STUDIES ON EXISTING AND NEW ISOLATES OF *CRYPTOPHLEBIA LEUCOTRETA* GRANULOVIRUS (CrleGV) ON *THAUMATOTIBIA LEUCOTRETA* POPULATIONS, FROM A RANGE OF GEOGRAPHIC REGIONS IN SOUTH AFRICA

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JOHN KWADWO OPOKU-DEBRAH

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Abstract

Baculoviruses are arthropod-specific DNA viruses that are highly virulent to most lepidopteran insects. Their host specificity and compatibility with IPM programmes has enabled their usage as safe microbial insecticides (biopesticides). Two baculovirus-based biopesticides, Cryptogran and Cryptex, which have been formulated with *Cryptophlebia leucotreta* granulovirus (CrleGV) have been registered for the control of false codling moth (FCM), *Thaumatotibia* (=*Cryptophlebia*) *leucotreta* (Meyrick) (Lepidoptera: Tortricidae) in South Africa and have been successfully incorporated into IPM programmes. However, several studies have indicated that insects can develop resistance to baculovirus-based biopesticide as was shown with field populations of codling moth (CM), *Cydia pomonella* (L.), which developed resistance to the biopesticide *Cydia pomonella* granulovirus (CpGV-M) in Europe. Other studies have shown that, under laboratory conditions, FCM populations differ in their susceptibility to Cryptogran and Cryptex. In order to investigate difference in susceptibility as well as protect against any future resistance by FCM to Cryptogran and Cryptex, a search for novel CrleGV-SA isolates from diseased insects from different geographic regions in South Africa was performed.

Six geographic populations (Addo, Citrusdal, Marble Hall, Nelspruit, Baths and Mixed colonies) of FCM were established and maintained in the laboratory. Studies on the comparative biological performance based on pupal mass, female fecundity, egg hatch, pupal survival, adult eclosion and duration of life cycle of the Addo, Citrusdal, Marble Hall, Nelspruit and Mixed colonies revealed a low biological performance for the Citrusdal colony. This was attributed to the fact that FCM populations found in the Citrusdal area are not indigenous and may have been introduced from a very limited gene pool from another region.

When insects from five colonies, excluding the Baths colony, were subjected to stress by overcrowding, a latent baculovirus resident in the Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colonies was brought into an overt lethal state. Transmission electron micrographs revealed the presence of GV occlusion bodies (OBs) in diseased insects. DNA profiles obtained by single restriction endonuclease analysis of viral genomic DNA using *Bam*H1, *Sal*1, *Xba*1, *Pst*1, *Xho*1, *Kpn*1, *Hind*III and *Eco*R1 revealed five CrleGV-SA isolates latent within the insect populations. The

new isolates were named CrleGV-SA Ado, CrleGV-SA Cit, CrleGV-SA Mbl, CrleGV-SA Nels and CrleGV-SA Mix isolates. The novelty of the five CrleGV-SA isolates was confirmed by the presence of unique submolar bands, indicating that each isolate was genetically different. PCR amplification and sequencing of the *granulin* and *egt* genes from the five isolates revealed several single nucleotide polymorphisms (SNPs) which, in some cases, resulted in amino acid substitutions. DNA profiles from RFLPs, as well as phylogenetic analysis based on *granulin* and *egt* sequencing showed the presence of two CrleGV-SA genome types for the CrleGV-SA isolate. Cryptex and CrleGV-SA Ado, CrleGV-SA Cit, CrleGV-SA Mbl and CrleGV-SA Mix were placed as members of Group one CrleGV-SA, and Cryptogran and CrleGV-SA Nels isolate were placed into Group two CrleGV-SA.

In droplet feeding bioassays, the median survival time (ST_{50}) for neonate larvae inoculated with Group one and two CrleGV-SA were determined to range from 80 – 88 hours (3.33 – 3.67 days), for all five colonies. LD_{50} values for Group one and two CrleGV-SA against neonates from the Addo, Citrusdal, Marble Hall, Nelspruit and Mixed colonies varied between some populations and ranged from 0.80 - 3.12 OBs per larva, indicating some level of variation in host susceptibility.

This is the first study reporting the existence of genetically distinct CrleGV baculovirus isolates infecting FCM in different geographical areas of South Africa. The results of this study have broad-ranging implications for our understanding of baculovirus-host interactions and for the application of baculovirus based-biopesticides.

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BV – budded virus

°C – Degrees celsius

CM – Codling moth

CpGV – *Cydia pomonella* granulovirus

CpGV-M – Mexican isolate of *Cydia pomonella* granulovirus

CrleGV – *Cryptophlebia leucotreta* granulovirus

CrleGV-CV – Cape Verde isolate of *Cryptophlebia leucotreta* granulovirus

CrleGV-CV3 – strain or genotype number 3 of a Cape Verde isolate of *Cryptophlebia leucotreta* granulovirus

CrleGV-SA – South African isolate of *Cryptophlebia leucotreta* granulovirus

DNA – Deoxyribose nucleic acid

e.g. – example

et al. – *et alia* (and others)

FCM – false codling moth

Fig. – figure

g – gram

GV – granulovirus

HabrGV – Harrisina *brillians* granulovirus

HearGV – *Helicoverpa armigera* granulovirus

IPM – integrated pest management programme

LC – lethal concentration

LC_{50} – median lethal concentration

LC_{90} – 90 % lethal concentration

$LC_{99.9}$ – 99.9 % lethal concentration

LD_{50} – median lethal dosage

LT – lethal time

LT_{50} – median lethal time

LT_{90} – 90 % lethal time

Ltd – Limited

min – minutes

ml – millilitre

mm – millimetre

MNPV – multiply enveloped nucleopolyhedrovirus

NPV – nucleopolyhedrovirus

SNPV –single nucleocapsids NPVs

ODV – occlusion derived viruses

OB – occlusion body

PiraGV – *Pieris brassicae* granulovirus

PrGV – *Pieris rapae* granulovirus

REN – restriction enzyme analysis

RFLP – restriction fragment length polymorphism

Rpm – revolutions per minute

SDS – sodium dodecyl sulphate

SE – standard error

SIT – sterile insect technique

SpliNPV – *Spodoptera littoralis* nucleopolyhedrovirus

SNPV – singly enveloped nucleopolyhedrovirus

SNP – single nucleotide polymorphism

SpfrGV – *Spodoptera frugiperda* granulovirus

TnGV - *Trichoplusia ni* granulovirus

sp. – species

µl – microlitre

µm – micron

XecnGV – *Xestia c-nigrum* granulovirus

° – degrees

% – percent

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CHAPTER ONE

BACKGROUND STUDY AND PROJECT PROPOSAL

1.0 INTRODUCTION

Insects possess remarkable adaptations to exploit their food and habitat. This has sometimes brought them into direct conflict with man. This study will focus on one notable insect pest of economic importance, the false codling moth (FCM), *Thaumatotibia* (=*Cryptophlebia*) *leucotreta* (Lepidoptera: Tortricidae) (Meyrick, 1912; Komai, 1999). FCM has been reported to attack a wide range of crops in sub-Saharan Africa and breeds all year round, which makes its control quite problematic (Newton, 1998). In some cases, fruit losses as a result of FCM attacks have been reported to range from lower than 2% to as high as 90% in South Africa (Newton, 1998). In another report it was estimated that the annual losses incurred by the citrus industry of South Africa in 2004 alone as a result of FCM infestation was about R100 million (US$14 million) (Moore *et al.*, 2004a). Since FCM does not occur in most countries to which citrus are exported from South Africa, it poses a serious international phytosanitory threat. As a result the detection of a single larva in fruit destined for export may lead to an entire consignment being rejected (Moore, 2002; Hattingh, 2006).

In order to control FCM in South Africa, a number of control options have been adopted. The conventional approach has been the use of chemical insecticides. However chemical insecticides although very effective, can be detrimental to the environment when misapplied. The most notable problems are the high residues left on fruit (post harvest), warranting stricter residue restriction limits and the non-target effects of sprays, which kills other beneficial organisms, leading to secondary pest outbreaks (Hofmeyr & Pringle, 1998; Hattingh, 2006). For example, the chemical insecticide Alsystin (triflumuron) was reported to be highly detrimental to an important parasitoid of FCM, *Trichogrammatoidea cryptophlebiae* (Hymenoptera: Trichogrammatoidea) (Hattingh & Tate, 1997). In addition FCM has developed resistance to Alsystin in South Africa (Hofmeyr & Pringle, 1998; Moore, 2002). Other problems include the safety and

environmental risks associated with insecticide usage (Moore, 2002; Hofmeyr & Pringle, 1998). Resistance development, coupled with other environmental concerns associated with pesticide usage, has resulted in growing interest towards safer control options. A group of naturally occurring invertebrate viruses, baculoviruses, of the family *Baculoviridae*, are known to be effective in controlling insects of the order Lepidoptera (Federici, 1997), Diptera, Coleoptera and Hymenoptera (Adams & McClintock, 1991). Baculoviruses have also shown good promise for use as microbial insecticides in the control of FCM in South Africa (Moore *et al.*, 2004a & b).

This chapter provides a review of literature on baculoviruses and on the biology of the host (FCM). In addition, the potential challenges associated with the use of baculoviruses as microbial insecticides and insect resistance will be explored, and within this context the objectives of this study are proposed.

1.1 THE HOST (FALSE CODLING MOTH, *Thaumatotibia leucotreta*)

1.1.1 History & Taxonomy

The first account of FCM was documented by Fuller (1901). Fuller referred to it as the Natal codling moth due to the nature of damage on citrus in KwaZulu-Natal (Province of South Africa). Eight years later it was recorded as the orange codling moth, *Enarmonia batrachopa* from the Transvaal by Howard (1909). Thereafter, it was generally referred to as the false codling moth and was described taxonomically by Meyrick (1912) as *Argyroploce leucotreta* (Eucosmidae: Olethreutidae). It was later re-named by Clarke (1958) and referred to as *Cryptophlebia leucotreta* (Meyrick) (Lepidoptera: Tortricidae). *Cryptophlebia leucotreta* is commonly referred to as the false codling moth, since its behaviour resembles that of the codling moth, *Cydia pomonella* (L.) (Reed, 1974). *Cryptophlebia leucotreta* has been re-classified by Komai (1999) and is currently referred to as *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) (Table 1.1).

Table 1.1 Classification of the false codling moth, FCM (Meyrick, 1912; Bloem *et al.*, 2010)

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Lepidoptera
Family	Tortricidae
Tribe	Grapholitini
Species	*Thaumatotibia leucotreta*
Synonym	*Cryptophlebia leucotreta*
Common name	False codling moth
Name easily confused with	Litchi moth (*Cryptophlebia peltastica*), Codling moth (*Cydia pomonella*), Macadamia nut borer (*Thaumatotibia batrachopa*)

1.1.2 Distribution

FCM is widely distributed in sub-Saharan Africa (Table 1.2). However, FCM is known to be endemic in Africa, where it is thought to have originated (Newton, 1998). It is mostly confined to the hot-tropics and sub-tropics (Karvonen, 1983). FCM has also been reported in Angola, Burkina Faso, Burundi, Cameroon, Eritrea, Gambia, Ghana, Mali, Niger, Rwanda, Senegal, Sierra Leone, Sudan, Swaziland, Tanzania, Togo and Zambia (Bloem *et al.*, 2010).

Table 1.2 Geographic distribution of FCM in sub-Saharan Africa and SW Asia

Author & year	**Country**
Bredo (1933)	Ethiopia & Congo
Catling (1969)	Swaziland
CIBC (1984)	Madagascar, Reunioun & Saint Helena
Gunn (1921)	Kenya
Hargreaves (1922)	Uganda
Hepburn (1947)	South Africa
Jack (1916)	Zimbabwe
Meyrick (1930)	Mauritius
Mück (1985)	Cape Verde Islands
Pearson (1958)	Ivory Coast
Stofberg (1954)	Mozambique
Sweeney (1962)	Malawi
Thompson (1946)	Somalia

Thompson (1946)	Nigeria
Wysoki (1986)	Israel

1.1.3 Host range

FCM is host to a wide range of cultivated plants in sub-Saharan Africa and the islands of the Atlantic and Indian oceans (Newton, 1998; Vennette, *et al.*, 2003; Bloem *et al.*, 2010) (Tables 1.3). FCM also attacks a wide range of wild plants, mostly serving as secondary host, which enables it to persist for long in the absence of its primary host (Tables 1.4) (Newton, 1998; Bloem *et al.*, 2010).

Table 1.3 Cultivated plants reported as FCM host (Pinhey, 1975; Daiber, 1980; Newton, 1998; Vennette, *et al.*, 2003)

Common Name	Scientific Name
Avocado	*Persea americana*
Apricot	*Prumus armeniciata*
Banana	*Musa paradisica*
Bean	*Phaseolus* spp.
Cacao	*Theobroma cacao*
Citrus	*Citrus sinensis, Citrus* spp.
Coffee	*Coffea Arabica, Coffea* spp.
Cola	*Cola nitida*
Corn	*Zea mays*
Cotton	*Gossypium hirsutum*
Grape	*Vitis* spp.
Guava	*Psidium guyjava*
Litchi	*Litchi chinensis*
Loquat	*Eribotrya japonica*
Macadamia nut	*Macadamia ternifolia*
Mango	*Mangifera indica*
Olive	*Olea europaea subsp. Europaea*
Pepper/pimeto	*Capsicum* spp.
Persimmon	*Diospyros* spp.
Peach & Plum	*Prunus* spp.
Pineapple	*Ananas comosus*
Pomegrade	*Punica granatum*
Sorghum	*Sorghum* spp.
Tea	*Camellia sinensis*

Table 1.4 Wild plants reported as FCM host (Schwartz, 1981; Vennette *et al.*, 2003; Kirkman & Moore; 2007)

Common Name	Scientific Name
Albuca	*Albuca* spp.
Asparagus fern	*Asparagus crassicladus*
Bur weed	*Triumfeta* spp.
Bluebush	*Diospyros lycoides*
Blue passion flower	*Passiflora caerulea,*
Bloubos	*Royena pallens*
Boerboon	*Schotia afra*
Buffalo thorn	*Zizyphus mucronata*
Carambola	*Averrhoa carambola*
Castorbean	*Ricinnus communis*
Chayote	*Sechium edule*
Cowpea	*Vigna unguiculata, Vigna* spp.
Custard apple	*Amona reticulate*
Elephant grass	*Pennisetum purpureum*
English walnut	*Juglans regia*
Governors plum	*Flacourtia indica*
Indian mallow	*Abutilon hybridium*
Jakkalsbessie	*Diospyros mespiliforms*
Jade/dollar plant	*Crassula ovata*
Jujube	*Zizyphus jujube*
Jute	*Abutilon* spp.
(Wild) Kaffir plum	*Harpephyllum caffum*
Kapok/copal	*Ceiba pentrandra*
Kei apple	*Dovyalis caffra*
Khat	*Catha edulis*
Kudu-berry	*Pseudolachnostylis maprouneifolia*
Lima bean	*Phaseolus lunatus*
Mallow	*Hibiscus* spp.
Mangosteen	*Garciania mangostana*
Marula	*Sclerocarya caffra, sclerocarya birrea*
Monkey pod	*Cassia petersiana*
Oak	*Quercus* spp.
Okra	*Ablemoschus esculentus*
Peacock flower	*Caesalpinia pulcherima*
Pride of De Kaap	*Bauhimia galpini*
Raasblaar	*Combretum zeyheri*
Red milkwood	*Mumisops zeyheri*
Rooibos/Bushwillow	*Combretum apiculatum*
Sida	*Sida* spp.
Snot apple	*Azanza agarkeana*
Stamvrugte	*Chrysophylulum palismontanum*
Sodom apple	*Calotropis procera*
Soursop	*Ammona muricata*
Stemfruit	*Englerophytum magaliesmontanum*
Surinum cherry	*Eugenia uniflora*

Suurpruim/large sour plum	*Ximenia caffra*
Water-bessie	*Syzygium cordatum*
Wag'n bietjie	*Capparis tomentosa*
Weeping boerboon	*Scotia brachypetala*
Wild fig	*Ficus capensis*
Wild medlar	*Vangueria infausta*
Wing bean	*Xeroderris stuhlmannii*
Yellow-wood berries	*Podocarpus falcatus*
Yellow-wood	*Podocarpus latifolius*

1.1.4 Nature and extent of injury

FCM causes considerable damage to fruits, which affects their quality and market acceptability (Newton, 1998; Moore, 2002; Moore *et al.*, 2004a). The female moth lays most of its eggs superficially on fruit, with the newly hatched larvae quickly boring into the rind of fruit, resulting in fruit damage and discolouration (Newton, 1998). Infested fruit already showing colour-break normally have a deeper tone than usual and the point of entry is much paler than the background colouration (Fig. 1.1) (Newton, 1998; Moore, 2002). Oviposition on physically damaged and early-ripening fruit is reported to be much greater than that on healthy ones in their normal stage of development (Newton, 1998).

Figure 1.1 FCM infested citrus fruit with rind discolouration and entry marks (black arrow) (Source: Moore, 2002).

The entrance of the larva in the fruit is conspicuous due to the characteristic 'frass' (excrement) produced by the insect (Fig. 1.2) (Myburgh, 1987; Newton, 1990 & 1998; Moore, 2002).

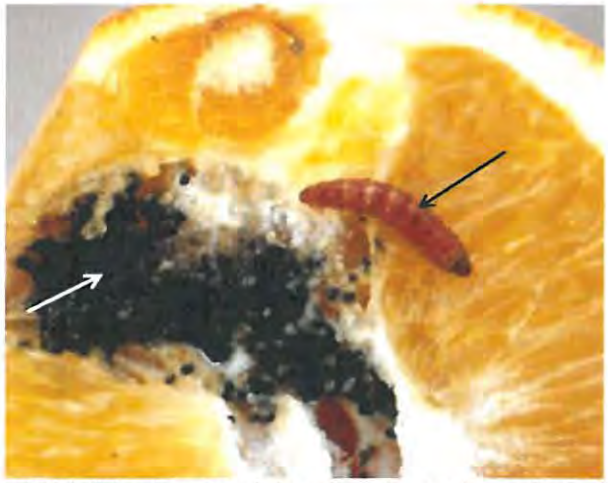

Figure 1.2 Fifth instar FCM (black arrow) feeding in a citrus fruit. Note the characteristic frass (white arrow) left behind by the larva (Source: Moore, 2002).

The larva continues to feed and stay in the fruit until it is fully grown (5th instar) (Newton, 1998). In avocados, the larva causes considerable damage which affects fruit quality (Fig. 1.3) leading to huge economic losses (Bloem *et al.*, 2010).

Figure 1.3 FCM larva on avocado (Source: Bloem *et al.*, 2010).

Once fully-grown, the larva bores its way out of the fruit to seek a site for pupation. This larval penetration adversely affects the physiological state of the fruit. Fruit ripens prematurely, leading to abscission (Newton, 1998). Once a fruit has dropped to the orchard floor, it plays host to a wide range of fungal invaders and vertebrate and invertebrate feeders (Newton, 1998).

1.1.5 The life cycle of FCM

In the field, the life cycle of FCM is quite variable and is seasonally dependent. For instance in summer, the complete life cycle (egg to adult) of FCM has been reported to last between 48 and 65 days, while in winter the complete life cycle lasts for 70 - 90 days (Stofberg, 1939). Temperature and humidity may also play a significant role in this variation. In citrus orchards, Stofberg (1939) found that the average duration for FCM to complete its life cycle was 66 days with between $5^1/_2$ to 6 generations per annum.

1.1.5.1 The ovum (egg): FCM eggs are laid singly and are mostly spaced a little distance from each other, although occasionally two eggs may be found in contact. In citrus, most of the eggs are laid on the rind of the fruit (Newton, 1998). However, oviposition on physically damaged and early ripening oranges has been reported to be much greater than that of healthy navel or valencia oranges in their normal stage of development (Newton, 1998). The incubation period is reported to last between 9 - 12 days in winter and 6 - 8 days in summer (Stofberg, 1939). Hatching occurs at all times during the day (Daiber, 1979a). According to Stofberg (1954) up to 65 eggs can be found on a single fruit, but such high numbers are considered rare.

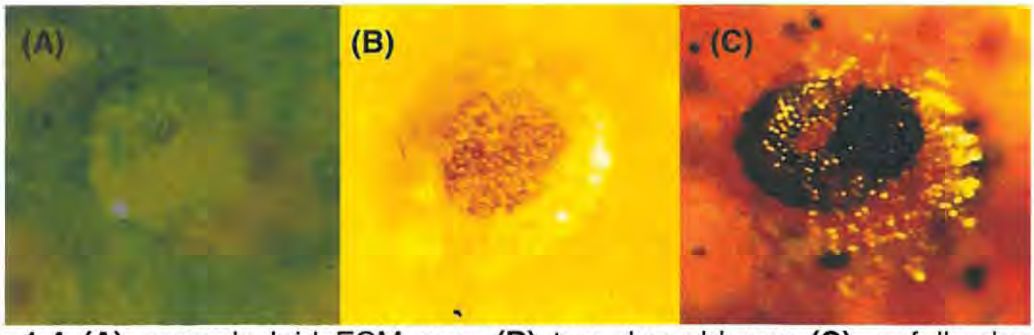

Figure 1.4 (A) a newly laid FCM egg; **(B)** two day old egg **(C)**; a fully developed neonate larvae about to hatch (Source: Moore, 2002).

Newton (1998) observed that in laboratory cultures female moths prefer to oviposit on any clean flat surface, whereas in the field eggs are laid inconspicuously in depressions of the rind of oranges. The incubation period lasts for 3 - 5 days at 25°C in laboratory cultures (Daiber, 1979a). The eggs are flat, translucent oval shaped discs with a granulated surface measuring approximately 0.77 mm X 0.60 mm (length and breadth)

and 1 mm in diameter (Daiber, 1979a). The eggs are white to cream coloured when laid (Fig. 1.4 A). Within a few days the eggs turn red (Fig. 1.4 B) and shortly before hatching, the eggs turn dark (Fig. 1.4 C). Prior to hatching the head capsule is noticeable through the transparent egg shell under the chorion (Daiber, 1979a; Moore, 2002).

1.1.5.2 Larva: FCM larvae have been reported to share some similarities with codling moth (CM), *Cydia pomonella* (L.), with both having dark head capsules. However, FCM larvae possess a characteristic dark anal comb that is absent in CM (Newton, 1990). FCM has five larval instars, of which the 1st instar (Fig. 1.5) is extremely delicate and suffers high mortality (Catling & Aschenborn, 1978). Under field conditions the 1st instar (neonate) larva hatches from the egg within 6 - 12 days and bores its way through the rind of the fruit (Fig. 1.5) (Hepburn, 1947). At this early stage, the legs and prolegs are normally similar in colour to the abdomen, with inconspicuous white hairs found on the body (Fuller, 1901). The larva is also noted for having a preference for boring through the navel end of citrus fruits (Newton, 1990). In laboratory cultures, low humidity levels are known to result in high mortality for both the egg and the 1st instar. In the field, low winter temperatures can prove fatal for these life stages (Catling & Aschenborn, 1978). Young larvae have been reported to be cannibalistic towards each other and their eggs (Newton, 1998).

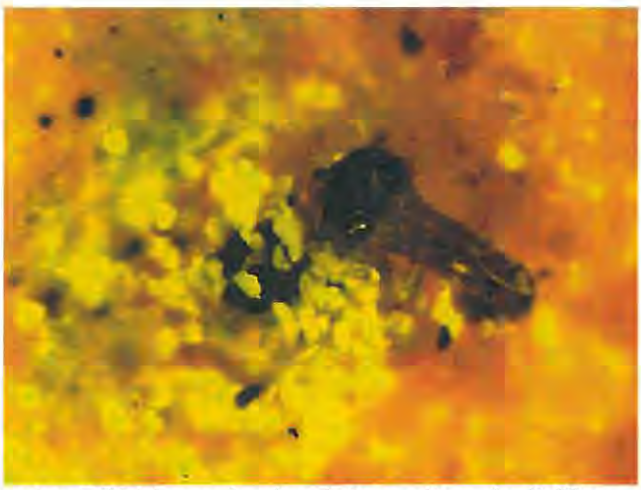

Figure 1.5 First instar FCM larva boring into a citrus fruit (Source: Moore, 2002).

The 1st instar is recorded to be approximately 1 - 1.2 mm in length with a dark pinacula giving it a spotted appearance (Catling & Aschenborn, 1978). The 5th instar larva has an orangey-pink colouration, (Fig. 1.6) which is paler on the sides and yellow on the ventral region (Catling & Aschenborn, 1978).

Figure 1.6 Fifth instar FCM larva (Source: Opoku-Debrah, 2008).

The 5th instar is approximately 12 - 18 mm long, with a brown head capsule (Catling & Aschenborn, 1978). The late 5th instar exits the fruit leaving behind a conspicuous frass-filled exit hole (Fig. 1.2 &1.3) (Catling & Aschenborn, 1978). Daiber (1979b) recorded the mean head capsule width for the 1st to the 5th instar larvae as; 0.22, 0.37, 0.61, 0.94 and 1.37 mm respectively. Larval development in the field is completed in 25 - 35 days and 35 - 67 days in summer and winter respectively (Newton, 1998). In laboratory cultures, hundreds of larvae can be reared in single containers using artificial diet (Moore, 2002; Opoku-Debrah, 2008). However, diet of poor quality has been reported to increase mortality (Daiber, 1979b; Moore, 2002).

1.1.5.3 Pupa: FCM pupal stage consists of two sub-stages, the pre-pupal (the fifth instar larva that has stopped feeding) and the pupal stage (Fig. 1.7) (Stofberg, 1954). The larva spins a cocoon around soil particles or debri (in the field) and with time enters the pre-pupal stage having a cream coloured appearance (Daiber, 1979c). The pre-pupa normally appears light brown and is soft skinned but the chitin hardens and appears dark brown when it enters the pupal stage (Daiber, 1979c). In laboratory cultures FCM will normally pupate in cotton wool stoppers used to cork the rearing

containers (Moore, 2002; Sishuba, 2003; Opoku-Debrah, 2008). According to Daiber (1979c), pupae from which female FCM moths emerge are normally larger than males.

Figure 1.7 FCM pupa

The pupal stage of FCM is gender and temperature regulated. Daiber (1979c) found the average duration of the pre-pupal stage to be 2 days at 25°C. At 25°C the duration of the pupal stage for the male moths was 12 days before hatching, whereas that of females was reported to be 11 days (Daiber, 1979c). In the field, the pupal stage lasted between 12 to 24 days in summer and 29 to 40 days in winter (Stofberg, 1954).

1.1.5.4 Adult: The adult stage begins once the moth ecloses from its cocoon. Some similarities have been observed between the CM and FCM. According to Fuller (1901), FCM share a superficial likeness both in size and tone of colour to the CM, except that, FCM lacks the coppery patches on the wings that are present in the CM. The adult FCM (Fig. 1.8) is a small dark brown to grey moth with mottled forewings. The hind wings are much paler than the forewings and evenly coloured but fringed with hairs (Annecke & Moran, 1982). The adult body length is normally 6 - 8 mm with a wingspan varying between 17 – 20 mm and 15 - 18 mm for the female and male moth respectively (Couilloud, 1988). The antennae are also setiform with distinct segments (Couilloud, 1988).

Figure 1.8 An adult FCM

Daiber (1980) found the sex ratio of FCM field populations to be close to unity. Females mate shortly after emergence with pre-oviposition periods lasting between 5 - 6 days in the field, and 1 - 2 days in laboratory cultures respectively (Diaber, 1980). Catling & Aschenborn (1978) observed that, the adult moth rarely feeds but water is essential for extending longevity. In the field, adults could live for a week or two (Annecke & Moran, 1982). However, in the laboratory the average life span of a male moth ranges from 14 days at 25°C to 34 days at 15°C and that of females from 16 days at 25°C and 48 days at 15°C (Daiber, 1980). Although moths can live this long, they do not continue laying eggs as their peak egg-laying regime is normally within their first 5 days (Sean Moore, pers. comm.). FCM is noted to be a predominantly warm climate pest with its development being limited by cold temperatures (Diaber, 1980; Van Der Geest, *et al.*, 1991).

1.1.6 Control options

A number of insect control options have been adopted for the management of FCM in South Africa. However, for the purposes of this study, emphasis will be placed on the application of baculoviruses as biological control agents. Cultural practices such as orchard sanitation have proven to be quite effective in reducing FCM infestation in the field - especially during seasons of high infestation rates (Ulyett, 1939; Stofberg, 1939).

This is normally followed up with more rigorous procedures such as the pulping of infested fruit in a hammer mill and burying the pulped fruits in deep trenches (Schwartz, 1974). Chemical insecticides registered for use in South Africa against FCM are Cypermethrin, Alsystin (triflumuron), Nomolt (triflubenzuron), Penncap-M (micro-encapsulated - methyl parathion) and Meothrin (fenpropathrin) (Moore, 2002). Other chemicals include Delegate (spinetoram) and Coragen (Rynaxapyr) (Sean Moore, pers. comm.) However, most of these chemicals have been reported to be incompatible with an integrated pest management programme (IPM) and some of them are also considered to be detrimental to natural enemies (Hofmeyr & Pringle, 1998).

Other studies have shown that natural enemies such as parasitoids can be used as biocontrol agents in the management of FCM. For example, the egg parasitoid *Trichogrammatoidea cryptophlebiae* (Hymenoptera: Trichogrammatoidea) (Newton & Odendal, 1990), and the larval parasitoid *Agathis bishopi* (Nixon) (Hymenoptera: Braconidae) have shown potential for use as biocontrol agents against FCM (Moore & Fourie, 1999; Moore & Richards, 2001; Sishuba, 2003; Gendall, 2007). Other natural enemies such as the predators *Orius insduosus* (Hemiptera: Anthocoridae) (Say) and *Rhynocorus albopunctatus* (Hemiptera: Reduviidae) (Stal) have also been reported to attack FCM (Nyiira, 1970). Entomopathogenic microbes such as nematodes (Malan & Moore, 2006) and the fungus, *Beauveria bassiana*, *Metarhizium anisopliae* var. *anisopliae* have also shown promise in use as biocontrol agents against FCM (Goble *et al.*, 2011).

