European Commission

Draft Assessment Report prepared according to the Commission Regulation (EU) N° 1107/2009

Spodoptera exigua multicapsid nucleopolyhedrovirus (SeMNPV)

Active substance data

Volume 3 – Annex B.2 Biological and physical properties

Rapporteur Member State: Spain

April 2020

Version History

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INTRODUCTION

The company Suisse AG (new Swiss subsidiary of Andermatt Biocontrol AG) submits the current dossier for the approval of the baculovirus (BV) *Spodoptera exigua* multi nucleopolyhedrovirus (SeMNPV) as a new microbial pest control agent (MPCA) and SPEXIT as its reference microbial pest control product (MPCP) to the European Authorities.

BVs used as MPCA in the EU are regulated as microorganism according to Regulation 1107/2009[1]. Data requirements for the registration of BVs as an active substance and their products are laid down in part B of the regulation documents 283/2013 [2]and 284/2013 [3]and the principles for evaluation and authorization of plant protection products contained microorganism according to regulation 546/2011[4].

BV isolates however, represent a unique case in which the wild type isolates are genetically heterogeneous (mixture of different genotypes or pool of isolates). These variations may influence in some biological properties, such as the virulence, but it has no consequences on the safety towards non-target organisms or the environment. Isolation of a single genotype is difficult and even not appropriated, since genetic variation is needed to account for variation in the target organisms and obtain better efficacy in the control of insect populations. Therefore, the BVs were not necessary evaluated at strain level (SANCO/0253/2008). [5]The high similarity between BVs justifies a general assessment at the level of the family *Baculoviridae*, considering species-specific information where necessary. The proposed procedure to include BVs at species level was adopted by the member states and the European Regulatory Authorities already in 2007, when the first BV species was included in Annex I, and for the REBECA proposal 2008[6], for a simplified inclusion of BVs on the species level into Annex I. Most of the formally required data are published and equal for all BVs, already assessed by MS and EU authorities and therefore, some data on the isolate or species level are not mandatory.

The BVs are included on species level in Annex I of directive 1107/2009 and the different pool of isolates were added after they have been evaluated to a separate list, to be maintained in the Review Report and to be amended by taking note in the Standing Committee (SANCO/0253/2008). This approach has been confirmed by a decision in the Standing Committee on May 15, 2007[7] were *S. exigua* NPV was listed at species level in Annex I. The experience that BVs present no risk for the environment have been confirmed by numerous studies during the last fifty years, since their first use as biocontrol agents. With regard to safety considerations, it is important to note that the whole *Baculoviridae* family are naturally present in our environment and are closely associated with their host occurrence. Therefore, their application in pest control would only produce a non-permanent fluctuation of the virus titre in the biotope of the pest insect. Due to their host specificity, BVs do not affect other organisms like vertebrates, arthropods other than their host species, microorganisms, or plants. BVs do not produce any metabolites at all.

For the BV specie *S. exigua* multicapsid nucleopolyhedrovirus (SeMNPV) a DAR with a reference isolate (Florida isolate SeNPV-F1, the first applied for) was approved in 2006 and the isolate SeNPV-F1 was listed on Annex I. Two new more isolates were further applied for at Member State level: the SeMNPV-SP2, approved in 2008 and the SeNPV-BV0004, approved in 2010. Conversely, the current dossier was based on the data already assessed by the MS and EU authorities:

- The previous DAR document for the approval of a new active substance SeNPV-F1 submitted by Mitsui Agri Science International S.A and evaluated by The Netherlands in 2007.

[1]*Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal of the European Union L 309, 1-50.*

[2] Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal of the European Union L 93, 1-84.

[3]*Commission Regulation (EU) No 284/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal of the European Union L 93, 85-152.*

[4]*Commission Regulation (EU) No 546/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products. Official Journal of the European Union L155, 127-175.*

[5]*SANCO/0253/2008 rev. 2, 22 January 2008. Guidance Document on the assessment of new isolates of baculovirus species already included in Annex I of Council Directive 91/414/EEC.*

[6]*Ehlers RU., 2011 Regulation of Biological Control Agents and the EU Policy Support Action REBECA. In: Ehlers RU. (eds) Regulation of Biological Control Agents. Springer, Dordrecht.*

[7]*Review report for the active substance Spodoptera exigua nuclear polyhedrosis virus. Finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 15 May 2007 in view of the inclusion of Spodoptera exigua nuclear polyhedrosis virus in Annex I of Directive 91/414/EEC. Spodoptera exigua NPV SANCO/T14/2007 - rev. final1 12 March 2007.*

- The evaluation report of the new isolate of SeMNPV, BV0004 previously submitted by the company Andermatt Biocontrol GmbH and evaluated by the Netherlands' in 2010.

Active substances are approved for maximum period of 10 years under Directive 91/414/EEC[8]. The active substance SeMNPV was under programme of renewal Regulation EU 686/2012 (AIR-III programme[9]). According to draft working document AIR III renewal programme SANCO/2012/11284[10], *Spodoptera exigua* nuclear polyhedrosis virus was included in Batch 9" Active substance *Spodoptera exigua* nuclear polyhedrosis virus No application for renewal of approval has been submitted. Previous expiry date 30/11/2017"

Commission implementing regulation (EU) No 844/2012[11] setting out the provisions necessary for the implementation of the renewal procedure for active substances, as provided for in Regulation (EC) No 1107/2009 establishes in its Art 1: "the application for the renewal of an approval of an active substance shall be submitted by a producer of the active substance to the rapporteur Member State, no later than three years before the expiry of the approval"

The application for the renewal of the active substance *Spodoptera exigua* nuclear polyhedrosis virus was not submitted before of three years before the expiry date of the approval of the active substance SeMNPV (30/11/2017).

The applicant then have submitted an application for SeMNPV as a new active substance.

In this RAR, the information submitted regarding *Spodoptera exigua* multicapside nucleopopyhedrovirus (SeMNPV) is evaluated as new active substance, therefore, all information is considered and evaluated as new.

Literature reference included by the applicant comes from a literature search according to EFSA (2011) [12]in order to identify relevant recent published peer reviewed references covering the last 10 years. The RMS has also included relevant studies considered important to support the application for the approval of *Spodoptera exigua* multipolyhedrivirus (SeMNPV) genotype pool BV-0004 and the microbial product SPEXIT.

[8] *Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market. OJ L 230 of 19.8.1991.C.*

[9] *Programme of renewal Regulation EU 686/2012 (AIR-III programme).*

[10] *SANCO/2012/11284 –rev. 22, December 2018. Draft working document AIR III renewal programme.*

[11] *Commission implementing regulation (EU) No 844/2012, of 18 September 2012. Setting out the provisions necessary for the implementation of the renewal procedure for active substances, as provided for in Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market.*

[12] *Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011; 9(2):2092.*

B.2 BIOLOGICAL AND PHYSICAL PROPERTIES OF THE MICROORGANISM

SeMNPV is a BV belong to the family Baculoviridae, which are arthropod-specific, enveloped viruses with a circular double-stranded DNA genome. BVs exclusively have been isolated from arthropods, primarily from the three insect orders Lepidoptera, Hymenoptera, and Diptera (OECD, 2002[13]). In general, the host range of most BVs is restricted to one or few species of the genus or family of the host where they were originally isolated.

SeMNPV is a naturally occurring virus worldwide and acts highly specific against larvae of the beet armyworm, *Spodoptera exigua*, therefore, the presence of SeMNPV in the environment is linked to the presence of the host, *S. exigua*. Thus, its application in pest control means only a fluctuation of the virus titre in the biotope of the pest insect (Krieg, 1976). The SeMNPV strain BV004 in use was originally isolated in China (Jianfeng, 2005). It is not supposed to have any harmful effects on organisms not belonging to the genus *Spodoptera*. SeMNPV does not produce antibiotics and secondary metabolites of toxicological and/or environmental, or ecotoxicological concern. Neither SeMNPV active ingredient produce nor the end-use product (SPEXIT) contains chemical compounds of critical toxicological, environmental, or ecotoxicological concern. The same would be applied for the semi-synthetic insect diet.

Spodoptera exigua multicapsid nucleopolyhedrovirus (SeMNPV) is the valid name of the virus species. In the literature, the term *Spodoptera exigua* nucleopolyhedrovirus (SeNPV) is also found to refer to the same species. Other BVs isolated from *Spodoptera* species, namely *S. litura* NPV (SpltNPV), *S. terricola* NPV (SpteNPV), and *S. littoralis* NPV (SpliNPV) also were previously classified to the group II NPVs, todays, they are all classified to genera *Alphabaculovirus*es. These species are very closely related to each other, but are more distantly related to SeMNPV (Jehle *et al*., 2006, submitted in KMA 1.3/02).

BVs are large dsDNA viruses that are occluded into proteinaceous occlusion bodies (OBs) for horizontal transmission. OBs are pathogenic to certain species of insects, especially those in the order Lepidoptera (Gröner, 1986; Martignoni and Iwai, 1986). These viruses form the basis for a number of biological insecticides employed in the control of caterpillar pests of forests and field crops (Moscardi, 1990). The effectiveness of biological insecticides and the prevalence of disease in natural insect populations depend in large part on the variation in traits relevant to horizontal transmission, such as OB pathogenicity and OB productivity, associated with particular genotypes (Myers and Cory, 2013; Erlandson, 2009).

There is no evidence that genetic transfers occurs from viruses used as MPCAs to any other organisms (EFSA supporting publication 2013: EN-518).

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During the biphasic replication cycle of BVs, two distinct viral phenotypes are formed: Occluded virions (ODV) and budded virions (BV) differing in the origin and composition of their envelopes and their roles in the virus life cycle. ODVs are released from the inclusion bodies and infect midgut epithelium cells (first round of virus replication), and then the newly produced nucleocapsids traverse the nuclear membrane, the cytosol and bud through the basal lamina of the midgut cells into the hemolymph. The now so-called BVs acquire a new envelope and are responsible for the systemic cell-to-cell infection in host larvae (OECD, 2002). The family Baculoviridae is divided into the four genera: Alphabaculovirus, Betabaculovirus, Gammabaculovirus and Deltabaculovirus. In the past, the family was divided into Nucleopolyhedroviruses (NPV) and Granulovirus (GV) and the classification was based on the morphology of the occlusion body (OB), nowadays on the basis of genome phylogeny. OBs are crystalline matrices embedding the virion(s) and serve to protect the virions against damaging environmental conditions and allow virions to remain viable for many years. The OBs of Alphabaculovirus, Gammabaculovirus, and Deltabaculovirus show a polyhedral shape with a size of 0.5 to 5 µm, containing many virions. The OBs of Betabaculovirus show an ovicylindrical shape with a size of 0.3 × 0.5 µm, containing only one, rarely two or more virions. Baculoviridae genomes encode for 100 to 200 proteins, whereby thirty gene homologs form the BVs core genes, which are shared among alpha-, beta-, gamma- and deltabaculoviruses. The conserved genes are involved any a variety of functions, including DNA replication, late gene transcription and virion

[13] Consensus document on information used in the assessment of environmental applications involving baculoviruses

structure. The matrix proteins (polyhedrin and granulin) of different Baculoviridae genera are serologically closely related.

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For clarity, the following definitions are used in this RAR (from Council Directive 2005/25/EC):

Infection: The introduction or entry of a pathogenic microorganism into a susceptible host, whether or not it causes pathological effects or disease. The organism must enter the body of the host, usually the cells, and be able to reproduce to form new infective units. Simply ingesting a pathogen does not imply infection.

Infectivity: The characteristics of a microorganism that allow it to infect a susceptible host.

Pathogenicity: the capacity of the virus to enter the host, establish infection, reproduce, and cause death (measured in terms of dose–mortality metrics).

Virulence: Measurement of the degree of disease producing ability of a micro-organism as indicated by the severity of the disease produced. Measure of the dosage (inoculum size) required to cause a specific degree of pathogenicity. It is measured experimentally by the median lethal dose (LD50) or median infective dose (ID50). The time elapsing between initial infection and after the death of the host. Virulence is an intrinsic property of parasites, defined as the deleterious effects of infection on the host fitness

B.2.1 HISTORY OF THE MICROORGANISM AND ITS USES. NATURAL OCCURRENCE AND GEOGRAPHICAL DISTRIBUTION

Baculoviridae is a family of insect-specific large DNA viruses widely used in biotechnology and biological control. Its applied value stems from millions of years of evolution influenced by interactions with their host and the environment. BVs are natural control agents of a wide range of insect pests. Owing to their high specificity and high virulence, BVs are natural control factors for a variety of insects. Due to their high host specificity and virulence, several BVs have been developed as biopesticides for control of agricultural, horticultural and forestry pests (Cunningham, 1995). At present, more than 600 BVs have been reported (Rohrmann, 2013). Natural occurring BVs seem especially promising as they combine reasonable efficacy to very few hosts with environmental safety and no hazards to man, domestic animals and wildlife, that fore, a number of these viruses have been used as biological insecticides. There has been a long-held interest in BVs as potential biocontrol agents for the management of insect pests. This interest motivated the field collection and acquisition of many BVs isolates by various laboratories beginning in the 1950s and early 1960s. The first attempts to use BVs for biological control can be dated back to the year 1892 to control an eruptive forest defoliator using a NPV against natural populations of its host, the nun moth (*Lymantria monacha* L.), in Europe (Huber 1986). During massive population increases of nun moths (*Lymantria monocha*, L.), a severe pine pest in Europe, the use of the infectious agent was intended to combat the insect pest. The first successful case of controlling an eruptive defoliator using a BVs occurred in Canada during the 1930s. In the decades that followed, numerous investigations were undertaken to examine the potential of different BVs for the control of eruptive insects using different methods (Cunningham 1995; Wallace and Cunningham 1995). In these investigations, BVs OBs were mass produced either by infecting larvae in laboratory rearing or by harvesting dead larvae from field populations that had been previously treated at high rates with the virus. After OB purification from dead larvae or grinding of freeze-dried cadavers to fine powder, OBs were generally suspended in a water solution containing molasses, sometimes sticker products. The successful use of BVs as a biopesticide is well-known (Mazid *et al*., 2011, Rohrmann 2013). Some examples of the most important BVs insecticides tested and used in the field are: *Adoxophyes orana* GV, *Agrotis segetum* GV, *Anticarsia gemmatilis* MNPV, *Autographa californica* MNPV, *Heliothis* (*Helicoverpa*) sp. NPV, *Helicoverpa armigera* NPV, *Lymantria dispar* NPV, *Mamestra brassicae* NPV, *Neodiprion sertifer* NPV, *Orgyia psuedotsugata* NPV and *Spodoptera* spp. NPV.

BVs exclusively have been isolated from arthropods, primarily from four insect orders: Lepidoptera, Hymenoptera, Diptera and Coleoptera (OECD 2002). Any intended use of BVs for insect pest management includes the screening for a virus isolate virulent for the particular species. Isolates from diseased insects in the application area frequently are the first choice. If available, such isolates are in general included in the screening, but the testing for suitable viruses is not conventionally limited to these indigenous agents.

Natural population of BVs tend to be very diverse, particularly in the case of lepidoptera nucleopolyhedroviruses in the gene of *Alphabaculovirus*, where diversity can vary across geographical regions, or more locally and even within isolates obtained from individual diseased insects (Cory *et al*. 2015; Virto *et al*., 2016). The diversity of Alphabaculovirus populations is transmitted and maintained because the emergent phenotypic traits that arise from genetic diversity favour the survival of pathogen. The value of retained genetic diversity present in virus-based biological products is well recognised. Recent studies involving the cloning of genotypes from highly pathogenetic natural isolates, followed by the characterization of novel genotype mixtures that have resulted in the identification of unique combinations with marked improvements in OB pathogenicity or speed of kill characteristics (Arrizubieta *et al*. 2015, Bernal *et al.* 2013). BVs are naturally present and ubiquitous in our environment and closely associated to the presence of their host species. Their geographic distribution usually corresponds to the distribution of their hosts. The application in pest control means only a fluctuation of the virus titre in the biotope of the pest insect (Krieg, 1971).

B.2.1.1 Historical background

SeMNPV is an important pathogen of *S. exigua* (Smits *et al*., 1987) that plays an important role in regulating its natural host (Gelernter and Federici 1986, Caballero *et al*. 1992). SeMNPV is a virus species from Group II of the genus Alphabaculovirus, differs significantly from other baculoviruses species in that it is a highly pathogenic host-specific virus.

SeMNPV is of particular interest for the bio insecticide industry owing to its very high pathogenicity, fast speed of kill, and total specificity (Smits *et al*. 1987, Jkel *et al*. 1999). For this reason, SeMNPV has been produced and commercially registered in several countries for control of *S. exigua* populations both in open-field (Smits and Vlak 1994, Kolodny-Hirsch *et al*. 1997) and greenhouse crop systems (Smits *et al*. 1987, Bianchi *et al*. 2002, Lasa *et al*. 2007, Virto *et al*., 2014).

The usefulness of SeMNPV in control of beet armyworm has focused interest in selecting strains with high pathogenicity and genetic stability. Different strains of naturally occurring of SeMNPV have been isolated from many geographic regions of the world. Since Steinhaus (1949) first isolated a SeMNPV in California, USA, several SeMNPV isolates, including SeMNPV-SP3, SeMNPV-SP1, SeMNPV-SP2, SeMNPV-UZB, SeMNPV-TH, and SeMNPV-608 etc. have been characterized from different regions of the world, including the Netherlands (Vlak, *et al*. 1981) , California (Gelernter and Federici, 1986), Spain (Caballero *et al*, 1992), Florida (Kolodny-Hirsch *et al*., 1993), Japan (Kondo *et al*., 1994), Thailand (Hara *et al*., 1995), China (Guo *et al*., 2013) and Mexico (Zamora-Avilés *et al*., 2017). The strains can be distinguished unequivocally from each other by one or more DNA restriction enzyme fragments. For example, for SeMNPV strains from three geographically very separate regions (from the United States, Spain, and Thailand), the PstI M fragment from each isolate served as a restriction fragment length polymorphic marker for their identification. Additionally, the biological activities of some of the various SeMNPV strains differed significantly from each other.

The selection of indigenous isolates that are suitable for development as biological control agents requires the characterization of the isolate present in each geographical region. Molecular and biological characteristics of these isolates have been widely reported. A high degree of specificity and infectivity of SeMNPV against *S. exigua* both *in vivo* and *in vitro* has been demonstrated.

Base on restriction endonuclease analysis of the viral genomes, all of the wildtypes SeMNPV isolates worldwide are closely related to the California isolate SeMNPV-SU1 (Caballero et al. 1992; Gelerter and Federici, 1986; Hara et al., 1995; Kondo et al., 1994). The SeMNPV-US1 from California was propose as the type of strain of SeMNPV (Caballero et al., 1992). The strain SeMNPV-US1 was completely sequenced and mapped, conversely the genome organisation was determined (Jkel et al., 1999). The genome contains 135,611 bp and 139 open reading frames larger than 150 bp.

There are three SeMNPV that have been developed as a MPCA and were commercialize in Europe during the last decade, including:

1) **SeNPV-F1** or Florida isolate SeU2, from the product SPOD-X was used in Europe since 2006 (Certis, USA).The SeMNPV-US2 from Florida is being used as active ingredient in the product Spod X (Smits & Vlak 1994), which is used in the USA and Thailand since 1995. This isolate contains 7 different, but closely related genotypes (Muñoz *et al*., 1998).

2) **SeMNPV-SP2**, a Spanish isolate from the product VIR-EX was used in Europe since 2008 (Biocolor, Spain). Isolate SeMNPV-SP2 contains the genotypes ALPst0935, ALPst1400 andALPst1033. These genotypes were collected from diseased *Spodoptera exigua* larvae on vegetables in greenhouses in El Ejido (Almeria), Spain, in 2002 and 2003 (Murillo *et al*, 2006). Identification of isolate SeMNPV-SP2, containing the genotypes ALPst0935, ALPst1400 and ALPst1033 can be achieved to a very high level by polymerase chain reaction (PCR) amplification of the hr1 genomic region followed by restriction cleavage with BglII (Murillo *et al*., 2007).

