# Chapter 1

# Introduction,
# Review of Literature,
# Objectives,
# Materials and Methods

# Section 1.1: Introduction

## 1.1.1 Tea cultivation in India

Tea, *Camellia sinensis* (L.) O. Kuntze is the most common and widely consumed refreshing drink throughout the world. It is a resolutely and skilfully managed monoculture crop cultivated on large as well as small-scale between latitudes 41°N and 16°S with annual precipitation of 1000-5000 mm and temperature of 8-35$^{0}$C (Hazarika et al., 2008). As per the 64$^{th}$ annual report (2017-18) of "Tea Board India", India is the World's second-largest producer of tea sharing 23% (1325.05 Million Kg) of global tea production, while China is the first producing 45%. However, India exported 14% of world tea which comes after Kenya (23%), China (20%) and Srilanka (16%). India is also one of the largest tea consumers in the world accounting for 19% of global tea consumption. Almost 76% of produced tea in India is consumed within the Country itself. During 2017-18, 256.57 Million Kg of tea was exported from India with a Cost, Insurance, and Freight (CIF) value of Rs. 5064.88 Crs, while 20.59 Million Kg with a CIF value of Rs. 288.56 Crs was imported into India (Anonymous, 2018).

Tea is the chief foliar crop in the northern part of West Bengal, Assam, Sikkim, Tripura, and Nilgiri of Tamil Nadu and is also grown on a small scale in Himachal Pradesh, Kerala, Karnataka and Odissa (**Figure 1-1**). Assam tea and Darjeeling tea are very popular in India and are exclusively cultivated in a large number of tea plantations in the Terai-Dooars region of West Bengal and Assam. In India, tea is cultivated over an area of 6,36,557.07 hectare, of which 3,37,690.35 hectare (53%) is situated in Assam and 1,48,121.74 hectare (23%) in West Bengal including both big and small growers. The economy of both of these states largely depends on the production of tea. In the northern part of West Bengal, concerning the Himalayan foothills and plains, the Terai plantation is on the Western flank of the mighty Teesta river and the Dooars plantation stretches on the eastern terrain of the river continuing up to the state of Assam.

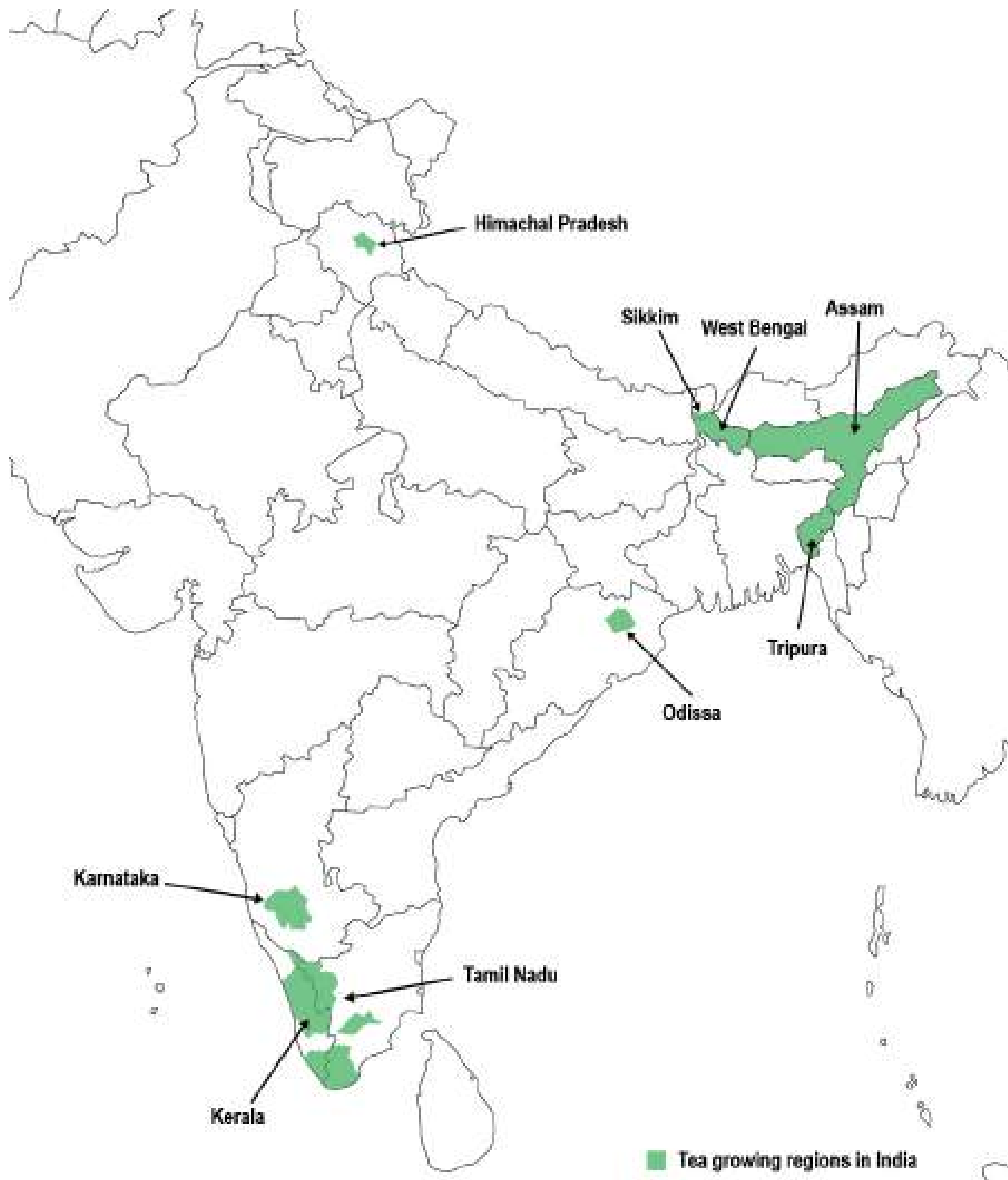


**Figure 1-1: Tea growing regions in India (Map not to scale).**

## 1.1.2 Looper pests in the Tea plantations of the Terai-Dooars region of India

Tea plantations of the Terai and Dooars region of West Bengal and Assam are experiencing severe attacks of different leaf-eating lepidopteran pests, among which, *Biston (Buzura) suppressaria* Guen (Lepidoptera: Geometridae) was reported as a major tea pest for several decades from Assam and the Terai-Dooars region of North Bengal. Recent studies reveal that a few other species of geometrid pests are attacking tea plantations among which the pest species, *Hyposidra talaca* Walker (Lepidoptera: Geometridae), the early caterpillar of which is commonly known as 'Black Inch Looper' (**Figure 1-2**), has taken over as the major defoliating pest in tea plantations (Das et al., 2010a). Loopers of this species that primarily feed on a number of forest plants and weeds in India, Malaysia and Thailand (Das and Mukhopadhyay, 2009; Mathew et al., 2005; Winotai et al., 2005) have turned to tea as an active defoliating insect pest in plantations of Assam and Darjeeling Terai-Dooars of North Bengal (Basumajumdar and Ghosh, 2004; Das et al., 2010b). A substantial loss in tea production due to this defoliating lepidopteran pest has been reported in these regions (Gurusubramanian et al., 2008; Hazarika et al., 2008), severely has affected the economy of the country (Roy and Muraleedharan, 2014).

**Figure 1-2: Caterpillar of *Hyposidra talaca.***

### 1.1.3 Management of Tea pest population

To manage pest infestations, there are two main methods of pest control: chemical and biological. The looper pests have so far been controlled by regular application of synthetic chemical pesticides especially (**Figure 1-3**), organophosphates and pyrethroids, but, the pests are gradually becoming less susceptible, and have developed some extent of resistance to the pesticides (Das et al., 2010b; Das and Mukhopadhyay, 2008) often resulting in their control failures (Sannigrahi and Talukdar, 2003). Moreover, the application of these synthetic chemical insecticides is one of the major sources of pollution of soil and water (Saravanan et al., 2009; Singh et al., 2017) and is reported to be hazardous to non-target organisms including humans (Azmi et al., 2006; Mobed et al., 1992; Saravanan et al., 2009; Velmurugan et al., 2006). Because of these harmful effects, the application of a number of these insecticides has been banned by the Environmental Protection Agency (EPA) of the USA and even by the Tea Research Organizations of India. The registration of many other insecticides has been reviewed (Chattopadhyay et al., 2004) and recommended under the Plant Protection Codes (PPC). Therefore, concerning the fact of development of pesticide resistance to insect pests and the hazardous effects of chemical pesticides, organic tea has become more acceptable, especially for export and health-conscious tea consumers than the chemically managed conventional tea. Because of this, the protection and production of tea need to be largely dependent on non-conventional including biological control methods.

**Figure 1-3: Application of Chemical pesticides in tea plantations.**

The use of biological agents as biopesticides in agricultural fields is an integral part of pest management. Biocontrol is thus being considered an alternative or supplementary means for reducing the use of synthetic chemicals in agriculture (Gerhardson, 2002; Postma et al., 2003; Welbaum et al., 2010). The use of genetically modified cotton with *Bt* toxin against bollworms in China, despite initial success, is ineffective due to having no direct benefit to consumers, no substantial economic benefits to small farmers and weak intellectual property rights (Pray et al., 2001). *Bt* brinjal has also caused great controversy about its use in India (Seetharam, 2010). Recent studies in India revealed that GM plants with *Bt* endotoxin Cry1Ac have detrimental effects on the regeneration, growth and development of cotton and tobacco plants (Rawat et al., 2011). So, as an alternative option to GM crop application, baculoviruses that infect a diverse group of insects can be tried as potential biopesticides which is target specific and safe for the environment and health.

### 1.1.4 Baculovirus as bioinsecticide

Out of 19 virus families pathogenic to insects (**Table 1-1**), Baculoviridae is most intensely studied because of their killing efficacy to insect hosts (King et al., 2012). Baculoviridae is globally known for their application as biopesticide/bioinsecticide to control insect pest populations (Sun and Peng, 2007). The family Baculoviridae includes a large group of DNA viruses that are pathogenic to several arthropod species, primarily to the insects of the order Lepidoptera and also to the Hymenoptera and Diptera (King et al., 2012). Before the 20$^{th}$ century, approximately 700 baculoviruses have been identified from different lepidopteran, hymenopteran, and dipteran insects (Moscardi, 1999).

Their application to crops can be an alternative eco-friendly approach for pest management to minimize the use of synthetic chemical pesticides. Moreover, baculoviruses have also proved themselves as a powerful expression vector for expressing genes of the insect as well as vertebrate origin (Hu, 2006, 2005), and are successfully applied in the production of beneficial proteins (Shrestha et al., 2008). Being host-specific, baculoviruses are very safe for industrial production and their field applications (Sumathy et al., 1996), as microbial insecticides in integrated pest management (IPM) programs (Kunimi, 2007).

