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Effect of netting on Cryptophlebia leucotreta granulovirus for the management of *Thaumatotibia leucotreta* in South Africa

A thesis submitted in fulfilment of

the requirements for the degree of

MASTER OF SCIENCE

of

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by

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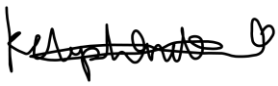
Declaration

I, Kurhula Califonia Luphondo (G19L1946) hereby declare that the thesis submitted is my own work. It is being submitted for the degree of Master of Science at Rhodes University. It has not been previously submitted for assessment of any degree at any other university or other body, organisation outside of the university.



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Abstract

Baculoviruses constitute a diverse group of insect viruses that serve as natural pathogens and biocontrol agents, playing a vital role in regulating insect populations and providing a potential alternative to chemical pesticides. *Thaumatotibia leucotreta*, commonly known as false codling moth (FCM), is a significant citrus pest in South Africa. Native to sub-Saharan Africa, this pest poses a serious threat to exports due to strict zero-tolerance policies by certain markets for larvae in shipments, making effective control essential. *Betabaculovirus cryleucotretae*, Cryptophlebia leucotreta granulovirus (CrleGV), is an integral part of the systems approach used to manage *T. leucotreta*. Currently, four commercially available biopesticides, namely Cryptogran®, CryptoMax, Cryptex®, and Gratham, utilise CrleGV as their active ingredient. While these biopesticides have been successfully used for years, a major limitation of baculoviruses is their sensitivity to UV radiation, which renders them inactive within hours or days. Consequently, developing new control measures or enhancing existing methods is crucial to improving their persistence in the field.

Shade nets are increasingly being adopted in South African citrus farming for multiple purposes, including protecting fruit from hail, sunburn, and wind. However, despite their widespread use, limited data are available on their impact on biotic factors, particularly entomopathogens. This study aimed to assess the impact of shade netting on CrleGV efficacy.

The virus was propagated in *T. leucotreta* third and fourth instars to produce two separate stocks, which were used in experiments conducted at different times. Occlusion bodies (OBs) from larval cadavers were purified using the glycerol gradient method for the first stock (CrleGV-1) and a crude extraction method for the second stock (CrleGV-2). The concentrations of the virus stocks were determined through OB quantification using darkfield microscopy and a counting chamber, yielding values of 8.13×10^{10} and 2.69×10^{11} OBs/ml for CrleGV-1 and CrleGV-2, respectively.

To assess the effectiveness of CrleGV, semi-field exposure trials were conducted under netted and non-netted conditions using virus-treated fruit. The virus was exposed to natural UV radiation for different durations, 1, 7, 14, and 21 days in the first experiment, and 1, 3, 5, and 7 days in the second. After exposure, the virus was washed from the fruit, enumerated, and subjected to surface-dose bioassays to evaluate its virulence and persistence and, as such, provide insights into the level of UV protection that netted orchards offer for CrleGV-SA.

Statistical analysis indicated that netting provided little protection to CrleGV against UV radiation in Experiment 1. However, the virus lost activity very quickly which resulted in low and inconsistent mortality of *T. leucotreta* neonates in surface dose bioassays. The highest average mortality recorded during this experiment was 58.33% and this was recorded under the netted treatment. To be able to make a better conclusion on the extent of protection shade nets provide to CrleGV, the second experiment was conducted. The results for this experiment confirmed those of Experiment 1, showing that CrleGV persisted longer under netting. The mortality recorded on bioassays conducted using the virus recovered from netted structures was consistently higher than that recorded from virus recovered from unnetted structures. Lethal concentrations, LC_{50} values, were also calculated. These were generally lower for the netted treatment than the unnetted.

The findings obtained in this study demonstrate the potential for improving the persistence of CrleGV in managing *T. leucotreta* in citrus. As the first study to examine the influence of shade netting on CrleGV against *T. leucotreta*, it lays the groundwork for future research to explore the effects of netting on entomopathogens for improved management of *T. leucotreta* and other pests.

Table of contents

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of tables

List of abbreviations

Abbreviations

AGE	Agarose gel electrophoresis
BLAST	Basic Local Alignment Search Tool
BV	Budded virus
C1	Control 1
C2	Control 2
CABI	Centre for Agriculture and Bioscience International
CBC	Centre for Biological Control
CE	Controlled environment
CGA	Citrus Growers' Association
CM	Codling moth
CRI	Citrus Research International
CTAB	Cetyltrimethylammonium bromide
D1	Dose 1
D2	Dose 2
D3	Dose 3
D4	Dose 4
D5	Dose 5
dH_2O	Distilled water
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
EPF	Entomopathogenic fungi
EPN	Entomopathogenic nematodes
EPPO	European and Mediterranean Plant Protection Organisation
EPV	Entomopathogenic viruses
et al	et alia (and others)
EU	European Union
FAO	Food and Agriculture Organization
FCM	False Codling Moth
GV	Granulovirus

HDPE	High-density polyethylene
i.e.	Id est (namely)
IPM	Integrated Pest Management
Lab	Laboratory
LC	Lethal concentration
LC_{50}	Lethal concentration (50%)
LC_{90}	Lethal concentration (90%)
LD	Lethal dose
LD_{50}	Lethal dose (50%)
LD_{90}	Lethal dose (90%)
Ltd	Limited
MCA	Microbial control agent
Na_2CO_3	Sodium carbonate
NPV	Nucleopolyhedrovirus
NTC	No template control
OB	Occlusion Body
ODV	Occlusion derived virion
ORF	Open reading frames
PCR	Polymerase chain reaction
Pty	Propriety
qPCR	Quantitative polymerase chain reaction
R	Rand
REN	Restriction endonuclease
RSA	Republic of South Africa
SA	South Africa
SA-DAFF	South African Department of Agriculture Forestry and Fisheries
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
SEM	Scanning electron microscope
SIT	Sterile Insect Technique
SNPs	Single nucleotide polymorphisms
TAE	Tris base, acetic acid and EDTA

TEM	Transmission electron microscope
TM	Trademark
UK	United Kingdom
US	United States
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
W1	Working suspension 1
W2	Working suspension 2
Xsit	X Sterile Insect Technique Pty. (Ltd)

Viruses

CpGV	Cydia pomonella granulovirus
CrleGV	Cryptophlebia leucotreta granulovirus
CrleGV-IC	Cryptophlebia leucotreta granulovirus, Ivory Coast isolate
CrleGV-CV3	Cryptophlebia leucotreta granulovirus, Cape Verde isolate
CrleGV-SA	Cryptophlebia leucotreta granulovirus, South African isolate
CrpeNPV	Cryptophlebia peltastica nucleopolyhedrovirus
TYLCV	Tomato yellow leaf curl virus

Units

Bp	Base Pair
Cm	Centimetre
℃	Degrees Celsius
G	Grams
H	Hour
Ha	Hectare
Hz	Hertz
Kb/kpb	Kilobase pairs
µl	Microliter
Ml	Millilitre
Mm	Millimetre
Min	Minutes
N	Number

Nm	Nanometre
OBs/ml	Occlusion bodies per millilitre
%	Percentage
pH	Potential of hydrogen
Rpm	Revolutions per minute
S	Seconds
×g	Times gravity
v/v	Volume per volume
w/v	Weight per volume

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Chapter 1

General Introduction

1.1 The South African citrus industry: an overview

Native to Southeast Asia, the genus *Citrus*, Family Rutaceae, is now one of the most cultivated and consumed crops worldwide (Jaouad et al., 2020). Citrus is defined as any group of evergreen (often thorny) trees or shrubs that bear berry-like juicy fruits with a thick rind (Tennant et al., 2009). It is particularly liked for its health improvement and nutritional benefits (Lee & Kader, 2000; Tennant et al., 2009; Lado et al., 2018). For example, citrus fruits are a great source of Vitamin C (Tennant et al., 2009; Nath & Deka, 2019), which helps to sustain healthy skin and blood vessels (Lee & Kader, 2000). Vitamin C is also an antioxidant that reduces the risk of various diseases, such as cancer (Huang et al., 2007). In South Africa, the cultivation of citrus fruit began in the mid-1600s, with the first export occurring in the early 1900s (Moore, 1962). Since then, the industry's fruit production and exports have grown steadily. Citrus in South Africa is grown in seven of the nine provinces, namely Limpopo, Eastern Cape, Western Cape, Mpumalanga, KwaZulu Natal, Northern Cape, and North West (Malan et al., 2018). By 2019, citrus trees were planted in a total of 88 569 hectares (ha) across South Africa and in Zimbabwe and Swaziland (Citrus Growers' Association (CGA), 2020). This increased by 8.7% (96 231 ha) in 2020 (CGA, 2021) and by a further 3.9% (99 969 ha) in 2021 (CGA, 2022b) (Figure 1.1). This growth is expected to increase in accordance with significant investments and new citrus plantings, especially of new cultivars.

Province	Area (ha)
Limpopo	40 383
Eastern Cape	25 226
Western Cape	18 952
Mpumalanga	7 877
KwaZulu-Natal	2 440
Northern Cape	1 838
Zimbabwe	1 987
Eswatini	432
North West	822
Free State	12
Grand Total	**99 969**

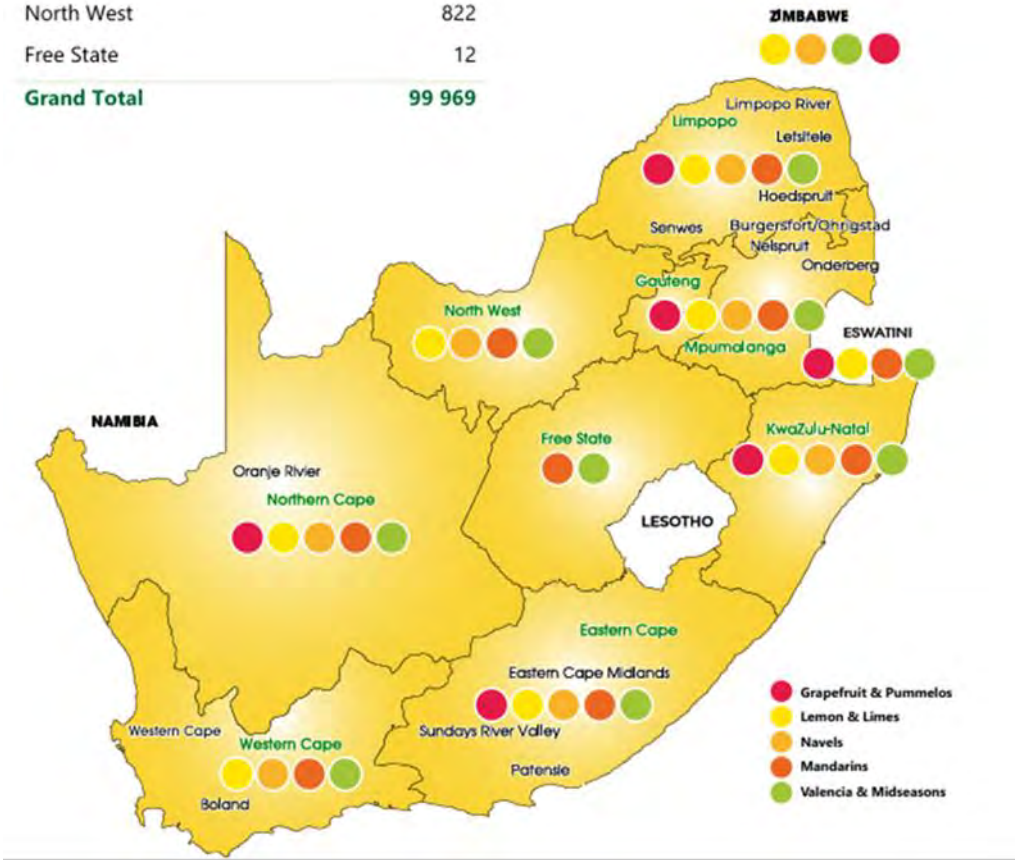


Figure 1.1: The citrus producing provinces in South Africa (including Zimbabwe and Eswatini) and the main types of citrus cultivars produced together with the area (ha) in which the fruit are produced (CGA, 2022b).

Citrus is in season for most of the year, as the citrus growing regions have variable climatic conditions, allowing for different varieties to be available at separate times of the year. The warmer provinces (Limpopo, KwaZulu-Natal and Mpumalanga) produce mostly Valencia oranges and grapefruit, while cooler provinces, including the Eastern Cape and Western Cape mainly grow Navel oranges, lemons, limes, and soft citrus (mandarins or tangerines). The bulk

of citrus in South Africa (~75%) is destined for export (CGA, 2016; Hattingh et al., 2020; Moore, 2021). Despite the difficulties that the citrus industry has faced in recent years, such as the consequences of the COVID-19 pandemic, electricity shortages (load shedding), labour strikes, shortage of shipping equipment, and an increase in shipping rates, 161.5 million cartons (15 kg per carton) of citrus were exported to foreign countries in the 2022 season (CGA, 2022a). Currently, South Africa is the 13th biggest citrus producer in the world, the leading citrus exporter in the Southern Hemisphere, and the second largest exporter worldwide, shipping fruit to 119 countries (Malan et al., 2018; Food and Agriculture Organization of the United Nations (FAO), 2021; Moore & Manrakhan, 2022; CGA, 2024). This industry contributes largely to the foreign exchange income of the country. It also provides employment to approximately 140,000 people (CGA, 2024) and contributes over R1.6 billion in wages and salaries, providing job and food security (CGA, 2017). Additionally, the South African citrus industry generates between R25 and R28 billion annually in export revenue, invests R180 million annually in citrus industry research and allocates R60 million annually to black grower development and human capital development (CGA, 2024). Despite its economic importance, the citrus industry faces many constraints that limit both the production and export of fruit. One of the constraints and a constant challenge that often needs to be managed effectively and sustainably is pests (Jaouad et al., 2020).

1.2 Pest insects that affect citrus

Citrus trees host many pest insects and are also susceptible to many plant diseases (Jaouad et al., 2020). Invasions of pest insects to new areas continue to increase as a result of international travel and trade (Urbaneja et al., 2020). Citrus pests are classified into three main groups, depending on the type of damage they cause to the crop, the part of the crop they damage, and how frequently they cause the damage (Smith & Peña, 2002). Firstly, there are key pests that cause the most damage and often occur in all the regions where the crop is grown. Therefore, control of these pests is crucial. Some pests are classified as occasional (sometimes referred to as minor pests), and usually occur in small numbers or cause minor damage. Therefore, they only need to be controlled occasionally. Lastly, there are secondary or sporadic pests, such as several kinds of scale insects. These are normally controlled by their natural enemies, except if their natural enemies are disrupted by the excessive use of chemical insecticides (Smith & Peña, 2002; Urbaneja et al., 2020).

It is important to note that other insects only gain pest status in certain seasons of the year, because they are affected by climatic conditions such as rainfall, temperature, and humidity (Bedford, 1998). Generally, pest richness and distribution increase with an increase in temperature and rainfall, as it provides a warm and humid environment that is suitable for their development. Some pests may also be considered cosmetic, since despite their widespread occurrence, they often have minimal effect on the quality and quantity of the crop's internal components. Instead, they only compromise the appearance of fruit reducing its monetary worth (Annecke & Moran, 1982). An example of such pests is citrus thrips and scale insects (Horton, 1918; Annecke & Moran 1982; Malik et al., 2021). However, it is important to note that severe infestation of scale insects can significantly harm the tree. These pests suck important fluids from the tree, resulting in yellowed leaves that eventually fall off (Moustafa, 2012). Furthermore, as scale insects feed on the tree, they inject toxic saliva into the tree, with many (excluding red scale) excreting honeydew that attracts ants, which encourages the development of sooty mould, resulting in poor tree health, low yield, and potential death of the tree (Moustafa, 2012; Almeida et al., 2018; Moore SD, personal communication). Certain pests cause indirect damage to the crop by creating pathways for different pathogens or acting as vectors of disease themselves (Annecke & Moran, 1982). Due to the significant damage some pests could cause if they spread from their native country to a new one, stringent biosecurity regulations are enforced by both importing and exporting parties to prevent their introduction, whether accidental or intentional (Moore & Manrakhan, 2022). Such pests are identified as phytosanitary or quarantine pests. Quarantine pests are defined as “pests of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled” (FAO, 2023).

In South Africa, there are approximately 100 known citrus pest species, of which only about six are considered key pests and 12 occasional pests (Bedford, 1998; Smith & Peña, 2002). *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), also known as the false codling moth (FCM) (the topic of this thesis), has been identified as one of the key citrus pests (Smith & Peña, 2002), and its management is very important. Left unchecked, it can cause major damage to the South African citrus industry, given its quarantine status for several export markets (Moore et al., 2004).

1.3 *Thaumatotibia leucotreta*

1.3.1 Taxonomy and distribution

Thaumatotibia leucotreta, commonly known as the false codling moth (FCM), is a member of the family Tortricidae in the order Lepidoptera. *Thaumatotibia leucotreta* is native to sub-Saharan Africa and the Afrotropical Region. It has also been reported in various islands outside the African continent, such as the Reunion Island, Cape Verde Island, the Mauritius Islands, and Israel (Wysoki, 1986; Hofmeyr et al., 2015). In South Africa, *T. leucotreta* was first discovered in the Kwa-Zulu Natal Province by Fuller (1901), who named it the Natal codling moth, *Carpocarpsa* genus (Newton, 1998), due to its close resemblance to *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae), also known as the true codling moth. A few years later, it was mistakenly identified as the orange codling moth, *Enarmonia batrachopa,* when found in what was known as the Transvaal area by Howard (1909). It was described as *Argyroploce leucotreta* (Eucosmidae, Olethreutidae) by Meyrick in 1912 and then moved to the genus *Cryptophlebia* by Clark in 1958 (Newton, 1998). It was only in 1999 that Komai removed it and put it in its current taxonomic rank, *Thaumatotibia leucotreta* (Table 1.1) (van den Berg, 2001; Venette et al., 2003).

Table 1.1: Taxonomic rank of *T. leucotreta*.

Taxa	Scientific classification
Domain	Eukaryota
Kingdom	Metazoa
Phylum	Arthropoda
Subphylum	Uniramia
Class	Insecta
Order	Lepidoptera
Family	Tortricidae
Genus	*Thaumatotibia*
Species	*Thaumatotibia leucotreta*

1.3.2 Host range

Thaumatotibia leucotreta has a broad range of host plants. Initially, this included only 35 species, consisting of both wild and cultivated plants, with cultivated plants accounting for the majority (21 species) (Schwartz, 1981). By 2003, Venette et al. (2003) expanded this list to 70 host plants. This was further expanded to 107 plants by the European and Mediterranean Plant Protection (EPPO) (2013). However, some of the reported host species are either inaccurate or were observed in no-choice laboratory trials i.e. forced associations. This led to more than 30 of the listed host plant species being disproved (Moore et al., 2015b). Additional host species from East Africa, specifically Kenya, have been recorded by Brown et al. (2014). This list encompasses mainly wild native, naturalised and several cultivated plants. The most recent and comprehensive *T. leucotreta* list can be found in Carstens & Moore (2022). Important crops that are affected by *T. leucotreta* include avocadoes (Erichsen & Schoeman, 1992; Newton, 1998; Grové et al., 1999), stone fruit (Daiber, 1978), nuts (La Croix & Thindwa, 1986), and also maize and flowering plants like roses and hibiscus (Carstens & Moore, 2022). In South Africa, citrus is considered a favoured host for *T. leucotreta*, with some cultivars being more susceptible than others (Kirkman & Moore, 2007; Malan et al., 2011; Carstens & Moore, 2022). *Thaumatotibia leucotreta* has gained a preference for Navel oranges and soft citrus (mandarins), while grapefruit and Valencia oranges are less susceptible (Love et al., 2014). Lemons and limes, on the other hand, are resistant to *T. leucotreta* damage, possibly owing to the fact that they are highly acidic (Newton, 1998; Moore & Hattingh, 2012; Grout & Moore, 2015; Moore et al., 2015a) and therefore provide an environment that is not favourable for larval development.

1.3.3 Life cycle and economic importance

The *T. leucotreta* life cycle consists of four life stages, the egg, larva, pupa, and adult (Stofberg, 1954). The whole life cycle ranges between 30 to 120 days, depending on environmental factors such as temperature and food quality (Newton, 1998; Bloem et al., 2003). Generally, the cycle is shorter in summer and longer in winter. The eggs are laid singly, usually in large numbers on the surface or rind of fruit by an adult female. The female oviposits around 450 eggs during her lifetime and the maximum number of eggs laid was recorded to be 799 (Daiber, 1980). The eggs are very small and oval in shape. Morphological changes are observed in the egg before hatching. They are cream-coloured when first laid and then become transparent (Figure 1.2a). They then change to a slightly red colour and subsequently, a black spot (larva's

head capsule) is visible (Newton, 1998) (Figure 1.2b). After hatching, the neonate larva will burrow into the fruit to feed. During its development, the larvae will then excrete, moult several times, and leave head capsules in the fruit until larval development is complete (Daiber, 1979b). Cannibalism has been observed in neonates, however, this is uncommon (Newton, 1998). A few larvae (usually one, but occasionally two or three) can survive per fruit.

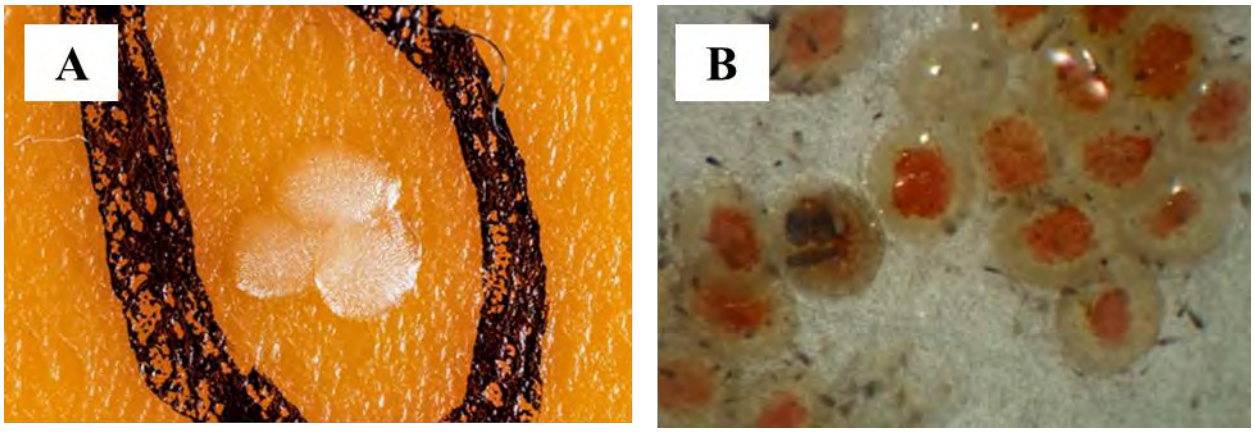


Figure 1.2: *Thaumatotibia leucotreta* eggs. Immediately after being laid, they are whitish or coloured (A), compared to a few days after being laid, where the colour changes to reddish (B) (Photo credit: David Taylor).

The larva develops through five instars, and while the other larval stages are creamy/white, the fifth instar is pink/red in colour (Figure 1.3a) (Daiber, 1979a). Even though the pinkish-red colour is more prominent in the fifth instar, the colour starts appearing in the third or fourth instar (Moore SD, personal communication). Once the fifth instar is ready to pupate, it will exit the fruit, and spin a cocoon in the soil, using silk, organic debris, and soil particles (Stofberg, 1954; Daiber, 1979b; Gilligan et al., 2011). The larva will thereafter stop feeding, at which point it is referred to as a pre-pupa. The pre-pupa will moult into a soft cream/light brown coloured young pupa (Daiber, 1979c), and then a dark brown coloured pupa with a harder chitin covering (Figure 1.3b) (Newton, 1998). Female *T. leucotreta* pupae tend to be larger and mature faster than their male counterparts. *Thaumatotibia leucotreta* adults also show sexual dimorphism, with males being smaller than the female. In addition, males can be differentiated from females by long scales on their hind tibiae, black anal tufts, and a scent organ on each hind wing's anal angle (van den Berg, 2001). *Thaumatotibia leucotreta* wings are black, brown to grey in colour with a wingspan of 1.5-2 cm (Figure 1.3c) (Newton, 1998). *Thaumatotibia*

leucotreta adults are nocturnal, and they find their mates by using pheromones. Females, on average, live longer than males. *Thaumatotibia leucotreta* does not undergo winter diapause and in South Africa, it can have up to six or more overlapping generations in one year (Daiber, 1980; Newton, 1998), owing to the fact that it is multivoltine (Boardman et al., 2012) and a polyphagous pest (Stofberg, 1954).

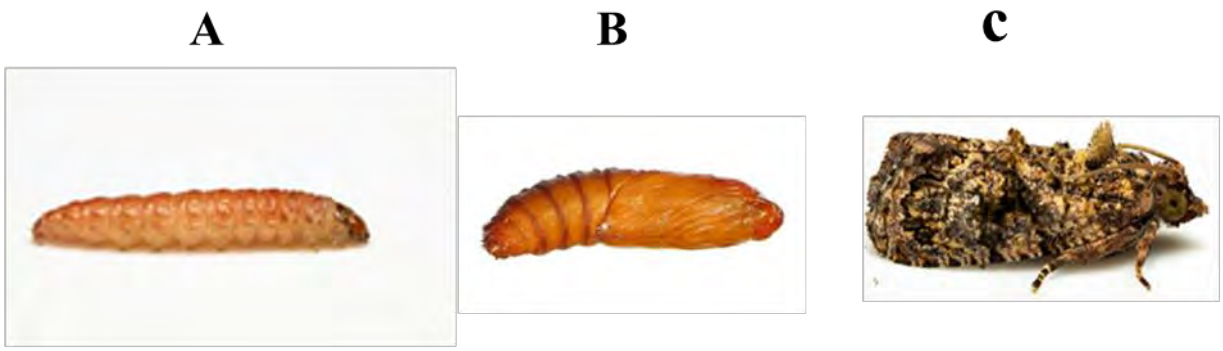


Figure 1.3: *Thaumatotibia leucotreta* larva (fifth instar) (A), pupa (B) and adult (C) (Photo credit: David Taylor).

The *T. leucotreta* larval stage is the one that causes the damage. When the neonate larva enters the fruit to feed, it results in early fruit drop and fruit decay (Hofmeyr et al., 2016). Even when the neonate fails to burrow into the fruit (which is very common), it will still cause lesions on the fruit rind, which might cause the fruit to decay (Hofmeyr et al., 2016). Moreover, the neonate larva creates an entry hole that not only allows other insects or disease-causing pathogens to infect the fruit, resulting in yield loss, but the biggest concern remains its phytosanitary status. The insect does not occur in the continents that South Africa exports to (Europe, Asia, North America, and the United Kingdom) (Moore, 2002), with the exception of Israel, where it was inadvertently introduced in the 1980s (Wysoki, 1986). This has led to a zero-tolerance policy for the presence of *T. leucotreta* in consignments destined for exports to some of these markets (Moore, 2021). This means that if a single live larva is detected in the consignment, the whole consignment can be rejected. This has implications for the generation of foreign income resulting in strict control of *T. leucotreta*.

1.4 *Thaumatotibia leucotreta* management

Thaumatotibia leucotreta is a key economic pest. Hence, several control measures have been developed over time to suppress its population below a set threshold for economic damage [thresholds are specified in Moore et al. (2016)]. The management of *T. leucotreta* follows a systems approach. A systems approach refers to "the integration of different risk management measures, at least two of which act independently, and which cumulatively achieve the appropriate level of protection against regulated pests" (FAO, 2007; Moore et al., 2016; Hattingh et al., 2020; Moore, 2021). Aluja & Mangan (2008) define a systems approach as "the integration of pre- and postharvest practices, from the production of a commodity to its distribution and end use, that cumulatively meet predetermined requirements for quarantine security". This programme was established as an alternative to a stand-alone cold treatment. Developed in the mid-1900s by Cottier (1950, 1952) and later by Myburgh (1963, 1965), a stand-alone treatment was included in certain export regulations (United States Department of Agriculture (USDA), 2019) because it is required by some nations (such as the United States and South Korea) that South Africa exports citrus fruits to as a phytosanitary measure to restrict risk of live *T. leucotreta* entering the importing country (South African Department of Agriculture Forestry and Fisheries [SA-DAFF], 2015). A standard cold treatment for *T. leucotreta* involves exposure of fruit to very cold temperatures (below 0°C) over 22 days (Hattingh et al., 2020). Some citrus varieties, such as white grapefruit and Satsuma mandarins, are, however, susceptible to damage caused by sub-zero temperatures, which cause chilling injury to the fruit and are therefore not acceptable for export (Lafuente et al., 2003; Lafuente & Zacarias 2006; Cronjé, 2007; Moore & Hattingh, 2016). Moore et al. (2017) improved on the original treatment by shortening the duration of fruit exposure to lower temperatures and increasing temperatures in other circumstances while maintaining the Probit 9 efficacy standard, (i.e., 99.9968% efficacy at the 95% confidence level) or zero survivors when treating a population of 93,613 insects. The improved treatments included: 16 days at 0.2°C, 18 days at 0.4°C, 20 days at 0.4°C and 19 day at 1.0°C (Moore et al., 2017; Moore, 2021). This would, in turn, be less expensive, safer, and simpler to implement, while reducing chilling injury and thus avoiding export sanctions. Regardless of the improvement, a stand-alone cold treatment is a significant challenge when exporting large volumes of fruit. For example, South Africa exports more than 40% of its citrus to Europe and the United Kingdom (UK), and in addition to chilling injury, infrastructural and logistical constraints make this initiative unfeasible (Hattingh et al., 2020; Moore, 2021). Nonetheless, a cold treatment of 1°C for 19 days is an

accepted International Plant Protection Convention (IPPC) schedule and was recently approved for citrus exports from South Africa to Vietnam (CGA, 2024; Moore SD, personal communication). Moore et al. (2016) and Hattingh et al. (2020) demonstrated that a systems approach is a suitable alternative to the cold treatment, with the efficacy being equivalent to the recommended Probit 9.