3) **SeMNPV isolate BV0004**, from the product product SPEXIT was used in Europe since 2010 (Andermatt Biocontrol, Switzerland). The *Spodoptera exigua* SeMNPV isolate in use (BV-0004) was isolated in 2000 from *Spodoptera exigua* larval cadavers infected with nuclear polyhedrosis from a green pepper field in Huaian City, Jiangsu province, China (Jianfeng, 2005). The SeMNPV isolate was characterised by analysis of restriction fragments obtained after enzymatic digestion and compared to the California isolate SeMNPV-US1 (Jehle 2007). SeMNPV-BV0004 is identical to the pattern of SeMNPV-US1. The isolate is preserved and maintained at -24°C since it was isolated. Phylogenetic analysis of marker genes revealed that SeMNPV belongs to the Group II Nucleopolyhedroviruses (Jehle *et al*., 2006).

The SeMNPV isolate used to produce SPEXIT does not have any morphological characteristics that differ from the classical description of the species.

SeNPV- F1 was the reference isolate (Florida isolate in Spod-X GH (Se NPV-F1), first included in ANNEX I as an active substance in 2007. Later on, in 2008 (SeMNPV-SP2) and 2010 (SeMNPV-BV0004) were assessed for the inclusion as new isolates of SeMNPV species already included in Annex I of Council Directive 91/414/EEC, and there been used as active ingredients until the end of the commercial period in 2017.

Other SeMNPV isolates were identified and analyzed as a potential biocontrol agent for *S. exigua*, as the isolates SfNIC-B and SfNIC-C in Mexico (Zamora-Avilés *et al*. 2017), isolate SeMNPV-QD in Chine (Chen *et al*., 2019) and the isolates SeSLP6, SeSLP8, SeSLN8 in Spain (Clavijo *et al* 2010, Luna-Espino 2018).

Two other viruses from *Spodoptera* species, namely *Spodoptera litura* NPV (SpltNPV) and *Spodoptera littoralis* NPV (SpliNPV) belong as well to the Group II NPVs, but are more distantly related to SeMNPV than to each other (Jehle *et al*., 2006).

B.2.1.2 Origin and natural occurrence

BVs are closely associated with their host, and occurrence of SeMNPV is linked to the presence of the host, *S. exigua*. SeMNPV is very host specific and affects no other species than its host *S. exigua*. SeMNPV was isolated from diseased larvae in The Netherlands, Spain, France, Egypt, China, Japan, Thailand, and the USA (Muñoz *et al*., 1997).

The BVs isolated from nature are generally a mixture of different genotypic variants. Thus, these variants can be cloned individually and their biological activities can be performed individually. The genetic variability in the isolates should be detected and differences of the genotypes can be performed by randomly picking plasmid colonies after a PCR amplification. Restriction enzyme analysis of the isolate is been used to detect its variability. In order to isolate genotypes, a cloning step is required followed by their characterization at the biological and genetic level. The study of variation in the replication speed and in the number of progeny virions produced is also essential to fully characterize the isolate.

The application of PCR and DNA sequencing methods to characterize Bvs isolates in collections has greatly expanded our comprehension of the genetic diversity in Baculoviridae. Even though nowadays there is the possibility of obtaining fully sequenced genomes, RFLP / REN analyses are still used to differentiate between *Baculoviridae* isolates and / or species (Zamora-Avilés *et al*., 2017). The species criterion defined by Jehle *et al*. (2006, submitted in KMA 2.1.1/05) is still appropriate and applied for *Baculoviridae*. However, newer research started to study genome data allowing mapping different behaviour in host to differences in the genomes. The genetic variations may be linked to possible phenotypic effects in different isolates. Thézé *et al*. (2014) applied a comparative genomic analysis of among seven European SeMNPV isolates (HT-SeG24, HT-SeG25 and HT-SeG26 isolated from green house soil substrate in Almería, Spain; the VT-SeAl1 and VT-SeAl2 isolated from virus-killed progeny of field-caught adults from Almeria and VT-SeOX4 from virus-killed progeny from chronically infected laboratory population from Oxford, UK), differing in virulence. He found a similar genome size and content, but a high number of polymorphic sites, however with relatively few sites in the genomes that were potentially involved in functional changes. According to Thézé *et al*. (2014, 2018), (See section 1, point B.1.3.3, Test procedures and criteria used for identification at strain level) observations, BVs populations naturally have high genomic variation at different levels of the interaction between virus and host during the course of an infection. The differences in BVs virulence and transmission phenotypes involves multiple molecular pathways.

B.2.2 INFORMATION ON TARGET ORGANISM(S)

B.2.2.1 Description of target organism(s)

Beet armyworm, *S. exigua* (Hübner) (Lepidoptera: Noctuidae) **(Table B.2.2.1-01)**, is one of the most destructive polyphagous important pest world-wide of a wide variety of crops in sub-tropical and tropical regions and in glass houses of temperate regions (Smagghe *et al*., 2003; Su *et al*., 2016), feeding on crops that include corn, pea nuts, tomatoes, peppers, and sweet peppers (Osorio *et al*., 2008). In recent years, it has caused serious economic losses in America, Asia, Europe, Oceania, and Africa (Bianchi *et al*., 2002; Saeed *et al*., 2010; Underwood, 2011; Zheng *et al*., 2011). Currently, there are no reports on taxonomical differences between the populations of different parts of Europe. *S. exigua* (Hübner), a polyphagous noctuid pest, can be found both in open air and in greenhouse crops in the Mediterranean area, but is mostly confined to greenhouse crops in Central and Northern Europe. In the Netherlands, *S. exigua* is frequently found as a pest in sweet pepper and other greenhouse crops since 1976.

At present, the control of *S. exigua* is primarily based on the use of a broad spectrum of chemical pesticides. However, several studies have shown that *S. exigua* has rapidly developed resistance to insecticides (Sayyed *et al*., 2012; Zamora-Avilés *et al*., 2017).

In the tropics, breeding can be continuous with four to six generations per year, while in northern regions normally only one or two generations develop. Beet armyworms overwinter in the warmer regions of the Mediterranean countries, North America and Africa and invade the cooler northern regions as temperatures permit. The climate determines the seasonal activity (Hill, 1983).

EPPO code	LAPHEG
Scientific name	*Spodoptera exigua* (Hübner, 1808)
Order	*Lepidoptera*
family	*Noctuidae: Acronictinae, Amphipyrinae, or Ipimorphinae*
Genera	*Spodoptera*
Synonyms	*Noctua fulgens*, *Spodoptera fulgens* (Geyer), *S. pygmaea* (Rambur), *S. junceti* (Zeller), *S.caradrinoides* (Walker), *S. flavimaculata* (Harvey), *S. sebghana* (Astaut), *S. venosa* (Butler*) Caradrina exigua (Hübner); Laphygma exigua (Hübner); Laphygma flavimaculata (Harvey); Noctua exigua (Hübner)*
English name	Beet armyworm, Small mottled willow, soldier worm

Table B.2.2.1-01 EPPO *S. exigua*

Biology of beet armyworm

Eggs are laid at night on the leaves of the host, generally stuck to the lower surface of the lower leaves, preferably of younger plants. Egg masses consist of tight clusters of 50 - 150 eggs, usually covered with a protective layer of abdominal bristles. Hatching usually requires 2 - 5 days. Young larvae gradually move from lower to upper leaves, thereby feeding on the lower side of the leaves leaving the upper epidermis intact. Five to six larval instars occur. In the last larval instar, larvae are 2.5 - 3 cm long and consume about 75 % of the leaf area eaten during their development.

The pupae are 1.2 - 1.5 cm in length, shiny brown and located within an earthen cell. Pupation requires 6 - 9 days. Adults emerge at night, and typically use their natural pre-oviposition period (1 - 3 days) to fly for considerable distances before they settle to oviposit. Adult beet armyworms have a body length of about 1.2 cm and wingspan of 2.5 cm. On average, adults live for 8 - 11 days. Given the short generation time of approximately 25 days, several generations can be completed in a cropping cycle under favorable conditions (24 - 28 °C).

Damage

Young beet armyworm larvae feed on the lower surface of leaves where they eat the lamina but often leave the upper epidermis and larger veins intact. Larger larvae make irregular holes in leaves and fully-grown larvae devour foliage completely, leaving only major veins.

Crops affected by beet armyworms

More than 200 different crops are attacked by *S. exigua* larvae, among these are ornamentals and crops like sugar beet, cabbage, lettuce, soybeans, cotton, maize, tomato, potato, legumes, citrus, strawberry, melon, leek, garlic, onion, rice, flax, and tobacco.

Because of resistances against numerous substances in many populations, the chemical control of *S. exigua* has become exceptionally difficult, in particular if they become well established. Moreover, chemical control is often impossible in greenhouses where it interferes with the use of pollinators or beneficial insects (Moulton *et al*. 2000, Wang *et al*. 2006).

B.2.2.2 Mode of action

The isolate SeMNPV-Bv0004 is closely related to the other SeMNPVs described in the literature, the mode of action of SeMNPV will be similar to the mode of action of other SeMNPVs. In order to be effective SeMNPV have to be ingested by larvae of the beet armyworm with the food. The virus particles dissolve under the alkaline conditions in the larval gut and liberated virions penetrate the gut cells and multiply. After most tissues are infected the larva dies (**Figure B2.2.1-**

01). No isolate-specific data of SeMNPV-BV0004 is submitted. The mechanisms of host invasion, viral spread, tissue tropism and gross pathology have however been shown to be broadly common among all nucleopolyhedrosis viruses infecting Lepidopteran hosts (Evans and Harrap, 1982, Martins *et al*., 2005).

Application of SeMNPV should be timed at hatching of larvae so that early-instar larvae meet the virus. The early instar larval stages of the insect life cycle are the most susceptible to infection with BVs (Evans and Harrap, 1982, Martins *et al*., 2005). Following the application of BVs-based insecticides, a portion of pest population often survives to adulthood and may reproduce and lay eggs on the same, or nearby crop. During this period, the original inoculum applied to the crop is rapidly inactivated by solar ultraviolet radiation **Figure B.2.2.1-02a, stress factors and virus inactivation)** and diluted by rainfall and the growth of the plants, leaving little inoculum available to infect and control the following generation of pest larvae. The three mixture-genotypes OB preparation presented in this studio had similar OB potency and speed of kill, since the time to death varied between virus treatments with HT and VT genotypes and their mixtures. The expected LC50 values were estimated in order to evaluate the potential interaction among genotypes.

B2.2.3 Transmission routes: Horizontal and vertical transmission of SeMNPV

BVs adopt a mixed-mode transmission strategy involving both horizontal and vertical transmission. Transmission of BVs can be horizontal (HT) among individuals through environmental contamination, or vertical (VT) from parents to offspring on or in the eggs. Upon death of an infected larva, OB are released and other larvae become infected if they ingest the OB on contaminated foliage. VT can occur if larvae consume occlusion bodies but pupate before death leading to sub lethally infected adults. Adults sub lethally infected as larvae might transmit virus to their offspring either on or in the eggs. This can lead to an active infection killing the offspring or potentially a covert infection that is passed on to offspring that will survive.

HT is usually risky if susceptible hosts are rare, while VT is safer, but is constrained by host survival and reproductive success. As a result, HT is selectively advantageous at high host densities, whereas VT is favored in low density host populations. In the case of BVs, these transmission strategies generally exclude one another because the production of massive numbers of OBs for HT results in host death prior to the adult stage. Recent studios evident that mixed-mode transmission involving long-lived viral OBs in the environment and VT from infected adult insects to their offspring has key implications for the persistence, spatial dispersal and genetic diversity of insect BVs (Cory, 2015, Williams *et al*., 2017).

After larvae ingest OBs while feeding on contaminated foliage a portion of the infected individuals, develop lethal disease and release OBs onto the host plant where they can be transmitted to a susceptible host **Figure B.2.2.3-01** (red arrow). OBs on foliage are also washed by rainfall into the soil, from which they can be transported back to plants by biotic and abiotic factors **B.2.2.3-1** (black arrows). Alternatively, insects that consume OBs but survive may continue to develop, pupate and emerge as covertly infected adults **Figure B.2.2.3-01** (blue arrows). These adults can disperse before laying eggs and passing the infection to their offspring. Vertical transmission can be sustain over several generations until some elicitor or stress factor triggers (orange arrow) the covert infection into lethal disease which returns to the HT cycle (red arrows).

Sublethal infections by SeMNPV are common in field populations of *S. exigua*. In laboratory assays, a significant reduction in pupal weight, adult emergence, fecundity, and fertility (egg hatch) and a significant increase in larval developmental time and duration of the preoviposition period, was found in *S. exigua* with a sublehtal infection. Such covert infections were found to persist for at least five generations. Individuals from a persistently infected experimental population were significantly more susceptible to SeMNPV OB inoculum than individuals without a sublethal infection. It was concluded that sublethal infections may benefit pest control programs, given that insects that do not die from an overt infection after consuming contaminated food have a high probability of reduced reproduction and that pest control level is improved from one generation to the next (Cabodevilla *et al*. 2011a). Further studies conducted by the same research group, detected the prevalence of sublethal infections in *S. exigua* populations and measured a positive infection in 16.1 % of the surveyed individuals (Cabodevilla *et al*. 2011b). Also in this study, it was observed that sublethally infected insects are more susceptible to infection than healthy insects. Vertical transmission (VT), were the BVs are transferred from one generation to the other, has been suggested as a pathogen survival strategy when opportunities for HT are highly restricted (Virto *et al*. 2016). Sampling analysis of distinct genotypes isolated from Almeria (Spain) (Virto

et al, 2017), revealed marked differences in their insecticidal phenotypes and their tendency to produce covert, sublethal infections in their host. These genotypes were associated with routes of transmission. Genotypes isolated from soil samples (**Figure B.2.2.1-02, soil OB reservoir)** were assumed to originate from larvae that had died of polyhedrosis disease and would likely be transmitted horizontally. In contrast, genotypes isolated from laboratory-reared progeny of field-caught army beet (**Figure B.2.2.1-02, VT to offspring)** were might have been transmitted vertically.

VT genotypes were generally capable of producing a high prevalence of persistent infection in adults that survived an inoculum challenge during the larva stage, since HT genotypes tended to have higher OB pathogenicity and faster speed of kill compared to VT genotypes (Caballero *et al*., 2011a).

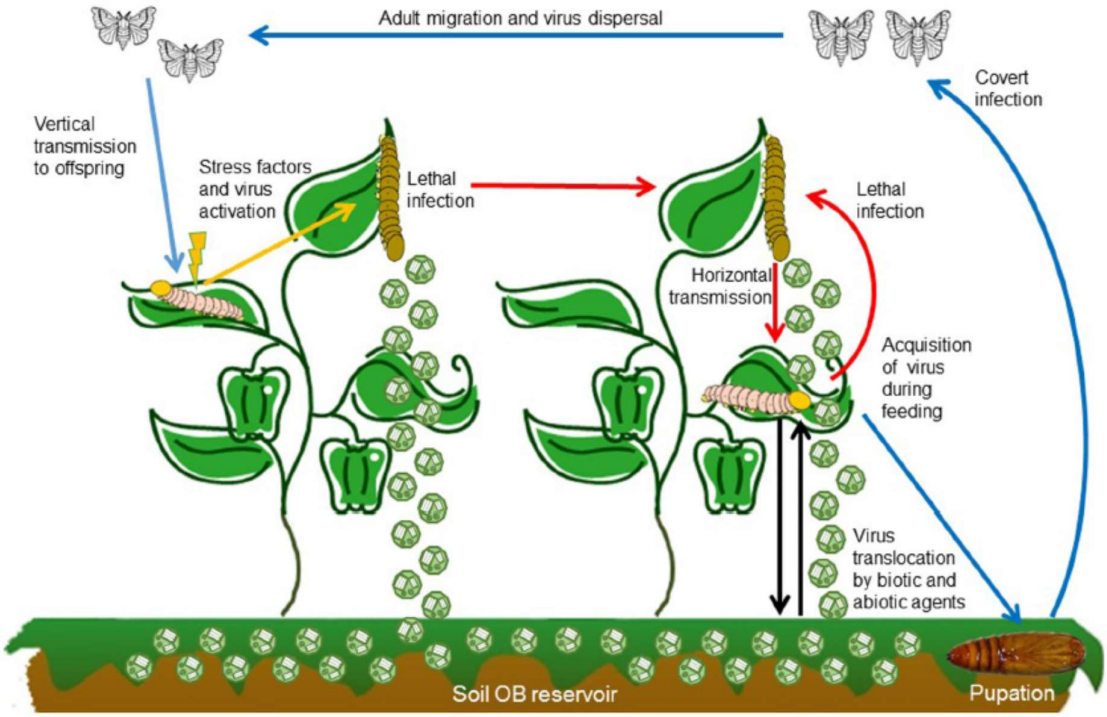

Figure B.2.2.3-01 BV transmission routes, mode of infection and dispersal pathways in the environment. (Williams *et al*. 2017).

Reference:	Cabodevilla, O., Villar, E., Virto, C., Murillo, R., Williams, T., Caballero, P. (2011a) Intra- and Intergenerational Persistence of an Insect Nucleopolyhedrovirus: Adverse Effects of Sublethal Disease on host Development, Reproduction, and Susceptibility to Superinfection
Report No.:	**KMA 2.2.2/06**
Guideline:	no
GLP:	no
Summary:	Sublethal infections by *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) are common in field populations of the beet armyworm (*S. exigua*, Hübner) in the Almerian horticultural region of Spain. Inoculation of second, third, and fourth instars with occlusion bodies (OBs) of an isolate (VT-SeA11) associated with vertically transmitted infections resulted in 15 to 100% of sublethal infection in adult survivors, as determined by reverse transcription-PCR (RT-PCR) detection of viral DNA polymerase transcripts, and quantitative PCR (qPCR) targeted at the DNA polymerase gene. The prevalence of adult sublethal infection was positively related to the inoculum OB concentration consumed during the larval stage. Sublethal infections persisted in OB treated insects for at least five generations. Viral transcripts were more frequently detected in adult insects than in third instars. qPCR analysis indicated a consistently higher prevalence of sublethal infection than RT-PCR. Sublethal infection was

	associated with significant reductions in pupal weight, adult emergence, fecundity, and fertility (egg hatch) and significant increases in larval development time and duration of the preoviposition period. Insects taken from a persistently infected experimental population were significantly more susceptible to the OB inoculum than control insects that originated from the same virus-free colony as the persistently infected insects. We conclude that OB treatment results in rapid establishment of sublethal infections that persist between generations and which incur costs in the development and reproductive capacity of the host insect.
M&M: **Test substances:**	• **Test Item**: OB of SeMNPV Spanish isolate (VT-SeAl1) with a single genotype (from green houses in Almeria 2007). Considered to have been vertically transmission from parents to offspring. • **Target organism**: beet armyworm (*S. exigua*, Hübner) second, third and fourth instars from Andermatt Biocontrol AG.
Test conditions	• **Bioassay:** OB production: Second, third and fourth instar larvae (L2, L3, and L4) were treated with OB concentration resulted in 20-80% mortality. OB from each instar were offered one of four OB concentration in 10-fold increments. Larvae ingested OB suspension within 10 minutes transferred to 24-well tissue culture plates with semisynthetic diet. 25±1ºC 50%±5 RH. • **Detection of viral DNA polymerase transcripts by RT-PCR** specific viral gene were target. Amplicon products were confirmed by sequencing. For the detection of viral genome DNA, total DNA was extracted from adult untreated control and adult survivor of OB treatments. • **Quantitative analysis by PCR (qPCR)** for the estimation of latent infections with SYBR fluorescence.
Study design & methods Experimental treatment:	1) Effect of larvae instar stage and OB inoculum concentration on larval mortality and adult emergence. 2) Detection of sublethal SeMNPV infections. 3) Transgenerational persistence of sublethal infections. 4) Cost of survival OB treatments on host development and reproduction. 5) Susceptibility of healthy and subhealthy infected insects to OB inoculum.
Replicates: Observations: Evaluation:	• Four suspension concentrations in 10-fold increments of OB from each instar (L2, L3 and L4). • Control larvae were fed with OB-free suspension. • 24 larvae ingested OB suspension four OB concentration for each of the three larvae instar. • Bioassay were performed 5 times for L2 and L3 and six for L4. • Daily observation of larvae until death or pupation. • 1) Fitting generalized linear models. • 2) Polymerase transcripts by RT-PCR. • 3) Quantitative analysis by PCR (qPCR).
Results:	• Prevalence of adult sublethal infection is positively related to the inoculum OB concentration consumed during the larval stage. • Sublethal infections persisted in OB treated insects for at least five generations. • Viral transcripts were more frequently detected in adult insects than in third instars. • Sublethal infection was associated with significant reductions in pupal weight, adult emergence, fecundity, and fertility (egg hatch) and significant increases in larval development time and duration of the preoviposition period.
Conclusions:	OB treatment results in rapid establishment of sublethal infections that persist between generations.