**Table 1-1: List of virus families and the corresponding genera pathogenic to insects (summarized from King et al. (2012).**

| Sl. No. | Virus family | Genus | Host |
|---|---|---|---|
| 1. | Ascoviruses | Unclassified | Lepidopteran larvae and pupae |
| 2. | Baculoviridae | Alphabaculovirus | Lepidoptera |
| | | Betabaculovirus | Lepidoptera |
| | | Gammabaculovirus | Hymenoptera |
| | | Deltabaculovirus | Diptera |
| 3. | Iridovirus | Unclassified | Insects in aquatic and damp habitats |
| 4. | Polydnavirus | Unclassified | Hymenoptera |
| 5. | Poxiviridae | Entomopoxiviridae | |
| 6. | Parvoviridae | Densoviridae | Dictyoptera, Diptera, Hemiptera, Homoptera, Lepidoptera, Odonata, Orthoptera |
| 7. | Metaviridae | Semotivirus | Diptera |
| 8. | Pseudoviridae | Hemivirus | Diptera |
| 9. | Birnaviridae | Entomobirnavirus | Diptera |
| 10. | Reoviridae | Cypoviruses | Lepidoptera |
| 11. | Rhabdoviridae | Ephimerovirus | Diptera |
| | | Vesiculovirus | Insect vector |
| 12. | Orthomyxoviridae | Thogovirus | Insect vector |
| 13. | Dicistroviridae | Cripavirus | Diptera, Lepidoptera, Orthoptera, Hemiptera and Hymenoptera |
| | | Aparavirus | Hymenoptera, Dendrobranchiata |
| 14. | Iflaviridae | Iflavirus | Lepidoptera |
| 15. | Flaviviridae | Flavivirus | Diptera, Dendrobranchiata |
| 16. | Nodaviridae | Alphanodavirus | Coleoptera, Diptera, Hemiptera etc. |
| 17. | Tetraviridae | Betatetravirus | Lepidoptera |
| | | Omegatetravirus | Lepidoptera |
| 18. | Togaviridae | Aphavirus | Insect vector |
| 19. | Tymoviridae | Marafivirus | Insect vector |

### 1.1.5 Genome structure of the family Baculoviridae

The family Baculoviridae, possesses a circular, covalently closed, double-stranded DNA genome ranging from 80-180 kb in length, encoding for 100-200 proteins (King et al., 2012).

Baculovirus genome contains several functional genes required for oral infectivity, host-specific interaction, replication, transcription, packaging of the virus, cell cycle regulation etc. (discussed later in the "Review of literature").

### 1.1.6 Classification of Baculoviridae

Based on the major matrix protein present in the occlusion body, baculoviruses are of two types, nucleopolyhedrovirus (NPV) having polyhedrin as the major matrix protein and granulovirus (GV) consisting of granulin as the major matrix protein.

Based on the core genes, baculoviruses are phylogenetically divided into four genera: **Alphabaculovirus** (lepidopteran-specific NPV), **Betabaculovirus** (lepidopteran-specific Granulovirus), **Gammabaculovirus** (hymenopteran-specific NPV), and **Deltabaculovirus** (dipteran-specific NPV) (King et al., 2012). Based on the presence of the *gp67/gp64* fusion protein gene, the alphabaculoviruses have been further divided into two groups (Herniou et al., 2003):

i) Group I Alphabaculovirus - *gp67/gp64* fusion protein gene(s) is present, and
ii) Group II Alphabaculovirus - *gp67/gp64* fusion protein gene(s) is absent, instead fusion protein F is present.

### 1.1.7 Nucleopolyhedroviruses in IPM

Nucleopolyhedroviruses are well accommodated with integrated pest management (IPM) strategies because of their compatibility with other biological and chemical agents. Baculoviruses also have a proven safety track record if applied wisely with minimal effects on non-target beneficial insects such as honey bees and silk worms. They require an alkaline (pH 9-11) digestive system for dissolving the protein matrix to release their virions from the occlusion body to initiate infection. As the proteolytic digestive system in humans and other vertebrates are primarily functional at acidic pH, baculoviruses do not appear to cause health hazards to human and other vertebrates (Persley, 1996). Moreover, NPVs have greater potential as biopesticides because of their greater killing efficiency and their continuous production in selective insect cell lines that support a high level of their multiplication (Bonning, 2005). Therefore, microbial insect pest control with NPV can be very effective as well as eco-friendly.

## 1.1.8 Morphological structure of NPV

NPV contains a circular, covalently closed, double-stranded DNA genome which is surrounded by a small basic protein that neutralizes the negative charge of the DNA. This structure is protected by proteins forming a rod-shaped nucleocapsid of 30-60 nm X 250-300 nm (King et al., 2012). On the basis of nucleocapsids per virion, NPVs can further be morphologically divided into two groups. The single nucleopolyhedroviruses (SNPVs) contain a single nucleocapsid per virion, whereas the multiple-nucleopolyhedroviruses (MNPVs) contain multiple nucleocapsids per virion (Rohrmann, 2011).

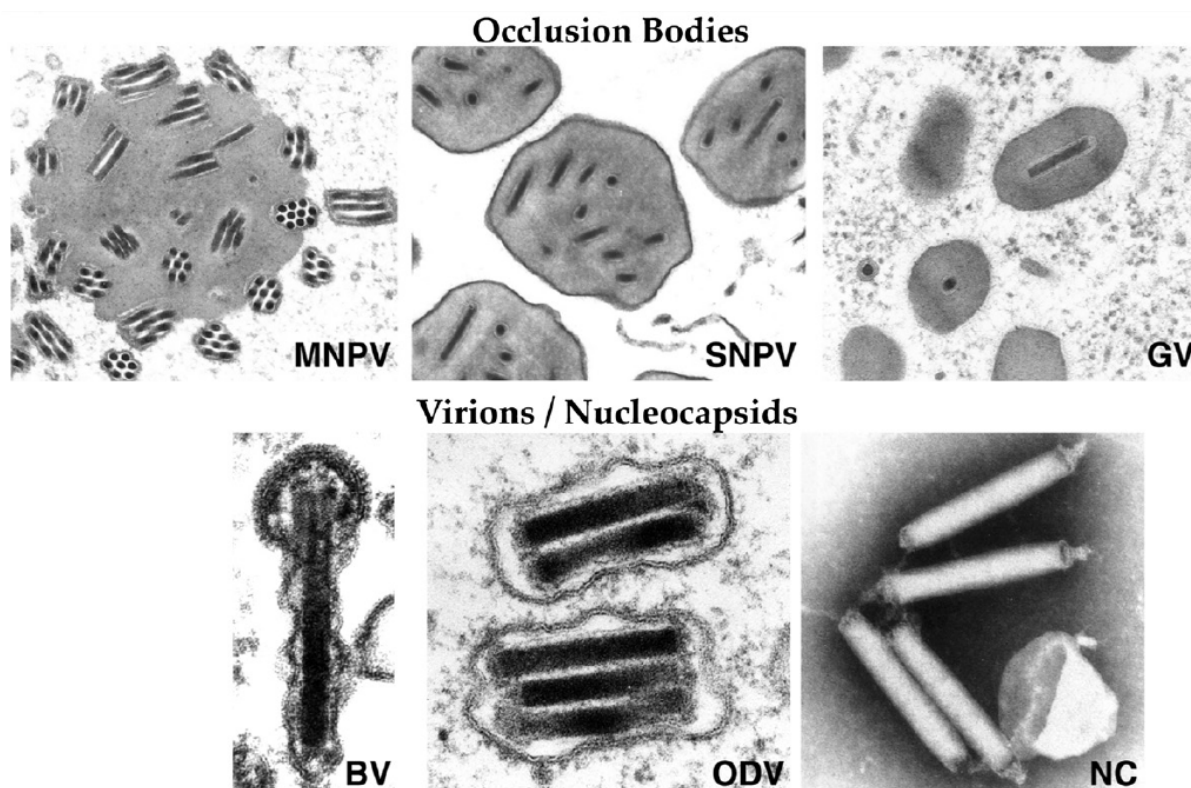


**Figure 1-4: Transmission electron micrographs of occlusion bodies (MNPV, SNPV and GV), and other forms of baculoviruses, BV (budded virions), ODV (occlusion-derived virions) and nucleocapsids (NC). The picture was taken from King et al. (2012).**

The virions are subsequently occluded in large protein crystals forming polyhedra or Occlusion body (OB) (**Figure 1-4**). The occluded form of NPVs is polyhedral in shape and each occlusion body is 0.15 to 15 µm in size, matures within nuclei of infected cells and characteristically contains many enveloped virions (King et al., 2012).

NPVs produce two types of virions during their infection cycle, which enable the virus to replicate efficiently within the infected body and spread its progeny among insects. Occlusion-derived viruses (ODVs) released in the environment from the dead insects

transmit infections among insects (oral infection), whereas budded viruses (BVs) within the insect body spread the infection to neighbouring cells (Keddie et al., 1989). At the late stage of infection, greatly enhanced dispersal behaviour of infected larva/ adult is observed (Goulson, 1997) followed by dramatic degradation of the host cadaver (Federici, 1997) and this pathogenicity is highly species-specific.

**Occlusion-derived virus (ODV)**: The ODV is encapsulated or occluded in a protein crystal or matrix predominantly composed of a single protein called polyhedrin. The occluded virus is highly stable, which allows it to persist in the environment and promotes seasonal infections of susceptible insects (**Figure 1-5**).

**Budded virus (BV)**: The budded virus is not occluded in any type of protein matrix and has an envelope distinct from ODV that facilitates systemic infection (Funk et al., 1997; King et al., 2012) (**Figure 1-5**).

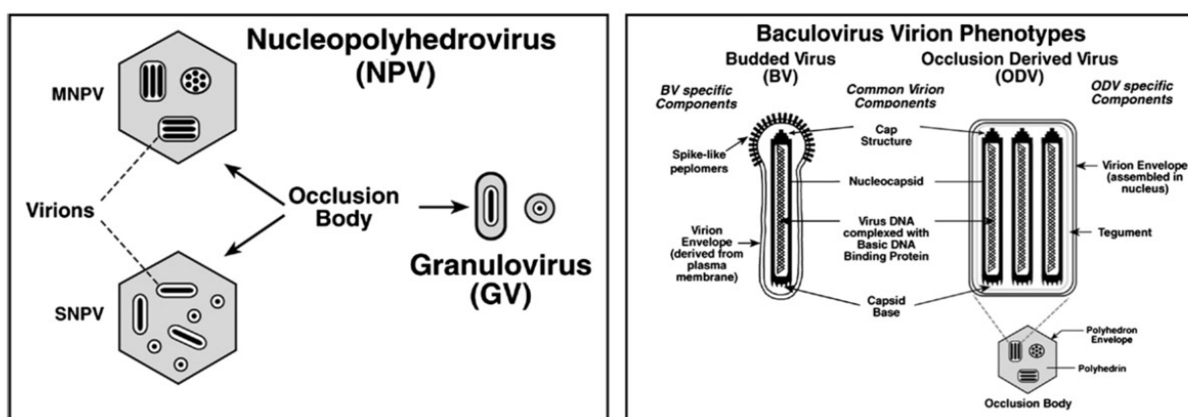


**Figure 1-5: Morphology of MNPV, SNPV, Budded virus, Occlusion derived virus and Occlusion body. The Picture was taken from King et al. (2012).**

## 1.1.9 The infection cycle of NPV

The infection cycle of NPV in nature starts when insect larvae ingest occlusion bodies (OBs) present on the contaminated foliage. When the occlusion body or polyhedron enters the mid gut of a larval host, it gets dissolved under alkaline pH (pH 9-11) releasing the virions (ODVs). These ODVs passing through the peritrophic membrane infect mid-gut cells and proceed to multiply in the nucleus. From this initial infection, budded virions (BVs) are produced which spread the infection to other body tissues such as haemocytes, tracheal cells,

fat body cells and hypodermis, where they cause a secondary infection (Tanada and Kaya, 1993).

During the later stages, the virions are retained within the nuclei of the cells of those tissues where they acquire specific proteins and are eventually embedded into the occlusion body (Tanada and Kaya, 1993)(**Figure 1-7**).

When the insect dies it ruptures to release the polyhedra to infect other insects. Insects killed by NPV infection usually contain up to 100 million OBs (Braunagel and Summers, 1994; Rohrmann, 2011).