Other commonly used methods involve the use of mating disruption, which involves the application of synthetic female moth pheromones as distractants. The synthetic female pheromones work by confusing the male moths to follow the false pheromones, thus making them unavailable for mating (Minks & Cardé, 1988; Hofmeyr *et al.*, 1991). In some cases, high concentrations of the pheromones have been reported to have an overwhelming effect, which desensitises male moths to an extent that they are no longer able to detect the natural pheromones released by the female moths (Minks & Cardé, 1988).

Another alternative control option is the use of the sterile insect technique (SIT). With this method, laboratory reared adult FCM males are selected and treated with irradiation. The irradiated males are then mass released into the field at an over flooding ratio to wild males, of at least 10:1. There is therefore a greater probability of a wild female mating with a sterile male than with a wild male. As a result, infertile eggs are produced which leads to a subsequent reduction in insect field populations (Myburgh, 1963; Carpenter *et al.,* 2004; Blomefield *et al.*, 2010).

Lastly, several post-harvest techniques, including the treatment of fruit with irradiation, methyl bromide, extreme temperatures and controlled atmospheres can also be used as effective sterilization mechanisms against insects (Pryke & Pringle, 2008). For example, in laboratory experiments cold treatment or the exposure of FCM to low temperature conditions below 10°C were shown to reduce survival and development time of FCM eggs and larvae (Van Der Geest, *et al.*, 1991). Lower temperatures at -4.5°C have also been shown to be detrimental to FCM adults (Stotter & Terblanche, 2009).

1.2 THE PATHOGEN (BACULOVIRUS)

Insect pathogens include members of several invertebrate viruses which infect arthropods in both terrestrial and marine ecosystems (Adams & McClintock, 1991; Miller, 1997). These include the the *Ascoviridae, Baculoviridae, Iridoviridae, Paryoviridae, Polydnaviridae, Poxyiridae, Birnaviridae, Calciviridae, Nodaviridae, Picornaviridae, Reoviridae, Rhabdoviridae* and *Tetraviridae* families (Hunter-Fujita *et al.*, 1998). For the purpose of this study, emphasis will be placed on the *Baculoviridae* family, which are considered as effective biological control agents used in the management of several Lepidopteran pests world-wide (Erlandson, 2009).

1.2.1 Taxonomy

Baculoviruses are named according to the characteristic shape of their viral nucleocapsids (Fauquet & Fargette, 2005). The term 'baculovirus' was first coined from the Latin word *baculum* which means 'a rod' or 'rod-shaped' (Francki *et al.*, 1991). Initially, the *Baculoviridae* family was divided into three subgroups, subgroup A, B and C

(Adams & McClintock, 1991). Subgroup A included the nuclear polyhedrosis viruses (NPVs), which were further divided into two: (a) the multiple-embedded nuclear polyhedrosis viruses (MNPVs) and the (b) single-embedded nuclear polyhedrosis viruses (SNPVs). Subgroup B included the granulosis viruses (GVs) with subgroup C reserved for the non-occluded viruses (Adams & McClintock, 1991).

Later on the *Baculoviridae* family was re-classified into only two genera on the basis of the morphology of their occlusion bodies (OBs). They are namely *Nucleopolyhedrovirus*, NPVs (formerly nuclear polyhedrosis virus), and *Granulovirus*, GVs (formerly granulosis virus) (Fig. 1.9 & 1.10 A) (Murphy *et al.,* 1995; Miller, 1997). The NPVs were further divided into two groups: the multiple nucleocapsid nucleopolyhedroviruses (MNPVs), with several nucleocapsids within their viral envelope (Fig. 1.9 & 1.10 B) and the single nucleocapsid nucleopolyhedroviruses (SNPVs) with a single virion within their viral envelope (Fig. 1.9 & 1.10 C) (Miller, 1997; Winstanley & O'Reilly, 1999; Van Regenmortel *et al.*, 2000).

At present the *Baculoviridae* family have been reclassified into four genera (Jehle *et al.*, 2006a). They are the *Alphabaculovirus* which represents all lepidopteran specific nucleopolyhedrovirus, which is also subdivided into groups I & II. The lepidopteran-specific granuloviruses have also been placed into the genus *Betabaculovirus*. The hymenopteran-specific baculoviruses have been placed into the genus *Gammabaculovirus*. The last group are the dipteran specific baculoviruses which have been designated as the genus *Deltabaculovirus*. This group currently comprises of CuniNPV infecting the *Culex nigripalpus* (Theobald), (Jehle *et al.*, 2006a; Miele *et al.*, 2011). This current classification system proposed by Jehle *et al.* (2006a) was based on molecular phylogenetics as opposed to morphological traits, for the classification and taxonomy of baculoviruses. Despite this, several baculoviruses and a large number of viruses in general remain unclassified (Fauquet & Fargette, 2005). To avoid ambiguity, the old system of GVs and NPVs will still be used throughout this dissertation as its application is common with most literature.

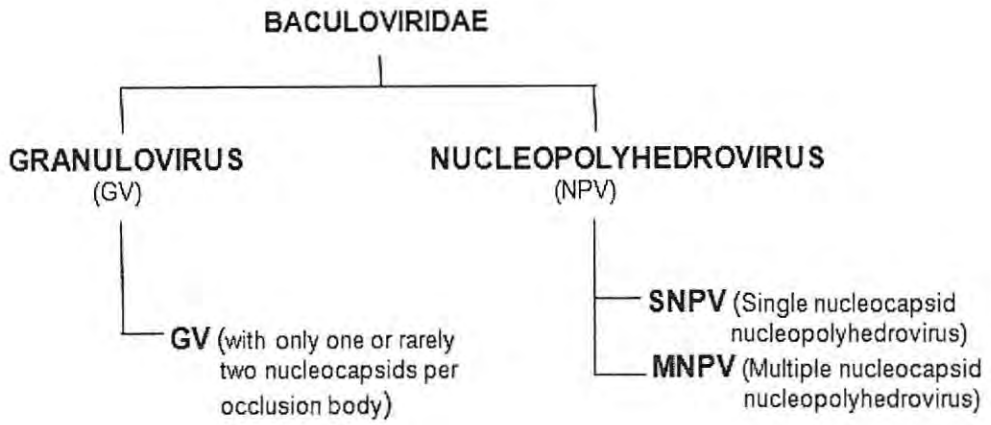

Figure 1.9 Taxonomy of baculoviruses (Source: Murphy *et al.*, 1995).

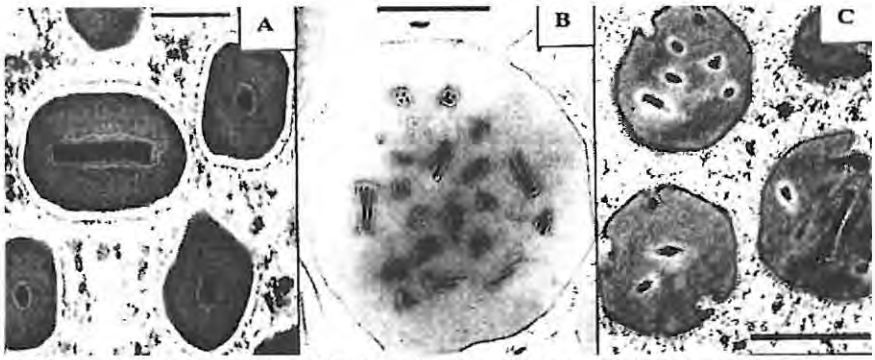

Figure 1.10 (A) A granulovirus with single nucleocapsids per occlusion body (Bar = 0.5µm), (B) a MNPV virus (Bar = 1.0µm) and (C) an SNPV with one nucleocapsid per virion embedded in the same occlusion body (Bar = 0.5µm) (Source: Maramarosch, 1977; Tanada & Kaya, 1993).

The nomenclature of baculoviruses is based firstly on the host species from which they are isolated and secondly on the type of occlusion body (NPV or GV) associated with them (Hunter-Fujita *et al.*, 1998; OECD, 2002; Bonsall, 2004; Fauquet & Fargette, 2005; Erlandson, 2009; ICTV, 2009). For example, *Cryptophlebia leucotreta* granulovirus, CrleGV, was named firstly after its host FCM, *Thaumatotibia* (=*Cryptophlebia*) *leucotreta* and secondly after the type of baculovirus (GV) infecting it. In some cases the country from which the virus was isolated is added as an acronym and suffix, as in CrleGV-SA, which denotes the *Cryptophlebia leucotreta* granulovirus isolated from South Africa (SA) (Jehle *et al.*, 1992; Singh *et al.*, 2003).

However, this system of nomenclature is considered problematic as the incidence of co-infection and cross-infection is quite common (Cory & Myers, 2003). Historically, this problem came into being as previously most insect pathologists held the opinion that insect viruses were particularly species-specific and argued strongly against the possibility of co-infection and cross-infection (Smith, 1967). However, cross-infection has been observed with some baculovirus species, which have been found to infect different insect species and orders (see section 1.2.10 on cross-infection). Other studies have also reported high levels of genetic variation among baculoviruses collected from the same insect species in different geographic locations (Briese, 1986; Fuxa, 1993; Cory *et al.,* 1997). Again, the incidence of latent infection (Il'inykh & Ul'yanova, 2005) and the synergistic (Tanada, 1959a & 1959b; Xu & Hukuhara, 1992; Corning, 1998) effects of baculoviruses make this system of naming baculoviruses after their host problematic.

1.2.2 History of baculoviruses

The first account of virus infection in insects was recorded in ancient Chinese literature. The viral symptoms that were observed with a silkworm, *Bombyx mori* (L.) culture were initially described as being 'jaundiced' (Smith, 1967). In western literature this disease of insects was documented in a poem by an Italian bishop, Marco Vida of Cremona, in 1527 (Smith, 1967 & 1973). In the poem, "*The silkworm*", Vida referred to these symptoms as 'wilting' or 'melting' (Smith, 1967 & 1973; Benz, 1986). These findings stimulated wide interest in the field of baculovirology (Bolle, 1894; Smith, 1973; Miller, 1997). Consequently, more research has been conducted on baculoviruses compared to that of other invertebrate viruses, owing to their effectiveness as biological control agents (Adams & McClintock, 1991; Miller, 1997; Hunter-Fujitta *et al.*, 1998).

1.2.3. Structure

The *Baculoviridae* family is described as a family of rod-shaped, enveloped viruses having a large circular, covalently closed, double-stranded DNA genome of 80 – 188 kilobase pairs (kbp) (Winstanley & O'Reilly, 1999; Theilmann *et al.*, 2005). The virions

consist of a protein matrix (coat), an envelope and a nucleocapsid (Fig. 1.11). The enveloped virions are occluded in a granulin matrix in GVs and polyhedrin for NPVs. The GV rods appear to be rather long and branch in an unlimited and uncontrolled fashion (Smith & Brown, 1965). The protein matrix also gives the virions some level of protection against ultraviolet light and mechanical stress - enabling them to persist in the environment (Van Regenmortel *et al.*, 2000). The envelope usually surrounds one capsid or nucleocapsid, but rarely multiple nucleocapsids, with the nucleocapsid being protected by a proteinaceous coat or occlusion body (OB) (Francki *et al.*, 1991; Theilmann *et al.*, 2005). The occlusion protein polyhedrin has a molecular mass of 25000 - 33000 Daltons (Da) (Theilmann *et al.*, 2005). The nucleocapsids (elongated with helical symmetry) measure approximately 200 - 450 nm in length and 30 - 100 nm in width (Theilmann *et al.*, 2005). The OBs are soluble in strong alkalis (pH 9.0 – 10) and strong acids, but rather insoluble in water and alcohol (Smith, 1967; Federici, 1997). The capsules are also resistant to the enzymatic activity associated with the decomposition of host larvae (Smith, 1967). Using an optical microscope Bolle (1894) was the first to determine the protein nature of the polyhedra, of the *B. mori*, NPV. NPVs have large polyhedral OBs with diameters between 0.15 µm x 15 µm, while GVs, on the other hand, are ovoid or ovocylindrical, averaging 0.13 µm in diameter by 0.4 µm – 0.5 µm nm in length (Murphy *et al.*, 1995; Hunter-Fujita *et al.*, 1998; Theilmann *et al.*, 2005).

1.2.4 Baculovirus life cycle

The best studied baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) infecting the alfafa looper, *Autographa californica* (speyer) (Lepidoptera: Noctuidae) is mostly used as a model for the baculovirus-insect cell system (O'Reillly *et al.*, 1992; Federici, 1997). Formerly, there was little consensus on the site of baculovirus replication in host cells. Some authors contended that virus multiplication commenced inside the cytoplasm of the cell – with the nucleus intact (Bird, 1958 & 1959). However, further studies found the nucleus to be the principal site for virus replication (Smith & Xeros, 1954; Smith & Rivers, 1956; Huger & Krieg, 1961; Huger, 1963; Tanada, 1959a; O'Reillly *et al.*, 1992). Other authors found similarities between the mode of replication of the GVs and NPVs (Smith & Hills, 1962).

Generally, baculovirus pathogenesis is considered to be varied between and within NPVs and GVs, with that of NPVs more widely studied than GVs (Federici, 1997). Their notable differences have been restricted to their tissue tropism (the host cells that support viral growth). But there is a general consensus on their initial infection pathway (Federici, 1997). The primary infection is initiated when a susceptible host feeds on leaves or artificial diet contaminated with the occluded form of the virus, thus infecting the midgut epithelial cells of the host (Fig. 1.12) (Van Regenmortel *et al.*, 2000). For example, in larvae of *Harrisina brillians* (B.) the midgut epithelium is the principal site of infection (Smith *et al.*, 1956). It has however, been reported that larvae of *Pieris brassicae* (L.) tend to feed on the liquefied cadavers of infected individuals (Smith, 1960; Wilson, 1960). Upon successful ingestion of OBs the protein coat of the virion is easily dissolved in the highly alkaline midgut of the host (Summers, 1971; Federici, 1997). Thereafter, the infection progresses by the release of virions which traverse the peritrophic membrane, a protective lining secreted by the midgut, of infected individuals (Federici, 1997).

There is also little consensus on the mechanism of viral passage of baculoviruses through the peritrophic membrane of many insect species (Federici, 1997). There is also considerable variation in the nature of the peritrophic membranes of different insects (Federici, 1997). For example, the peritrophic membrane of the Douglas fir tussock moth, *Orgyia pseudotsugata* (McD) larva is fibrous, allowing particles less than 800 nm to pass through, while that of the cabbage looper, *Trichoplusia ni* (Hubner) are multilayered and channeled (Adang & Spence, 1981 & 1983). The peritrophic membrane is not considered the critical barrier to infection since it is known to be shed during moulting, making newly moulted larvae more susceptible to infection as virions come into direct contact with the microvilli of the midgut (Washburn *et al.*, 1995; Federici, 1997). It has therefore been speculated that most of the virus particles are rather ingested by larvae after the intermoult period (Washburn *et al.*, 1995; Federici, 1997). According to Summers (1971) and Federici (1997), for GVs, the viral DNA is usually injected by the nucleocapsids into the nuclear pore of the host cell to initiate replication. But unlike GVs, the NPVs have been reported to enter directly into the

nuclear pore before uncoating to release their viral DNA (Federici, 1997). Enzymes have also been reported to play a role in facilitating the passage of virions, although not known for all baculoviruses, through the peritrophic membrane of most insects (Derkseen & Granados, 1988; Wang *et al.*, 1994). Studies by Lepore *et al.* (1996) showed that enzymes (enhancins) released by *Trichoplusia ni* (TnGVs) facilitated the localized digestion of the peritrophic membrane, creating lesions for virion passage.

The replicative stage (Fig. 1.11) and life cycle of baculoviruses (Fig. 1.12) involves two distinct viral forms and the production of two virion phenotypes (Van Regenmortel *et al.*, 2000). The first are the occlusion derived viruses (ODVs), which are noticeable by their characteristic thick crystalline protein coat, polyhedrin or granulin (Fig. 1.11 B) enveloping their electron dense nucleocapsids (Fig. 1.11 B) (O'Reillly *et al.*, 1992; Winstanely & O'Reillly, 1999). The ODVs initiate the primary infection in the host and are also responsible for horizontal infection.

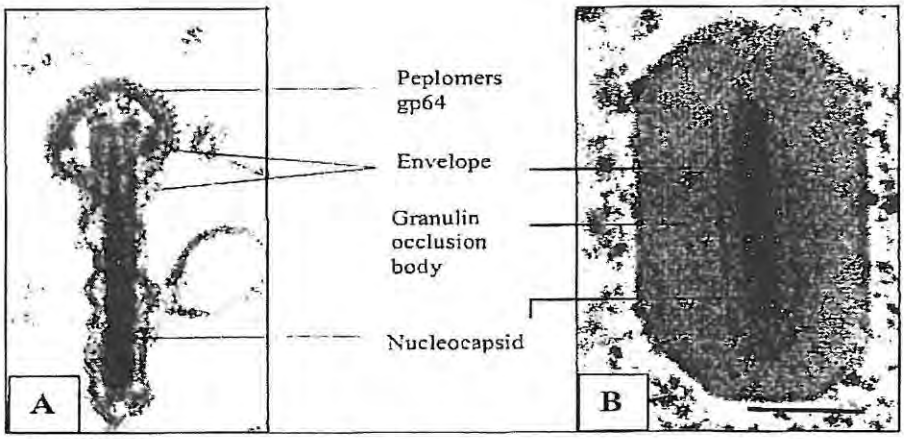

Figure 1.11 (A) A longitudinal section through the budded virion phenotype. **(B)** The characteristic thick protein coat enveloping the electron dense nucleocapsids of GVs (Winstanely & O'Reillly, 1999).

The secondary infection is initiated by the second viral phenotype, the budded virus (BV) (Fig. 1.11 A). Infection in the polarized midgut cells created by the ODVs subsequently results in the release of numerous BVs from the basement membrane side of the cell (Keddie *et al.*, 1989). The BVs then traverse the nuclear membrane (via endocytosis) into the cytosol and then bud through the basal lamina of the midgut cells into the haemocoel and are transported via the haemolymph to other tissues in the

insect (Keddie *et al.*, 1989). The BVs also infect the epithelial cells of the tracheoles (which provide oxygen to the midgut) and spread the infection along the tracheal network of the insect (Keddie *et al.*, 1989; Van Regenmortel *et al.*, 2000). Therefore, the BVs are only responsible for cell-to-cell transmission of viral infection within susceptible host tissues (Theilmann *et al.*, 2005). By this time they will have acquired a new virion envelope, consisting of a plasma membrane containing polymers of a viral encoded glycoprotein, called gp64 (O'Reillly *et al.*, 1992; Theilmann *et al.*, 2005). Afterwards, the BVs acquire an occlusion body (polyhedrin or granulin) around the viral envelope and new OBs are released into the environment when the insect dies – the life cycle is then repeated (Fig. 1.12) (Federici, 1997; Theilmann *et al.*, 2005).

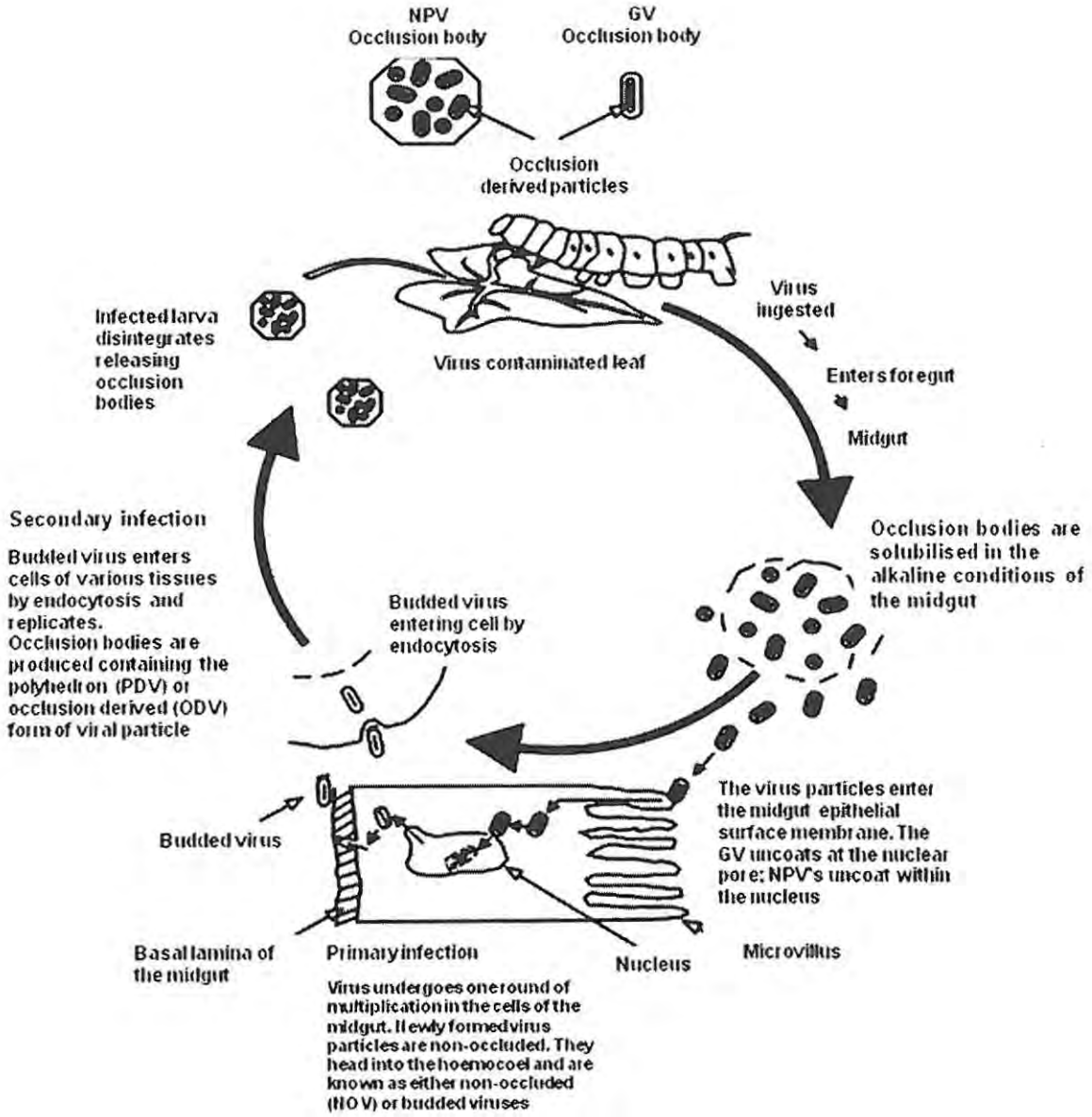

Figure 1.12 The life-cycle of a baculovirus through its host (Hunter-Fujita *et al.*, 1998).

1.2.5 Granulovirus

Paillot (1926) was the first to report on the incidence of a granulovirus infection. The virus, which appeared 'granule-like', was found in larvae of the cabbage white butterfly, *Pieris brassicae* (L.) and was referred to as "granules" (Paillot, 1926). The GV was later on isolated from the pine shoot roller, *Christoneura murinana* (Hübner), and confirmed by Bergold (1948), using an electron microscope. Bergold (1948) referred to the virus

particles (causative agent) as "Viruskapseln" and the disease "Kapselvirus-Krankheit". Due to the nature of the virus particles, which appeared as single small 'granules', Steinhaus (1947) coined the term 'granulosis'.

1.2.5.1 Granulovirus host range: GV infections have been recorded in more than 100 insect species (Murphy *et al.*, 1995) and are considered to have a relatively narrow host range compared to NPVs, with infection being confined to one or more species within the same family as the original host (O'Reillly *et al.*, 1992; Federici, 1997). However, in a few instances some GVs have been reported in laboratory assays as being able to infect more than one insect host specie. For example, CpGV (*Cydia pomonella* GV) has been reported to infect both CM and FCM (Fritsch *et al.*, 1990; Jehle *et al.*, 1992). In another report Goto *et al.* (1992), showed that GVs recovered from six noctuid species, namely *Xestia c-nigrum* (L.) (Lepidoptera: Noctuidae), *Autographa gamma* (L.) (Lepidoptera: Noctuidae), *Hydraecia amurensis* (Staudinger) (Lepidoptera: Noctuidae), *Celaena leucostigma* (Hubner) (Lepidoptera: Noctuidae), *Aletia pallens* (L.) (Lepidoptera: Noctuidae) and *Pseudaletia separata* (Walker) (Lepidoptera: Noctuidae) were all genetic variants of XecnGV (Xestia c-nigrum GV).

1.2.5.2 Gross pathology and symptomatology: The symptoms of GV infection in most lepidopteran larvae are confined to the fat body. During infection, larvae stop feeding and become lethargic (O'Reillly *et al.*, 1992). The cuticle melanizes due to a polyphenol oxidase-mediated process which results in cuticle discolouration and eventually the rupturing or disintegration of the cuticle, sometimes referred to as 'wilting' or 'melting' (Smith, 1967 & 1973; Benz, 1986; O'Reillly *et al.*, 1992). Based on disease, three different types of GVs have been identified namely: type 1, type 2 and type 3 GVs (Federici, 1997).

With the type 1 GV, only the fat body tissue is attacked via the midgut epithelium, an example is the TnGV (*Tricloplusia ni* GV) (Federici, 1997). Infected *T. ni* fourth instar larvae live much longer and last between 10 to 14 days post infection and are known to grow much larger than uninfected larvae, as other important tissues are not attacked (Federici, 1997). Larvae become sluggish within a day or two of death with a creamy or

yellowish appearance, due to the accumulation of viral granules in the fat body. With this type of infection the epidermis remains intact with no liquefaction, as it is not affected larvae turn dark brown or black and may desiccate (Federici, 1997). The type 1 GVs have been reported to have a much higher LD_{50} value, and are normally referred to as "slow" GVs – owing to their prolonged infection (Winstanley & O'Reilly, 1999).

The type 2 GVs infection and gross pathology is parallel to that of a typical lepidopteran NPV disease. Unlike the type 1 GV, the type 2 GV has a much wider tissue tropism, with infection occurring in the midgut, epidermis, fat body, tracheal matrix and possibly the malpighian tubules (Tanada & Kaya, 1993; Federici, 1997).

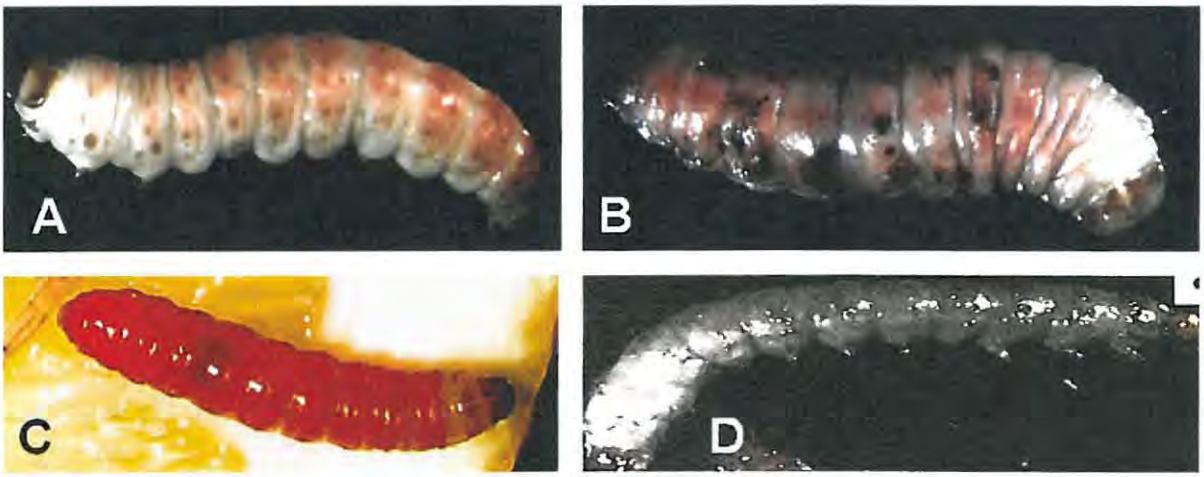

Figure 1.13 (A) Symptoms of a CrleGV infected fifth instar FCM (still alive), with inner body mass appearing whitish; **(B)** a 5th instar larva with brownish lesions due to infection; **(C)** a healthy 5th instar larvae; **(D)** a dead and distended virus infected 5th instar FCM larva (Source: Moore, 2002; Opoku-Debrah, 2008).

The type 2 GV is more acute than the type 1 GV, typically lasting between 5 - 10 days in larvae infected during the fourth instar (Federici, 1997). Normal symptoms of the disease involve the development of irregular white to yellow patches below the cuticle (Fig. 1.13). Infected larvae swell and distend slightly with the body liquefying due to the injured epidermis (Federici, 1997). The *Cryptophlebia leuotreta* granulovirus (CrleGV) and *Cydia pomonella* granulovirus (CpGV) are examples of GVs that fall into this category.

This last type, the type 3 GV, is unique to HabrGV (*Harrisina brillians*, GV), and normally attacks the Western grapeleaf skeletoniser, *Harrisina brillians* (B. & McD) (Lepidoptera: Zygaenidae) (Federici, 1997). Unlike the type 1 and 2 GVs, this GVs tissue tropism is restricted to the midgut epithelium. *H. brillians* infected larvae tend to change in colour from a lemon yellow to grey and then finally to brown or black (Smith *et al.*, 1956). As a result the HabrGV replicates only in the midgut with virions and occlusion bodies attacking both the larvae and adult. The disease is very acute and normally lasts between 4 to 7 days in the third or fourth instar. Larvae shrivel after the fourth or fifth day post infection, and do not liquefy due to their intact epidermis (Federici, 1997).