With the possibility to obtain relatively easily genomic data, first studies were conducted focusing on different genetic features of BVs. Also SeMNPV data was generated and studied. Thereby, it was observed, that distinct genotypes of SeMNPV differ in their insecticidal properties and can be associated with horizontal or vertical routes of transmission. Thereby, research described, that novel combinations of horizontally and vertically transmitted genotypes in an Alphabaculovirus-based insecticide could provide immediate pest control and contribute to transgenerational pest suppression of *S. exigua* larval populations in agro-ecosystems (Virto *et al*. 2016, 2017). Also the study conducted by Caballero *et al*. (2009) described the insecticidal potential of mixtures of certain SeMNPV genotypes and measured a promising field efficacy.

Next to the molecular studies, which were conducted in recent years, research also focused on behavioural objectives. It is widely known, that different parasites alter host behaviour in order to enhance transmission. BVs trigger behavioural alteration of their caterpillar host by inducing hyperactivity and by causing infected hosts to migrate to the top of the plant prior to death (so called tree-top disease). This allows an optimal distribution of the progeny virus, respectively occlusion bodies, onto lower foliage and increases the probability of transmission to healthy conspecific larvae (van Houte *et al*. 2014, Dobson *et al*. 2015, van Houte *et al*. 2015, and Rebolledo *et al*. 2015). The circumstances around this behaviour were surveyed in different studies. Van Houte *et al*. (2014/2015) concluded based on their observations from the experiments that the tree-trop disease in SeMNPV infected caterpillars is the result of positive phototaxis prior to death and that this response is specifically triggered during virus infection. However, other researchers (Dubson *et al*. 2015) criticised the study design and interpretation basis and determined this phenomenon to result rather from optimally timed larval killing. Rebolledo *et al*. (2015) concluded, that BVs-induced climbing behaviour increases the incidence of intraspecific necrophagy in *S. exigua*, which is considered the most efficient mechanism of virus transmission. They tested for evidence for a volatile attractant or feeding stimulant in SeMNPV-killed insects. In laboratory choice tests and olfactometer studies they did not find an indication for a virus-associated olfactory or phagostimulant factor that might lead to necrophagic behaviour, but observations in greenhouse indicated that virus-infected cadavers were attractive to healthy *S. exigua* larvae.

Phytochemicals may affect the performance of insecticides in the tritrophic interaction involving an herbivorous insect, a host plant and the pest control agent. The better the host chemistry is surveyed, the better the phytochemical variation leading to possible differences in the pest mortality can be understood. This fact was observed to be an important factor in the management of pest insects. In addition, the infectivity of BVs to herbivores is affected by phytochemicals, which are ingested during the acquisition of viral inoculum on the foliage of the host plant (Wan *et al*. 2016). Wan *et al*. (2016) tested the effects of different host plant species on the infectivity of SeMNPV to *S. exigua* larvae and detected a significant effect for host plant and virus dose on the corrected mortality and mean time to death. The lowest LD_{50} values were measured for the host plants Chinese cabbage (*Brassica chinensis*), corn (*Zea mays*) and soya bean (*Glycine max*). The highest LD_{50} values were measured for water convolvulus (*Ipomoea aquatica*), collards (*Brassica oleracea*) and radish (*Raphanus sativus*).

Reference:	Cabodevilla, O., Ibañez, I., Simón, O., Murillo, R., Caballero, P., Williams, T. (2011b) Occlusion body pathogenicity, virulence and productivity traits vary with transmission strategy in a nucleopolyheddrovirus
Report No.:	**MA.2.2.2/07**
Guideline:	NO
GLP:	NO
Summary:	The prevalence of sublethal infections of *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) was quantified in natural populations of *S. exigua* in Almería, Spain, during 2006 and 2007. Of 1045 adults collected, 167 (16.1%) proved positive for viral polyhedrin gene transcripts by RT-PCR. The prevalence of covert infection varied significantly according to sex and sample date. Of 1660 progeny of field-collected insects, lethal disease was observed in 10–33% of offspring of transcript-positive females and 9–49% of offspring of transcript-negative females. Isolates associated with vertically transmitted infections were characterized by restriction endonuclease analysis using BglII or EcoRV and compared with isolates originating from greenhouse soil-substrate believed to be horizontally transmitted. Insects from a sublethally infected Almerian colony were between 2.3-fold and 4.6-fold more susceptible to infection than healthy insects from a Swiss colony, depending on isolate. Horizontally transmitted isolates were significantly more pathogenic than vertically transmitted isolates in insects from both

	colonies. Mean speed of kill in second instars (Swiss colony) varied between isolates by >20 h, whereas mean OB production in fourth instars (Swiss colony) varied by 3.8-fold among isolates. Intriguingly, all three horizontally transmitted isolates were very similar in speed of kill and OB production, whereas all three vertically transmitted isolates differed significantly from one another in both variables, and also differed significantly from the group of horizontally transmitted isolates in speed of kill (one isolate) or both variables (two isolates). We conclude that key pathogenicity and virulence traits of SeMNPV isolates vary according to their principal transmission strategy.
M&M: **Test substances:**	• **Test Item**: (1) horizontal transmitted isolates: OB of SeMNPV (SeG24, SeG25, SeG26), from greenhouses soil-substrate environment in Almeria. (2) Vertical transmitted isolates: Considered to have been vertically transmission from parents to the patently disease progeny of field collected.
Test conditions:	• **Target organism**: beet armyworm (*S. exigua*, Hübner) from 3 different sources: (1)Almería colonies, short term culture from Almería (Spanish) (2) Oxford colonies, long-term laboratory cultures from Oxford (UK) and (3) Switzerland colonies, provided by Andermatt Biocontrol AG.
Study design & methods Experimental treatment:	• **Field collection of *S. exigua* and identification of VT viruses.** • **Field collected of vertically transmitted *S. exigua*** by UV light tramps (July to September). • **RT-PCR analysis** for detection of SeMNPV transcripts in *S. exigua* • **Genotype variability of VT** • **Bioassay for the induction of covert infection**
Replicates:	• 20 neonate larvae from each female selected randomly, fed and daily monitored for viral disease, and those selected for REN of viral DNA (VT)
Observations:	• Determination of OB dose-response • Determination of mean time death • Determination of OB production
Objective:	• To quantify the incidence of covert infections and the prevalence of VT in natural insect population in Almería.
Evaluation:	• Studio of the relationship between the genetic diversity and transmission strategies. • Assessment of the abundance and diversity present in natural *S. exigua* population by characterization of genotypes associated with covert infections (likely to be vertical transmission).
Results:	• The prevalence of covert infection varied significantly according to sex and sample date. • Insects from a sublethally infected Almerian colony were between 2.3-fold and 4.6-fold more susceptible to infection than healthy insects from a Swiss colony. • Horizontally transmitted isolates were significantly more pathogenic than vertically transmitted isolates in insects from both colonies. • Intriguingly, all three horizontally transmitted isolates were very similar in speed of kill and OB production.
Conclusions:	• Key pathogenicity and virulence traits of SeMNPV isolates vary according to their principal transmission strategy.

Reference:	Virto, C., Williams, T., Navarro, D., Tellez, M.M., Murillo, R., Caballero, P. (2017) Can mixtures of horizontally and vertically transmitted nucleopolyhedrovirus genotypes be effective for biological control of *S. exigua*?
Report No.:	**KMA 2.2.2/08**
Guideline:	NO
GLP:	NO

Summary: Previous studies identified distinct genotypes of *S. exigua multiple nucleopolyhedrovirus* (SeMNPV) that were associated with HT(named HT-SeG25) or vertical transmission (named VT-SeAl1) in the host insect, *S. exigua* (Lepidoptera: Noctuidae). We examined the use of mixtures of occlusion bodies (OBs) of these genotypes as the basis for a virus preparation that could provide immediate pest control and establish a persistent sublethal infection in the survivors of an OB application for transgenerational pest suppression. Mixtures of HT-SeG25 + VT-SeAl1 comprising 25:75 or 75:25 % of each genotype, respectively, resulted in improved OB pathogenicity in terms of concentration-mortality metrics compared to OBs of VT-SeAl1 alone or similar values compared to OBs of the HT-SeG25 genotype alone. In contrast, no significant differences were observed in speed of kill or mean OB production per larva. Laboratory and greenhouse trials revealed that the prevalence of sublethal infection in adults that survived OB treatments in the larval stage increased with the proportion of VT-SeAl1 present in the inoculum, as determined by qPCR. Greenhouse trials indicated that the 75 % VT-SeAl1 + 25 % HT-SeG25 mixture was as effective as methoxyfenozide in preventing pest damage to pepper fruits. The potential contribution of vertically transmitted genotypes to transgenerational suppression of pest populations is discussed.

M&M:

Test substances:

- **Test Item**: OB of SeMNPV vertical transmission isolate VT-SeAl1 from offspring of field caught-adults and horizontal transmission isolate HT-SeG25 isolated from soil. Three combination of OB(75% VT-SeAl1+25% HT-SeG25); (25% VT-SeAl1+75% HT-SeG25); (50% VT-SeAl1+50% HT-SeG25)
- **Target organism**: beet armyworm (*S. exigua*, Hübner) virus free (from Andermatt Biocontrol)

Test conditions:

- Insecticidal properties of mixtures of OB: to evaluate the influence of possible interactions between genotypes on the insecticidal properties of each mixture. Speed mortality; time-mortality;
- Covert infection in survivors of an inoculum challenge. Quantify by q-PCR.
- Efficacy of single genotype OBs on pepper plants. Effect of genotypes and OB application rate on larval mortality.
- Lethal and sublethal infection by single genotype OBs and mixtures in greenhouse trial.
- Susceptibility of the progeny of treated insects to OBs.

Study design & methods

Experimental treatment:

Evaluation:

Results:

- Mixtures of each genotype, resulted d in improved OB pathogenicity in terms of concentration-mortality metrics compared to OBs of VT-SeAl1 alone or similar values compared to OBs of the HT-SeG25 genotype alone.
- No significant differences were observed in speed of kill or mean OB production per larva.
- Sublethal infection in adults that survived OB treatments in the larval stage increased with the proportion of VT-SeAl1 present in the inoculum.
- Virus OB involving a mixture of 75% of VT+25%HT genotypes were effective as chemical insecticidal for the control of *S. exigua* feeding damage to pepper fruits.
- Mixtures of OB of horizontal and vertical transmitted genotypes were as pathogenic as HT OBs alone and induced covert infection in 60-72%of the adults that survived expose to OB inocula in the larva stage in laboratory and green house conditions.
- The 75:25 mixture was an effective crop protection treatment on greenhouse grown sweet pepper.

Conclusions:

- Distinct genotypes of SeMNPV are associated with horizontal or VT routes.
- The inclusion of VT genotypes in combination to highly pathogenic HT genotypes as components of the active ingredient of BVs based insecticides could provide a means of promoting transgenerational pest suppression in agro-ecosystems.
- Further studios are required to quantify long-term effects on pest suppression.

Treatment	LC_{50} ($\times 10^4$) (OBs/ml)	95 % confidence interval ($\times 10^4$)		Relative potency	Expected LC_{50} ($\times 10^4$) (OBs/ml)	MTD (h)	95 % confidence interval		Median OB yield ($\times 10^8$ OBs/larva)	Interquartile range ($\times 10^8$ OBs/larva)
		Low	High				Low	High		
VT-SeAl1	5.98 a	4.43	8.08	1	–	86.1 a	84.6	87.8	5.8 a	5.4
75:25	2.55 b	1.82	3.59	2.4	3.94	84.3 a	82.6	86.1	5.0 a	5.4
50:50	3.45 a,b	2.48	4.85	1.7	2.94	85.2 a	83.6	86.9	5.6 a	5.1
25:75	2.45 b	1.75	3.42	2.5	2.34	86.0 a	84.3	87.8	6.8 a	5.9
HT-SeG25	1.95 b	1.40	2.74	3.1	–	85.0 a	83.4	86.6	4.9 a	5.0

Table B.2.2.3-01. Mean lethal concentration LC50, confidence interval (95%), relative potency expected LC50 using the method Tabashnik, mean time to death (MTT) and OB yield of the VT-SeAl1, 75:25, 50:50, 25:75, and HT-SeG25 in the second instar *S. exigua* larvae (Virto *et al*., 2017).

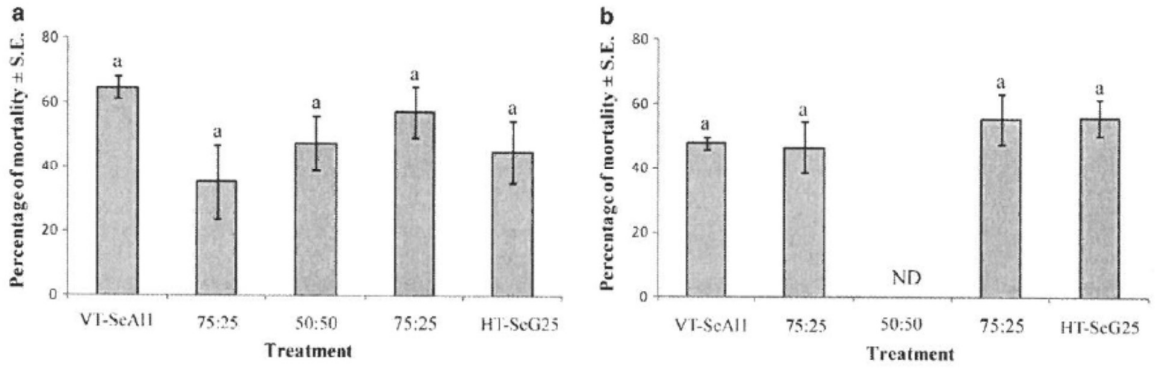

Figure B.2.2.3-02 Mean percentage of the viral mortality in *S. exigua* larvae in the covert infection bioassay performed in (a) laboratory and (b) greenhouse conditions. Colum's labeled with identical letters did not differ significantly (ANOVA). ND no data (Virto *et al*., 2017).

RMS comments: Report KMA 2.2/08 Virto *et al* 2017 and document KMA2.2.2/09 Virto *et al*., 20017 since to be the same document, with mixture of data. RMS have eliminated KMA 2.2.2/08 and has only included a corrected report for KMA 2.2/09.

RMS conclusions: The combination of HT and VT isolates could provide immediate pest control and contribute to transgenerational control *of S. exigua* larval populations. Natural population of BVs tend to be very diverse, particularly in the case of lepidoptera nucleopolyhedroviruses in the gene of Alphabaculovirus, where diversity can vary across geographical regions, or more locally and even within isolates obtained from individual diseased insects. The diversity of Alphabaculovirus populations is transmitted and maintained because the emergent phenotypic traits that arise from genetic diversity favor the survival of pathogen. The four above studios have provided important information on SeMNPV virus transmission and its importance in the management of insect control by combination of horizontally and vertically transmitted genotypes. Nevertheless, the value of retained genetic diversity present in virus-based biological products is well recognized. The presented studies involving the cloning of genotypes from highly pathogenetic natural isolates, followed by the characterization of novel genotype mixtures have resulted in the identification of unique combinations with marked improvements in OB pathogenicity and speed of kill characteristics.

The mechanism of virus transmission in SeMNPV isolate Bv-0004 is unknown. RMS recommend the application of similar studios in the isolate of studio SeMNPV-Bv0004. The mechanism of virus transmission in SeMNPV isolate Bv-0004 is unknown.

A data gap is therefore identified.

B2.2.4 Pathogenicity, virulence and production of progeny virus OBs

The mixtures of certain genotypes have greater insecticidal potential than pure genotypes in BVs insecticidal products. Mortality was determined for different isolates and genotypes of SeMNPV and LD_{50} varied between 9.2 and 49 OB/larva

for 2nd stage *S. exigua* larvae (Muñoz *et al*., 1997; Muñoz *et al*., 1998). Since in Virto *et al*.217 studios mean virus-induced mortality was similar among treatments with different mixtures of genotypes, ranging from 64.6±3.4% to 35.4±11.5%, in **Figure B.2.2.3-01** The percentage of larvae that succumbed to the infection was similar among treatments, ranging from 46.4±8.0% to 56.5.7%, **Figure B.2.2.3-02a.** Since in **Table B.2.2.3-01** OB pathogenicity significantly differed across virus treatments.

Moreover, efforts have been made to identify SeMNPV genes involved in pathogenicity and virulence. Genome sequence analysis of different SeMNPV isolates enabled to find a set of genes with an impact on the pathogenicity of the OBs and the speed of kill: se4, se5, se28, se76, se87 and se129. Via biological assays it could be confirmed that several of the genes had an effect on virus insecticidal phenotype. Thereby, the gene se5 was recognized as the most promising one for further investigations since it affects both mean lethal concentration and mean time to death (Serrano *et al.* 2015).

Reference:	Serrano, A., Pijlman, G.P., Vlak, J.M., Muñoz, D., Williams, T., Caballero, P. (2015), Identification of *S. exigua* nucleopolyhedrovirus genes involved in pathogenicity and virulence
Report No.:	KMA 2.7/6
Guideline:	NO
GLP:	NO
Summary:	Genome sequence analysis of seven different *S.* exigua multiple nucleopolyhedrovirus (SeMNPV) isolates that differed in insecticidal phenotype permitted the identification of genes likely to be involved in pathogenicity of occlusion bodies (OBs) and speed of kill (virulence) of this virus: se4 (hoar), se5 (unknown function), se28 (unknown function), se76 (cg30), se87 (p26) and se129 (p26). To study the role of these genes experimentally on the insecticidal phenotype, a bacmid-based recombination system was constructed to delete selected genes from a SeMNPV isolate, VT-SeAL1, designated as SeBacAL1. All of the knockout viruses were viable and the repair viruses behaved like the wild-type control, vSeBacAL1. Deletion of se4, se5, se76 and se129 resulted in decreased OB pathogenicity compared to vSeBacAL1 OBs. In contrast, deletion of se87 did not significantly affect OB pathogenicity, whereas deletion of se28 resulted in significantly increased OB pathogenicity. Deletion of se4, se28, se76, se87 and se129 did not affect speed of kill compared to the bacmid vSeBacAL1, whereas speed of kill was significantly extended following deletion of se5 and in the wild-type isolate (SeAL1), compared to that of the bacmid. Therefore, biological assays confirmed that several genes had effects on virus insecticidal phenotype. Se5 is an attractive candidate gene for further studies, as it affects both biological parameters of this important biocontrol virus.