### 1.1.10 Signs and symptoms

NPV-infected caterpillars show some typical symptoms 2-4 days after ingestion of the virus. The following symptoms appear sequentially (Tanada and Kaya, 1993).

i) At first, the larvae progressively cease feeding and become less active.

ii) During advanced stages of the infection as the epidermis is infected the skin becomes very fragile and ruptures easily.

iii) Subsequently, the larvae become wilted, swollen sometimes liquefied containing a mass of decomposed tissue and occlusion bodies (**Figure 1-6**).

iv) Just before death, infected larvae often climb to the highest part of the substrate on which they are located e.g., on top of the plant and attach themselves by their prolegs (Goulson, 1997). On death, they hang in a characteristic inverted V-shaped form.

**Figure 1-6: NPV infected dead caterpillar of *H. talaca*.**

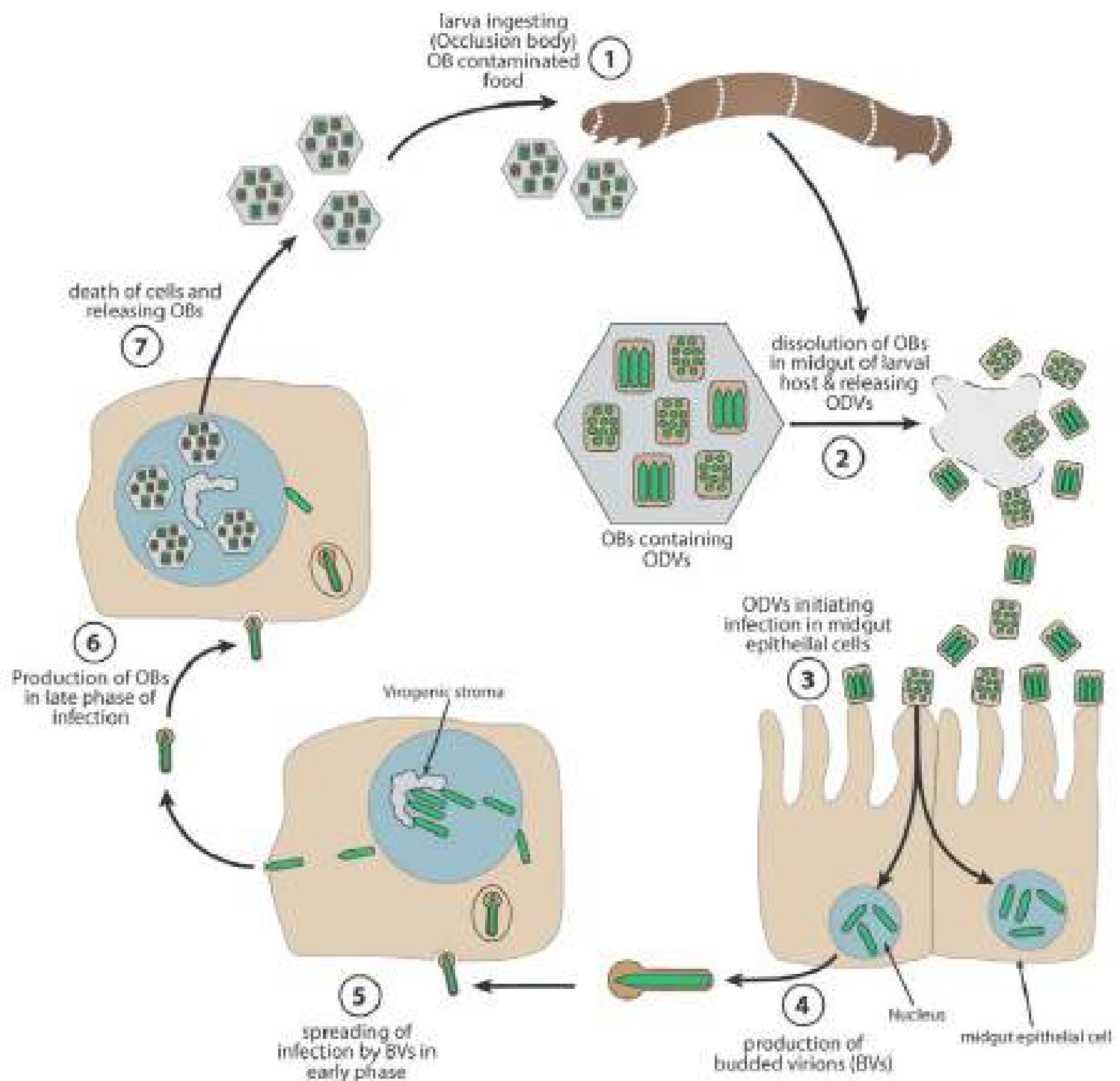


**Figure 1-7: Infection cycle of nucleopolyhedrovirus through insect larva.**

## 1.1.11 NPV as bioinsecticides

Selection of a virus adapted to a particular host or ecosystem as a bioinsecticide is done by isolating local strains of NPVs. The majority of NPVs have been isolated from the order Lepidoptera. Development of most pest-control programs utilizing baculoviruses (Cory and Bishop, 1995) is usually done by screening and determining the killing efficacy based on bioassay ($LD_{50}$/ $LC_{50}$/ $LT_{50}$) for their application as potential control agents. Subsequently, large-scale *in vivo* production, and suitable formulation of the virus are done for field use.

Knowledge of pest biology and behaviour is also helpful for an integrated pest management approach to pest control.

#### 1.1.11.1 Disadvantages of the use of Natural NPVs as insecticides

Although numerous naturally occurring baculoviruses have been tested or even commercialized in a few cases, their use has not expanded as greatly as their development. Their global use has been hampered due to the slower speed of killing, narrow host range, product stability, registration and patentability.

### 1.1.12 Genetically modified NPVs as Bioinsecticides

Because baculoviruses are slower in killing efficiency than synthetic chemical pesticides, genetic modifications which increase the killing efficacy may be helpful for the commercialization of baculoviruses as a bioinsecticide. Introduction of insect-specific toxin gene(s) such as, exogenous toxin genes isolated from the scorpion or spiders, in the NPV genome were shown to increase the killing efficacy of that genetically modified NPV to its natural host (Bonning and Hammock, 1996; Inceoglu et al., 2001, 2006). Infection with a genetically modified NPV expressing a toxin isolated from scorpion *Androctonus australis* showed comparatively more feeding damage in infected larvae than the infection with wild-type baculovirus (Inceoglu et al., 2001). Toxin genes isolated from straw itch mite *Pyemotes tritici* (Burden et al., 2000), *Leiurus quinquestriatus hebraeus* (Froy et al., 2000), ants (Szolajska et al., 2004) or spiders (Hughes et al., 1997), have also been studied to increase the pathogenicity of the baculoviruses. NPV recombinants producing occlusion bodies with *Bacillus thuringiensis* (*Bt*) toxin, were constructed by incorporating a fusion protein consisting of polyhedrin and *Bacillus thuringiensis* (*Bt*) toxin, in the NPV genome (Chang et al., 2003).

Altering the physiology of the insect host by introducing gene(s) expressing some insect hormones or hormone-modifying enzymes into the NPV genome, can also be an approach to increase the killing efficacy of the virus. Such an approach was applied by incorporating genes like juvenile hormone esterase into the NPV genome to decrease the concentration of juvenile hormone in the host which in turn produces a signal for the caterpillar to starve and pupate (Hammock et al., 1990; Inceoglu et al., 2001). Another approach is the deletion of the

baculovirus-encoded ecdysteroid glucosyltransferase (*egt*) gene (O'reilly and Miller, 1991). Deletion of the *egt* gene from the NPV genome results in 30% faster killing of caterpillars altering the normal moulting of larva and indirectly increasing the time of feeding of infected caterpillars. The *egt* gene is not associated with viral replication and replacing it with an exogenous gene, such as any toxin gene, may increase the insecticidal activity of the recombinant virus (Sun et al., 2004).

### 1.1.12.1 Advantages of genetically modified NPVs as bioinsecticides

Genetically modified (GM) baculovirus can kill up to 30% faster than naturally occurring NPVs and are more stable and efficient than natural NPVs. Moreover, GM baculoviruses are non-pathogenic to vertebrate species (Sun et al., 2004) as well as to the natural enemies of larvae such as parasitoids and predators (Boughton et al., 2003).

Therefore, the genetically modified baculovirus strains can be used as potential biopesticides in future due to their eco-friendly and target-specific application.

## 1.1.13 *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV)

Recent studies in the tea plantations of Darjeeling foothills and Terai and the Dooars of north-eastern India revealed that the looper activities especially of *Hyposidra talaca* (Lepidoptera: Geometridae), a major defoliating insect pest in tea plantations, have greatly increased (Das et al., 2010a). A nucleopolyhedrovirus isolated from the loopers of *Hyposidra talaca* has been found pathogenic to this insect pest (Mukhopadhyay et al., 2011). Hence, *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) has the potential of being used as a bioinsecticide, especially as an alternative to synthetic chemical pesticides, both in organic as well as in bio-rationally managed plantations. So, to increase the pest control efficacy of HytaNPV and develop this NPV into a more efficient recombinant baculovirus, genetic characterization and comparison of the locally occurring isolates of HytaNPV from the Terai-Dooars belt are much required. The contemplated investigation done in this study is intended for a better understanding of variants of the viral genome.

# Section 1.2: Review of literature

Because of the control failure of insect pests, their increased resistance and biohazards resulting from the overuse of chemical pesticides, baculovirus formulation are evolving as one of the promising biopesticides which is target specific, and largely non-hazardous to the environment. Its use as an efficient biopesticide is expected to reduce the application of synthetic chemical pesticides. In this regard, genome characterization, as well as modification of some baculoviruses, has been carried out globally to understand their degree of virulence and killing efficacy. This chapter of the thesis mainly consists of a review of literature on the baculovirus genome, the application of baculoviruses as bioinsecticides and the scope of genetic characterization of HytaNPV.

## 1.2.1 Molecular characterization of Baculovirus genome by Restriction endonuclease (REN) fragment analyses

One of the most traditional and common approaches to characterizing a virus genome and identifying, comparing and differentiating different isolates or variants of a virus is to perform a restriction endonuclease fragment analysis of its genome (Hu et al., 1998). Physical mapping of the baculovirus genome has been carried out since the 1980s. A physical map of the genome of *Autographa californica* NPV had been constructed by Miller and Dawes (1979) with *Bam*HI, *Xma*I, *Eco*RI and *Hin*dIII. A restriction map of the *Heliothis zea* single NPV (HzSNPV) genome was concocted with a set of six restriction endonucleases by Knell and Summers (1984). The Physical map of *Heliothis zea* single NPV (Hz-1) baculovirus genome by restriction mapping was studied by Chao et al. (1990). Crawford et al. (1985) reported the physical map of *Oryctes* baculovirus with four restriction endonucleases, *Bam*HI, *Eco*Rl, *Hin*dlII and *Pst*I and estimated the genome size to be 127.03 kb. A physical map of the *Mamestra configurata* NPV genome was prepared by Li et al. (1997) and the genome size was estimated to be about 156 kb.

Chen et al. (2000) characterized the genome organization of *Helicoverpa armigera* NPV (HearNPV) to study the infectivity of the virus to be used as biopesticide and reported the genome size of HearNPV to be 130.1 kb, whereas the NPV isolated from *Helicoverpa assulta*, the closest relative of HearNPV was found to have a genome size of 138 kb (Woo et al., 2006) which is 8 kb higher than that of the former. The Restriction endonuclease

fragment profile along with restriction mapping and estimation of genome size of BusuNPV isolated from another looper pest of tea, *Buzura suppressaria* has been reported in China with a mean genome size of 129 kb (Liu et al., 1993), however, Hu et al. (1998) and his collaborators documented its genome size to be 120.9 kb.