The systems approach consists of three measures: 1) preharvest controls and measurements and post-picking sampling, inspection, and packinghouse procedures, 2) post-packing sampling and inspection, and 3) shipping conditions (Moore, 2021). Within these measures, there are 13 components that are all important in eliminating risks associated with *T. leucotreta* (Moore et al., 2016; Hattingh et al., 2020). Thus, it is critical that each step is adequately followed. The pre-harvest control options that are currently registered and commercially available for *T. leucotreta* can be categorised into cultural control, semiochemical technology, genetic control, chemical control, and biological control (Figure 1.4) (Moore & Hattingh, 2012; Moore & Hattingh, 2016; Malan et al., 2018). Cultural control entails making the crop environment unsuitable for the pest insect. Against citrus pests, the best way to achieve this is with orchard sanitation, which is considered the cornerstone or foundation of all the control options (Moore & Hattingh, 2012; Moore, 2021). Orchard sanitation involves removing and disposing of (destroying) infested and damaged fruit (outside of the orchard), both from trees and fallen fruit (Moore, 2021). Stofberg (1954) indicated that a consistent sanitation programme could prevent between 24 and 60 fruit per tree from being infested by *T. leucotreta*. Additionally, Moore & Kirkman (2008) found that implementing weekly sanitation from December to June could lower fruit infestation by an average of 75%. Therefore, maintaining orchard sanitation is crucial, particularly the removal of out-of-season fruit, as it may contribute to the pest population in the following season (Malan et al., 2018).

There are currently two behavioural options available for the mitigation of *T. leucotreta* and they are mating disruption and the attract-and-kill method (Malan et al., 2018). These two methods are similar in the way that they both use female sex pheromones to attract or confuse the male moth. The mating disruption distracts or confuses the male moth from finding a female to mate with, which eventually causes their population to decline (Miller & Gut, 2015), while in the attract-and-kill method, the pheromone is combined with a chemical insecticide, sterilant, or insect pathogen, which leads to the death of the male moth (El-Sayed et al., 2009; Moore, 2021). There are four registered mating disruption products, namely Isomate (Shin-Etsu, Japan), Checkmate FCM-F (Suttera, Bend, OR, USA), Splat-FCM (ISCA Technologies,

Riverside, CA, USA), and X-Mate (Insect Science, Tzaneen, South Africa) and one attract-and-kill product named Last Call FCM (Insect Science, Tzaneen, South Africa (Moore & Hattingh, 2012; Moore, 2021). Both mating disruption and attract-and-kill methods are effective. However, they are density-dependent (Malan et al., 2018). Therefore, they should not be used as a standalone method unless *T. leucotreta* populations are low (Kirkman & Moore, 2007; Moore & Hattingh, 2012).

Chemical control is a common management tool, because it is readily available, is familiar to farmers, is simple to administer, and effective with instant results. However, it also has the most detrimental side effects. These include the development of resistance by the pest insect (Hofmeyr & Hofmeyr, 2005; Moore et al., 2004), environmental concerns, human health concerns, and non-target effects (Moore, 2002). As a result, there are strict regulations set by importing parties for chemical residues allowed in fruit destined for export, meaning that there is a need for alternative control measures that will be as effective in the control of *T. leucotreta* (Moore & Hattingh, 2012).

One of the most successful strategies for combating *T. leucotreta* is the sterile insect technique (SIT) (Malan et al., 2018). SIT is a form of genetic control that entails mass rearing, sterilisation, and release of male *T. leucotreta* moths to mate with wild females, causing them to lay sterile eggs and, as a result, reducing the *T. leucotreta* population. For SIT to be successful, there should be more sterile male moths than wild mates. This is also referred to as an overflooding ratio, which in *T. leucotreta* has been determined to be at least 10 sterile males to 1 wild male (Knipling, 1955; Hofmeyr et al., 2005, 2015). However, results from some field trials involving *T. leucotreta* SIT programmes suggest that even higher ratios may yield better outcomes (Hofmeyr & Hofmeyr, 2009, 2010; Moore, 2011). This is supported by a recent study by Githae et al. (2024), which demonstrated that overflooding ratios of 40:1 and 60:1 were significantly more effective. SIT was commercialized in 2007 following a successful semi-commercial trial conducted in 2005, where it was used as a standalone treatment (Hofmeyr et al., 2016). During this trial, *T. leucotreta* populations were effectively suppressed, resulting in a 95.2% decrease in fruit damage in treated orchards compared to an untreated control (Hofmeyer et al., 2016). Since its commercial launch, the implementation of SIT as part of an integrated management programme has achieved significant results, including a 99% reduction in moth catches, a 96% decrease in fruit infestation, and reduction in export rejections by 89% (Barnes et al., 2015; Hofmeyr et al., 2015; Moore, 2021). Sterile insects are classified as beneficial organisms, which is why SIT is now categorised as a form of biological control

(Moore, 2021). The SIT technique has been successful as it is easy for farmers to implement because most of the work is handled by service providers (Moore, 2021). It is important to highlight that SIT is not a standalone technology and should be integrated with other control methods (Barnes et al., 2015).

Although not faster than its chemical alternatives, biological control is an effective and more sustainable option for controlling *T. leucotreta* (Knox et al., 2015; Moore, 2021). Biological control is the use of natural enemies of a pest, that is, predators, parasitoids, and pathogens (viruses, bacteria, nematodes, microsporidia, and fungi), to control the pest population (Doutt, 1967; Knox et al., 2015). There are only a few predators known to prey on *T. leucotreta*, and these include *Orius* bugs (Hemiptera: Anthocoridae), which have been recorded preying on *T. leucotreta* eggs, and assassin bugs (Hemiptera: Reduviidae), which can feed on larvae (Bedford, 1998). Predatory ants of the species *Anoplolepis custodiens* (Smit) and *Pheidole megacephala* (Fabricius) (Hymenoptera: Formicidae) are also effective natural predators of *T. leucotreta*. They mainly attack the late fifth instar, pre-pupa, pupal stage, and newly emerging adults (i.e., soil-dwelling life stages) (Samways et al., 1982). In a study by Bownes et al. (2014), pupae of *T. leucotreta*, *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae), and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) were placed in a citrus orchard across various plot treatments. These included plots where *A. custodiens* and *P. megacephala* ants were poisoned, plots where ant foraging activity was restricted using sticky barriers that separated arboreal and epigeic trophic interactions by preventing ground-dwelling ants from climbing trees, and control plots where ants remained active. The results showed reduced pupal survival across all three species in both the ant-banded and control plots, suggesting notable predation pressure from ants. Notably, *T. leucotreta* pupae experienced the highest predation rates, with survival dropping to nearly zero, significantly lower than that of *H. armigera* and *C. capitata*. The downside of ants is their mutualistic relationship with honeydew-producing insects (such as aphids, scale insects, and planthoppers), which cause crop damage (Samways et al., 1982). Previously, the base of tree trunks in citrus orchards was sprayed with poison to prevent ants from climbing the tree. However, Bownes et al. (2014) recommended using ant bands as an alternative. Their research demonstrated that sticky ant bands successfully served as a barrier, limiting the movement of ants up and down the tree trunk. This is advantageous as the bands allow ants to continue preying on *T. leucotreta* pupae in the soil while simultaneously being blocked from nurturing other pest insects (Bownes et al., 2014; Malan et al., 2018). Despite being the best method, very few farmers actually utilise ant bands (Moore SD, personal

communication). The egg parasitoid *Trichogrammatoidea cryptophlebiae* Nagaraja (Hymenoptera: Trichogrammatidae) is the most effective parasitoid of *T. leucotreta* (Catling & Aschernborn, 1974; Kirkman & Moore, 2007). It can parasitise up to 80% of *T. leucotreta* eggs and decrease the loss of citrus to *T. leucotreta* by 60% (Kirkman & Moore, 2007). Other important parasitoids are *Agathis bishopi* (Nixon) (Hymenoptera: Braconidae) and *Apophua leucotreta* (Wilkinson) (Hymenoptera: Ichneumonidae) (Moore, 2002; Kirkman & Moore, 2007).

Entomopathogens are also an essential part of biological control. There are many naturally occurring entomopathogens, but only a select few have been developed for use as microbial control agents (MCAs), which control various kinds of arthropods, including forest and agricultural insect pests. Many of these MCAs are registered and commercially available for pest management (Haase et al., 2015). The sub-species *Bacillus thuringiensis* subsp. *kurstaki* is one of the few bacterial pathogens that have been developed and have been successful in the management of pests for many years (Baum et al., 1999; Lacey & Shapiro-Ilan, 2008; Lacey et al., 2015). However, this is not very effective against *T. leucotreta* in the field (Moore SD, personal communication). Nematodes have only recently been employed to manage pests in agriculture. Nematodes have been highly effective in protected horticultural systems, but their use in large-scale or broadacre agriculture remains less developed (Lacey & Georgis, 2012). Malan et al. (2011) identified six nematode isolates that are virulent to *T. leucotreta* in South African citrus orchards. Through laboratory biological assays (bioassays), they found that the genera *Steinernema* and *Heterorhabditis* resulted in the highest deaths of *T. leucotreta* larvae. A nematode-based biopesticide, Cryptonem™ (River Bioscience, South Africa), has been formulated and is registered for the control of *T. leucotreta* stages found in the soil. In addition, it was also discovered that emerging adult moths can also be infected, which is helpful in that the nematodes can be dispersed over large distances by the adults (Malan et al., 2011, 2018). Fungi are favourable agents, since they differ from other microbial control agents in that infection does not require ingestion, but it is through penetration of the pest cuticle (Shah & Pell, 2003). Their use is practical, especially for controlling non-feeding life stages such as pupal stages of *T. leucotreta* and sap-sucking insects like mealybug and scale (Lacey & Shapiro-Ilan, 2008; Coombes et al., 2018). Fungal species like *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Cordycipitaceae) and *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) are persistent and effective in reducing the levels of *T. leucotreta* in the field (Coombes et al., 2013, 2015, 2016).

Baculoviruses, the focus of this study, are among the most important MCAs used to combat insect pests, particularly those in the order Lepidoptera (Moscardi, 1999). Baculoviruses against *T. leucotreta* have been the subject of extensive research, leading to significant advancements such as the development of biopesticides. These include Cryptogran®, Cryptomax (both River Bioscience (Pty) Ltd, South Africa), Cryptex®, and Gratham (both Andermatt Biocontrol, Switzerland), all of which contain the baculovirus *Betabaculovirus cryleucotretae*, commonly known as Cryptophlebia leucotreta granulovirus (CrleGV), and hereafter referred to as CrleGV, as their active ingredient (Moscardi et al., 2011; Rodriguez et al., 2012; Knox et al., 2015; Haase et al., 2015; Moore & Jukes, 2023; van Oers et al., 2023). Baculovirus biopesticides have been demonstrated to be as effective and sometimes outperforming traditional conventional chemical insecticides (Moore et al., 2015b; Moore, 2021). They are eco-friendly, have a very narrow host range and leave no residues on the fruit, making them ideal biological control agents. This is particularly important as it can lead to reduced reliance on chemical insecticides, which are more likely to cause harm to the environment, non-target species and humans. What is of greater significance is the ongoing pursuit of novel methods or the refinement of existing control measures for effectively addressing evolving challenges, safeguarding the environment, and protecting public health, despite the existence of numerous strategies to manage *T. leucotreta*. The upcoming sections will provide a more detailed discussion on baculoviruses.

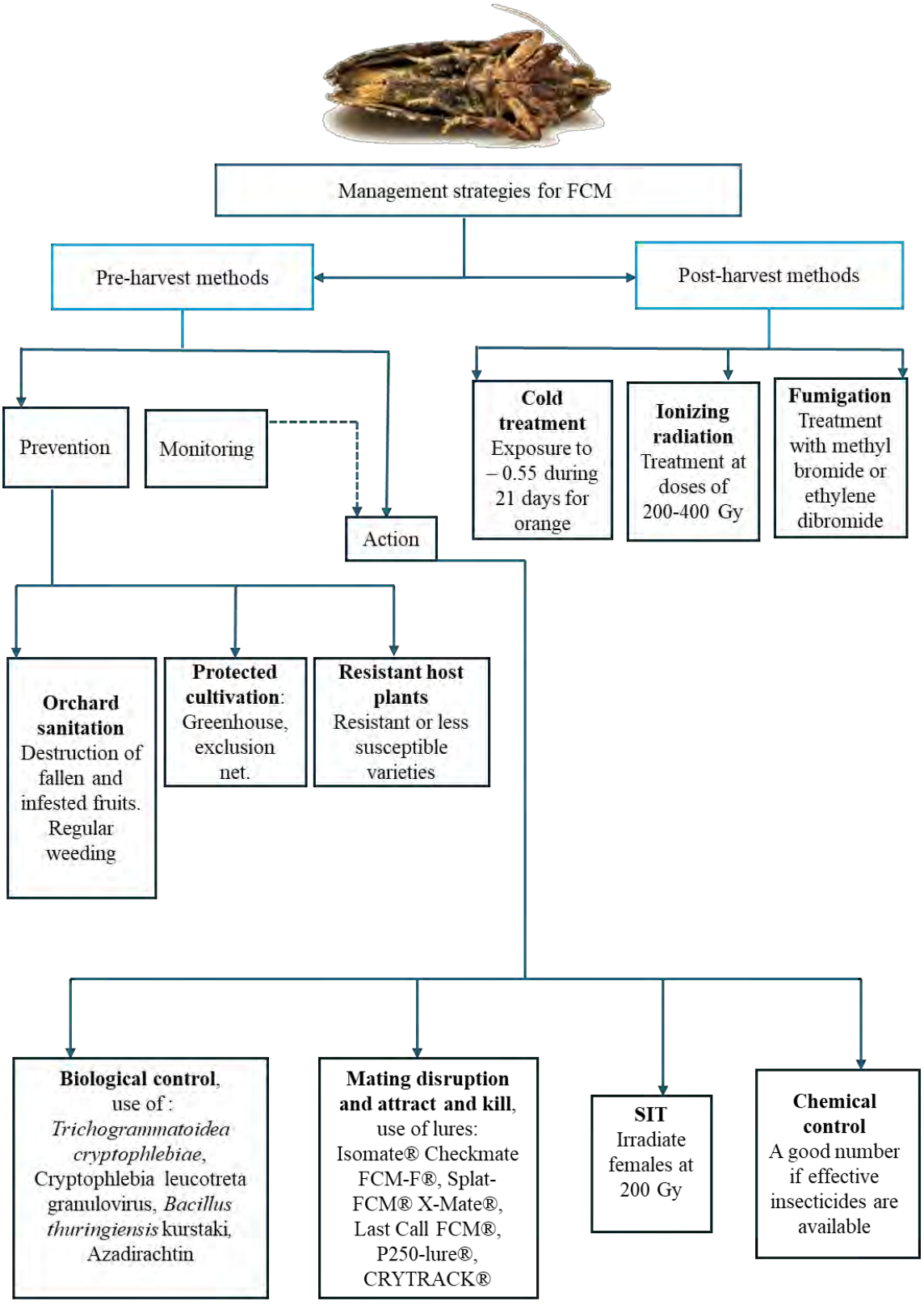


Figure 1.4: An overview of the control measures and potential control measures for *T. leucotreta*. Adapted from Adom et al., 2021; Myburgh (1963, 1965); Moore & Kirkman (2008); EPPO (2013); Moore & Hattingh (2016); Fening & Billah (2019).

1.5 Baculoviruses: an overview

Baculoviruses are an interesting group of insect viruses that have gained considerable attention for their distinctive biology and their application in different fields. Apart from their use as biological insecticides, baculoviruses have been applied to biotechnological and therapeutic domains, including being used as gene expression vectors, serving as delivery systems in gene therapy, and the development of new vaccines (Szewczyk et al., 2006; Moscardi et al., 2011; Knox et al., 2015). This significantly contributed to our understanding of their biology, lifecycle, and genetics. There are almost 1000 baculovirus species described worldwide (Szewczyk et al., 2011), and these are known to have over 600 host species, most of which fall under the orders Lepidoptera, Diptera, and Hymenoptera (Szewczyk et al., 2011; Rohrmann, 2019). Baculoviruses have a very narrow host range, typically infecting a single species or closely related species (Lacey et al., 2001; Szewczyk et al., 2006). They do not replicate in humans or other vertebrate species, plants, microorganisms and even in predators and parasitoids of their hosts (Szewczyk et al., 2006, 2011). These traits make them a suitable biological control agent and useful within the *T. leucotreta* systems approach (Gelaye & Negash, 2023). Fifteen virus families are recognised to infect insects. However, only viruses of the family *Baculoviridae* have been exploited as bioinsecticides, as they pose no threat to other organisms (Szewczyk et al., 2006; Knox et al., 2015).

1.5.1 Taxonomy and characteristics

Baculoviruses were first discovered in China over 5000 years ago, due to their association with a disease affecting silkworms (silkworm's wilting disease) in the silk production industry (Rohrmann, 2019). In an attempt to identify the causative agent of the disease, distinctive highly refractile occlusion bodies (OBs) were observed in affected larvae when examined under a light microscope (Rohrmann, 2019). OBs are protein-rich structures that surround viruses (Jehle et al., 2006). They are highly stable and typically function to protect the virions when they are outside of their hosts, allowing them to survive and remain resilient in harsh environments and infectious when ingested by the next host (Kost et al., 2005; Szewczyk et al., 2006). The OBs observed were polyhedron-shaped, and therefore, the disease associated with the OBs was named polyhedrosis (Rohrmann, 2019). Subsequently, with the development of electron microscopy, infectious particles called virions were uncovered within the OBs (Rohrmann, 2008). Two distinct forms of polyhedrosis diseases were later identified: nuclear polyhedroses (NPVs), where the polyhedra form within the cell nucleus, and cytoplasmic

polyhedroses (CPVs), where occlusion bodies are located in the cytoplasm (Smith et al., 1983). Unlike NPVs, which are rod-shaped and contain DNA, CPVs possess icosahedral capsids and were placed in the *Reoviridae* family (genus *Cypovirus*), characterized by segmented, double-stranded RNA genomes (Rohrmann, 2019). Additionally, a second group of baculoviruses, recognized by their small, granular, ellipsoidal occlusion bodies, was first documented in the 1920s and later classified as granulosis viruses (GVs). The two main morphological groupings of the family *Baculoviridae* were then called Nucleopolyhedrovirus (NPV) and Granulovirus (GV) (Rohrmann, 2008). OBs in NPV are called polyhedra and granules or capsules in GVs (Jehle et al., 2006). The polyhedra are large, about 0.6–2 μM in diameter and they contain many rod-shaped virions that develop in the nuclei (Ackermann & Smirnoff, 1983; Rohrmann, 2008, 2013). Moreover, the polyhedron is covered by a crystalline protein matrix called polyhedrin. The granules, in contrast, are smaller and ovoid, about 0.2–0.4 μM in diameter (Ackermann & Smirnoff, 1983; Rohrmann, 2008, 2013). These normally have only one virion and the protein matrix that encloses the granules is called granulin. The proteins of polyhedrin and granulin are related, despite their distinct names (Rohrmann, 2019). Baculoviruses have a genome size ranging from 80 to 180 Kbp and encode 90 to 180 open reading frames (ORF) or proteins. (Lange & Jehle, 2003; Herniou et al., 2003). As a result of nucleotide sequencing and phylogenetic analysis, baculoviruses have been recently reclassified into four genera: *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus* (Jehle et al., 2006). This classification was proposed by Jehle et al. (2006) after comparing 29 fully sequenced baculoviral genomes and finding that the phylogenetic analysis of baculoviruses resembled the classification of their respective hosts more than the morphological traits traditionally employed for categorising this virus family. Alphabaculoviruses and Betabaculoviruses are lepidopteran-specific NPVs and GVs, respectively, while Gammabaculoviruses and Deltabaculoviruses are NPVs that affect hymenopterans and dipterans, respectively (Jehle et al., 2006). The conventional division of NPVs and GVs is still commonly employed (Jehle et al., 2006). Baculoviruses have two viral forms, called budded virions (BV) and occlusion derived virions (ODVs) (Szewczyk et al., 2006). The genetic make-up of these two is the same, however, they have different phenotypes and are produced at distinct times post-infection.

1.5.2 Replication and infection cycle of baculoviruses

The life cycle of baculoviruses involves both the two different viral forms: ODVs and BVs (Szewczyk et al., 2011) (Figure 1.5). It starts when a vulnerable insect feeds on foliage that contains OBs left by a previously infected insect. The host insect's gut is alkaline, and it causes the crystalline polyhedron matrix that occludes the ODVs to dissolve in the midgut of the insect, thereby releasing ODVs in the gut. Subsequently, the released ODVs pass through the peritrophic membrane, attach themselves to the microvilli and infect the columnar epithelial cells. This is called the primary infection. The secondary infection begins when BVs are produced in the epithelial cells, and these spread throughout other tissues of the insect's body. The endosomes release the nucleocapsid, which is transferred to the nucleus where BVs and ODVs are produced as a result of the process of transcription, replication of the DNA, and assembly of progeny nucleocapsids. In brief, in the context of baculovirus secondary infections, new viral OBs are generated and then released upon the death of the infected insect. These baculoviruses induce a state in the host insect where it becomes liquefied, making it easier for the OBs to burst forth and initiate infection in a new host. During this secondary infection phase, the affected insect exhibits relentless feeding behaviour, subsequently driving it to ascend to higher parts of plants or tree branches, even venturing into exposed areas it would typically avoid due to predators. This behaviour is of great importance as it aids in the widespread dispersion of OBs. Additionally, the insect may fall prey to predators like birds, serving as carriers that can transport the virus over considerable distances. An intensive review of the baculovirus life cycle is given by Rohmann (2013, 2019).

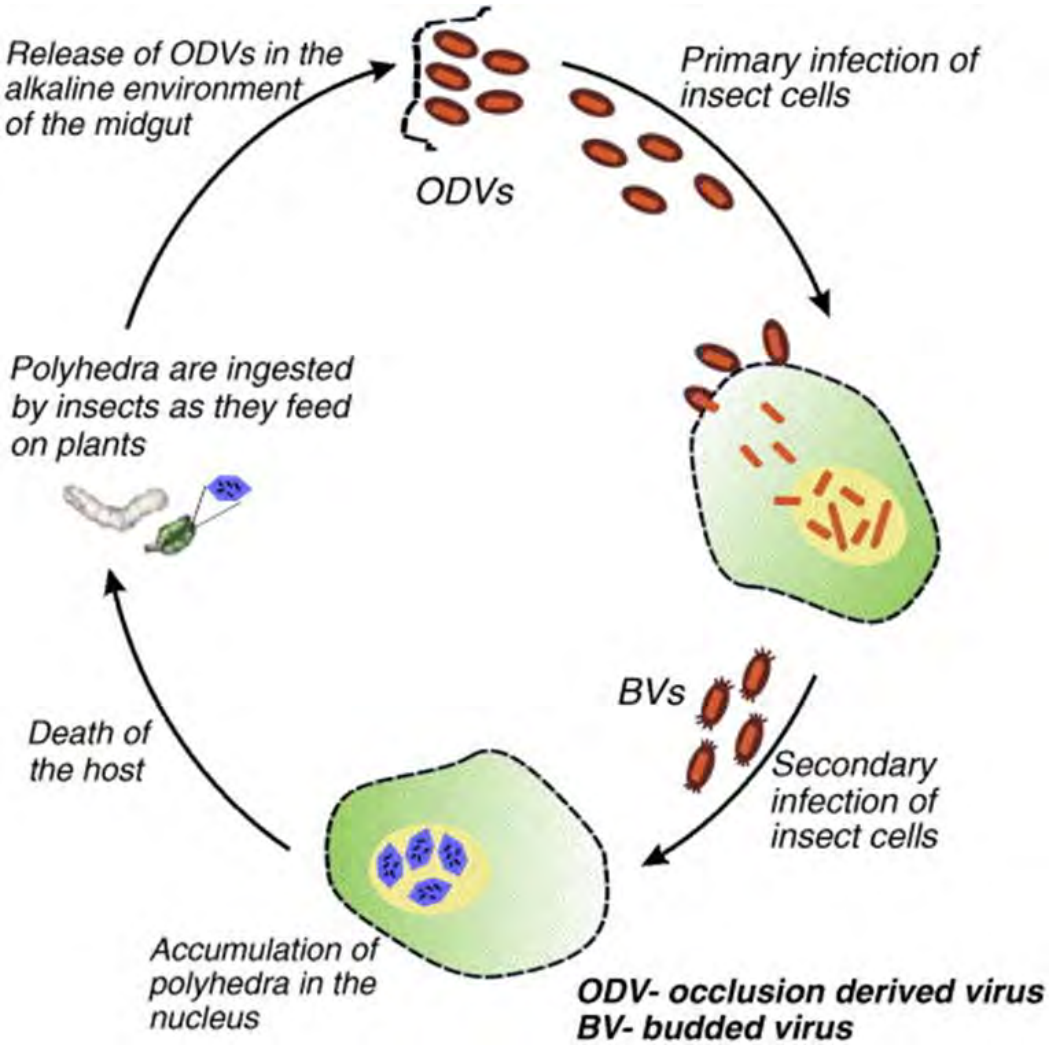


Figure 1.5: The life cycle of baculoviruses (Szewczyk et al., 2006).

1.5.3 Limitations of baculoviruses

Despite their widespread usage in agricultural pest control, baculoviruses have several limitations, including a longer speed of kill than synthetic insecticides (Szewczyk et al., 2006). This slow speed of kill is disadvantageous since the insect will continue to feed on the fruit, even after ingesting the virus (Rai & Ingle, 2012). This causes fruit deterioration and, eventually, early fruit drop. Another drawback is their narrow host range. While this trait makes them desirable and ideal for use in IPM programmes owing to no non-target effects, many farmers often prefer an "all-in-one" pest control strategy that targets multiple pest insect species simultaneously. Having multiple pesticides for different insects is not only costly but also time-consuming and labour-intensive. The narrow host range of baculoviruses also means a smaller market which can reduce the attractiveness for commercial investment (Knox et al., 2015;

Gelaye & Negash, 2023). The intensive use of baculoviruses can lead to resistance development by the target host. This has been recorded for *T. leucotreta* 's closely related species, the codling moth (CM), to *Betabaculovirus cypomonellae*, commonly known as Cydia pomonella granulovirus (CpGV), which is an important biocontrol agent of *C. pomonella* (Huber, 1998; Fritsch et al., 2005; van Oers et al., 2023). Host resistance reduces the biopesticide's effectiveness, resulting in a decrease in crop yield (Schulze-Bopp & Jehle, 2013). Currently, baculoviruses are commercially produced *in vivo*. They are produced by either rearing the target pest insect on artificial diet in the laboratory or spraying the virus against the pest insect in the field and collecting infected or dead larvae (Moscardi et al., 2011). The aforementioned is the most commonly used method of baculovirus production in various countries (Moscardi et al., 2011). Biopesticides are difficult to produce *in vitro* because repeated viral passages in cell culture can cause genetic changes that ultimately cause a decrease in virulence (Gelaye & Negash, 2023). Possibly the main downside of baculoviruses is that they are sensitive to ultraviolet (UV) radiation from the sun (Shapiro et al., 2002; Fuller et al., 2012). UV radiation causes the virus to degrade rapidly in the field. Although baculoviruses have OBs that offer them protection from harsh environments, they do not protect the virions from UV radiation. UV radiation obstructs DNA replication and causes mutations as a result of cross-linking of adjacent pyrimidine residues in the DNA (Tyrrell et al., 1974; Rohrmann, 2013). This means that farmers may have to spray the biopesticide when the sun is down and may need to apply it frequently. Shade nets are a well-established practice in commercial agriculture. They offer a potential solution for protecting OBs from the damaging effects of sunlight, and the following sections will explore shade nets in greater detail.

1.6 Protective nets or exclusion barriers

1.6.1 Background

The practice of using exclusion netting began in the mid-20th century (Scarascia-Mugnozza et al., 2011; Chouinard et al., 2016) and gained widespread adoption in the 1990s, particularly after *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), commonly known as the sweet potato, cotton, or tobacco whitefly, became a major issue for the tomato industry in Israel (Berlinger et al., 2002). This whitefly species served as a vector for the tomato yellow leaf curl virus (TYLCV). Chemical pesticides were initially used exclusively to control the pest (Berlinger et al., 2002), however, due to the whitefly's short lifespan, high mobility, and rapid

development of resistance to nearly all available pesticides, alternative methods became necessary (Berlinger et al., 2002). Exclusion netting emerged as a cost-effective solution (Berlinger et al., 1991), leading to its increased use for the control of the pest. Since then, the use of exclusion barriers in agriculture has gradually increased across various countries, particularly for annual crops and tree nurseries (Stander & Cronjé, 2016). The exclusion barrier technology operates by preventing access to the crop (Chouinard et al., 2016). Traditionally, the primary environmental factors against which nets afforded citrus fruits protection, include extreme temperatures, hail, sunburn, frugivorous bats (and other small mammals), birds, and sometimes insects (Briassoulis et al., 2007b; Castellano et al., 2008).

1.6.2 Types of nets

The use of shade netting has become increasingly prominent in citrus farming in South Africa (Marsberg et al., 2024). There are currently many different types of nets used for various crops, each distinguished by specific structural features such as mesh size, texture, shading factor, colour, air permeability, durability, and more (Castellano et al., 2008). For instance, anti-wind nets, which are primarily used to decrease airflow and reduce average wind velocity within the nets (Manja & Aoun, 2019), are typically dark in colour with a mesh size ranging from 1 to 3 mm (Briassoulis et al., 2007b). In contrast, biological nets (also known as bio-nets) are designed to prevent virus-vector insects from accessing crops and are usually made from transparent thread with a mesh size of approximately 0.5 mm, though this may vary depending on the targeted insect (Briassoulis et al., 2007b). Shade nets, the focus of this study, are used to protect plants from high temperatures and excessive solar radiation (Briassoulis et al., 2007b). Shade nets have the ability to absorb various spectral bands of solar radiation, including visible, UV, far-red and infrared, while also allowing for an increase in the amount of scattered and diffused light (Shahak et al., 2004; Shahak, 2006; Chouinard et al., 2016). In other words, shade netting selectively filters out sunlight wavelengths that are not suitable for photosynthesis, allowing only those that promote growth to reach the crop (Eyouagro, 2023). These normally have a mesh size between 0.6 and 4 mm, with light transmittance ranging from 20% to 70% (Briassoulis et al., 2007b). Shade nets used are generally transparent. However, different colours such as yellow, blue, red, white, pearl, and grey are available (the coloured nets are also called photo-selective nets) (Shahak et al., 2004). Additionally, the lifespan of different types of nets varies; white nets typically last around 10 years, while black nets can last about 15 years under field conditions (Amarante et al., 2009; Manja & Aoun, 2019).