Reference:	Pascual, L., Jakubowska, A.K., Blanca, J.M., Cañizares, J., Ferré, J., Gloeckner, G., Vogel, H., Herrero, S. (2012). The transcriptome of *S. exigua* larvae exposed to different types of microbes. Published report.
Report No.:	KMA 2.2.1/04
Guideline:	NO
GLP:	NO
Summary:	We have obtained and characterized the transcriptome of *S. exigua* larvae with special emphasis on pathogen-induced genes. In order to obtain a highly representative transcriptome, we have pooled RNA from diverse insect colonies, conditions and tissues. Sequenced cDNA included samples from 3 geographically different colonies. Enrichment of RNA from pathogen-related genes was accomplished by exposing larvae to different pathogenic and non-pathogenic microbial agents such as the bacteria *Bacillus thuringiensis, Micrococcus luteus, and Escherichia coli, the yeast Saccharomyces cerevisiae, and the S. exigua nucleopolyhedrovirus (SeMNPV*). In addition, to avoid the loss of tissue-specific genes we included cDNA from the midgut, fat body, hemocytes and integument derived from pathogen exposed insects. RNA obtained from the different types of samples was pooled, normalized and sequenced. Analysis of the sequences obtained using the Roche 454 FLX and Sanger methods has allowed the generation of the largest public set of ESTs from *S. exigua*, including a large group of immune genes, and the identification of an important number of SSR (simple sequence repeats) and SNVs (single nucleotide variants: SNPs and INDELs) with potential use as genetic markers. Moreover, data mining has allowed the discovery of novel RNA viruses with potential influence in the insect

	population dynamics and the larval interactions with the microbial pesticides that are currently in use for the biological control of this pest.

RMS conclusions: The study provide information on the transcriptome of *S. exigua* larvae, especially on the pathogen-induced genes. The RNA from pathogen genes was obtain from larvae exposed to SeMNPV. This information on the RNA of the target organism *S. exigua* is important for the study of the mode of action of the virus microbial pesticide, as well as in the analysis of the mechanism of insect resistance. The effectiveness of the infection depends not only in the virulence of the isolate, but also on the mechanism developed by the insect to repress the progress of the infection or to reduce the detrimental effects produced by the virulence factors. In that sense, the insecticidal activity is modulated by the insect immune system.

Reference:	Caballero, P., Murillo, R., Muñoz, D., Williams, T. (2009) El nucleopolyhedrovirus de *S. exigua* (*Lepidoptera: Noctuidae*) como bioplaguicida: análisis de avances recientes en España
Report No.:	**KMA 2.2.2/10**
Guideline:	NO
GLP:	NO
Summary:	The greenhouses of Almeria, Spain, represent the largest extension of covered crops in Europe. Larvae of *S. exigua* are an important pest in many of these crops and have developed resistance to the majority of insecticides registered in Europe. The nucleopolyhedrovirus (SeMNPV; Baculoviridae) of *S. exigua* is a native pathogen of this insect. The persistence of the virus is high in the soil of all crops, although its incidence is highest in spring and summer. As many as nine genotypic variants of the virus have been identified in this zone. In terms of pathogenicity, virulence and production of progeny virus occlusion bodies (OBs), mixtures of certain genotypes have greater insecticidal potential than pure genotypes. Production of OBs *in vivo* can be up to three-fold greater in larvae treated with juvenile hormone analogues. Compounds derived from stilbene have a synergistic activity with OBs in the laboratory and reduce the lethal doses in the field. The field efficacy of a simple formulation was greater than that offered by treatments of various synthetic insecticides. The virus is currently being mass-produced in a commercial production facility and the process of registration has begun for its use in sweet pepper crops in Almerian greenhouses.
Results:	• Greenhouse results showed that a bioinsecticide based on native isolates of SeMNPV have and excellent control of *S.exigua* populations in sweet green pepper crops.
Conclusions:	• The results of these investigations should facilitate the development of SeMNPV as a biological insecticide in other parts of the world.

RMS conclusions: The study is relevant for the study of natural population of virus and its efficacy in the control of *S.exigua*. There were no statistical data analysis performed.

B2.2.5 Host behaviour effects

BVs are natural disease agents that are adapted to both their hosts and the pertinent ecosystem. Larvae usually become infected by feeding on foliage contaminated with virus OBs. During the infection process larvae change color, development and feeding activity slows and mobility is reduced. Shortly prior to death, infected larvae migrate to the top of the plant, where they die in a characteristic form hanging from the pseudopods, a behavior induced by the BVs.

BVs alter the behavior of their caterpillar hosts by inducing hyperactivity and by causing infected caterpillars to migrate to the top of the plant prior to death. This behavior is thought to be adaptive for the virus, as it ensures optimal dissemination of progeny virus onto lower foliage and enhances visibility for birds, spreading the virus over longer distance.

Reference:	Van Houte, S., van Oers, M.M., Han, Y., Vlak, J.M., Ros, V.I.D. (2014). Baculovirus infection triggers a positive phototactic response in caterpillars to induce 'tree-top' disease
Report No.:	**KMA 2.2.2/11**

Guideline:	NO
GLP:	NO
Summary:	Many parasites manipulate host behavior to enhance parasite transmission and survival. A fascinating example is baculoviruses, which often induce death in caterpillar hosts at elevated positions ('tree-top' disease). To date, little is known about the underlying processes leading to this adaptive host manipulation. Here, we show that the BV *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) triggers a positive phototactic response in *S. exigua* larvae prior to death and causes the caterpillars to die at elevated positions. This light-dependent climbing behavior is specific for infected larvae, as movement of uninfected caterpillars during larval development was light-independent. We hypothesize that upon infection, SeMNPV captures a host pathway involved in phototaxis and/or light perception to induce this remarkable behavioral change.
Results:	• Hypothesis that 'tree-top' disease results from a positive phototactic response, i.e. attraction to light.
Conclusions:	• SeMNPV triggers a positive phototactic response in *S. exigua* larvae prior to death and causes the caterpillars to die at elevated positions.

RMS conclusions: Infection (SeMNPV) causes *S. exigua* larvae to die in an elevated position. This result would improve dissemination of viral occlusion bodies over plant foliage and would increase probability of transmission to healthy conspecific larvae. The study does provide information of host behavior in the environment (effect on light expose), but does not indicate any relationship between climbing behavior and infected insects. It is not relevant for the intended used of SeMNPV as a bioinsecticide. There were no statistical data analysis performed.

Reference:	Dobson, A.D.M., Auld, S.K.J.R., Tinsley, M.C. (2015) Insufficient evidence of infection-induced phototactic behaviour in *S. exigua*: a comment on van Houte *et al*. (2014)
Report No.:	**KMA 2.2.2/12**
Guideline:	NO
GLP:	NO
Summary:	A recent paper by van Houte *et al*. (2014) claims to demonstrate that (i) infection with the BV *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) causes *S. exigua* larvae to die in an elevated position; and (ii) this is achieved by the virus triggering a positive phototactic response in its larval host. Their study is grounded in knowledge that baculoviruses manipulate climbing behavior in some lepidopteran species. Dobson *et al*. (2015) argue van Houte *et al*.'s study would have significant limitations: the experimental design cannot test the authors' hypotheses, and the data presented are open to other interpretations that do not support the authors' conclusions.

RMS conclusions: No relevant results.

Reference:	van Houte, S., van Oers, M.M., Han, Y., Vlak, J.M., Ros, V.I.D. (2015) Baculovirus infection triggers a positive phototactic response in caterpillars: a response to Dobson *et al.* (2015)
Report No.:	**KMA 2.2.2/13**
Guideline:	
GLP:	YES
Summary:	The research group recently reported that BV *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) triggers positive phototaxis in *S. exigua* larvae, leading to death at elevated positions. However, the experimental set up and the conclusions from the results were criticized by another research team. In this paper van Houte's team is explaining more detailed their experiments and in summary conclude, that the other research team comments would not convince them does not invalidate their main conclusion that light is the cue for "tree-top" disease.

RMS conclusions: No relevant results. There were no statistical data analysis performed.

Reference:	Rebolledo, D. Lasa, R., Guevara, R., Murillo, R., Williams, T. (2015) Baculovirus-Induced Climbing Behaviour Favours Intraspecific Necrophagy and Efficient Disease Transmission in *S. exigua*
Report No.:	**KMA 2.2.2/14**
Guideline:	NO
GLP:	NO

Summary:	Shortly prior to death, many species of Lepidoptera infected with nucleopolyhedrovirus climb upwards on the host plant. This results in improved dissemination of viral occlusion bodies over plant foliage and an increased probability of transmission to healthy conspecific larvae. Following applications of *S. exigua* multiple nucleopolyhedrovirus for control of *S. exigua* on greenhouse-grown sweet pepper crops, necrophagy was observed by healthy *S. exigua* larvae that fed on virus-killed conspecifics. We examined whether this risky behavior was induced by olfactory or phagostimulant compounds associated with infected cadavers. Laboratory choice tests and olfactometer studies, involving infected and non-infected cadavers placed on spinach leaf discs, revealed no evidence for greater attraction of healthy larvae to virus-killed over non-infected cadavers. Physical contact or feeding on infected cadavers resulted in a very high incidence of transmission (82–93% lethal disease). Observations on the behavior of *S. exigua* larvae on pepper plants revealed that infected insects died on the uppermost 10% of foliage and closer to the plant stem than healthy conspecifics of the same stage, which we considered clear evidence of BV-induced climbing behavior. Healthy larvae that subsequently foraged on the plant were more frequently observed closer to the infected than the non-infected cadaver. Healthy larvae also encountered and fed on infected cadavers significantly more frequently and more rapidly than larvae that fed on non-infected cadavers. Intraspecific necrophagy on infected cadavers invariably resulted in virus transmission and death of the necrophagous insect. We conclude that, in addition to improving the dissemination of virus particles over plant foliage, BV-induced climbing behavior increases the incidence of intraspecific necrophagy in *S. exigua*, which is the most efficient mechanism of transmission of this lethal pathogen.

M&M: **Test substances:**	• **Test Item**: insect infected cadavers of *S. exigua* infected with SeMNPV-SU2 • OB of SeMNPV isolate US2 • **Target organism**: beet armyworm (*S. exigua*, Hübner) from maize fields (from Monterey, Mexico)
Study design & methods **Experimental treatment:**	• **Crop:** insects were fed with Spinach leafs. • **Petri dish bioassay** contained spinach leaf, infected cadaver and larvae.
Evaluation:	• Assessment of larvae behaviour to infected cadavers (97 larvae replicates). • Assessment of feeding stimulant effect of infected cadavers (45 larvae replicates). • Response to volatile components of infected cadavers (55 larvae replicates). • Assessment of BVs climbing and foraging -induced behaviour of larvae on plants (30 larvae replicates).
Results:	• Laboratory studies in Petri dish arenas indicated no differences in the frequencies of selection, contact or necrophagous feeding on infected and non-infected cadavers. • Physical contact and feeding on infected cadavers resulted in a high prevalence of lethal virus infection in experimental insects. • Low levels of virus infection were observed in insects that had no direct contact with the infected cadaver. • No evidence for differential responses to volatiles emitted by infected and non-infected cadavers placed on leaf discs. • Insect pathogenic viruses have not been reported to produce volatile compounds that favor their transmission. • Greenhouse observations indicating that SeMNPV-infected cadavers were attractive to healthy conspecifics, laboratory tests and olfactometer studies provided no evidence for the existence of

	virus-associated olfactory or phago-stimulant factors that might induce intraspecific necrophagy in *S. exigua* larvae. • BV-induced climbing behavior resulted in infected insects dying in the upper 10% of the plant, which was significantly higher up the plant than the site at which non-infected conspecifics were located at the moment of death of the diseased insect.
Conclusions:	• Climbing in BVs infected insects has been shown to be a pathogen induced behavior that increases the dispersal of viral OBs on the host plant as the insect cadaver disintegrates and OBs fall, or are washed by rainfall, over inferior plant foliage. • BV-induced climbing behavior, involving an increase in the height of infected larvae on the plant and their movement close to the central plant stem, increases the frequency of encounters between viruses infected cadavers and healthy larvae foraging for young foliage. This resulted in a very high incidence of intraspecific necrophagy; a behavior that resulted in transmission of SeMNPV.

RMS conclusions: The study is interesting in term of virus transmission and dispersion.

B2.2.6 Tritrophic interaction BVs-Host plant-insect

Trittrophic interactions have become widely recognised as an important factor in the management of herbivorous insects with entomopathogen biocontrol agents. The ability of the host plants to modified the infectivity of the BVs in the lepidoptera insects have been widely documented (Hoover *et al*, 2000; Ali *et al*., 2004). The different host plant, the leaf chemistry and plant architecture, can influence in BVs persistence and can inhibited or enhance the infectivity of BV within the insect guts (Shikano *et al*., 2010) (**Figure 2.2.2-04) Wan *et al*, 2016.**

Reference:	Wan, N.-F., Jiang, J.-X., Li, B. (2016) Effect of host plants on the infectivity of nucleopolyhedrovirus to *S. exigua* larvae.
Report No.:	**KMA 2.2.2/15**
Guideline:	NO
GLP:	NO
Summary:	Previous studies have shown that the infectivity of BV to herbivores is affected by phytochemicals ingested during the acquisition of viral inoculum on the foliage of host plants. Here, we measured the effects of 14 host plant species on the infectivity of *S. exigua* nucleopolyhedrovirus (SeNPV) to its larvae. The order of the LD_{50} values of SeNPV among the host plants was *Ipomoea aquatica* > *Brassica oleracea* > *Raphanus sativus* > *Amaranthus tricolor* > *Spinacia oleracea* > *Vigna unguiculata* > *Solanum melongena* > *Capsicum annuum* > *Apium graveolens* > *Allium fistulosum* > *Lactuca sativa* > *Brassica chinensis* > *Zea mays* > *Glycine max*, with 940.1 ± 2.26, 424.0 ± 0.60, 295.2 ± 1.13, 147.3 ± 0.63, 138.6 ± 0.22, 119.9 ± 0.07, 119.8 ± 0.02, 109.2 ± 0.18, 104.8 ± 0.62, 102.1 ± 0.66, 97.9 ± 0.22, 89.9 ± 0.32, 79.0 ± 0.13 and 64.0 ± 0.38 OBs per larva, respectively, and the values of mean time to death of virus-infected larvae were 6.21 ± 0.11, 7.12 ± 0.10, 7.33 ± 0.21, 6.97 ± 0.02, 7.06 ± 0.01, 7.29 ± 0.03, 7.32 ± 0.05, 7.07 ± 0.08, 7.24 ± 0.11, 7.09 ± 0.13, 7.50 ± 0.06, 7.23 ± 0.01, 7.30 ± 0.02 and 7.19 ± 0.07 days, respectively. The mean time to death of larvae decreased with increasing viral dose, and corrected mortality decreased as the larval mean time to death increased. These findings have significance for understanding the effects of host plants on the infectivity of BV to noctuids.
M&M: **Test substances:**	• **Test Item**: SeMNPV isolate from cabbage field collected in Shanghai, China. • **Target organism**: beet armyworm (*S. exigua*, Hübner) from cabbage fields collected in Shanghai, China. • **Host plants:** fourteen crop species.
Study design & methods **Experimental treatment:**	• **Leaf disc method** • **Statistical analysis:**

Evaluation:	- The infectivity indices of SeMNPV-corrected mortality and mean time of death of larvae by two ways ANOVA. - LD50 and LT50 by fitted equations. - Differences between host plant in above two infectivity indices - Correlated mortality and virus dose by linear regression
Results:	• Host plant virus and virus dose have a significant effect on the correlated mortality and mean time death (**Figure B.2.2.6-01)**. • Host plant foliage has a significant effect on the correlated mortality and mean time death of beet armyworm treated with 5 different virus doses.
Conclusions:	• The mean time to death of larvae decreased with increasing viral dose, and corrected mortality decreased as the larval mean time to death increase.

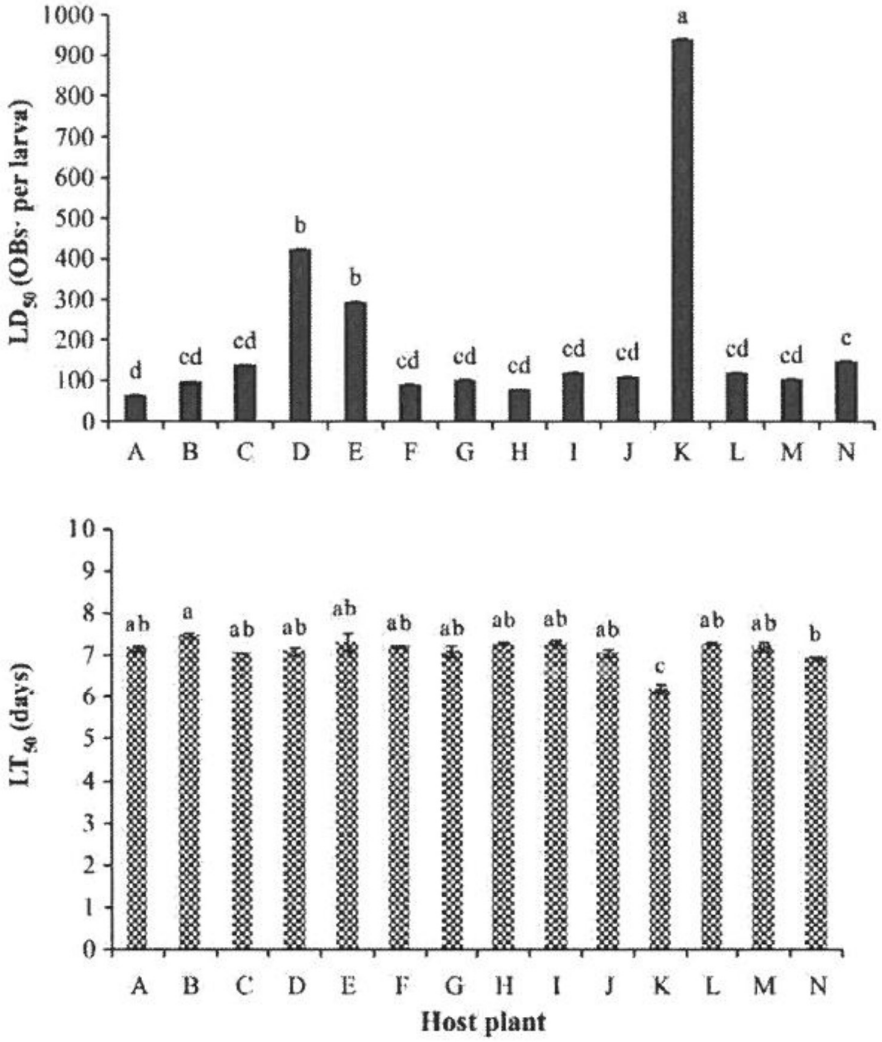

Figure B.2.2.6-01 Median lethal dose (LD_{50}) (A) and median lethal time (LT50) (B) of beet armyworm larvae consuming aliquots of SeMNPV on foliage of 14 crop plant species. Vertical bars represented the standard errors; different lowercase letters on the bars indicate significant difference among crops species at $p<0.05$ (Tukey´s test, ANOVA), (Wan *et al.*, 2016).

RMS conclusions: The study clearly showed that host plant species played an important role in mediating the infectivity of SeMNPV to *S. exigua*. These examples demonstrate the modulation of SeMNPV virulence by the plant environment on which target insect larvae are feeding. This environment either indirectly alters insect response and tolerance, or interferes with the first steps of virus infection through variation of the milieu of the midgut lumen (e.g. pH), and/or directly modulates the specific activity.

B.2.3 HOST SPECIFICITY RANGE AND EFFECTS ON SPECIES OTHER THAN THE TARGET HARMFUL ORGANISM

BVs have been found only in arthropods. No member of this family is known to infest vertebrates or plants. In general, the host range of one BVs species is restricted to few species within one family (Burges *et al*., 1980). SeMNPV differs from other BVs in the fact that it infects only a single insect species (**Table B2.3-01)** and is highly virulent for larvae of the beet armyworm *S. exigua*.