In India similar study was carried out on *Spodoptera littura* NPV where Das and Prasad (1996) reported the restriction endonuclease fragment profile of the viral genome. *S. littura* is a polyphagous insect pest of tobacco, cotton, soybean, beet etc.

Christian et al. (2001) proposed a rapid method for the identification and differentiation of nucleopolyhedroviruses pathogenic to *Helicoverpa* by RFLP analysis. Therefore, REN fragment analysis was found and established as a useful molecular tool for the identification and comparative differentiation of baculovirus isolates.

### 1.2.2 Molecular characterization of Baculovirus genome by sequencing

To date genomes of about 70 nucleopolyhedroviruses infecting hosts of different insect species have been sequenced (NCBI databases) and many have been analysed and published (King et al., 2012; Rohrmann, 2011).

The first NPV to be completely sequenced was *Autographa californica* NPV (AcMNPV) (Ayres et al., 1994). Each complete sequence has provided a wealth of data about the complement of genes present in each virus. A preliminary physical map of the genome of AcMNPV was constructed in the order of *Bam*HI and *Xma*I restriction sites and partial order of *Eco*RI and *Hin*dIII restriction sites (Lee and Miller, 1978).

With the advent of new and sophisticated tools and techniques in the field of molecular biology, the genomes of the different host-specific NPVs were extensively analysed and characterized. Names and genome sizes of different host-specific NPVs are presented in **Table 1-2**.

**Table 1-2: Genome size of different host-specific baculovirus.**

| Sl. No. | Name of NPV | Abbreviation | Genome Size (bp) | References |
|---|---|---|---|---|
| **Alphabaculovirus: Group I** | | | | |
| 1 | *Autographa californica* NPV | AcNPV | 133,894 | (Ayres et al., 1994) |
| 2 | *Bombyx mori* NPV | BmNPV | 128,413 | (Gomi et al., 1999) |
| 3 | *Orgyia pseudotsugata* MNPV | OpMNPV | 131,990 | (Ahrens et al., 1997) |
| 4 | *Epiphyas postvittana* MNPV | EppoMNPV | 118,584 | (Hyink et al., 2002) |
| 5 | *Rachiplusia ou* MNPV | RoMNPV | 1315,00 | (Harrison and Bonning, 2003) |
| 6 | *Choristoneura fumiferana* MNPV | CfMNPV | 129,593 | (de Jong et al., 2005) |
| 7 | *Choristoneura fumiferana* defective NPV | CfDEFNPV | 131,160 | (Lauzon et al., 2005) |
| 8 | *Hyphantria cunea* NPV | HycuNPV | 132 959 | (Ikeda et al., 2006) |
| 9 | *Antheraea pernyi* NPV | AnpeNPV | 126,629 | (Nie et al., 2007) |
| 10 | *Plutella xylostella* MNPV | PlxyMNPV | 134,417 | (Harrison and Lynn, 2007) |
| 11 | *Anticarsia gemmatalis* MNPV | AgMNPV | 131,68 | (de Brito et al., 2016) |
| 12 | *Maruca vitrata* NPV | MaviNPV | 111,953 | (Chen et al., 2008) |
| 13 | *Bombyx mandarina* NPV | BomaNPV | 126,770 | (Xu et al., 2010) |
| 14 | *Catopsilia pomona* NPV | CapoNPV | 128,058 | (Wang et al., 2016) |
| 15 | *Thysanoplusia orichalcea* NPV | ThorNPV | 132,978 | (Wang et al., 2012) |
| 16 | *Lonomia obliqua* NPV | LoobNPV | 120,023 | (Aragão-Silva et al., 2016) |
| 17 | *Philosamia cynthia ricini NPV* | PhcyNPV | 125,376 | (Qian et al., 2013) |
| 18 | *Choristoneura murinana NPV* | ChmuNPV | 124,688 | (Rohrmann et al., 2014) |
| 19 | *Choristoneura occidentalis NPV* | ChocNPV | 128,446 | (Thumbi et al., 2013) |
| 20 | *Choristoneura rosaceana NPV* | ChroNPV | 129,052 | (Thumbi et al., 2013) |
| **Alphabaculovirus: Group II** | | | | |
| 1 | *Mamestra configurata* NPV | MacoNPV | 155,060 | (Li et al., 2002) |
| 2 | *Lymantria dispar* MNPV | LdMNPV | 161,046 | (Kuzio et al., 1999) |
| 3 | *Spodoptera exigua* MNPV | SeMNPV | 135,610 | (Ijkel et al., 1999) |
| 4 | *Helicoverpa armigera* SNPV G4 isolates | HearSNPV-G4 | 131,403 | (Chen et al., 2001) |
| 5 | *Spodoptera litura* MNPV | SpltMNPV | 139,342 | (Pang et al., 2001) |
| 6 | *Helicoverpa zea* SNPV | HzSNPV | 130,869 | (Chen et al., 2002) |
| 7 | *Adoxophyes honmai* NPV | AdhoNPV | 113,220 | (Nakai et al., 2003) |

**Table 1-2: Genome size of different host-specific baculovirus, continued from page 25.**

| Sl. No. | Name of NPV | Abbreviation | Genome Size (bp) | References |
|---|---|---|---|---|
| 8 | *Chrysodeixis chalcites* NPV | ChchNPV | 149,622 | (Van Oers et al., 2004) |
| 9 | *Helicoverpa armigera* ANPV C1 isolates | HearSNPV-C1 | 130,759 | (Zhang et al., 2005) |
| 10 | *Trichoplusia ni* SNPV | TnSNPV | 134,394 | (Willis et al., 2005) |
| 11 | *Agrotis segetum* NPV | AgseNPV-A | 147,544 | (Jakubowska et al., 2006) |
| 12 | *Clanis bilineata* nucleopolyhedrovirus | ClbiNPV | 135,454 | (Zhu et al., 2009) |
| 13 | *Leucania seperata* NPV | LsNPV | 168,041 | (Xiao and Qi, 2007) |
| 14 | *Spodoptera frugiperda* MNPV | SfMNPV | 131,330 | (Harrison et al., 2008) |
| 15 | *Agrotis ipsilon* MNPV | AgipMNPV | 155,122 | (Harrison, 2009) |
| 16 | *Adoxophyes orana* NPV | AdorNPV | 113,220 | (Hilton and Winstanley, 2008) |
| 17 | *Helicoverpa armigera* NPV NNg1 strain | HearNPV-NNg1 | 132,425 | (Ogembo et al., 2009) |
| 18 | *Lymantria xylina* MNPV | LyxyMNPV | 156,344 | (Nai et al., 2010) |
| 19 | *Ectropis obliqua* NPV | EcobNPV | 131,204 | (Ma et al., 2007) |
| 20 | *Euproctis pseudoconspersa* NPV | EupsNPV | 141,291 | (Tang et al., 2009) |
| 21 | *Apocheima cinerarium NPV* | ApCiNPV | 123,876 | Zhang et al, 2009 (Unpublished) |
| 22 | *Sucra jujuba NPV* | SujuNPV | 135,952 | (Liu et al., 2014) |
| 23 | *Hyposidra talaca NPV* | HytaNPV | 139,089 | (Nguyen et al., 2018) |
| 24 | *Buzura suppressaria NPV* | BusuNPV | 120,420 | (Zhu et al., 2014) |
| 25 | *Lambdina fiscellaria* NPV | LafiNPV | 157,977 | (Rohrmann et al., 2015) |
| 26 | *Orgyia leucostigma* NPV | OrleNPV | 157,179 | (Thumbi et al., 2011) |
| **Deltabaculovirus** | | | | |
| 1 | *Culex nigripalpus* NPV | CuniNPV | 108,252 | (Afonso et al., 2001) |
| **Gammabaculovirus** | | | | |
| 1 | *Neodiprion lecontei* NPV | NeleNPV | 81,755 | (Lauzon et al., 2004) |
| 2 | *Neodiprion sertifer* NPV | NeseNPV | 86,462 | (Garcia-Maruniak et al., 2004) |
| 3 | *Neodiprion abietis* NPV | NeabNPV | 84,264 | (Duffy et al., 2006) |

## 1.2.3 Phylogenetic analyses of baculoviruses

The application of baculoviruses as bioinsecticide in different agricultural fields and orchards to curb the pest populations encourages their constant study in an attempt to

understand the molecular mechanisms of their evolution and the phylogenetic relationship among them.

The genes whose orthologs are present in all baculovirus genera are considered core genes which are more conserved than the other baculovirus genes. Therefore, one of the effective and traditional markers to assess the phylogenetic relationships of baculoviruses was the amino acid or nucleotide sequence analyses of a single conserved gene such as *polyhedrin/granulin*, the envelope fusion polypeptides known as F protein and GP64, DNA polymerase or late expression factors etc. (Cowan et al., 1994; Paolo et al., 1993).

*Polyhedrin* is the most conserved gene present in all nucleopolyhedroviruses and is used to study the phylogenetic divergence of different NPVs (Antony et al., 2011; Bulach et al., 1999; Dasgupta et al., 2016; Paolo et al., 1993; Woo et al., 2006). Orthologs of *polyhedrin* are found in all baculoviral genomes either in the form of *polyhedrin* in nucleopolyhedroviruses (NPVs) or *granulin* in granuloviruses (GVs), except in the dipteran-specific NPVs, where it has an occlusion body protein unrelated in the primary amino acid sequence of the *polyhedrin* gene of the other NPVs and is about three times larger than the other forms (Afonso et al., 2001; Perera et al., 2006).

*Dnapol* is another conserved gene used to detect the phylogenetic relationships of different baculoviruses (Bulach et al., 1999). Single gene phylogenetic analysis of *Bombyx mori* NPV was conducted by Liang et al. (2013) based on *p10*, another core gene in baculoviruses. The evolutionary divergence of *Spodoptera littura* NPV was analysed based on *lef-8*, another conserved gene in baculoviruses (Wang et al., 2001).

Robust phylogenetic inferences have been obtained based on the sets of genes with homologous sequences in all baculoviruses. Therefore, most of the modern approaches were to find out the core baculovirus genes and the phylogenetic analyses based on core-protein concatemers (Jehle et al., 2006). Recently parsimonious phylogenetic inferences were procured based on the analyses of concatenated amino acid alignment of the core genes obtained from the complete genome sequences of baculoviruses (Nguyen et al., 2018; Zhu et al., 2014).

To date, 37 core genes have been identified in baculoviruses (Nguyen et al., 2018) and concatenated sequences of a combination of core genes can be used for phylogenetic

analyses. In a comparative study of 57 baculovirus strains comprising 31 core genes, Miele et al. (2011) showed that *pif-2*, *lef-9*, *lef-8* etc. were more highly conserved compared to the others.

## 1.2.4 Baculovirus core genes

Baculovirus contains double-stranded circular DNA genomes of 80 to 180 kb. A key feature to explore and manipulate the baculovirus genome for different biotechnological applications is to determine the core genes. The core genes have been classified according to their function such as genes involved in replication, transcription, packaging of the virus, cell cycle regulation and oral infectivity of the virus (**Table 1-3**) (Rohrmann, 2011).