Greenhouse covering nets, which are preferred over glass-covered greenhouses as they are cheaper, lighter, and larger, generally last 3 to 4 years (Scarascia-Mugnozza et al., 2011).

The primary material used for agricultural nets is high-density polyethylene (HDPE), which is favoured due to its non-toxic, environmentally friendly, recyclable, waterproof, and durable properties (Castellano et al., 2008). While polypropylene is also utilised in net production, it is mainly used for creating non-woven layers (Castellano et al., 2008). The design, construction structures, and characteristics, including chromatic properties of different types of netting, significantly influence crop response and production (Shahak, 2006; Castellano et al., 2008). Factors such as fruit quality, colour, firmness, weight, and flavour can vary depending on the specific netting conditions (Manja & Aoun, 2019). Overall, the different exclusion techniques are categorised as either complete or incomplete exclusion netting (Figure 1.6) (Chouinard et al., 2016). In complete exclusion netting, the soil is excluded from the enclosed zone. An example would be a row-by-row systems or “tunnel” netting. The netting structures are removed at harvest time. In the incomplete exclusion, however, the soil is not excluded from the system (Rigden et al., 2008). An example of this type of netting is full block netting systems, with more permanent structures that are built. Both systems have their own disadvantages, such as favouring the development of secondary pests in complete exclusion and allowing several key pest species to complete their life cycle and remain inside the enclosed area (Chouinard et al., 2016).

Figure 1.6: Examples of exclusion nets used for stone and pome fruit: (A) Alt'Carpo incomplete exclusion system used for the management of the codling moth (Sévérac & Romet, 2008); (B) incomplete exclusion dual system used to exclude cherry flies and to prevent fruit cracking (Charlot et al., 2014); (C) and (D) complete exclusion system examples for the management of various pests of apples (Photo credit: G. Chouinard in Chouinard et al., 2016).

1.6.3 Advantages of shade nets

Although the primary function of shade netting is crop protection against high temperature extremes, shade netting has many other advantages. In agriculture, shade netting is among the safest methods of crop protection as it is environmentally friendly (Manja & Aoun, 2019). Most importantly, shade nets can alter the microclimate in orchards, which can result in an increase in crop quality and production (Briassoulis et al., 2007a; Manja & Aoun, 2019). Shade nets

also promote uniformity of the crop owing to their ability to disperse sunlight, such that it reaches the plants equally from all sides (Briassoulis et al., 2007a). This will, therefore, allow fruit to reach maturity at the same time. In other cases, the fruit ripening may be delayed or slowed down to increase the market value of the fruit produced (Briassoulis et al., 2007a; Castellano et al., 2008). Another advantage of shade nets is that they increase water use efficiency. Maximum temperatures can be decreased under netted structures, leading to a decrease in the overall amount of water needed by the plant and lost through evapotranspiration (Wachsmann et al., 2014; Manja & Aoun, 2019). A study by Wachsmann et al. (2014), found that the use of nets resulted in an up to two-fold increase in fruit yield (kg fruit per tree) and significantly increased tree water usage efficiency and fruit quality. Other farmers reported up to a 30% decrease in the use of water for irrigation. Moreover, shade nets erected in a 'Star Ruby' grapefruit farm in the Kakamas area (Northern Cape, South Africa) led to a reduction of 12% and 20% in sunburn damage and irrigation volume, respectively (Stander & Cronjé, 2016).

Shade nets allow for a significant reduction in the number of pesticide (synthetic or organic-approved) applications needed to protect the crop from pests (Briassoulis et al., 2007a; Chouinard et al., 2016). This is beneficial, especially on organic farms (Granatstein et al., 2016). Most importantly, shade netting could be a crucial tool for a more concerning problem, climate change, which is anticipated to be a threat to the global production of crops and food security (Wheeler & Von Braun, 2013; Stander & Cronjé, 2016; Grünig et al., 2020). The global surface temperature has increased by approximately 0.2°C every decade for the past three decades (Hansen et al., 2006). Global minimum and maximum daily temperatures have also increased (Alexander et al., 2006). Consequently, many parts of the world experience unpredictable weather patterns, such as high temperature extremes and heat waves, frequent wind, and hailstorms (Midgeley, 2016; Dovjik et al., 2021). Global climate change is a huge problem, especially in already warmer regions, because it also leads to a severe increase in warmer winter nights annually (Alexander et al., 2006). Warmer winters, unlike heat waves and warmer summers, which can be partially solved by a good irrigation system, pose a threat to fruit tree production (Dovjik et al., 2021). Flower induction of citrus trees depends on the accumulation of cold hours during the winter and is hindered by warmer environmental conditions (Samach & Smith, 2013). This is the same for the external development of citrus fruits, which is determined by peel colouration (influenced mainly by the accumulation of carotenoids and the breakdown of chlorophyll) (Rodrigo et al., 2013; Lado et al., 2018).

However, the internal maturation of the fruit is encouraged by warmer nights in winter. This can be problematic, as it shortens the harvesting period of the fruit, because the fruit may be over-matured internally and yet still have a green rind (Dovjek et al., 2020). An effective solution for this is to breed fruit cultivars that require fewer cold hours. However, the process is very long, costly and labour intensive. Shade netting may therefore be a practical, readily available solution to the climate change problem.

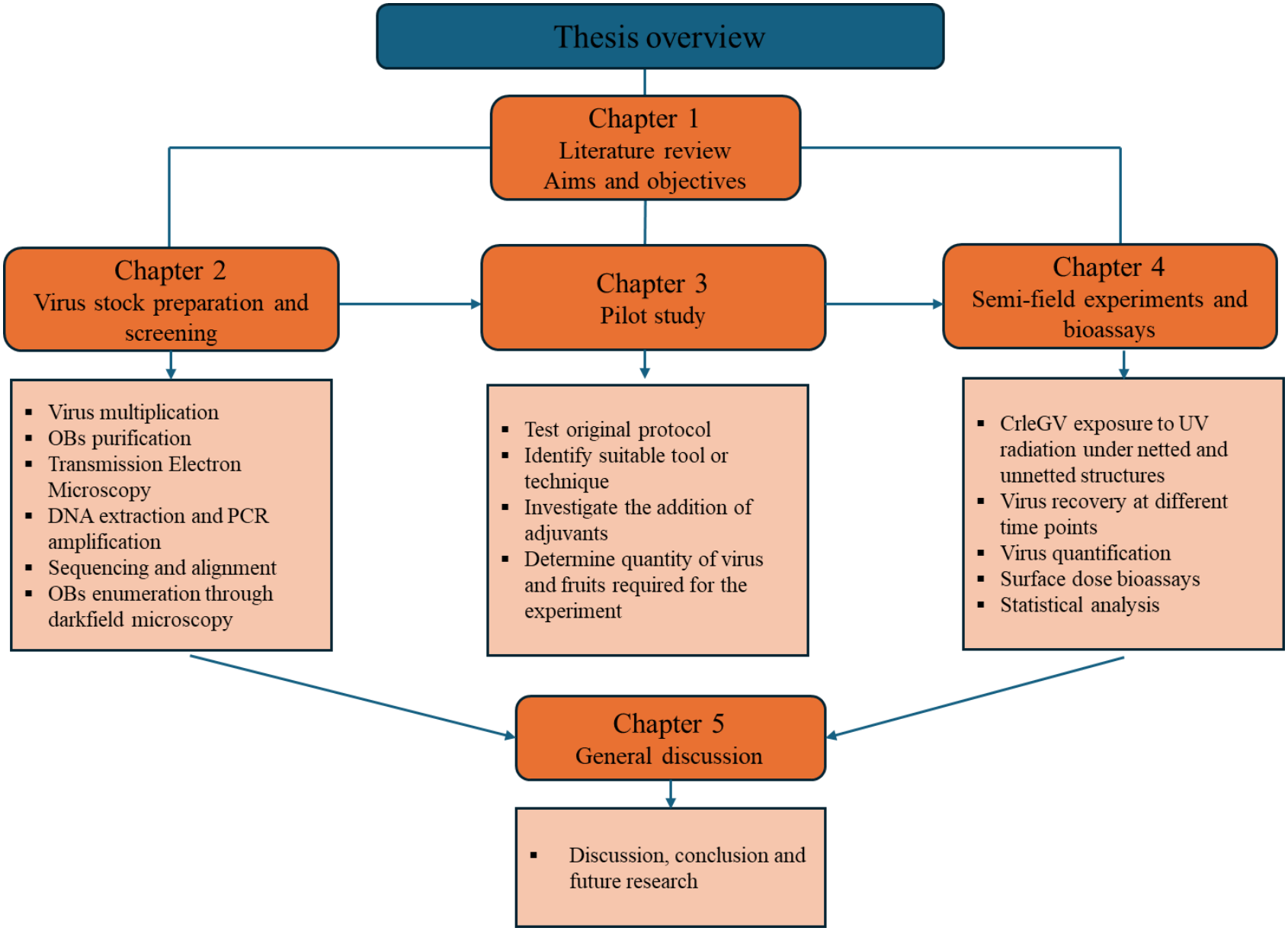
1.6.4 The effect of UV radiation on baculoviruses

The half-life of baculoviruses ranges from 10 h to 10 days under field conditions (Entwistle & Evans, 1985). In full sunlight exposure, however, the average half-life is only 24 h (Burges & Jones, 1998). The overall amount of UV incidence varies in an orchard according to the season and topography (Barker, 1968). Moore (2002) and Mwanza (2015) demonstrated that CrleGV degrades faster on the sun-facing (northern) side of the crop than on the shaded (southern) side. Mwanza (2015) also showed in a field experiment following CrleGV biopesticide application that samples from the shaded side had a lower LD_{50} and gave a clear biological assay concentration response compared to the sun-facing side. This pattern is presumed to be because of the architecture of the citrus trees, which provide greater shade than most crops where baculovirus biopesticides are used (Moore et al., 2004; Mwanza et al., 2022). Baculoviruses tend to persist longer in plants with a branch architecture and leaf arrangement that limit sunlight exposure. In contrast, virus deposits on leaves at the outer edges are less shielded from solar radiation, resulting in reduced longevity (Jacques, 1985). For example, when the cotton bollworm NPV was applied to the calyx, bracts, blooms, or the underside of cotton leaves, its persistence increased 10-fold compared to when it was applied to the upper surface of the terminal leaves (Ignoffo & Couch, 1981). Finding ways to increase the viral activity or residual efficacy of entomopathogenic viruses has been the focus of a lot of research (Knox et al., 2015). Many substances such as Zinc-oxide, Titanium oxide, dyes and fluorescent brighteners, have been tested as UV protectants for different viruses (Shapiro, 1995; Burges & Jones, 1998; Moscardi, 1999). In a study on improving the residual efficacy of CrleGV, Kirkman (2007) discovered that lignin was an effective additive that enhanced the virus activity. Nevertheless, applying the insecticide in the evening as opposed to during the day produced a comparable outcome, which was also cheaper. For CpGV, the addition of lignin only improved UV protection in the laboratory but not in the field (Arthurs et al., 2006). The improvement of biopesticide formulation to enhance important factors like shelf-life, and viral activity is

preferable, but improved protection may not necessarily lead to significant gains, especially if it means increasing the cost of the product (Moscardi, 1999). For this reason, perhaps shade netting may play a significant role in providing UV protection to plants in the field. It is important to note that shade nets have their drawbacks, including their often high cost and the potential difficulty in managing pests under the nets. Stander & Cronjé (2016) highlight key factors to consider when using shade nets. Manja & Aoun (2019) review various types of nets and their effects on crop production, considering factors such as the crop's geographical location, type, growing conditions, and desired plant response. Additionally, Mditshwa et al. (2019) reviewed the effect of shade netting on subtropical fruit, focusing mainly on the environmental conditions, tree physiology and fruit quality.

1.6.5 Research aims

Even though the use of netting is rising in many citrus-growing regions across South Africa, there are a lot of important research questions that need to be prioritised and addressed (emphasised by Stander & Cronjé (2016)). There is limited literature on netting, with the majority of studies concentrating on the effects of photo-selection, temperature, humidity, and variations in microclimates in orchards under nets. This study aims to address one key question, assessing the impact of shade netting on CrleGV, which could enhance the biological control of *T. leucotreta*. More specifically, the objectives of this research are (A) multiplication of CrleGV in *T. leucotreta* larvae and purification of occlusion bodies, (B) quantification of CrleGV occlusion bodies and genetic verification of viral samples, (C) execution of a pilot study for experimental design and methodology refinement, (D) semi-field exposure of CrleGV both under and without netting, and (E) execution of surface dose biological assays to assess virulence of recovered CrleGV from semi-field exposure under netting and without netting.


Thesis overview
Chapter 1
Literature review
Aims and objectives
Chapter 2
Virus stock preparation and screening
Chapter 3
Pilot study
Chapter 4
Semi-field experiments and bioassays
Virus multiplication
OBs purification
Transmission Electron Microscopy
DNA extraction and PCR amplification
Sequencing and alignment
OBs enumeration through darkfield microscopy
Test original protocol
Identify suitable tool or technique
Investigate the addition of adjuvants
Determine quantity of virus and fruits required for the experiment
CrleGV exposure to UV radiation under netted and unnetted structures
Virus recovery at different time points
Virus quantification
Surface dose bioassays
Statistical analysis
Chapter 5
General discussion
Discussion, conclusion and future research

Chapter 2

Virus stock preparation and screening

2.1 Introduction

The use of entomopathogenic viruses as microbial control agents (MCAs) in place of synthetic chemical insecticides has long been recognised (Moscardi, 1999). While over 150 years of research has been dedicated to exploring entomopathogens as MCAs (Davidson, 2012), and more than 30 years have been spent developing mass production methods for host organisms to cultivate these viruses, scaling up to the level necessary for commercial viability for many has yet to be achieved (Granados et al, 2007; Moscardi et al., 2011; Lacey et al., 2015). The most fundamental limiting factors for successful large-scale commercialisation of MCAs include existing knowledge of the pathogens and mass production technology (Moscardi, 1999; Lacey et al., 2015). Although insect viruses are highly diverse, only those from the family Baculoviridae have been formulated and registered as bioinsecticides (Moscardi et al., 2011). This can be attributed to the fact that, in comparison to other families of invertebrate viruses, the Baculoviridae family has been thoroughly studied, and the basic taxonomy, biology, pathology, and ecology of these viruses are understood (Lacey et al., 2015). In South Africa, the baculovirus CrleGV plays a crucial role in the systems approach used to manage *T. leucotreta.*

Several isolates of CrleGV have been identified in association with *T. leucotreta* across three different countries (Mwanza et al., 2022; Opoku-Debrah et al., 2013). The first isolate was originally discovered from infected larvae in the Ivory Coast by Angélini et al. (1965). A second isolate was obtained from diseased larvae collected in the Cape Verde Islands (Möck, 1985). The third isolate originated from a *T. leucotreta* laboratory culture established in 1980 using field collected samples from South Africa, maintained by the Hoechst Corporation in Germany (Jehle et al., 1992). Fritsch & Huber (1986), Fritsch (1989), and Jehle et al. (1992) confirmed that these three isolates were genetically distinct through restriction endonuclease (REN) analysis of viral DNA, leading to their designation as CrleGV-IC (Ivory Coast), CrleGV-CV (Cape Verde), and CrleGV-SA (South Africa) (referred to as just CrleGV in this study). Moore (2002) later reported the discovery and development of another novel CrleGV isolate, which was found infecting *T. leucotreta* larvae in the Goedhoop citrus insectary at Citrusdal, Western

Cape, South Africa. Singh et al. (2003) conducted the genetic characterisation of this isolate. Further research by Opoku-Debrah et al. (2013) successfully induced CrleGV outbreaks in five geographically distinct *T. leucotreta* laboratory cultures through larval overcrowding, leading to the isolation and genetic characterisation of five novel CrleGV isolates from South Africa. These differences were confirmed through single REN analysis of viral DNA, partial sequencing of the *granulin* and *egt* genes, and multiple sequence alignments, resulting in the classification of two phylogenetic CrleGV-SA groups (Opoku-Debrah et al., 2013). In total, at least seven genetically distinct CrleGV-SA isolates have been identified in South Africa, one of which is utilised in the formulation of a commercial biopesticide produced in the country (Opoku-Debrah et al., 2013).

Currently, the main method of virus production uses the *in vivo* approach (Burges & Jones, 1998; Moscardi, 1999; Moscardi et al., 2011; Reid et al., 2014; Lacey et al., 2015). This is achieved by mass-rearing of host insects on an artificial diet to an optimum stage, followed by infection with the virus (Jones, 2000). This method has been effectively used by the South African company River Bioscience for the past 20 years to produce viruses, such as CrleGV, enabled by a monthly production of approximately 5.38 million moths (Moore et al., 2014). This rearing technology plays a major role in the distribution of viral products into southern African agricultural markets (Knox et al., 2015). However, mass production of virus products could potentially be more efficient through *in vitro* methods, using insect cells in bioreactors (Moscardi et al., 2011). This technique aids in subduing some of the challenges faced by *in vivo* production, such as reducing contamination from unwanted pathogens (Burges & Jones, 1998; Szewczyk et al., 2006) and would also alleviate the amount of labour effort and space needed for large-scale production (Harrison & Hoover, 2012). Most importantly, the *in vitro* method would enable easier selection of cell clones with higher baculovirus yield. Despite these advantages, *in vitro* production remains technically and economically challenging (Moscardi, 1999; Grzywacz & Moore, 2017), and the timeline for the commercial implementation of this approach is still uncertain (Reid et al., 2014). Details about the limitations of *in vitro* production are beyond the scope of this study, however, several studies have reviewed this, including work by Weiss et al., (1994); Black et al., (1997); Jem et al., (1997); Moscardi, (1999); Jones, (2000); Moscardi et al., (2011); Reid et al., (2014); Lacey et al., (2015) and Grzywacz & Moore, (2017).

On the positive side, the *in vivo* method for virus production has been well-established and is capable of meeting current market demands (Lacey et al., 2015). Additionally, both small- and large-scale production use a similar protocol (Jones, 2000).

According to Jones (2000), virus samples that are to be used for biological assays should be purified, as these may contain contaminants, such as microorganisms, that may interfere with the infection process. Purifying baculovirus OBs generally involves centrifugation and ultra-centrifugation, which help separate virus particles from insect debris in the sample (Opoku-Debrah et al., 2013; Jukes et al., 2014; Jukes, 2018). Various methods, including glycerol gradient, sucrose cushion purification, and crude extraction, are employed for OB purification (Grzywacz et al., 2004; Wennmann & Jehle, 2014; Jukes, 2018).

Accurate identification of virus types can only be achieved at the molecular level, as Jones (2000) highlighted. For the purpose of this study, genomic DNA was extracted using a commercial DNA extraction kit. However, laboratory protocols such as the cetyltrimethylammonium bromide (CTAB) method could also have been employed as an alternative (Opoku-Debrah et al., 2013). Additionally, a polymerase chain reaction (PCR) was used to determine whether other non-target viruses are present in the purified virus (Garibyan & Avashia, 2013).

Following virus identification, the determination of stock concentration is essential for downstream experimentation. This is important because in the field, there is a minimum concentration that is applied to achieve sufficient pest mortality. For example, the CrleGV based formulations that are currently used in South Africa (Cryptogran®, Cryptex® and Gratham®) are registered at a minimum concentration of 5×10^{10} OBs/ml for Cryptogran® and 2×10^{10} OBs/ml for both Cryptex® and Gratham®. Moore et al. (2004) also established that CrleGV should not be applied at less than $\sim 2 \times 10^{13}$ OBs/ha to avoid compromised efficacy. Whilst other methods of determining concentrations are known e.g., real time quantitative polymerase chain reaction (qPCR) and scanning electron microscopy (SEM), in this study, the purified virus stock was quantified using the most common technique, dark-field microscopy (Dhladhla et al., 2018; Jukes, 2018).

This chapter focused on the multiplication and screening of a virus intended for use in two separate semi-field experiments: a long-term study (Experiment 1) and a short-term study (Experiment 2). These experiments were conducted at different times, with the following key objectives: (1) virus multiplication in *T. leucotreta* third and fourth instars, (2) Purification of

OBs from larval cadavers using the glycerol gradient method for Experiment 1 and purification using the crude extraction method for Experiment 2, (3) Virus sample visualisation using transmission electron microscopy as the first step of virus identification, (4) DNA extraction using the PCR technique and agarose gel electrophoresis, (5) PCR amplicon sequencing and sequence alignment, and (6) OB enumeration through dark field microscopy.

2.2 Methods

2.2.1 Virus preparation

2.2.1.1 Virus Multiplication

For Experiment 1, a purified stock suspension of a known isolate of the CrleGV-SA was provided by River Bioscience (Pty) Ltd (South Africa). Prior to the passage assay, the virus was quantified (using the enumeration protocol described below) to calculate the concentration of the stock. The concentration was calculated to be 7.67×10^{10} OBs/ml, which was diluted to 3.83×10^{7} OBs/ml, a suitable lethal dose for the serial passage assay determined in preliminary assays in this study. The assay was carried out with approximately nine day old third and fourth instar (approximately nine days old post-egg hatch when reared at 26°C) *T. leucotreta* using a diet-plug method described by Jones (2000) and Wennmann & Jehle (2014), with modification. These life stages were used as they represent active feeding periods, and the larvae are large enough to generate sufficient viral quantities.

A total of 720 (distributed across 30 × 24 well plates) third/fourth instars were gently collected from their rearing jars (rearing information is described in Chapter 4) and placed into well plates using a fine paintbrush (R000) or sterile soft forceps. These plates, covered with a glass plate and plastic lids, were sealed with Parafilm® M, and placed in a CE room (± 25°C) to starve the larvae for 8 h. Subsequently, fresh diet was prepared (see Chapter 4) into small plugs measuring 3 × 3 × 3 mm using a modified grid, which is pressed into the diet (Figure 2.1). The diet plugs were transferred to new 24-well plates with a sterile toothpick, with each well receiving a single diet plug, inoculated with a 1 µl aliquot of the CrleGV OB suspension applied directly to the surface of each diet plug in each well, using a micropipette. The larvae were then placed singly into the wells, and the plates were again covered with glass plates, plastic lids and sealed with parafilm. The plates were stored in the CE room overnight at ± 25°C and thereafter, larvae that consumed the entire diet plug were transferred to glass vials containing approximately 1-2 g of fresh diet, which was cut out using a modified sterile 10 ml syringe and

carefully pressed and levelled. These vials, closed with cotton wool, were further incubated at ± 25°C for 7 to 14 days. CrleGV infected larvae can be identified by swelling and their milky-glazed or brownish-grey appearance (Moore, 2002; Opoku-Debrah et al., 2013). Cadavers of symptomatic larvae were collected immediately after death to ensure maximum viral load, as the virus reaches peak infectivity at this stage, having completed much of the viral replication process. Since infected larvae often become flaccid due to liquefaction of internal tissues (Harrison & Hoover, 2012), the larvae were monitored daily at an arbitrary time, with minimal disturbance, to identify those displaying symptoms of infection and to collect cadavers before liquefaction. These were grouped together per assay for OB extraction or stored at -20°C for later use.

In Experiment 2, the virus used for multiplication was sourced from the virus stock produced in Experiment 1 and was propagated following the steps outlined above.

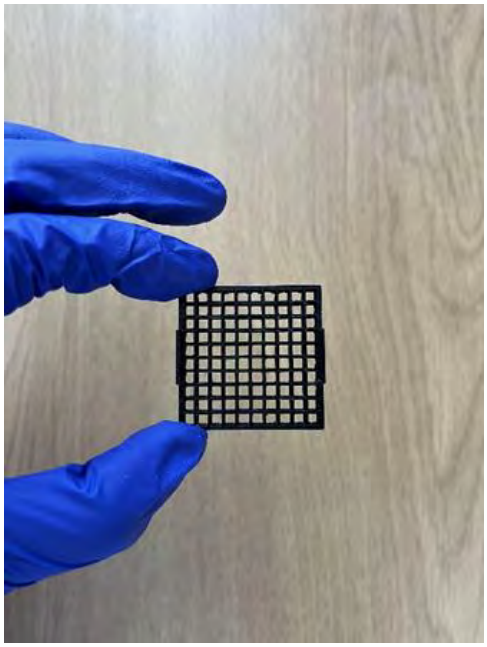


Figure 2.1: Modified grid (10 × 10 grid with each having dimensions: 3 × 3 × 3 mm) used for cutting *T. leucotreta* artificial diet into 3 × 3 × 3 mm cubes.

2.2.1.2 Virus purification

In this study, both the glycerol gradient method and crude extraction methods were used as they yield a sufficiently pure stock for transmission electron microscopy (TEM) (Taylor, 2021). TEM was used as an initial step to determine whether the virus in the sample was a granulovirus (GV) or nucleopolyhedrovirus (NPV), based on their morphological differences.

The baculovirus Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) is known to infect *T. leucotreta* and can be isolated from cadavers of larvae that have died from a baculovirus infection (Grzywacz et al., 2004). These infections occur either when a stressed larva triggers a covert infection to become overt or when the larva ingests OBs of a baculovirus to which it is susceptible (Hughes et al., 1993; Opoku-Debrah et al., 2013). As a result, it was essential to explore the potential for infection by this virus.

Glycerol gradient purification

For Experiment 1, the virus particles from larval cadavers were purified using the glycerol gradient protocol described by Hunter-Fujita et al. (1998), Opoku-Debrah et al. (2013), and Jukes et al. (2014). The purification process was as follows: Two gradients were prepared in SW28 ultracentrifuge tubes by layering 80-70-60-50-40-30% (v/v) glycerol solutions prepared according to Table 2.1. Each solution was made using 0.1% SDS and 100% glycerol. In the ultracentrifuge tubes, 6.1 ml of each solution was added, starting with the 80% at the bottom and ending with the 30% solution on top. This was then stored at 4°C prior to use. Approximately 20 infected insect cadavers were homogenised in 6 ml of 0.1% (w/v) SDS using a mortar and pestle. The liquid was filtered through a cheesecloth into a clean JA-20 centrifuge tube. The JA-20 tubes were filled with double-distilled water (ddH_2O), balanced, and centrifuged at 7,840 ×g (8,000 rpm) for 30 min at 4°C in a Beckman Coulter Avanti® J-E centrifuge. Supernatants from each were discarded and pellets resuspend in ddH_2O. The centrifugation step was repeated. The supernatants were once again discarded, and the pellets were resuspended in 1.5 ml ddH_2O. The virus suspension was then added on top of each of the glycerol gradients. These were centrifuged at 27,783 ×g (12,500 rpm) for 15 min at 4°C in a Beckman Coulter Optima™ L-90 K ultracentrifuge. Subsequently, the OBs, which form a whitish brown band across the middle of the tube, were carefully removed using an auto-pipette and distributed into two clean JA-20 tubes. The JA-20 tubes were filled with ddH_20 and centrifuged at 7,840 ×g (8,000 rpm) for 30 min at 4°C. The supernatant was then discarded, and the pellets were resuspended in 1.5 ml ddH_2O and then combined into one JA-20 tube. The tube was filled with ddH_2O and centrifuged again at 7,840 ×g (8,000 rpm) for 30 min at 4°C. The supernatant was discarded, and the resultant pellet was suspended in 500 to 750 µl of ddH_2O in a 1.5 ml tube. All OBs that were extracted from the larvae were subsequently pooled together into a 15 ml tube. This was stored at -20°C for later use. The stock is hereafter referred to as CrleGV-1.

Table 2.1: Volumes used to prepare 30-80% glycerol gradients for OBs purification.

Glycerol percentage (%)	Glycerol (ml)	0.1% SDS (ml)
30	9	21
40	12	18
50	15	15
60	18	12
70	21	9
80	24	6

Crude extraction

During Experiment 2, the Beckman Coulter Optima™ L-90 K ultracentrifuge, a key instrument for glycerol gradient virus purification, was unavailable. Consequently, a crude extraction method was employed as an alternative. The purification of *T. leucotreta* OBs from larval cadavers followed a modified version of Jukes (2018), which was originally adapted from Wennmann & Jehle (2014). Larval cadavers were transferred to a 2 ml tube, followed by the addition of 1 ml of 0.07% SDS. The mixture was thoroughly homogenised using a pipette tip and vortexed to ensure uniformity. The sample was then centrifuged at 100 ×g (1,200 rpm) for 30 seconds. The resulting supernatant was transferred to a clean 2 ml tube, while the pellet was resuspended in another 1 ml of 0.07% SDS and centrifuged again at 100 ×g (1,200 rpm) for 30 seconds. The newly obtained supernatant was pooled with the previously collected supernatant. The pooled supernatant was then centrifuged at 10,000 ×g (12,200 rpm) for 10 min. The supernatant was discarded, and the remaining pellet was resuspended in 1 ml of 0.07% SDS followed by another round of centrifugation at 10,000 ×g (12,200 rpm) for 10 min. After discarding the supernatant, the pellet was resuspended in 1 ml of ddH_2O and centrifuged again at 10,000 ×g (12,200 rpm) for 10 min. Finally, the supernatant was discarded, and the remaining pellet was resuspended in 200 µl of ddH_2O and stored at −20°C for future use. The stock is hereafter referred to as CrleGV-2.