SeMNPV only replicates in *S. exigua* cells. Simón *et al*. (2004) determined the course of infection processes and virus replication in larvae of *S. littoralis*, *S. frugiperda*, and *S. exigua* after oral intake of occlusion bodies or after injection of occlusion derived virions into the haemocoel. None of these treatments resulted in virus progeny in the heterologous hosts. Block of virus proliferation occurs after the haemocoel transmission stage and the virus was then cleared from larval tissues (Simón *et al*., 2004).

The host range of SeMNPV was determined in cross-infection experiments (summarized by Gröner, 1986) concluding in being infective only for *S. exigua and* not for other noctuid lepidopteran species (**Table B.2.3-01).**

Replication in animals other than arthropods or in plants was never observed for BVs. No adverse effects on human health has been observed indicating that the use of BVs is safe and does not cause any health hazards (OECD, 2002).

Alternative host species	Family	Result*	Reference
Agrotis segetum	*Noctuidae*	-	Gröner, 1986
Amathes c-nigrum	*Noctuidae*	-	Gröner, 1986
Mamestra brassicae	*Noctuidae*	-	Gröner, 1986
Mamestra oleracea	*Noctuidae*	-	Gröner, 1986
Spodoptera frugiperda	*Noctuidae*	-	Simón *et al*., 2004
Spodoptera littoralis	*Noctuidae*	-	Gröner, 1986, Simón *et al*., 2004
Plutella xylostella	*Plutellidae*	-	Gröner, 1986
Galleria mellonella	*Pyralidae*	-	Gröner, 1986
Ostrinia nubilalis	*Pyralidae*	-	Gröner, 1986
Adoxophyes orana	*Tortricidae*	-	Gröner, 1986
Cydia pomonella	*Tortricidae*	-	Gröner, 1986

* (+) successful attempt
(-) Unsuccessful attempt of cross-transmission

Table B2.3-01: Results of experiments to infect larvae or cell cultures from Lepidopteran species with SeMNPV

Infectivity of different virus species from *Spodoptera* species was analysed in *S. exigua*, *S. littoralis*, and *S.frugiperda*. In this context, SeMNPV was fed to 2 nd instar larvae or injected into the haemocoel of 4th instar larvae of *S. littoralis* and *S. frugiperda*. No symptoms were observed after feeding or injection into the haemocoel and no virus replication occurred in heterologous species. Initial infection occurred in *S. littoralis* and *S. frugiperda*, but infection stopped and the virus

was cleared from larvae (Simón *et al*., 2004). SeMNPV is considered among the most specific BVs as it only infects one single insect species, *S. exigua* (Ijkel *et al*., 1999). This extremely high host-specificity is especially important for assessing the side-effects on beneficial arthropods and other non-target organisms.

Recent host range experiments (Chen et al., 2019) were carried out on *S. exigua*, *S. litura, A. ipsilon*, *A. segetum, B. mori, H. cunea*, and *S. salicis* using SeMNPV-QD and SeMNPV-US1isolates. On the third day after the inoculation, larvae of *S. exigua* showed obvious symptoms of viral infection and began to die. Mortality reached 100% on day 7. The other insect species showed no symptoms of infection. Most of them pupated normally, and only a few died. No viral polyhedra were detected in the dead larvae. One of the characteristics of the insect BV is a narrow host range but host range varies among different species of nucleopolyhedrovirus. For example, *A. californica* multiple nucleopolyhedrovirus (AcMNPV) can infect dozens of insect species, while most nucleopolyhedroviruses infect one or a few host species. Vlak et al. (1981) reported that SeMNPV isolated in a Dutch greenhouse could cause polyhedrosis in *S. exigua*, but not in the congeneric species S. littoralis. Cross transmission of SeMNPV to other noctuid's, such as *Mamestra brassicae*, *M. oleracea,* A. c-*nigrum, A. segetum*, or members of other families, *Ostrinia nubilalis, Galleria mellonella, Plutella maculipennis*, Adoxophyes orana, and Laspeyresia pomonella, did not occur. *S. exigua* was the only species among those tested insects that was susceptible to infection with SeMNPV-608 (Gelernter and Federici, 1986), and the tobacco budworm, (*Heliothis virescens*), cabbage looper (*Trichoplusia ni*), and fall armyworm (*S. frugiperda*) larvae all survived to pupation. Its bee confirmed the high degree of host specificity of SeMNPV.

RMS comments: All document provide information on the high host specificity of BVs family. Even though, there is no studios with the isolate SeMNPV-Bv0004 of the effect in non-target related arthropods (*S. litura, Agrotis ipsilon, A. segetum, Bombyx mori, Hyphantria cunea, or Stilpnotia salicis*).

There is no scientific evidences confirmed SeMNPV-Bv-0004 only infects the larvae of *S. exigua.*

It cannot be confirmed the high host specificity of SeMNPV-Bv0004. A data gap is therefore identified.

B.2.4 DEVELOPMENT STAGES/LIFE CYCLE OF THE MICRO-ORGANISM

Occlusion bodies are ingested by the larvae. Subsequently, the occlusion bodies are dissolved in the alkaline midgut and occlusion-derived virions (ODV) are released. The nucleocapsids attach to the microvilli and enter the midgut epithelium cells. The nucleocapsids are transported to the nucleus and release their DNA, followed by DNA replication and expression. After virus replication in the midgut cells, budded virus are released through the basal lamina into the hemocoel and subsequently spread to other organs, like the fat body, epidermis, tracheal matrix, muscle, nerve, Malpighian tubules, and glandular tissues. Finally, occlusion bodies are formed, leading to hypertrophy of cells and the swollen appearance of larvae at late infection stages. Disintegration of the larval cadavers leads to release of new occlusion bodies which can again infect larvae (OECD, 2002).

The OB protects the virion and makes it quite stable at moderate and low temperatures, in soil under humid surroundings, and resistant to various chemicals. This ensures the existence of the virus even when no hosts are present. Occlusion bodies can be transported by insects, e.g. adult *S. exigua*, but also predators or parasitoids. Furthermore, transport by birds is possible (Entwistle *et al*., 1978).

B2.4.1 Sequential steps of nucleopolyhedrovirus transmission and replication

Infection only occurs after ingestion of polyhedra by larvae. Gross pathology is characterised by loss of reactivity of larvae to external stimuli and swelling of the larvae, followed by glossy and moribund appearance 4 to 6 days after infection. From day 6 on, larvae begin to die and subsequently liquefy, releasing further NPV that are able to infect other larvae (OECD, 2002). During primary infection, **(Figure B.2.4.1-01)** (A) OBs are ingested during feeding on contaminated foliage. (B) OBs are solubilized in the insect midgut and release ODVs that pass through the peritrophic membrane (C) and fuse with the microvilli of midgut epithelial cells (D). Nucleocapsids travel to the nucleus where they release the viral genome to initiate replication. (E) Virus replication occurs in virogenic stroma. Progeny nucleocapsids assemble and bud through the basal membrane (F) during which they acquire an envelope containing GP64 or F fusion protein present in the virus-modified cell membrane. During the secondary phase of infection these budded virions (BVs) disperse in the hemolymph or along the cells of the insect traqueal system (traqueoblasts) to spread the infection to the cells of other tissues in the insect. (G) BVs enter cells by endocytosis and replicate in the nucleus. Newly assembled

nucleocapsids (H) may bud out of the cell or may be enveloped to form ODVs that are occluded into OBs (I). At the end of the infectious cycle OBs accumulate in the nucleus (J). Upon death the larvae typically hang from the uppermost leaves of the host plant (K), the larval tegument ruptures and releases OBs that contaminate foliage for further cycles of horizontal transmission.

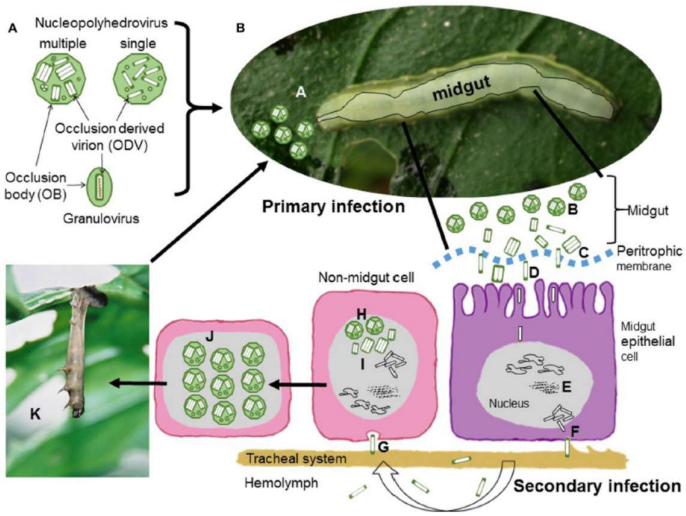

Figure B.2.4-01 BV Infection process (Williams *et al*. 2017).

The improvement of BVs biological activity against pests is closely related to understanding their genome organization. To associate the BVs biology with the genes involved in BVs genome and understand their life cycle contribute toward an improvement in their efficacy as biopesticides and help their effective use (Toprak *et al*., 2005). To date, complete genome sequences of *S. exigua* is available, therefore, transcriptome data of *S. exigua* is also available. Analysis of the mechanism of insect resistance to control agent or in general studies on the mode of action of chemical and microbial pesticides, were previously limited through the lack of genetic information on the target species. Next generation technologies have strongly increased the ability to acquire genome information. Thus, for example, it is possible to characterize the transcriptome of *S. exigua* larvae with special emphasis on pathogen-induced genes (Pascual *et al*. 2012). The insecticidal activity is modulated by the insect immune system. New insights obtained by next generation sequencing data enable to understand resistance development better in the future.

B.2.5 INFECTIVENESS, DISPERSAL AND COLONISATION ABILITY

BVs are only infective to arthropods, and mainly to insects from the orders Lepidoptera and Hymenoptera. SeMNPV can only infect and multiply on species of the specie *S. exigua*. No infectivity and replication was ever observed in any non-arthropod. BVs are only passively dispersed and active colonisation does not take place as multiplication is dependent on susceptible hosts (Krieg, 1976).

B2.5.1 Replication strategies

The BVs replication strategy involves a series of temporally coordinated events that begin when the infecting nucleocapsids release the viral genome into the nucleus (Rohrmann, 2013). During the first _6 h post-infection, the host RNA polymerase II transcribes immediate-early viral genes (ie-0, ie-1, ie-2, pe38) that are expressed in the absence of any other viral proteins and which encode transcription factors and delay dearly genes that promote genome replication and the expression of late genes and block apoptosis. The late genes (6–12 h post-infection) include a virally encoded DNA polymerase for genome replication, a virally encoded RNA polymerase, structural proteins and a range of late expression factors (left) involved in genome replication and transcription, as well as many genes with auxiliary functions. Viral DNA replication is concurrent with the expression of structural components necessary for the assembly of new nucleocapsids at 6–24 h post-infection and ODVs and OBs at 18–72 h post-infection. These temporal gene classes are mainly coordinated through DNA promoter elements (Rohrmann, 2013). BVs infection induces a plethora of changes in

host cell functions including cytoskeletal remodeling, cell cycle arrest, modulation of cellular stress responses and marked changes in cellular metabolism (Monteiro *et al*., 2012).

B2.5.2 Mixed-mode transmission

BVs adopt a mixed-mode transmission strategy involving both HT and VT that is common across a broad range of viruses, parasites, symbionts, and microbiota (Ebert, 2013). HT is usually risky if susceptible hosts are rare, while VT is safer, but is constrained by host survival and reproductive success. As a result, HT is selectively advantageous at high host densities, whereas VT is favored in low density host populations. In the case of BVs, these transmission strategies generally exclude one another because the production of massive numbers of OBs for ht results in host death prior to the adult stage. Through the examples provided in Williams *et al* 2017 document it have been proved that mixed-mode transmission involving long-lived viral OBs in the environment and VT from infected adult insects to their offspring has key implications for the persistence, spatial dispersal and genetic diversity of insect BVs.

B.2.6 RELATIONSHIPS TO KNOWN PLANT OR ANIMAL OR HUMAN PATHOGENS

Known BVs have been exclusively isolated from arthropods ((OECD) 2002) and not from other animals, humans or plants.

SeMNPV as well as all other BVs are not related to any known plant, animal (other than arthropods) or human pathogen.

B.2.7 GENETIC STABILITY AND FACTORS AFFECTING IT

The used strain of SeMNPV is maintained at –24°C since its isolation. No variation in infectivity to the host was observed.

BVs isolates always contain a mixture of slightly different genotypes, with proportions of the different genotypes being stable during multiplication in the same host population (Muñoz et al. 1998). This micro-heterogeneity helps to account for variation in the host insects.

RMS comments: According to data requirement on 283/2013 2.7“information on genetic stability (e.g. mutation rate of traits related to the mode of action or uptake of exogenous genetic material) under the environmental conditions of proposed use must be provided”

Muñoz et al. 1998 provides general information on genotype stability of BVs. There is no information on genetic stability of the specific isolate SeMNPV BV-0004 during the production process, the storage or under environment condition of use.

B2.7.1 Genetic stability in cell culture serial passages

It has been reported that the passage of SeMNPV in *S. exigua* cell lines leads to the rapid accumulation of deletion mutants, some of which have been demonstrated to have defective interfering properties. The OBs of these mutants are significantly less virulent in *S. exigua* larvae. This phenomenon is due to a deletion of about 25 kbp from the SeMNPV genome. Moreover, the genotypic alterations can result in changes in phenotypic characteristics, including virus morphology and biological activity. The occurrence and accumulation of defective or mutant viruses upon passage of NPVs in cell culture have been reported in several studies. The most common mutants are few polyhedra and defective interfering particles. Both mutant viruses cause significant reduction in OB formation and virus infectivity.

Serial passages of different BVs in cell culture often result in “Few Polyhedra” mutants where only a low number of polyhedra is produced within one cell. For example, few polyhedra mutants were observed after serial passage of a *Helicoverpa armigera* NPV isolate in cell culture (Lua *et al*., 2002). This reduction in polyhedra number directly results in reduced infectivity.

A single passage of SeMNPV in *S. exigua* cell cultures resulted in mutants that lacked infectivity to larvae upon feeding or injection. This is associated to a deletion in the genome (Heldens *et al*., 1997).

In order to evaluate the potential of this cell line for the production of SeMNPV, Chaeychomsri (2018) have propagated the virus in cell monolayers to determine the kinetics of virus replication. The phenotypic changes in the SeMNPV OBs observed in this study were likely due to the influence of viral genes, thus limiting the nucleocapsid organization during ODV and OB assembly and occlusion. The present study provides more information on the in vitro propagation of the SeMNPV in homologous cell line and the abnormalities in virion envelopment and occlusion within OBs, leading to significant reduction in the assembly of virions. The findings in the present study suggest that the size of OBs and virus morphogenic features resulted from some factors both in host cells and in the viral genomes. These factors may play an important structural or accessory role in determining the size and shape of OBs, nucleocapsid assembly and ODV formation.

Horizontal gene transfer commonly occurs from cells to viruses but rarely occurs from viruses to their host cells, with the exception of retroviruses and some DNA viruses (Liu *et al*., 2015). Transduction is a way for bacteria to exchange genetic material using a virus that takes up a piece of DNA from its bacterial host and incorporates it into its own viral genome. After the virus has multiplied, many copies of the virus erupt from the infected cell. Depending on the kind of transduction, some or all of the daughter viruses take copies of parts of the bacterial DNA with them. When one of them infects a new cell, it inserts the stolen DNA into the new cell, where the stolen piece becomes integrated into the new cell's DNA. Transduction by viruses works in eukaryotic organisms as well.

BVs have the ability to efficiently transduce non-insect cells with: BV-mediated in vitro and ex vivo gene delivery into dormant and dividing vertebrate cells of diverse origin (human, monkey, pig, rabbit, rat, feline, mouse, fish, avian, frog, etc.) has been described convincingly by many authors (Airenne *et al.,* 2010; Condreay *et al.,* 1999; Hu, 2008; Cheng *et al*., 2004; Airenne *et al*., 2013). The most widely studied BV, *A. californica*, a multiple polyhedrovirus, is able to penetrate and deliver genes into non-target cells (Airenne et al., 2010), but in the non-target cells, the virus is incapable for replication or proper viral gene expression and is thus nontoxic for the vertebrate cells.

However, no information has been found on the potential transfer of genetic material to other organisms from viruses used as biocontrol agents. Sun *et al.* (2005) showed that *Helicoverpa armigera* nucleopolyhedrovirus cannot transfer a foreign gene to organisms in the same ecological niche (the fungus *Verticillium dahliae Lleb and the ladybeetle Propylaea japonica Thunberg*). However, a study showed that horizontal transfer of gene exists from the insects hosts *Cydia pomonella* and *Cryptophlebia leucotreta* to the *Cydia pomonella granulovirus* that infected them (Arends *et al.,* 2005), and also that baculoviruses acquired the chitinase gene from the bacterium *Serratia marcescens* (Kang *et al.,* 1998). In the same manner, Lauzon *et al*. (2006) showed that Neodiprion sertifer nuclear polyhedrosis virus have acquired genes by HGT from their insect hosts, as well as BV GV (Rohrmann, 2011).

RMS comments: Genetic stability of the target BV SeMNPV BV000-4 is unclear. No information was submitted.
Taken together the general results on BVs, studies show that changes in phenotypic characteristics of SeMNPV can be affected by host as well as viral factors. Even though, no information has been found on the potential transfer of genetic material to other organisms from viruses used as biocontrol agents.

B2.7.2 Genotypic variation in SeMNPV isolates

BVs population naturally harbor high genomic variation located in genes involved at different levels of the complex interaction between virus and host during the course of the infection process. It was confirmed by several studios with different SeMNPV isolates by genomic comparative analysis, that the differences in BVs virulence and transmission phenotypes involved multiple molecular pathways.

The first SeMNPV genome that was completely sequenced was that of American strain **SeMNPVUS1** (Ijkel *et al*., 1999). This genome contains 135,611 bp and 139 ORFs. However, the genome lacks homologues of the major budded virus glycoprotein gene gp64, the immediate-early transactivator ie-2 and BV repeat ORF (bro) genes. Subsequently, the genomes of a number of other SeMNPV strains were completely sequenced (Thézé *et al*., 2014). This study of genomic diversity among several isolaes of SeMNPV revealed a high number of polymorphic sites Thézé *et al*. (2014) compared the genomes of seven European isolates with that of the isolate from the USA (SeMNPV-US1). Although these isolates

all have similar genome size and G+C content, the mutations in some of them caused changes in gene function, resulting in different virulence and transmission characteristics among them. There is a high level of genotypic heterogeneity in natural SeMNPV populations that was demonstrated in groups of virus-killed larvae collected from greenhouse soil. The key traits of the virus, such as virulence and transmission strategies, mainly depend on variations of the gene (Thézé *et al*., 2014). In the study (Chen et al., 2019) investigated the morphological features and the host range of a new BVs of SEMNPV, the SeMNPV-QD isolated from naturally diseased larvae of *S. exigua* in the field in Qingdao, China. The entire genome of SeMNPV-QD was sequenced. The genome sequence analysis showed that the genome, compared with the genome of SeMNPV-US1, have large differences. Among the sequenced SeMNPV isolates, there are one American strain, SeMNPV-US1 and seven European isolates, including VTSeAl1, VT-SeAl2, VT-SeOx4, HT-SeG24, HT-SeG25, HT-SeG26 and HT-SeSP2A. The genome of SeMNPV-US1 was assembled with a contiguous sequence of 135,611 bp. One hundred and thirty-nine ORFs, defined as methionine initiated ORFs encoding more than 50 amino acids (IJkel *et al*., 1999). The VT-SeOx4 genome is the longest with 142,709 bp, while the VT-SeAl2 genome is the shortest with 134,972 bp. The six isolates, including VTSeAl1, VT-SeAl2, VT-SeOx4, HT-SeG24, HTSeG25, and HT-SeG26, contain 137 ORFs, and the isolate HT-SeSP2A genome is 142,709 bp, encoding 138 ORFs (Thézé *et al*., 2014). The genomic similarity between SeMNPV-QD and SeMNPV-US1 was 45.8%, and that between SeMNPV-US1 and the seven European strains was 97.3%. The similarity among European strains was 98.7%.