**Table 1-3: Core genes found in Baculoviruses [Adapted form Rohrmann, (2011)].**

| Genes | AcMNPV ORF | References |
|---|---|---|
| **Matrix gene** | | |
| *polyhedrin/ granulin* | ORF - 8 | (Rohrmann, 2011) |
| **Envelope gene of BVs** | | |
| *GP64 / Fusion protein* | ORF - 128 | (Herniou et al., 2003; Ijkel et al., 2000) |
| **Replication** | | |
| *lef-1* | ORF - 14 | (Evans et al., 1997) |
| *lef-2* | ORF - 6 | (Evans et al., 1997) |
| *DNA pol* | ORF - 65 | (Vanarsdall et al., 2005) |
| *Helicase* | ORF - 95 | (Bideshi and Federici, 2000; McDougal and Guarino, 2000) |
| **Transcription** | | |
| *lef-4* | ORF - 90 | (Gross and Shuman, 1998a; Guarino et al., 1998a) |
| *lef-8* | ORF - 50 | (Titterington et al., 2003) |
| *lef-9* | ORF - 62 | (Iorio et al., 1998) |
| *p47* | ORF - 40 | (Guarino et al., 1998b; McLachlin and Miller, 1994) |
| *lef-5* | ORF - 99 | (Todd et al., 1996) |
| **Cell cycle arrest and/or interaction with host proteins** | | |
| *odv-e27* | ORF - 144 | (Belyavskyi et al., 1998; Braunagel et al., 1996b) |
| *ac81* | ORF - 81 | (Chen et al., 2007) |

***AcMNPV ORF = *Autographa californica* multiple nucleopolyhedrovirus (Genbank acc. no. L22858.1) open reading frame**

**Table 1-3: Core genes found in Baculoviruses [Adapted from Rohrmann, (2011)], continued from page 28.**

| Genes | AcMNPV ORF | References |
|---|---|---|
| | **Packaging, assembly and release** | |
| *ODV-E25* | ORF - 94 | (Hong et al., 1997; Nguyen et al., 2018) |
| *ODV-E43* | ORF - 109 | (Fang et al., 2003) |
| *ODV-E18* | ORF - 143 | (Braunagel et al., 2003; Deng et al., 2007) |
| *p6.9* | ORF - 100 | (Wang et al., 2010; Wilson et al., 1987) |
| *vp39* | ORF - 89 | (Wang et al., 2010) |
| *vlf-1* | ORF - 77 | (McLachlin and Miller, 1994; Mikhailov and Rohrmann, 2002a) |
| *alk-exo* | ORF - 113 | (Mikhailov and Rohrmann, 2002b) |
| *vp1054* | ORF - 54 | (Olszewski and Miller, 1997a) |
| *vp91/p95* | ORF - 83 | (Russell and Rohrmann, 1997) |
| *gp41* | ORF - 80 | (Whitford and Faulkner, 1992a, 1992b) |
| *38k* | ORF - 98 | (Woo et al., 2006) |
| *49k* | ORF - 142 | (Vanarsdall et al., 2007; Yang et al., 2008) |
| *odv-nc42* | ORF - 101 | (Braunagel et al., 2003) |
| *desmoplakin* | ORF - 66 | (Ke et al., 2008) |
| *p33* | ORF - 92 | (Nie et al., 2011; Wu and Passarelli, 2010) |
| | **Oral infectivity** | |
| *pif-0/p74* | ORF - 138 | (Haas-Stapleton et al., 2004; Simón et al., 2005) |
| *pif-1* | ORF - 119 | (Braunagel et al., 2003; Ohkawa et al., 2005) |
| *pif-2* | ORF - 22 | (Braunagel et al., 2003; Simón et al., 2005) |
| *pif-3* | ORF - 115 | (Braunagel et al., 2003) |
| *pif-4/19k/odv-e28* | ORF - 96 | (Fang et al., 2009) |
| *pif-5/odv-e56* | ORF - 148 | (Braunagel et al., 1996a; Sparks et al., 2011) |
| *pif-6* | ORF - 68 | (Nguyen et al., 2018; Zhu et al., 2014) |

***AcMNPV ORF = *Autographa californica* multiple nucleopolyhedrovirus (Genbank acc. no. L22858.1) open reading frame.**

## 1.2.5 Baculovirus Core Proteins

Several core proteins have been identified in baculoviruses involved in structural integrity and the process of infection, packaging, assembly, and release of OBs. A brief description of the core baculoviral proteins has been given below.

### 1.2.5.1 Matrix Protein

**Polyhedrin:** Polyhedrins are the major structural components of occlusion bodies found in all nucleopolyhedroviruses (NPV). NPVs produce polyhedrin protein at a very high level in the late phase of infection. Polyhedrin is about 250 amino acids long with a molecular weight of about 30kDa. The 30 kDa subunits form trimers that are arranged into dodecamers (four trimers) via disulfide bonds (Rohrmann, 2011).

### 1.2.5.2 Envelope proteins of budded virions

**GP64:** GP64 is a characteristic protein and major distinguishing feature of group I Alphabaculovirus (Herniou and Jehle, 2007; Jiang et al., 2009). It is a fatty acid acylated glycoprotein (Roberts and Faulkner, 1989), activated at low pH and functions as a fusion protein (Hohmann and Faulkner, 1983).

**Fusion protein-F:** Orthologs of this protein are present in Group II alphabaculovirus, betabaculovirus and deltabaculovirus, but absent in group I alphabaculovirus, where GP64 acts as an active fusion protein (Herniou and Jehle, 2007). The only exception was gammabaculovirus (hymenopteran NPVs) which lack homologs of both the F and GP64 proteins. F proteins of Group II NPVs function as low-pH envelope fusion proteins (Ijkel et al., 2000).

### 1.2.5.3 Oral infectivity factors/ *per os* infectivity factors (*pif*) (additional envelope proteins of ODVs)

Seven conserved *pif* genes, so far, have been identified as involved in the process of oral infection in several nucleopolyhedroviruses which are: *p74* (Kuzio et al., 1989; Yao et al., 2004), *pif-1* (Kikhno et al., 2002), *pif-2* (Fang et al., 2006; Pijlman et al., 2003), *pif-3* (Li et al., 2007; Ohkawa et al., 2005), *pif-4* (Fang et al., 2009; Huang et al., 2012; Zhu et al., 2014), *pif-5* (Zhu et al., 2014) and *pif-6* (Nie et al., 2012; Zhu et al., 2014). These genes are commonly known as *per os* infectivity factors (PIFs) and are required for oral infection of insects but superfluous for infection in cultured cells. These factors or proteins are only present in ODVs. Mutations causing lack of expression of *pif-1* (Kikhno et al., 2002), *pif-2* (Fang et al., 2006), *pif-3* (Li et al., 2007), *pif-4* (Fang et al., 2009) and *p74* (Yao et al., 2004) restrict viral oral infection prior to gene expression in midgut cells suggesting that PIFs are

important for ODV oral infection and are involved in early infection events (Fang et al., 2006). A recent study showed that the PIF1, PIF2, PIF3, PIF4 and P74 form a PIF complex during early infection events (Huang et al., 2012). Moreover, PIF1, PIF2 and P74 bring about the specific binding of ODV to the midgut cells of the host, and their involvement in virus-cell interaction in the initial step of infection (Haas-Stapleton et al., 2004; Ohkawa et al., 2005). Though PIF3 is found as an important ODV protein its role in specific binding in midgut cells was not identified instead, it is found to mediate nucleocapsid translocation along the microvilli (Ohkawa et al., 2005). PIFs have strict host specificities and are crucial for deceiving the host range of baculoviruses (Song et al., 2016).

Homologs of *Pif-6* appear to be present in all NPVs (Zhu et al., 2014). Deletion of *pif-6* in AcMNPV causes no major effect other than a longer lethal time in larvae (Li et al., 2008). Similar results were obtained when the ortholog of *pif-6* in BmNPV was deleted from a bacmid. However, the OBs produced by the mutant BmNPV bacmid were abnormal and lacked virions, indicating a role of *pif-6* in polyhedron morphogenesis (Xu et al., 2008).

### 1.2.5.4 Proteins involved in packaging, assembly, and release

**ODV-E25:** N-terminal 24 amino acids of ODV-E25 appear to contain a nuclear targeting signal (Hong et al., 1997). Its homologs are present in all baculoviruses (Nguyen et al., 2018).

**ODV-EC43:** Homologs of ODV-EC43 are present in all baculovirus genomes (Braunagel et al., 2003; Fang et al., 2003; Nguyen et al., 2018). Although deletion of ODV-EC43 in AcMNPV did not affect DNA replication, the virions were not infective (Fang et al., 2009).

**ODV-E18:** It was found to be associated with ODV by proteomic analysis (Braunagel et al., 2003; Deng et al., 2007). Homologs of ODV-E18 are present in all baculoviruses (McCarthy and Theilmann, 2008). In addition to ODV, it is also associated with BV (Wang et al., 2010).

**P6.9:** It is a 55 amino acid long arginine/serine/threonine-rich DNA binding protein (Wang et al., 2010; Wilson et al., 1987) which is not essential for DNA replication, but the viruses deleted for the gene are not viable (Wang et al., 2010).

**VLF-1:** The very late factor (VLF-1), a member of the lambda integrase family of proteins, was reported to influence the hyperexpression of very late genes (McLachlin and Miller, 1994) possibly by binding to their regulatory regions (Yang and Miller, 1999). Mutations that affect the transcription of the very late gene were not lethal, whereas, other mutations required for integrase activity produce inactive viruses (Yang and Miller, 1998).

**VP39:** Proteomic analysis revealed that vp39 is one of the three most abundant capsid proteins of BV (Wang et al., 2010). Homologs of vp39 are present in all baculovirus genomes (Nguyen et al., 2018).

**GP41:** It is present in the tegument, a structure found between the virion envelope and capsid (Whitford and Faulkner, 1992a, 1992b). Homologs of GP41 are present in all baculovirus genomes. GP41 is found to have an important role in the release of nucleocapsids from the nucleus and morphogenesis of ODVs (Olszewski and Miller, 1997b).

**38K:** It interacts with VP1054, VP39, and VP80 (Broyles and Moss, 1986). Deletion of this gene leads to the formation of tube-like structures devoid of DNA (Wu et al., 2006).

**49k:** Deletion of 49k in AcNPV and BmNPV affects nucleocapsid formation but does not found to affect DNA synthesis (McCarthy et al., 2008; Vanarsdall et al., 2007).

**BV/ODV-C42:** It is conserved in all baculovirus except *Culex nigripalpus* nucleopolyhedrovirus (CuNiNPV) and expresses a capsid associated proteins in both, BV and ODV (Braunagel et al., 2003).

**Desmoplakin**: It was reported to play an important role in releasing processes of nucleocapsids from the virogenic stroma to the cytoplasm (Ke et al., 2008).

**Alk-exo:** This is an alkaline nuclease having 5′-3′ exonuclease and possibly endonuclease activity also (Okano et al., 2004) and appeared to be associated with a DNA binding protein LEF-3 (Mikhailov and Rohrmann, 2002a).

**Vp1054**: This protein is reported as a virus structural protein required for nucleocapsid assembly (Olszewski and Miller, 1997a).

**Vp91/p95**: This protein was reported as a structural component in both the capsid and its surrounding envelope of ODVs (Russell and Rohrmann, 1997).

**P33:** It is a flavin adenine dinucleotide-linked sulfhydryl oxidase which appeared to be involved in the efficient production of virions in the nucleus of infected cells (Deng et al., 2007; Nie et al., 2011; Wu and Passarelli, 2010).

### 1.2.5.5 Proteins involved in replication

**LEF-1**: LEF-1 was reported to have a primase-like motif and is important to carry out transient DNA replication (Evans et al., 1997).

**LEF-2**: LEF-2 was found to interact with LEF-1 and any mutation leading to abortion of this interaction resulted in reduced levels of transient DNA replication (Evans et al., 1997).

**DNA Polymerase**: Baculovirus genome also expresses DNA polymerase which is conserved in all baculoviruses. Baculovirus lacking *dnapol* was unable to replicate its DNA in the host cell (Vanarsdall et al., 2005).