2.2.1.3 Transmission electron microscopy

The carbon grids for the transmission electron microscopy were prepared according to the method described by Jukes (2018), originally adapted from methods described by Abdulkadir et al. (2013) and Opoku-Debrah et al. (2013). In this process, 5 µl of purified OBs were aliquoted onto the carbon formvar grid using a micropipette and left for 60 seconds. Afterwards, excess liquid from the grid was removed using a filter paper and then 5 µl 1% uranyl acetate (w/v) was pipetted onto the grid to stain the OBs and left for 60 seconds. Excess uranyl acetate was removed from the grid using filter paper. The grids were left overnight to dry. A JEOL JEM-2100 transmission electron microscope at The Centre for High Resolution Transmission Electron Microscopy (Nelson Mandela University, South Africa) was used to visualise and capture images of the OBs. This was performed only for CrleGV-1.

2.2.2 Molecular screening of the CrleGV stock

2.2.2.1 Genomic DNA extraction

Genomic DNA was extracted from purified OBs of CrleGV-1 and CrleGV-2 by transferring 50 µl of OBs into a sterile 1.5 ml microcentrifuge tube followed by 50 µl ddH_2O before adding 45 µl 1M Na_2CO_3 to the tube and incubated at 37°C for 15 min. To neutralise the suspension, 60 µl of 1M Tris-HCl (pH 6.8) was added to the suspension. The extraction process continued as per the Quick-DNA Miniprep kit (Zymo Research, USA) instructions. 200 µl BioFluid & Cell Buffer (Red) and 20 µl Proteinase K were added to the suspension, mixed well, and incubated at 55°C for 10 min. Subsequently, 420 µl of Genomic Binding Buffer was added, and the sample was transferred to a Zymo-Spin ™ IIC-XLR column within a collection tube. The sample was centrifuged at 12,000 ×g (13,400 rpm) for 1 min, and the collection tube was discarded with the flow through. The column was placed in a new collection tube and 400 µl DNA Pre-Wash Buffer was added. The sample was centrifuged again at 12,000 ×g (13,400 rpm) for 1 min, and the flow through was discarded. The column was returned to the collection tube. Next, 700 µl g-DNA Wash Buffer was added to the tube and centrifuged at 12,000 ×g (13,400 rpm) for 1 min. This was followed by adding 200 µl g-DNA Wash Buffer to the column and centrifuging at 12,000 ×g (13,400 rpm) for 1 min. Finally, the spin column was then placed in a sterile 1.5 ml microcentrifuge tube where 50 µl of a pre-heated (at 65 °C) DNA Elution Buffer was added. This was then incubated for 5 min at room temperature, centrifuged at 12,000 ×g for 1 min and stored at 4°C for short-term storage or -20°C for long-term storage.

2.2.2.2 Polymerase chain reaction

A polymerase chain reaction (PCR) was carried out using a SimpliAmp Thermal Cycler (Applied Biosystems, USA) and each sample was set up based on the PCR master mix outlined in Table 2.2. In the control reactions, ddH_2O replaced the DNA template. Oligonucleotide optimisation (Table 2.3) was conducted at 50°C, 53°C, and 55°C, with 55°C selected as the annealing temperature. The PCR protocol began with an initial denaturation at 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, ending with a final elongation at 72°C for 3 min.

Table 2.2: PCR reaction set-up for each PCR cycle.

Reagents	Sample	No Template Control (NTC)
2× Taq DNA Polymerase Master Mix Red (Ampliqon A/S, Denmark)	12.5 µl	12.5 µl
Forward primer (10 µM)	2 µl	2 µl
Reverse primer (10 µM)	2 µl	2 µl
DNA Template	4 µl	_
ddH_2O	4.5 µl	8.5 µl
Total	**25 µl**	**25 µl**

2.2.2.3 Agarose gel electrophoresis

The PCR amplicons were analysed by 1% (w/v) agarose gel electrophoresis (AGE) prepared with TopVision Agarose Tablets (Thermo Scientific, USA) and 1 × TAE buffer (1 mM EDTA, 20 mM acetic acid, 40 mM Tris-acetate). The agarose gel was stained in UView 6 × gel loading dye (Bio-Rad Laboratories, USA) and separated at 90 v for 30 minutes. The amplicon size was estimated by using the GeneRuler™ 1Kb DNA ladder (Thermo Fisher, USA) and a ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad Laboratories, USA) was utilised to visualise and capture images of the gel.

2.2.2.4 PCR amplicon sequencing and sequence alignment

The PCR amplicons were sequenced by Inqaba Biotechnical Industries (Pty) Ltd in both forward and reverse directions using M13 forward and M13 reverse sequencing

oligonucleotides, binding to the corresponding M13 sequences designed into the oligonucleotides (Table 2.3). The ABI Base Recall software (Elyazghi et al., 2017) was used to auto-trim the 5' and 3' regions of each sequence and to resolve any ambiguities by referencing sequence chromatograms. The sequences were then submitted to NCBI BLAST using the blastn algorithm to search for somewhat similar sequences. Subsequently, a ClustalW alignment was performed in Geneious R11.1.5 against the target region, which was extracted from the published CrleGV-SA genome sequence [GenBank accession number: MF984563; (van der Merwe et al., 2017)].

Table 2.3: Oligonucleotides designed to target unique regions in CrleGV-SA and CrpeNPV genome sequences (Tole, 2024).

Name	Length (bp)	Sequence (5'– 3')	Amplicon size (bp)	Tm (°C)
CrleGV-F	37	GTAAAACGACGGCCAGTTTGTGACGGGTGTTACAGGT	687	59.17
CrleGV-R	37	CAGGAAACAGCTATGACTCTGGGACGTGACCAAAAAC		58.33
CrpeNPV-F	37	GTAAAACGACGGCCAGTCTCCTTCTCGTGTGGCCATT	756	60.04
CrpeNPV-R	37	CAGGAAACAGCTATGACTCGACTTCGCGATTAGGTCG		59.97

* The underlined sequences are M13 universal forward and reverse linker sequences integrated into the unique oligonucleotides to facilitate sequencing in both forward and reverse directions.

2.2.3 Virus enumeration

The CrleGV OB samples were counted using dark field microscopy in order to calculate the concentration of the virus. Virus samples were prepared in 0.07% sodium dodecyl sulphate (SDS w/v) in double distilled water at various dilutions ranging from 2000 × to 1250×. In a 1.5 ml tube, 10 µl of virus OBs were diluted in 40 µl of sterile ddH_2O to make a 1:5 dilution (D1 – Total 5× Dilution). A homogenous suspension was achieved by vortexing the mixture for approximately 5 sec. A further 1:5 dilution (D2 – Total 25× Dilution) was made by adding 200 µl of 0.07% SDS solution to D1. The suspension was homogenised by shaking and then sonicated using a sonication probe at 60 Hz in 4 × 15 sec bursts (not longer than 60 seconds) to avoid damage to the virus particles. The sample was kept in ice during sonication to prevent

heating and ear protection was worn. A further 1:10 dilution (D3 – Total 250×) of the suspension was made in a new 1.5 ml tube by adding 900 µl of ddH_2O and 100 µl of the sonicated sample and vortexed to ensure homogenous dilution. Final dilutions ranging from 1:8 to 1:5 (Total 2000× - 1250×) were prepared by adding 125 µl of D3 to 875 µl, 750 µl¸ 625 µl, and 500 µl ddH_2O respectively, in new tubes. Final dilutions were vortexed and approximately 5 µl of the first dilution (starting with the 1250× dilution) was pipetted onto a clean Thoma bacterial counting chamber (Hawksley Medical and Laboratory Equipment, Lancing, UK) (0.02 mm depth), covered with a cover slip, and the slide was left to rest for 5 minutes before enumeration to allow Brownian movement of non-virus particles to settle. Subsequently, only vibrating virus particles were counted at 400× magnification (40× lens) under dark field microscopy, as Hunter-Fujita et al. (1998) described using an Olympus BX 51 TF microscope (Olympus, Tokyo, Japan). Five large squares, which consist of 16 small squares in each, are counted. These are the squares on the top right, top left, bottom left and bottom right corners of the chamber, and one random large square in the middle (Figure 2.2). The counts were performed in triplicate, and the average count was used to calculate the concentration of the virus OB, using Equation 2.1.

Equation 2.1: Formula for calculating the concentration of the virus sample using a counting chamber.

$$\textbf{Concentration (OB mL}^{-1}\textbf{)} = \textbf{(dilution} \times \textbf{mean OB count) / (80} \times (5 \times 10^{-8})\textbf{)},$$

where 80 is the number of small squares counted and 5×10^{-8} is the volume in millilitres of the virus suspension within these small squares.

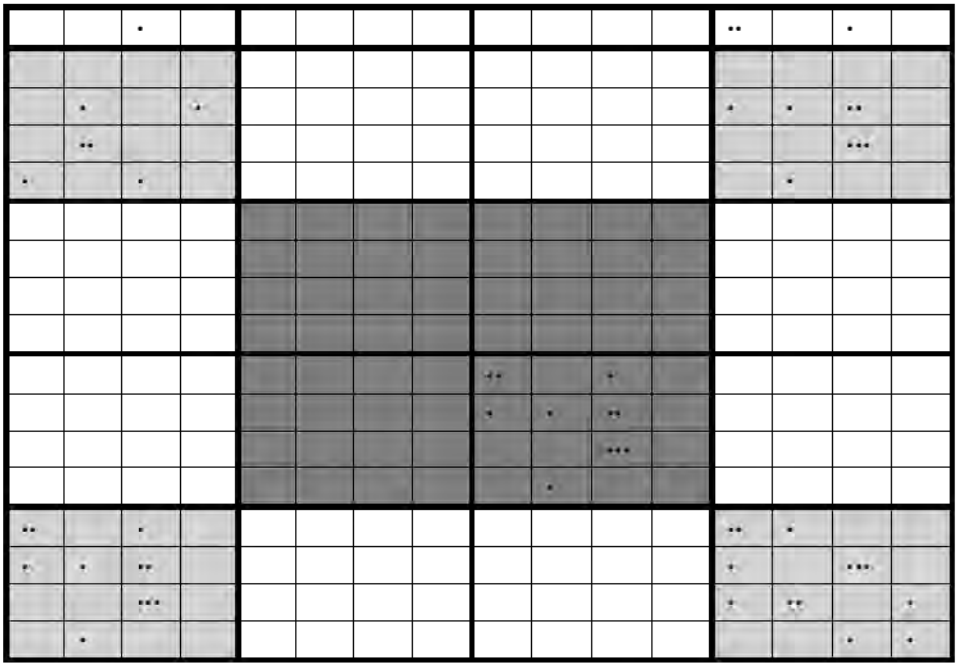


Figure 2.2: Position of counts on a Thoma chamber haemocytometer. Five large squares (16 small squares in each) are counted: one at each corner of the chamber (light grey) and a random large square in the middle (dark grey).

2.3 Results

2.3.1 Virus multiplication, purification and OB enumeration

In Experiment 1, CrleGV was propagated in 669 third and fourth instar *T. leucotreta*, utilising 669 glass vials, to generate sufficient virus stock for subsequent semi-field experiments. Symptoms of baculovirus infection were observed in the infected larvae (Figure 2.3). The CrleGV OBs were effectively purified through glycerol gradient ultracentrifugation (Figure 2.4). The concentration of the produced virus stock was determined using dark-field microscopy (Table 2.4). In Experiment 2, CrleGV-1 was multiplied in 701 third and fourth instar *T. leucotreta*, and the larval cadavers were purified using the crude extraction process.

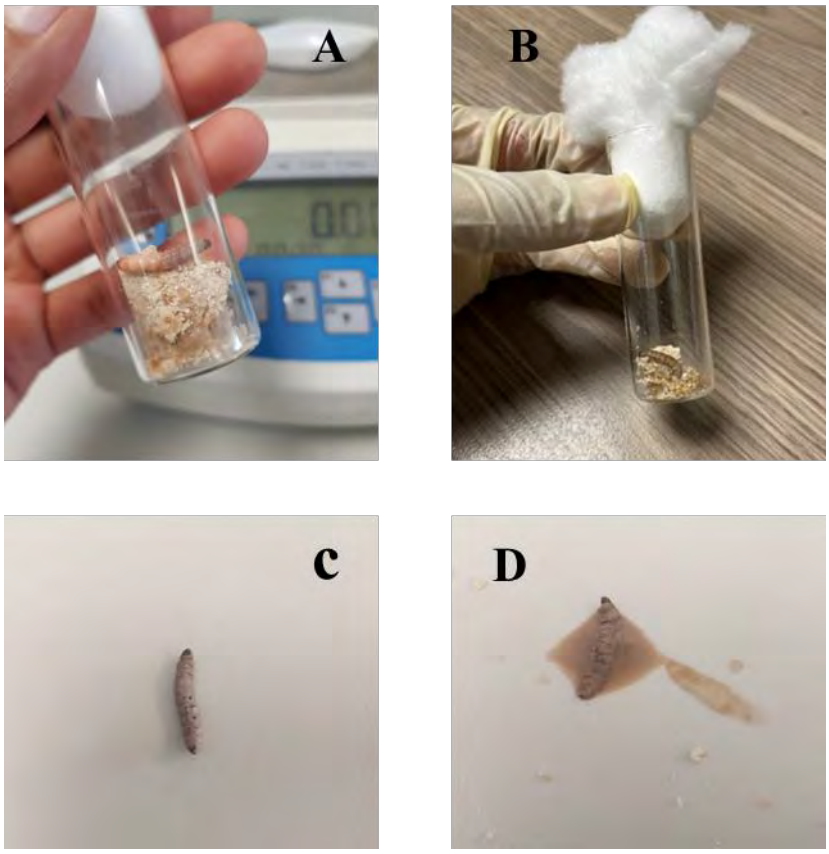


Figure 2.3: *Thaumatotibia leucotreta* larvae displaying symptoms of CrleGV infection, including larval cadavers on the diet surface with a creamy appearance (A) and a greyish to pale brown colouration (B). Infected larvae several hours post-death are shown in (C) and (D), with the larval body ruptured, releasing a milky-brown liquid containing a high concentration of occlusion bodies.

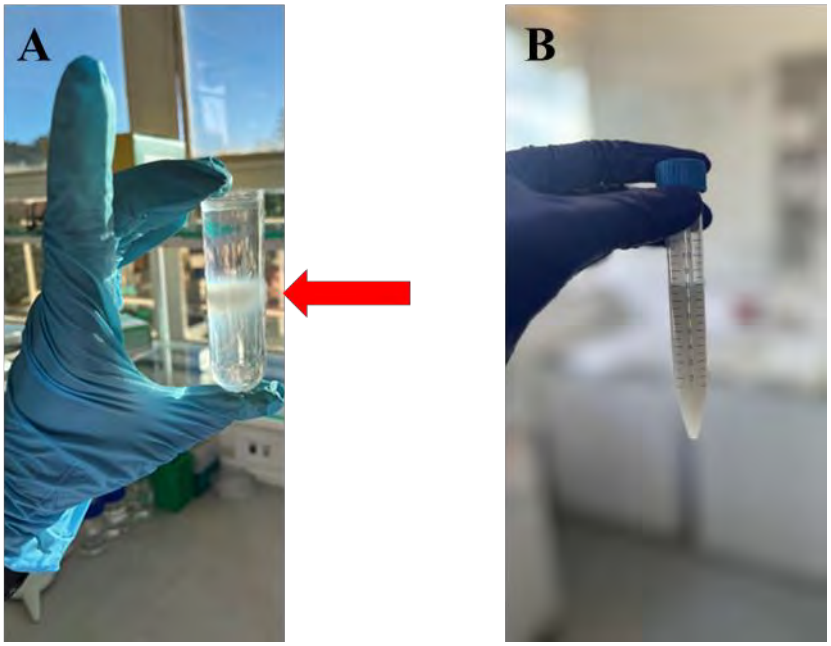


Figure 2.4: Glycerol gradient ultracentrifugation: with the CrleGV OB band indicated by the red arrow (A) and the resulting virus stock obtained from multiple OB bands pooled together (B).

Table 2.4: Summary of CrleGV multiplication, detailing the number of larvae collected, the quantity of tissue used for OB extraction, the concentration of OBs as determined by dark-field microscopy, and the total volume of the stock.

Sample	Number of larvae collected	Total tissue mass (g)	Concentration (OBs/ml)	Volume (ml)
CrleGV-1	669	6.600	8.13×10^{10}	10
CrleGV-2	701	6.902	2.69×10^{11}	10.4

2.3.2 Transmission electron microscopy

The purified CrleGV OBs were analysed using TEM (Figure 2.5). The observed OBs confirmed the presence of a GV, displaying external morphology consistent with CrleGV. They were roughly oval in shape, with a length ranging from 307 nm to 374 nm (average 348 nm) (n = 10) and a width ranging from 187 nm to 253 nm (average 224 nm) (n = 10).

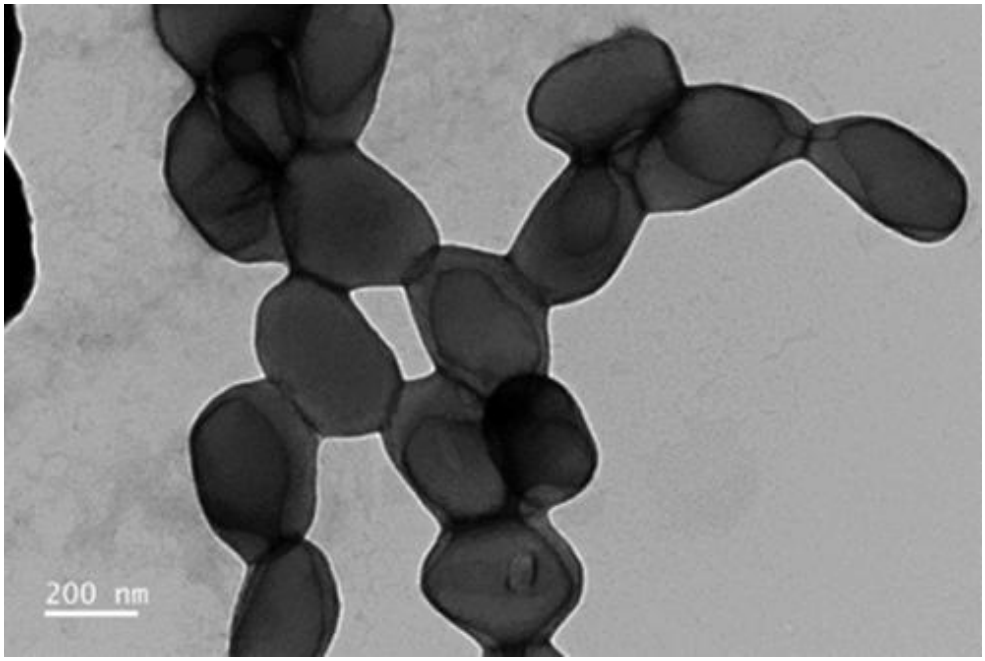


Figure 2.5: Transmission Electron Micrograph of purified CrleGV OBs (Experiment 1). Captured at the CHRTEM, NMU, South Africa.

2.3.3 Molecular screening of CrleGV stock

2.3.3.1 Genomic DNA Extraction and PCR

Genomic DNA was extracted from purified CrleGV OBs using Quick-DNA Miniprep kit and analysed by PCR to evaluate the specificity of the CrleGV F/R and CrpeNPV F/R oligonucleotides (Figure 2.6 and Figure 2.7). The analysis was conducted using 1% AGE stained with 6× gel loading dye.

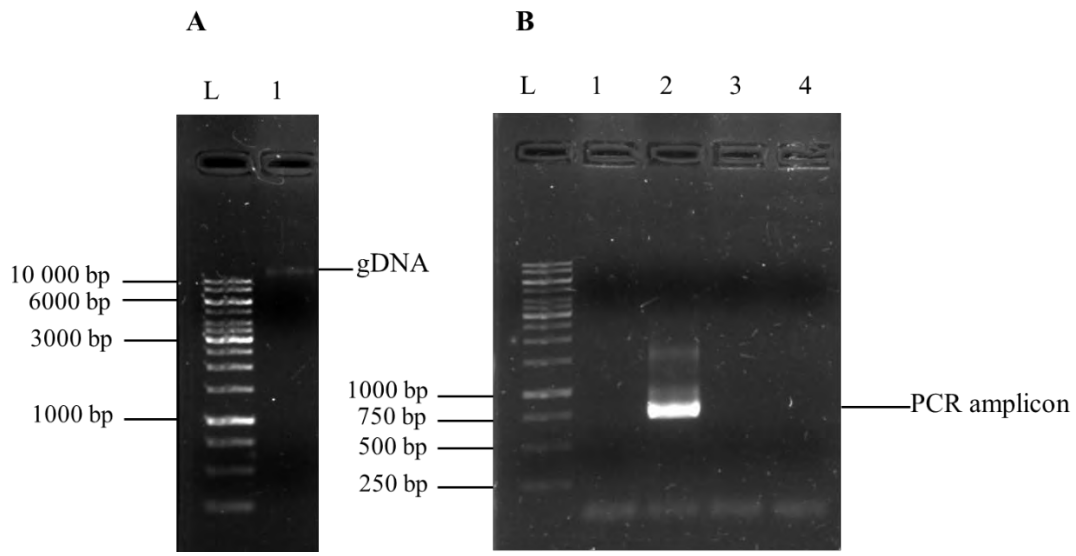


Figure 2.6: AGE image showing the results of genomic DNA extraction of CrleGV 1. The abbreviation "bp" represents base pair. Gel A, Lane L – GeneRuler 1kb ladder; Lane 1 – genomic DNA from the propagated sample. Gel B, Lane L – GeneRuler 1kb ladder; Lane 1 – NTC with CrleGV oligonucleotides; Lane 2 – CrleGV oligonucleotides; Lane 3 – NTC with CrpeNPV oligonucleotides; Lane 4 – CrpeNPV oligonucleotides.

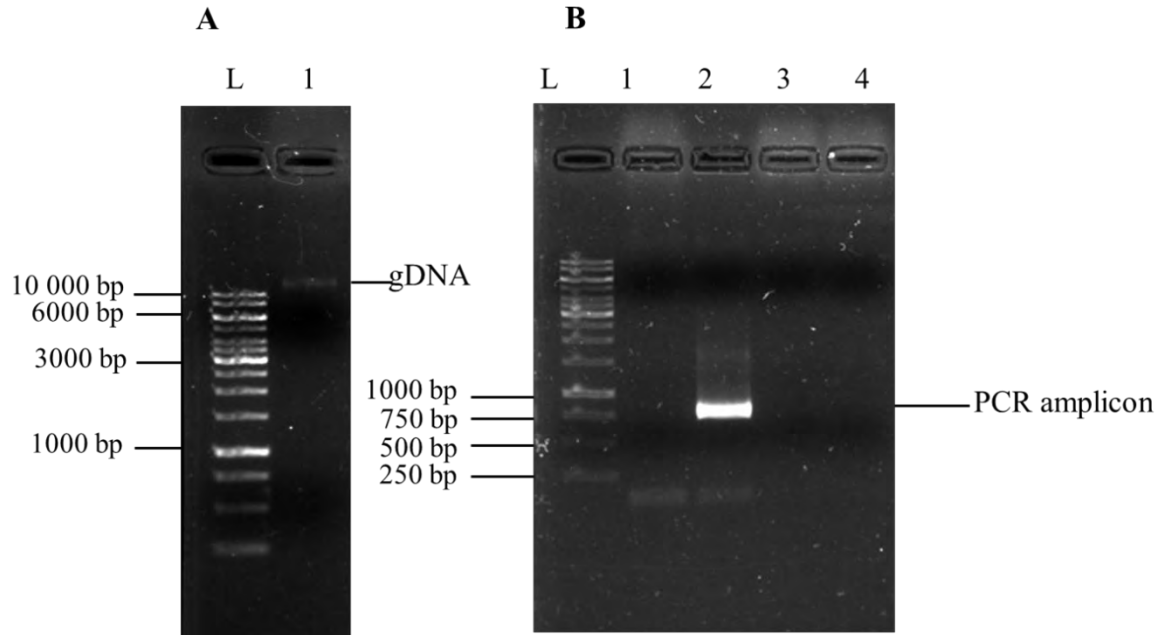


Figure 2.7: AGE image showing the results of genomic DNA extraction of CrleGV 2. Gel A, Lane L – GeneRuler 1kb ladder; Lane 1 – genomic DNA from the propagated sample. Gel B, Lane L – GeneRuler 1kb ladder; Lane 1 – NTC with CrleGV oligonucleotides; Lane 2 – CrleGV oligonucleotides; Lane 3 – NTC with CrpeNPV oligonucleotides; Lane 4 – CrpeNPV oligonucleotides.

For both CrleGV-1 and CrleGV-2, the genomic DNA band was observed above 10 000 bp in lane 1 of gel A. PCR analysis of the extracted DNA produced a single amplicon between 500 –750 bp in lane 2 of gel B. No amplification was detected in the no template control (NTC) (lanes 1 and 3) or in lane 4, where the CrpeNPV F/R oligonucleotides were used.

2.3.3.2 Sanger sequencing of PCR amplicons

The CrleGV PCR product was sequenced in both forward and reverse directions using M13 forward and reverse sequencing oligonucleotides. After automatic trimming of the 5' and 3' ends and recall of ambiguous nucleotides using ABI base recall, the resultant sequence length was 580 bp. The sequence was analysed using BLAST (blastn) to search for somewhat similar sequences, and the top three matches are summarised in Table 2.5 for CrleGV-1 and in Table 2.6 for CrleGV-2. Subsequently, the sequence was aligned to the target region using the ClustalW alignment tool in Geneious R11.1.5. It was compared to the region spanning 22 283 bp (position 1 in the alignment) to 22 969 bp (position 687 in the alignment), this being the region to which the CrleGV primers target, with the resulting alignment shown in Figure 2.8 and Figure 2.9 for CrleGV-1 and CrleGV-2, respectively.

Table 2.5: The top three matches resulting from the BLAST analysis for CrleGV-1.

Description	Query subject start – end	E value	Query Cover	Percentage Identity	Accession Number
Cryptophlebia leucotreta granulovirus isolate CrleGV-SA, complete genome	22 913 – 22 339	0.0	99%	99.48%	MF974563.1
Cryptophlebia leucotreta granulovirus isolate CrleGV-CV3, complete genome	22 567 – 22 045	0.0	98%	85.10%	NC_005068.1
Cryptophlebia leucotreta granulovirus, CV4 orf427 gene, partial cds	2 567 – 2 393	3e-47	31%	86.96%	AY096243.1

Table 2.6: The top three matches resulting from the BLAST analysis for CrleGV-2.

Description	**Query subject start – end**	**E value**	**Query Cover**	**Percentage Identity**	**Accession Number**
Cryptophlebia leucotreta granulovirus isolate CrleGV-SA, complete genome	22 913 – 22 326	0.0	98%	98.81%	MF974563.1
Cryptophlebia leucotreta granulovirus islolate CrleGV-CV3, complete genome	22 567 – 22 025	0.0	98%	84.87%	NC_005068.1
Cryptophlebia leucotreta granulovirus, CV4 orf427 gene, partial cds	2 567 – 2 393	4e-46	30%	86.41%	AY096243.1

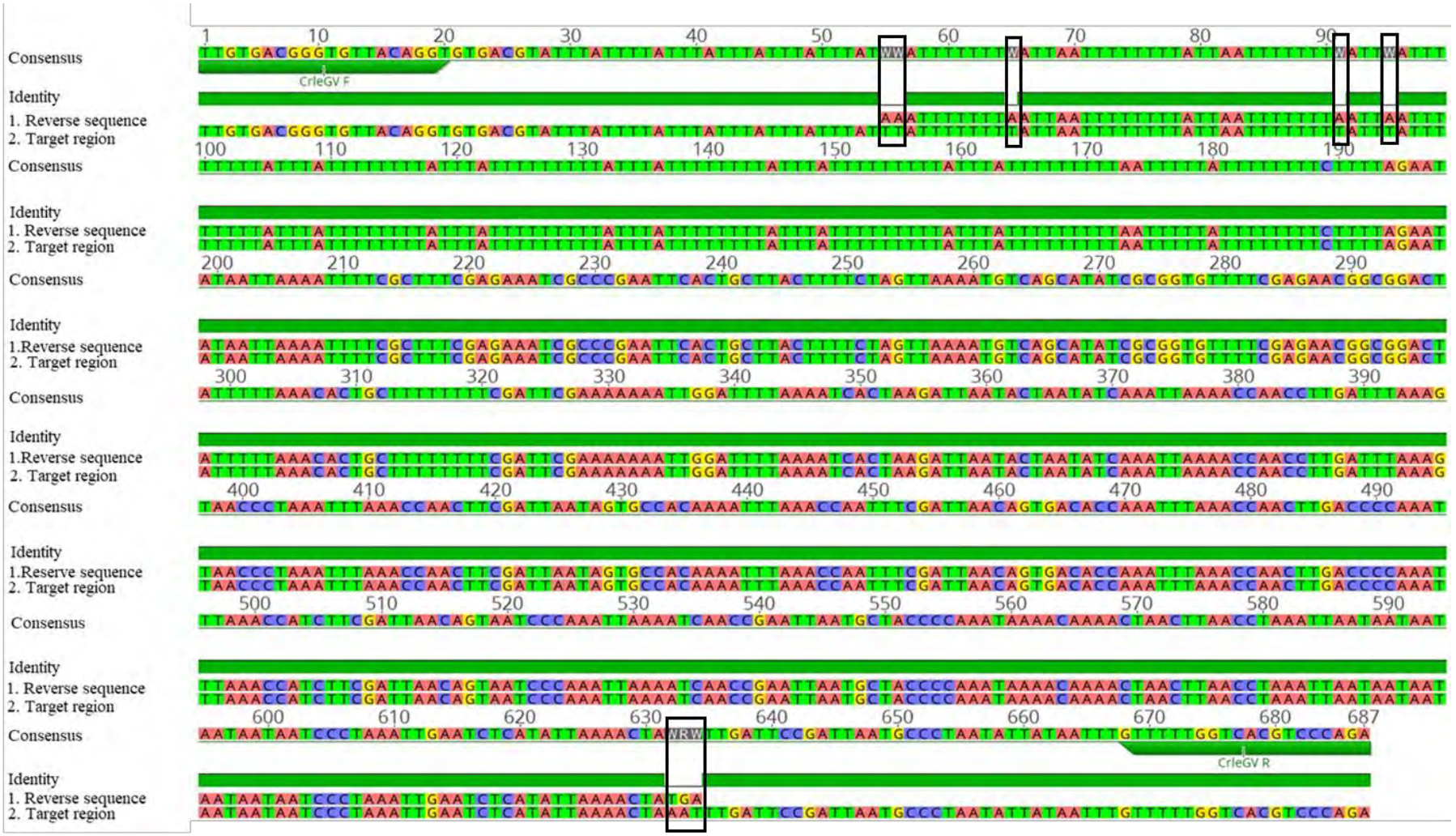


Figure 2.8: Sequence alignment of the CrleGV-1 amplicon against the target region in the CrleGV-SA genome sequence. The black boxes highlight the targeted SNPs.