1.2.5.3 *Cryptophlebia leucotreta* granulovirus (CrleGV): The granulovirus infecting FCM, *Cryptophlebia leucotreta* granulovirus (CrleGV), was first described by Angelini *et al.* (1965). The virus was isolated from infected larvae in the Ivory Coast (CrleGV- IC) (Angelini *et al.*, 1965). Another CrleGV isolate was recovered from the Cape Verde Islands (CrleGV-CV) (Mück, 1985). Using restriction endonucleases to digest the DNA of the Ivory Coast and Cape Verde isolates, Jehle *et al.* (1992) showed that the Ivory Coast and Cape Verde isolates were genetically different. Another CrleGV isolate was reported in a laboratory culture of FCM (FCM material collected from South Africa) held by the Hoechst Corporation in Germany (Jehle *et al.*, 1992). Fritsch (1989) was the first to report and identify a South African isolate (CrleGV-SA). However, she did not characterise this isolate. Thereafter, another CrleGV-SA isolate was recovered by Moore (2002). Classification of CrleGV according to the International Committee on Taxonomy of Viruses (ICTV) is indicated in table 1.5 below (ICTV, 2009).

Table 1.5 Classification of *Cryptophlebia leucotreta* granulovirus (Jehle *et al.*, 2006a; ICTV, 2009)

Order	unassigned
Family	*Baculoviridae*
Sub family	-
Genus	Betabaculovirus
Species	*Cryptophlebia leucotreta* granulovirus
Host	*Arthropoda*

Thereafter, some of these CrleGV isolates were successfully developed as biopesticides for use in the management of FCM in South Africa. Moore (2002) was the first to develop and evaluate *Cryptophlebia leucotreta* granulovirus (CrleGV-SA, South African isolate) as a biological control agent for the management of FCM in South Africa. The granulovirus was identified from Goedehoop citrus insectary at Citrusdal (Moore, 2002; Moore *et al.*, 2004a & 2004b). The CrleGV-SA isolate was subsequently characterized by Singh *et al.* (2003). The novel CrleGV-SA isolate has been developed into the biopesticide, Cryptogran (manufactured by River Bioscience (Pty) Ltd., South Africa) (Moore *et al.,* 2004a & 2004b). Another CrleGV biopesticide, Cryptex, produced by Andermatt Biocontrol (Switzerland) has also been registered for the control of FCM in South Africa (Kessler & Zingg, 2008).

1.2.6 Pathogenicity and virulence

Pathogenicity and virulence are important terminologies used in biology to describe virus-host interactions (Tanada & Kaya, 1993). The terms virulence and pathogenicity are mostly used interchangeably to describe both the severity and speed of impact that a parasite exhibits on its host (Cory *et al.*, 1997). However, considerable ambiguities surrounding the terms pathogenicity and virulence exist within different disciplines (Thomas & Elkinton, 2004). Pathogenicity is defined as "the ability of an organism to infect a host and cause disease" and can be genetically determined (Onstad *et al.*, 2006). Pathogenicity is also considered to be qualitative, an all-or-none concept (Onstad *et al.*, 2006). Virulence, on the other hand, is used to describe the degree of pathogenicity and can be quantified (Onstad *et al.*, 2006). These terms as defined by Onstad *et al.* (2006) will therefore be employed, with their stipulated meanings,

throughout the thesis. Marked differences in the virulence of baculoviruses to their host has been widely reported (Parnell *et al.*, 2002; Takatsuka *et al.*, 2003; Eberle *et al.*, 2008 & Jehle *et al.*, 2008). Moore (2002) found that CrleGV-SA was highly virulent to FCM neonates with a virus concentration required to kill 50% (LC_{50}) of neonates being estimated at 4.095 x 10^3 OBs/ml. The time required to kill 50% (LT_{50}) of FCM neonates was also recorded as 4 days 22 hours, indicating that CrleGV-SA is a fast GV (Moore *et al.*, 2011).

1.2.7 Virus transmission

Generally two routes of baculovirus transmission between infected and susceptible hosts have been identified (Cory *et al.*, 1997). The first and most common type of virus transmission is normally referred to as "horizontal transmission" - which occurs when susceptible individuals ingest OBs from the environment. The second type of virus transmission occurs across several insect generations; within populations and is referred to as "vertical transmission" (Smith, 1967; Cory *et al.*, 1997). These two modes of transmission both occur in the field and in the laboratory. However, there is little consensus on the pathway for vertical transmission of the virus. Early accounts on vertical transmission were thought to be either transmitted via transovarial (transmission inside the egg) or transovum (adsorption of virus on the egg surface) (Smith, 1967). The former (transovarial) was first suggested by Conte (1907) and Bolle (1908). Some authors indicated that the virus was transmitted via transovarial and not transovum (Smith & Wycloff, 1951; Smith, 1967). In other studies, NPVs were reported to transmit infection through the egg and spiracles of insects (Tanada & Kaya, 1993; Kirkpatrick *et al.*, 1994). Vertical transmission of a cytoplasmic polyhedrovirus (CPV) in *B. mori* has also been reported (Hukuhara, 1962). However, Il'inykh & Ul'yanova (2005) argue that the supposed transmission of the virus through the egg or with the egg content is improbable since although insect embryos or neonates are known to be highly sensitive to exogenous viruses, this does not seem to be the case with baculoviruses. Moreover, Il'inykh & Ul'yanova (2005) found that, there was little or no data supporting the confirmation (diagnostic) of an insect embryonic death from a baculovirus.

In the field, the most common means of virus spread is through the migratory movement of infected female adults that tend to pass on the disease to their progeny (Tanada *et al.*, 1964; Elmore & Howland, 1964). Artificial inoculation by spray application of virus inocula is considered an efficient way of achieving mass infection in the field (Elmore & Howland, 1964). Other insect parasites have been reported to play a significant role in virus transmission. For example, Thompson & Steinhaus (1950) observed that an adult *Apanteles* spp. parasite could transmit virus to three successive larvae per hour. Franz *et al.* (1955) found that certain NPVs could traverse the gut of the bug, *Rhinocerrus annulatus* (L.) and the bird, *Erithacus rubecula* (L.) with ease. Other abiotic factors such as wind and rain have also been noted to play a vital role in virus dissemination in the field (Smith, 1967).

Other researchers have reported artificial types of virus transmission such as the direct injection of virus into the host (via the haemocoel) using micro injection needles, as an efficient way to achieve optimum viral infection (Maramorosch, 1951; Martignoni, 1955 & 1959). However, Martignoni (1957) found peroral inoculation of host larvae with granulovirus suspensions to be more effective than injections.

1.2.8 Genetic variation

Generally, baculoviruses have been reported to exhibit high levels of variation in their genetic profile. For example, baculoviruses collected in the same geographic area have been reported to vary considerably within isolates (Goto *et al.*, 1992). According to Cory *et al.* (1997) baculoviruses isolated from one insect, or a group of insects from a single species at one time and location, are normally referred to as 'isolates'. Several authors have also reported the occurrence of field-collected isolates frequently showing differences in virus genotype (Lee & Miller, 1978; Knell & Summers, 1981; Smith & Crook, 1988; Maeda *et al.*, 1990). Other researchers found that baculoviruses isolated from a single insect host may exhibit high levels of genotypic variation (Cooper *et al.*, 2003; Cory *et al.*, 2005). Differences in the virulence of some strains of NPVs against wattle bagworm, *Kotochalia junodi* (Heyl.) populations were also observed (Ossowski, 1958 & 1960). Brown *et al.* (1985) attributed this genetic variation within baculoviruses

to small mutations, sequence duplications and in some cases the acquisition of host DNA.

1.2.9 Host resistance and lower susceptibility

In the field, when insect populations become exceedingly abundant, virus epizootics are a common phenomenon (Glaser, 1915). Glaser (1915) suggests that insects that survive these epizootics may possess certain mechanisms for resistance. For example, Bird & Elgee (1957) found that despite an epizootic by an NPV affecting some populations of the European pine sawfly, *Neodiprion sertifer* (Geoffroy) there were very low levels of mortality. Bird & Elgee (1957) suggested that this low mortality was as a result of some *N. sertifer* populations developing resistance to the virus. In another study, Briese & Mende, (1981) found that certain laboratory strains of the potato tuber moth, *Phthorimaea operculella* (Zeller), exhibited a significantly greater resistance to the potato tuber moth virus, *Phthorimaea operculella* granulovirus by having an LC_{50} value 30 times greater than that of the field populations.

It was initially thought that adult insects were particularly resistant to virus infection (Smith, 1963). However, Martignoni (1964) showed that it was possible to infect healthy adults of the variegated cutworm, *Peridroma saucia* (Hubner) with an NPV. They observed that, the diseased moths lived shorter than the healthy moths. They also found the site of infection in the fat body, tracheal matrix and epidermis of diseased adult moths to be similar to that of diseased larvae (Martignoni, 1964). Other studies have shown that adult insects can be indirectly infected by inoculating their larval stages (Smith, 1963; Neilson, 1965).

Insects have developed several mechanisms to evade baculoviral infection. By encapsulating foreign bodies insects can use this as an effective immune response mechanism against invading organisms (Salt, 1970). In one study, Washburn *et al.* (1995) showed that some haemocytes produced by larvae of the cabbage looper, *Trichoplusia ni* (Hubner), were able to encapsulate and destroy virus infected *T. ni* cells. Encapsulation has also been used by larvae of the *Drosophila melanogaster* (Meigen)

as an effective defensive mechanism against invading parasitoid attack (Fellows *et al.*, 1999). Insects can also fight off infection by the self destruction or programmed cell death (apoptosis) of baculovirus infected cells (Zhang *et al.*, 2002). Phenoloxidase has also been noted to be a key mediator of immune function in insects (Cotter & Wilson, 2002).

Stairs (1965) alluded that when insects undergo metamorphosis the accompanied breakdown and disintegration of organic tissues (histolysis) can lead to the destruction of baculovirus infected cells, thereby conferring some resistance to the host. Another way that insects develop resistance to viruses is by sloughing off their midgut epithelial cells during molting (Federici, 1997; Sun, 2005; Jehle *et al.*, 2006b). However, older larvae (late instars) are known to be particularly more resistant to virus infection since they develop a much thicker peritrophic membrane (a protective lining secreted by the midgut) which makes it difficult for the virus to penetrate and get access to the midgut epithelium in order to initiate infection. Immature larvae (lower instars), on the other hand, having a thiner peritrophic membrane, are more susceptible to virus infection, since their new epithelial cells generated are rather thin and easily accessible by the virus (Federici, 1997). Evans (1983), however, attributed this age-related increase in larval resistance to the developmental increase in larval weight when larvae move from one instar to the next during moulting.

1.2.10 Cross infection

Although baculoviruses are known to be particularly species specific, with regards to their host, it is not uncommon to find the same virus infecting insect host's belonging to different species or even different orders. This phenomenon is normally referred to as cross-infection. Previously, most insect virologists held the opinion that insect viruses were particularly species-specific and argued strongly against the possibility of cross-infection (Smith, 1967). Research conducted mostly on NPVs showed that, baculoviruses could cause infection within as well as between insect hosts belonging to the same order, family or genus (Smith, 1967).

In a related experiment with the tent caterpillar, *Malacosoma disstria* (Hubner) (Lepidoptera: Lasiocampidae), Stairs (1964) found that NPVs could infect several members within the same genus. Stairs (1964) observed that the *M. alpica* (Staudinger - European isolate) NPV was equally virulent against *M. disstria*, but rather less virulent against its American isolates, *M. americanum* (Fabricius) and *M. pluvial* (Dyar). In another instance, Smirnoff (1963) reported that an NPV infecting *Erannis tiliaria* (Hufnagel) was able to infect *Alsophila pometaria* (Harris) and *Phigalia titea* (Cramer) larvae which both belonged to the same order (Lepidoptera) and family (Geometridae) as *E. tiliaria*. There was a noticeable similarity in the shape of the polyhedral crystals of the two geometrids (*A. pometaria* and *P. titea*) and the source insect (*E. tiliaria*) (Smirnoff, 1963). However, subsequent attempts by Smirnoff (1963) to transmit *E. tiliaria* NPVs to six other species of Lepidoptera proved unsuccessful. However, Sidor (1960) and Smith *et al.* (1959) report that not only do baculoviruses transmit infection between insect species, but also between different orders. They found that NPVs isolated from the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) larvae were able to infect larvae of *Hemerobius* spp. belonging to the order Neuroptera (Sidor, 1960; Smith *et al.,* 1959).

In another study on GVs, Smith (1959 & 1960) showed that one GV was able to co-infect larvae of *P. brassicae, P. rapae* and *P. napi* (Lepidoptera: Pierinae). However, similar attempts to infect different species of cutworms (Noctuidae) with a cutworm GV proved unsuccessful (Smith *et al.,* 1964). Huger (1963) argues that in general GVs are more host specific than NPVs.

Conversely, subsequent studies found that different baculovirus species can co-infect more than one host (Fritsch *et al.*, 1990; Jehle *et al.*, 1992; Lacey *et al.*, 2002). For instance, CpGV has been reported to infect both CM and FCM. However, CpGV is noted to be more pathogenic to CM than to FCM. On the other hand, CM is not susceptible to the FCM GV, CrleGV (Fritsch *et al.*, 1990; Jehle *et al.*, 1992). Again, in most cases, baculoviruses have been noted to be more pathogenic to their homologous host (Lacey *et al.*, 2002).

1.2.11 Synergism

Corning (1998) defines synergism as "two or more parts, agents or individuals working together to produce a result not obtainable by any of the agents independently." Some co-infection by baculoviruses has been reported to have a synergistic effect (Smith, 1967). Tanada (1959a) observed that when *Pseudaletia unipuncta* (Haworth) larvae were simultaneously infected with a GV and an NPV there was a synergistic effect which resulted in an overall mortality much higher than the mortalities of the individual viruses. In another study, Shapiro & Shepard (2006) showed that, LdNPV (*Lymantria dispar* NPV) when used in combination with HzSNPV (*Helicoverpa zea* SNPV), SeMNPV (*Spodoptera exigua* MNPV) and SfMNPV (*Spodoptera frugiperda* MNPV) had a synergistic effect.

1.2.12 Sub-lethal effects

Although, most baculovirus infections normally lead to the ultimate death of their host this is not always the case, as some infections may alter the developmental process of their host. These infections are normally referred to as sub-lethal infections (Cory *et al.*, 1997). Sub-lethal effects such as changes in development time, reduction in fecundity, reduced egg viability and changes in sex ratio are a common phenomenon in most baculovirus-host interactions (Cory *et al.*, 1997; Cory & Myers, 2003). For example, Myers & Kukan (1995) found that in field observations of the western tent caterpillar, *Malacosoma californicum pluvial* (Dyar), and its NPV, several shifts in the frequencies of large and small egg masses was frequent, with mean fecundity changing rapidly between different sites.

Neilson (1965) reported that, when adults of *Alsophila pometaria* (Haris), *Nymphalis antiopa* (L.), *Operophtera brumata* (L.) and *Paleacrita vernata* (Peck) were infected with a CPV, sub-lethal effects such as reduced fecundity (in females) and reduced moth size (in both males and females) was very common in all four insect species. Neilson (1965) noted that most of the *A. pometaria*, *Operophtera brumata* (L.) and *Paleacrita vernata* (Peck) adults had tumor-like structures with the prevalence of wing malformation appearing in most of the diseased *A. pometaria* and *O. brumata* males. Therefore, in

addition to the normal fatality resulting from baculovirus infection, sub-lethal infections contribute in suppressing insect populations (Cory *et al.*, 1997; Cory & Myers, 2003).

1.2.13 Latency in baculoviruses

With insects reared in captivity the occurrence of latent virus infections in their larval populations is common (Smith, 1967). Even though, the mechanisms involved with the occurrence of latent baculoviruses, in general, are still not well understood (Il'inykh, 2007) the pathogen which initially appears dormant (latent) in their host can become infective under favourable conditions such as host stress (Adams & McClintock, 1991). Therefore, a latent virus can be stimulated into its virulent form by subjecting the host to conditions of stress (Steinhaus, 1958a & 1958b). Steinhaus (1958a & 1958b) referred to these stress inducing mechanisms as "stressors". Some notable examples of stressors include the following.

1.2.13.1 Temperature: Some authors contend that, under laboratory conditions, manipulating the environmental temperature of the host may serve as a useful stress mechanism (Kitajima, 1926; Hukuhara & Aruga, 1959; Smith, 1967). For example, Smith (1967) found that subjecting insects to low temperature conditions, unlike high temperature, proved to be an effective stressor. In another instance the induction of a latent CPV infection in silkworm, *Bombyx mori* cultures, using low temperature treatment, was successfully achieved by Kurusi (1955) and Aruga (1957) and Aruga *et al.* (1961). By contrast, some authors have successfully used high temperature to induce stress (Kitajima, 1926; Hukuhara & Aruga, 1959). For example, Kitajima (1926) showed that an NPV could be easily induced in *B. mori* populations when subjected to temperatures between 45°C to 50°C for 10 minutes. Hukura & Aruga (1959) also report of similar results with the induction of a CPV in *B. mori*. They suggested that, physiological changes associated with cold and hot treatment in host larvae, served as a precursor for converting the virus from a non-infective form (latent form) into a virulent form (Hukura & Aruga, 1959).

1.2.13.2 Overcrowding: In the field, when insect populations become exceedingly abundant the incidence of virus epizootics is a common phenomenon (Adams & McClintock, 1991). An example is the field epizootics of NPVs infecting *Lymantria dispar* (L.) (Steinhaus & Dineen, 1960). The incidence of field epizootics can easily be simulated in the laboratory via overcrowding. According to some authors, overcrowding is an effective latent virus induction mechanism which may work best with solitary insects (Steinhaus, 1958b; Smith, 1967). This method involves increasing the population of insects per unit area (rearing chamber) or varying the size of the rearing chamber per unit population of insects (Steinhaus, 1958b). Overcrowding was reported to be effective in the stimulation into virulence a latent CPV of the alfafa larvae, *Colias eurytheme* (Boisduval) (Steinhaus & Dineen, 1960).

1.2.13.3 Changes in diet: Alteration of insect diets can be used as an effective stressing mechanism (Smith, 1967). In a related study, the incidence of an NPV was reported in *B. mori* cultures when larvae were fed on black oyster plant, *Scorzonera hispanica* (Ripper, 1915) and Osage orange, *Maclura aurantiaca* rather than its preferred mulberry leaves (Vago, 1951 & 1955).

1.2.13.4 Introduction of a foreign virus: Feeding insect larvae with a foreign virus has been suggested as a good stressor. Smith (1967) reports of the successful stimulation into virulence, of a CPV affecting the winter moth, *Operophtera brumata* (L.) and the pine looper, *Bupalus piniarius* (L.) out of their latent forms when larvae were initially challenged with an NPV. Krieg (1957) also reports of the stimulation into virulence of an NPV infecting the European pine sawfly, *Neodiprion sertifer* (Geoffroy) when it was challenged with a CPV from the pale tussock moth, *Dasychira pudibunda* (L.).

1.2.13.5 Induction with chemicals: In a study conducted by Aruga & Hukuhara (1960) with 18 chemicals, six of these chemicals (sodium cyanide, sodium fluoride, arsenic acid, monoiodoacetic acid, sodium azide, and ethylenediamine tetraacetic acid, EDTA) were successful in the induction of a CPV in *B. mori* cultures. However, EDTA and sodium azide showed a higher efficacy in the induction of the *B. mori* CPV, in

comparison to the other chemicals (Aruga & Hukuhara, 1960). Aruga & Hukuhara (1960) indicated that when these two chemicals were fed to *B. mori* larvae, the 5th instars exhibited a higher induction frequency than the 4th instars. Ether has also been used in the induction of an NPV in *Peridroma saucia* (Hübner) larvae (Tanada, 1959b; Steinhaus & Dineen, 1960).

1.2.13.6 Combination of stressors: Some authors suggest that combining stressors may prove to be more effective than individual stressors. In one study, Aruga *et al.* (1963) found that when *B. mori* larvae were initially dosed with lower doses of a CPV and later on treated with a combination of stressors such as, low temperature (5°C for 3 to 5 hours), heat (37°C for 5 hours), formalin (0.01 -1%), EDTA (powder) and acetic acid (0.1 - 0.5M), there was a significant increase in the incidence of a CPV in all cases. They argued that, these stressors either accelerated virus multiplication in host cells or made it easier for the virus to penetrate cells (Aruga *et al.*, 1963). In contrast, when larvae were treated with an after dose of calcium hydroxide [Ca $(OH)_2$] there was a decreased incidence of the disease (Aruga *et al.*, 1963). In another experiment, Jaques (1960) observed that when cabbage looper, *T. ni* larvae were treated with vibration after an initial dose of its NPV administered at a very low dosage, there was a high incidence of the disease in the treated larvae than in the controls. The control larvae were fed with a low virus dosage without being treated with vibration.

1.2.13.7 Identification of latent infection in insect populations: Since latent infections do not lead to the death of their host, with infected individuals being known to exhibit no viral symptoms its identification, especially in laboratory cultures, is very important (Il'inykh & Ul'yanova, 2005). A number of methods have been developed for the identification of latent infections of baculoviruses in laboratory colonies. Some examples include the use of molecular methods such as standard polymerase chain reaction (PCR) techniques (Hughes *et al.*, 1997; Burden *et al.*, 2003) to identify the presence of latent baculovirus infections in insect cultures. Methods such as DNA re-association kinetics and quantitative PCR (q-PCR) can also be used in quantifying the

amount of viral DNA found in insect cellular genomes (Miryuta *et al.*, 1985; Murillo *et al.*, 2011).

1.3 BIOASSAY OF ENTOMOPATHOGENIC VIRUSES

1.3.1 Introduction

Bioassay or biological assays are common terms used in toxicological studies. The main objectives of these bioassays are to quantify the responses that arise when a biological system receives a "stimulus" (Hawcroft *et al.*, 1987). Hawcroft *et al.* (1987) refer to a stimulus as 'a standard or test sample containing the analyte or biologically active substance to be applied to a biological system or subject.' Therefore, the stimulus is applied at a specified dose (concentration, weight or time) of which the subject elicits the desired response (death, growth or a score) to be quantified (measured statistically) (Finney, 1971). In this study the insect, FCM (subject) is stimulated with a baculovirus (applied at a specified dose) in order to induce mortality (response).

1.3.2 Bioassay techniques

The objective of most bioassays with entomopathogenic viruses are to measure dose or time response relationships (Finney, 1971; Jones, 2000). Bioassays are also important in assessing the comparative pathogenicity of different virus isolates or one virus isolate (Hughes & Shapiro, 1997). In most bioassays with entomopathogenic viruses, the viral inoculum is usually multiplied in the host (*in vivo*) or grown in cell culture systems (*in vitro*) (Jones, 2000). But the former (*in vivo*) is normally the preferred choice as it is considered to be a much cheaper option (Black *et al.*, 1997). Jones (2000) outlines that, one advantage in the use of live insects over the cell culture system, is the ability to test for all stages involved in the infection process. Bioassays are mostly used to determine the LC_{50} (the concentration required to kill 50% of a given population of test insects), LD_{50} (the lethal dose required to kill 50% of a given population of test insects) or LT_{50} values (the time required to kill 50% of a given population of test insects) (Finney, 1971; Hughes & Shapiro, 1997; Hunter-Fujita *et al.*, 1998; Jones, 2000).

According to Hughes & Shapiro (1997) the quantity of inoculated diet administered to the test insects could be used in determining the lethal dose (LD) or lethal concentration (LC) of the virus sample. With the LD method a known amount of virus inoculated diet, of known concentration, is fed to the host of which the larvae consume the entire diet-virus aliquot (Hughes & Shapiro, 1997). Therefore, the dosage of virus ingested by a single larva can be estimated. The diet plug method is the most common procedure used under this category (Hughes & Shapiro, 1997). However, this method is considered to be only suitable for very small or gregarious larvae (Hughes & Shapiro, 1997; Hunter-Fujita *et al.,* 1998; Jones, 2000). Other more accurate methods such as droplet feeding bioassays are used to determine the LD value (Hughes & Wood, 1987; Jones, 2000). Results obtained from the LD method are considered to be less variable than the LC method (Jones, 2000). Different mass dosing bioassay techniques have been developed to measure dose-response relationships with entomopathogenic viruses. The most notable methods are the surface dosing bioassay, diet incorporation bioassay, droplet feeding bioassay and egg-dipping bioassays.

1.3.2.1 Surface dosing bioassay: With this method a known concentration of the virus suspension is spread on an artificial diet or leaf surface, at a previously determined volume, known to adequately cover the entire diet surface. The inoculum is then allowed to dry out thoroughly on the diet surface. The virus medium could be sterile distilled water or any suitable virus carrier medium. For leaf-eating larvae, the leaves are normally maintained in a flat position for uniform spread of the virus suspension (Jones, 2000). This method seeks to mimic the feeding habits of larvae that tend to feed on the surface of their substrate (diet). Some examples are the cotton leaf worm, *Spodoptera littoralis* (Boisduval) and the Indianmeal moth, *Plodia interpunctella* (Hubner) larvae which tend to feed on the surface of leaves (Hunter-Fujita *et al.*, 1998; Jones, 2000). In most LD or LT (lethal time) studies, a single larva is placed on the diet to feed until quantal response (death) or pupation takes place (Hughes & Shapiro, 1997; Hunter-Fujita *et al.*, 1998; Jones, 2000).

1.3.2.2 Diet incorporation bioassay: The diet incorporation bioassay is synonymous to the surface dosing method, except that with the former the virus is incorporated into the diet rather than on the surface (Hughes & Shapiro, 1997; Hunter-Fujita *et al.*, 1998; Jones, 2000). This method also aims at mimicking the feeding behaviour of larvae that tend to feed internally (fruit borers, such as FCM). However, this method is not considered to be very practicable when compared to a normal field scenario. Since in most field applications of baculoviruses there is little chance of larvae, such as FCM, ingesting virus inside a fruit. Virus ingestion is exclusive to the fruit surface (Moore, 2002).

1.3.2.3 Droplet feeding bioassay: This method also called 'synchronous peroral method' was first developed by Hughes & Wood (1981). With this method, the inoculum is administered directly in droplets of virus suspension to the test larvae. This increases the probability of larvae ingesting the virus (Hunter-Fujita *et al.*, 1998; Jones, 2000). Normally a dye such as Brilliant Blue R or food dye is mixed with the viral suspension, making it easy to identify larvae that have ingested the inoculum (Hunter-Fujita *et al.*, 1998; Jones, 2000). Jones (2000) points out that, this method has the added advantage of allowing a large number of insects to be assayed. Moreover, since the volume ingested by the test larvae virtually remains constant it facilitates a good estimation of the LD value from a given LC value during an assay. It is also considered to be more appropriate for LT studies (Jones, 2000).

1.3.2.4 Egg dipping bioassay: The egg dipping bioassay method was developed for neonate larvae that tend to eat away the chorion of their eggs. Hence this method takes advantage of the fact that, with larvae that tend to eat away their chorion after hatching, dosing unhatched eggs with the viral inoculum will increase the chances of larvae ingesting the inocula alongside the chorion (Jones, 2000). Sometimes a wetting agent such as 0.1% Tween or Teepol is added to the virus suspension for ease of spread. However, since there is the possibility that not all the larvae will consume (or completely consume) their chorion after hatching, the accuracy and reliability of these experiments are suspect (Jones, 2000). Another problem is the difficulty in achieving an even spread

of the virus inoculum on the entire egg surface, thereby leading to further increased variation in experimental results (Jones, 2000).

1.4 MICROBIAL CONTROL

1.4.1 Virus production

Two main routes for commercial production of baculoviruses have been identified. The virus is either multiplied (grown) inside the insect, *in vivo* or grown in insect cell culture systems (*in vitro*) (Shuler *et al.*, 1995). The *in vitro* propagation involves the adoption of an appropriate medium for the culturing of insect cell lines. The Grace's medium (Grace, 1962), IPL-41 (Weiss & Vaughn, 1986) and the TC-100 (Gardiner & Stockdale, 1975) are examples of media used to support the growth of lepidopteran cell lines for subsequent propagation of baculoviruses. However, the most common insect culture medium used is the TNM-FH medium (Hink, 1970), which is composed of the Graces medium (Grace, 1962) supplemented with lactalbumin hydrolysate and yeastolate (O'Reillly *et al.*, 1992). The risk of fungal and bacterial growth in the propagating medium could easily be minimized by the addition of an antibiotic-antimycotic solution (O'Reillly *et al.*, 1992). Significant progress has been made with regard to the *in vitro* technique owing to its usefulness for studies on genetically engineered baculoviruses. The low contamination involved in its production, unlike the *in vivo* method, which is mostly contaminated with insect matter and more importantly bacteria has also encouraged interest (Black *et al.*, 1997). However, the *in vivo* method is still widely used as the *in vitro* method requires much more expertise and is rather costly. With regard to the *in vivo* production of baculoviruses a proportional relationship between host weight at death and virus (OB) production has been established. Therefore, in order to optimize virus yield, older larval stages of insects are normally used for virus propagation (Hunter-Fujita *et al.*, 1998).