**Helicase: The** DNA helicase gene was also present in all baculovirus genomes and is one of the core genes (McDougal and Guarino, 2000).

### 1.2.5.6 Proteins involved in Transcription

**LEF-4**: It was reported as a virus-encoded RNA polymerase subunit, having RNA 5′-triphosphatase, nucleoside triphosphatase, and guanylyltransferase activities (Gross and Shuman, 1998b; Guarino et al., 1998a). It has also been shown to be a capping enzyme but the host enzyme can carry out the triphosphatase reaction during capping (Li and Guarino, 2008).

**LEF-8**: It is a subunit of the baculovirus late RNA polymerase complex (Crouch et al., 2007) required for transient late gene expression (Passarelli et al., 1994). Homologs are found in all baculoviruses and contain a conserved motif found in other RNA polymerases (Passarelli et al., 1994; Titterington et al., 2003).

**LEF-9**: It was also reported as a subunit of the baculovirus RNA polymerase complex (Guarino et al., 1998a) and was essential for the short-term expression of the late gene (Lu and Miller, 1994).

**p47**: It is also a subunit of the baculovirus RNA polymerase complex (Guarino et al., 1998b) and was reported as an essential factor for transient late gene transcription (Todd et al., 1995).

**LEF-5**: LEF-5 is another factor required for transient late gene expression. Previously, it was reported to interact with itself having a domain similar to the elongation factor TFIIS of RNA polymerase II (Harwood et al., 1998). But later experiments revealed that it acts as an initiation factor rather than an elongation factor (Guarino et al., 2002).

### 1.2.5.7 Proteins involved in cell cycle arrest and/or interaction with host proteins

**Odv-ec27:** This protein is found to act as multifunctional cyclin and is reported to be involved in the regulation of the cell cycle during virus infection (Belyavskyi et al., 1998; Braunagel et al., 1996b) and was shown to interact with p40 (ODV-C42) solely or in combination with p78/83 (Braunagel et al., 2001).

**Ac81:** This protein does not appear in ODVs or BVs, but interacts with Actin A3 in the cytoplasm (Chen et al., 2007).

## 1.2.6 Application of NPVs as bioinsecticides

One of the most important tasks in agricultural pest control is the ability to sustain pest insect population level below the economic injury threshold cost-effectively so that the cost of pest control operations justifies the income generated. The application of microorganisms pathogenic to insects is an environmentally benign method of insect pest control. In this criterion, baculoviruses have several inherent advantages as biopesticides and have so far been deemed successful. In this context, due to their inherent insecticidal activities the nucleopolyhedroviruses, a group of the Baculovirus family, have been registered and successfully used as safe and effective biopesticides for the protection of field and orchard

crops and forests in America, Europe and Asia (Black et al., 1997; Copping and Menn, 2000).

Baculoviruses have been used against insect pests since the late 1930s. *Gilpinia hercyniae*, the European spruce sawfly, after its introduction to North America in the early 20th century, became established as a major pest of spruce trees. In 1938, approximately 12,000 square miles of spruce were infested leading to serious damage. The introduction of an NPV pathogenic to this insect was reported to control the infestation and resulted in the disappearance of the pest within a few years (Balch and Bird, 1944).

Elcar™, a preparation of *Heliothis zea* Single NPV (HzSNPV), was the first baculovirus-based commercial insecticide (Ignoffo and Couch, 1981). It was pathogenic to a broad range of insect pests belonging to the genera *Helicoverpa* and *Heliothis*. HzSNPV not only controlled the pest population of cotton bollworms but also the population of pests attacking soybean, sorghum, maize, tomato and beans. The development of resistance to many chemical insecticides including pyrethroids in the pest population revived the interest in HzSNPV-based biopesticide and subsequently, the same baculovirus was registered under the name GemStar™. Another baculovirus, *Helicoverpa armigera* single NPV (HaSNPV) similar to HzSNPV was registered in China as a bioinsecticide in 1993 (Zhang et al., 1995) and has been extensively applied in cotton fields. A large range of bioinsecticides based on HaSNPV has also been used in India (Srinivasa et al., 2008).

The forests of temperate regions are frequently attacked by lepidopteran (most commonly *Lymantria dispar*, *Lymantria monacha*, *Orgiya pseudotsugata* and *Panolis flammea*) and hymenopteran (mainly *Neodiprion sertifer* and *Diprion pini*). The formulation of MNPV isolated from *L. dispar* commercialized under the trade names Gypchek, Disparivirus, Virin-ENSH, and the MNPV of *O. pseudotsugata* under the trade names TM BioControl-1 and Virtuss (Reardon et al., 1996) are sometimes used for forest protection.

Caterpillars of the genus *Spodoptera* are the major concern for agricultural fields in many countries. Two NPV-based biopesticides, SPOD-X™ containing *Spodoptera exigua* NPV to control insects on vegetable crops and Spodopterin™ containing *Spodoptera littolaris* NPV which is used to protect cotton, corn and tomatoes are commercially available. By the use of SPOD-X™ insect pest population of *Spodoptera*, commonly known as a cotton pest, was successfully controlled (Inceoglu et al., 2001). Application of *Spodoptera frugiperda* NPV over 20,000 ha in Brazil has controlled the pest population in a maize field (Moscardi, 1999), but later it has been discontinued due to technical problems in the virus production under

laboratory conditions. In Brazil, an NPV pathogenic to *Anticarsia gemmatalis*, a major caterpillar pest of soybeans, is used to curb the pest population of over a million hectares of this crop (Moscardi, 1999).

NPV isolated from *Perigonia lusca*, a tea pest in Paraguay, was used to control the insect over 900 ha of tea plantations (Sosa-Gomez et al., 1994). NPV infecting *B. suppressaria* was effectively used to control the pest in tea plantations and tung oil trees over 20,000 ha in China (Yi P. and Li Z., 1989).

NPVs pathogenic to *Trichoplusia* sp. (*Brassica* pest) and *Spodoptera litura* (cotton pest) have also been commercially produced under the name biotrol-VTN and biotrol-VSE, respectively (Nutrilite Products Inc., U.S.A.) (Dubey, 2006). NPVs isolated from *Autographa californica* and *Anagrapha falcifera* were also registered and evaluated in the field on a limited scale (Sahayaraj, 2014). The presence of NPV in geometrid loopers of Indian tea has been shown by Mukhopadhyay and workers (Mukhopadhyay et al., 2011, 2007).

### 1.2.7 Molecular Characterization of *Hyposidra talaca* NPV genome

Pathogenicity of *Hyposidra talaca* NPV to the respective host was reported by Mukhopadhyay et al. (2011). A dose-mortality bioassay study of OBs of HytaNPV isolated from the Terai population of loopers was conducted on the second instar caterpillars of *H. talaca*. Results showed a median lethal concentration ($LC_{50}$) value of 2.8 x $10^3$ OBs/ml with a median lethal time ($LT_{50}$) of 5.45 days for 1 x $10^4$ OBs/ml, 4.15 days for 1 x $10^5$ OBs/ml and 4.05 days for 1 x $10^6$ OBs/ml concentrations. These results encouraged us to take up a further study on the genetic characterization of HytaNPV to develop it in future as a potential bioinsecticide against *H. talaca*. The *polyhedrin* gene of HytaNPV was sequenced (Sinu et al., 2011) and based on the partial sequence of the *polyhedrin* gene, Antony et al. (2011) reported a close relation between *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) and *Biston* (=*Buzura) suppressaria* nucleopolyhedrovirus (BusuNPV), another NPV infecting the tea pest *Buzura suppressaria* mainly recorded in China (Hu et al., 1993) and was subsequently reported from the Terai-Dooars and North-East region of India (Das et al., 2010a; Dasgupta et al., 2016; Mukhopadhyay et al., 2007; Sinu et al., 2011). Preliminary RFLP analysis of the HytaNPV genome (Ghosh et al., 2015) showed that the restriction

profile and genome size of HytaNPV differ from that of BusuNPV (Hu et al., 1998). Dasgupta et al. (2016) also analyzed the phylogenetic relationship of HytaNPV based on the *polyhedrin* gene and reported it to be the same HytaNPV variant as documented by Antony et al. (2011). They concluded that the variant is predominant in the northeastern part of India having the potential of pathogenicity as an alternative to chemical pesticides to control the *H. talaca* pest population. Recently, the whole genome organization and sequence of HytaNPV of the Dooars isolate was reported by Nguyen et al. (2018). Phylogenetic analyses based on 37 core baculovirus genes revealed that the Dooars isolate of HytaNPV is a closer relative of BusuNPV of China than other NPVs infecting the other lepidopteran pest specimens.

# Section 1.3: Objectives

1. To survey, sample and maintain the virus culture *in vivo* drawn from *Hyposidra talaca* looper populations of different tea plantations of Sub-Himalayan foothills and Terai region of West Bengal.

2. To isolate and purify HytaNPV polyhedral occlusion bodies (POBs) from cadavers of *Hyposidra talaca* looper using differential sucrose centrifugation method.

3. To determine the approximate genome size by restriction digestion analysis of the strains of HytaNPV.

4. To study restriction profile of the genome of HytaNPV strain(s) collected from Terai region of Darjeeling foothills.

5. To characterize HytaNPV gene(s) such as *pif* or some others, related to the pest control property of the virus.

6. To construct a phylogenetic tree of the different strains of HytaNPV based on the similarities and differences.

# Section 1.4: Materials and Methods

## 1.4.1 Survey

Different tea plantations of Terai and the Dooars regions of West Bengal, India were surveyed around the year during 2014-2018 to observe the occurrence of the *Hyposidra talaca* caterpillars (loopers) before the spray of pesticides in the tea garden.

## 1.4.2 Sample collection

**a) Collection of NPV-infected caterpillars**

Cadavers of *Hyposidra talaca* larvae show typical symptoms of NPV infection such as caterpillars perched at the top of the tea shoots, attached and hanging head down on dying with their prolegs, with a characteristic inverted V-shape, and liquefaction of the body with light brown fluid (Federici, 1997). Such moribund specimens were collected separately for the study from different tea plantations in the Terai-Dooars region of Darjeeling foothills. The NPV-infected dead caterpillars were also collected from the Dooars region of West Bengal, India for comparison.

**b) Collection of healthy caterpillar and adult moths**

Healthy $3^{rd}$-$5^{th}$ instar larvae and adult moths of *Hyposidra talaca* were also collected to establish and maintain a culture in the laboratory.

### 1.4.2.1 Collection time

The adult moths were collected in the early morning usually between 5:30 AM to 7:00 AM as they are active in the dimmed light during this period and the larvae were collected usually in the late morning between 7:00 AM to 9:00 AM as the loopers start climbing up to the top of the tea plants during this period.

Sample collection was mainly done from March to November. The collection of caterpillars was mostly avoided during the rainy season to avoid any contamination and secondary infection.

The looper caterpillars were identified in the field and subsequently in the laboratory using the taxonomic key provided by Das and Mukhopadhyay (2009).

### 1.4.2.2 Sampling sites

Both NPV-infected and non-infected loopers of *H. talaca* were collected from different tea plantations in the Terai region of Darjeeling foothills. Later for better comparison, the Dooars region was also included as a collection site. Details of the sampling sites were given below (**Figure 1-8**).

**a) Terai region** - Matigara Tea Estate (26°42'41.1"N latitude, 88°22'30.8"E longitude), Atal Tea Estate (26°41'35.8"N latitude, 88°15'03.5"E longitude), Sathbhaiya Tea Estate (26°40'18.5"N latitude, 88°13'09.3"E longitude), and Kamalpur Tea Estate (26°42'26.3"N latitude, 88°18'24.5"E longitude) (**Figure 1-8**).