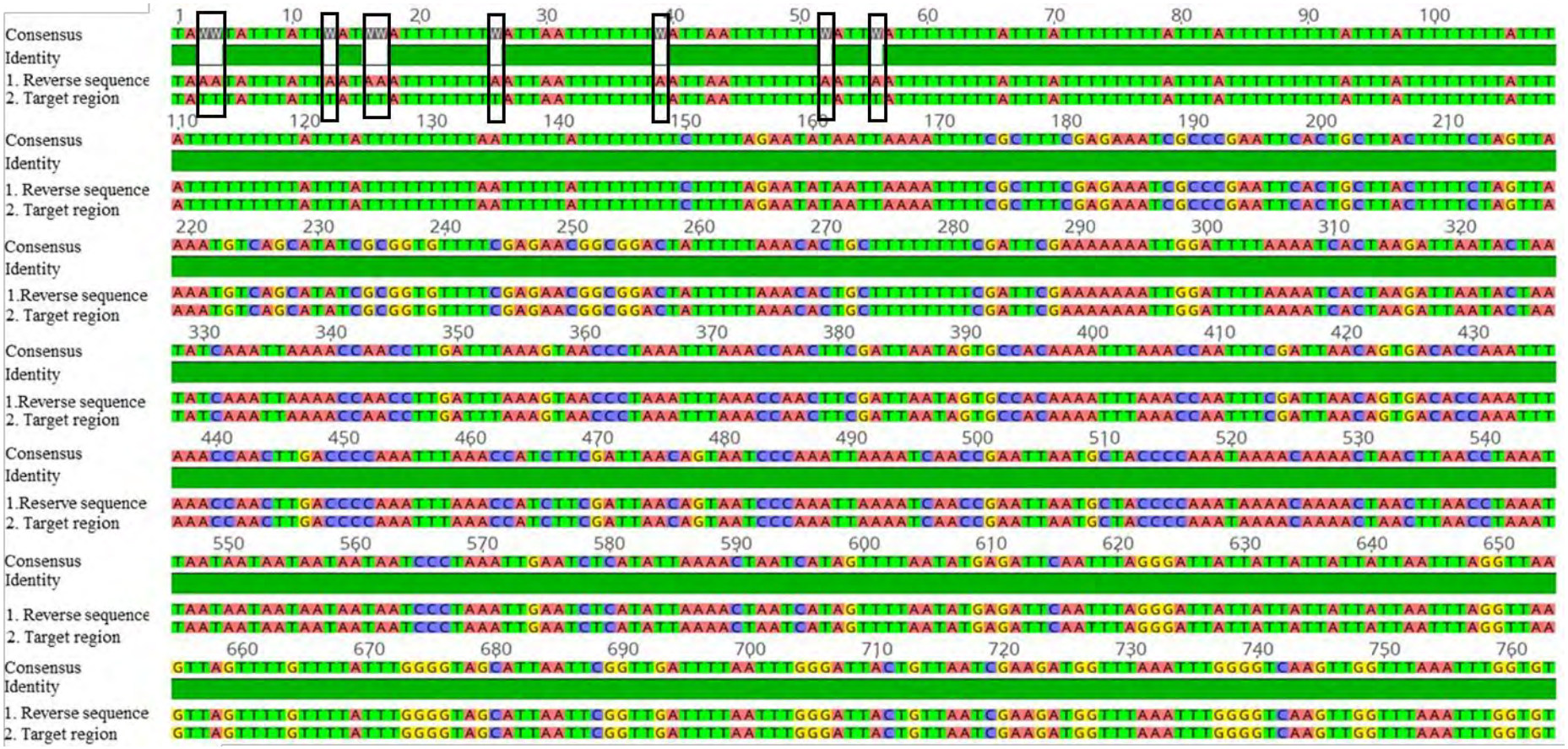


Figure 2.9: Sequence alignment of the CrleGV-2 amplicon against the target region in the CrleGV-SA genome sequence.

The top BLAST match for the sequence was CrleGV-SA, with an identity of 99.48% calculated for CrleGV-1 (Table 2.5) and an identity of 98.81% for CrleGV 2 (Table 2.6). Alignment of the sequence to the target region in CrleGV confirmed the correct target region had been amplified. Only 8 (SNPs) were observed for CrleGV-1, five of which were located in the T/A rich region and the remaining three toward the 3' region of the sequence. For CrleGV-2, 9 SNPs were observed, and all were located in the T/A rich region.

2.4 Discussion

The primary objective of this chapter was to generate CrleGV virus stocks for use in upcoming semi-field experiments. A CrleGV stock with a known concentration, provided by River Bioscience (Pty) Ltd, was successfully propagated in third and fourth instar *T. leucotreta*. The virus was subsequently extracted from the infected larval cadavers using the glycerol gradient purification method (CrleGV-1). The purified stock was further multiplied in third and fourth instar *T. leucotreta* using the crude extraction method to produce another virus stock (CrleGV-2). The presence of CrleGV OBs in the CrleGV-1 stock was verified using a TEM. TEM micrographs confirmed the presence of CrleGV, with external morphology aligning with descriptions by Moore (2002) and Jehle et al. (2006), indicating roughly oval-shaped OBs. Furthermore, the OB dimensions measured in this study were consistent with the size range of betabaculoviruses reported by Jehle et al. (2006) and Herniou et al. (2011), which specifies lengths of 300-500 nm and widths of 130-250 nm or 300-400 nm and 120-250 nm, respectively. Due to time constraints and the unavailability of the TEM microscope during the multiplication of CrleGV-2, visualising this stock under the TEM microscope was not conducted.

Virus screening progressed with DNA extraction, PCR amplification, and sequencing of the PCR products to confirm the presence of the target virus and the absence of contamination in the virus stocks. AGE results from the DNA extraction showed genomic DNA exceeding 10 000 bp, confirming successful extraction, as baculovirus genomes typically range between 80 and 180 kbp (van Oers & Vlak, 2007). PCR results validated the presence of the CrleGV genome, with an amplicon of the expected size produced for the CrleGV template. No amplification was observed when using the CrpeNPV F/R oligonucleotides or in the NTC. The CrleGV amplicon measured between 500 bp and 750 bp, consistent with the target region in the genome with a size of 687 bp, plus the M13 forward and reverse adapter sequences (34 bp), resulting in a total expected size of 721 bp.

Although the PCR test yielded the expected results, sequencing of the products was essential to confirm the presence of the desired virus and to rule out contamination. This is because, despite its numerous advantages, PCR has certain limitations (Garibyan & Avashia, 2013). The genes detected during the PCR process were dependent on the primers used. Although the oligonucleotides were specifically designed to target regions in either the CrleGV-SA or CrpeNPV genomes, it is possible that other regions within baculoviral genomes or contaminating DNA were amplified, resulting in non-specific amplicons. Consequently, other viruses or DNA targeted by these primers could have been overlooked. The PCR amplicons were subsequently sequenced, and BLAST analysis confirmed that the stock contained the desired virus. The various CrleGV isolates displayed high percentage identity scores and extremely low E-values, signifying a more significant match and indicating a very small probability that the results were due to random chance. Notably, the top three matches for both CrleGV-1 and CrleGV-2 amplicons were different isolates of CrleGV, which is a common occurrence, with the top hit identified as CrleGV-SA. The variation observed between these top hits may be because baculoviruses that occur naturally are typically mixed populations of diverse genotypes, displaying genetic variation caused by indel mutations (Crook et al., 1985; Thézé et al., 2014) and single nucleotide polymorphisms (SNPs) (Chateigner et al., 2015; Wennmann et al., 2017; Larem et al., 2019). The target regions were successfully matched with the sequence. It should be noted that the target region had fewer ambiguities and aligned better with the reverse complement of the sequence generated with the CrleGV-R oligonucleotide. This is likely due to the A/T rich sequence found in the 5' region of the amplified DNA.

Finally, the concentrations of the virus stocks were established and found to be sufficient for carrying out further experiments. The CrleGV-1 stock had a concentration of 8.13×10^{10} OBs/ml, and the CrleGV-2 stock had a concentration of 2.69×10^{11} OBs/ml. These concentrations are greater than the highest concentration of 3×10^{6} OBs/ml required to kill 90% of *T. leucotreta* neonates (LC_{90}) (Jukes, 2018).

All the objectives outlined in this chapter were successfully accomplished. The virus stock was propagated in *T. leucotreta* larvae, followed by extraction and purification. Multiple verification steps were performed to confirm the presence of the desired virus and the absence of contamination. All results indicated that the propagated virus was CrleGV, with no detectable presence of CrpeNPV, which is known to infect *T. leucotreta* (Marsberg, 2016; Marsberg et al., 2017), or only at quantities too low to be identified using the applied techniques. Additionally, the virus stock concentrations were adequate to support the subsequent experiments planned

for this study. The next chapter focused on designing and refining the methodology for the semi-field experiments detailed in Chapter 4.

Chapter 3

Semi-field experiment pilot study for experimental design and methodology refinement

3.1 Introduction

In the previous chapter, CrleGV was propagated in *T. leucotreta* larvae to generate a stock for use in semi-field experiments. The virus was extracted from infected larval cadavers and screened through various techniques to ensure the stock was uncontaminated. The next step was to conduct semi-field experiments where the virus produced in the previous chapter would be applied to fruit under both netted and unnetted structures to evaluate whether netting could protect CrleGV from UV degradation, which is the primary aim of this study. However, before the full-scale experiments could be conducted, a pilot study or small sample size study was necessary to refine the methodology.

This preliminary step was particularly important for two reasons. First, there are specific periods when sufficient quantities of oranges can be sourced from farms, making it crucial for the methodology to function effectively during this time to prevent wasting these limited opportunities. Secondly, once the experiment begins and insecticide is applied, the oranges cannot be reused to prevent contamination, ensuring accurate results and avoiding unnecessary costs and time wastage. Thus, this chapter focuses on designing the experiment and testing different tools and techniques to optimise the approach prior to initiating the full-scale study.

The key aspects of the study involve (1) applying the virus to fruit and (2) recovering the virus from the fruit. In essence, the methodology must ensure the virus is evenly distributed, adheres effectively to the fruit, and can later be recovered for use in surface dose-response bioassays against *T. leucotreta* neonates (see Chapter 4). This methodology will help determine if there are differences in mortality rates caused by the virus recovered from netted versus unnetted structures over time. The original protocol was adapted from Mwanza (2015), which is a modified version of that from Stevenson et al. (2010). This method was specifically chosen for its focus on the field application of CrleGV and its wash-off technique for sprayed fruit, both of which are integral to the study's objectives.

Several factors, such as the equipment and virus formulation, can affect the success of a virus application (Moscardi, 1999). Pesticide application methods in the field range from basic tools, such as spray bottles and simple pressure sprayers, to advanced equipment such as hand-gun spray machines, electrically powered devices, and fixed spraying systems (Futch, 2014; Chen et al., 2023). Selecting the right application tool is crucial to ensure the pesticide reaches the target with adequate coverage and at the appropriate rate (Futch, 2014). For a smaller-scale study (such as this pilot study), simpler equipment can be evaluated, including small spray bottles, sprayers, and the dip method using a beaker. Baculoviruses, unlike non-occluded insect viruses, are more resilient or can persist in the environment for extended periods, making them easier to formulate, apply, and store for long periods (Lacey et al., 2015; Grzywacz & Moore, 2017). While they can be applied without special formulations (Gan-Mor & Matthews, 2003), their effectiveness is often enhanced by incorporating additives, such as UV protectants, wetting agents, stickers, and phagostimulants (Burges & Jones, 1998). These can be included in the formulation or mixed into spray tanks before application.

Cryptogran, a commercial microbial insecticide containing CrleGV as its active ingredient, is registered for use with molasses and a surfactant and a wetting agent like BREAK-THRU® S240 (250 g/L Polyether, 750 g/L Polyether-modified trisiloxane) (Evonik Industries AG, Germany) (Moore et al., 2015b), hereafter simply referred to as Breakthru. Molasses is widely used in microbial insecticides, due to its multifunctionality, serving as a phagostimulant, sticker, and UV protectant (Burges & Jones, 1998). However, its role as a UV protectant is not well-supported. For instance, molasses provided no UV protection to Cryptogran when exposed to artificial UV light (Kirkman, 2007). On the other hand, molasses has been shown to act as a larval attractant. Ballard et al. (2000) found that the efficacy of CpGV in the field improved when applied with molasses. Similarly, Moore et al. (2015b) found that incorporating molasses into CrleGV notably, and in some cases significantly, improved its effectiveness against *T. leucotreta* on citrus, even at low concentrations of 0.5% and 0.25%. Mwanza et al. (2016) further observed that *T. leucotreta* larvae fed more actively on molasses-treated fruit compared to untreated fruit. More recently, Coombes et al. (2024) investigated the impact of molasses on *T. leucotreta* first instars and found that larvae exposed to molasses-treated oranges (at concentrations of 0.25% or 0.50%) exhibited reduced movement and penetration of the fruit compared to those on untreated oranges.

These studies suggest that molasses acts as a phagostimulant, promoting faster and prolonged feeding when combined with Cryptogran. Such an effect enhances virus uptake by the larvae, increasing virus-induced mortality, while also providing sufficient time for the virus to act before significant fruit damage occurs, ultimately helping to preserve yield and fruit quality (Mwanza et al., 2016; Coombes et al., 2024). Although there is limited recent literature on the use of molasses as a sticker for baculoviruses and it may have minimal impact on virus recovery (Coombes CA & Jukes MD, personal communication), molasses does alter spray viscosity, which affects droplet size and distribution (Burges & Jones, 1998). Additionally, the spray composition influences droplet spread upon impact, determining the thickness of the dried residue and, consequently, the level of protective coverage. These factors may potentially impact the recovery of virus particles (Jones, 1988; Burges & Jones, 1998).

In a study by Bennett (2022), detached fruit bioassays were used to compare a UV-tolerant isolate, and the CrleGV-SA isolate under natural UV exposure. No significant differences were found between the isolates, and it was suggested that OBs may not have adhered effectively to the fruit. This could be attributed to the absence of molasses and an adjuvant, which are part of the registered tank formulation. The study recommended including these components in future trials to improve outcomes. For this reason and because molasses and a surfactant were used in the original protocol (Mwanza, 2015), it is worth investigating whether the addition of molasses and a surfactant could enhance virus recovery.

The primary goal of this chapter was to outline the methodology for the full-scale study. The objectives were to (1) test the original protocol and modify it, if needed, (2) identify the most suitable virus application tool or technique for the experiment, (3) evaluate whether the virus should be applied alone, with molasses, with a surfactant, or with a combination of both, and (4) determine the required quantity of virus and fruit for the experiment.

3.2 Methodology refinement

3.2.1 Virus application and recovery from fruit

The methodology for virus application and subsequent recovery from fruit was adapted from Mwanza (2015) (see section 3.2.1.1 below). In the initial trial, the procedure was followed as described in the protocol, with adjustments made to the volumes of virus, water, and/or additives to align with the pilot study's standards. The primary objective at the start of the experiments was to ensure a sufficient concentration of the virus was recovered, exceeding 3×10^6 OBs/ml, which is the highest concentration reported by Jukes (2018) as necessary to achieve 90% mortality in *T. leucotreta* neonates (LC_{90}).

The second objective was to identify the most effective tools for maximising OB recovery in an efficient manner and to test if the addition of the molasses and/ or Breakthru is necessary.

3.2.1.1 Original protocol adapted from Mwanza (2015)

The experiments were conducted inside the laboratory under normal conditions (i.e., at room temperature). The oranges for the experiment were carefully cleaned using a small amount of dish soap, then rinsed with water, wiped, and allowed to dry overnight to eliminate any residues or contaminants. In a 1.5 L spray bottle (Figure 3.1a), 250 µl of purified virus (with a concentration of 5.84×10^{10} OBs/ml) was mixed with 999.75 ml of ddH_2O (Total 1000 ml). This mixture was sprayed onto the oranges placed in a plastic crate lined with a refuse bag underneath to avoid water runoff (Figure 3.2). After 24 hours, the virus was removed from the oranges using the following procedure: each orange was placed in a beaker and immersed in 50 ml of 1% sodium dodecyl sulphate (SDS) solution. The beakers were then sonicated for 1 min at 40% power for 5 cycles using a Bandelin sonicator (ProfiLab24 GmbH, Germany) to dislodge the virus. Next, the beakers were placed on a shaker and allowed to shake at 130 rpm for 30 min. Each orange was carefully removed, and the liquid was filtered through muslin cloth into a 50 ml tube. The filtered liquid was centrifuged in an Eppendorf 5804R desktop centrifuge at 2,934 ×g (4,000 rpm) for 15 min. After discarding the supernatant, the pellet was washed with sterile distilled water and centrifuged again under the same conditions. This washing step was repeated once more. Finally, the pellet was resuspended in 1 ml of ddH_2O and stored at 4°C.

To determine the concentration of the recovered virus, five samples were pooled together, and the OBs were counted using the enumeration protocol described in section 2.2.3.

A B

Figure 3.1: Spray bottles tested in the pilot study. A: Normal trigger spray bottle and B: Pesticide pump-up pressure spray bottle.

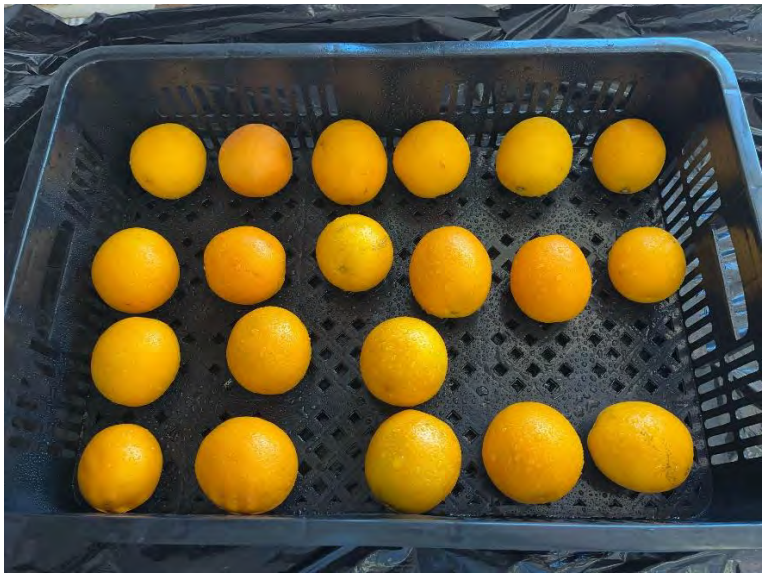


Figure 3.2: Oranges sprayed with purified CrleGV and water.

Outcome

The resulting pellet recovered from the oranges was far smaller than anticipated, indicating that a high number of OBs had been lost during the recovery process. Following resuspension of

the pellet and subsequent attempt to enumerate using dark field microscopy, no viral OBs were observed.

The experiment was, therefore, repeated with modifications to the original protocol, which was performed using an iterative process. It is important to note that in the case where no OBs were recorded or the concentration recovered was calculated to be less than 3×10^6 OBs/ml, the result was not reported here.

3.2.1.2 Iterative methodology development

Iteration 1

In the initial modification of the original protocol, the first adjustment involved reducing the volumes of the virus and water used. Specifically, the volume of purified virus was reduced from 250 µl to 62.5 µl, and the volume of ddH_2O was reduced from 999.75 ml to 249.94 ml. Furthermore, a second test was conducted whereby the virus volume was doubled while maintaining the same total volume of 250 ml made up with ddH_2O. The virus concentration used was 9.45×10^{10} OBs/ml, and this level was maintained across all iterations.

For the virus wash-off process, several changes were made, such as doubling the volume of the 1% SDS solution used to immerse the oranges. This adjustment ensured the oranges were fully submerged in the beaker, allowing the liquid to coat the fruit adequately.

Additionally, the effect of using muslin cloth for filtering the OBs was investigated to determine its impact on the number of OBs transferred to the 50 ml tube. For this, part of the liquid recovered from the oranges after shaking was filtered through the muslin cloth, while another portion was left unfiltered.

Outcome

The concentration of OBs following the modified recovery process remained very low with no major difference measured between the liquid that was filtered through the muslin cloth (1.89×10^3 OBs/ml) and that which was not (1.61×10^3 OBs/ml). It is important to note that bioassays were not conducted for all iterations due to time constraints.

Iteration 2

In the second modification, the volume of virus added was increased to 187.5 µl, while the total volume remained unchanged at 250 ml, made up using ddH_2O. The key adjustment in this attempt involved replacing the Bandelin sonicator (ProfiLab24 GmbH, Germany), previously used for dislodging the virus, with a sonicating probe (Vibra-Cell™ VC50-1 Ultrasonic Liquid Processor, Sonics & Materials, Inc, USA). This replacement improved the vibrations in the liquid, allowing the oranges to rotate more effectively during sonication and ensuring comprehensive treatment of the entire fruit. Additionally, the beaker shaking duration was extended from 30 minutes to 45 minutes, with the shaker speed increased during the final 15 minutes, from 130 rpm to 150 rpm.

Outcome

The OB concentration recovered following these modifications showed an increase in resulting virus as compared to the first protocol (iteration 1), however, it remained below the required target of 3×10^6 OBs/ml. The concentration recovered was 9.45×10^5 OBs/ml.

3.2.1.3 Final protocol

In the third and final iteration, the protocol was slightly adjusted from the original. Key modifications included increasing both the volume of virus added and the number of oranges used in the experiment, eliminating the rinse step, and raising the centrifugation speed.

The revised method involved spraying 10 oranges with 250 µl of purified virus mixed with 249.75 ml of ddH_2O (Total 250 ml). This was done in two layers: half the volume was sprayed initially, and the second half was applied an hour later after the first layer had partially dried. After 24 hours, the oranges were placed in either 500 ml or 600 ml glass beakers filled with 100 ml 1% SDS solution and sonicated using a sonicator probe (Vibra-Cell™ VC50-1 Ultrasonic Liquid Processor, Sonics & Materials, Inc, USA) at 60 Hz to ensure thorough treatment on all sides of the oranges. The beakers were then placed on a shaker, shaking at 130 rpm for 30 minutes, followed by 150 rpm for 15 minutes. The liquid from 10 oranges was pooled into a 1000 ml beaker, stirred with a clean glass rod, and filtered through a muslin cloth into four JA-14 250 ml Beckman centrifuge bottles. These bottles were centrifuged using a Beckman Coulter Avanti J-E refrigerated centrifuge at 10,500 ×g (12,500 rpm) and 4°C for 30

minutes. After centrifugation, 200 ml of the supernatant from each bottle was removed using a 50 ml sterile syringe. The bottles were then vortexed and mixed to ensure any pellet was resuspended. The remaining liquid was combined into a single clean centrifuge tube and centrifuged again at 2,934 ×g (4,000) at 4°C for 30 minutes. Following this, the supernatant was discarded, and 5–10 ml of the remaining liquid was transferred into a 15 ml tube for storage at 4°C.

To evaluate the application method, the procedure was repeated using a pesticide pressure sprayer (Figure 3.1b) and a dipping method (as described by Bennett, 2022) to determine which approach yielded better results. For the dipping method, oranges were placed in a 500 ml or 600 ml beakers containing 250 ml of either the virus treatment (250 µl of purified virus diluted in 249.75 ml of ddH_2O) or the control solution (ddH_2O). The fruit was immersed for approximately 20 seconds, turning after 10 seconds to ensure complete coverage. After 24 hours, the virus was washed off the fruit as described above.

The most effective application tool was then used to investigate the impact of molasses and the surfactant on virus recovery from the fruit. Experiments followed the final protocol with three treatments: (1) 625 µl of molasses, (2) 125 µl of Breakthru, and (3) a combination of 625 µl molasses and 125 µl Breakthru. Each treatment was combined with 250 µl of the purified virus and supplemented with 249.1 ml, 249.6 ml, and 249 ml of ddH_2O, respectively.

Outcome

Following virus enumeration of the recovered virus using the final protocol (just virus and ddH_2O), the concentration was calculated to be 7.83×10^9 OBs/ml. All application methods (i.e., the normal trigger spray bottle, pressure pump-up spray bottle and dipping method) produced comparable results (Table 3.1), but the pressure spray bottle was chosen for future experiments as it was easier to use compared to the trigger spray bottle, the nozzle can be altered to ensure that the right droplet size is distributed to the fruit and most importantly, it allows for a thorough mixing of the virus and water compared to the dipping method. This makes it the most effective tool for application in the full-scale study. Even though the treatment in combination with molasses and Breakthru had a slightly higher concentration of 9.83×10^9 OBs/ml (Table 3.1), other small particles were seen floating on the slide as viewed under darkfield microscopy, which made it difficult to count and differentiate between the virus

particles and those caused by molasses (particles were also observed in the virus, water and molasses mixture).

Table 3.1: Comparison of tools and treatments for optimising OB recovery efficiency.

	Treatment	Concentration of OBs recovered (OBs/ml) ± 95% CI
Application method	Trigger spray bottle	$7.67 \times 10^9 \pm 3.03 \times 10^8$
	Pressure pump-up spray bottle	$7.83 \times 10^9 \pm 3.07 \times 10^8$
	Dipping method	$7.33 \times 10^9 \pm 2.97 \times 10^8$
Formulation method	Virus + water	$7.42 \times 10^9 \pm 2.98 \times 10^8$
	Virus + water + molasses	$9.08 \times 10^9 \pm 3.30 \times 10^8$
	Virus + water + Breakthru	$7.50 \times 10^9 \pm 3.00 \times 10^8$
	Virus + water + molasses + Breakthru	$9.83 \times 10^9 \pm 3.44 \times 10^8$

3.3 Discussion and recommendations

Pilot studies are commonly utilised across scientific fields as preparatory steps for larger research projects (Polit et al., 2001). They play a vital role in identifying potential problems that could jeopardise the success of the main study, making them a critical aspect of effective study design (van Teijlingen & Hundley, 2001; Thabane et al., 2010). The study outlined in this chapter aimed to refine and evaluate the methodology before conducting the full-scale study. The primary objectives were: (1) testing and modifying the virus application and recovery methods adapted from Mwanza (2015), (2) determining the appropriate volumes of virus and quantities of fruit required, (3) identifying the most effective application tool, and (4) assessing whether the virus should be applied with or without additives.

The experiment followed the protocol described by Mwanza (2015), but the outcomes were not as expected. Several modifications were made, ultimately resulting in a virus concentration sufficient for future experiments. The main differences between the original and modified

protocols included adjustments to the virus volume, the quantity of fruit used, the volume of SDS solution for virus recovery, the centrifugation speed and duration, and the wash steps.

The modified protocol required more virus, likely because the virus was purified, unlike the one used as a commercially formulated suspension, as in the original protocol. While GVs strongly adhere to leaf surfaces and are resistant to being washed off by rain (David & Gardiner, 1966; Entwistle & Evans, 1985; Young & Yearian, 1986; Burgerjon, 1989; Kirkman, 2007), purifying virus suspensions can weaken this adhesion. In unpurified suspensions, insect proteins and debris act as natural adhesives, improving their stickiness and significantly reducing wash-off—by 2 to 10 times—even after exposure to up to 13 cm of rainfall (Jones, 1988; Entwistle & Carruthers, 1989). Consequently, a higher quantity of purified virus was necessary to ensure adequate recovery. During the initial virus recovery stage, oranges were immersed in 50 ml of 1% SDS solution, which was insufficient to coat even half of the fruit, potentially hindering the removal of OBs. The volume of SDS was therefore doubled, and the number of oranges pooled into a single sample was also increased to maximise OB recovery. Additionally, initial centrifugation yielded little to no visible pellet. To address this, a higher centrifugation speed and longer duration were implemented, improving the separation of virus OBs from the SDS solution. Since the pellet size was still very small, even with the modified protocol, the wash steps using ddH_2O were eliminated and a syringe was used to carefully remove the supernatant, with all remaining liquid pooled prior to a final centrifugation step to obtain the stock.

The pilot study also investigated whether additives such as Breakthru and molasses could improve OB recovery. Although the results showed that the addition of molasses and molasses and Breakthru did improve recovery, the increase in recovery was small, leading to the decision to use only virus and water in subsequent experiments. While unformulated viruses are rarely used in the field, they can still produce favourable outcomes. For example, unformulated CrleGV was tested by Moore et al. (2015b), and despite the lack of formulation, it achieved up to 82% reduction in *T. leucotreta* larvae infestation on fruit. However, the virus application rates in these experiments were very high, up to approximately 10^{15} OBs/ha, significantly exceeding the rates typically used in commercial application and in trials involving formulated viruses with adjuvants.

While pilot studies are essential for optimising study design and increasing the likelihood of success in the main study (van Teijlingen & Hundley, 2001; Thabane et al., 2010), they do not

guarantee it. Nonetheless, conducting a pilot study in this case was valuable, as it highlighted the need for modifications to the original protocol, ultimately saving time, effort, and resources. The next chapter will present the full-scale study, including semi-field experiments conducted using the modified protocol. Surface dose bioassays were performed to assess virus virulence or stability under netted and unnetted conditions across various time points.

Chapter 4

Semi-field experiments and bioassays: full-scale study

4.1 Introduction

Shade nets are being increasingly adopted across various citrus production regions in South Africa. However, their impact on the field efficacy of entomopathogens, such as CrleGV, particularly in terms of protection against sunlight, remains untested. Therefore, this chapter aimed to evaluate the extent to which shade nets protect CrleGV from UV radiation compared to its application without shade nets. This was investigated through semi-field experiments and surface dose bioassays to further assess the virulence of CrleGV over time.