1.4.2 Virus formulation and field persistence

Another important component of most baculovirus insecticides is product formulation. The main objective is to protect the virus from environmental degradation, thereby

allowing the virus enough time to be ingested by the host and also preserve the biological activity of the product (Black *et al.*, 1997). Some common components of most viral insecticides are the inclusion of flowables and wettable powders that are compatible with conventional spray equipments. The behaviour and feeding pattern of the host are also taken into account during product formulation and spray application. For instance, a standard fan-jet application of the virus has been noted to give a good coverage of the upper leaf surface of plants such as cotton, but rather poorly covers the leaf undersurface where early instars of species like *Spodoptera litoralis* (Boisduval) are known to feed (Black *et al.*, 1997). Young & Yearian (1974) indicated that products that have a high level of protection from the sun's ultraviolet light (UV) may have some added effect, since baculoviruses are easily denatured by UV rays. Several components such as, gustatory stimulants, thickening agents, emulsifiers, viral synergists and spreader thickener agents may be used during product formulation (Black *et al.*, 1997). For example, molasses has been reported to provide some level of UV protection in Cryptogran (Moore *et al.*, 2004b). However, in some laboratory and field studies, when lignin, molasses, wetcit, oil, yeast and milk powder were added to Cryptogran as a possible UV protectant, lignin was found to show a better level of UV protection than the other additives, although its effect was not exceptional (Kirkman, 2007). Their usage, however, in terms of added cost to product formulation is not considered to be satisfactory, when compared with other applications which use the crude forms of virus and insect matter (Kirkman, 2007). Insect host tissues and pigments have been reported to produce some level of UV protection (Shapiro *et al.*, 2002).

1.4.3 Commercialisation of baculoviruses

Initial accounts on the production and usage of the first commercial baculovirus product, Elcar, containing *H. zea* NPV, were reported in the 1970's (Black *et al.*, 1997). Thereafter, other baculovirus preparations such as, Gypcheck (*Lymantria dispar* nucleopolyhedrovirus), TM Biocontrol-1 (*Orgyia pseudotsuga* nucleopolyhedrovirus) and Neocheck-S (*Neodiprion sertifer* nucleopolyhedrovirus) were introduced (Black *et al.*, 1997). Initially, due to the challenges with product formulation, high production costs and field persistence, subsequent viral insecticides did not fare well in comparison to

chemical insecticides (Black *et al.*, 1997). The speed of kill of baculoviruses was also noted to be one of the main weaknesses of commercially applied baculoviruses, when compared to their chemical counterparts (Moscardi, 1999). This is due to the fact that, baculoviruses take a much longer time to induce mortality in their host, leading to considerable losses in crop damage beforehand. For example, the *Lymantria dispar* NPV has been reported to take up to about three weeks (between 18 to 21 days) to cause mortality (Black *et al.*, 1997). Nonetheless, in recent times several improvements have been made and a large number of baculovirus insecticides are now on the market. A few examples are Elcar™ (HzSNPV, Sandoz Agro Inc., USA), CYD-X® (CpGV, Certis Inc., USA), (Moscardi, 1999), Helicovex® (HearNPV, Andermatt-Biocontrol AG, Switzerland), Cryptex (CrleGV, Andermatt-Biocontrol AG, Switzerland) (Kessler & Zingg, 2008) and Cryptogran (CrleGV, River Bioscience (Pty) Ltd., South Africa) (Moore *et al.*, 2004a).

Other alternatives such as the usage of genetically engineered baculoviruses have been explored. With this approach, the main focus has been the removal of viral genes known to prolong the life span of the host (O'Reillly *et al.*, 1992). These "non-essential genes" are considered not vital for the growth of the virus per se, but are thought to play a role in the evolutionary fitness of the virus in its natural habitat (Black *et al.*, 1997). An example of a gene that falls into this category is the *egt* (ecdysteroid glucosyltransferase) gene of baculoviruses. The *egt* gene prolongs the life span of the host, which enables the virus to replicate much longer. Therefore, by modifying or deleting this gene the speed of kill of the virus is greatly enhanced, as the host dies much quicker (O'Reillly *et al.*, 1992). This has been the focus of most genetically engineered baculovirus insecticides. However, the viral yield is compromised as the virus replicates for a shorter period in the host, which serves as a trade-off (Black *et al.*, 1997). The use of molecular techniques and the development of baculovirus expression systems, has also led to the construction of fast-killing recombinant baculoviruses, such as those that express insect-specific toxins that are comparable with some chemical insecticides (Black *et al.*, 1997). However, at present genetically engineered baculovirus insecticides are not used commercially.

Therefore, owing to the ease and lower cost involved in the production of wild-type baculovirus insecticides, they are the main method of choice. A good spraying regime of baculovirus insecticides also creates a good reservoir of the virus in the field, which further perpetuates the occurrence of field epizootics (Cory *et al.*, 1997; Hunter-Fujita *et al.*, 1998).

1.5 PROJECT PROPOSAL

1.5.1 Justification, aims and objectives

Since the discovery of baculoviruses they have found numerous applications in both research and industry. Baculoviruses play an important role as they serve as models for studying most host-pathogen interactions (Il'inykh & Ul'yanova, 2005) and influence the population dynamics of their host (Bonsal, 2004). Their compatibility with IPM programmes and their environmental friendliness also makes them attractive as alternatives to other insect control methods (Bishop *et al.*, 1995; Moscardi, 1999).

However, in spite of their numerous benefits, some notable challenges with regards to differences in host susceptibility to the virus and insect resistance can be problematic. In the past, the occurrence of insect resistance to baculoviruses was considered unlikely and was only observed under laboratory conditions (Briese & Mende, 1983). Laboratory resistant populations were recovered when selection was conducted by challenging insects with medium doses of the virus. Resistance was not stable and was lost within one or a few generations (Cory *et al.*, 1997; Jehle *et al.*, 2006b). Generally, levels of decreased host susceptibility to baculoviruses following selection pressure were considered to be low (Fuxa, 1993) and field observations of resistance were rare and not systematically investigated (Cory & Meyrs, 2003).

Just a few years back, the first account of field insects developing resistance to a commercial baculovirus product was reported. This was observed in Europe, where increased resistance by both field and laboratory reared CM to a commercial biopesticide, CpGV-M was reported (Fritsch *et al.*, 2005; Eberle & Jehle, 2006; Jehle *et al.*, 2006b). CpGV and CrleGV are also very closely related (Wormleaton & Winstanley,

2001), with CpGV being reported to infect both CM and FCM (Fritsch *et al.*, 1990; Jehle *et al.*, 1992).

Other studies have shown that genetic variation between different FCM populations exists (Timm, 2005a & 2005b), with laboratory reared FCM populations being found to show considerable differences in their biological activity to Cryptogran and Cryptex (Opoku-Debrah, 2008). It is also widely known that high levels of genetic variation between baculovirus isolates exists (Crook *et al.*, 1985; Takatsuka *et al.*, 2003; Cory *et al.*, 2005; Eberle *et al.*, 2009), with isolates collected from the same insect species from different geographic locations, frequently showing differences in their biological activity (Rezapanah *et al.*, 2008; Espinel-Correal *et al.*, 2010; Patel *et al.*, 2010).

Therefore, considering that Cryptogran and Cryptex are widely used in South Africa for the management of FCM, any future development of resistance by FCM to Cryptogran and Cryptex (the two formulated CrleGV products used in the management of FCM in South Africa) would be very unfortunate. Moreover, with the recent global interest in organic fruit (Wahab, 2009), the incidence of such unforeseen resistance development by insects to biocontrol agents may pose a serious challenge to invertebrate pathologists and other researchers in biocontrol, as this could lead to a loss of confidence in baculovirus usage by farmers and a subsequent reduction in other concerted efforts aimed at promoting baculovirus based biopesticides.

In addition, it is significantly possible that generally geographically distinct FCM populations may differ in their susceptibility to Cryptogran and Cryptex. If such a situation becomes imminent then it would be important to understand these differences in host susceptibility to the virus. Subsequently, this information would also assist in making informed decisions regarding the efficient utilisation of CrleGV based biopesticides. Yet again, considering the possibility that some FCM populations could develop resistance to CrleGV, as was the case with CpGV against CM, it would also be an added benefit if several more CrleGV isolates, especially those found to show a superior virulence to certain FCM populations, be recovered to help prepare for any possible resistance by FCM to Cryptogran and Cryptex.

In view of the above, the overall aim of this study is to investigate the biological activity of CrleGV against different geographic populations of FCM and bioprospect for more CrleGV isolates.

In order to achieve this aim, a number of objectives will be explored.

1. Firstly, to establish and maintain geographically distinct laboratory populations of the host (FCM). This would increase the probability of obtaining genetically different CrleGV isolates from these insects.

2. The second objective is to conduct a study on the biological performance of geographic FCM populations. This would shed more light on the comparative fitness of insects originating from different geographic regions.

3. The third objective is to develop a protocol for the induction of a latent CrleGV in these laboratory populations. This would facilitate the recovery of new CrleGV isolates from these colonies, once established.

4. The fourth objective is to characterise any new CrleGV isolates obtained from the latent induction protocol. New virus isolates found could serve as a gene bank for different CrleGV genotypes.

5. The fifth objective is to investigate the genetic diversity of some conserved baculovirus genes, such as the *granulin* and *egt* genes, in CrleGV.

6. The final objective is to investigate the biological activity of Cryptex and Cryptogran, as well as any CrleGV isolates recovered against different geographic populations of FCM. This would shed more light on the comparative susceptibility of geographically distinct FCM populations to CrleGV.

CHAPTER TWO

COLONY ESTABLISHMENT AND INVESTIGATION OF HOST BIOLOGICAL FITNESS

2.0 INTRODUCTION

Insects are reared for a variety of reasons. Some insects may be reared as biocontrol agents for the control of aquatic weeds in water bodies (Hill & McConnachie, 2009). Other insects such as parasitoids may be mass reared in the laboratory and released into the field as biocontrol agents to control other insect pests considered to be of economic importance (Wei *et al.*, 2005). Insects are also used to conduct toxicological tests such as investigating insect resistance to chemical insecticides (Cohen, 2001; Ceccatti, 2004). Other notable insects like the fruit fly, *Drosophila melanogaster* (Meigen) are widely used by geneticists to serve as models for studying genetic applications in eukaryotic organisms (Reiter *et al.*, 2001; Lu *et al.*, 2009).

In order to rear insects, a reliable source of fresh host material is required. An artificial diet can be used if the host material is not readily available. The type of diet and insect rearing technique used depends on the species of insect as well as its habitat and food preference (Hunter-Fujita *et al.*, 1998). For rearing of the false codling moth (FCM) *Thaumatotibia leucotreta*, artificial diet is the preferred method (Ripley *et al.* 1939; Theron, 1948; Schwartz, 1971). Early accounts on the history of the development of an artificial diet for mass rearing FCM were reported by Ripley *et al.* (1939). The rearing technique was provided by Theron (1948) which involved the inoculation of maize meal diets with the fungus, *Rhizopus* spp. However, due to high fungal contamination of the diet coupled with its high labour intensity and other hygiene related issues, critical modifications of some aspects of the host diet were suggested by some authors (Couilloud & Giret, 1980; Moore, 2002). Based on extensive trials with different diet recipes developed by Bot (1965), Guennelon *et al.* (1981) and Huber (1981) an innovative FCM diet, which was less labour intensive was subsequently developed by Moore (2002). The diet excluded the fungus *Rhizopus* spp. and included anti-microbial agents like nipagin and sorbic acid (Moore, 2002). Moore's diet has been successfully used by various researchers to rear FCM (Sishuba, 2003; Gendall, 2007; Opoku-Debrah, 2008).

In order to conduct experiments in line with the proposed aims of this study (Chapter One, section 1.5.1), it was important that a permanent source of the host material be established. The first objective of this chapter, therefore, was to establish and maintain several geographic populations of the host (FCM) using Moore's artificial diet. These geographic populations would also serve as a stock for the execution of future experiments on the induction of latent baculoviruses. The increased geographic distribution of the insects would also increase the probability of obtaining genetically different CrleGV isolates.

The second objective was to conduct a brief study on the biology and fitness of the different geographic populations. This would shed more light on the comparative fitness of insects originating from different geographic regions

2.1 MATERIALS AND METHODS

2.1.1 Geographically distinct laboratory populations of FCM

FCM from five colonies initially held in a laboratory facility at Citrus Research International (CRI) in Port Elizabeth was transported to an insectary at Rhodes University for maintenance. These colonies were established by Moore (2002) and Opoku-Debrah (2008). The first colony was established by Sean Moore in 1996 using field collected FCM material from Citrusdal (Western Cape Province), Zebediela (Limpopo Province) and the Eastern Cape (Sean Moore, pers. comm.). The colony had been maintained for over 166 generations estimated at a rate of one generation per month. The colony was therefore of heterogeneous origin or a mixed colony. The other four colonies were established by Opoku-Debrah (2008) using field collected FCM material from Addo (Eastern Cape), Citrusdal (Western Cape), Marble Hall (Limpopo) and Nelspruit (Mpumalanga), which were in their 32^{nd}, 31^{st}, 30^{th} and 30^{th} generations respectively (Fig. 2.1).

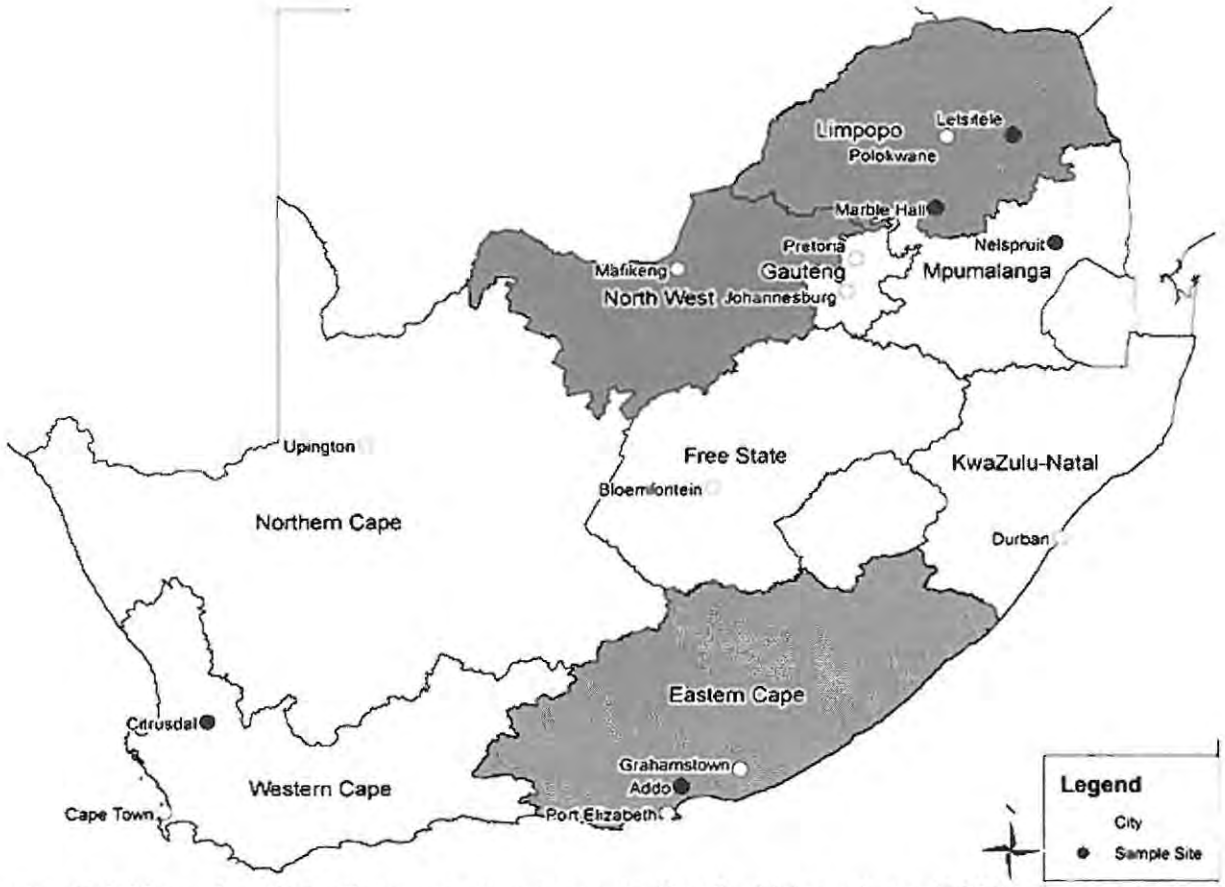

Figure 2.1 Map showing citrus growing areas in South Africa where FCM-infested fruit were collected for establishment on artificial diet. Sample sites are indicated by shaded dots.

In this study, two additional colonies were established to augment the other five colonies. The first colony was established using FCM infested citrus fruit from Letsitele (Limpopo). The second colony, which is a relatively new colony in its third parental generation ($P_{3,}$), was kindly supplied by Rob Stotter. This colony was established using field collected FCM material from Baths farm in the Citrusdal area (Rob Stotter, pers. comm.).

These seven colonies are referred to as Addo, Nelspruit, Citrusdal, Marble Hall, Letsitele, Baths and Mixed colony throughout this thesis. Passport data for the colonies are shown in table 2.1 below.

Table 2.1 Passport data for the Addo, Nelspruit, Citrusdal, Marble Hall, Letsitele and Baths colonies

	Addo colony	Citrusdal colony	Nelspruit colony	Marble Hall colony	Baths colony	Letsitele colony
Farm name	Lone Tree Farm	Noordhoek	Crocodile Valley	Schoeman Boerdery	The Baths	-
Area	Addo – Sundays River Valley (SRV)	Citrusdal (bo-rivier)	Nelspruit	Marble Hall	Citrusdal	Letsitele
Geographic coordinates	33°34'S, 25°41'E	32°36'S, 19°01'E	25°28'S, 30°58'E	24°58'S, 24°18'E	32°43'S, 19°02'E	32°53'S, 30°24'E
Farmer	Danie Bouwer	Hardy van der Merwe	Johan Kellerman	Oubaas van Zyl	Allan Hall	-
Orchard	36	-	Bridge 3	Block J11	-	-
Variety	Lane Late navel oranges	Washington navel oranges	Lane late navel oranges	Washington navel oranges	Washing - ton navel oranges	Washing -ton navel oranges
Tree spacing (rows x trees)	6 m x 3 m	6 m x 4 m	8.3 m x 2.8 m	5.5 m x 5.5 m	-	-
Rootstock	-	Rough lemon	Empress mandarin	Rough lemon	-	-
Year planted	1999	1978	1978	1968	-	-
Age of trees	8 yrs	30 yrs	30 yrs	40 yrs	-	-
Number of trees	2590	458	586	-	-	-
Size (hectares)	4.66	1.1	1.36	3.84	-	-
Date of fruit collection	3-8 /04/2008	17-24 /4/2008	26 /06/2008	23/06/2008	28 /01/2010	29 /05/2009

*Dash (-) indicates data / information not available.

2.1.2 Protocol for the establishment of field populations of FCM on artificial diet

Field populations of FCM were established on artificial diet as illustrated in Fig 2.2. Firstly, Moore's artificial diet was prepared beforehand. The diet consisted of maize meal, wheat germ, milk powder, brewers' yeast, nipagin and sorbic acid (Moore, 2002). An amount of 150 g dry ingredients of Moore's diet was uniformly mixed with 150 ml of sterile distilled water (ddH_20) to form a paste. The contents were mixed in an aluminium baking tray and baked in

an oven at 180°C for 25 minutes. The cooked diet was allowed to cool under a laminar flow cabinet. Once cool, individual diet plugs (approximately 5 to 6 mm thickness) were cut using the lip of a glass vial (28 ml capacity, Bibby Sterilin®) and inserted into the vials using sterile glass rods. Fruit were individually cut open with sharp knives to locate larvae (Fig. 2.2 B) and larvae were transferred onto diet plugs using a small paint brush sterilised in 5% (v/v) sodium hypochlorite. Larvae were held individually per vial in order to avoid possible disease contamination which could lead to the total collapse of the colonies. The vials were corked with cotton wool and then sent to an incubation chamber for development and growth to take place (Fig 2.2 C). Larvae pupated inside the cotton wool stoppers and emerging adults (both males and females) were then captured.

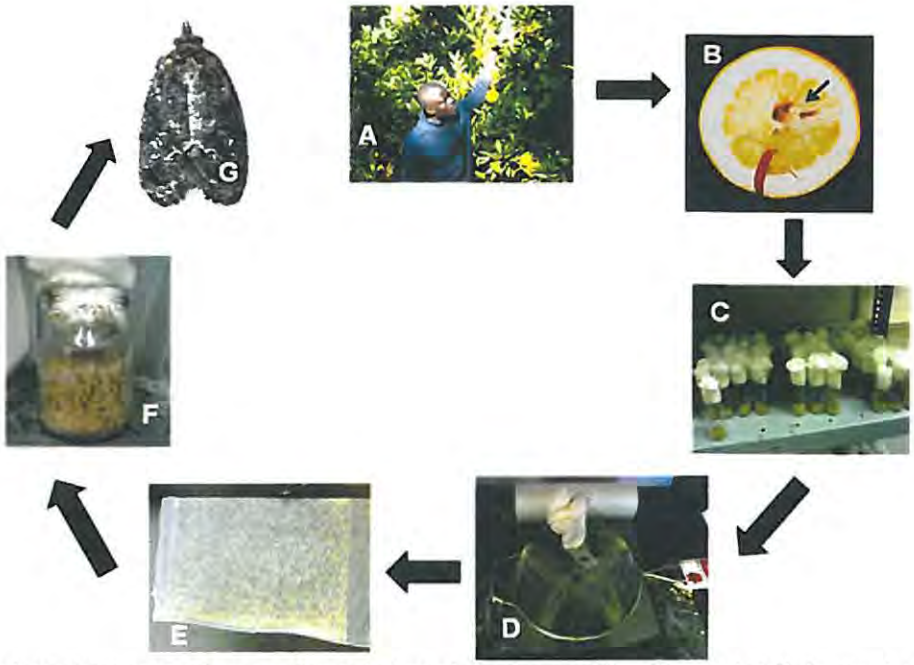

Figure 2.2 Pictorial image of experimental protocol used for the establishment of a laboratory colony of FCM from field lines. **(A)** Field collection of FCM infested citrus fruit. **(B)** Dissected fruit infested with 5th instar larva. **(C)** Individual vials filled with artificial FCM diet, with each holding a single larva. **(D)** Adult moths held under sieve. **(E)** FCM eggs. **(F)** FCM larvae growing in artificial diet held in jars. **(G)** An adult FCM.

To collect moths, a second vial was inverted over the first one in order to allow moths to climb into the top of the inverted vial. Moths were transferred into a sieve with a hole cut in the middle. By gently tapping the vial with a finger, moths were encouraged to fly or drop into the sieve (Fig. 2.2 D). The sieve was inverted onto a wax paper sheet which served as a

substrate for the female moths to oviposit (Fig. 2.2 D). Wet cotton wool, which was plugged into the hole, served as a source of water for the moths. The sieve was finally secured at both edges with Sellotape in order to prevent moths from escaping. The females laid eggs (Fig. 2.2 E) on the wax paper sheets which were pulled out daily, sterilised and put on autoclaved Moore's diet for growth to take place. Thereafter, subsequent generations were maintained according to the protocols described below.

2.1.3 Laboratory maintenance of colonies

Moths were reared in an insectary facility that had mosquito-fabric cages constructed inside to house the seven colonies (Fig. 2.3 A). It was considered important that the colonies be separated, in order to prevent interbreeding among them. Cages were constructed using mosquito net fabric that was secured around metal frames. The cage entrance was constructed by cutting portions of the net and securing the edges with a fabric zipper (Fig. 2.3 B). As an additional measure to avoid cross contamination between colonies, moths that had escaped or were found outside the cages were immediately killed.

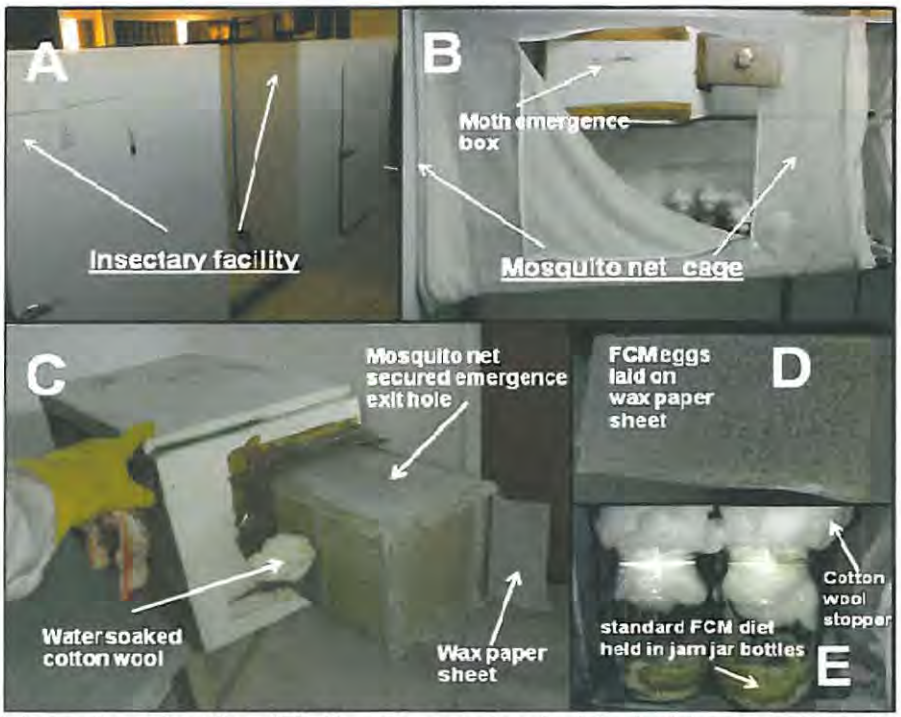

Figure 2.3 (A) Insectary facility. **(B)** Cages constructed to hold separate colonies. **(C)** Moth emergence box. **(D)** a wax paper sheet with FCM eggs. **(E)** Preserve jars with FCM diet.

To maintain and ensure the adaptation of the colonies to artificial diet, colonies were reared in the following manner. Approximately 40 g of Moore's diet was mixed with 40 ml of ddH_20 in preserve jars (380 ml, Pattesons Glass Ltd.). The jars were stoppered with cotton wool, autoclaved at 120°C for 20 minutes and placed under a laminar flow hood to cool (Fig. 2.3 E). Once the diet had sufficiently cooled down, approximately 400 - 500 FCM eggs were taken and sterilised in 25% (v/v) formaldehyde (37% stock solution, Minema®) and inoculated onto Moore's diet. The jars were then sent to the incubation chamber for development and growth to take place. About 14 days later, late instars normally pupated inside the cotton wool stoppers corked in the jars (Fig. 2.2 F). The pupae in cotton wool were placed into a wooden moth emergence box. At one end of the emergence box, a small chamber (exit hole) was created using mosquito net fabric secured around a metallic frame. The transparent nature of the fabric allowed in light, which attracted emerging moths to congregate and mate inside the exit hole. The net had a hole punctured on one side which was plugged with wet cotton wool for moths to drink from. The moths were therefore fed with water. The wax paper was run along one side of the emergence cage (Fig. 2.3 C). Mated female moths oviposited on the wax paper sheet (Fig. 2.3 D). These sheets were collected daily and inoculated on Moore's diet for the subsequent rearing of the next generation. Thereafter, the moth rearing process was repeated for the maintenance of the colonies.

2.1.4 Host biology

Data on host biology (egg to adult) and fitness of the different host populations was recorded. Fecundity, egg hatch, pupal weight and length, pupal survival, duration of life cycle and adult eclosion were used as parameters to test host fitness. However, host fitness data was only collected for the Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colonies, since these colonies had been established for a longer duration on Moore's diet, they were considered to be very stable populations and ideal for these trials. Similar data on the Baths colony and Letsitele colony were not collected. Since the Baths colony is a relatively new colony it was decided that host material be reserved for colony maintenance. The Letsitele colony on the other hand failed to establish. Therefore, only data on larval and adult generations during initial colony establishment were recorded for these two colonies. The images of eggs, larvae,

pupae and adults were taken using a dissecting microscope (Leica® EZ4D). A digital analytical balance (UniBloc®) with 0.01mg precision was used to record pupal weight.

2.1.4.1 The egg: The mean number of eggs laid per female moth was determined. This was done by placing one male and one female virgin moth in an empty glass vial for the pair to mate. The vials were corked with wet cotton wool, which served as a source of water for the moths. After three days when females had laid eggs the pair were removed and placed in a new vial, which was also corked with wet cotton wool. The number of eggs laid in each vial was then recorded. This process was continued until all moths had died. Eggs from adults that were injured or had died pre-maturely were excluded from the results. Fifteen pairs of moths (male and female) were used per colony per replicate and experiments replicated three times.

2.1.4.2 The larva: The five distinct FCM larval stages were identified based on measurements of their head capsule width (mm). According to Daiber (1979b) FCM instars can be distinguished from each other based on the width (mm) of their head capsules. Therefore, after each molting stage, 5 larvae that had recently shed their head capsule and were a day old were selected from each colony and used in trials. Trials were not replicated. Other parameters such as colour (judged visually) and body size measurements (mm) were also used in guiding instar identification.

2.1.4.3 The pupa: Pupal mass of the five colonies was determined. Daiber (1979c) found that pupae from which female moths emerged appeared to be much larger in size than that of males. Pupae were therefore selected based on visual judgement of size, from each of the five colonies, and their mean weight (g) and length (mm) measured. Thirty one pupae were selected per colony and experiments replicated three times.

2.1.4.4 The adult: According to Catling & Aschenborn (1978) the adult male FCM is much smaller than the female and is easily distinguished by the presence of densely packed elongated scales found on their hind tibia. The sex of the adult FCM was therefore determined based on the presence or absence of these morphological features.