**b) Dooars region** - Elenbari Tea Estate (26°52'09.2"N latitude, 88°32'41.5"E longitude) and Dumdim Tea Estate (26°50'35.2"N latitude, 88°39'39.7"E longitude) (**Figure 1-8**).

**Figure 1-8: Map showing the sampling sites of Terai and the Dooars region in the present study.**

## 1.4.3 Rearing and culture of *Hyposidra talaca*

The healthy 3$^{rd}$-5$^{th}$ instar larvae were reared in the laboratory in sterilized plastic containers (15 cm height x 8 cm diameter) with pesticide-free fresh tea leaves (*Camellia sinensis*). The pesticide-free leaves were collected from the tea plantation maintained by the Entomological Research Unit of the Department of Zoology, University of North Bengal. The cultures were maintained at room temperature with a daily change of fresh tea leaves washed in distilled water and the stalks of the twig immersed in the water-filled small 2.0 ml micro-centrifuge

tube or in the conical flask (**Figure 1-9**). The twigs were arranged in such a way that enough space was available for the free movement of larvae.

The adult moths developed from the pupa in the laboratory were reared in large plastic containers (30 cm height x 15 cm diameter) with a 2:1 male-female ratio for successful mating to maintain the culture for the next generation.

### 1.4.4 Isolation and purification of OBs (virus particles) from NPV-infected cadavers

Stocks of OBs were built-up separately from the cadavers of *H. talaca* following the method of (Kawarabata and Matsumoto, 1973) with some modifications. The methods were described as follows.

The cadavers were stored in distilled water and left to putrefy for about 15 days to enable the release of OBs from the larval tissues. The putrefied suspension of infected larvae was homogenized using a glass tissue homogenizer. The homogenate was filtered through four layers of cheesecloth and the filtrate was immediately centrifuged at 1000 x g for 20 minutes at room temperature. The supernatant was removed carefully by a Pasteur pipette and the sedimented OBs were suspended in 25% (w/v) sucrose dissolved in distilled water. The suspension was centrifuged at 1000 x g for 20 minutes at room temperature. The supernatant was discarded and the sedimented OBs were resuspended in 10 ml of 25% sucrose solution. The suspended OBs (1 volume) were slowly layered on 3 volumes of 50% sucrose solution and centrifuged at 1800 x g for 40 minutes at room temperature. The differential centrifugation was repeated twice and the OBs were washed 7-8 times with deionised distilled water. The pellet containing pure OBs was resuspended in distilled water and stored at 4°C until further use.

**Figure 1-9: Culture of *H. talaca* larvae in the laboratory. (a) culture of healthy larvae ($3^{rd}$-$4^{th}$ instar), (b) culture of infected larvae ($3^{rd}$ – $5^{th}$ instar).**

## 1.4.5 Recognition of virus by Phase contrast microscopy

The crystalline white precipitate (OBs) isolated from each cadaver was examined at 1000X magnification in a bright field compound microscope (Nikkon Eclipse 200, Nikkon, Tokyo, Japan) and photographed. The OBs were evident as bright particles under the compound microscope **Figure 2-5**.

## 1.4.6 Counting Polyhedra Occlusion Bodies (OBs)

The number of OBs/ml was determined by using a Neubauer haemocytometer.

## 1.4.7 Maintenance and Mass production of HytaNPV OBs under laboratory conditions

The prepared polyhedral OBs were used to infect the healthy *H. talaca* larvae reared in the laboratory. The tea leaves were brushed with the solution of polyhedral OBs ($10^5$ OBs/ml) and were used as feed for the $3^{rd}$-$5^{th}$ instar larvae of *H. talaca* for mass production of HytaNPV. The larvae showing symptoms of viral infection were collected for further isolation of polyhedral OBs (Virus particles).

## 1.4.8 Extraction and Purification of DNA from Polyhedra Occlusion Bodies of *H. talaca* NPV

Viral DNA was extracted as described by O'reilly and Miller (1991). The procedure was described below.

**a) DNA extraction:**

The suspension of Occlusion bodies (OBs) was centrifuged at 1800 x g for 20 min and the sedimented polyhedra were suspended in Tris-EDTA (10mM Tris, 1mM EDTA, pH 8.0). Proteinase K (10 mg/ml) was added to the suspension to a final concentration of 1mg/ml and incubated for 1 hour at $37^0$C. Following proteinase k digestion, the suspension was dissolved in dissolution buffer (0.1M $Na_2CO_3$, 0.01M EDTA, 0.17M NaCl, pH 10.8). After dissolution 10% SDS was added into the suspension to a final concentration of 1%, mixed thoroughly and incubated for 1 hour at $37^0$C. The suspension was centrifuged at 11,000 x g for 3 min and the supernatant was transferred to a fresh tube.

**b) Purification of extracted DNA**

An equal volume of Tris-equilibrated phenol (pH-8.0) was added to the supernatant, mixed gently for at least 5 min followed by centrifugation at 8000 x g for 10 mins at $4^0$C. The top aqueous phase was transferred to a fresh tube. To it, an equal volume of a mixture of Tris-equilibrated phenol: chloroform: iso-amyl alcohol (25:24:1) was added, gently mixed and centrifuged at 8000 x g for 10 mins at $4^0$C. The top aqueous phase was transferred to a fresh tube, and an equal volume of a mixture of chloroform: iso-amyl alcohol (24:1) was added and gently mixed followed by centrifugation at 8000 x g for 10 min at $4^0$C. The top aqueous phase was carefully transferred to a fresh tube and twice the volume of absolute ethanol was added, incubated for 1 hour at $4^0$C and centrifuged at 15,000 x g for 15 min at $4^0$C. The supernatant was discarded. The sedimented DNA pellet was washed in 500µl of 70% ethanol following centrifugation at 15,000 x g for 15 min at $4^0$C. The ethanol was discarded and the DNA pellet was dried. Finally, the DNA was suspended in Tris-EDTA (10mM Tris, 1mM EDTA, pH-8.0) and stored at $-20^0$C.

## 1.4.9 *In vivo* Restriction Endonuclease Digestion

HytaNPV DNA was digested by *Eco*RI, *Xho*I, *Kpn*I, *Bam*HI, *Hind*III, *Bgl*I and *Pst*I following the method of Sambrook and Russell (2001) with some modifications. 1.5-2 µg of

HytaNPV DNA was set up for digestion with 10 units of restriction enzyme in the presence of 1X restriction buffer and 1X acylated-BSA at 37°C for 4 hours for complete digestion.

The reaction mixture was set as per the manufacturer's specifications. Each component and its amount have been given below.

| Substrate | Quantity | Final Concentration |
|---|---|---|
| DNA | 1.5-2 µg | 75-100ng/µl |
| Restriction Buffer (10X) | 2 µl | 1X |
| Acylated BSA (100X) | 0.2 µl | 1X (0.1mg/ml) |
| Restriction Endonuclease | 10 units | 0.5 unit/ µl |
| **Final Volume** | **20 µl (adjusted by adding Milli-Q water)** | |

### 1.4.9.1 Electrophoresis of digested samples

The digested DNA samples were separated on 0.4-0.7% agarose gel (depending on expected fragment size) (0.4-0.7% agarose in 1x TAE buffer) at 70 volt for 4-6 hours in 1X TAE buffer (40mM Trishydroxymethylaminomethane pH 8.0, 1mM EDTA pH 8.0, 20mM acetic acid). A λ DNA *Hind*III digest molecular weight marker and a High Range DNA ruler (Thermofisher) were used to estimate the size of the digested fragments (bands). After electrophoresis, the gels were stained in ethidium bromide solution (0.5µg/ml). The gels were viewed and photographed in Gel Documentation System (SPECTROLINE BI-O-VISION, UV/WHITE Light Transilluminator) with transmitted UV light. The photographs were analyzed using the software: ImageAide: Version 3.06.

## 1.4.10 Construction of *in silico* Restriction map

*In silico* restriction map of the HytaNPV genome (Nguyen et al., 2018) and obtained sequences of the HytaNPV isolates of the present study were prepared in SnapGene viewer.

## 1.4.11 Designing of Primers

The set of primers to amplify the *polyhedrin* gene was used from Antony et al. (2011).

To amplify *pif-1* and *lef-8* gene BusuNPV (KF611977.1), EcobNPV (DQ837165.1), ApciNPV (FJ914221.1), SujuNPV (KJ676450.1) and EupsNPV (FJ227128.1) were used to design the degenerate primers, during the year of 2015-2018 as there was no sequence available for those genes of HytaNPV at that time.

After the publication of the complete genome sequence of HytaNPV by Nguyen et al. (2018) the oligonucleotide primers to amplify *pif-2*, *pif-2* and *lef-9* genes were designed based on the sequence alignment of HytaNPV (MH261376.1), BusuNPV (KF611977.1), EcobNPV (DQ837165.1), ApciNPV (FJ914221.1) and SujuNPV (KJ676450.1).

**Table 1-4: List of the primers used to amplify different genes and respective annealing temperature and duration of extension in each cycle of the PCR program.**

| Target Gene | Primer set | Primer Name | Primer sequence (5′-3′) | Annealing temperature | Extension time in Cycle |
|---|---|---|---|---|---|
| *polyhedrin* | SET 1 | PHF1 | GGACCSGGYAARAAYCAAAAA | 42 | 50 sec |
| | | PHR1 | GCRTCWGGYGCAAAYTCYTT | | |
| *pif-1* | SET 1 | P1SF1 | GARGGNCTNGCNAAYTGYCA | 50 | 70 sec |
| | | P1SR1 | TNGGRTANCGNGTNGCNGG | | |
| *pif-2* | SET 1 | P2SF1 | CTGGTCAAAAACCTACCGAC | 54 | 60 sec |
| | | P2SR1 | GATCCGCGTTATTTTGCCG | | |
| | SET 2 | P2SF2 | GGCAAAATAACGCGGATCT | 54 | 60 sec |
| | | P2SR2 | ACGAACAACACGCAAAAATG | | |
| *pif-3* | SET 1 | P3SF1 | CAAGAAACGTGCAGGCAAA | 54.5 | 60 sec |
| | | P3SR1 | ATCAACAATCGCAATACGGC | | |
| *lef-8* | SET 1 | L8SF1 | GGCACTTTCATGATHGACGG | 55 | 70 sec |
| | | L8SR1 | CCACCGTCATTTCNCCGTG | | |
| | SET 2 | L8SF2 | ACGGNGAAATGACGGTGGC | 55 | 70 sec |
| | | L8SR2 | GGDATRTANGGRTCTTCGGC | | |
| | SET 3 | L8SF3 | GCCGAAGAYCCNTAYATHCC | 55 | 70 sec |
| | | L8SR3 | GATTGRTTDATNGTCCATTGATC | | |
| *lef-9* | SET 1 | L9SF1 | CGCTTTCGGATTTTGTCTTCA | 54.5 | 80 sec |
| | | L9SR1 | CCGACTTTACCGACTGGAA | | |
| | SET 2 | L9SF2 | TCCAGTCGGTAAAGTCGGC | 54.5 | 80 sec |
| | | L9SR2 | CCCGTAAATTTCGACGCTACT | | |

## 1.4.12 Polymerase Chain Reaction (PCR)

The PCR amplification of the target gene was carried out with 50ng of HytaNPV DNA in the presence of 1X GoTaq Flexi Buffer, 2mM $MgCl_2$, 250µM dNTPs mixture, 0.5µM each

of the forward and reverse primers and 2 units of GoTaq Flexi DNA Polymerase (Promega) with a PCR programme of 94ºC for 5 min (initial denaturation) followed by 35 cycles of 94ºC for 50 sec (denaturation), optimum annealing temperature for 40 sec and extension at 72ºC for the required period (**Table 1-4**); then the final extension of 7 min at 72ºC.

