to NPV, GVs have only been recorded from Lepidoptera. There are three major genetic types of GV. Type 1 GVs, such as that isolated from the cabbage looper, *Trichoplusia ni*, infects the midgut and fat body cells. Since it does not infect many tissues (such as the tracheal matrix or epidermis), the infected larvae typically live longer than NPV-infected larvae. Type 2 GVs are similar to NPV infections while Type 3 GVs, only known from the codling moth *Cydia pomonella*; infects the midgut tissue.

During GV infection, nuclear membrane ruptures and causes merging of the nuclear and cytoplasmic regions of the infected cell. OBs of GV, termed capsules will be formed throughout the cells, in contrast to NPV infection, where OBs formation only occurs in the nucleus of the infected cell.

2.2.2 Virion Phenotypes

Baculoviruses exist in two forms, budded virus (BV), (also called extracellular virus, ECV, or nonoccluded virus, NOV) and occluded virus (OV) which is also called polyhedral-derived (PDV) or occlusion-body derived virus (ODV) after it is released from the OBs. The budded form of the virus contains a single rod-shaped nucleocapsid surrounded by a loosely fitted lipid bilayer membrane envelope, which has a surface projection or peplomer structure at one end. This type of virus possesses only one nucleocapsid per virion, irrespective of whether it is an SNPV, MNPV or GV. BV is highly infectious to tissues within the hemocoel and in cell culture (Volkman *et al.*, 1976), and is responsible for the systemic spread within an individual insect, and apparently for lateral or cell-cell transmissions.

Although both BV and OV consist of enveloped nucleocapsids, their envelopes differ in source and composition (Braunagel and Summers, 1994). They serve different roles in the life cycle of the virus and are assembled by different mechanisms and at different sites in the infected cell. By definition, OV are occluded within a crystalline matrix, largely comprising a single 28 kDa protein to form OBs (formerly called polyhedra inclusion bodies, PIBs). OBs from NPV are also called polyhedra because most reveal a polyhedral shape when viewed by microscopy. They range in size from 0.5 to 15μm in diameter. However, in the case of GV, the OBs are known as capsules or granules and are cylindrical bodies, 0.05 x 0.25 to 0.08x 0.4μm).

Polyhedra have an outer layer known as calyx, which is rich in carbohydrates and are believed to increase the stability of the inclusion bodies. The carbohydrates are linked to the polyhedral matrix by proteins (Zuidema *et al*., 1989). OBs give a refractile and shiny appearance under the light, phase contrast, dark or bright-field microscope. This form of virus is stable in the environment and is protected to some degree from light, ultraviolet rays, desiccation and other hazardous environmental conditions and physical abuse. This explains its ability to act as biological control agent especially in integrated pest management fields programmes. Hence, OBs play a role in the horizontal transmission between insects.

2.3 Infection Cycle

An early description of baculovirus infection was in the silkworm, B*ombyx mori* and cause major losses in silk production (Bilimoria, 1991; Van Oers and Vlak, 1997). Baculoviruses have an interesting biphasic life cycle and produce two functionally different forms of virus throughout their replication mechanism (Kawarabata and Aratake, 1978). Since viral genes are expressed sequentially, infections occur in three stages; early, late and very late stages (Friesen and Miller, 1986), which correspond to the initiation of viral replication, BV and OB production, respectively (Tanada and Maeda, 1983). During early stages, the nucleocapsid migrates through the cytoplasm to the nucleus of the host cell and through the nuclear pore where it uncoats (Granados and Williams, 1986). The