The application of CrleGV for managing *T. leucotreta* commenced in the year 2000. Between 2000 and 2015, more than 50 field trials were conducted to evaluate its effectiveness against *T. leucotreta* on citrus in South Africa (Moore, 2002; Moore et al., 2004; Kirkman, 2007; Moore et al., 2015b). The trials conducted in the first three years resulted in the registration of the first commercially available CrleGV-based biopesticide, Cryptogran, in 2004 for use on citrus, avocadoes, grapes and other crops (Moore et al., 2015b). A sample of 13 field trials conducted over this 15-year period showed that its efficacy, determined by the reduced number of larvae infesting the fruit, ranged between 30% and 92%. Moreover, the efficacy remained at a level of 70% for up to 17 weeks after application. (Moore et al., 2015b). This was, however, the best possible outcome and was only achieved when the virus was applied in blocks rather than on individual trees (Moore et al., 2015b). While achieving the highest possible level of efficacy is important, it is just as essential to ensure that the efficacy of the applied virus remains effective on the plant surface for an extended period (Grzywacz & Moore, 2017). This is particularly important considering the natural environmental factors such as UV radiation, heat, plant architecture, plant chemistry, wind and rain, all of which can limit the persistence of baculoviruses in the field (Grzywacz & Moore, 2017; Moore & Jukes, 2019). Among these factors, UV radiation is the most damaging (Shapiro, 1995) and has the greatest impact on whether a baculovirus product maintains its effectiveness in the field (Grzywacz & Moore, 2017).

The half-life of CrleGV in the field is estimated to be two to three days (Fritsch & Huber, 1989). However, Moore (2002) showed that CrleGV lost more than 50% of its initial activity within three to six days. This was recorded on the northern (sunny) side of the citrus trees, while on the southern (shady) side, the activity had not decreased as much, even 21 days after application. Mwanza (2015) later supported this in an investigation to determine the reapplication frequency of CrleGV in the field. Mwanza found that the LD_{50} (the lethal dose required to cause the death of 50% of *T. leucotreta* neonates) of CrleGV recovered from the northern sides of trees was 15 times higher than that from the southern sides at 21 days after application (Mwanza, 2015; Mwanza et al., 2016). Additionally, the virulence of CrleGV on the northern sides of trees was undetectable, while on the southern sides, a clear dose-response was still evident 28 days after application.

Grzywacz & Moore (2017) highlighted that the necessity for UV protection of a virus depends on the plant's architecture and the feeding location of the pest. Dickler & Huber (1988) found that for codling moth GV to be effective against codling moth on apples, reapplication was required every 7–14 days, a frequency significantly higher than that of Cryptogran on citrus. Moore et al. (2004) proposed four reasons for this difference and the two primary reasons were that the dense canopy of citrus trees limits solar radiation exposure compared to other crops where viruses have been tested, and observations during the growing season showed that *T. leucotreta* larvae enter navel oranges through the navel end, and this is exactly where the CrleGV could be protected from sunlight and perhaps even rainfall. The high efficacy of over 70% recorded over a 17-week period in navel orchards may partly be attributed to this factor (Moore et al., 2015b; Grzywacz & Moore, 2017). However, challenges arise with young, smaller trees, which provide less shade due to their sparse canopy. Additionally, citrus cultivars other than navel oranges lack a navel end where OBs can be protected from direct sunlight and where *T. leucotreta* larvae preferentially penetrate (Moore et al., 2015b; Mwanza et al., 2022). Many studies have shown that baculovirus activity can be enhanced by UV protectants (e.g., Dougherty et al., 1996; Farrar et al., 2003; Asano, 2005, Arthurs et al., 2006; Wu et al., 2015) and possibly the selection of a UV-resistant isolate (e.g., Mwanza, 2019; Mwanza et al., 2022). However, these results were obtained under laboratory conditions and there is currently little evidence to support that this may have a significant impact in the field under commercial operations (Arthurs et al., 2008; Moore et al., 2015b). Therefore, it is crucial to explore the potential effects of shade nets, as they could impact CrleGV efficacy by modifying environmental conditions such as UV radiation exposure, which influences the virus's stability,

persistence and virulence. The term virulence has been defined in various ways across different scientific disciplines (Thomas & Elkinton, 2004; Shapiro-Ilan et al., 2005). In this study, it is defined, according to Thomas & Elkinton (2004), as the proportion of dead individuals relative to those infected. Virulence is quantitative (Burpee, 1983) and can be assessed through bioassays (Thomas & Elkinton, 2004).

Jones (2000) provided an overview of the various bioassays that can be conducted for entomopathogenic viruses to evaluate their virulence against target pests. Bioassays of baculoviruses are essentially used to examine the interaction between the virus and insect by measuring factors such as overall mortality or the rate at which the insect is killed (Cory & Bishop, 1997). Selecting the appropriate bioassay is crucial, particularly when developing new or improved biological control strategies (Bennett, 2022). Both laboratory and field bioassays play an essential role, but there are often disparities between the two (Moore et al., 2011). According to the Hajek & Butler (2000), many comparisons of laboratory, which they refer to as physiological host range, and field, which they refer to as ecological host range are anecdotal. They point out that in many cases, there is a lack of systematic experimental evidence linking the two, and much of the available information comes from isolated observations or case studies rather than controlled, replicated research. These variations often arise due to environmental factors such as wind, rain, or UV exposure, which are typically absent in laboratory conditions. Semi-field assays, including detached fruit bioassays, are commonly used to assess factors like the impact of novel pathogens, new pesticides, or the addition of adjuvants on the control of pests (for example Moore et al., 2011; Fullard & Hill, 2013; van der Merwe et al., 2022; Coombes et al., 2024). These are frequently selected as they better simulate field conditions than do laboratory tests, while minimising interference from external abiotic and biotic factors, such as birds and spray programmes in the orchard. Detached fruit bioassays generally involve placing the targetable feeding stage (often neonates) of the target pest on fruit to evaluate penetration. However, in this study, this approach is not used. Instead, the virus was applied to the fruit, and its effectiveness was assessed through dose-response bioassays.

There are three main types of dose-response bioassays: droplet feeding, diet incorporation, and surface dose bioassays (Grzywacz et al., 2004). The primary distinction among these methods lies in how the virus is administered to the larvae, with each approach having its own advantages and limitations. In the droplet feeding method, larvae are starved before being given a small droplet of virus mixed with a brightly coloured dye. This technique increases the

likelihood that larvae consume the entire virus droplet and allows for precise identification of individuals that have ingested the virus through the presence of the dye, thereby enhancing the accuracy of the assay (Pereira-da-Conceição et al., 2012). However, a major drawback is that starvation induces stress, which can affect larval physiology. Additionally, this method can be difficult to apply to very small larvae, such as *T. leucotreta* neonates. Diet incorporation involves mixing the virus directly into the diet, which the larvae then consume (Fritsch et al., 2007). This approach is suitable for small larvae, however, the amount of virus ingested increases as feeding continues, making it challenging to quantify their exact exposure. In surface dose bioassays, the virus is applied to the surface of the diet, and larvae are carefully placed to feed on it. The larvae become infected upon initiating feeding, receiving a single dose of the virus as they burrow through the surface. Similar to diet incorporation, surface dose assays are suitable for small larvae, but it is difficult to accurately determine the amount of virus ingested by the larvae. However, this method has the advantage of inducing less stress on the larvae and more closely resembles the field application of pesticides. Surface dose bioassays have been widely used for various baculoviruses, including CrleGV against *T. leucotreta*, making it the preferred method for this study.

Bioassay data can be analysed using various methods, depending on the type of bioassay conducted. A dose-response curve is often used to illustrate the relationship between dosage and insect response, allowing for the calculation of parameters such as lethal concentration (LC), lethal dose (LD), or lethal time (LT) to assess virulence of the virus (Cory & Bishop, 1997; Grzywacz et al., 2004). Since this study will utilise surface dose response bioassays, LC values (LC_{50}) will be determined to evaluate the correlation between the virus concentration and insect mortality.

This chapter aimed to assess the impact of netting on CrleGV through two separate experiments: a long-term study (Experiment 1) and a short-term study (Experiment 2). The main objectives included: (1) evaluating CrleGV exposure in semi-field conditions under both netted and unnetted structures using virus-treated fruits, (2) virus recovery both for Experiment 1 and Experiment 2 following natural UV exposure, (3) quantifying the recovered virus, and (4) conducting surface dose bioassays to assess the virulence of recovered virus.

4.2 Materials and methods

4.2.1 Semi-field exposure of CrleGV under netted and unnetted structures

4.2.1.1 Building netted and unnetted structures

Experiments took place at the Waainek mass rearing facility at Rhodes University, located in the Eastern Cape province. White shade nets, supplied by Haygrove Tunnels (Haygrove South Africa, Pty Ltd), were used to construct three netted cages (462 cm × 276 cm × 163.5 cm) (Figure 4.1a). The top nets offered 20% shading, while the side nets provided 40% shading. The mesh size and shading percentage used in this study align with the standards set by Agri-Netting (Marsberg et al., 2024). Within each netted cage, a galvanised table was set up for pesticide application on fruit. For the non-netted treatments, three galvanised tables (242 cm × 81.5 cm × 70 cm) were covered with a hex netting (chicken wire) (Figure 4.1b) to prevent birds or other small mammals from interfering with the experiment. All the tables were washed using dishwashing liquid, a scrubbing brush and a pressure sprayer prior to conducting the experiment.

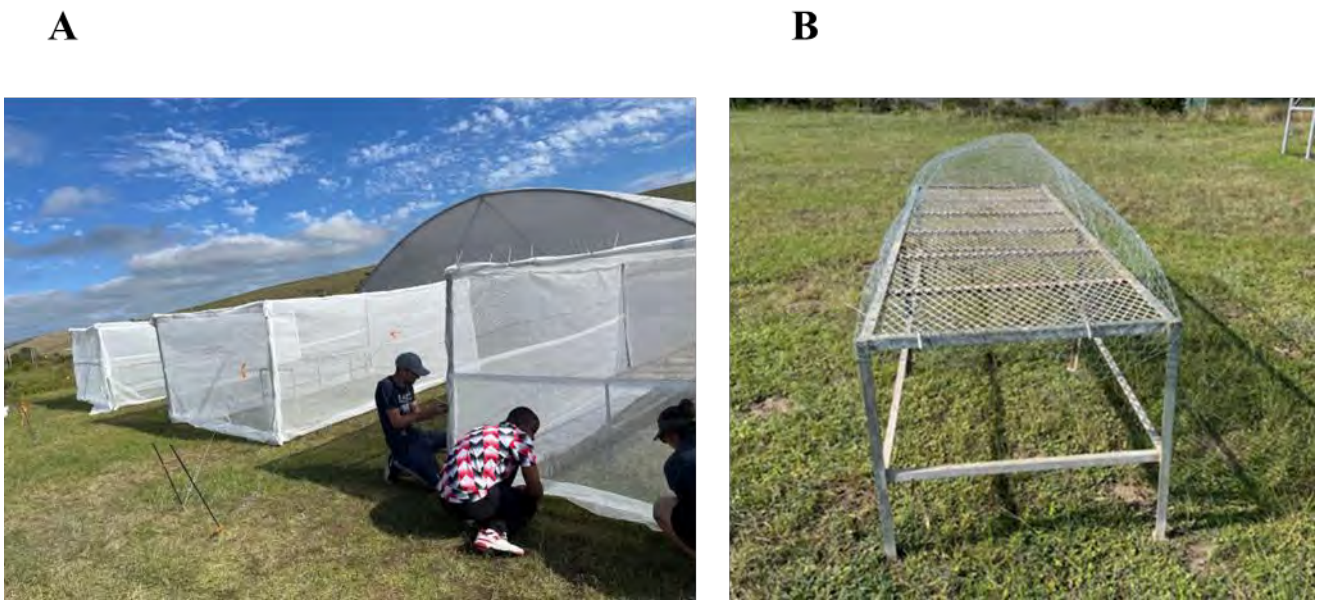


Figure 4.1: Cages used for the experiment: (A) Three netted cages (462 cm × 276 cm × 163.5 cm) with 20% shading on top and 40% shading on the sides, (B) galvanised table (242 cm × 81.5 cm × 70 cm) covered with a hex netting for the unnetted treatment.

4.2.1.2 Fruit collection and preparation

For Experiment 1, oranges from Orangelus Farm, orchard 66, in Sundays River Valley, Eastern Cape, South Africa, were used. The selected fruits were late navel oranges (Cambria cultivar)

that were still green in colour and smaller to ensure that they lasted longer in the field and fitted into the beakers used during the virus recovery process. For Experiment 2, Valencia oranges were purchased at local grocery stores, and these were yellow and larger in size.

To remove any residues or contaminants, the oranges were thoroughly washed with water and a small amount of dishwashing liquid, rinsed with water, wiped, and left to dry overnight. They were then placed on galvanised tables under both netted and non-netted structures. Each table contained both treated and untreated fruit, separated by a rebar steel rod wrapped in a refuse bag. For the long-term experiment (Experiment 1), a total of 120 oranges were placed on each table, calculated as follows: 10 fruit per treatment (plus 5 extra) at four intervals (60 fruit), and 10 fruit per control (plus 5 extra) at four intervals (60 fruit). For the short-term experiment (Experiment 2), 3 extra oranges were included per treatment in the four intervals (total per table = 104 oranges). Additional oranges were placed to mitigate any loss due to decay and ensure that sufficient fruit was available for downstream virus recovery procedures.

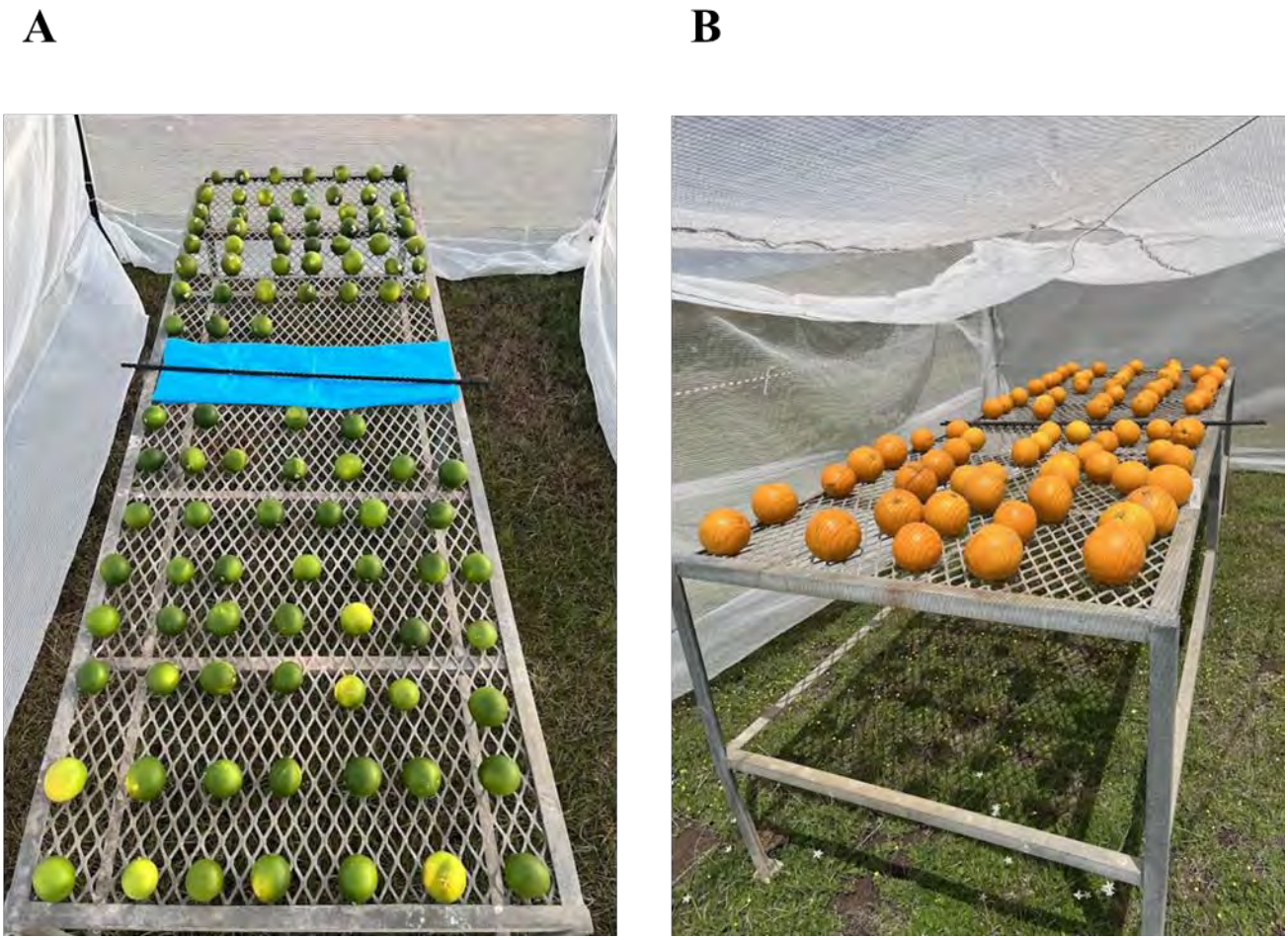


Figure 4.2: A layout of the oranges on the tables, (A) oranges used in Experiment 1 and (B) oranges used in Experiment 2.

4.2.1.3 Virus preparation and application

In a pesticide pressure spray bottle, 750 µl of the purified virus (CrleGV-1 for Experiment 1 or ClreGV-2 for Experiment 2) described in Chapter 2 was mixed with 749 ml of type 2 water (dH_2O). For Experiment 1, spraying took place in the late afternoon at 18:00 on February 15, 2024, following Kirkman's (2007) recommendation to spray later in the day to minimise sunlight inactivation. The process involved placing all the fruit designated for the treatments on the tables, excluding the control fruit (Figure 4.3a). The virus-water mixture was then sprayed onto the fruit to run off, while a plastic bag was positioned upright to shield the area in which the control fruits were to be placed (Figure 4.3b) from potential spray drift. Tables/cages were labelled from 1 to 6, and the spraying proceeded sequentially from the first to the last table. A second application of the virus (i.e., a second layer similar to the pilot study), using the same volumes as before, was then applied. Once spraying was completed, the control fruits were placed on the tables and sprayed solely with dH_2O. Sampling was conducted as follows: 20 fruits (10 treated and 10 control) were randomly collected from each table on days 1, 7, 14, and 21 post-application at approximately 13:00.

For Experiment 2, spraying was carried out on October 25, 2024, following the same procedure as Experiment 1. However, fruit collection occurred on days 1, 3, 5, and 7 after spraying at approximately 13:00.

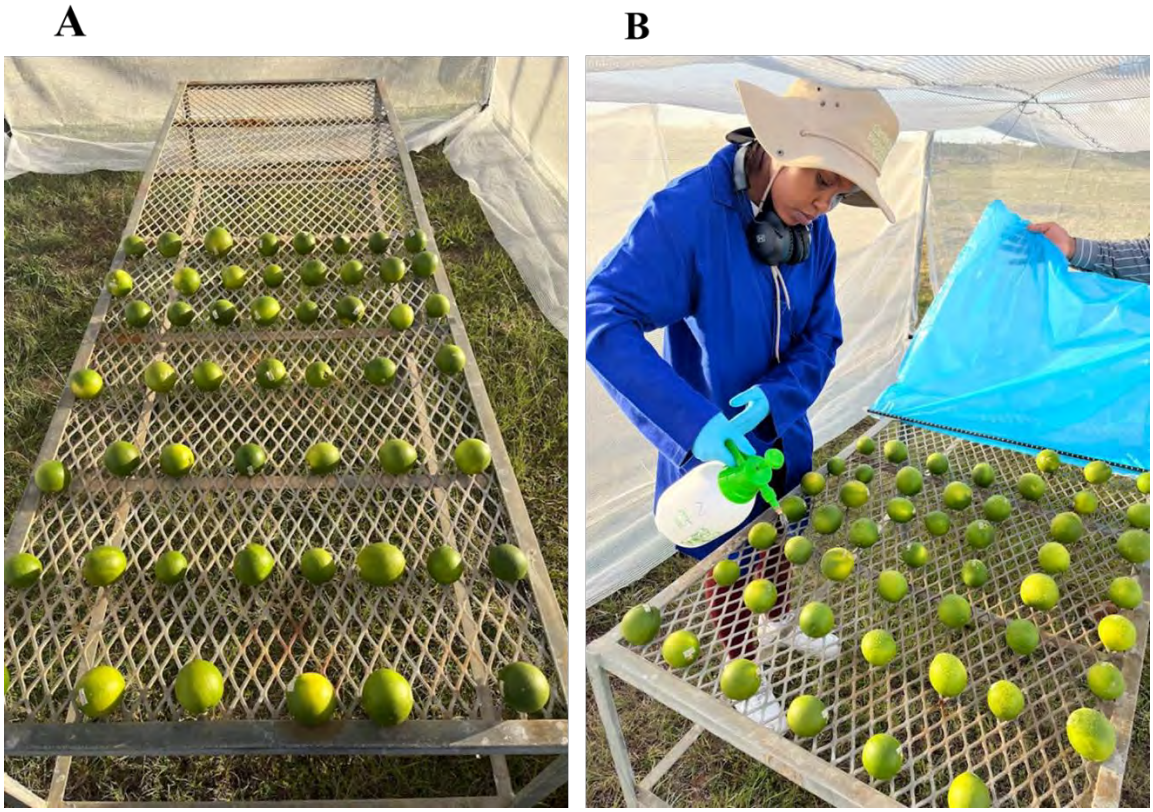


Figure 4.3: Virus spraying procedure: (A) The fruit designated for treatment was first placed on the table and sprayed, with a plastic barrier positioned upright to limit virus dispersion onto the non-treatment side (B).

4.2.2 Virus recovery

The virus was recovered according to the virus wash-off protocol described in Chapter 3, section 3.1.2.3. Each fruit was placed in 500/ 600 ml glass beakers to which 100 ml of 1% SDS solution was added. This was sonicated using a sonicator probe (Vibra-Cell™ VC50-1 Ultrasonic Liquid Processor, Sonics & Materials, Inc, USA) at 60 Hz for about 2-4 min or until all sides of the fruit were covered. The beaker was then transferred to a shaker and allowed to shake for 30 min at 130 rpm, followed by 150 rpm for 15 min. Subsequently, the fruit was removed from the beaker and using a muslin or cheese cloth, the liquid was filtered into a 1000 ml beaker. Liquid collected from 10 fruit was pooled into one tube to make one sample. The liquid was homogenised using a clean rod and then equally divided into four 250 ml JA-14 Beckman tubes. The tubes were centrifuged at 10,500 ×g (12,500 rpm) for 30 min at 4°C. The supernatant was carefully discarded using a 50 ml syringe until about 10-15 ml of the suspension was left. The tubes were vortexed and mixed to fully resuspend any pellet. The

resulting residue was then pooled in a JA-20 tube and centrifuged at 2,934 ×g (4000 rpm) for 30 minutes at 4°C. The majority of the supernatant was discarded using a pipette, leaving about 5-10 ml of the suspension, which was used to resuspend any pellet before being transferred into a 15 ml centrifuge tube.

Slight adjustments were made in Experiment 2 with the centrifugation speed and duration. The duration was increased from 30 minutes to 45 minutes, and both centrifugation rounds were at the speed of 10,500 ×g (12,500 rpm). These modifications were implemented to enhance the final concentration of the recovered virus.

4.2.3 Virus enumeration

The virus samples recovered from the oranges, including the control, were quantified following the protocol outlined in Chapter 2, Section 2.2.3. The virus samples were prepared using 0.07% SDS in ddH_2O at a single dilution factor of 1000×. Initially, 10 µl of virus OBs were diluted in 40 µl of sterile ddH_2O in a 1.5 ml tube, making a 1:5 dilution (D1 – Total 5× Dilution). The mixture was vortexed for approximately 5 seconds to achieve a homogeneous suspension. A subsequent 1:5 dilution (D2 – Total 25× Dilution) was prepared by adding 200 µl of 0.07% SDS solution to D1, followed by shaking for homogenisation. The sample was then sonicated using a probe at 60 Hz in four bursts of 15 seconds each (not exceeding 60 seconds in total) to prevent virus particle damage. The sample was kept on ice during sonication to minimise heating. For further dilution, 100 µl of the sonicated sample was added to 900 µl of ddH_2O in a new 1.5 ml tube, resulting in a 1:10 dilution (D3 – Total 250× Dilution). The solution was vortexed to ensure uniformity. To achieve the final 1000× dilution, 125 µl of D3 was combined with 375 µl of ddH_2O in a fresh tube and vortexed again. A 5 µl aliquot of the final dilution was pipetted onto a clean Thoma bacterial counting chamber, covered with a coverslip, and left undisturbed for 5 minutes to allow non-virus particles to settle through Brownian motion. Only vibrating virus particles were counted under 400× magnification (40× objective) using dark-field microscopy. Triplicate counts were performed, and the concentration was calculated using Equation 2.1.

4.2.4 Dose response bioassays

4.2.4.1 Thaumatotibia leucotreta egg preparation

The egg sheets used in this experiment were provided by the insect-rearing facility at the Department of Zoology and Entomology, Rhodes University. The insects were reared on an artificial maize-based diet under controlled laboratory conditions (Moore et al., 2014). Adult female moths oviposit eggs on wax paper which was cut into small rectangles and surface sterilised by dipping in a mixture of 0.4% spore kill (poly-dimethyl-ammonium chloride; Hygrotech International, South Africa) and 0.15% liquid bleach (NaOCl) for 1 min before rinsing in ddH_2O for 5 min before performing the bioassays to prevent contamination from other pathogens. The egg sheets were then placed in petri dishes and left under a laminar flow cabinet to dry. Once dry, each petri dish was sealed with Parafilm® and incubated in a CE room at 25°C.

4.2.4.2 Thaumatotibia leucotreta diet preparation

The *T. leucotreta* artificial diet (as described in Moore et al., 2014) was combined with ddH_2O in a 1:1 ratio in a glass Pyrex dish. The dish was covered with tin foil and placed in an oven to bake at 200°C for 20 min. Thereafter, the baked diet was removed and allowed to cool under a laminar flow before use.

4.2.4.3 Virus dose preparation

The quantified virus stocks were used to prepare the doses for the surface dose-response bioassays. Since the virus stocks had varying concentrations, working suspensions (W1 and W2) were used to adjust the stock volumes to ensure a consistent concentration across all doses (Table 4.1). The concentrations applied were established based on preliminary experiments conducted as part of this study. Two controls were included C1, representing the control from recovered fruit (to detect any potential contamination of the fruits occurring during the spraying process), and C2, containing only ddH_2O (negative control). The dilution steps are illustrated in Figure 4.4, and the final concentrations are presented in Table 4.1.

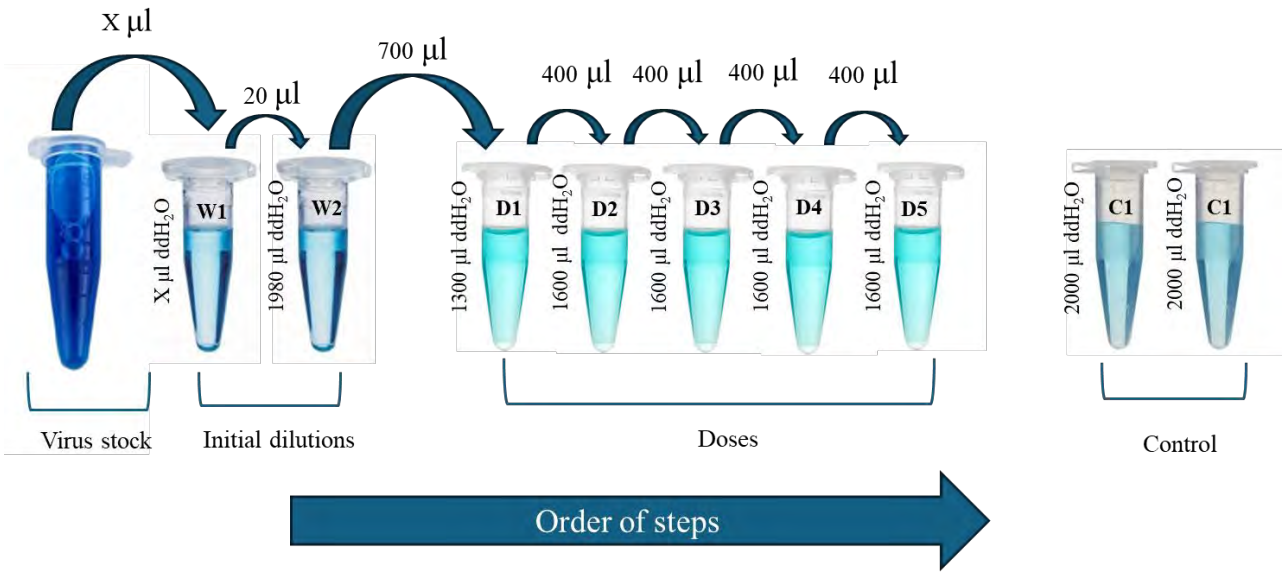


Figure 4.4: Preparation of the doses for surface dose-response bioassays against *T. leucotreta* neonates. X denotes different volumes used to prepare a standardised suspension (across all samples), based on the concentration of the virus stocks. W stands for working suspension. C1 represents control recovered from fruit and C2 represents control with ddH_2O. D1 to D5 represents virus doses 1 to 5.

Table 4.1: Concentrations used for surface dose bioassays against *T. leucotreta* neonates for Experiments 1 and 2.