### 1.4.13 Detection and purification of PCR amplified product for sequencing

The amplified PCR products were subjected to electrophoresis in a 1% agarose gel (1% agarose in 1x TAE and ethidium bromide 0.5µg/ml). The electrophoresis was carried out at 80 volts for 1 hour in 1x TAE buffer with a 100bp DNA ladder as a molecular weight marker. After electrophoresis, the gels were observed and photographed in the Gel Documentation System (SPECTROLINE BI-O-VISION, UV/WHITE Light Transilluminator) with transmitted UV light. The amplified products of the expected size were purified with the SureExtract Spin PCR Clean up/ Gel extraction kit (Brand: Geneticx).

### 1.4.14 Sequencing of the amplified products

The purified PCR products were sequenced from both directions using Applied Biosystems 3730xl/ABI3730XL-15104-028 capillary sequencer.

### 1.4.15 Sequence alignment and BLAST search

The gene sequences obtained were aligned and joined using ClustalW in MEGAX (Kumar et al., 2018). The correct nucleotide sequences were obtained by aligning and comparing them with the reported sequences of HytaNPV (GenBank Accession No. MH261376.1; Nguyen et al., 2018) or BusuNPV (GenBank Accession No. KF611977.1; Zhu et al., 2014) using ClustalW Program with MEGAX. BLAST analyses were performed using BLASTN 2.9.0+ (Zhang et al., 2000), and BLASTX 2.9.0+ (Altschul, 1997). The representations of the aligned sequences were prepared using BioEdit version 7.2.5 (Hall, 1999) or GeneDoc (Nicholas and Nicholas, 1997). The obtained sequences were submitted to the NCBI GenBank database.

## 1.4.16 Sequence representations

Representations of the obtained sequences were prepared in SnapGene viewer and the alignments were prepared in BioEdit (Hall, 1999).

## 1.4.17 Sequence comparison

Nucleotide and amino acid sequences were compared with the Dooars isolate of HytaNPV (Accession No. MH261376.1; Nguyen et al., 2018) to detect nucleotide and amino acid substitution(s) using MEGAX (Kumar et al., 2018).

## 1.4.18 Sequence Divergence

The p-distance model was used to estimate the nucleotide sequence and amino acid sequence divergence by using MEGAX (Kumar et al., 2018). The ratio of transitional to transversional differences was also estimated using MEGAX. Nucleotide positions 1st, 2nd, 3rd of the codon and non-coding position were included. All positions containing gaps and missing data were eliminated.

## 1.4.19 Phylogenetic analysis

All the retrieved sequences were aligned using ClustalW in MEGAX applying default parameters. The phylogenetic trees were constructed by MEGAX using the maximum likelihood method (Kumar et al., 2018; Nei and Kumar, 2000). All the phylogenetic trees were tested by the bootstrap method with 1000 replicates. Positions containing gaps and missing data were not considered.

Phylogenetic analysis was carried out with nucleotide sequences of 77 baculoviruses comprising 75 sequences (1 deltabaculovirus, 3 gammabaculoviruses, 18 betabaculoviruses, 22 group I alphabaculoviruses and 31 group II alphabaculoviruses) retrieved from NCBI GenBank databases (**Table 1-5**) and the sequences of two isolates of HytaNPV of the present study, HytaNPV-ITK1 (Terai) and HytaNPV-ID1 (Dooars) based on a concatenated sequence alignment of six genes (*polyhedrin/granulin, lef-8, lef-9, pif-1, pif-2*, and *pif-3*) together. Both the amino acid and nucleotide substitution models were used for analysis. Analyses were carried out by extracting the total reading frame of each gene from the complete genome sequences of 75 baculoviruses. All the gaps and missing data were not considered for maximum likelihood analysis. A test of maximum likelihood fit was

performed for each phylogeny to find the considerably best method/model, rates and pattern of substitution to be used for constructing the trees (Nei and Kumar, 2000). Initial trees were designed by using Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated by the Maximum Composite Likelihood approach.

The General time reversible model (GTR) for nucleotide substitutions and Le Gascuel (2008) model for amino acids substitutions were used for the construction of phylogenetic trees applying non-uniformity of evolutionary rates considering discrete gamma distribution with invariant sites.

**Table 1-5: List of baculovirus sequences used for phylogenetic analysis in the present study.**

| Sl. No. | Baculovirus | GenBank acc No. | Designation |
|---|---|---|---|
| 1. | *Hyposidra talaca* NPV | MH261376.1 | HytaNPV-R |
| 2. | *Buzura suppressaria* NPV | KF611977.1 | BusuNPV |
| 3. | *Sucra jujuba* NPV | KJ676450.1 | SujuNPV |
| 4. | *Apocheima cinerarium* NPV | FJ914221.1 | ApciNPV |
| 5. | *Ectropis Obliqua* NPV | DQ837165.1 | EcobNPV |
| 6. | *Lambdina fiscellaria* NPV | KP752043.1 | LafiNPV |
| 7. | *Orgyia leucostigma* NPV | EU309041.1 | OrleNPV |
| 8. | *Euproctis (=Arna) pseudoconspersa* NPV | FJ227128.1 | EuprNPV |
| 9. | *Lymantria xylina* NPV | GQ202541.1 | LyxyNPV |
| 10. | *Lymantria dispar* NPV | AF081810.1 | LdNPV |
| 11. | *Adoxophyes orana* NPV | EU591746.1 | AdorNPV |
| 12. | *Adoxophyes honmai* NPV | AP006270.1 | AdhoNPV |
| 13. | *Mamestra configurata* NPV-A | AF539999.1 | MacoNPV-A |
| 14. | *Mamestra configurata* NPV-B | AY126275.1 | MacoNPV-B |
| 15. | *Mamestra brassicae* NPV | JQ798165.1 | MabrNPV |
| 16. | *Agrotis segetum* NPV | DQ123841.1 | AgseNPV |
| 17. | *Agrotis ipsilon* NPV | EU839994.1 | AgipNPV |
| 18. | *Spodoptera frugiperda* NPV | EF035042.2 | SfNPV |
| 19. | *Spodoptera exigua* NPV | AF169823.1 | SeNPV |
| 20. | *Spodoptera litura* NPV | AF325155.1 | SpliNPV-A |
| 21. | *Spodoptera littoralis* NPV | NC_038369.1 | SpliNPV-B |
| 22. | *Helicoverpa zea* NPV | KM596835.1 | HzNPV |
| 23. | *Helicoverpa armigera* NPV | KT013224.1 | HearNPV |
| 24. | *Leucania separata* NPV | AY394490.1 | LeseNPV |

**Table 1-5: List of baculovirus sequences used for phylogenetic analysis in the present study, continued from page 50.**

| Sl. No. | Baculovirus | GenBank acc No. | Designation |
|---|---|---|---|
| 25. | *Chrysodeixis chalcites* NPV | AY864330.1 | ChchNPV |
| 26. | *Chrysodeixis (=Pseudoplusia) includens* NPV | KJ631622.1 | PsinNPV |
| 27. | *Trichoplusia ni* NPV | DQ017380.1 | TnNPV |
| 28. | *Peridroma sp* NPV | KM009991.1 | PespNPV |
| 29. | *Perigonia lusca* NPV | KM596836.1 | PeluNPV |
| 30. | *Clanis bilineata* NPV | DQ504428.1 | ClbiNPV |
| 31. | *Urbanus proteus* NPV | KR011717.2 | UrprNPV |
| 32. | *Autographa californica* NPV | L22858.1 | AcNPV |
| 33. | *Plutella xylostella* NPV | DQ457003.1 | PlxyNPV |
| 34. | *Catopsilia pomona* NPV | KU565883.1 | CapoNPV |
| 35. | *Thysanoplusia orichalcea* NPV | JX467702.1 | ThorNPV |
| 36. | *Rachiplusia ou* NPV | AY145471.1 | RoNPV |
| 37. | *Bombyx mori* NPV | JQ991010.1 | BmNPV |
| 38. | *Bombyx mandarina* NPV | JQ071499.1 | BomaNPV |
| 39. | *Lonomia obliqua* NPV | NC_043520.1 | LoobNPV |
| 40. | *Antheraea pernyi* NPV | DQ486030.3 | AnpeNPV |
| 41. | *Maruca vitrata* NPV | EF125867.1 | MaviNPV |
| 42. | *Philosamia cynthia ricini* NPV | JX404026.1 | PhcyNPV |
| 43. | *Anticarsia gemmatalis* NPV | KR815455.1 | AngeNPV |
| 44. | *Epiphyas postvittana* NPV | AY043265.1 | EppoNPV |
| 45. | *Hyphantria cunea* NPV | AP009046.1 | HycuNPV |
| 46. | *Condylorrhiza vestigialis* NPV | KJ631623.1 | CoveNPV |
| 47. | *Choristoneura murinana* NPV | KF894742.1 | ChmuNPV |
| 48. | *Choristoneura occidentalis* NPV | KC961303.1 | ChocNPV |
| 49. | *Choristoneura fumiferana* NPV | AF512031.3 | CfNPV |
| 50. | *Choristoneura rosaceana* NPV | KC961304.1 | ChroNPV |
| 51. | *Dasychira pudibunda* NPV | KP747440.1 | DapuNPV |
| 52. | *Orgyia pseudotsugata* NPV | U75930.2 | OpNPV |
| 53. | *Dendrolimus kikuchii Matsumura* NPV | JX193905.1 | DekiNPV |
| 54. | *Cydia pomonella* GV | U53466.2 | CypoGV |
| 55. | *Pieris rapae* GV | JX968491.1 | PiraGV |
| 56. | *Clostera anachoreta* GV | HQ116624.1 | ClanGV-H |

**Table 1-5: List of baculovirus sequences used for phylogenetic analysis in the present study, continued from page 50.**

| Sl. No. | Baculovirus | GenBank acc No. | Designation |
|---|---|---|---|
| 57. | *Clostera anastomosis* GV | KC179784.1 | ClanGV-HB |
| 58. | *Adoxophyes orana* GV | AF547984.1 | AdorGV |
| 59. | *Erinnyis ello* GV | KJ406702.1 | ErelGV |
| 60. | *Choristoneura fumiferana* GV | DQ333351.1 | CfGV |
| 61. | *Agrotis segetum* GV | AY522332.4 | AgseGV |
| 62. | *Epinotia aporema* GV | JN408834.1 | EpapGV |
| 63. | *Diatraea saccharalis* GV | KP296186.1 | DisaGV |
| 64. | *Spodoptera frugiperda* GV | KM371112.1 | SfGV |
| 65. | *Helicoverpa armigera* GV | EU255577.1 | HearGV |
| 66. | *Pseudaletia unipuncta* GV | EU678671.1 | PsunGV |
| 67. | *Trichoplusia ni* GV | KU752557.1 | TnGV |
| 68. | *Mocis latipes* GV | KR011718.1 | MolaGV |
| 69. | *Plutella xylostella* GV | AF270937.1 | PlxyGV |
| 70. | *Cnaphalocrocis medinalis* GV | KP658210.1 | CnmeGV |
| 71. | *Cryptophlebia leucotreta* GV | AY229987.1 | CrluGV |
| 72. | *Neodiprion sertifer* NPV | AY430810.1 | NeseNPV |
| 73. | *Neodiprion abietis* NPV | DQ317692.1 | NeabNPV |
| 74. | *Neodiprion lecontei* NPV | AY349019.1 | NeleNPV |
| 75. | *Culex nigripalpus* NPV | AF403738.1 | CuniNPV |