B2.7.3 Recombination in SeMNPV isolates

The possibility that MPCP introduced BVs might recombine with indigenous strains and produce recombinants with novel characteristics is an important consideration for the introduction of BVs geographically distinct strains.

The results reported in Muñoz et al, 1997 add further evidence to the high frequency of recombination among BVs. *S. exigua* larvae were co-infected with two different isolates from the USA, SeMNPVUS1 and from Spain, SeMNPVSP2, respectively. A new recombinant isolate, **SeMNPV-SUR1** was detected after the first infection cycle and successively replaced both parental isolates (Muñoz *et al*., 1997). All three isolates did not differ significantly in the dose required to kill larvae or in the time between inoculation and killing of larvae. Differentiation of isolates was possible through mapping of restriction fragments. The identity and relative location of diagnostic markers from Se-US and Se-SP2 that were found in Se-SUR1 suggest that Se-SUR1 was generated as a result of recombination involving at least four crossovers **(Figure B2.7.3-01**).

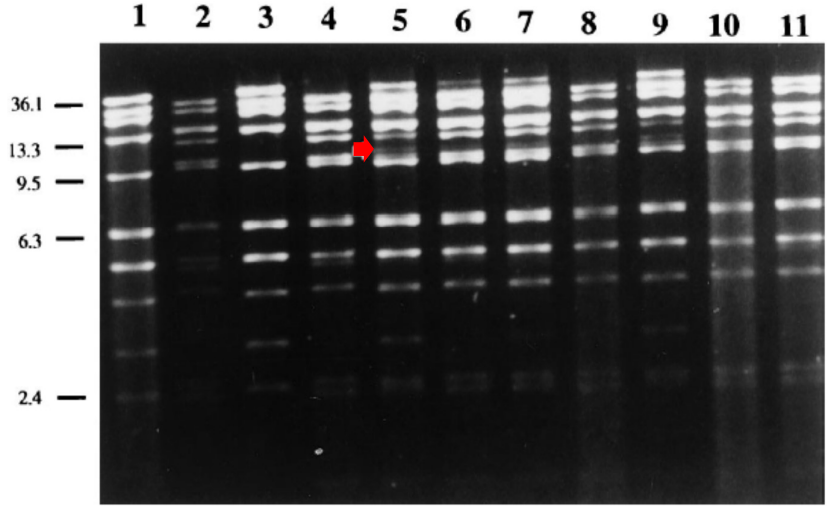

Figure B2.7.3-01 Bg/II profiles of viral DNA from the first coinfection of larvae with Se-US, lane (1) and Se-SP2 Lanes (1)-to (11), lanes (2) –(10) viral DNA from individual coinfected larvae and lane (11) SeSP2. A novel 10.6Kb Bg/II submolar fragment not present for either SeSP2 or SeUS is indicated by an arrow in lane (5). (Muñoz *et al.,* 1997).

It was demonstrated that recombination can also occur in the natural host indicates that (i) introduction of foreign strains of SeMNPV in regions where natural strains already exist may result in the appearance of new genotypes from recombination between the parental viruses, (ii) the recombinants may replace the parental strains, and (iii) the recombinants may have an altered biological activity. For all these reasons, epidemiological and ecological studies are strongly recommended before the release of biological control agent BVs bioinsecticides with active components different from the natural population strains. It is also recommended that extensive field sampling be carried out prior to releasing new strains to preserve the natural diversity of BVs strains.

Conversely, genetic stability have been demonstrated in the isolate from Florida SeMNPV-US2, contains 7 different genotypes, with two of these considered as "parasitic" genotypes that are not able to replicate alone in the absence of other genotypes (Muñoz *et al*., 1998). Cloned variants of US2 were able to kill *S. exigua* larvae independently. Moreover, the REN profiles for each variants were stable through 10 consecutive passages of each variant in insects. The presence of these parasitic genotypes demonstrates the stability of the genotype composition of the BVs isolate.

RMS comments: According to the above documented studios on different isolates of SeMNPV (none of them have been done with the target isolate), it was concluded that variation in infectivity and modification in the genome structure of the BVs can easily occur. These variations can be produced during the production process, the subculture cell culture, or during the storage. Even in the environment, when the virus is exposed to aggressive environmental conditions (UV, high temperatures, and desiccation) or even to other different isolates. The studio of the genetic stability is necessary for the isolate SeMNPV BV-0004. There applicant have no confirmed genetic stability of the specific isolate SeMNPV BV-0004 (variation in infectivity or in genetic structure). The analysis of the genetic variation at the genome level is important for understanding the diversity of SeMNPV infectivity and the relationships between the SeMNPV and its host *S. exigua*.

An important feature of SeMNPV viruses is also its capacity to replicate in and spread through host populations. Consequently, applications of anexotic SeMNPV strain to reduce damage by *S. exigua* replace or alter other indigenous SeMNPV populations that are present at enzootic or epizootic levels. To better evaluate and ascertain this possibility, it is important to know genetic structure of the virus in order to be able to monitor possible genetic alteration in virus populations.

Mass production of BV for insecticidal purposes is done in the insect host with wt virus isolates, which most frequently contain heterogeneous viral populations. While the coexistence of such a range of genotypic variants may be important for virus survival under field conditions, a quality control of commercial virus products would prevent the spread of undesirable parasitic viral variants.

Biological studies of the different variants will help select the most competitive variants for biopesticide design, since those affected by large deletions or insertions, which might disrupt the expression of essential genes, may actually interfere in the biological activity of regular variants.

- Document KMA 2.7-5 is interesting in terms of evolutionary mechanism, but doesn´t report further information on genetic stability of the isolate SeMNPV. The document Serrano et al. (2013) compared a SeMNPV with a SfMNPV isolate and found similar population structures in the distinct BVs species from different hosts indicating a shared evolutionary mechanism.

- Document KMA 2.7-6 is interesting in terms of the identification of genes involved in pathogenicity and virulence but doesn´t report further information on genetic stability of the isolate SeMNPV, therefore, it was moved to section B2.2.2, mode of action.

B.2.8 EFFECT OF ENVIRONMENTAL PARAMETERS ON GROWTH, INFECTIVITY, DISPERSAL AND COLONIZATION ABILITY: TEMPERATURE, SUNLIGHT, HUMIDITY, PH AND HOST REQUIREMENTS.

An excellent summary on the environmental stability of BVs has been reviewed by Jaques (1977), which includes the results of all older laboratory and field studies of importance. The summary of Jaques has been further compressed and augmented with supplementary results of newer studies here.

B2.8.1 Environmental factor that can influence in environment survival outside the host

Temperature

The effect of temperature on BVs is significant regard to their stability in storage and in the field following application. Whereas expose to low temperatures has little effect on activity of the OB, expose to high temperatures would cause inactivation **(table B2.8.1-01).** Test by several works summarized in table (**table B2.8.1-01)** show that a 10-min expose to temperatures of 70-80ºC would expected to inactivate the virus.

Virus suspension	Temperature (ºC)	Period of exposure	Loss of activity (Estimated %)	Reference
Heliothis NPV	37.7	2 hr	<10	Stuermer and Bullock 1968
	60	2 hr	<10	
	71	15 min	>50	
	71	2 hr	>75	
	82	15 min	>75	
Heliothis NPV	70	10 min	<10	Gudauskas and Cannerday 1968
	80	10 min	>90	
Trichoplusia ni NPV	75	10 min	<10	Gudauskas and Cannerday 1968
	80	10 min	75	
	90	10 min	>95	
Pieris brassicae GV	20	1 yr	25	David and Gardiner 1967b
	40	20 days	75	
	50	5 days	<90	
	60	24 hr	>90	
	65	1 hr	>90	
	70	10 min	>90	
Pieris rapae GV	70	10 min	10	Tanada 1953
	75	10 min	>90	

Table B2.8.1-01 The effect of high temperature on viral activity. (Jaques, 1977).

Low temperature. The stability of BVs stored under refrigeration is indicate in **Table B2.8.1-02**. These studies on the effect of temperature indicate that the virus should withstand maximum temperature normally encountered in the field environment (air temperature 31C) or soil temperature (50ºC), at least for short periods. The maximum temperature of surface soil not covered by plant growth could exceeded 50ºC, but when the plant is present, the soil temperature would not reach this level.

Virus	Storage temperature (ºC)	Period of storage (years)	Loss of activity (Estimated %)	Reference
Bombyx mori NPV (Ampules)	4	20	<50	Steinhaus 1960
Neodiprion hercyniae NPV (Cadavers)	4	6	<25	Neilson and Elgee 1960
Lambdina f. fiscellaria NPV (Suspension)	4	6	>90	Cunningham 1970
Trichoplusia ni NPV (Suspension)	4	4	<10	Jaques (Unpublished)
Pieris brassicae GV (Dry)	0	4	<10	David and Gardiner 1967b
Pieris rapae GV (Suspension)	4	4	<10	Jaques (Unpublished)
Panonychus citri virus (Dry)	4	6.5	<10	Shaw et al. 1972.

Table B2.8.1-02 The effect of low storage temperature on viral activity. (Jaques, 1977).

Sixteen temperature-sensitive mutants of *A. californica* nuclear polyhedrosis virus were isolated. Several interesting phenotypes were observed. A large proportion of the mutants were unable to form polyhedral occlusion bodies (polyhedra) at the nonpermissive temperature (32.5°C). At 32.5°C, one mutant formed plaques in which the cells lacked polyhedra. Another mutant type was defective in the production of progeny extracellular no occluded virus and produced a “plaque” consisting of only a single cell containing polyhedra at 32.5°C. One mutant was defective in plaque formation, progeny no occluded virus formation, and polyhedra formation at 32.5°C. Several mutants produced non-occluded virus but failed to produce plaques or polyhedra at 32.5°C. Other phenotypes were also distinguished. Complementation analyses, performed by either measuring the increase in extracellular non-occluded virus formation or by observing

polyhedra formation in mixed infections at 32.5°C, indicated the presence of 15 complementation groups. A high frequency of recombination was observed. Four of the mutants were found to be host dependent in their temperature sensitivity for polyhedra formation.

Sunlight

Sunlight is considered the most important factor contributing to the inactivation of viral inclusion bodies. *Trichoplusia ni* NPV (TnNPV) and *Pieris rapae* GV (PiraGV) applied to leaves of cabbage in field plots lost at least 50% of original activity within 2 days and retained less than 15% of original activity after 10 days **(Figure B.2.5-01**). Similar results of inactivation were obtained with *Heliothis* sp. NPV on cotton, corn silks, soybean foliage, and on glass plates.

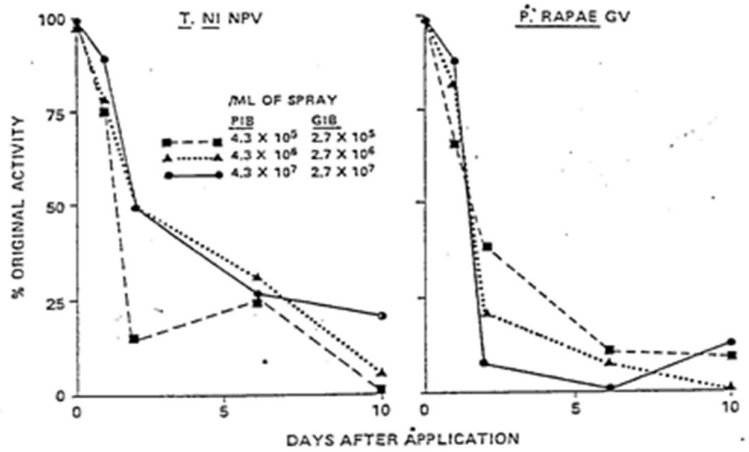

Figure B.2.8.1-01 Activity of *Trichoplusia ni* NPV and *Pieris rapae* GV following application of suspensions of occlusion bodies to leaves of cabbage in field plots (Jaques, 1977).

There is considerable evidence that it is the ultraviolet portion of sunlight that inactivates insect viruses. Krieg *et al*. (1981) determined the sensitivity of insect pathogens to short-wave UV light (254 nm) and to longer-wave UV light (285-380 nm). The results are summarized in Tables B. 2.5 2.5-1 and B2.5-2.

Pathogen (irradiated object)	**Inactivation rate (%)**	**Inactivation dose (mW sec/cm^2)**
Mamestra brassicae NPV	90.0	11.9
(polyhedra)	99.0	46.9
Cydia pomonella GV	99.0	32.0
(granula)	99.9	195.6

Table B.2.8.1-03 Comparison of the stability of certain insect pathogens after irradiation with a “Sterisol” lamp (far UV: 254 nm; illumination rate: 0.046 mW/cm2), **(**Krieg *et al*., 1981).

Pathogen (irradiated object)	**Inactivation rate (%)**	**Inactivation dose (mW sec/cm^2)**
Mamestra brassicae NPV	90.0	321 + 1669
(polyhedra)	99.0	918 + 4774
Cydia pomonella GV	90.0	189 + 983
(granula)	99.0	615 + 3198

Table B 2.8.1-04 Comparison of the stability of certain insect pathogens after irradiation with "Ultra-Vitalux" lamps (near UV: 285-380 nm; illumination rate: 0.5 mW/cm2 at 285-315 nm and 2.5 mW/cm^2 at 315-380 nm**), (**Krieg *et al*., 1981).

Rapid inactivation at short-wave UV light (254 nm) was also observed with *Heliothis* NPV, *Trichoplusia ni* NPV, and *Zeiraphera diniana* GV. Exposure of suspensions of *Heliothis* NPV to UV light at 254 nm reduced activity more than UV light at 307.5 nm while exposure to longer-wave UV (364 nm) and a broad-band mixture of visible and IR light did not affect activity. Likewise, the effect of UV light on *P. brassicae* GV decreased as the wavelength was increased from 250 nm to 320 nm. Exposure to high dosages of longer wavelengths had no detectable effect on this virus.

Exposition of *Lambdina fiscellaria lugubrosa* NPV to UV light (366 nm) for long periods did not cause appreciable effect on the virus. The intensity of the UV portion of sunlight as well as its wavelength affected the rate of inactivation of *Neodiprion swainei* NPV. A reason could be a morphological difference between the genera *Alphabaculovirus* and *Gammabaculovirus*.

The inactivation of virus by exposure to sunlight is the most important factor. Naturally, deposits of *Heliothis* NPV on leaves/fruits on the inside of the foliage canopy are more protected than those of the periphery. Certain substances have negative impacts on the stability of *Heliothis* NPV. Leaf exudates may produce alkaline layers of pH up to 10.1 and high concentrations of metallic ions (Evans and Harrap, 1982).

Humidity

It is generally accepted that humidity has less effect on stability of insect viruses than on stability of other types of pathogens. Irrigation of *Heliothis* NPV on cotton leaves did not lead to a loss in activity. However, there may be an indirect influence by affecting chemical action on the virus, by increasing the inactivation rate by sunlight, and by washing off the virus from leaves.

Virus suspensions or dried powders remain active for long periods if they are kept at low temperatures. Freezing and thawing suspensions of *P. brassicae* GV 10 times in 12 days did not cause a significant loss of activity indicating that repeated freezing and thawing of a virus in the field environment would not affect activity appreciably.

pH

Viruses can persist in soil for longer periods. The pH of the soil may affect persistence of viruses. Extreme hydrogen ion concentration buffers have an adverse effect on the infectivity of NPVs. Thomas *et al*. (1973) bioassayed *Trichoplusia ni.* NPV extracts of a loamy sand of various pH at three-monthly intervals and showed a correlation between pH and virus activity: the lower the pH, the more rapidly the virus was inactivated. In general, it is not clear whether, or how much, virus in soil is available for initiating infection in pests.

While it is known that most viruses in intact occlusion bodies are reasonably stable in aqueous suspension, little is known on their persistence in natural aquatic environment. It is supposed that the pH and salt concentration of water would influence stability.

B2.8.2 Effect of environmental substrates

Host Foliage

The leaf is the most important substrate to consider due to SeMNPV target organism, beet armyworm, are leaf-eating insects. The inactivation of SeMNPV by exposure to sun light is the most important factor affecting stability of SeMNPV deposit on leaf. Inactivation by sunlight is influenced by substrate, in that, a broad thick leaf such as cabbage leaf would protect virus deposit in other part of the plant from sunlight by shading.

There are evidence that certain substance on or in the leaves may also influence the stability of SeMNPV. An example is the deposits of the virus *Heliothis* VNP on cotton leaves were inactivated during the night due to the alkalinity of the dew (pH8.2-9.1) and the washing of the leaves surface pH were 9.7-10.1. In other study, a suspension of NPV in buffer pH9.0 had no effect on virus stability, since pH>12 inactivated the BVs. They found that the polyhedral did not dissolved, probably because prolonged and repeated exposure to dew would contribute to dissolution of the OB protein and subsequently to the inactivation of the virus.

Various chemicals, as bicarbonates, carbonates, sodium, potassium and calcium found in dew on leaves could have been responsible for the effects of the dew on deposits of the BVs.

BVs cannot reproduce outside the host.

RMS comments: There is no available document Krieg, A., Gröner, A., Huber, J., Zimmermann, G. Inactivation of certain Insect Pathogens by Ultraviolet Radiation. Journal of Plant Diseases and Protection, 88 (1), 38-48. KMA 2.5/02. Tables from this document cannot be evaluated.

Soil

Several studios have confirmed that BVs may persist for long periods in soil. Jaques (1967) found that T. ni NPV retain about 15% of its original activity 6 years after application to surface soil in field plots. The stability of these BVs in soil is presented in **figure B2.8-04,** where the BV stability remain stable during the 200 week of the experiment. Activity remain in the topc0-0.25cm on the undisturbed soil in the 223 weeks of observation. Little viral activity was observed at the 10-12.5 cm depth. The BV activity were bee reduced by the soil depth. OB appear to be adsorbed quite firmly onto soil particles and are not leached through the soil by rain.

The pH of the soil may also affect persistence of the virus. It was reported that. Ni t NPV was inactivated more quickly in soils with low pH than in neutral soils. In addition, some laboratory studies pointed that the BVs are not affected by soil pH range between 5.5 to 9.0.

BVs can persist and accumulate on soil following application or natural epizootics of disease.

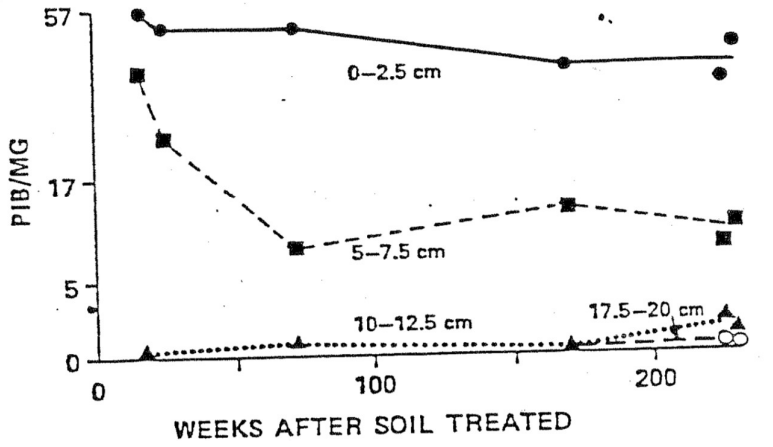

Figure B2.8.2-01 The concentration of NPV at four depth (0-2.5cn, 5-7.5cm, 10-12.5cm and 17.5-20cm) in soil in field plots after treatment of soil at rate of 3.2x 104 PIB/ha (Jaques, 1969).