2.1.4.5 Egg hatch, pupal survival, adult eclosion and duration of FCM life cycle: Egg hatch was determined by placing 50 FCM eggs in sterile vials. Vials were then corked with their lids and the number of larvae hatching out were removed and counted daily. To improve ventilation, cotton wool was plugged into holes that had been cut in the centre of each vial lid. The number of larvae recovered indicated by the proportion of egg hatch, was expressed as a percentage. Fifty eggs were used per trial and were replicated six times for all colonies.

Pupal survival and adult eclosion was determined by incubating 100 FCM eggs on Moore's diet, held in preserve jars. Larvae hatching from eggs were allowed to develop in the diet until they had reached the pupal stage. Pupae were then removed and counted. The number of pupae recovered was expressed as a percentage, which is indicated as percentage pupal survival. Subsequently, the number of adults successfully emerging from these pupae was also recorded, and indicated as percentage adult eclosion. Six jars were used per replicate and experiments replicated six times for each of the five colonies. The mean temperature and humidity readings recorded during this period was 24.68 ± 2.08°C and 45.90 ± 13.09%, respectively. The duration (days) of the various FCM life stages were also recorded for six generations for each of the five colonies.

2.1.5 Temperature and humidity

Temperature (°C) and humidity (%) readings in the insectary were recorded at least three times weekly, using a digital thermo-hygrometer (Major-Tech®, MT662). A heater (7 fin oil heater, platinum design®) was also placed in the insectary in order to maintain a temperature range of between 22°C and 29°C. Occasionally a bucket of water was placed in the insectary in order to increase the humidity levels.

2.1.6 Quality control

In order to ensure that insects were reared in a disease free environment a number of strict hygiene control practices were adopted. Firstly, the insides of the insectary cages were sterilised bi-monthly using bleach (5% v/v, sodium hypochlorite) and Virkon® (10% w/v, potassium peroxomonosulphate). Once moths from a particular batch had stopped laying eggs, the insides of the moth emergence boxes were disinfected with bleach. The cotton wool

stoppers used by pupating insects were also discarded or recycled by autoclaving them at 120°C for 30 minutes. Other materials used in the rearing process such as forceps, scissors, petri-dishes and test-tubes were either sterilised by autoclaving or using bleach. Any leftover insect pupal cases, moth scales, wings and left over diet were also removed from the insectary, as these attracted mites, ants and cockroaches. Ant repellants were placed on the outsides of the insectary in order to prevent ants from damaging the colonies by feeding on moths or pupae. Moths that had escaped outside the insectary were killed either with a "fly swatter" or by spraying them with 70% ethanol. The floor of the insectary was mopped once every month using Dettol® (active ingredient, chloroxylenol) antiseptic liquid diluted with water (5%, v/v).

2.1.7 Statistical analysis

SPSS (for Windows, version 16.0) statistical analysis software was used to analyse biological data sampled from the five colonies. Descriptive analysis tests were carried out in order to determine the means and standard error. Data were then subjected to a one-way analysis of variance (ANOVA). If a significant difference was established between means, a follow up post-hoc test was carried out in order to determine which means were significantly different from each other. The Scheffé post-hoc test for multiple comparisons of means was employed. Levels of significance were established at $P < 0.05$.

2.2 RESULTS

2.2.1 Temperature and humidity readings in experimental chamber and insecatry

Temperature and relative humidity (RH) readings in the insectary were recorded for two years. In the 1st year (June, 2009 - 2010) the mean temperature and RH readings were recorded to be 25.45 ± 2.41°C and 42.33 ± 11.84% respectively. In the 2nd year (June, 2010 - 2011) mean temperature and RH readings were recorded to be 24.68 ± 2.08°C and 45.90 ± 13.09% respectively. There were fluctuations in the annual temperature and RH readings in the insectary (Fig. 2.4).

Experiments on biological data of the host were conducted between January and March, 2011. During this period the mean temperature and RH readings were recorded to be 26.67 ±

1.39°C and 60.53 ± 6.24% respectively. This is indicated by the broken rectangular line in figure 2.4.

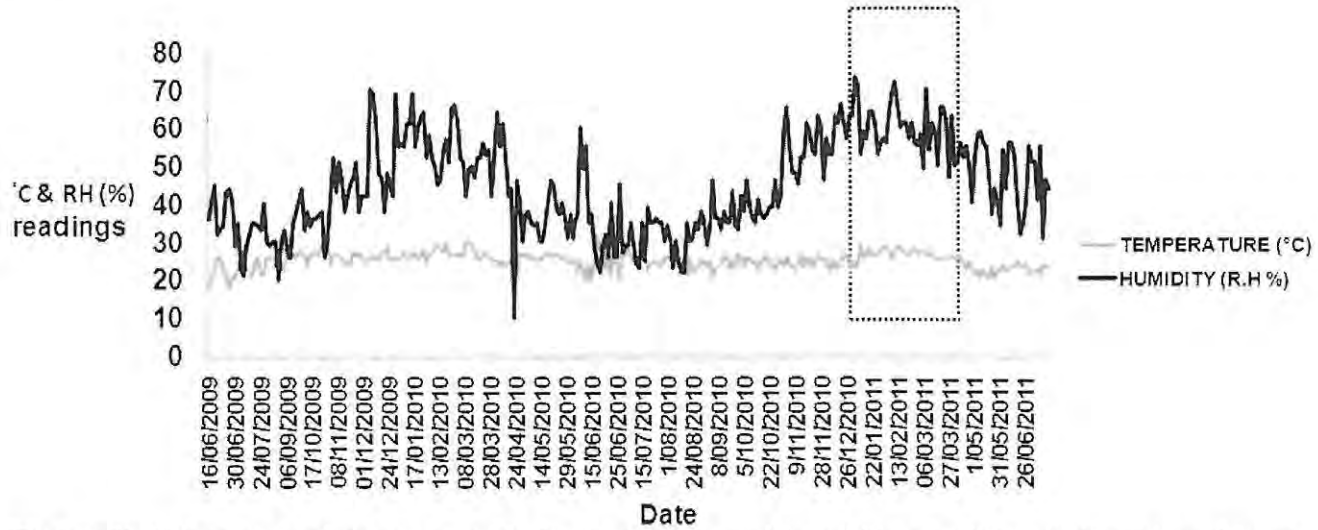

Figure 2.4 Two year data on weekly temperature and humidity readings in insectary (June, 2009 - July, 2011). The period of collection of biological data is shown in the rectangle.

2.2.2 Establishment of the Letsitele and Baths colony

2.2.2.1 Letsitele colony: From 367 infested citrus fruit collected from Letsitele, 118 larvae were recovered (see Table 2.2). However, all larvae died before reaching adulthood. The cause of death was not investigated.

Table 2.2 Larvae recovered from navel oranges collected from Letsitele

FCM larval stage	Number
1st	0
2nd	34
3rd	44
4th	7
5th	33
Total	118

2.2.2.2 Baths colony: Initial colony insects which were in their third parental generation (P_3,) were monitored for two subsequent generations and the number of adults emerging as well as eggs laid were recorded (Table 2.3).

Table 2.3 Population dynamics of three FCM generations from the Baths colony

Generation of adult	Number of moths	Total number of eggs laid	Average number of eggs laid per female
P_3	49	2048	41.79
P_4	104	6051	58.18
P_5	320	9387	29.33

The number of moths in the P_3, P_4 and P_5 generations was recorded to be 49, 104 and 320 respectively. The number of eggs laid by adult females in the P_3, P_4 and P_5 generations was recorded to be 2048, 6051 and 9387 respectively. The moth population increased significantly after each subsequent generation. Oviposition in females also increased rapidly after successive generations leading to an improved fecundity (Fig. 2.4).

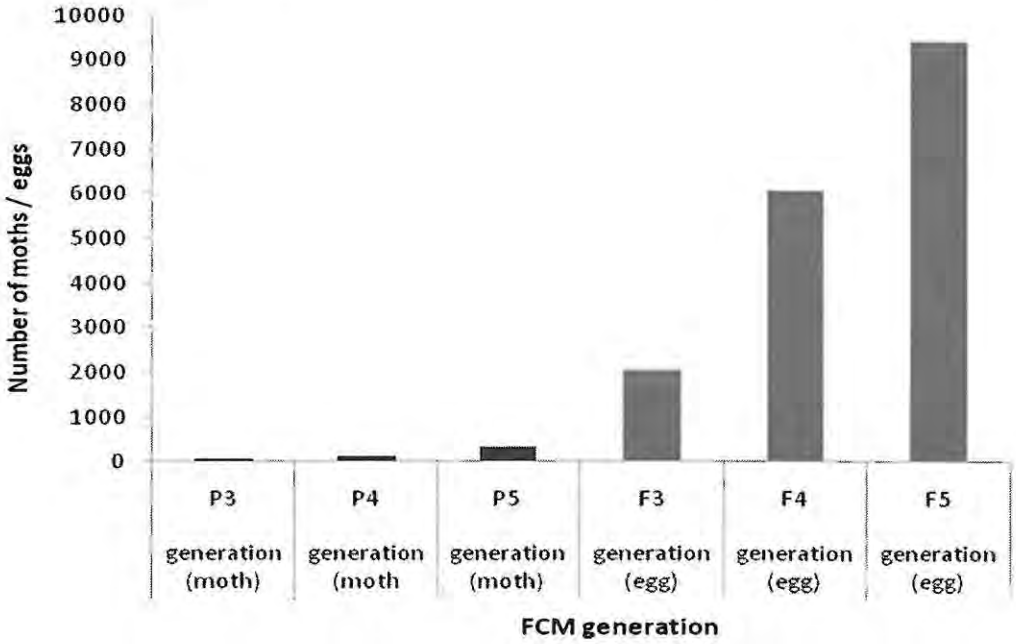

Figure 2.5 Population of FCM parental and filial generations for the Bath colony

2.2.3 Host biology

2.2.3.1 The egg: Under the dissecting microscope, newly hatched eggs were oval shaped and cream coloured (Fig. 2.6 A). On the second day, some eggs had a reddish tint in the centre (Fig. 2.6 B). On the third day most of the eggs had a reddish tint in the centre (Fig. 2.6 C). On the fourth day the head capsules of developing neonate larvae were visible through the chorion (Fig. 2.6 D). At this stage some neonates could be observed hatching out, but

most larvae hatched out on the fifth day (Fig. 2.6 E). Initially, eggs were laid singly and were widely spaced apart. However, as the egg mass increased subsequent eggs were laid in congregates. Adult females oviposited 2 to 3 days after mating.

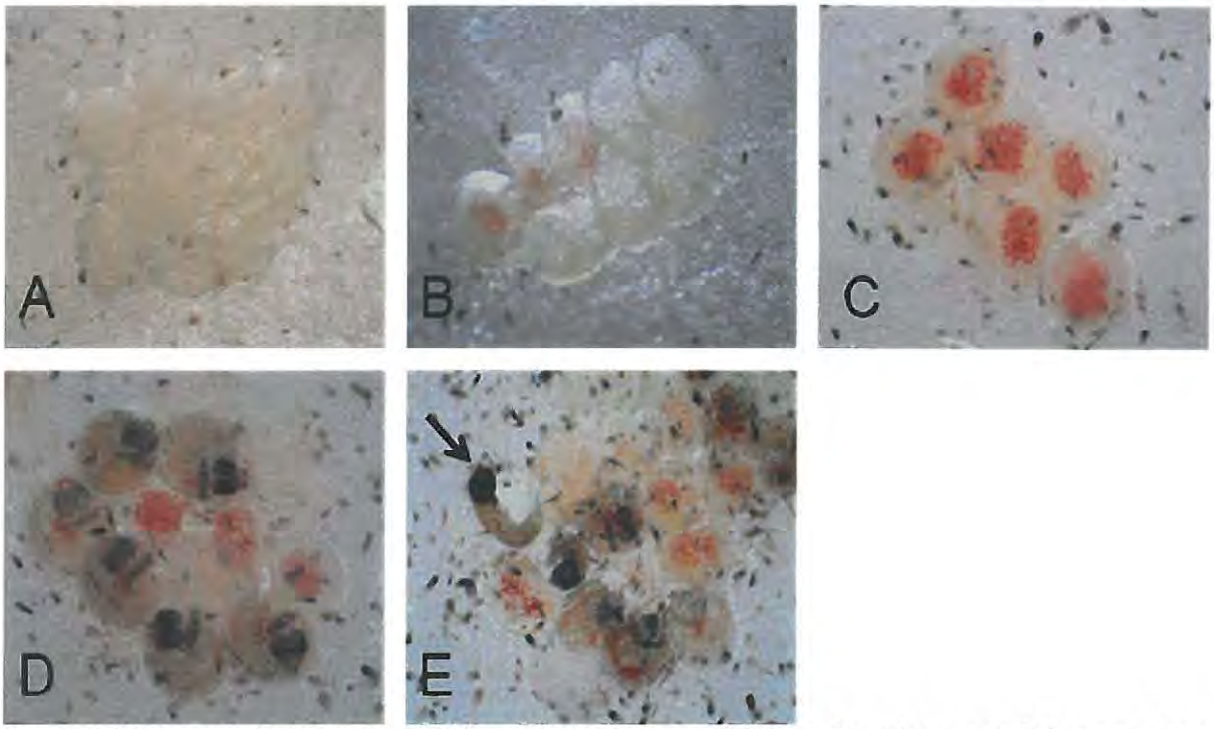

Figure 2.6 Microscopic images of **(A)** newly laid eggs; **(B)** two day old eggs; **(C)** three day old eggs; **(D)** fully developed neonate larvae about to hatch and; **(E)** five day old egg with a neonate larva emerging from the egg (black arrow).

The highest and lowest numbers of eggs per female were recorded for the Mixed colony and Citrusdal colony respectively. However, the number of eggs laid by females from the Citrusdal colony was significantly lower than the other four colonies ($F_{4,10}$=35.24; P<0.001) (Table 2.4).

Table 2.4 Mean number (mean ± SE) of eggs from the Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colony

	Mixed colony	Addo colony	Citrusdal colony	Marble Hall colony	Nelspruit colony
number of eggs ± (SE)	391.73a ± 6.08	370.93a ± 3.29	314.40b ± 2.32	387.07a ± 4.16	368.33a ± 8.01

NB: 15 pairs of moths (male and female) were used per trial and experiments replicated three times *Values followed by different letters are significantly different.

2.2.3.2 The larva: Body lengths of the 1st, 2nd, 3rd, 4th and 5th instar larvae from the Mixed, Addo, Citrusdal, Marble Hall and Nelspruit colonies ranged between 1 – 1.13 mm, 4.13 – 6.35 mm, 5.92 – 8.24 mm, 9.49 – 12.28 and 12.13 – 15.41 mm, respectively (Table 2.5). Head capsule widths of 1st, 2nd, 3rd, 4th and 5th instar larvae from the Mixed, Addo, Citrusdal, Marble Hall and Nelspruit colonies ranged between, 0.25 – 0.30, 0.51 – 0.71, 1.01 – 1.17, 1.27 – 1.48 and 1.52 – 2.07 respectively (Table 2.5). Larval age was found to increase with body size and head capsule width.

Table 2.5 Body length and head capsule of the five FCM larval instars

	Average body length (mm)				
FCM colony	**1st instar** mean ± (CI)	**2nd instar** mean ± (CI)	**3rd instar** mean ± (CI)	**4th instar** mean ± (CI)	**5th instar** mean ± (CI)
Mixed	1.02 ± 0.05	5.0 ± 0.87	7.0 ± 1.24	11.0 ± 1.24	13.2 ± 1.61
Addo	1.0 ± 0.0	5.5 ± 0.87	7.2 ± 1.04	11.0 ± 0.88	13.4 ± 1.66
Citrusdal	1.0 ± 0.0	5.2 ± 0.55	7.0 ± 1.24	10.6 ± 1.11	13.0 ± 0.87
Marble Hall	1.06 ± 0.07	5.8 ± 0.55	6.6 ± 0.68	10.8 ± 1.04	13.0 ± 1.52
Nelspruit	1.02 ± 0.05	5.5 ± 0.87	7.0 ± 1.24	11.6 ± 0.68	13.8 ± 1.61
	Head capsule width (mm)				
Mixed	0.26 ± 0.03	0.60 ± 0.09	1.10 ± 0.09	1.34 ± 0.41	1.86 ± 0.21
Addo	0.27 ± 0.03	0.62 ± 0.05	1.12 ± 0.05	1.32 ± 0.05	1.74 ± 0.22
Citrusdal	0.26 ± 0.03	0.64 ± 0.07	1.12 ± 0.05	1.32 ± 0.11	1.82 ± 0.24
Marble Hall	0.26 ± 0.03	0.62 ± 0.10	1.06 ± 0.11	1.40 ± 0.08	1.86 ± 0.11
Nelspruit	0.25 ± 0.0	0.62 ± 0.05	1.1 ± 0.0	1.34 ± 0.11	1.84 ± 0.14

NB: 5 larvae were used for experiments without replicates.

The 1st, 2nd and 3rd instars had a cream coloured appearance (Fig. 2.7 A, B & C). The 4th instar had a brownish colouration (Fig. 2.7 D). The 5th instars, on the other hand, had a brownish-orange colouration (Fig. 2.7 E).

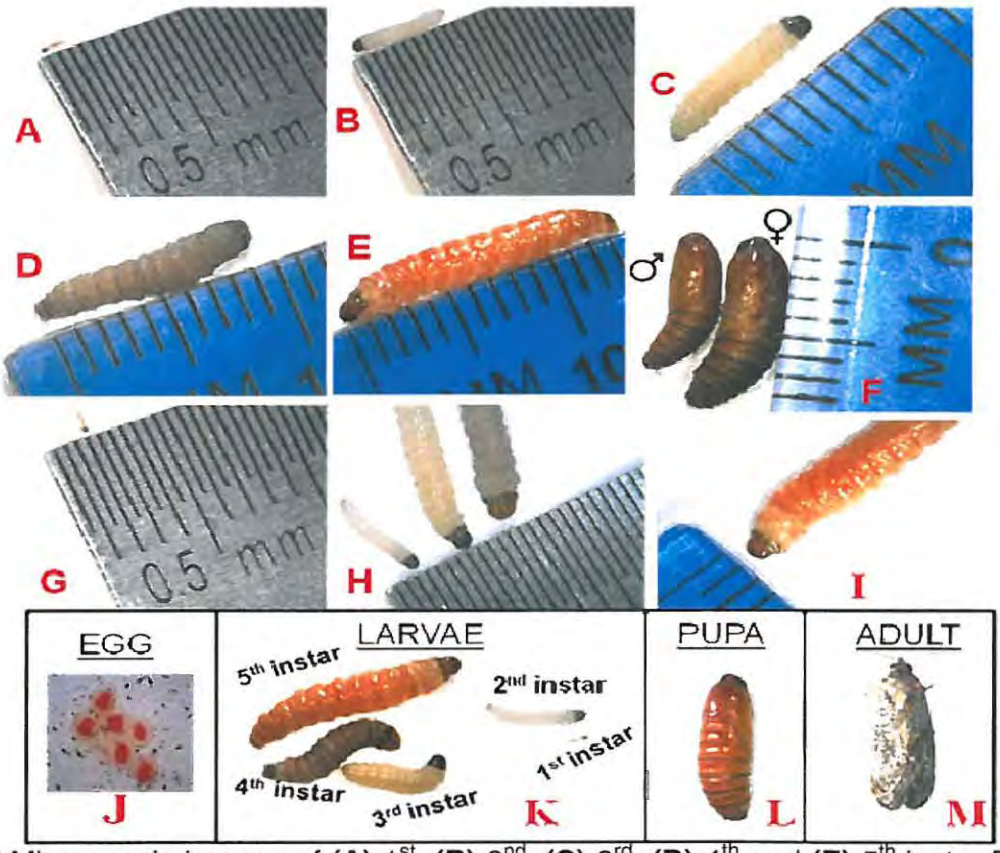

Figure 2.7 Microscopic images of **(A)** 1st, **(B)** 2nd, **(C)** 3rd, **(D)** 4th and **(E)** 5th instar FCM larva. **(F)** Male and female pupae showing differences in length and size. Head capsule lengths of **(G)** 1st instar, **(H)** 2nd, **(H)** 3rd, **(H)** 4th and **(I)** 5th instar larva. Life cycle of FCM from: egg **(J)**, larvae (**K**), pupa **(L)** and **(M)** adult.

2.2.3.3 The pupa: The lowest and highest male pupal weights were recorded from the Citrusdal and Nelspruit colonies respectively. Mean pupal weight of males from the Citrusdal colony was significantly ($F_{4,10}$=16.82; P<0.001) less than the other four colonies. The Citrusdal and Mixed colony recorded the lowest and highest female pupal weights respectively. Female pupae from the Citrusdal colony also weighed significantly ($F_{4,10}$=51.35; P<0.001) less than the other four colonies. However, female pupae from the Mixed colony weighed significantly ($F_{4,10}$=51.35; P<0.001) more than the other four colonies. Pupal weights of females from the Nelspruit, Addo and Marble Hall colonies were also significantly ($F_{4,10}$=51.35; P=0.001) higher than the Citrusdal colony. However, there were no significant

($F_{4,10}$=51.35; P=0.386) differences between female pupal weights from the Nelspruit, Addo and Marble Hall colonies (Table 2.6).

There was no significant ($F_{4,10}$=1.896; P=0.188) difference between the pupal lengths of males from all colonies. However, there was a significant ($F_{4,10}$=1.896; P=0.011) difference between the pupal lengths of females. The Citrusdal and Mixed colony recorded the longest and shortest female pupal lengths respectively (Table 2.6). Pupal length of females from the Citrusdal colony was significantly ($F_{4,10}$=1.896; P=0.037) shorter than the Mixed colony. However, there was no significance ($F_{4,10}$=1.896; P=0.080) difference between the pupal length of females from the Citrusdal, Addo, Marble Hall and Nelspruit colonies (Table 2.6).

Table 2.6 Data on weight (g) and length (mm) (mean ± SE) of male and female pupae from the Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colonies

	Mixed colony	**Addo colony**	**Citrusdal colony**	**Marble Hall colony**	**Nelspruit colony**
Male pupal weight (g) ± (SE)	0.0321b ± 0.0003	0.0325b ± 0.0004	0.0295a ± 0.0003	0.0320b ± 0.0004	0.0326b ± 0.0001
Female pupal weight (g) ± (SE)	0.0481c ± 0.0001	0.0452b ± 0.0005	0.0405a ± 0.0001	0.0440b ± 0.0003	0.0449b ± 0.0005
Male pupal length (mm) ± (SE)	7.823a ± 0.049	7.839a ± 0.043	7.742a ± 0.01	7.726a ± 0.02	7.801a ± 0.043
Female pupal length (mm) ± (SE)	9.064b ± 0.070	9.032ab ± 0.01	8.839a ± 0.037	8.952ab ± 0.01	8.871ab ± 0.041

NB: 31 pupae were used per trial and experiments replicated three times. *Values followed by different letters are significantly different.

2.2.3.4 The adult: By studying the morphological features described by Catling & Aschenborn (1978), accompanying a few others, for the male and female adult, differences between them were observed. Firstly, females were larger than the males (Fig. 2.8 C & D). The anal tuft of males was bigger and fringed with more hairs than the females (Fig. 2.8 A & B). The hind tibia of females was also much longer than the males. However, the most distinct feature which separated the two sexes was the presence of densely packed black fringes of hair protruding from the hind tibia of males (Fig. 2.8 A). This unique feature described by Catling & Aschenborn (1978) was clearly absent in the female. These above features therefore served as reliable tools for adult sexing.

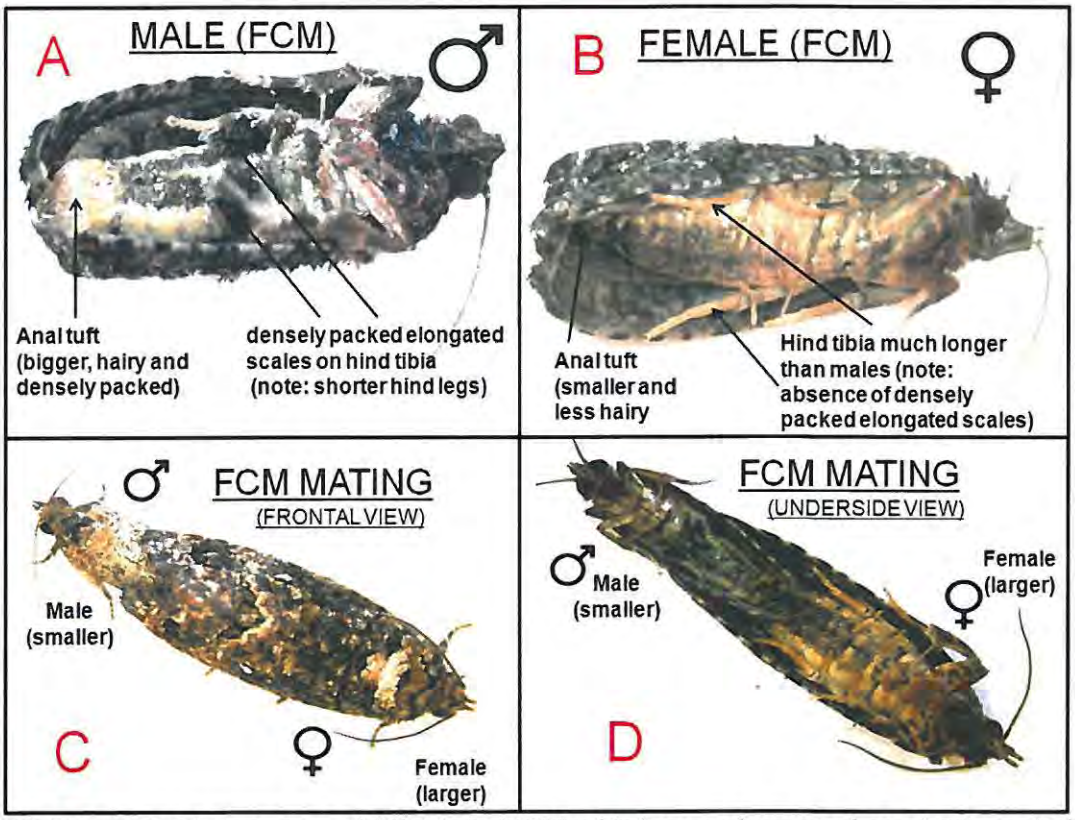

Figure 2.8 Microscopic images of **(A)** Male FCM with large elongated anal tuft and elongated scales found on their hind tibia (see black arrow pointers). **(B)** Female FCM with a smaller anal tuft and the absence of elongated scales on hind tibia (see black arrow pointers). **(C)** Frontal view of a male and female mating. **(D)** Underside view of a male and female mating.

2.2.3.5 Egg hatch, pupae survival and adult eclosion: The Marble Hall colony and Mixed colony recorded the lowest and highest percentage of egg hatch respectively. Egg hatch for the Mixed colony was significantly ($F_{4,25}$=11.198; P=0.008) higher than the Citrusdal, Nelspruit and Marble Hall colonies. However, there was no significant ($F_{4,25}$=11.198; P=0.894) difference between egg hatch for the Mixed colony and Addo colony. Egg hatch for the Addo colony was also significantly ($F_{4,25}$=11.198; P=0.003) higher than the Marble Hall colony. However, there was no significant ($F_{4,25}$=11.198; P=0.74) difference between egg hatch for the Addo, Citrusdal and Marble Hall colonies. There was also no significant ($F_{4,25}$=11.198; P=0.714) difference between egg hatchability for the Marble Hall, Nelspruit and Citrusdal colonies (Table 2.7).

The lowest and highest percentage pupae survival was recorded for the Citrusdal and Mixed colony respectively. Pupae survival from the Citrusdal colony was significantly ($F_{4,25}$=94.801; P<0.001) lower than the other four colonies. However, there was no significant ($F_{4,25}$=94.8; P=0.229) difference between pupae survival for the Addo, Nelspruit and Marble Hall colonies. The Mixed colony also recorded a significantly higher pupae survival than the other four colonies ($F_{4,25}$=94.801; P<0.003) (Table 2.7).

Table 2.7 Percentage egg hatch (mean ± SE) and pupal and adult eclosion (mean ± SE) of the Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colonies

	Mixed colony	Addo colony	Citrusdal colony	Marble Hall colony	Nelspruit colony
% Egg hatch	50.67c ± 1.36	49.00bc ± 1.23	44.00ab ± 0.76	41.67a ± 1.39	44.00ab ± 0.72
% Pupal survival	71.67c ± 1.18	65.69b ± 0.62	47.50a ± 0.78	63.67b ± 0.99	62.50b ± 0.92
% Adult eclosion	55.83d ± 1.70	53.67cd ± 1.45	31.17a ± 2.17	49.33b ± 1.52	50.83bc ± 1.94

NB: For percentage egg hatch, 50 eggs were used per trial and experiments replicated six times. For percentage pupal survival and adult eclosion, 6 jars were used per trial and experiments replicate six times. *Values followed by different letters are significantly different.

The lowest and highest percentage adult eclosion was recorded from the Citrusdal and Mixed colony respectively. Adult eclosion from the Citrusdal colony was significantly lower than the other four colonies ($F_{4,25}$=120.56; P<0.001). Adult eclosion from the Marble Hall and Nelspruit colony were also significantly higher than the Citrusdal colony ($F_{4,25}$=120.56; P<0.001), however there was no significant difference between them ($F_{4,25}$=120.56; P=0.841) (Table 2.7). Percent eclosion of adults from the Addo colony was significantly higher than for both the Citrusdal ($F_{4,25}$=120.56; P<0.001) and Marble Hall colonies ($F_{4,25}$=120.56; P=0.041). However, there was no significant difference between the number of adults emerging from the Addo and Nelspruit colonies ($F_{4,25}$=120.56; P=0.315) (Table 2.7). On the other hand, percent eclosion of adults from the Mixed colony was significantly higher than the Citrusdal ($F_{4,25}$=120.56; P<0.001), Marble Hall ($F_{4,25}$=120.56; P=0.001) and Nelspruit colonies ($F_{4,25}$=120.56; P=0.014). There was no significant difference between the number of adults emerging from both the Mixed colony and Addo colony ($F_{4,25}$=120.56; P=0.579) (Table 2.7).