Dilutions	Concentration (OBs/ml)
Working suspension 1	8.13×10^8
Working suspension 2	8.13×10^6
Dose 1	2.84×10^6
Dose 2	5.69×10^5
Dose 3	1.14×10^5
Dose 4	2.28×10^4
Dose 5	4.55×10^3
Control 1 (Recovered from fruit)	0
Control 2 (ddH_2O)	0

4.2.4.4 Surface dose-response bioassays

Surface dose-response bioassays were conducted following protocols adapted from Moore et al. (2011) and Jukes (2018). The *T. leucotreta* diet was prepared as described in Section 4.2.4.2. A modified 10 ml syringe, cut at the 2–3 ml mark, was used to portion the diet into sterile 24-well plates. Five doses, as shown in Table 4.1, were prepared from the recovered virus, along with two controls, one containing ddH_2O and another with water recovered from the control fruit. Each dose or control treatment was applied to a 24-well plate, with each well receiving 50 µl aliquots of the respective treatment using a pipette. The plates were then placed under a laminar flow hood to allow slight drying, preventing excess moisture on the diet. Newly hatched neonate larvae (less than 18 hours old) were individually placed onto the surface of the treated diet in each well using a fine paintbrush (R000) or sterile soft forceps, with plates then covered with a glass sheet, a plastic lid, and sealed with Parafilm®. The plates were carefully labelled and incubated in a CE room at 25°C. After 10 days, the diet was carefully inspected for live and dead larvae, with the number of dead larvae recorded for each treatment.

4.2.5 Statistical analysis

Weather data for each natural exposure of CrleGV-1 and CrleGV-2 was obtained from the National Centers for Environmental Information (NOAA) (Asheville, USA) and analysed using Microsoft Excel. A Generalised Linear Model paired *t*-test was conducted to evaluate differences between the two controls in both Experiment 1 and Experiment 2. Statistical significance was set at $P < 0.05$ to determine differences between controls or treatments.

Mortality data from the bioassays was analysed using Microsoft Excel. For each treatment, the mean mortality rate was calculated, and trendlines were generated to identify mortality patterns across both experiments for comparative analysis. To determine and compare the effective doses (LC_{50}) between netted and non-netted treatments in Experiment 2, data was analysed using R (version 4.4.3) and RStudio (version 2024.12.0+467, © 2009-2024 Posit Software, PBC), employing the "drc" package (Ritz et al., 2015) with the Weibull model (Weibull, 1951). Enumeration data was analysed in R and RStudio using an ANOVA followed by pairwise comparison using the emmeans package (Searle et al., 1980). Statistical significance levels were defined as follows: $P < 0.05$ ("*", marginally significant), $P < 0.01$ ("**", significant), $P < 0.001$ ("***", highly significant), and $P \geq 0.05$ (not significant), to determine which treatments differed significantly at the level of LC_{50}. Additionally, the "ggplot2" package

(Wickham, 2016) was used to generate a mortality curve from the analysed data, and the "dplyr" package was utilised to restructure and organise the dataset.

4.3 Results

4.3.1 Weather data

The minimum and maximum temperature, along with precipitation data, were obtained from the National Centers for Environmental Information (NOAA). In Experiment 1, the highest recorded temperature was 36.7°C on days 14 and 15 (Figure 4.5). Rainfall occurred on days 8 and 10, measuring 0.04 mm and 0.16 mm, respectively. For Experiment 2, the highest temperature recorded was 27.8°C on the final day (day 7) (Figure 4.6). Rainfall was recorded on days 4 and 5, with measurements of 0.75 mm and 1.14 mm, respectively.

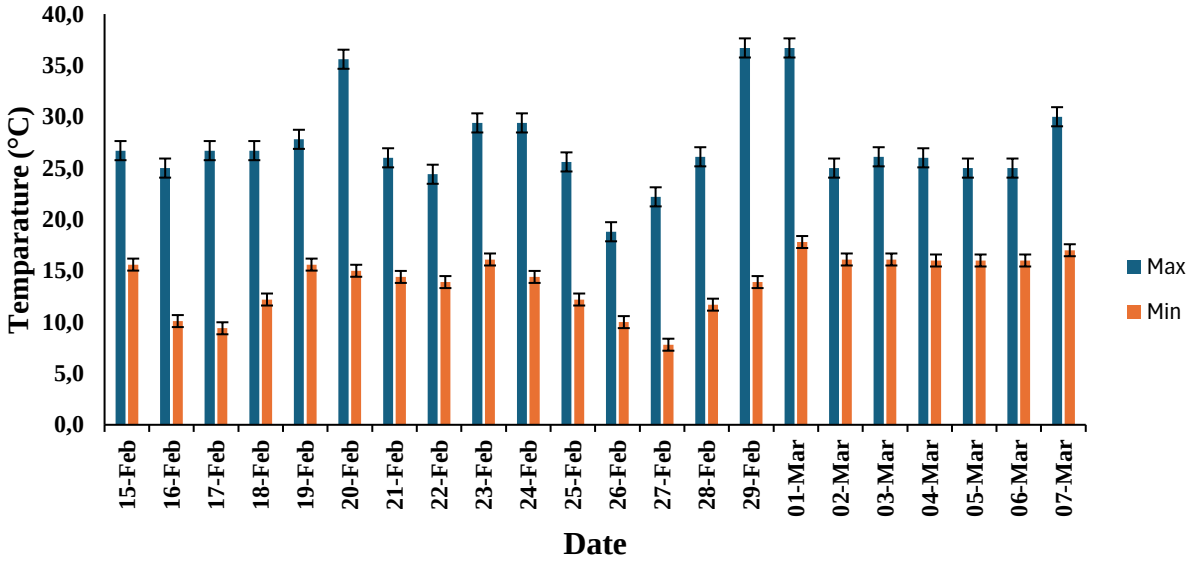


Figure 4.5: The recorded minimum (±SE) and maximum (±SE) temperatures during the UV exposure period for Experiment 1.

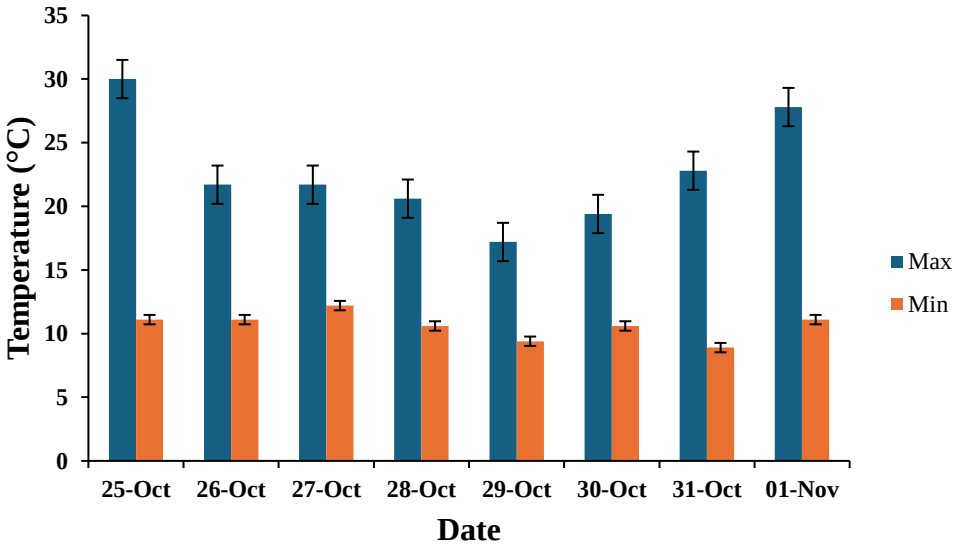


Figure 4.6: The recorded minimum (±SE) and maximum (±SE) temperatures during the UV exposure period for Experiment 2.

4.3.2 Enumeration of recovered viral stocks

The virus was recovered from the fruit at 1, 7, 14, and 21 days post-spray in Experiment 1, and at 1, 3, 5, and 7 days post-spray in Experiment 2. The recovered viral stocks were quantified using darkfield microscopy (see Section 2.2.3), and their concentrations for both Experiment 1 and Experiment 2 are presented in Tables 4.2 and 4.3, respectively. No significant differences ($P > 0.05$) in the concentration of virus recovered from fruit in Experiment 1 (Table 4.2) or Experiment 2 (Table 4.3) were measured.

Table 4.2: Concentrations of recovered CrleGV-1viral stock (8.13×10^{10} OBs/ml) at different time intervals in the long-term Experiment 1, under netted and unnetted treatments, with 95% confidence intervals.

Time (days)	Condition	Treatment	Concentration (OBs/ml) ± 95% CI
Day 1	Netted	Cage 1	$7.42 \times 10^9 \pm 2.98 \times 10^8$
		Cage 2	$1.75 \times 10^9 \pm 1.45 \times 10^8$
		Cage 3	$3.17 \times 10^9 \pm 1.95 \times 10^8$
	Unnetted	Cage 4	$5.33 \times 10^9 \pm 3.58 \times 10^8$
		Cage 5	$1.58 \times 10^9 \pm 1.38 \times 10^8$
		Cage 6	$1.17 \times 10^9 \pm 1.18 \times 10^8$
Day 7	Netted	Cage 1	$2.42 \times 10^9 \pm 1.70 \times 10^8$
		Cage 2	$1.83 \times 10^9 \pm 1.48 \times 10^8$
		Cage 3	$1.92 \times 10^9 \pm 1.52 \times 10^8$
	Unnetted	Cage 4	$4.00 \times 10^9 \pm 2.19 \times 10^8$
		Cage 5	$3.08 \times 10^9 \pm 1.92 \times 10^8$
		Cage 6	$3.00 \times 10^9 \pm 1.90 \times 10^8$
Day 14	Netted	Cage 1	$3.33 \times 10^9 \pm 2.00 \times 10^8$
		Cage 2	$3.17 \times 10^9 \pm 1.95 \times 10^8$
		Cage 3	$2.00 \times 10^9 \pm 1.55 \times 10^8$
	Unnetted	Cage 4	$2.42 \times 10^9 \pm 1.70 \times 10^8$
		Cage 5	$3.42 \times 10^9 \pm 2.03 \times 10^8$
		Cage 6	$2.67 \times 10^9 \pm 1.79 \times 10^8$
Day 21	Netted	Cage 1	$2.58 \times 10^9 \pm 1.76 \times 10^8$
		Cage 2	$1.33 \times 10^9 \pm 1.27 \times 10^8$
		Cage 3	$1.08 \times 10^9 \pm 1.14 \times 10^8$
	Unnetted	Cage 4	$2.00 \times 10^9 \pm 1.55 \times 10^8$
		Cage 5	$2.50 \times 10^8 \pm 5.48 \times 10^7$
		Cage 6	$6.67 \times 10^8 \pm 8.95 \times 107$

Table 4.3: Concentrations of recovered CrleGV-2 viral stock (2.69×10^{11} OBs/ml) at different time intervals in the short-term Experiment 2, under netted and unnetted treatments, with 95% confidence intervals.

Time (days)	Condition	Treatment	Concentration (OBs/ml) ± 95% CI
Day 1	Netted	Cage 1	$8.08 \times 10^9 \pm 3.12 \times 10^8$
		Cage 2	$7.50 \times 10^9 \pm 3.00 \times 10^8$
		Cage 3	$7.83 \times 10^9 \pm 3.07 \times 10^8$
	Unnetted	Cage 4	$6.08 \times 10^9 \pm 2.70 \times 10^8$
		Cage 5	$6.92 \times 10^9 \pm 2.88 \times 10^8$
		Cage 6	$7.33 \times 10^9 \pm 2.97 \times 10^8$
Day 3	Netted	Cage 1	$6.08 \times 10^9 \pm 2.70 \times 10^8$
		Cage 2	$5.33 \times 10^9 \pm 2.53 \times 10^8$
		Cage 3	$5.25 \times 10^9 \pm 2.51 \times 10^8$
	Unnetted	Cage 4	$4.75 \times 10^9 \pm 2.39 \times 10^8$
		Cage 5	$5.25 \times 10^9 \pm 2.51 \times 10^8$
		Cage 6	$3.00 \times 10^9 \pm 1.90 \times 10^8$
Day 5	Netted	Cage 1	$2.75 \times 10^9 \pm 1.82 \times 10^8$
		Cage 2	$2.50 \times 10^9 \pm 1.73 \times 10^8$
		Cage 3	$2.83 \times 10^9 \pm 1.84 \times 10^8$
	Unnetted	Cage 4	$2.42 \times 10^9 \pm 1.70 \times 10^8$
		Cage 5	$3.50 \times 10^9 \pm 2.05 \times 10^8$
		Cage 6	$2.17 \times 10^9 \pm 1.61 \times 10^8$
Day 7	Netted	Cage 1	$2.17 \times 10^9 \pm 1.61 \times 10^8$
		Cage 2	$1.92 \times 10^9 \pm 1.52 \times 10^8$
		Cage 3	$2.00 \times 10^9 \pm 1.55 \times 10^8$
	Unnetted	Cage 4	$1.50 \times 10^9 \pm 1.34 \times 10^8$
		Cage 5	$1.25 \times 10^9 \pm 1.23 \times 10^8$
		Cage 6	$9.17 \times 10^8 \pm 1.05 \times 10^8$

4.3.3 Surface dose-response bioassay to evaluate the biological activity of CrleGV-1 and CrleGV-2

Following the bioassays, no statistically significant differences were detected between the control groups within Experiment 1 (P = 0.144) or within Experiment 2 (P = 0.106). Accordingly, the average control mortality for each experiment was calculated and applied independently in the statistical analysis of the respective experiment.

The average mortality rate for each dose across all replicates in Experiment 1 was determined (Table 4.4). The highest recorded mortality occurred in netted cages, with a mean mortality rate of 58.33%. The lowest mortalities were recorded on days 14 and 21, primarily in the unnetted cages. Netted cages provided some protection to the virus against UV radiation, as indicated by higher average mortality rates for virus recovered from oranges under nets compared to virus recovered from oranges in unnetted structures (Figure 4.7). However, these differences may not have been statistically significant, and LC_{50} and LC_{90} values could not be calculated.

Table 4.4: Average percentage mortality of *T. leucotreta* across different doses in surface dose bioassays for the long-term Experiment 1, conducted under netted (cage 1-3) and unnetted (cage 3-4) treatments.

Time (days)	**Treatment**	**Average mortality (%)**					
		Dose 1	**Dose 2**	**Dose 3**	**Dose 4**	**Dose 5**	**Control**
Day 1	Netted	58.33	33.34	47.22	44.44	41.67	23.61
	Unnetted	46.61	29.17	30.56	27.78	16.67	23.61
Day 7	Netted	43.06	25.00	19.44	25.00	12.50	23.61
	Unnetted	53.33	22.22	8.33	2.78	9.72	8.33
Day 14	Netted	29.17	13.89	18.06	5.56	6.94	12.50
	Unnetted	13.89	8.33	2.78	5.56	1.39	5.56
Day 21	Netted	32.00	21.00	21.00	15.00	15.00	10.00
	Unnetted	16.67	13.89	6.94	4.17	5.56	2.78

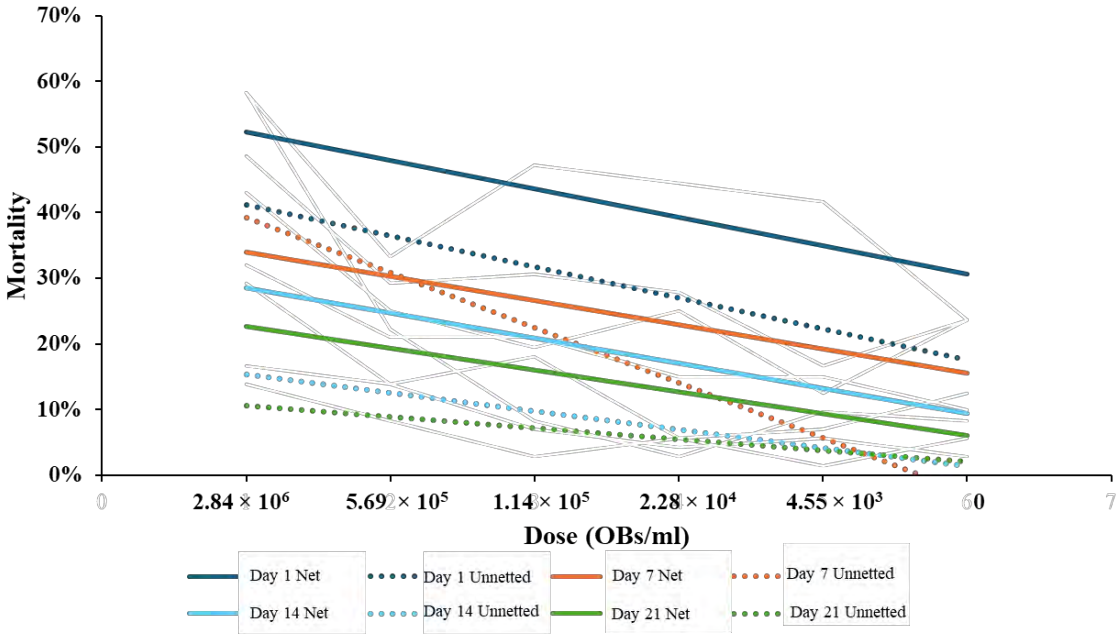


Figure 4.7: Dose-response trends showing the difference between average mortality rates of CrleGV-1 under netted and non-netted structures determined by surface dose-response bioassays against *T. leucotreta* neonates at various time intervals post-exposure for Experiment 1.

The mean mortality rate for each dose across all replicates in Experiment 2 was calculated (Table 4.5). The highest observed mortality occurred in the netted cages, with an average mortality rate of 75%. The increased mortality in these treatments suggests that the netted enclosures offered some degree of protection to the virus from UV radiation, as evidenced by the higher average mortality rates of virus recovered from oranges under nets compared to those from unnetted structures (Figure 4.8).

Table 4.5: Average percentage mortality of *T. leucotreta* across different doses in surface dose bioassays for Experiment 2.

Time (days)	Treatment	Average mortality (%)					
		Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Control
Day 1	Netted	75.00	63.89	44.44	26.39	13.89	12.50
	Unnetted	72.22	55.56	41.67	26.39	18.06	6.25
Day 3	Netted	61.11	43.06	27.78	19.44	15.28	11.81
	Unnetted	59.72	38.89	27.78	19.44	6.94	8.33
Day 5	Netted	40.28	31.94	20.83	18.04	12.50	11.81
	Unnetted	23.61	22.22	15.28	9.72	4.17	8.33
Day 7	Netted	31.94	19.44	16.67	11.11	4.17	6.94
	Unnetted	27.78	18.06	9.72	4.17	6.94	10.42

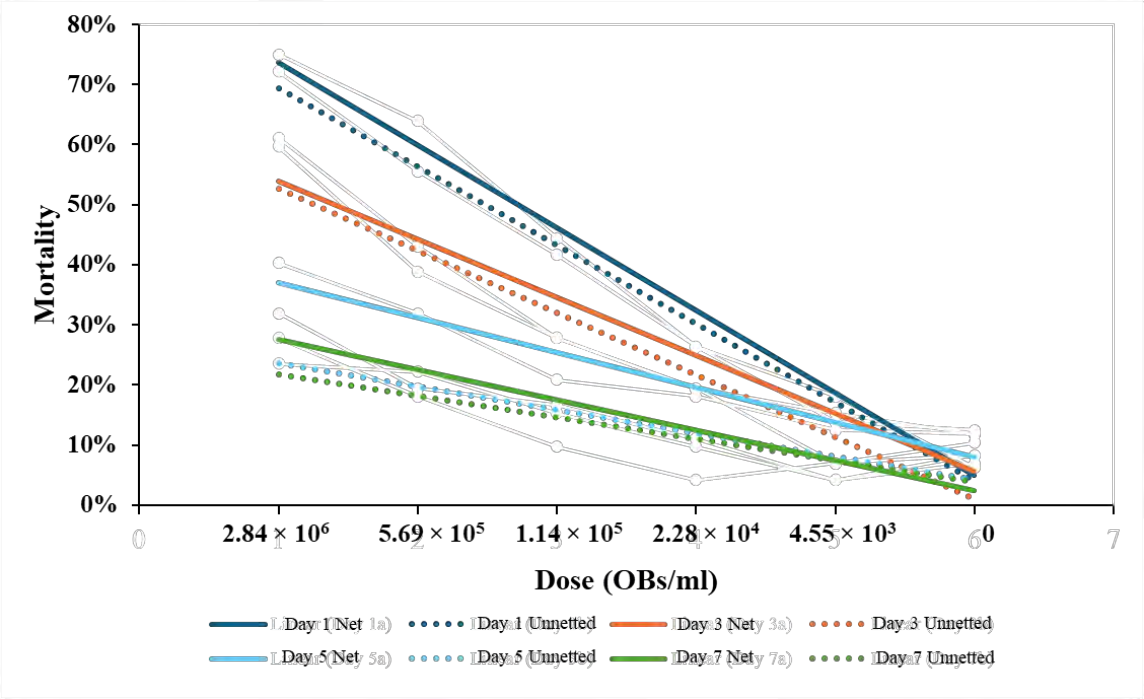


Figure 4.8: Dose-response trends showing the difference between average mortality rates of CrleGV-2 under netted and non-netted structures determined by surface dose-response bioassays against *T. leucotreta* neonates at various time intervals post-exposure for Experiment 2.

A mortality curve for CrleGV-2 was generated using data obtained from surface dose bioassays, demonstrating that the netted treatment consistently outperformed the unnetted treatment (Figure 4.9).

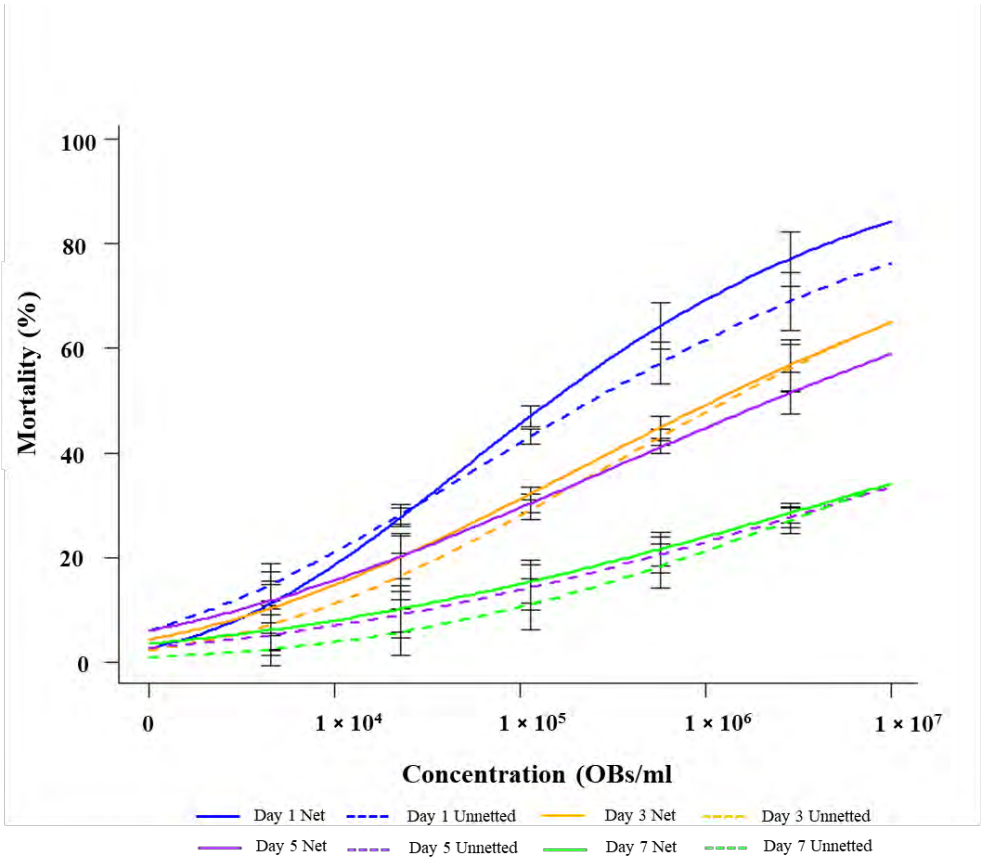


Figure 4.9: Dose-mortality response of *T. leucotreta* first instars to CrleGV-2, based on surface dose bioassays conducted 10 days post-infection for Experiment 2.

Lethal concentrations (LC_{50}) were calculated based on bioassay data and were generally lower for netted treatments compared to unnetted treatments (Table 4.6). As mortality did not reach 50% on days 5 and 7, LC_{50} values for these time points were estimated by extrapolation.

Table 4.6: Lethal concentrations (LC_{50}) and the 95% confidence upper and lower limits for CrleGV-2 treatments using surface dose bioassays against *T. leucotreta* first instars.

Time (days)	Treatment	LC_{50}	95% Confidence intervals	
			Lower	Upper
Day 1	Netted	1.47×10^5	1.15×10^5	1.78×10^5
	Unnetted	2.43×10^5	1.67×10^5	3.19×10^5
Day 3	Netted	1.11×10^6	6.02×10^5	1.61×10^6
	Unnetted	1.30×10^6	7.88×10^5	1.82×10^6
Day 5	Netted	2.24×10^6	9.54×10^5	3.53×10^6
	Unnetted	3.34×10^8	1.17×10^8	5.51×10^8
Day 7	Netted	3.40×10^8	1.11×10^7	5.70×10^8
	Unnetted	1.43×10^8	6.09×10^7	2.25×10^8

Statistical comparison of LC_{50} values was conducted to assess significant differences between treatments (Figure 4.10). Significant differences in LC_{50} values were recorded between netted and unnetted treatments on day 1 post-application (SE = 2.2813×10^{-4}, $P = 9.45 \times 10^{-230}$), day 3 (SE = 4.4986×10^{-5}, $P < 2.2 \times 10^{-16}$), day 5 (SE = 5.1024×10^{-7}, $P < 2.2 \times 10^{-16}$), and day 7 (SE = 1.0489×10^{-6}, $P < 2.2 \times 10^{-16}$). Additionally, significant differences were noted when all treatments were compared collectively as indicated in Appendix A.

Further analysis of the bioassay results revealed a 15-fold increase in LC_{50} for CrleGV-2 between the netted treatment of day 1 and the netted treatment on day 5 post UV exposure, and a 2274-fold increase in LC_{50} for CrleGV-2 when comparing the netted treatment on day 1 to the unnetted treatment on day 5. These findings highlight the substantial protective effect provided by the netting against UV degradation.

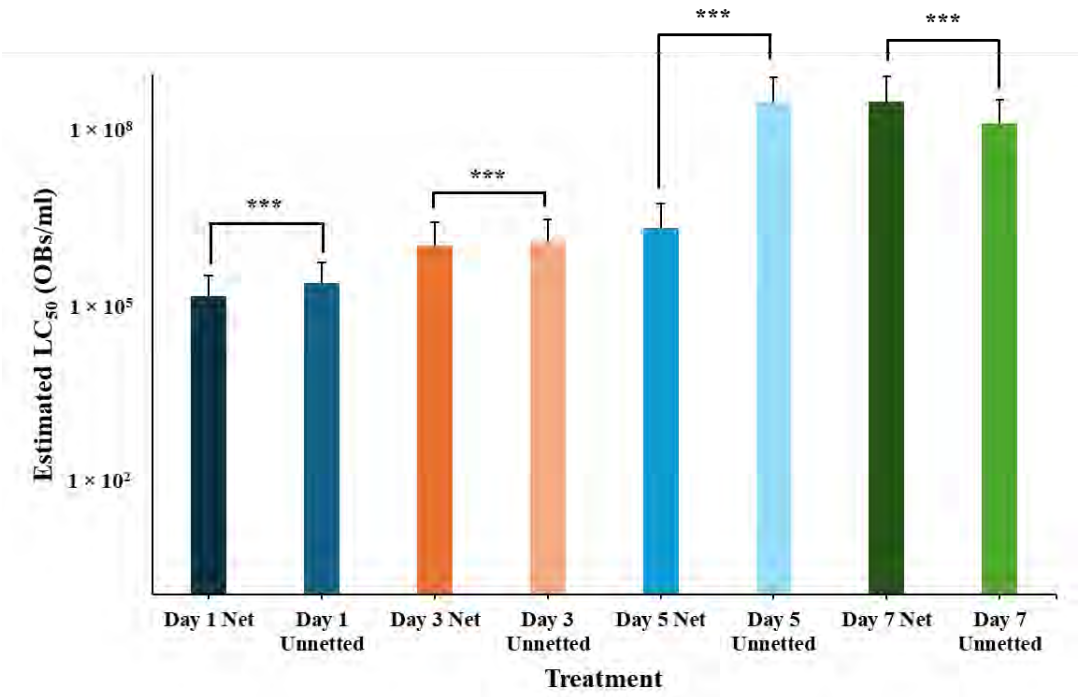


Figure 4.10: Comparison of LC_{50} between treatments. Significant differences were recorded between netted and unnetted treatments on day 1 (SE = 2.2813×10^{-4}, $P < 0.001$), day 3 (SE = 4.4986×10^{-5}, $P < 0.001$), day 5 (SE = 5.1024×10^{-7}, $P < 0.001$), and day 7 post-application (SE = 1.0489×10^{-6}, $P < 0.001$).

4.4 Discussion

A study by Mwanza (2015) evaluated the degradation rate of Cryptogran on the sun-exposed northern side versus the shaded southern side of citrus trees under field conditions over 28 days. By the end of the trial, the virus's efficacy was undetectable on the northern side, whereas a dose-response remained evident on the southern side. This finding influenced the decision to set the current study's maximum duration at 21 days. However, the anticipated persistence of the virus was not observed, as CrleGV degraded at a faster rate than expected. This result was most likely a product of the experimental design, where there is no doubt that under these conditions, the virus was exposed to far higher UV than would be expected in the canopy of the trees. While the data indicated that netted structures provided some degree of protection to CrleGV, as evidenced by the higher overall virus concentrations recovered from netted structures, as well as increased mortality rates of *T. leucotreta* neonates in surface dose-response bioassays compared to unnetted structures, the highest recorded mortality was only 58.33% under netted cages one day after application, becoming inconsistent from day 7 post-

application. Additionally, these differences may not have been significant. The recorded mortalities were too low and variable, and this prevented the generation of reliable mortality curves and calculation of LC values (Jones, 2000). Several factors may have contributed to the rapid degradation of CrleGV, including rainfall, insufficient viral concentrations and exposure to UV radiation, and lack of natural UV protection, such as shade provided by the tree canopy.