Water

Most viruses in intact inclusion bodies are reasonably stable in aqueous suspension (Jaques, 1977). *Heliothis* sp. NPV was stable in water at 30°C for one year (Ignoffo, 1992). However, Jaques (1977) continues that it is apparent that the pH and salt concentration of water influences stability. Moreover, once introduced into a water body, the viral particles are likely to deposit and are absorbed by sediments. Furthermore, there is evidence of deactivation/destruction by the UV portion of sunlight in aqueous suspensions. Moreover, it is assumed that the protein of the virus will be completely mineralised by bacterial action in water and sediment. There are no risks of pollution of surface or groundwater expected due to the high level of retention of the viral particles by the soil.

B2.8.3 Dispersal and Persistence in the environment outside the host

Dispersal of BVs can occur through small animals and birds (their feces are able to contain infective viruses), predators, wind blow of dry soil and rain splash at canopy edges. Knowledge of the importance of such mechanisms is scant (Evans and Harrap, 1982).

Occlusion bodies of BVs are able to persist for many years in soil.

B2.8.4 Factors affecting virus activation

Covert infections were first proposed to explain the spontaneous outbreaks of BVs disease that occurred in apparently healthy insects **(Figure B.2.2-03).** Early studies concluded that physiological stress was a major contributor to virus activation. Specifically, overcrowded rearing conditions marked changes in temperature or relative humidity (Williams *et al*., 2017). The ingestion of mildly toxic chemical compounds (Virto *et al*., 2017), parasitism or changes in nutrient availability, have all been reported as potential activators of overt disease, although insect responses are often unpredictable.

The ability to respond to changes in expected host survival and anticipated reproduction, and adopt the transmission pathway that will maximize the fitness derived from each infected host, provides a unique evolutionary advantage to these viruses. As such, phenotypic plasticity in transmission strategy means that viruses with mixed-mode transmission can persist under a wider range of ecological conditions and at a higher prevalence than viruses that adopt strict single-mode transmission (Ebert, 2013).

Covert infection in combination with mixed-mode transmission provides a mechanism by which the BVs can survive when opportunities for HT are scant, such as during periods of low host population densities, during diapause or non-overlapping generations. However, when host densities exceed a threshold that allows sustained HT, the virus has the opportunity to reactive and produce patent disease that kills the host and releases progeny OBs for HT (Cooper *et al*., 2003). This can trigger epizootics of disease in high-density lepidopteran populations in field crops that rapidly reduce the host population to below the threshold density (Myers and Cory, 2016). Covert infection in highly mobile or migratory species also provides a mechanism for virus dispersal over large distances (Hostetter and Bell, 1985; Burden *et al*., 2003; Vilaplana *et al*., 2010). Transgenerational host–pathogen interactions can also convey benefits to the host as a defense against further infections (Jones *et al*., 2011). For example, the vertically transmitted bacterial symbiont, *Wolbachia*, provided protection against infection by a small RNA virus (*Dicistroviridae*) (Hedges *et al*., 2008). Similarly, infection of *Helicoverpa armigera* by a densovirus (Parvoviridae) appears to protect against a second infection by an alphabaculovirus or the bacterial pathogen Bacillus thuringiensis (Xu *et al*., 2014). Conversely, S. exempta larvae infected by Wolbachia were markedly more susceptible to SpexNPV than Wolbachia-free larvae (Graham *et al*., 2012). At the population level, reduced fecundity of covertly infected insects was identified as the most likely cause for delayed recovery in populations of the Western tent caterpillar, M*alacosoma* C*alifornicum pluviale* that crashed following epizootics of alphabaculovirus disease (Cory and Myers, 2009). Unfortunately testing this hypothesis using molecular methods was problematic due the paucity of insects present in low-density populations (Myers and Cory*, 2016*). Population models make several clear predictions regarding the ecological impact of covert infections. In age structured insect populations covert infections were predicted to affect the periodicity and amplitude of population cycles (Bonsall *et al*., 2005). In a subsequent model, a low prevalence of covert infection was favored if opportunities for transmission varied, for example seasonally. In contrast, a high prevalence of covert infection was predicted under three specific

scenarios: (i) when covert infection was due to host immune suppression, (ii) when covert infection directly improved the probability of transmission through patent lethal disease, and (iii) when covert infection protected against lethal infection by other pathogens (Sorrell *et al*., 2009). These models have provided clear predictions that can be tested empirically in laboratory and field populations.

Finally, from a pest control perspective, sublethal effects in insect survivors after field application may be desirable and benefit pest control in subsequent generations as covertly infected insects might be more susceptible to a second virus application, so that effective pest control could be achieved with lower rates of OBs applications (Virto *et al*., 2017).

B.2.9 INFORMATION ON THE PRODUCTION OF METABOLITES (ESPECIALLY TOXINS)

BVs do not have any potential to form toxins and are not able to produce secondary metabolites or metabolites of concern for human health or the environment (OECD, 2002).

B.2.10 ANTIBIOTICS AND OTHER ANTI-MICROBIAL AGENTS

Viruses are sensitive to disinfectants that act by a chemical or physical principle and for which resistance is not a case.

Viruses can be also susceptible to virucidal or virustatic drugs acting mostly by inhibiting certain enzymes. Indeed, viruses may develop resistance to these compounds. However, the mechanism behind the occurrence of viral resistance is different from those in, e.g., bacteria and there is no evidence that resistance may be transmitted from one virus species to another.

B.2.11 REFERENCES RELIED ON

A literature search according to EFSA (2011)[14] was conducted to identify relevant recent published peer reviewed references covering the last 10 years (Gueli Alletti, 2018). The literature research was conducted on the search-engine ProQuest Dialog™. The data requirement "Biological properties of the micro-organism" was covered using a focused search encompassing BVs in general but focused on specific search terms related to biological properties. This focused search retrieved a large number of references (240) which were sorted manually for relevance for the data requirements. After a first check for relevance, 22 references were submitted to full text analysis. According to the full text analysis 17 references were regarded relevant for M-MA Section 1, Section 2 and Section 3 of this dossier. For all details on the selection process, please refer to the literature review report submitted in KMA 2.1.1/01.

RMS comments:

- RMS has considered all document as new information on the current Draft Assessment Report for the new microbial pest control agent SeMNPV.
- In the opinion of the RMS, the literature research made by the applicant according to EFSA 2011 guidance covered the most relevant news for SeMNPV. The RMS has also included some new references considered important for the evaluation.

ABA – Andermatt Biocontrol AG

Data point	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
KMA 1.3/02 MA B.2/01 MAB.2.1.1/22	Jehle, J.A., Lange, M., Wang, H., Hu, Z., Wang, Y., Hauschild	2006	Molecular identification and phylogenetic analysis of baculoviruses from Lepidoptera not available, not applicable Virology, 346, 180-193 GLP/GEP: no Published: yes	N	N	not protected	
KMA 2.3/03 MA B.2/02 MA B.2.3/01/03	Gröner, A.	1986	Specificity and safety of baculoviruses. not available, not applicable The Biology of Baculoviruses, Volume I, Biological Properties and Molecular Biologie, Chapter 9, 177-201 GLP/GEP: no Published: yes	N	N	Open literature	
KMA 2.3/03 MA B.2/3	Martignoni, M.E. and Iwai, P.J.	1986	Propagation of multinucleocapsid nuclear polyhedrosis virus of Orygia pseudotsugata in larvae of Trichoplusia ni. Journal of Invertebrate Pathology 47, 32-41.	N	N	Open literature	

[14] Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092

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KMA 2.3/03 MA B.2/04	Moscardi, F.	1990	Development and use of soybean caterpillar baculovirus in Brazil. pp. 184-187 In: Proceedings and Abstracts, Vth International Colloquium on Invertebrate Pathology and Microbial Control, Adelaide, Australia, 20-24	N	N	Open literature	
KMA 2.3/03 MA B.2/05 MA B.2.1/07 MA B2.8.4/05	Myers, J. H., and J. S. Cory.	2013	.Population Cycles in Forest Lepidoptera Revisited. Pages 565-592 in D. J. Futuyma, editor. Annual Review of Ecology, Evolution, and Systematics, Vol 44.	N	N	Open literature	
KMA 2.3/03 MA B.2/06	Erlandson, M. A.	2009	Genetic variation in field populations of baculoviruses: mechanisms for generating variation and its potential role in baculovirus epizootiology. Virol Sin 24, 458–469.	N	N	Open literature	
KMA 2.3/03 MA B.2/07	EFSA supporting publication	2013	Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products –Lot 1 Environmental Risk characterization (2013:EN-518).	N	N	Open literature	
KMA 1.3/01 KMA 2.1.1/01 KMA 2.3/03 MA B.2/08 MA B.2.1/06 MA B.2.3/01/04 MA B.2.4/01 MA B.2.6/01 MA B.2.9/01	OCDE document	2002	Consensus document on information used in the assessment of environmental applications involving baculoviruses not available, not applicable OECD Organisation for Economic Co-operation and Development, 2002 GLP/GEP: no Published: yes	N	N	Open literature	
KMA 2.1.1/06 MA B.2/09 MA B.2.7.2/01	Ijkel, W.F.J., van Strien, E.A., Heldens, J.G.M., Broer, R., Zuidema, D., Goldbach, R.W., Vlak, J.M.	1999	Sequence and organization of the *Spodoptera exigua* multicapsid necleopolyhedrovirus genome not available, not applicable Journal of General Virology, 80, 3289-3304 GLP/GEP: no Published: yes	N	N	Open literature	

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MA B.2.1/01	Cunningham J.C.	1995	Baculoviruses as microbial insecticides. In R. Reuveni (ed.).Novel Approaches to Integrated Pest Management. pp. 261–292. Lewis Publishers, Boca Raton, FL.	N	N	Open literature	
KMA 2.1.1/03 MA B.2.1/02 MA B.2.5.1/01	Rohrmann, G.F.	2013	Chapter 1: Introduction to the baculoviruses, their taxonomy and evolution not available, not applicable Baculovirus Molecular Biology, 3rd edition, 1-24 GLP/GEP: no Published: yes	N	N	Open literature	
MA B.2.1/03	Huber, J.	1986	Use of baculovirus in pest management programs. In R.R. Granados and B.A. Federici (eds.). The Biology of Baculoviruses, Vol. 2, Practical Applications for Insect Control. pp. 181–202. CRC Press, Boca Raton, FL.	N	N	Open literature	
MA B.2.1/04	Wallace and Cunningham	1995	Wallace, D.R. and J.C. Cunningham. 1995. Diprionid sawflies. In J.A.Armstrong and W.G.H. Ives (eds.). Forest Pest Insects in Canada. pp. 193–232. Natural Resources Canada, Ottawa, ON.	N	N	Open literature	
KMA 2.1.1/02 MA B.2.1/05	Mazid, S., Kalita, J.C., Rajkhowa, R.C.	2011	A REVIEW ON THE USE OF BIOPESTICIDES IN INSECT PEST MANAGEMENT not available, not available International Journal of Science and Advanced Technology, 1, 169 - 178 GLP/GEP: no Published: yes	N	N	Open literature	
MA B2.1/07 MA B.2.2.3/01	Cory J.S	2015	Insect virus transmission: different routes to persistence. Curr Opin Insect Sci 8:1-6	N	N	Open literature	

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KMA 2.2.2/08 MA B.2.1/08 MA B.2.2.3/05	Virto, C., Navarro, D., del Mar Tellez, M., Williams, T., Murillo, R., Caballero, P.	2016	Mixtures of vertically and horizontally transmitted variants of *Spodoptera exigua* multiple nucleopolyhedrovirs (SeMNPV) as the basis for biological insecticides not available, not applicable IOBC/wprs Bulletin, 113, 131-135 GLP/GEP: no Published: yes	N	N	Open literature	
MA B2.1/09	Arrizubieta, M., Simón O., Williams T., Caballero, P	2015	A novel binary mixture of Helicoverpa armigena single nucleopolyhedrivirus (HearSNPV) genotypic variants has iMAroved insecticidal characteristics for control of cotton bollworms. Appl. Environ Microbiol 81:3984-3993	N	N	Open literature	
MA B2.1/10	Bernal A., Simon O., Williams T., Muñoz D., Caballero P.	2013	A. Chrysodeixis chalcites single-nucleocapsid nucleeopolyhefdrovirus population from the Canary islands is genetically structured to maximize survival. Appl Environ Microbiol 79:7709-7718	N	N	not protected	
KMA 2.1.2/02 MA B.2.1/11 MA B.2.5/01	Krieg, A.	1976	Granulosis and nuclear polyhedrosis viruses: safety aspects concerning their production and application not available, not applicable Z Angew Entomol, 82, 129-134 GLP/GEP: no Published: yes	N	N	not protected	-
MA B2.1.1/01	Smits, P.H.	1987	Smits, P.H., 1987. Nuclear polyhedrosis virus as biological control agent Of *Spodoptera exigua*. PhD Thesis, Wageningen Agricultural University, Wageningen, The Netherlands	N	N	not protected	
MA B2.1.1/02	Gelernterw. W.D. and Federici B.A.	1986	Isolation, identification and determination of virulence of a nuclear polyhedrosis virus from the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) Environ. Entomol., vol. 15, pp. 240-245, Apr. 1986	N	N	not protected	

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MA B2.1.1/03	Caballero P., Aldebis H. K., Vargas-Osuna E., and Santiago-Alvarez C.	1992	Epizootics caused by a nuclear polyhedrosis virus in populations of *Spodoptera exigua* in southern Spain. Biocontrol Sci. Technol., vol. 2, pp. 35-38	N	N	not protected	
KMA 2.3/04 MA B2.1.1/04 MA B.2.3/05	Ijkel, W.F.J., van Strien, E., Heldens, J.G.M., Broer, R., Zuidema, D., Goldbach, R.W., Vlak, J.M.	1999	Sequence and organisation of the *Spodoptora exigua* multicapsid nucleopolyhedrovirus genome not available, not applicable Journal of General Virology, 80, 3289 - 3304 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.1.1/07 MA B2.1.1/05	Smits P.H., Vlak, J.M.	1994	Registration of the first viral insecticide in the Netherlands: The development of Spod-X, based on *Spodoptera exigua* nuclear polyhedrosis virus not available, not applicable Med Fac Landbouww Univ Gent, 59/2a, 385-392 GLP/GEP: no Published: yes	N	N	not protected	-
MA B2.1.1/06	Kolodny-Hirsch, D. M., T. Sitchawat, T. Jansiri, A. Chenrchaivachirakul, and U. Ketunuti	1997	Field evaluation of a commercial formulation of the *Spodoptera exigua* (Lepidoptera: Noctuidae) nuclear polyhedrosis virus for Control of beet armyworm on vegetable crops in Thailand. Biocontrol Sci. Technol. 7: 475–488.	N	N	not protected	
MA B2.1.1/07 MA B.2.2.1/04	Bianchi, F. J., J. M. Vlak, R. Rabbinge, andW. Van der Werf.	2002	Biological control of beet armyworm, *Spodoptera exigua*, with baculoviruses in greenhouses: Development of a coMArehensive process-based model. Biol. Control 23: 35–46.	N	N	not protected	
MA B2.1.1/08	Lasa, R., I. Pagola, I. Iba~nez, J. E. Belda, T. Williams, and P. Caballero	1997	Efficacy of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) as a biological insecticide for beet armyworm in greenhouse of southern Spain. Biocontrol Sci. Technol. 17: 221–232.	N	N	not protected	

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MA B2.1.1/09	Virto, C., Navarro, D., Tellez, M.M., Herrero, S., Williams, T., Murillo, R., Caballero, P	2014	Natural populations of *Spodoptera exigua* are infected by multiple viruses that are transmitted to their offspring. J. Invertebr. Pathol. 122, 22–27.	N	N	not protected	
MA B2.1.1/10	Steinhaus, E.A	1949	Nomenclature and classification of insect viruses. Bacteriol. Rev. 13, 203–223.	N	N	not protected	
MA B2.1.1/11 MA B.2.3/07	Vlak, J.M., Van Frankenhuyzen, K., Peters, D., Gröner, A	1981	Idengtification of a new nuclear polyhedrosis virus from *Spodoptera exigua*. J. Invertebr. Pathol. 38, 297–298.	N	N		
MA B2.1.1/12 MA B.2.3/08	Gelernter, W.D., Federici, B.A.,	1986	Isolation, identification, and determination of virulence of a nuclear polyhedrosis virus from the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). Environ. Entomol. 15, 24–245.	N	N	not protected	
MA B2.1.1/13	Caballero, P., Zuidema, D., Santiago-Alvarez, C., Vlak, J.M.,	1992	Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. Biocontrol Sci. Techn. 2, 145–157.	N	N	not protected	
MA B2.1.1/14	Kolodny-Hirsch	1993	D. M. Kolodny-Hirsch, D. L. Warkentin, B. Alvarado-Rodrıguez, and R. Kirkland, "*Spodoptera exigua* nuclear polyhedrosis virus as a candidate viral insecticide for the beet armyworm (Lepidoptera: Noctuidae)," J. Econ. Entomol., vol. 86, pp. 314-321, Apr. 1993.	N	N	not protected	
MA B2.1.1/15	A. Kondo, M. Yamamoto, S. Takashi, and S. Maeda,	1994.	"Isolation and characterization of nuclear polyhedrosis viruses from the beet armyworm *Spodoptera exigua* (Lepidoptera: Noctuidae) found in Shiga, Japan," Appl. Entomol. Zool., vol. 29, pp. 105-111, Jan.	N	N	not protected	
MA B2.1.1/16	K. Hara, M. Funakoshi, and T. Kawarabata	1995	, "In vivo and in vitro characterization of several isolates of *Spodoptera exigua* nuclear polyhedrosis virus," Acta Virol., vol. 39, pp. 215-222	N	N	not protected	
MA B2.1.1/17	H. F. Guo, J. C. Fang, W. F. Zhong, and B. S. Liu	2013	,Interactions between Meteorus pulchricornis and *Spodoptera exigua* multiple nucleopolyhedrovirus," Insect Sci., vol. 13, pp. 1-12	N	N	not protected	

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MA B2.1.1/18 MA B.2.2.1/9	Zamora-Avilés N. Zamora-Avilés, R. Murillo, R. Lasa, S. Pineda, J. I. Figueroa, A. Bravo-Patiño, O. Díaz, J. L. Corrales, and A. M. Martínez	2017	Genetic and biological characterization of four nucleopolyhedrovirus isolates collected in Mexico for the control of *Spodoptera exigua* (Lepidoptera: Noctuidae),” J. Econ. Entomol., vol. 110, pp. 1465-1475				
KMA 2.1.1/08 MA B2.1.1/19 MA B.2.2.4/01 MA B.2.7/01 MA B.2.7.3/02	Muñoz, D., Castillejo, J.I., Caballero, P	1998	Naturally Occuring Deletion Mutans are Parasitic Genotypes in a wild-type Nucleopolyhedrovirus Population of *Spodoptera exigua* not available, not applicable Applied and Environmental Microbiology, 64, No. 11, 4372-4377 GLP/GEP: no Published: yes	N	N	not protected	
MA B2.1.1/20	Murillo, R., D. Muñoz, C. Ruız-Portero, D. M. Alcazar, E. J. Belda, T. Williams, and P. Caballero.	2007	Abundance and genetic structure of nucleopolyhedrovirus populations in greenhouse substrate reservoirs. Biol. Control 42: 216–225.	N	N	not protected	
KMA2.1.1/04 MA B2.1.1/21	Jianfeng, Z.	2005	The identity of *Spodoptera exigua* Nuclear Polyhedrosis Virus (SeNPV) strain Andermatt Biocontrol AG, CH, not applicable not available GLP/GEP: no Published: no	N	N		ABA
MA B.2.2.1/01	Smagghe, G., Pineda, S., Carton, B., Del Estal, P., Budia, F., Viñiela, E.	2003	Toxicity and kinetics of methoxyfenozide in greenhouse-selected *Spodoptera exigua* (Lepidoptera: Noctuidae). Pest Manag. Sci. 59, 1203–1209	N	N	not protected	-
MA B.2.2.1/02	Su, R., Zheng, G.L., Wan, F.H., Li, C.Y.	2016	Establishment and characterization of three embryonic cell lines of beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). Cytotechnology 68, 1223–1232.	N	N	not protected	