2.2.3.6 Duration of FCM life cycle (egg to adult): The incubation period of FCM eggs did not differ significantly ($F_{4,25}$=1.10; P=0.377) for all five colonies. The duration of development from 1st – 5th instar between the Nelspruit, Marble Hall, Addo and Mixed colonies did not differ significantly ($F_{3,20}$=0.35; P=0.786) from each other. However, the duration of development from 1st – 5th instar, for the Citrusdal colony, differed significantly ($F_{4,25}$=2.94; P=0.0399) from the other four colonies. The life span of 5th instars did not differ significantly ($F_{4,25}$=0.07; P=0.989) between colonies. The duration from pupal stage to adult hood did not differ significantly ($F_{3,20}$=0.43; P=0.735) between the Nelspruit, Marble Hall, Addo and Mixed colonies. However, the duration from pupal stage to adult hood, for the Citrusdal colony differed significantly ($F_{4,25}$=4.01; P=0.012) from the other four colonies. There was no significant ($F_{4,25}$=0.18; P=0.947) difference between the adult longevity for all five colonies. The total life span, from egg to adult, for the Mixed, Addo, Citrusdal, Marble Hall and Nelspruit colonies was determined to be 42.99, 43.50, 46.0, 43.34 and 42.50 days respectively.

Table 2.8 Duration (days) of FCM life cycle (egg to adult) for the Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colony

	Mixed colony mean ± (SE)	**Addo Colony** mean ± (SE)	**Citrusdal Colony** mean ± (SE)	**Marble Hall Colony** mean ± (SE)	**Nelspruit Colony** mean ± (SE)
Incubation period of egg to 1st instar	4.33a ± 0.21	4.67a ± 0.21	4.83a ± 0.16	4.67a ± 0.21	4.83a ± 0.17
Duration of development 1st to 5th instar	11.0a ± 0.35	10.83a ± 0.4	12.0b ± 0.26	10.67a ± 0.33	10.50a ± 0.34
Lifespan of 5th instar	4.50a ± 0.34	4.67a ± 0.33	4.67a ± 0.33	4.50a ± 0.34	4.67a ± 0.33
Duration of pupal (pre-pupa to adulthood)	8.83a ± 0.31	9.0a ± 0.36	10.33b ± 0.33	9.17a ± 0.31	8.67a ± 0.33
Adult longevity	14.33a ± 0.56	14.33a ± 0.42	14.17a ± 0.47	14.33a ± 0.56	13.83a ± 0.54
Total life span (egg to adult)	42.99	43.50	46.0	43.34	42.50

NB: Four jars were used per generation per colony, and experiments replicated six times (or six generations).
*Values followed by different letters are significantly different.

2.3 DISCUSSION

In this chapter, six laboratory colonies of FCM were successfully established and studies on their biology and fitness was conducted. The diet recipe developed by Moore (2002) proved ideal for the establishment and maintenance of the Addo, Nelspruit, Citrusdal, Marble Hall, Baths and Mixed colonies. The Letsitele colony, on the other hand, failed to establish on Moore's diet. Although the causative factors leading to the non-establishment of the Letsitele colony were not investigated, a number of factors may have contributed to its failure.

Firstly, the sudden change in diet from the insect's natural diet to Moore's diet may have played a role in its non-establishment. Quite commonly, when field collected FCM larvae are reared on Moore's diet, the initial difficulties with insects adapting to the diet may result in larval mortality (Sishuba, 2003; Gendall *et al.*, 2006; Opoku-Debrah, 2008). In order to improve larval survival, it has been suggested that high numbers of matured larvae, preferably 5^{th} instars which are in an advanced physiological state and less susceptible to stress, be sought during field collections (Opoku-Debrah, 2008). Therefore, the small number of 5^{th} instars collected for the Letsitele colony may have contributed to its non-establishment.

A number of observations were also made regarding the biology of the insects. Firstly, pupae from which females emerged were observed to be larger in size and weighed more than the males. Similar findings have been reported by Daiber (1979c). Daiber (1979c) proposed these differences in pupal weight as a useful guide for FCM sexing. However, this method was found not to be always reliable as some females emerged from pupae much smaller or similar in size to males. For the proper sexing of FCM pupae, more reliable techniques that use scanning electron micrographs to distinguish and differentiate between the male and female pupae, based on the appearance of certain morphological features have been proposed (Timm *et al.*, 2007).

Adult males were smaller in size than females. Similar observations have been reported (Catling & Aschenborn, 1978; Daiber, 1980; Couilloud, 1988; Moore, 2002). Catling & Aschenborn (1978) indicated that adult male FCM are easily distinguished by the presence of densely packed elongated scales found on their hind tibia. However, they did not provide visual identifications for these features. In accordance with their findings, and as shown in this

study, the densely packed black fringes of hairs protruding from the hind tibia of FCM males proved to be a reliable tool for adult sexing. The males also possessed a short hind tibia and highly dense elongated anal tuft which was abbreviated in the females.

Studies on the comparative biological fitness of the Addo, Nelspruit, Marble Hall, Citrusdal and Mixed colonies also revealed some differences between them. These differences were mostly observed with insects from the Citrusdal colony. Firstly, the Citrusdal colony recorded the lowest pupal weight for both males and females. In a similar study with *Pieris rapae* (L.), Gilbert (1984) speculated that, differences in pupal weight could influence growth, development and adult fecundity, with temperature playing some role in insect fitness. Temperature generally affects food utilisation efficiency in insects, which may cause differences in their growth rate (Scriber & Slansky, 1981; Scriber & Lederhouse, 1983; Reynolds & Nottingham, 1985; Levesque *et al.*, 2002).

However, since the other four colonies performed well in comparison to the Citrusdal colony under similar rearing conditions, including temperature, it is difficult to attribute these differences to temperature. On the other hand, even though temperature may not have contributed to the reduced fitness of the Citrusdal colony, generally temperature plays a significant role in speeding up the growth rate of all the FCM life stages (Daiber, 1980).

The Citrusdal colony also recorded the lowest female fecundity, pupal and adult eclosion as well as the longest duration in larval and pupal development. One possible reason for this low fitness of the Citrusdal colony could be attributed to earlier difficulties in colony establishment. For instance, even though the Addo and Citrusdal colony seemed to have been established around the same period (Opoku-Debrah, 2008), from field lines, the Citrusdal colony was a generation slower than the Addo colony. Another reason could be that, FCM is not considered indigenous to the Western Cape and may have been introduced from a very limited gene pool from other regions in South Africa (Sean Moore, pers. comm.). This may have influenced the evolutionary fitness level of these insects. Other reasons could be attributed to differences in the genetic distribution of FCM in South Africa (Timm, 2005b) which may have affected their performance. For example, in a study on the genetic diversity (using amplified fragment length polymorphism, AFLP) of field populations of FCM sampled from the Western Cape,

Eastern Cape and Mpumalanga, Timm (2005b) found a significantly high genetic diversity within populations sampled from the Western Cape in comparison to the other regions.

2.4 CONCLUSION

In this study, six geographically distinct FCM laboratory populations namely, the Addo, Nelspruit, Marble Hall, Citrusdal, Baths and Mixed colony were successfully established and maintained. In addition, the visual guide for the rapid dentification of all the various FCM life stages would help augment other existing methods. These visual identification aids would also prove useful to farmers who constantly monitor insect populations to determine population levels, pest pressure and accurate timing of pest control measures against the appropriate life stages.

It also appears that under similar rearing conditions, geographic FCM populations may differ in their biological fitness. However, due to the difficulty in relating laboratory observations to field scenarios, one cannot say with certainty that this phenomenon exists in the field.

Lastly, when insects are reared under laboratory conditions, the incidence of latent baculovirus infections in their larval populations has been reported. This phenomenon is the subject of the next chapter.

CHAPTER THREE

BIOPROSPECTING FOR NEW *CRYPTOPHLEBIA LEUCOTRETA* GRANULOVIRUS (CrleGV-SA) ISOLATES

3.0 INTRODUCTION

Baculoviruses are regarded as one of the most beneficial viruses known to mankind (Miller, 1997). They are widely used in numerous research applications and, due to their host specificity and negligible environmental impacts, some of them have been successfully developed as biopesticides (Moscardi, 1999; Szewczyk *et al.*, 2006). A few examples are CAPEX (*Adoxophyes orana* GV, Andermatt-Biocontrol), MAMESTRIN (*Mamestra brassicae* NPV, Calliope S.A.), SPOD-X (*Spodoptera exigua* NPV, Brinkmann B.V) (Steineke, 2004), Helicover® (*Helicoverpa armigera*, NPV) (Moore, *et al.*, 2004c; Moore & Kirkman, 2010), Cryptex (CrleGV, Andermatt-Biocontrol AG) (Kessler & Zingg, 2008), and Cryptogran® (CrleGV, River Bioscience (Pty) Ltd.) (Moore *et al.*, 2004a).

Although baculoviruses have numerous benefits, they also have some notable disadvantages. Firstly, owing to their host specificity their usage as broad spectrum biopesticides is limited (Moscardi, 1999). Secondly, compared to chemical insecticides, they have a slow speed of kill, requiring 4 to 14 days to kill their host (Adams & McClintock, 1991; Moscardi, 1999; Chen *et al.*, 2000). This can be problematic as it reduces their attractiveness to farmers who prefer much faster acting insecticides. Another disadvantage of baculoviruses, although rare but relevant to this study, is that of insect resistance.

Recent reports of field insects developing resistance to a commercially applied baculovirus product has brought into light some potential challenges accompanied with their use. In 2002 and 2003, the first indications of some field populations of codling moth (CM), *Cydia pomonella* (L.) in Germany which had developed resistance to a commercially applied Mexican isolate of the CM virus, *Cydia pomonella* granulovirus (CpGV-M) used in the control of CM on apples in Europe, was reported (Fritsch *et al.*,

2005). In 2005, two CM populations with up to 1000-fold reduced susceptibility to CpGV-M were reported in southern Germany (Fritsch *et al.*, 2005). By 2006, another resistant population was recorded in France (Sauphanor *et al.*, 2006). Thereafter, 30 orchards with CpGV-M resistance were recorded across Europe (Jehle *et al.*, 2008). The resistance was confirmed from several CM populations in organic orchards where CpGV-M application had failed (Eberle & Jehle, 2006; Jehle *et al.*, 2006b; Asser-Kaiser *et al.*, 2007). Studies on a laboratory population established from CpGV-M resistant field lines, which had been reared for more than a year, showed no significant loss of resistance and resistance was found not to be restricted to a specific larval instar (Jehle *et al.*, 2006b).

One possible reason for the development of resistance could be attributed to repeated field application of the biopesticide CpGV-M, which led to a natural selection for insects with a lower susceptibility to the virus (Eberle & Jehle, 2006; Asser-Kaiser *et al.*, 2007). Some mechanism such as changes in the midgut cell physiology can impair primary infections of midgut cells as well as the immune status of insects thereby preventing secondary infection in other tissues which may confer to the insects some level of resistance (Evans, 1983; Federici, 1997; Sun, 2005). The fact that insects develop resistance to a virus is problematic (Berling *et al.*, 2009) because it can hinder concerted efforts aimed at promoting baculovirus based biopesticides.

A resistance management strategy that involved the introduction of genetically different baculoviruses known to be highly virulent against the CpGV-M resistance CM populations was proposed (Jehle *et al.*, 2006b; Asser-Kaiser *et al.*, 2007). One candidate of choice was an Iranian CpGV isolate, CpGV-I12, which was shown in laboratory bioassays as being able to partially increase the mortality of the CpGV–M resistant CM laboratory populations (Eberle *et al.*, 2008 & Jehle *et al.*, 2008; Rezapanah *et al.*, 2008). A later study by Berling *et al.* (2009) showed that another CpGV isolate, NPP-R1 appeared to be more virulent than the CpGV-I12 in controlling the CpGV–M resistant CMs. Therefore, by challenging virus resistant insects with genetically different and highly virulent viral isolates, these laboratory studies have shown that it is possible to manage resistance in the laboratory (Asser-Kaiser *et al.*, 2007; Eberle *et al.*, 2008;

Berling *et al.*, 2009). Subsequently, the CpGV–M which had showed reduced efficacy in controlling CM populations in Europe, as previously indicated, has led to a commercial replacement of products containing CpGV-M with those containing CpGV-R5 (MADEX Plus, Andermatt-Biocontrol AG) (Besse *et al.*, 2011; Zingg *et al.*, 2011).

Conventional approaches for recovering genetic isolates of baculoviruses can be used by scouting for diseased insects showing baculovirus symptoms during field epizootics (Cory *et al.*, 1997). Occlusion bodies (OBs) of genetically modified baculovirus can be recovered from soil samples in both terrestrial and aquatic habitats (Ebling & Holmes, 2002). During baculovirus epizootics, field populations of insects infected with an NPV or GV have been reported to carry several mixed wild-type, which mostly consist of a mixture of different genotypes, within and among geographic and temporal isolates, with some showing higher virulence than others (Parnell *et al.*, 2002; Takatsuka *et al.*, 2003; Cory *et al.*, 2005). The occurrence of these mixed wild-type isolates during baculovirus epizootics has been observed with most forest insects (Lepidoptera), during population explosions (Tanada & Fuxa, 1987; Fuxa, 1989; Adams & McClintock, 1991; Cory *et al.*, 1997; Cooper *et al.*, 2003; Il'inykh *et al.*, 2004; Il'inykh, 2007). The underlying factors leading to the occurrence of baculovirus epizootics are widely speculated to be caused by a dormant or latent baculovirus that becomes infective when the insects are subjected to stress conditions (Steinhaus & Dineen, 1960; Fuxa, 1989; Adams & McClintock, 1991). This phenomenon has been demonstrated in laboratory experiments where larvae of insects subjected to conditions of stress have exhibited baculoviral symptoms (Vago, 1951 & 1955; Steinhaus & Dineen, 1960; Smith, 1967; Il'inykh, 2007). Therefore it appears that, if insects are artificially subjected to extreme conditions of stress, a latent baculovirus present in an insect population can be stimulated into its virulent form (Steinhaus, 1958a & 1958b; Smith, 1967). Viral OBs extracted from diseased insects can then be screened by conducting biological assays in order to recover the most virulent baculovirus isolates for use in the development of biopesticides (Cory *et al.*, 1997).

The main objective of this chapter is to describe the development of a protocol for the induction of artificial epizootics in several geographic populations of the false codling moth (FCM), *Thaumatotibia leucotreta*. The approach was to expose larvae from each of the laboratory colonies (Chapter Two) to conditions of stress, thereby increasing the probability of recovering genetically different CrleGV isolates from each colony. Since geographic populations of FCM may differ in their susceptibility to Cryptex and Cryptogran, any new CrleGV isolates of superior virulence may be selected for use in the development of other CrleGV based biopesticides - particularly in the event of resistance occurring to either of the two commercial products.

3.1 MATERIALS AND METHODS

3.1.1 Induction of a latent baculovirus infection in laboratory colonies of FCM

To recover latent baculoviruses from insect colonies, a protocol for the induction and isolation of latent baculoviruses was developed. The previously established geographic FCM colonies (Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colony) (Chapter Two), were used as a stock for latent virus induction trials. The Baths colony was excluded from these trials as stable colonies were not obtained during this study. Changes in rearing diet, increased rearing temperature and overcrowding were used as stressing mechanisms and the experiment conducted for a period of 3 to 4 months.

3.1.1.1 Overcrowding: Standard laboratory conditions for rearing FCM involve incubating approximately 300 - 400 eggs on 40 g of Moore's diet in preserve jars, as described in Chapter two. Using overcrowding, the number of eggs was increased four-fold. Approximately 1200 - 1600 eggs incubated on 40 g Moore's diet were used in treatments, in all experiments. For controls, approximately 400 - 500 eggs were incubated on 40 g of Moore's diet. Several jars from each of the five colonies were used in the trials. Treatment and control jars were incubated under normal rearing conditions (Chapter Two, section 2.1.3) and larvae were observed for any disease symptoms until adulthood.

3.1.1.2 Increased rearing temperature: Jars with 3rd instar larvae were selected for trials. Treatment jars were placed in a 37°C constant environment room and larvae were observed daily for disease symptoms. Controls were maintained at normal rearing temperature (25.45 ± 2.41°C) and larvae observed daily for disease symptoms. All five colonies were subjected to this treatment.

3.1.1.3 Change in rearing diet: Since FCM had been reared successfully on Moore's diet without the incidence of a baculovirus infection, another diet recipe was adopted. For the treatment jars, approximately 400 - 500 eggs were incubated on 40 g of Morvite® (Tiger Brands Ltd.) consisting of pre-cooked sorghum, sugar, salt, vitamins, citric acid, sweeteners and food colourant. For the controls, approximately 400 - 500 eggs were incubated on 40 g Moore's diet. Larvae were observed daily for disease symptoms until adulthood. This process was conducted for each of the five colonies.

3.1.2 Symptomatology

According to Moore (2002), the normal symptoms of CrleGV infection are the appearance of a milky glazed colouration in both younger and matured larvae. Matured larvae, however, tend to maintain their pinkish colour during infection. During the late stages of infection, infected larvae normally climb up the inside walls of their rearing containers (Moore, 2002). Larvae used in latent virus induction experiments were closely monitored each day for the presence of the above symptoms and behaviour. Infection was confirmed using a dissecting microscope (Leica, EZ4D). Insects were immediately collected and stored at -25°C for subsequent experiments involving baculovirus occlusion body (OBs) purification.

3.1.3 Occlusion body purification

To recover baculovirus OBs from CrleGV infected larvae, the purification protocol described by Hunter-Fujita *et al.* (1998) and Moore (2002) was used, with few modifications. Approximately 2 g of larval cadavers were macerated in 6 ml of 0.1% sodium dodecyl sulfate (SDS) and filtered through cheese cloth. An equal volume of

0.1% SDS was added and the process repeated. The resulting filtrate was placed in two JA-20 (Beckman®) centrifuge tubes, in 3 ml aliquots. For OB purification from Cryptex and Cryptogran, 6 ml of formulated product was dispensed into two JA-20 tubes.

JA-20 tubes filled to the brim with 0.1% SDS were centrifuged at 7840 x *g* for 30 minutes at 4°C in a Beckman® coulter centrifuge (J-E Avanti). The supernatant was discarded and the pellet re-suspended in 3 ml of sterile ddH_20 (double distilled water). A 30 - 80% (v/v) glycerol gradient was prepared in two SW 28 Beckman® Ultra-clear centrifuge tubes using a Gradient-maker (Amersham Biosciences Inc.). With the aid of a magnetic stirrer, the two solutions were mixed to form a continuous gradient. The tubes were sealed with parafilm and incubated at 4°C. The pellets were loaded on top of the glycerol gradients and centrifuged for 15 min, at 29774 x *g* at 4°C in a Beckman® coulter optima (L-90 K) ultracentrifuge. The OBs were visualised as thick milky white or brown concentric bands appearing in the middle of the tube. The OBs were extracted using a pipette and placed into two sterile JA-20 tubes, which were filled to the brim with ddH_20 and centrifuged for 30 minutes at 29774 x *g* at 4°C. This process was repeated three times, in order to remove all traces of glycerol. The final pellets were re-suspended in 1 ml of ddH_20, vortexed briefly, and stored at -20°C.

3.1.4. Light microscopy for virus identification and OB enumeration

OBs were viewed and counted using the light microscopy method described by Hunter-Fujita *et al.*, (1998) and Jones (2000). For viewing, approximately 5 µl of OBs were pipetted onto a Helber counting chamber (0.02 mm depth, Hawksley®). Prior to counting on the Helber, OBs were diluted in ddH_20 and sonicated for 50 s to disperse them. The chamber was covered with a glass slip, the slide was allowed to stand for 5 minutes, and OBs visualised at 400x magnification under dark field microscopy.

3.1.5 Virus identification by transmission electron microscopy

Electron microscopy was performed using a modified version of the method described by Dezianian (2010). Approximately 5 µl of purified OBs were pipetted onto a carbon grid and the virus suspension incubated for 20 s and dried using filter paper. The grids

were viewed using a JEOL JEM-1210 transmission electron microscope (at 100 kV). The images were captured using Scandium® image analysis software and processed using Microsoft® Office Picture Manager.

3.2 RESULTS

3.2.1 Induction of a latent baculovirus infection in FCM laboratory colonies

Insects from the Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colonies were subjected to overcrowding, increased environmental temperature and changes in rearing diet. Results of virus induction with the different methods are presented in table 3.1.

Table 3.1 Virus induction in five colonies subjected to overcrowding, changes in rearing temperature and diet as stressors, versus untreated control larvae

FCM population	Overcrowding				Changes in rearing temperature				Change in rearing diet			
	treatment		control		treatment		control		treatment		control	
	Total number of jars	Jars with symptomatic larvae	Total number of jars	Jars with symptomatic larvae	Total number of jars	Jars with symptomatic larvae	Total number of jars	Jars with symptomatic larvae	Total number of jars	Jars with symptomatic larvae	Total number of jars	Jars with symptomatic larvae
Addo colony	28	6	12	0	13	0	6	0	12	0	6	0
Mixed colony	30	8	12	0	15	0	6	0	12	0	6	0
Nelspruit colony	27	7	12	0	12	0	6	0	12	0	6	0
Marble Hall colony	27	4	12	0	12	0	6	0	12	0	6	0
Citrusdal colony	27	5	12	0	12	0	6	0	12	0	6	0

Only insects subjected to overcrowding as a stressing mechanism showed disease symptoms (Table 3.1 & Fig. 3.1 B). No disease symptoms were recorded in the controls (Table 3.1 & Fig. 3.1 A). The number of jars with symptomatic larvae from the

overcrowding experiment, when expressed as a percentage was 21.43%, 26.67%, 25.93%, 14.81% and 18.52% for the Addo, Mixed, Nelspruit, Marble Hall, and Citrusdal colonies, respectively.

Within the jars containing diseased larvae, it was not uncommon to find some larvae showing disease symptoms while others were asymptomatic. Symptomatic larvae were observed to crawl upwards and hang upside down from the cotton wool stoppers (Fig. 3.1 B). There were also fewer asymptomatic than symptomatic larvae. Although it was not possible to count larvae, by observation approximately 25% of larvae present in the jars were asymptomatic. The asymptomatic larvae were able to pupate and successfully emerge as adults (Fig. 3.1 B).

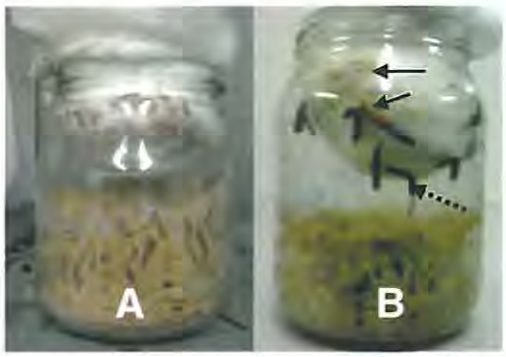

Figure 3.1 (A) Larvae in one of the control jars. **(B)** Symptomatic 5th instar larvae (broken arrow), with some 5th instars successfully pupating (solid arrows).

3.2.2 Symptomatology

Both control and symptomatic larvae collected from the overcrowding experiment were visualised under a dissecting microscope and differences were observed between them. Firstly, symptomatic larvae normally appeared lethargic and stopped feeding. Symptomatic larvae (Fig. 3.2 B & D) were also generally larger than the controls (Fig. 3.2 A & C). Secondly, there was a noticeable change in the colour of the epidermis of symptomatic larvae. Younger symptomatic larvae, such as 3rd instars (Fig. 3.2 B) normally had a shiny whitish appearance, while asymptomatic larvae maintained their cream colouration (Fig. 3.2 A). Symptomatic fifth instars or matured larvae also had a shiny whitish appearance (Fig. 3.2 D). However, both symptomatic and asymptomatic

5th instars maintained their brownish-orange colouration (Fig. 3.2 C & D). At this stage, live larvae pricked with a needle exuded a thick mass of whitish fluid. Dead larvae exuded a brown fluid (Fig. 3.2).

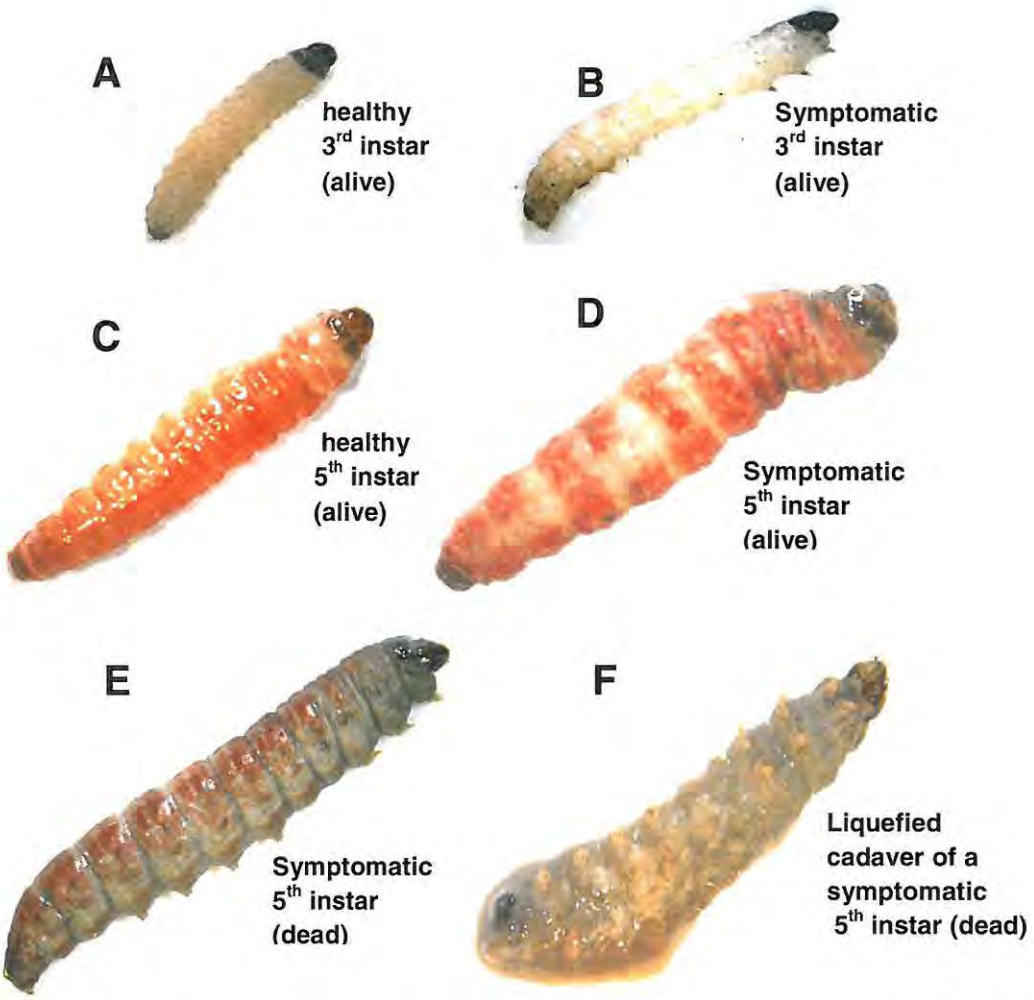

Figure 3.2 (A) Healthy 3rd instar larva **(B)** Symptomatic 3rd instar larva. **(C)** Healthy 5th instar larva. **(D)** Symptomatic 5th instar larva (alive). **(E)** Symptomatic 5th instar larvae (dead). **(F)** Liquified cadaver of Symptomatic 5th instar larva (dead) (Magnification: 25x).

3.2.3 Occlusion body purification

Following gradient purification, OBs obtained either from symptomatic larvae or from formulated products such as Cryptex and Cryptogran separated according to their densities. By illuminating the ultracentrifuge tubes, a clear virus band appearing as concentric rings was visible (Fig. 3.3).

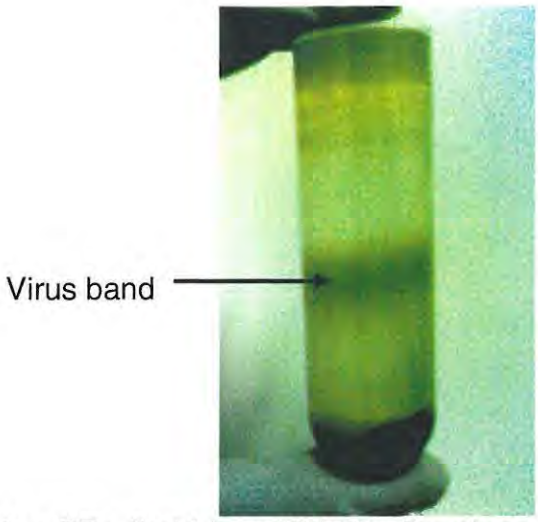

Figure 3.3 Cryptex OBs (held in a SW 28 ultra-clear centrifuge tube) appearing as a band in a glycerol in rate zonal gradient (30 - 80%, v/v).

3.2.4 Light microscopy for virus identification and OB enumeration

Under a light microscope, OBs in suspension appeared as bright shiny particles moving at random in the counting chamber (Fig. 3.4 B).