Rainfall can influence the persistence of baculoviruses on plant surfaces. In Experiment 1, weather data recorded precipitation on at least two days during the exposure period. Given that the treated oranges remained outside to ensure exposure to natural conditions, it is important to consider the possibility that rain may have washed the virus off the fruit. However, Kirkman (2007) conducted laboratory bioassays to assess the rain-fastness of Cryptogran, a formulated product with CrleGV as the active ingredient, and found that even heavy rainfall did not significantly reduce the efficacy of the virus. The study concluded that if Cryptogran could effectively control *T. leucotreta* after exposure to high-intensity rainfall, it could be deemed rain-fast under commercial conditions. As unformulated virus was used in this experiment, it is possible that there was some washing off of virus during rain events during the trial. In the present study, rainfall was only recorded on days 14 and 15, whereas rapid degradation of CrleGV was already evident by days 1 and 7. Additionally, the recovered virus was counted and evaluated at a target concentration (i.e., concentration that was used in bioassays with the purified virus prior to its application in the semi-field experiments, to confirm its viability and ability to induce mortality levels of 90% or higher (data not shown). This suggests that rainfall was unlikely to be a major contributing factor to the observed decline in virus persistence.

The possibility that insufficient viral concentrations were applied or that virus loss occurred during application and recovery, cannot be disregarded. The concentration used in this study was based on that used by Mwanza (2015). However, while the previous study incorporated molasses and Breakthru to enhance virus retention, it also used a formulated virus, meaning it was not purified like the one in the present study. This difference in formulation may explain the rapid degradation of CrleGV recorded in this study. Moore et al. (2015b) reported a 60–82% reduction in *T. leucotreta* larval infestation using unformulated CrleGV, though the virus concentration used in their study was exceptionally high. In contrast, the current study used a lower concentration, however, the concentration recovered was adequate for subsequent bioassays. It is therefore plausible that reduced virus viability, potentially due to UV degradation, contributed to the lower mortality rates recorded.

Ultra-violet (UV) radiation is a well-documented factor in the degradation of baculoviruses. Research has shown that direct sunlight can significantly inactivate baculoviruses within hours (Ignoffo & Garcia, 1992; Arthurs et al., 2006). UV-B radiation, in particular, damages viral DNA, leading to a loss of infectivity. Even under netted structures, some level of UV exposure will still occur, accelerating the breakdown of CrleGV and reducing its persistence over time. It is essential to recognise that this study was conducted under what may be considered a worst-case conditions. In the field, fruit remain attached to the trees, where the shade provided by the tree canopy helps shield the virus from UV radiation. As previously mentioned, the dense foliage of citrus trees reduces solar exposure. However, the experimental setup in this study lacked such protection. Here, only netting provided shade. Whilst the data generated from Experiment 1 made it challenging to determine if netting provided any protection against virus degradation, the results from Experiment 2, were far more positive.

The second experiment yielded greatly improved results, allowing for the generation of mortality curves and calculation of LC values. The highest mean mortality recorded under netted cages was 75%. The mortality curve indicated that the virus recovered from netted cages resulted in significantly higher *T. leucotreta* mortality than that from unnetted cages. Additionally, LC_{50} values were generally lower for the virus recovered from netted cages, further supporting the protective effect of shade nets. Rainfall was also recorded during this period; however, the results were better than those obtained in the first experiment, further supporting that rainfall did not result in a major loss of virus. These findings indicate promising potential for utilising this virus, along with other UV-sensitive microbial insecticides, to manage FCM populations. Positively, given that this exposure may be considered the harshest, when combined with protective factors commonly found in fruit orchards such as canopy coverage, potentially lower temperatures, and higher humidity (Teixeira & Reis, 2004; Yumnam et al., 2022), CrleGV is expected to be more effective in a netted environment than an open one. This will be discussed in more detail in Chapter 5.

It is important to consider the variation in mortality rates recorded between the two experiments, particularly on day 1 post-application, despite both experiments following the same protocol. A key difference between them was the number of OBs recovered, with higher OB recovery in Experiment 2 compared to Experiment 1. There are four possible explanations for this discrepancy. First, the centrifugation speed was increased in the second experiment, which may have influenced the quantity of virus recovered. Second, the oranges used in Experiment 2 were generally larger than those in Experiment 1. The increased surface area may

have allowed more OBs to adhere to the fruit, potentially leading to a higher recovery rate. Thirdly, different purification methods were used to produce the virus stocks for each experiment. A glycerol gradient purification was applied for CrleGV-1 in Experiment 1, while a crude extraction method was used for CrleGV-2 in Experiment 2. The glycerol gradient technique is known for yielding a highly purified virus preparation compared to the crude extraction method, however, crude extracts may enhance baculovirus persistence (Burges & Jones, 1998). Lastly, the two experiments used fruit at different stages of ripeness. In Experiment 2, where ripe orange fruit was used, higher concentrations of recovered virus and increased neonate mortalities were observed compared to Experiment 1, which used unripe green fruit. This difference may be partly due to higher UV reflectance from the ripe fruit; however, this was not assessed in the present study, and its potential impact remains uncertain. Silva & Moscardi (2002) conducted a study to investigate factors contributing to the reduced quality and effectiveness of the nucleopolyhedrovirus of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) (AgMNPV) in certain regions of Brazil during the 1993/94 season. Several factors were identified as potential influences on the stability and efficacy of the virus under field conditions, including UV radiation (Jaques, 1977; Silva, 1987), relative humidity and precipitation (Jaques, 1977), host insect age and population density (Silva, 1987), pH of the aqueous viral suspension in the spray tank (Young et al., 1977; Keating et al., 1988), temperature (Jaques, 1977), as well as virus formulation, equipment, and application technology (Silva, 1986). The study found that AgMNPV performed most effectively in its crude form, as plots treated with this preparation had significantly higher numbers of virus-infected or dead larvae compared to those treated with formulated biological products. This finding was consistent with Silva (1991), who reported a faster larval mortality rate and a tendency for higher soybean yields in plots treated with crude AgMNPV rather than commercial virus formulations.

It is worth noting that while control mortality was generally below the 20% threshold considered acceptable for robust bioassays (O'Callaghan et al., 2012), some assays exhibited high control mortality, in certain cases exceeding that of the treatment groups. Such occurrences may have influenced the interpretation of treatment effects and highlight potential challenges in maintaining optimal assay conditions. Possible causes could include handling effects, desiccation of the diet, or undetected background infections. Future assays could be improved by ensuring stricter environmental control and reducing handling stress to minimise larval mortality.

In summary, this chapter demonstrated that shade nets effectively protected CrleGV from UV radiation, leading to a significant difference in virus persistence between netted and unnetted treatments in Experiment 2. The environmental conditions in this study were more extreme than those typically found in the field, resulting in rapid virus degradation. However, a clear distinction in results was noted between the netted and unnetted structures. This suggests that CrleGV would likely perform even better when applied to trees in netted orchards, which offer additional shade, increased humidity, and other favourable conditions. The next chapter will provide a more detailed discussion of these findings.

Chapter 5

General Discussion

5.1 Thesis Overview

Implementing good agricultural practices is essential for ensuring safe and healthy food, protecting the environment, and supporting economic growth. In South Africa, the shift away from chemical insecticides toward safer alternatives has been ongoing for over 50 years (Department of Science and Technology (DST), 2013; Moore, 2021). While chemical residues may not pose direct risks to consumers, their improper use can increase health hazards for users (Kongtip et al., 2020), negatively impact the environment (Kosamu et al., 2020), and lead to the development of resistance in the target pest. Additionally, importers have set stricter residue limits, and this has led to a decline of 8.8% in fruit and vegetable trade (Hejazi et al., 2022). This makes it crucial to develop new pest control methods and enhance existing ones as alternatives to chemical insecticides to keep pest populations below economic thresholds and, in the case of phytosanitary pests, to manage them to non-detectable levels (Lacey et al., 2015; Moore, 2021). Shade netting could be a valuable addition to citrus orchards to not only improve yield, but also the performance of biopesticides, such as baculovirus products, in the field and thus enhance the management of targeted pests.

This study aimed to assess the impact of shade nets on the persistence of CrleGV for controlling *T. leucotreta*. The first objective, outlined in Chapter 2, involved producing virus stocks for use in semi-field experiments. CrleGV was propagated in *T. leucotreta* larvae, after which the larval cadavers were purified, screened, and quantified to determine viral concentration. A portion of the stock was used for a pilot study to refine the methodology (Chapter 3). Semi-field experiments were then conducted over 21 days, using virus-treated fruit, with recovered virus evaluated by surface dose-response bioassays (Chapter 4). While results indicated that shade netting provided some protection to CrleGV, rapid virus degradation led to inconsistent outcomes, when measured over 21 days. Consequently, a second experiment was conducted over a shorter 7-day period, revealing that shade nets significantly improved CrleGV persistence (Chapter 4). This chapter will discuss the study's findings in relation to existing literature, along with key insights and recommendations.

5.2 The importance of netting on biopesticide efficacy

The main limitation of using baculoviruses as biopesticides in commercial applications is their vulnerability to UV radiation from sunlight (Shapiro, 1995). Mwanza (2015) observed a clear response on day 28 in the shaded areas of citrus trees, and a similar result was expected in this study on day 21 under netted cages, but this did not materialise. Despite this, in the initial experiment, the highest recorded average mortality was 58.33% under netted structures on day 1 post-application, aligning with Entwistle & Evans (1985), who reported that the half-life of baculoviruses ranges between 10 hours and 10 days. The second experiment yielded improved results, with the highest average mortality reaching 75% under netted cages on day 1 post-application. However, any control level below the high 90% range may be insufficient against a pest with phytosanitary significance (Moore et al., 2015b). Nevertheless, since *T. leucotreta* is managed through a systems approach integrating multiple control methods (Section 1.4), if each contributes around 50% control, the cumulative efficacy could reach approximately 97% (Moore & Hattingh, 2012; Moore et al., 2015b). The results from Experiment 2 of this study indicate that the virus performed better up to the five day mark. This finding aligns with Moore (2002) and Moore et al. (2011), who reported that CrleGV activity decreased to less than 50% of its original effectiveness within 3 to 6 days.

The microclimate within an orchard differs significantly from the external environment, due to factors such as tree canopy, soil cover, and orchard management practices (Wilken, 1972). Variations in temperature and humidity within the orchard can influence the persistence of the virus. The tree canopy provides shade, helping to regulate extreme temperatures, while also reducing direct sunlight reaching the ground, making the orchard cooler than open areas. Additionally, humidity levels are higher inside orchards, due to lower evaporation and transpiration rates. Trees release moisture into the air, and the shaded conditions slow down soil moisture loss. Teixeira & Reis (2004) conducted a study in a mango orchard, and they observed that air temperatures within the orchard were up to 0.5°C lower during the hottest hours and 0.3°C lower during colder hours. This research also indicated that relative humidity inside the orchard was 9% higher in hotter hours and 6% higher in colder hours, compared to outside conditions. Similarly, Yumnam et al. (2022) reported that air temperatures in apple orchards were between 1.9°C and 4.4°C cooler, with relative humidity increasing by 9.2% to 27.5% relative to open-field conditions. Their study further highlighted that wind speeds and solar radiation levels were reduced due to the canopy's shading effect. Frequent irrigation methods, such as micro-sprinklers or drip irrigation, further enhance localised humidity by

adding moisture to both the soil and air and influence temperature changes in the orchard (Lakatos, 2016). This likely explains why a clear response was observed even 28 days after spraying in Mwanza (2015) and why CrleGV remained effective for 17 weeks at approximately 80% efficacy achievable only when applied in blocks rather than on individual trees (Moore et al., 2015b). Previous studies have reported that certain types of nets contribute to lower temperatures and higher humidity levels in orchards (Shahak et al., 2004; Wachsmann et al., 2014, Campi et al., 2020). Shade nets contribute to the already lower temperatures within the orchard compared to open areas and further increase humidity due to reduced wind speed under the netting system (Wachsmann et al., 2014) and the retention of moisture from transpiration within the enclosed environment (Tanny & Cohen, 2013). In the present study, nets were evaluated independently, and despite the harsh conditions, they provided some protection for the virus. This suggests that CrleGV would likely perform well under netted conditions in the field, particularly on the southern-facing side, and potentially even on the northern-facing side, where the netting could offer additional protection. This will not just be restricted to CrleGV, but other baculovirus-based biopesticides may also benefit. Currently, eleven baculovirus-based biopesticides, including CrleGV, are registered and commercially available in Africa (Moore & Jukes, 2023). Shade nets may also enhance the persistence of certain viruses such as CpGV, which are used on sparser trees that typically require more frequent applications (Moore et al., 2004). By prolonging the virus's effectiveness, shade nets could help reduce both spray frequency and overall costs.

5.3 The importance of netting on other control technologies

The standard *T. leucotreta* control programme recommended for citrus orchards in South Africa is based on consistent orchard sanitation, supplemented by area-wide control strategies, such as the sterile insect technique (SIT) or mating disruption, along with targeted pesticide applications, preferably virus-based products, timed according to predicted egg hatch peaks, based on moth flight activity (Moore, 2019, 2021). Moore (2021) noted that all these control methods are compatible, including the combination of mating disruption and SIT, which was previously thought to be conflicting. Given this, it is important to understand how the microclimates created by shade nets may influence current management strategies, particularly if they reduce efficacy.

Different entomopathogens vary in their susceptibility to environmental factors such as temperature, humidity, and UV radiation (Ignoffo & Garcia, 1992; Shapiro et al., 2002; Shapiro-Ilan et al., 2015; Akhanaev et al., 2017). However, most of these pathogens are affected by the same environmental conditions as baculoviruses, particularly UV exposure. For instance, the effectiveness of entomopathogenic fungi, like *Beauveria bassiana* and *Metarhizium anisopliae*, as well as the nematode *Heterorhabditis bacteriophora* (Heterorhabditidae: Rhabditida) used to control the soil-dwelling life stages of *T. leucotreta*, can be diminished by UV radiation (Ignoffo et al., 1977; Shapiro-Ilan et al., 2015; Fernandes et al., 2015; Rodrigues et al., 2016). Moreover, both pathogen types rely on climatic factors, especially temperature and moisture (Coombes et al., 2016; Malan et al., 2018), and the cooler temperatures and increased humidity and soil water retention provided by shade nets could enhance their environmental persistence.

A recent study by Marsberg et al. (2024) examined the impact of nets on major citrus pests in the Eastern Cape Province and this was assessed by using pheromone traps and SIT. This study found that *T. leucotreta* infestation levels were higher in netted orchards than in open orchards. This was attributed to the protective environment created by nets, which allowed existing *T. leucotreta* populations to persist at higher levels. This study also reported the elevation under nets of other important pests, particularly the citrus mealybug, *Planococcus citri* (Risso, 1813) (Hemiptera: Pseudococcidae) and California red scale, *Aonidiella aurantii* (Maskell, 1879) (Hemiptera: Diaspididae). Citrus thrips, *Scirtothrips aurantii* (Faure, 1929) (Thysanoptera: Thripidae) and the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae), also considered key pests of citrus fruit (Grout & Moore, 2015; Abd-Elgawad, 2021) conversely seemed to be lower under netted orchards than open orchards. This is important because it can lead to reduced reliance on harmful insecticides for the control of these pests.

Marsberg et al. (2024) mentioned that the variations in pest levels between netted and open orchards raise an important discussion about whether shade nets enhance or hinder the effective implementation of an integrated pest management (IPM) programme. However, considering the discussed benefits of nets and the findings of this current study, nets seem to have an advantage over open orchards, certainly for baculovirus based products, and possibly also other microbial based products. The findings of the current study are encouraging, suggesting that if shade nets create favourable conditions for *T. leucotreta* but also enhance CrleGV persistence, the pest could still be managed effectively as close to non-detectable levels as possible.

Nevertheless, extensive field trials are necessary to evaluate the effectiveness of this control method on CrleGV under real-world biotic and abiotic conditions. The study by Marsberg et al. (2024) also found that although netted orchards recorded higher catches of wild moths, they also had a higher number of sterile moths, leading to a greater ratio of sterile to wild moths. This suggests that SIT could be even more effective under nets than in open orchards, which is a positive outcome for managing *T. leucotreta*. It is worth noting that shade nets may also introduce some challenges for SIT. For instance, large-scale aerial or drone releases become impossible under netted structures, often requiring manual releases, which are labour-intensive and less efficient. Additionally, nets could restrict the natural dispersal of sterile males, limiting their ability to locate and mate with wild females. As a result, careful planning is required to successfully integrate SIT into netted orchards.

There is limited research on the direct effects of netting on biological agents like the egg parasitoid *Trichogrammatoidea cryptophlebiae* Nagaraja. However, a study by Wang et al. (2014) found that the efficacy of this parasitoid is influenced by ambient temperature. When exposed to a high temperature of 39°C for 8 hours during the pupal stage, only 19.90% of adults successfully emerged from host eggs, and over 14% displayed deformities, such as folded or incomplete wings. Additionally, female parasitoids exposed to 39°C for 8 hours as prepupae had a significantly reduced lifespan of just 1.45 days and parasitised around 23.5 host eggs. These findings suggest that the parasitoid is more effective at controlling lepidopteran pests in cooler conditions. A similar response is expected for the egg parasitoid *T. cryptophlebiae* and the larval parasitoid *Agathis bishopi* (Nixon), both of which are important biological control agents for *T. leucotreta*. Since shade nets help moderate extreme temperatures and increase humidity, they may improve the survival and longevity of these parasitoids, thereby enhancing the control of *T. leucotreta* within netted environments.

5.4 Future prospects

Three straightforward and effective strategies can improve the persistence of baculoviruses in the field. These include applying the biological product early in the morning or late in the afternoon (Silva & Moscardi, 2002; Kirkman, 2007), increasing the application rate (OB concentration) (Grzywacz & Moore, 2017), and applying the virus more frequently (Moore et al., 2015b). As previously noted, the virus suspension concentrations used in this study were lower than the standard rates of commercially available products. The LC_{50} values obtained in

this study ranged from 1.47×10^5 to 3.40×10^8 OBs/ml. However, the treatments, both netted and unnetted, at 5 and 7 days post-application did not achieve ≥ 50% mortality. As a result, the LC_{50} values for these time points (especially the unnetted treatment on day 5 and both treatments on day 7), are extrapolated and may not precisely reflect the actual lethal concentrations. Moore et al. (2011) assessed the virulence of CrleGV-SA and reported LC_{50} values of 9.310×10^7 OBs/ml from detached fruit bioassays. Therefore, future studies investigating the effects of shade nets on CrleGV and other baculovirus products should consider using higher virus concentrations, particularly when applying unformulated virus without adjuvants. However, it is recommended that future research utilise formulated biological products in combination with adjuvants to enhance efficacy. According to O'Callaghan et al. (2012), the design of a robust bioassay requires selecting sample sizes and concentrations that achieve the desired precision for parameter estimates, such as LC_{50} or LD_{50}. They also stated that LC_{50} is a standard measure for expressing virulence, and a preliminary or pilot bioassay can provide valuable insight into the lethal concentrations corresponding to specific mortality levels of interest. In this study, due to time constraints, a pilot bioassay was not conducted prior to field trials, however, incorporating such a step in future work would be highly beneficial. The volume of virus applied in this study was established based on a preliminary pilot study conducted under controlled laboratory conditions. However, when applied in field settings, environmental factors such as wind, even gentle wind, as spraying is recommended to never be conducted in strong wind conditions, may influence viral deposition and subsequently affect its efficacy. Silva & Moscardi (2002) and Yearian (1978) found that higher aqueous spray volumes improved baculovirus deposition on plants, reducing losses caused by spray drift and evaporation. Therefore, future studies should be conducted at the field level and using higher volumes of the suspension should be considered to enhance performance.

A factor that was not tested in experiments in the current study but has the potential to influence results, is the pH of both the virus stock and water. Baculoviruses degrade under alkaline conditions, making pH a critical factor (Cory & Bishop, 1997). Occlusion bodies are highly sensitive to the alkaline environment of the insect midgut (pH > 9.5), where they rapidly dissolve, releasing occlusion derived virions (ODVs) (McWilliam, 2007). Gotoh et al. (2008) examined baculovirus inactivation under different conditions, using the envelope-labelled virus assay (ELVA) and found that deviating from a neutral pH significantly reduced virus titer. Their study also suggested that the optimal storage pH for baculoviruses is around 6.4. In another

study, Keating et al. (1990) investigated the effect of diet pH and buffering capacity on the midgut pH of gypsy moth (*Lymantria dispar*; Lepidoptera: Lymantriidae) larvae and its impact on virus susceptibility across different host plant species. Their findings indicated that the pH of the foliage fed to larvae influenced midgut pH, with acidic foliage significantly lowering midgut pH. Furthermore, larvae consuming virus on highly acidic foliage or artificial diets exhibited reduced susceptibility to the gypsy moth nuclear polyhedrosis virus. They concluded that diet pH could impact virus activity by modifying midgut pH and altering the rate and location of virion release in the midgut lumen. Furthermore, Silva & Moscardi (2002) also reported that AgMNPV effectiveness was significantly influenced by the water pH in the spray tank. Plots treated with a viral suspension at pH 6 had a significantly higher number of infected or dead larvae compared to those treated at pH 2 or 10. It is important to highlight that all virus products registered in South Africa are recommended for use with water maintained at an optimal pH, typically between 5 and 8, as stated by River Bioscience. However, since pH requirements can vary between products, it is crucial to refer to the specific product label for accurate guidelines. Following these instructions is key to ensuring the biopesticide's effectiveness. In this study, the pH of the water or virus suspensions was not measured, which raises the possibility that suboptimal pH levels may have caused virus inactivation, particularly in Experiment 1. Future research should incorporate a pH indicator in the virus suspensions, following the approach used in the detached fruit bioassay protocol described by Moore et al. (2011).

5.4 Conclusion

The main objective of this study was to assess the impact of shade nets on CrleGV efficacy against *T. leucotreta* in South African citrus orchards. CrleGV was propagated in *T. leucotreta* larvae, followed by purification and verification using TEM, PCR, and sequencing, which confirmed the presence of a suitable virus. Before conducting the main experiments, a pilot study was carried out to refine the methodology, leading to the successful development of a virus recovery protocol. Two semi-field experiments were then performed, where purified virus stocks were exposed to natural UV radiation using virus-treated citrus fruits. The virus was later washed off the fruit at different time intervals, and surface dose-response bioassays were conducted on *T. leucotreta* neonates. These bioassays revealed a significant difference between the netted and non-netted treatments. This study's findings provided insight on the impact of shade nets on the persistence of CrleGV. The results indicate promising potential for utilising

this virus, along with other UV-sensitive microbial insecticides, to manage FCM populations in South African citrus orchards. Furthermore, if shade netting effectively provides UV protection, it could contribute to reducing formulation costs, particularly when combined with UV protectants. Fewer additives could mean less expenses and possibly greater market appeal.

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Appendix A

Table 4.7: Comparison between LC_{50} values to assess statistical significance between treatments in the short-term Experiment 2 using surface dose bioassays against *T. leucotreta* first instars.

	Estimate	**Std Error**	**t-value**	**p-value**
Day 1 Net/Day 1 Unnetted	8.4525×10^{-1}	2.2813×10^{-4}	-678.34	$< 2.2 \times 10^{-16}$ ***
Day 1 Net/Day 3 Net	2.3970×10^{-1}	5.0806×10^{-5}	-14964.80	$< 2.2 \times 10^{-16}$ ***
Day 1 Net/Day 3 Unnetted	1.7594×10^{-1}	3.6822×10^{-5}	-22379.22	$< 2.2 \times 10^{-16}$ ***
Day 1 Net/Day 5 Net	1.6258×10^{-1}	3.3951×10^{-5}	-24665.34	$< 2.2 \times 10^{-16}$ ***
Day 1 Net/Day 5 Unnetted	2.4751×10^{-3}	5.1018×10^{-7}	-1955237.24	$< 2.2 \times 10^{-16}$ ***
Day 1 Net/Day 7 Net	2.7486×10^{-3}	5.6656×10^{-7}	-1760200.37	$< 2.2 \times 10^{-16}$ ***
Day 1 Net/Day 7 Unnetted	3.2727×10^{-3}	6.7457×10^{-7}	-1477579.12	$< 2.2 \times 10^{-16}$ ***
Day 1 Unnetted/Day 3 Net	2.8358×10^{-3}	5.1355×10^{-5}	-13950.42	$< 2.2 \times 10^{-16}$ ***
Day 1 Unnetted/Day 3 Unnetted	2.0815×10^{-1}	3.7043×10^{-5}	-21376.58	$< 2.2 \times 10^{-16}$ ***
Day 1 Unnetted/Day 5 Net	1.9234×10^{-1}	$3.4126e \times 10^{-5}$	-23667.17	$< 2.2 \times 10^{-16}$ ***
Day 1 Unnetted/Day 5 Unnetted	2.9283×10^{-3}	5.1018×10^{-7}	-1954346.30	$< 2.2 \times 10^{-16}$ ***
Day 1 Unnetted/Day 7 Net	3.2518×10^{-3}	5.6656×10^{-7}	-1759309.21	$< 2.2 \times 10^{-16}$ ***
Day 1 Unnetted/Day 7 Unnetted	3.8718×10^{-3}	6.7457×10^{-7}	-1476687.51	$< 2.2 \times 10^{-16}$ ***
Day 3 Net/Day 3 Unnetted	7.3402×10^{-1}	4.4986×10^{-5}	-5912.45	$< 2.2 \times 10^{-16}$ ***
Day 3 Net/Day 5 Net	6.7828×10^{-1}	4.0493×10^{-5}	-7945.27	$< 2.2 \times 10^{-16}$ ***
Day 3 Net/ Day 5 Unnetted	1.0326×10^{-2}	5.1021×10^{-7}	-1939750.43	$< 2.2 \times 10^{-16}$ ***
Day 3 Net/Day 7 Net	1.1467×10^{-2}	5.6659×10^{-7}	-1744703.00	$< 2.2 \times 10^{-16}$ ***
Day 3 Net/Day 7 Unnetted	1.3653×10^{-2}	6.7463×10^{-7}	-1462061.56	$< 2.2 \times 10^{-16}$ ***
Day 3 Unnetted/Day 5 Net	9.2406×10^{-1}	4.5628×10^{-5}	-1664.42	$< 2.2 \times 10^{-16}$ ***
Day 3 Unnetted/Day 5 Unnetted	1.4068×10^{-2}	5.1023×10^{-7}	-1932328.38	$< 2.2 \times 10^{-16}$ ***
Day 3 Unnetted/Day 7 Net	1.5622×10^{-2}	5.6662×10^{-7}	-1737271.45	$< 2.2 \times 10^{-16}$ ***
Day 3 Unnetted/Day 7 Unnetted	1.8601×10^{-2}	6.7468×10^{-7}	-1454611.94	$< 2.2 \times 10^{-16}$ ***

Day 5 Net/Day 5 Unnetted	1.5224×10^{-2}	5.1024×10^{-7}	-1930029.69	$< 2.2 \times 10^{-16}$ ***
Day 5 Net/Day 7 Net	1.6906×10^{-2}	5.6663×10^{-7}	-1734969.27	$< 2.2 \times 10^{-16}$ ***
Day 5 Net/Day 7 Unnetted	2.0129×10^{-2}	6.7470×10^{-7}	-1452303.12	$< 2.2 \times 10^{-16}$ ***
Day 5 Unnetted/Day 7 Net	1.1105	8.4665×10^{-7}	130512.05	$< 2.2 \times 10^{-16}$ ***
Day 5 Unnetted/Day 7 Unnetted	1.3222	1.1183×10^{-6}	288129.70	$< 2.2 \times 10^{-16}$ ***
Day 7/Day 7 Unnetted	1.1906	1.0489×10^{-6}	181763.73	$< 2.2 \times 10^{-16}$ ***

Appendix B

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6 October 2023

DR Iain Paterson

Email: I.Paterson@ru.ac.za

Review Reference: 2023-3842-8102

Dear DR Iain Paterson

Re: Animal ethics application: Centre for Biological Control Oct

Principal Investigator:

Collaborators: ,

This letter confirms that the above Annual Report has been reviewed and **APPROVED** by the Rhodes University Animal Research Ethics Committee (RU-AREC). Your Approval number is: 2023-3842-8102.

Approval has been granted for one year from the date of this letter. **An annual progress report** will be required in order to renew approval for an additional period of one year. Ethics approval for a project can be renewed twice for a project (i.e. the maximum number of years that a project can receive ethics approval for is 3 years). Thereafter, the project will need to undergo a full application should you require ethics clearance for longer than 3 years.

Sincerely

Dr. Roman Tandlich

Chair: Rhodes University Animal Research Ethics Committee, RU-AREC

cc: Ethics Coordinator

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8 September 2024

DR Iain Paterson

Email: I.Paterson@ru.ac.za

Review Reference: 2024-8120-9068

Dear DR Iain Paterson

Re: Animal ethics application: Centre for Biological Control (2024)

Principal Investigator: Prof Iain Paterson

Collaborators: As specified in the application

This letter confirms that the above research proposal has been reviewed and **APPROVED** by the Rhodes University Animal Research Ethics Committee (RU-AREC). Your Approval number is 2024-8120-9068.

Your approval is depending on the following conditions:

The applicants and the CBC staff are requested to document the major activities of the centre through regular collection of photographic or video evidence. This evidence is requested to be collected throughout the period of approval and submitted to RUAREC with the next annual progress report.

Approval has been granted for one year from the date of this letter. **An annual progress report** will be required in order to renew approval for an additional period of one year.

Please ensure that the RU-AREC committee is notified should any substantive change(s) be made, for whatever reason, during the research process. This includes changes in investigators. Please also ensure that a brief report is submitted to the ethics committee on the completion of the research. The purpose of this report is to indicate whether the research was conducted successfully, if any aspects could not be completed, or if any problems arose that the RU-AREC should be aware of. If a thesis or dissertation arising from this research is submitted to the library's electronic theses and dissertations (ETD) repository, please notify the committee of the date of submission and/or any reference or cataloging number allocated.

Sincerely

Dr. Roman Tandlich

Chair: Rhodes University Animal Research Ethics Committee, RU-AREC

cc: Ethics Coordinator