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MA b.2.2.1/03	Osorio, A., Martinez, A.M., Schneider, M.I., Diaz, O., Corrales, L.J., Aviles, C.M., Pineda, S.	2008	Monitoring of beet armyworm resistance to spinosad and methoxyfenozide in Mexico. Pest Manag. Sci. 64, 1001–1007.	N	N	not protected	
MA B.2.2.1/05	Saeed, S., Sayyed, A.H., Ahmad, I.	2010	Effect of host plants on life-history traits of *Spodoptera exigua* (Lepidoptera: Noctuidae). J. Pest. Sci. 83, 165–172.	N	N	not protected	
MA B.2.2.1/06	Underwood, N	2011	Density dependence in insect performance within individual plants: induced resistance to *Spodoptera exigua* in tomato. Oikos 119, 1993–1999	N	N	not protected	
MA B.2.2.1/07	Zheng et al Zheng, X.L., Cong, P.X., Wang, P.X., Lei, L.C.	2011	A review of geographic distribution, overwintering and migration in *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae). J. Entomol. Res. Soc. 13, 39–48.	N	N	not protected	
MA B.2.2.1/08	Sayyed, H.A., Naveed, M., Rafique, M., Arif, M.J.	2012	Detection of insecticides resistance in *Spodoptera exigua* (Lepidoptera: Noctuidae) depends upon insect collection methods. Pakistan Entomol. 34, 7–15.	N	N	not protected	
KMA 2.2.1/01 MA B.2.2.1/10	Hill, D.S.	1983	Agricultural insect pests of the tropics and their control not available, not available Camebridge University Press, 376 GLP/GEP: no Published: yes no no not protected	N	N	not protected	
KMA 2.2.1/02 MA B.2.2.1/11	Moulton, J.K., Pepper, D.A., Dennehy, T.J.	2000	Beet armyworm (*Spodoptera exigua*) resistance to spinosad not available, not applicable Pest Management Science, 56, 842-848 GLP/GEP: no Published: yes	N	N	not protected	-

Data point	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
KMA 2.2.1/03 MA B.2.2.1/12	Wang, W., Mo, J., Cheng, J. Zhunang, P., Tang, Z.	2006	Selection and characterization of spinosad resistance in *Spodoptera exigua* (hübner) (lepidoptera: noctuidae) not available, not available Pesticide Biochemistry and Physiology, 84, 180-187 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.2.2/01 MA B.2.2.2/01	Evans, H.F., Harrap, K.A.	1982	Persistence of insect viruses not available, not applicable Virus Persistence, Publisher: Cambridge University Press, 58-96 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.2.2/02 MA B.2.2.2/02	Martins, T., Montiel, R., Medeiros, J., Oliveira, L., Simones, N.	2005	Occurence and characterization of a nucleopolyhedrovirus from spodoptera littoralis (lepidoptera: noctuidae) isolated in the azores not available, not applicable Journal of invertebrate Pathology, 89, 185-192 GLP/GEP: no Published: yes	N	N	not protected	-
MA B.2.2.3/02 MA B.2.4.1/01 MA B.2.8.4/01	Williams T, Virto C, Murillo R and Caballero P	2017	Covert Infection Of Insects by Baculoviruses. Front. Microbiol. 8:1337. doi: 10.3389/fmicb.2017.01337	N	N	not protected	
KMA 2.2.2/06 MA B.2.2.3/03	Cabodevilla, O., Villar, E., Virto, C., Murillo, R., Williams, T., Caballero, P.	2011a	Intra- and intergenerational persistence of an insect nucleopolyhedrovirus: adverse effects of sublethal disease on host development, reproduction, and susceptibility to superinfection not available, not applicable Applied and Environmental Microbiology, 77(9), 2954-2960 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.2.2/07 MA B.2.2.3/04	Cabodevilla, O., Ibañez, I., Simón, O., Murillo, R., Caballero, P., Williams, T.	2011b	Occlusion body pathogenicity, virulence and productivity traits vary with transmission strategy in a nucleopolyhedrovirus not available, not applicable Biological Control, 56, 184-192 GLP/GEP: no Published: yes	N	N	not protected	-

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MA B.2.2.3/6 MA B.2.8.4/03 MA B2.8.4/07	Virto, C., Williams, T., Navarro, D., Tellez, M. M., Murillo, R., and Caballero, P.	2017	Can mixtures of horizontally and vertically transmitted Nucleopolyhedrovirus genotypes be effective for biological control of *Spodoptera exigua*? J. Pest Sci. 90, 331–343. doi: 10.1007/s10340-016-0 743-x	N	N	not protected	
KMA 2.2.2/10 MA B.2.2.3/07 MA B.2.2.4/05	Caballero, P., Murillo, R., Munoz, D., Williams, T.	2009	El nucleopoliedrovirus de *Spodoptera exigua* (Lepidoptera: Noctuidae) como bioplaguicida: análisis de avances recientes en España not available, not applicable REvista Colombiana de Entomología, 35(2), 105-115 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.2.2/13 MA B.2.2.3/08 MA B.2.2.5/03	van Houte, S., van Oers, M.M., Han, Y., Vlak, J.M., Ros, V.I.D.	2015	Baculovirus infection triggers a positive phototactic response in caterpillars: a response to Dobson et al. (2015) not available, not applicable Biology Letters, 11, 1-4 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.2.2/11 KMA 7.2/07 MA B.2.2.3/09 MA B.2.2.5/01	van Houte, S., Ros, V. I. D., van Oers, M. M.	2014	Hyperactivity and tree-top disease induced by the baculovirus AcMNPV in *Spodoptera exigua* larvae are governed by independent mechanisms not available, not available Naturwissenschaften, 101, 347-350 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.2.2/14 MA B.2.2.3/10 MA B.2.2.5/04	Rebolledo, D. Lasa, R., Guevara, R., Murillo, R., Williams, T.	2015	Baculovirus-Induced Climbing Behavior Favors Intraspecific Necrophagy and Efficient Disease Transmission in Spodoptera exigua not available, not applicable PloS ONE, 1-16 GLP/GEP: no Published: yes	N	N	not protected	-

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KMA 2.2.2/12 MA B.2.2.3/11 MA B.2.2.5/02	Dobson, A.D.M., Auld, S.K.J.R., Tinsley, M.C.	2015	Insufficient evidence of infection-induced phototactic behaviour in Spodoptera exigua: a comment on van Houte et al. (2014) not available, not applicable Biology Letters, 11, 1-3 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.2.2/15 MA B.2.2.3/12 MA B.2.2.6/04	Wan, N.-F., Jiang, J.-X., Li, B.	2016	Effect of host plants on the infectivity of nucleopolyhedrovirus to *Spodoptera exigua* larvae not available, not applicable Journal of Applied Entomology, 140, 636-644 GLP/GEP: no Published: yes	N	N	not protected	-
MA B.2.2.4/02 MA B.2.7.3/01	Muñoz, D., J. M. Vlak, and P. Caballero	1997	In vivo recombination between two strains of the genus Nucleopolyhedrovirus in its natural host, *Spodoptera exigua*. Appl. Environ. Microbiol. 63:3025–3031.	N	N	not protected	
KMA 2.7/06 MA B.2.2.4/03	Serrano, A., Pijlman, G.P., Vlak, J.M., Muñoz, D., Williams, T., Caballero, P.	2015	Identification of *Spodoptera exigua* nucleopolyhedrovirus genes involved in pathogenicity and virulence not available, not applicable Journal of Invertebrate Pathology, 126, 43-50 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.2.1/04 MA B.2.2.4/04 MA B.2.4.1/02	Pascual, L., Jakubowska, A.K., Blanca, J.M., Cañizares, J., Ferré, J., Gloeckner, G., Vogel, H., Herrero, S.	2012	The transcriptome of *Spodoptera exigua* larvae exposed to different types of microbes not available, not applicable Insect Biochem Molec Biol, 42, 557-570 GLP/GEP: no Published: yes	N	N	not protected	-
MA B.2.2.6/01	Hoover, K., Washburn J.O., Volkman L.E.	2000	Midgut-based resistance of *Heliothis virescens* to baculovirus infection mediated by phytochemicals in cotton. J. Insect Physiol., 46,999-1007	N	N	not protected	

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MA B.2.2.6/02	Ali M.I., Young S.Y., McNew R.C.	2004	Host plant influence on activity of *Bacillus thuringiensis* Berliner against lepidopterus pests of crops. J. Entomol Sci, 39	N	N	not protected	
MA B.2.2.6/03	Shikano I., Ericsson J.D., Cory J.S., Myers J.H.,	2010	Indirect Plant-mediated effects on insect inmunity and disease resistance in a tritrophic system. Basic Appl Ecol, 11,15-22	N	N	not protected	
KMA 2.3/01 MA B.2.3/01/01	Burges, H.D., Croizier, G., Huber, J.	1980	A review of safety tests on baculoviruses not available, not applicable Entomophaga, 329-339 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.3/02 MA B.2.3/01/02	Simón, O., Williams, T., López-Ferber, M., Caballero, P.	2004	Virus entry or the primary infection cycle are not the principal determinants of host specificity of Spodoptera spp. nucleopolyhedroviruses not available, not applicable Journal of General Virology, 85, 2845 - 2855 GLP/GEP: no Published: yes	N	N	not protected	-
MA B.2.3/01/06	Chen Y., Qia B., Zheng G., Zhang Y., Deng F., Wan, F., Li C.	2019	Identification and genomic sequence analysis of a new *Spodoptera exigua* Multiple nucleopolyhedrovirus, SeMNPV-QD, isolated from Qingdao, China. Journal of Invertebrate Pathology 160 (2019) 8–17.	N	N	not protected	-
KMA 2.4/02 MA B.2.4/02	Entwistle, P.F., Adams, P.H.W., Evans, H.F.	1978	Epizootiology of a nuclear polyhedrosis virus in european spruce sawfly (Gilpinia hercyniae): The rate of passage of infective virus through the gut of birds during cage tests not available, not applicable Journal of Invertebrate Pathology 31, 307-312, 1978 GLP/GEP: no Published: yes	N	N	not protected	-

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MA B.2.2.5.1/02	Monteiro, F., Carinhas, N., Carrondo, M. J., Bernal, V., and Alves, P. M.	2012	Toward system-level understanding of baculovirus-host cell interactions: from Molecular fundamental studies to large-scale proteomics approaches. Front. Microbiol. 3:391. doi: 10.3389/fmicb.2012.00391	N	N	not protected	
MA B.2.7.1/01	Lua, L. H. L., Pedrini, M. R. S., Reid, S., Robertson, A., Tribe, D.E.	2002	Phenotypic and genotypic analysis of *Helicoverpa armigera* nucleopolyhedrovirus serially passed in cell culture. Journal of General Virology, 83: 945-955	N	N	not protected	
MA B.2.7.1/02	Heldens, J. G., Broer, R., Zuidema, D., Goldbach, R. W. & Vlak, J. M	1997	Identification and functional analysis of a non-hr origin of DNA replication in the genome of *Spodoptera exigua* multicapsid Nucleopolyhedrovirus. J Gen Virol 78, 1497–1506.	N	N	not protected	
MA B.2.7.1/03	Chaeychomsri S.	2018	Replication and Occlusion Body Formation of *Spodoptera exigua* Multicapsid Nucleopolyhedrovirus in a Homologous Cell Line Journal of Advanced Agricultural Technologies doi: 10.18178/joaat.5.3.236-244	N	N	not protected	
MA B.2.7.1/04	Liu, S., Chen, Y., and Bonning, B. C.	2015	RNA virus discovery in insects. Curr. Opin. Insect Sci. 8, 54–61. doi: 10.1016/j.cois.2014.12.005	N	N	not protected	
MA B.2.7.1/05	Airenne KJ, Makkonen KE, Mähönen AJ and Ylä-Herttuala S.	2010	Baculoviruses Mediate Efficient Gene Expression in a Wide Range of Vertebrate Cells. In: Merten OW and Al-Rubeai M. (eds.), Viral Vectors for Gene Therapy: Methods and Protocols, Methods in Molecular Biology, 737, 279-303.	N	N	not protected	
MA B.2.7.1/06	Condreay JP, Witherspoon SM, Clay WC and Kost TA.	1999	Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. Proc. Natl. Acad. Sci. USA, 96, 127-132.	N	N	not protected	
MA B.2.7.1/07	Hu, YC.	2008	Baculovirus vectors for gene delivery: a review. Current Genetic Therapy, 8, 54-65.	N	N	not protected	
MA B.2.7.1/08	Cheng T, Xu CY, Wang YB, Chen M, Wu T, Zhang J and Xia NS.	2004	A rapid and efficient method to express target genes in mammalian cells by baculovirus. World J Gastroenterology., 10, 1612-1618.	N	N	not protected	

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MA B.2.7.1/09	Airenne KJ, Hu YC, Kost TA, Smith RH and Kotin RM.	2013	Baculovirus: an insect-derived vector for diverse gene transfer applications. Mol Ther, 21, 739-49.	N	N	not protected	
MA B.2.7.1/10	Sun XC, Cheng GY, Zhou MZ, Hu ZH and Sun XL.	2005	Transfer of the AaIT Gene of a Recombinant *Helicoverpa armigera* Nucleopolyhedrovirus to its Surrounding Organisms. Virologica Sinica, 20, 420-423.	N	N	not protected	
MA B.2.7.1/11	Arends	2005	http://vir.sgmjournals.org/content/86/10/2731.full - aff-1 HM, Winstanley D and Jehle JA. Virulence and coMAetitiveness of *Cydia pomonell*a granulovirus mutants: parameters that do not match . J Gen Virol, 86, 2731-2738.	N	N	not protected	
MA B.2.7.1/12	Kang WK,Tristem M, Maeda S, Crook NE and O'Reilly DR.	1998	Identification and characterization of the *Cydia pomonella* granulovirus cathepsin and chitinase genes. Journal of General Virology, 79, 2283–2292.	N	N	not protected	
MA B.2.7.1/13	Lauzon HAM, Garcia-Maruniak A, Zanotto PMA, Clemente JC, Herniou EA, Lucarotti CJ, Arif BM and Maruniak JE.	2006	Genomic coMAarison of *Neodiprion sertifer* and *Neodiprion lecontei* nucleopolyhedroviruses and identification of potential hymenopteran baculovirus-specific open reading frames. J Gen Virol, 87, 1477-1489.	N	N	not protected	
MA B.2.7.1/14	Rohrmann G	2011	Introduction to the baculoviruses, their taxonomy, and evolution. in: Baculovirus Molecular Biology: Second Edition.	N	N	not protected	
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KMA 2.5/01 MA B2.8/01 MA B2.8.3/01	Jaques, R.A.	1977	Stability of entomopathogenic viruses not available, not applicable Misc Publ Entomological Soc America, 10(3), 99-119 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.5/02 MA B2.8/02	Krieg, A., Gröner, A., Huber, J., Zimmermann, G.	1981	Inactivation of certain Insect Pathogens by Ultraviolet Radiation not available, not applicable Journal of Plant Diseases and Protection, 88 (1), 38-48 GLP/GEP: no Published: yes	N	N	not protected	-
KMA 2.5/03 MA B2.8/03 MA B2.8.3/02	Evans, H.F., Harrap, K.A.	1982	Persistence of insect viruses not available, not applicable Virus Persistence, Publisher: Cambridge University Press, 58-96 GLP/GEP: no Published: yes Submitted in: KMA 2.2.2/01	N	N	not protected	-
KMA 2.5/04 MA B2.8/04	Thomas, E.D., Reichelderfer, C.F., HeiMAel, A.M.	1973	The effect of soil pH on the persistence of cabbage looper nuclear polyhedrosis virus in soil not available, not applicable Journal of invertebrate Pathology, 21, 21-25 GLP/GEP: no Published: yes	N	N	not protected	-
MA B2.8.4/03	Ebert, D.	2013	The epidemiology and evolution of symbionts with mixed-mode Transmission. Annu. Rev. Ecol. Evol. Syst. 44, 623–643. doi: 10.1146/annurevecolsys-032513-100555	N	N	not protected	
MA B2.8/0	Ignoffo Ignoffo, C. M	1992	Environmental factors affecting persistence of entomopathogens. Fla. Entomol. 75: 516-525.	N	N	not protected	
MA B2.8.4/04	Cooper, D., Cory, J. S., Theilmann, D. A., and Myers, J. H.	2003	Nucleopolyhedroviruses of forest and western tent caterpillars: cross-infectivity And evidence for activation of latent virus in high-density field populations. Ecol. Entomol. 28, 41–50. doi: 10.1046/j.1365-2311.2003.00474.x	N	N	not protected	

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MA B2.8.4/06	Hostetter, D. L., and Bell, M. R.	1985	"Natural dispersal of baculoviruses in the environment," in Viral Insecticides for Biological Control, eds K. Maramorosch and K. E. Sherman (Orlando, FL: Academic Press), 249–284. doi: 10.1016/B978-0-12-470295-0.50014-8	N	N	not protected	
MA B2.8.4/07	Burden, J. P., Griffiths, C. M., Cory, J. S., Smith, P., and Sait, S. M	2003	Vertical transmission of sublethal granulovirus infection in the Indian meal moth, Plodia interpunctella. Mol. Ecol. 11, 547-555.	N	N	not protected	
MA B2.8.4/08	Vilaplana, L., Wilson, K., Redman, E., and Cory, J.	2010	Pathogen persistence in migratory insects: high levels of vertically-transmitted virus infection in field populations of the African armyworm. Evol. Ecol. 24, 147-160.	N	N	not protected	
MA B2.8.4/09	Jones, E. O., White, A., and Boots, M.	2011	The evolution of host protection by vertically transmitted parasites. Proc. R. Soc. B 278, 863–870. doi: 10.1098/rspb.2010.1397	N	N	not protected	
MA B2.8.4/10	Hedges, L. M., Brownlie, J. C., O'Neill, S. L., and Johnson, K. N	2008	Walachia and virus protection in insects. Science 322:702. doi: 10.1126/science.1162418	N	N	not protected	
MA B2.8.4/11	Xu, P., Liu, Y., Graham, R. I., Wilson, K., and Wu, K	2013	Densovirus is a mutualistic symbiont of a global crop pest (Helicoverpa armigera) and Protects against a baculovirus and Bt biopesticide. PLoS Pathog. 10:e1004490. doi: 10.1371/journal.ppat.1004490	N	N	not protected	
MA B.2.8/12	Graham, R.I., Grzywacz, D., Mushobozi, W.L., Wilson, K.	2012	Wolbachia in a major 522 African crop pest increases susceptibility to viral disease rather than protects. 523 Ecol. Lett. 15, 993–1000.	N	N	not protected	
MA B.2.8/14	Bonsall, M. B., Sait, S. M., and Hails, R. S.B	2005	Invasion and dynamics of covert Infection strategies in structured insect–pathogen populations.	N	N	not protected	
MA B.2.8/15	Sorrell, I., White, A., Pedersen, A. B., Hails, R. S., and Boots, M.	2009	The evolution of covert, silent infection as a parasite strategy. Proc. R. Soc. B 276, 2217–2226. doi: 10.1098/rspb.2008.1915	N	N	not protected	

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