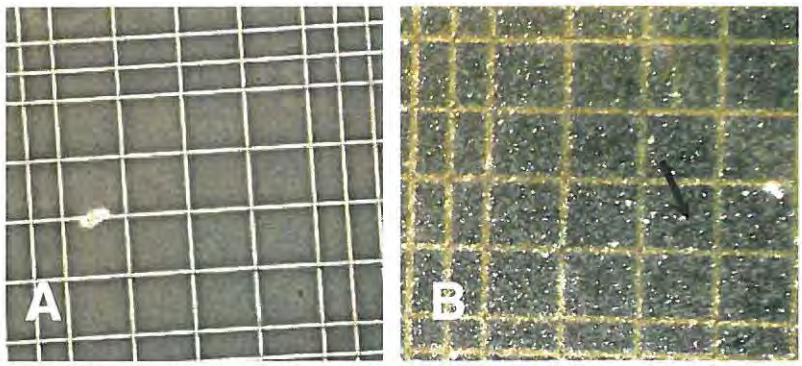

Figure 3.4 (A) An empty Helber counting chamber (Hawksley®). **(B)** Chamber filled with OBs (black arrow) in suspension.

The OB concentration extracted either from symptomatic larvae or formulated products (Cryptogram and Cryptex) ranged between 2.12 x 10^{11} and 3.56 x 10^{11} OBs/ml (Table 3.2). By observation, there was not much difference between these concentration values. This indicates that OBs extracted from larvae can be used for product formulation. The OB concentrations are represented in table 3.2.

Table 3.2 Concentration of OBs extracted from diseased insects or formulated products

Source of virus inoculum	Virus concentration (OBs/ml)
Cryptex	3.26×10^{11}
Cryptogran	3.56×10^{11}
Symptomatic Addo colony larvae	2.12×10^{11}
Symptomatic Nelspruit colony larvae	2.59×10^{11}
Symptomatic Marble Hall colony larvae	3.21×10^{11}
Symptomatic Citrusdal colony larvae	2.76×10^{11}
Symptomatic Mixed colony larvae	2.58×10^{11}

3.2.5 Virus identification by transmission electron microscopy

Figure 3.5 shows the size and morphology of OBs from diseased insects or formulated products observed under transmission electron microscopy. In some samples analysed, OBs were found to be attached to residual glycerol extracts used in the purification process (Fig. 3.5 A). However, further washes with sterile ddH_20 proved effective in removing all traces of glycerol (Fig. 3.5 C & D). This justified the importance of subjecting OBs to at least three washes during the purification process (see section 3.1.3, above).

Similarities in size and morphology of OBs extracted from Cryptex (Fig. 3. 5 A & C) and symptomatic larvae from the Addo colony (Fig. 3. 5 B) and Nelspruit colony (Fig. 3.5 D) were apparent. The individual size of the OBs from both samples ranged between 315 nm - 395 nm (Fig. 3.5). This is in accordance with that described for GV's (Hunter-Fujita *et al.*, 1998). The viral nucleocapsid was observed in some particles (Fig. 3.5 C). The particle size of OBs extracted from the Marble Hall and Citrusdal colony were also similar in size to that of Cryptex and Cryptogran. Pictures of OB samples from these samples are not shown due to poor picture quality.

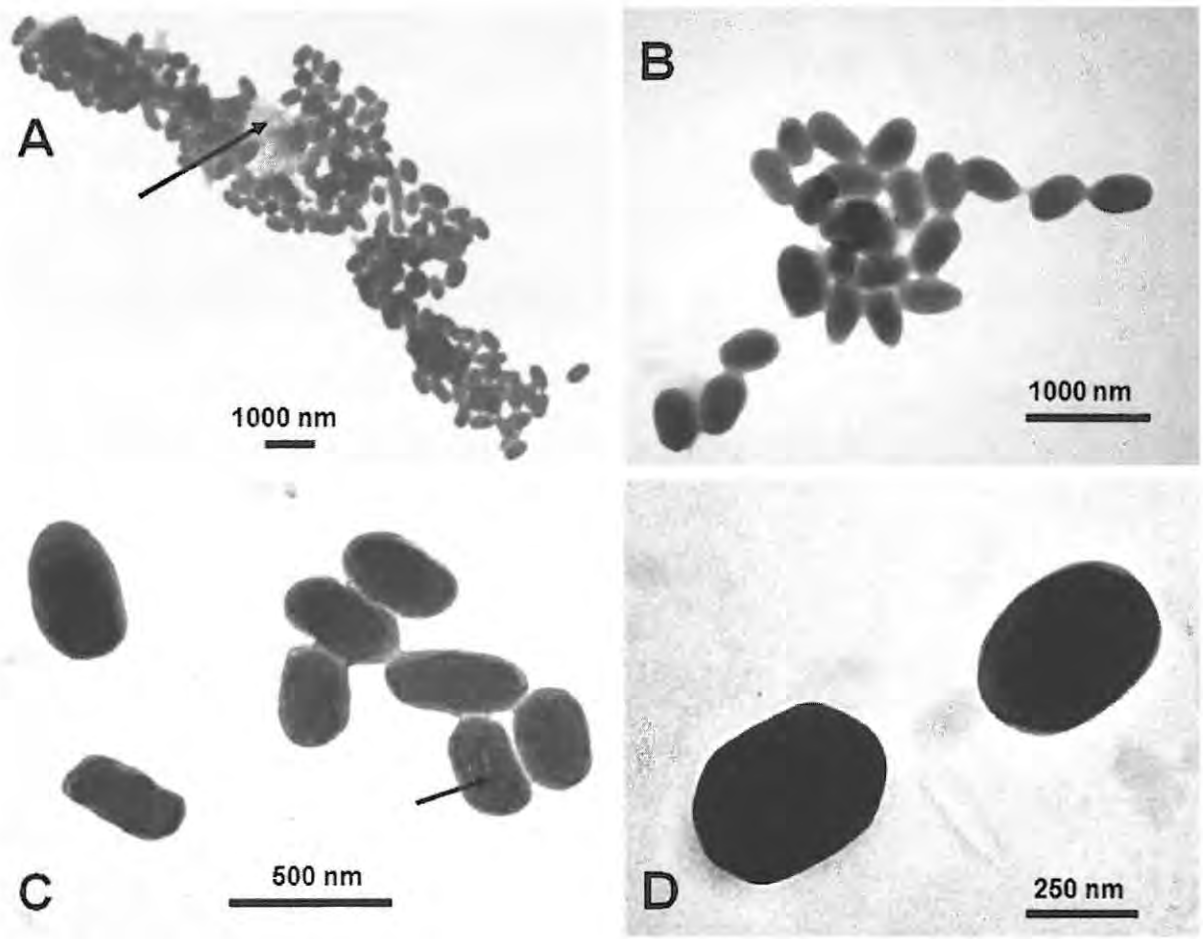

Figure 3.5 (A) Transmission electron micrograph of semi-purified OBs from Cryptex, with some residual glycerol adhering to OBs (arrow) (magnification: 8000x). **(B)** TEM of OBs from symptomatic Addo larvae (magnification: 10000x). **(C)** TEM of OBs from Cryptex, with OB showing viral nucleocapsid (arrow) (magnification: X 25000). **(D)** TEM of OBs from symptomatic Nelspruit larvae (magnification: 50000x).

3.3 DISCUSSION

This chapter describes the successful induction of latent baculovirus from five geographically distinct FCM laboratory colonies namely Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colony, using overcrowding as a stressor. Symptoms observed for diseased FCM larvae were similar to that reported by Moore (2002). However, in Moore's study, the diseased larvae were serendipitously obtained when insects were reared on a non-sterile *Rhizopus* sp. fungus inoculated diet (Moore *et al.*, 2011). Diseased dead larvae observed in this study were also noted to have a dark brown

appearance. This has been linked to a cuticle melanization process that is caused by a polyphenol oxidase mediated process, which results in cuticle discolouration and eventual rupturing or disintegration of the cuticle (O'Reillly *et al.*, 1992). This rupturing or disintegration of the cuticle is sometimes referred to as 'wilting' or 'melting' (Benz, 1986).

The inclusion of SDS in the OB purification process proved to be effective, as treating virus suspensions with 0.1 - 2% SDS helps to disrupt cells and reduces contamination (Thompkins, 1991). The size of OBs extracted from symptomatic larvae was 300 nm - 500 nm in length, which is within the range reported for GVs (Hunter-Fujita *et al.*, 1998). The OBs were also similar in their morphology and size to that reported for CrleGV by other authors (Whitlock, 1980; Jehle *et al.*, 1992; Moore, 2002). On this basis, it could be seen that the causative agent of symptoms observed in diseased FCM larvae was that of a GV, however final confirmation of the virus as that of CrleGV was established in chapter four using molecular techniques.

In this study, overcrowding was the only induction method that stimulated a latent baculovirus infection resident in the Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colonies. Overcrowding involves increasing the population of insects per unit area or varying the size of the rearing chamber per unit population of insects (Steinhaus, 1958b). This increased population density or overcrowding of insects, most notably with lepidopteran insects, has been reported to lead to an outbreak of several baculoviral diseases (Steinhaus, 1958b; Milks & Myers, 2001). However, although a change in rearing diet and temperature were not successful in this study, other researchers found them to be effective (Kitajima, 1926; Vago, 1951 & 1955; Hukuhara & Aruga, 1959; Smith, 1967). Some authors have suggested a combination of stressors as a more favourable option (Jaques, 1960; Aruga *et al.*, 1963; Smith, 1967).

Since no incidence of a CrleGV infection in the laboratory colonies was observed during rearing (Chapter Two), the virus was probably able to maintain itself in the FCM laboratory colonies for several generations via vertical transmission. This has been reported for a nucleopolyhedrovirus, NPV infection in the cabbage moth, *Mamestra*

brassicae (L.), which was able to persist in laboratory insect populations due to a vertical transmission of the virus over several generations (Hughes *et al.*, 1993). It is possible that, in this study once the virus was induced in some larvae, it was then transferred to other susceptible larvae via horizontal infection. This may have occurred due to susceptible individuals ingesting CrleGV on contaminated FCM diet or frass, as well as exudates from dead or diseased larvae. This is supported by the fact that, during infection, crowding increases contact between infected and susceptible individuals hence increasing the rate of viral transmission (Steinhaus, 1958b).

Successful recovery of latent CrleGV in the five laboratory colonies cannot be attributed entirely to overcrowding as the process involves a multiplicity of factors. For instance, the biological activity of the virus and its multiplicity of infection as well as the biological properties and physiological state of the host including other environmental factors are known to influence the efficiency of host-virus interactions (Il'inykh, 2007).

Although overcrowding proved to be a successful stressor, only a small number of jars containing diseased insects were recovered (<27%). It is therefore possible that not all individuals from the five colonies haboured the virus or may have escaped infection because they were not susceptible to the latent induction method employed. Interestingly, within the same jars that contained diseased larvae it was not uncommon to find some larvae successfully pupating and emerging as adults whiles other insects succumbed to infection in their larval stages. More importantly, it was noted that once larvae had reached their 5^{th} instar stage without any visible disease symptoms, these larvae normally pupated successfully and emerged as adults. These 5^{th} instars that had escaped infection may have developed some mechanisms to evade baculoviral infection. For example, one study found that when field collected 5^{th} instar FCM larvae were fed with artificial diet, which was surface inoculated with CrleGV OBs at a high concentration (1.661×10^8 OBs/ml), some larvae succumbed to infection while other insects appeared to be asymptomatic and were able to successfully pupate (Opoku-Debrah, 2008).

Some authors have suggested that there appears to be an age-related increase in larval resistance of insects to baculoviral infection. This argument is also supported by other studies, which have shown that older larval stages of insects appear to be more resistant to virus infection than their immature larval stages (Evans, 1983; Federici, 1997; Sun, 2005; Cory & Myers, 2003). The explanation given for this phenomenon is that, since mature or older larvae possess a thicker peritrophic membrane, a protective lining secreted by the midgut, this makes it difficult for the virus to penetrate and get access to the midgut epithelium in order to initiate infection (Federici, 1997). Subsequently, this age-related increase in resistance may have enabled these 5^{th} instars to escape infection. The low susceptibility or probable resistance observed in this study did not, however, prevent an overt lethal state in these colonies.

3.4 CONCLUSION

A protocol for the induction of latent baculovirus from FCM laboratory colonies was developed. Using overcrowding as a latent induction mechanism, five new CrleGV isolates were obtained. The next chapter describes the genetic characterisation of the isolates.

CHAPTER FOUR

CHARACTERISATION OF *CRYPTOPHLEBIA LEUCOTRETA* GRANULOVIRUS (CrleGV) ISOLATES

4.0 INTRODUCTION

In the previous chapter, a latent induction study successfully yielded five new CrleGV-SA isolates namely Addo, Nelspruit, Marble Hall, Citrusdal and Mixed colony isolates from five false codling moth (FCM) *Thaumatotibia leucotreta* (Meyrick) laboratory colonies. This chapter describes the genetic characterisation of the isolates obtained.

At present, three CrleGV isolates from three different countries have been reported with FCM, namely the Ivory Coast isolate (CrleGV-IC) (Angelini *et al.*, 1965), the Cape Verde isolate (CrleGV-CV) (Mück, 1985) and the South African isolate (CrleGV-SA) (Fristch *et al.,* 1989; Singh *et al.*, 2003). These three isolates are also genetically different (Fritsch *et al.,* 1989). However, of relevance to this study is the South African isolate (CrleGV-SA). The South African isolate was first discovered by Fristch (1989), and was recovered from FCM infected larvae from South Africa, held in an insectary by the Hoechst Corporation, Germany (Jehle *et al.*, 1992). Thereafter, another South African isolate was recovered from diseased FCM larvae held in an insectary by Citrus Research International in Port Elizabeth, South Africa (Moore, 2002). This CrleGV-SA isolate was subsequently characterised by Singh *et al.* (2003) and developed into the biopesticide Cryptogran (Moore *et al.,* 2004a & 2004b; Moore *et al.*, 2011). Another CrleGV-SA isolate was also developed into the biopesticide Cryptex (Kessler & Zingg, 2008).

Several molecular techniques can be used to establish the identity of baculovirus species. The most popular and conventional methods involve the use of restriction endonuclease (REN) analysis of total genomic DNA. The method is based on the fact that restriction endonucleases or restriction enzymes (REs) work by recognising a specific target sequence in double stranded DNA (dsDNA). The REs cleave to dsDNA within its target sequence, which results in fragmentation of DNA and the generation of

enzyme specific DNA profiles or "genetic fingerprints" of discrete fragments of defined length and sequence (Primrose *et al.*, 2001). Since several RE recognition sites are widely distributed in the genome of organisms, differences in their DNA fragments as generated by the REs can be used to establish genetic differences (Ahern, 1991). A few examples of REs are *Sac*1, *Nde*1, *Pst*1, *Bam*H1, *Eco*R1, *Xho*1, *Hind*III, *Sal*1, *Xba*1 and *Kpn*1, with each RE having a unique recognition site. Errors caused during DNA replication by DNA replication enzymes can also change the distribution of the restriction sites leading to genetic differences between organisms (Primrose *et al.*, 2001). REN analysis has been successfully used in establishing genetic differences between and within several baculovirus species (Parnell *et al.*, 2002; Cory & Myers, 2003; Jehle *et al.*, 2006a; Miele *et al.*, 2011).

Full genomic DNA sequencing techniques can also be used to identify isolates. With this approach the entire genome of the organism is fully sequenced, which results in an enormous amount of DNA sequence data being made available. By aligning the nucleotide sequence data generated and comparing them to other closely related organisms, any single nucleotide polymorphisms (SNPs) can be used to track mutations and polymorphisms between organisms. The most commonly used methods here include conventional methods such as the GS20 sequencing and advanced methods such as 454 GS-FLX Titanium sequencing (Gilles *et al.*, 2011).

Alternatively, specific genes of interest can be targeted for sequencing. By using oligonucleotides that flank a particular gene, the sequence can be amplified using targeted polymerase chain reaction (PCR) techniques. Thereafter, amplicons of genes obtained can be sequenced in order to obtain their nucleotide sequence. Several baculovirus genes such as the *polyhedrin* or *granulin*, *egt*, *lef*-5, *lef*-8 and *lef*-9 genes can be targeted for sequencing (Kuzio *et al.*, 1999; Jehle *et al.*, 2006a).

The objective of this chapter is to establish the genetic identities of the five new CrleGV-SA isolates and compare them with those used to formulate Cryptogran and Cryptex as well as other documented CrleGV isolates. For the purpose of this study, REN analysis and PCR amplification of specific gene sequences was performed. Full genome

sequencing of the isolates, on the other hand, was not conducted as it was beyond the scope of this study.

4.1 METHODOLOGY

In order to characterise the five new CrleGV-SA isolates (Addo, Nelspruit, Marble Hall, Citrusdal and Mixed colony isolates), occlusion bodies (OBs) were purified and extracted according to the methods described in Chapter three, section 3.1.3. OBs obtained were then subjected to a DNA purification protocol in order to obtain genomic DNA for REN analysis or PCR. PCR was used to amplify two conserved genes namely, *granulin* and *egt* genes. The protocols for DNA extraction, REN analysis and PCR are described below. Cryptogran and Cryptex isolates were also subjected to the same analysis and served as reference isolates. The phenol: chloroform (PC) and CTAB DNA extraction protocols were used for genomic DNA extraction.

4.1.1 Phenol: Chloroform (PC) DNA extraction protocol

The PC DNA extraction protocol as described by Hunter-Fujita *et al.* (1998) and Hilton & Winstanley (2008), with a few modifications was used. To extract DNA, 40 µl of sodium carbonate, Na_2C0_3 (1 M) was dispensed into a microcentrifuge tube containing 500 µl of OBs. This was incubated at 37°C for 30 min, until a clear suspension was obtained. More Na_2C0_3 was added to the reaction if the suspension did not clarify. Afterwards, 30 µl of TE (10 mM Tris/HCL, pH 8.0, 0.1 M EDTA) and 25 µl of RNaseA (100 mg/ml) buffer were added and the mixture incubated at 37°C for 1 hour. Thereafter, 60 µl of 10% (w/v) sodium dodecyl sulphate (SDS) and 50 µl of Proteinase K (25 mg/ml) were added and the mixture incubated for another hour. An equivalent volume of Tris-buffered phenol (Sigma-Aldrich®, Germany) was added. The tubes were inverted several times and centrifuged at 14000 rpm for 3 min, using a tabletop laboratory centrifuge (BIO-RAD, model 16K). The upper phase was extracted and transferred into a new tube and the lower phase discarded. Another equivalent volume of phenol: chloroform: Isoamyl alcohol mixture, in the ratio of 25:24:1 was added to the supernatant. The tubes were inverted several times and centrifuged at 14000 rpm for 3

min. The upper phase was again extracted and dispensed into new tubes. This process was repeated. A volume equivalent to that extracted from the upper aqueous phase of chloroform: Isoamyl alcohol (24:1) was added to the aqueous solution and the mixture inverted several times to mix. The tubes were centrifuged at 14000 rpm for 3 min. Approximately 200 µl upper aqueous phase was transferred into new tubes and the lower phase discarded. The DNA was precipitated by adding 20 µl of sodium acetate (7 M) and 400 µl of 95% cold ethanol (stored at -25°C). The solution was mixed by inverting 2 or 3 times and incubated overnight at -20°C. The next day, the tubes were centrifuged at 14000 rpm for 5 min. The supernatant was discarded and the resulting pellet was re-suspended in 1 ml of 70% cold ethanol. The tubes were again centrifuged at 14000 rpm for 5 min. The ethanol was discarded leaving the DNA pellet. The tubes, containing the DNA pellet were inverted over a paper towel for 3 to 4 min in order to drain off the ethanol, and incubated at 50°C until all remaining traces of ethanol had evaporated. The DNA pellet was re-suspended by pipetting up and down a few times in 25 µl of 10 mM Tris-HCl buffer (pH 8.0). The DNA was stored for a few days at 4°C, or for longer periods at -25°C.

4.1.2 CTAB total genomic DNA extraction

A modified version of the CTAB DNA extraction protocol described by Aspinall *et al.* (2002) was employed. To extract DNA using this method, 200 µl of OBs was pipetted into sterile microcentrifuge tubes and 90 µl of Na_2CO_3 (1 M) was added to the suspension. More Na_2C0_3 was added to the reaction if the suspension did not clarify. The contents were incubated at 37°C for 30 min and the suspension neutralized with 120 µl of Tris-HCL (1 M, pH 6.8). Thereafter, 90 µl of SDS (10% w/v) and 50 µl of Proteinase K (25mg/ml) was added. Samples were incubated for 30 min at 37°C before addition of 10 µl of RNaseA (10mg/ml) and incubation for a further 30 min at 37°C. The tubes were centrifuged at 14000 rpm for 3 min in a tabletop laboratory centrifuge (BIO-RAD, model 16K). The supernatant was transferred into a new tube and the pellets discarded. Afterwards, 400 µl of pre-warmed (at 70°C) CTAB buffer (2% w/v, CTAB, 100 mM Tris, pH 8.0, 20 mM Na_2EDTA, 1.4 M NaCl) was added to the supernatant and samples incubated at 70°C for 1 hour. Every 10 min, the tubes were inverted several

times to allow the contents to mix. Thereafter, 400 µl of chloroform stored at 4°C was added. The tubes were again inverted briefly and centrifuged for 10 min at 10000 rpm. The upper aqueous phase was transferred into a new tube and 400 µl of cold isopropanol (stored at -25°C) was added. The tubes were incubated overnight, at -25°C and then centrifuged at 14000 rpm for 20 min. The pellet was re-suspended in 1 ml of 70% ice cold ethanol. The tubes were centrifuged at 14000 rpm for 5 min and the ethanol gently poured off, without discarding the DNA pellet. The tubes were incubated until all remaining traces of ethanol had evaporated. The DNA pellet was re-suspended overnight at 4°C in 20 µl of RNase free water or 10mM Tris-HCL (pH 8.0) buffer. DNA was stored for a few days at 4°C or for longer periods at -25°C before use.

4.1.3 Determination of DNA concentration

DNA concentration was determined using a spectrophotometer (Genequant, Pharmacia Biotech®). A 1:100 dilution of DNA in sterile distilled water was made. The absorbance was read at 260 nm and the concentration of DNA was calculated according to the standard equation: 1 absorbance unit at OD_{260}nm = 50 µg /ml.

4.1.4 Single restriction enzyme digestion and agarose gel electrophoresis

Single REN digest reactions were performed in a total volume of 30 µl, containing 20 µl (10 – 12 ug) genomic DNA, 3 µl 1x RE buffer and 3 µl (30 U) of either: *Bam*H1, *Sal*1, *Xba*1, *Pst*1, *Xho*1, *Kpn*1, *Hind*III and *Eco*R1 (Fermentas®), with ddH_20 added to make the final volume. The contents of the reaction were briefly centrifuged and the tubes were incubated at 37°C for 4 hours.

Digests were analysed by 0.6% agarose gel electrophoresis (AGE) with ethidium bromide staining in 1x TAE buffer (40 mM Tris-acetate, 20 mM acetic acid, 1 mM EDTA) at 30 V for 16 hours. Four DNA markers namely λ mix 19, GeneRuler High Range, DNA marker II and 1 Kb DNA marker (Fermentas®, Thermo Fisher Scientific) were loaded into the gels and served as a reference for measuring the size of the DNA fragments. The gels were photographed under a UV trans-illuminator, Uviprochemi (Uvitec®, UK) and gel images captured using UVIprochemi (version 12.4 for Windows) software.

The size of the DNA fragments was estimated by comparison with DNA markers, by plotting the distance (cm) migrated by the DNA bands against the log of their known sizes in kilobase pairs (kbp), and a standard curve was generated. The sizes of the four DNA makers are indicated in figure 4.1. The distance migrated by the DNA fragments from CrleGV-SA samples was measured and the values obtained were inserted into the standard curve equation in order to determine their sizes. Based on the RE fragments generated by *Bam*H1, *Sal*1, *Xba*1, *Pst*1, *Xho*1, *Kpn*1, *Hind*III and *Eco*R1, restriction maps were constructed for the seven CrleGV-SA isolates.

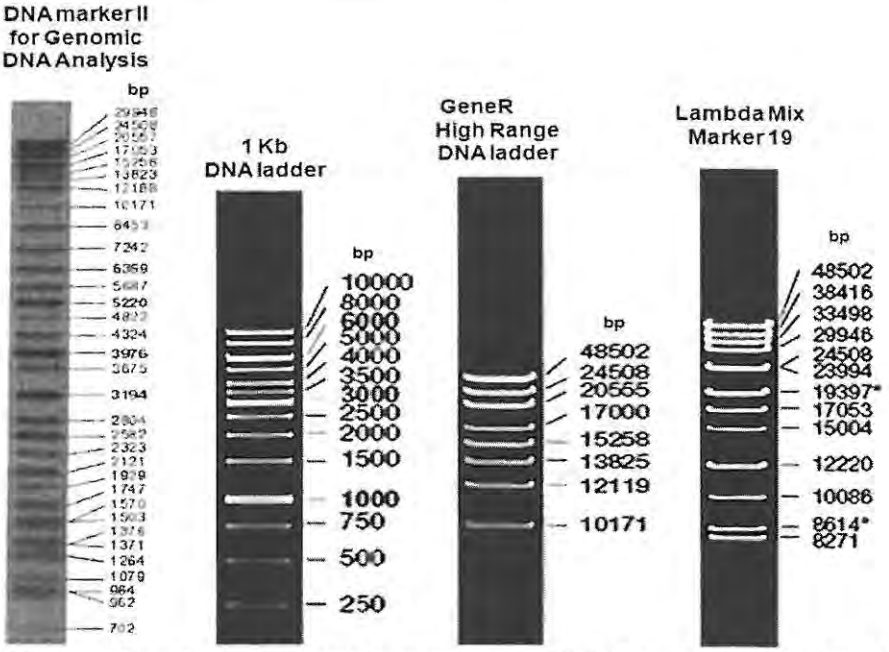

Figure 4.1 DNA profiles from four DNA markers. **(A)** DNA marker II (702 bp to 29946 bp), **(B)** 1 Kb DNA marker (250 bp to 10000 bp), **(C)** GeneRuler High Range marker (10171 bp to 48502 bp) and **(D)** λ mix marker 19 (8271 bp to 48502 bp) (Source: www.fermentas.com).

4.1.5 Amplification of the *granulin* and *egt* genes from CrleGV-SA samples using polymerase chain reaction (PCR)

The *granulin* and *egt* genes were amplified from genomic DNA of the seven CrleGV-SA isolates using PCR. Oligonucleotide primers used for amplifying the *granulin* and *egt* genes were designed according to published sequences found in the NCBI (Pubmed) database by Lange & Jehle (2003) (Table 4.1). The PCR reaction components are described in table 4.2. Amplifications were carried out on a thermocycler (BIO-RAD®)

as indicated in table 4.3. Amplified products were analysed by 0.6 % AGE. The 1 Kb DNA marker (Fig. 4.1 B) was used to estimate the size of the amplified products. Amplified products were sent to Inqaba Biotechnical Industries (Pty) Ltd (South Africa) for sequencing, using the oligonucleotide primers indicated in table 4.1.

Table 4.1 Oligonucleotide primers for amplification of the *granulin* and *egt* genes of CrleGV-SA

Oligonucleotide name	Sequence (5' to 3')
Granulin forward	ATG GGA TAT AAC AAA TCT TTG AGG
Granulin reverse	TTA ATA GGC TGG ACC GGT GAA TAG G
Egt forward	TTA TTT ATT TTC GTT AAA CAT AAA CAT TAC
Egt reverse	TGT ATA GTA TCT TTG TTG TGC TGT TG

Table 4.2 Volume of reagents used for PCR amplification of the *granulin* and *egt* gene of CrleGV-SA isolates

***Granulin* gene**	***Egt* gene**
36 µl of sterile distilled water (ddH_20)	36 µl of sterile distilled water (ddH_20)
5 µl of Taq buffer (with $MgCl_2$)	5 µl of Taq buffer (in $MgCl_2$)
2 µl of *Granulin* forward primer (10 µM stock)	2 µl of *Egt* forward primer (10 µM stock)
2 µl of *Granulin* reverse primer (10 µM stock)	2 µl of *Egt* reverse primer (10 µM stock)
2 µl of dNTPs (10 mM stock)	2 µl of dNTPs (10 mM stock)
2.5 µl of template (DNA)	2.5 µl of template (DNA)
0.5 µl of Taq (*Thermus aquaticus*) polymerase	0.5 µl of Taq polymerase
50 µl total reaction	50 µl total reaction

Table 4.3 Cycling parameters used for amplification of the *granulin* and *egt* gene of CrleGV-SA isolates

		Temperature °C	Duration	Cycle
Stage 1	Step 1	95°C	1 min	1 x
Stage 2	Step 1	95°C	1 min	30 x
	Step 2	50°C	1:30 min	
	Step 3	72°C	1:30 min	
Stage 3	Step 1	72°C	5 min	1 x

*Denaturing (95°C): Annealing (50°C): Extension or elongation (72°C)

4.1.6 Sequence alignments and phylogenetic analysis of the *granulin* and *egt* genes of CrleGV-SA samples

Forward and reverse sequences obtained from both the *granulin* and *egt* genes of the seven CrleGV-SA samples were assembled and edited using DNA Dragon® (version 1.3.0) and aligned using Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0 (Tamura *et al*, 2007 & 2011).

The *granulin* and *egt* gene sequences obtained were compared with other GV sequences on the National Centre for Biotechnology Information (NCBI) database. This was conducted either as multiple alignments in MEGA, or by individual BLAST comparisons on the NCBI web page. Details of the GV isolates obtained from the NCBI database that were was used in making comparisons are shown in table 4.4.

For the *granulin* gene, multiple alignments of nucleotide sequence data and their associated amino acid translations for the seven CrleGV-SA isolates used in this study and the CrleGV-SA (AY293731) isolate were conducted in MEGA. Individual BLAST comparisons were then conducted on the NCBI web page with the *granulin* gene sequences from each of the seven CrleGV-SA isolates, against CrleGV-SA (AY293731), CrleGV-CV3 (AY229987), CpGV (U53466) and PlscGV (AY706675) isolates. The accession numbers of the GV isolates obtained from the NCBI database are indicated in brackets and their details are shown in table 4.4.

For the *egt* gene, multiple alignments of nucleotide sequence data and their associated amino acid translations for both the seven CrleGV-SA isolates, and CrleGV-CV3 (AY229987) isolate were conducted in MEGA. Sequence data for CrleGV-CV3 (AY229987) isolate was imported from the NCBI database into MEGA for analysis. Further, individual BLAST comparisons were conducted on the NCBI web page with the *egt* gene sequences from each of the seven CrleGV-SA isolates against the CrleGV-CV3 (AY229987) isolate. No CrleGV-SA (AY293731) *egt* gene sequence was found on the NCBI database.

Table 4.4 *Granulin* and *egt* genes from GV species obtained from the NCBI database used in multiple alignments or individual BLAST comparisons

GV specie	Accession number	Authors
South African isolate of *Cryptophlebia leucotreta* GV, CrleGV-SA	AY293731	Singh *et al.* (2003)
Cape Verde isolate of *Cryptophlebia leucotreta* GV, CrleGV-CV3	AY229987	Lange & Jehle (2003)
Cydia pomonella GV, CpGV	U53466	Luque *et al.* (2001)
Plathypena scabra GV, PlscGV	AY706675	Jehle *et al.* (2006c)

(Source: www.ncbi.nlm.nih.gov/).

Molecular phylogenetic analysis was performed in MEGA using sequence data obtained from the *granulin* and *egt* genes of the seven CrleGV-SA isolates. Their sequence data was compared against the *granulin* gene sequence of CrleGV-SA (AY293731) and the *egt* gene sequence of the CrleGV-CV3 (AY229987) isolate. Phylogenetic trees were constructed using the minimum evolution (ME) method (Rzhetsky & Nei, 1992).

4.2 RESULTS

4.2.1 CrleGV-SA genomic DNA extracted using CTAB and PC methods

The PC and CTAB DNA extraction protocols were successful for extracting genomic DNA from the seven CrleGV-SA samples (Fig. 4.2). However, high molecular weight DNA extracted using the PC method was occasionally found to show some level of degradation before and after restriction analysis (Fig. 4.2 B).

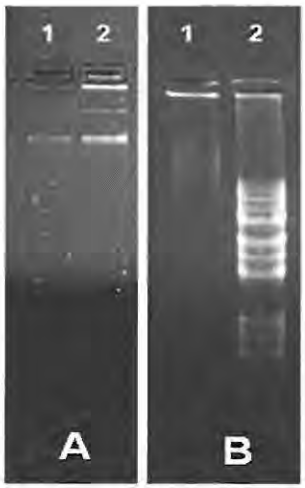

Figure 4.2 (A) High molecular weight CrleGV-SA genomic DNA extracted using the CTAB method (lane 1 & 2). **(B)** CrleGV-SA genomic DNA extracted using the PC method, with DNA before (lane 1) and after REN analysis with *Eco*R1 (lane 2).

4.2.2 CrleGV-SA genomic DNA digested with the restriction enzyme *Bam*H1

REN analysis of the genomic DNA extracted from Cryptex, Cryptogran and the Addo, Mixed, Citrusdal, Marble Hall and Nelspruit colony isolates was conducted using *Bam*H1, and the number of bands observed (Fig. 4.3 & 4.4). Fragment sizes were estimated according to the methods described in section 4.1.3. *Bam*H1 digestion

generated 9 prominent fragments for all samples (A – I) (Table 4.5, Fig. 4.4). It was observed that fragments E and G which were shared by Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates, were located as fragments G and H in Cryptogran and the Nelspruit colony isolate. However, fragments B and I were shared among all samples.

Based on their *Bam*H1 profile (Fig. 4.3) and fragment sizes (Table 4.5, Fig. 4.4), there appeared to be two distinct CrleGV-SA genome types. Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates could be placed as members of the Group one CrleGV-SA, and Cryptogran and the Nelspruit colony isolate formed the Group two CrleGV-SA. The genome sizes for Groups one and two CrleGV-SA were estimated to be 112.21 and 112.22 kbp, respectively.

Several submolar REN bands were present in all CrleGV-SA samples tested and it was observed that the Citrusdal colony isolate and Cryptex shared a similar submolar banding pattern, while all other samples showed a unique submolar banding pattern. The submolar profiles also differed from each other in terms of band position and the number of fragments generated (Fig. 4.3 & 4.4), but the bands (marked with asterisk) were always located between fragments B and C (Fig. 4.3 & 4.4). The appearance of submolar bands in restriction profiles of baculoviruses are commonly used to establish the presence of different genotypes in an isolate (Crook, 1986; Crook *et al.*, 1997). Based on the presence of these submolar bands in all samples tested, genetic differences could be established between samples with each isolate consisting of a mixture of more than one CrleGV-SA genotype.

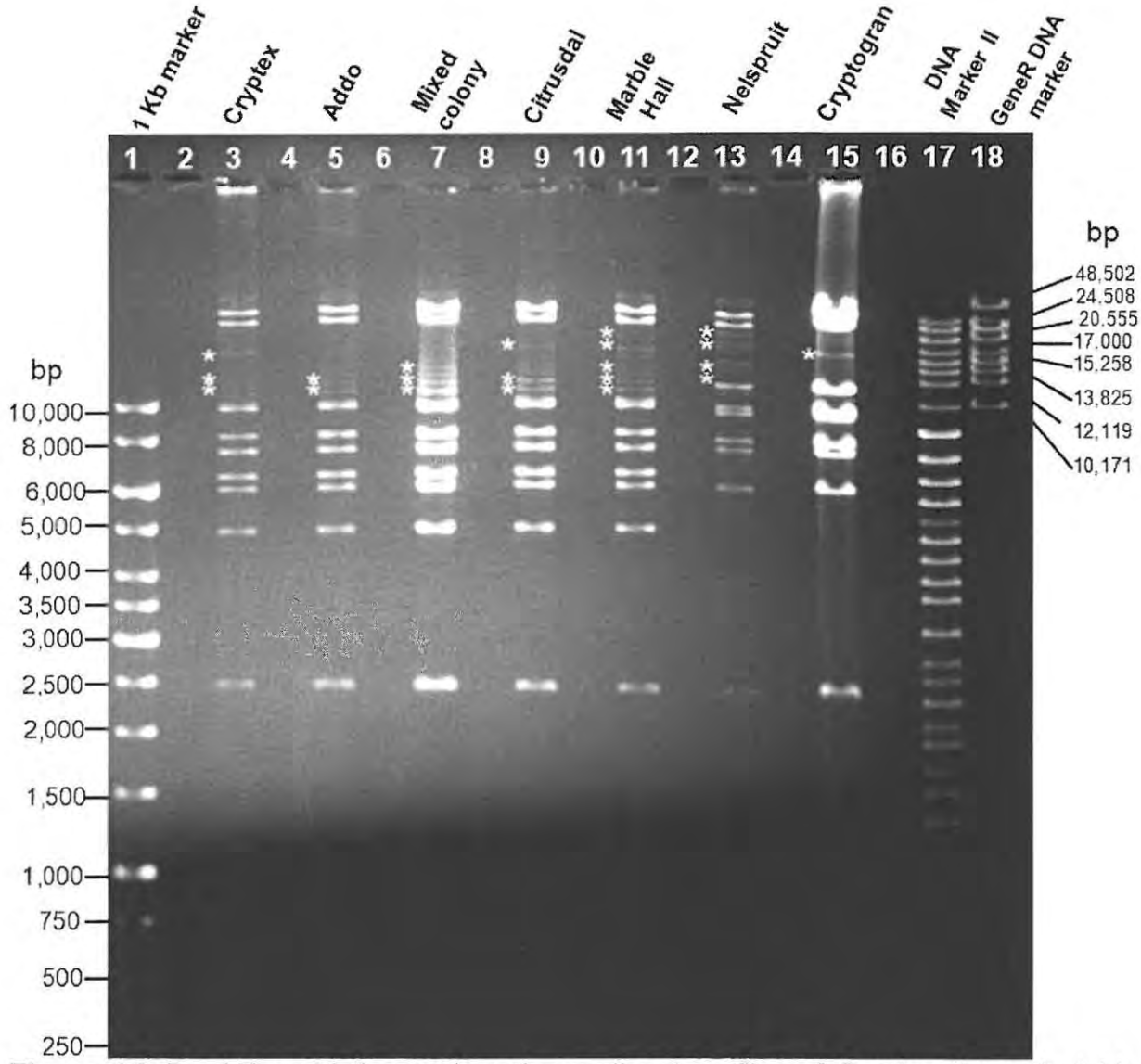

Figure 4.3 *Bam*H1 restriction endonuclease digest profiles of Cryptex (lane 3), Addo colony isolate (lane 5), Mixed colony isolate (lane 7), Citrusdal colony isolate (lane 9), Marble Hall colony isolate (lane 11), Nelspruit colony isolate (lane 13) and Cryptogran (lane 15) samples analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: 1 Kb DNA marker (lane 1), DNA marker II (lane 17) and GeneRuler High range (lane 18) were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

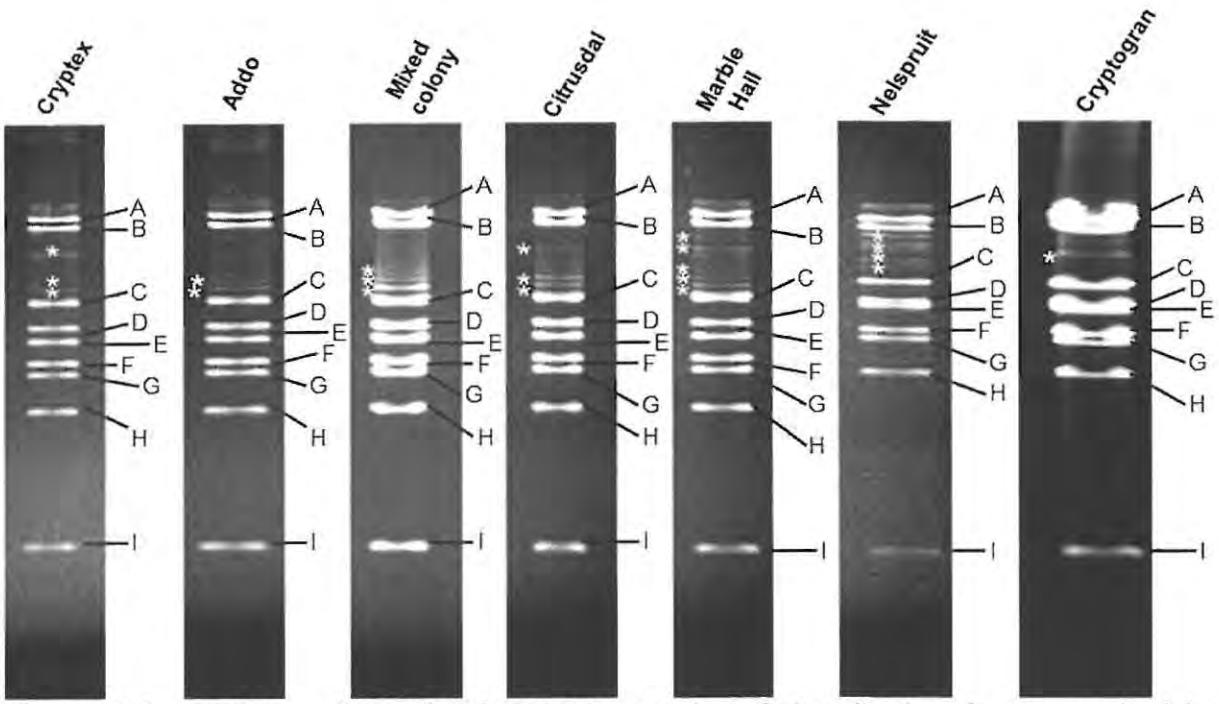

Figure 4.4 *Bam*H1 profiles of CrleGV-SA samples. Submolar bands are marked by asterisks (*).

Table 4.5 DNA restriction fragment sizes (kb) of CrleGV-SA samples after restriction endonuclease digestion with *Bam*H1

	Commercial isolate	New CrleGV-SA isolates					Commercial isolate
Fragment	Cryptex Isolate	Addo isolate	Mixed Colony isolate	Citrusdal isolate	Marble Hall isolate	Nelspruit isolate	Cryptogran isolate
A	40.94	40.94	40.94	40.94	40.94	32.04	32.04
B	**24.95**	**24.95**	**24.95**	**24.95**	**24.95**	**24.95**	**24.95**
C	10.15	10.15	10.15	10.15	10.15	11.55	11.55
D	8.37	8.37	8.37	8.37	8.37	9.75	9.75
E	**7.80**	**7.80**	**7.80**	**7.80**	**7.80**	9.37	9.37
F	6.40	6.40	6.40	6.40	6.40	8.08	8.08
G	**6.11**	**6.11**	**6.11**	**6.11**	**6.11**	**7.80**	**7.80**
H	4.92	4.92	4.92	4.92	4.92	**6.11**	**6.11**
I	**2.58**	**2.58**	**2.58**	**2.58**	**2.58**	**2.58**	**2.58**
Total	112.21	112.21	112.21	112.21	112.21	112.22	112.22

Fragments are labelled in order of decreasing size, "A" being the largest. Fragment sizes appearing in all samples are shown in bold.

4.2.3 CrleGV-SA genomic DNA digested with the restriction enzyme *Sal*I

*Sal*I profiles for Cryptex, Cryptogran and the Addo, Mixed, Citrusdal, Marble Hall and Nelspruit colony isolates generated 14 prominent bands (Fig. 4.5 & 4.6) which are indicated as fragments A – N (Table 4.6 & Fig. 4.6). It was observed that fragments G, J, K, L, M and N which were shared by Cryptex and the Addo, Mixed, Citrusdal and Marble Hall isolates was located as fragments F, H, I, J, M and N in Cryptogran and the Nelspruit colony isolate. However, fragments M and N were located in the same position for all samples (Table 4.6).

The *Sal*I profile (Fig. 4.5) and fragment sizes (Table 4.6 & Fig. 4.6) showed the presence of two distinct CrleGV-SA genome types. The genome size for the Group one (Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates) and two (Cryptogran and the Nelspruit colony isolate) CrleGV-SA was estimated to be 112.13 and 112.21 kbp, respectively.

One submolar band was observed in Cryptex and both the Addo and Mixed colony isolates. The submolar bands for all three samples were identical in terms of banding position and were located between fragments I and J, which are marked with asterisks (Fig. 4.5 & 4.6). These submolar bands may indicate the presence of mixed genotypes of CrleGV-SA, within these samples.

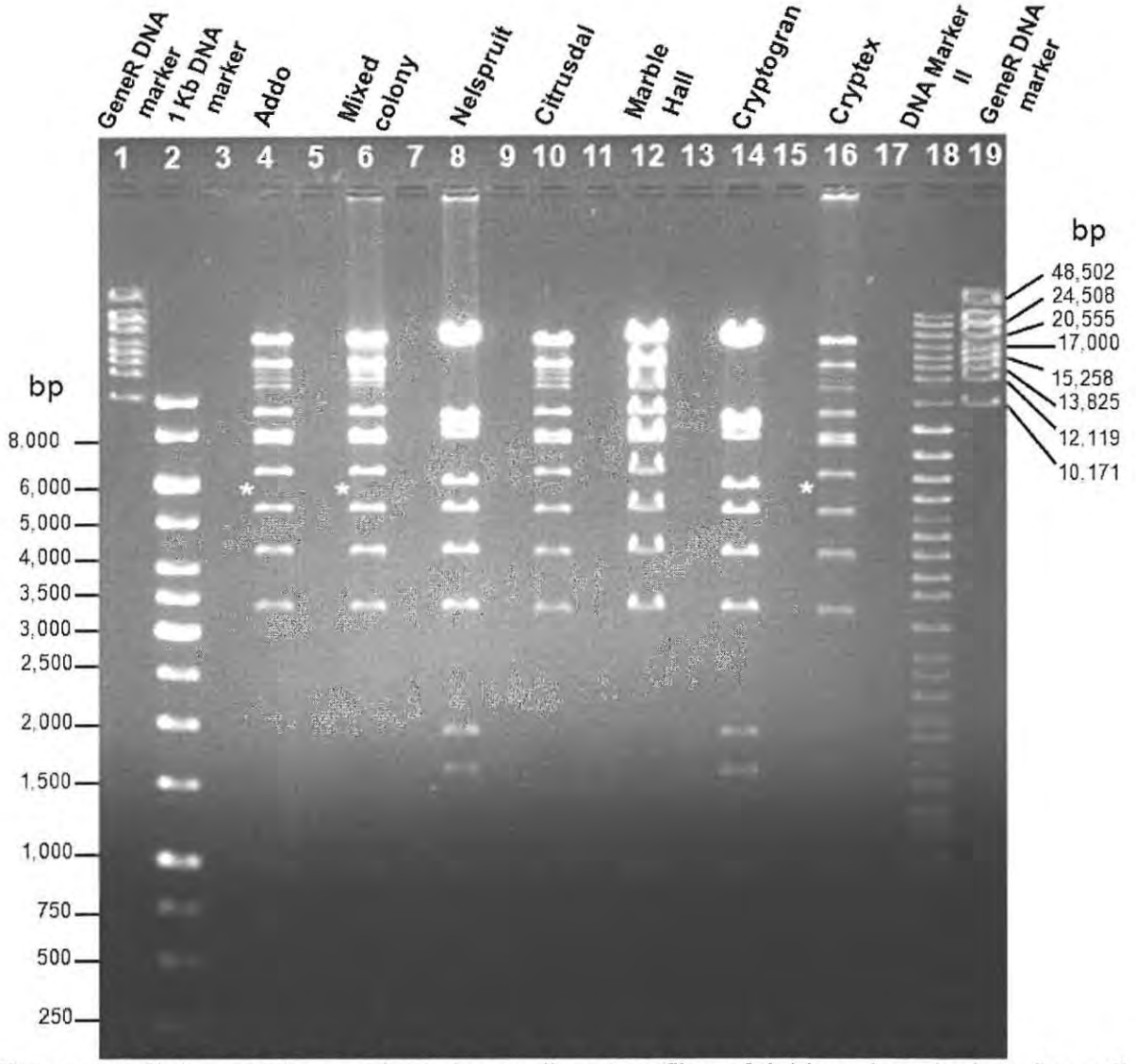

Figure 4.5 *Sal*I restriction endonuclease digest profiles of Addo colony isolate (lane 4), Mixed colony isolate (lane 6), Nelspruit colony isolate (lane 8), Citrusdal colony isolate (lane 10), Marble Hall colony isolate (lane 12), Cryptogran (lane 14) and Cryptex (lane 16) samples analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: GeneRuler High range (lane 1 & 19), 1 Kb DNA marker (lane 2), DNA marker II (lane 18) and were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

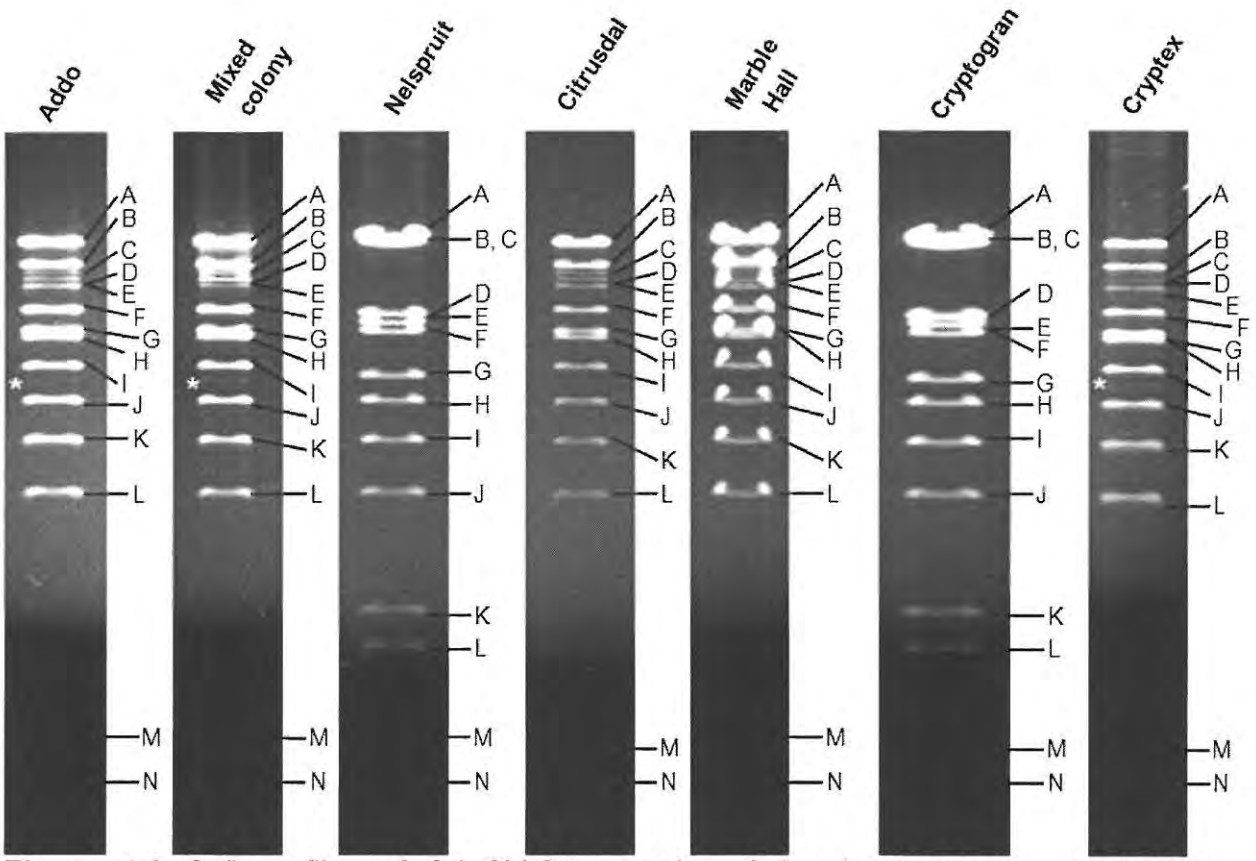

Figure 4.6 *SalI* profiles of CrleGV-SA samples. Submolar bands are marked by asterisks (*).

Table 4.6 DNA restriction fragment sizes (kb) of CrleGV-SA samples after restriction endonuclease digestion with *Sal*1

	Commercial isolate	New CrleGV-SA isolates					Commercial isolate
Fragment	Cryptex Isolate	Addo isolate	Mixed Colony isolate	Citrusdal isolate	Marble Hall isolate	Nelspruit isolate	Cryptogran isolate
A	18.18	18.18	18.18	18.18	18.18	22.17	22.17
B	12.54	12.54	12.54	12.54	12.54	20.34	20.34
C	11.97	11.97	11.97	11.97	11.97	19.66	19.66
D	11.44	11.44	11.44	11.44	11.44	8.95	8.95
E	10.95	10.95	10.95	10.95	10.95	8.62	8.62
F	9.67	9.67	9.67	9.67	9.67	**8.31**	**8.31**
G	**8.31**	**8.31**	**8.31**	**8.31**	**8.31**	5.81	5.81
H	8.02	8.02	8.02	8.02	8.02	**5.32**	**5.32**
I	6.36	6.36	6.36	6.36	6.36	**4.28**	**4.28**
J	**5.32**	**5.32**	**5.32**	**5.32**	**5.32**	**3.25**	**3.25**
K	**4.28**	**4.28**	**4.28**	**4.28**	**4.28**	1.96	1.96
L	**3.25**	**3.25**	**3.25**	**3.25**	**3.25**	1.71	1.71
M	**1.01**	**1.01**	**1.01**	**1.01**	**1.01**	**1.01**	**1.01**
N	**0.82**	**0.82**	**0.82**	**0.82**	**0.82**	**0.82**	**0.82**
Total	112.13	112.13	112.13	112.13	112.13	112.21	112.21

Fragments are labelled in order of decreasing size, "A" being the largest. Fragment sizes appearing in all samples are shown in bold.

4.2.4 CrleGV-SA genomic DNA digested with the restriction enzyme *Xba*I

*Xba*I profiles for Cryptex, Cryptogran and the Addo, Mixed, Citrusdal, Marble Hall and Nelspruit colony isolates generated 17 prominent bands (Fig. 4.7 & 4.8) which are indicated as fragments A – Q (Table 4.7 & Fig. 4.8). It was noted that fragments F, G, I, J, K, L, M, N, O and P, which were shared by Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates was located as fragments E, F, J, K, L, M, N, O, P and Q in Cryptogran and the Nelspruit colony isolate. However, fragment H was in the same position for all samples. *Xba*I profile (Fig. 4.7) and fragment sizes (Table 4.7 & Fig. 4.8) generated two distinct CrleGV-SA genome types. The genome size for Group one (Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates) and two (Cryptogran and the Nelspruit colony isolate) CrleGV-SA was estimated to be 112.13 and 112.02 kbp respectively.

Three and two unique submolar bands were observed in the Citrusdal and Nelspruit colony isolates respectively. However, the positions of two of the submolar bands were similar in terms of their banding position (Fig. 4.7 & 4.8). The submolar bands were located between fragments G and K for the Nelspruit isolate and fragments F and J for the Citrusdal isolate (Fig. 4.8). This suggests that these two isolates each consist of multiple genotypes of CrleGV-SA.

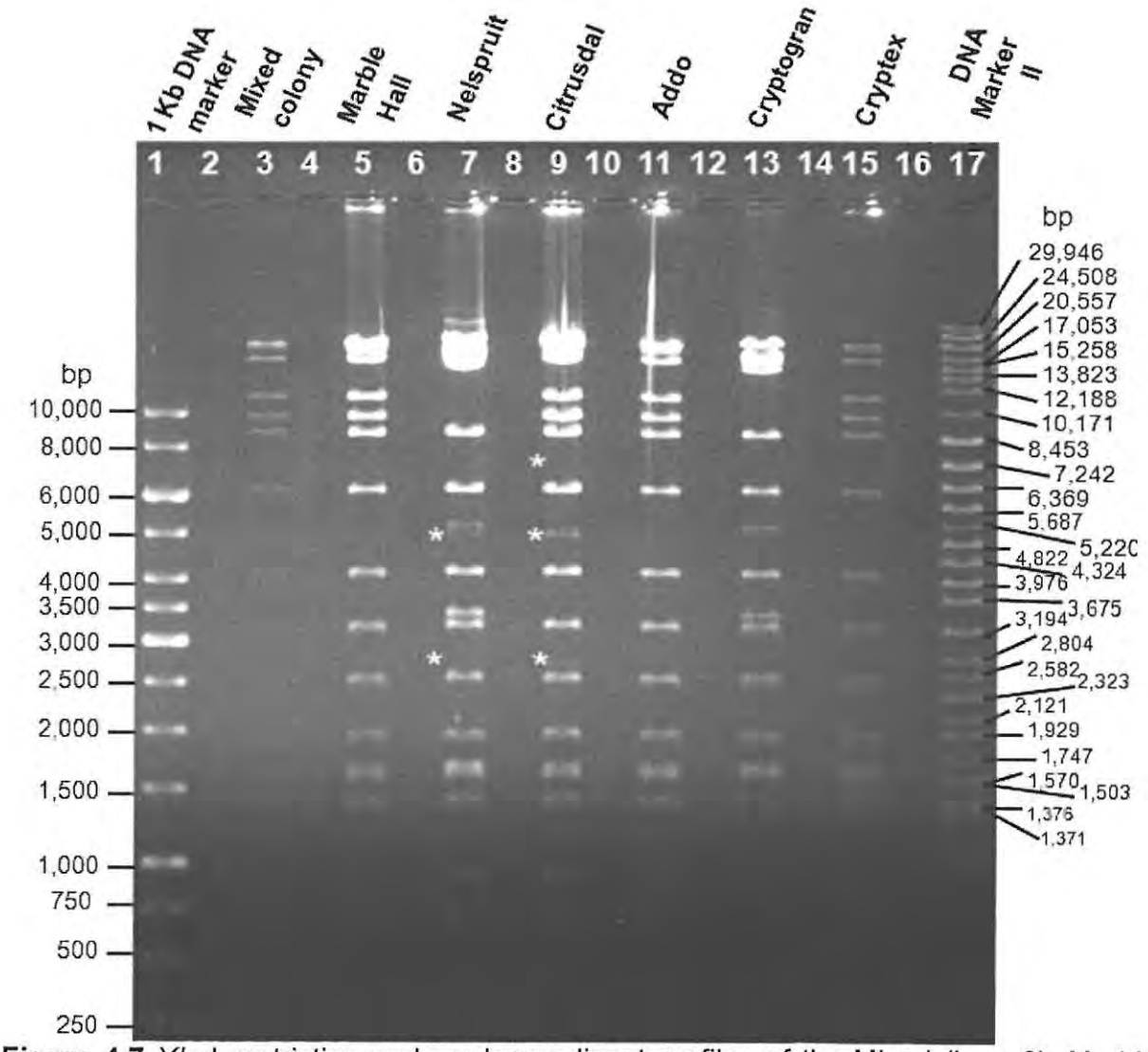

Figure 4.7 *Xba*I restriction endonuclease digest profiles of the Mixed (lane 3), Marble Hall (lane 5), Nelspruit (lane 7), Citrusdal (lane 9), and Addo (lane 11) colony isolates with Cryptogran (lane 13) and Cryptex (lane 15) analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: 1 Kb DNA marker (lane 1) and DNA marker II (lane 17) and were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

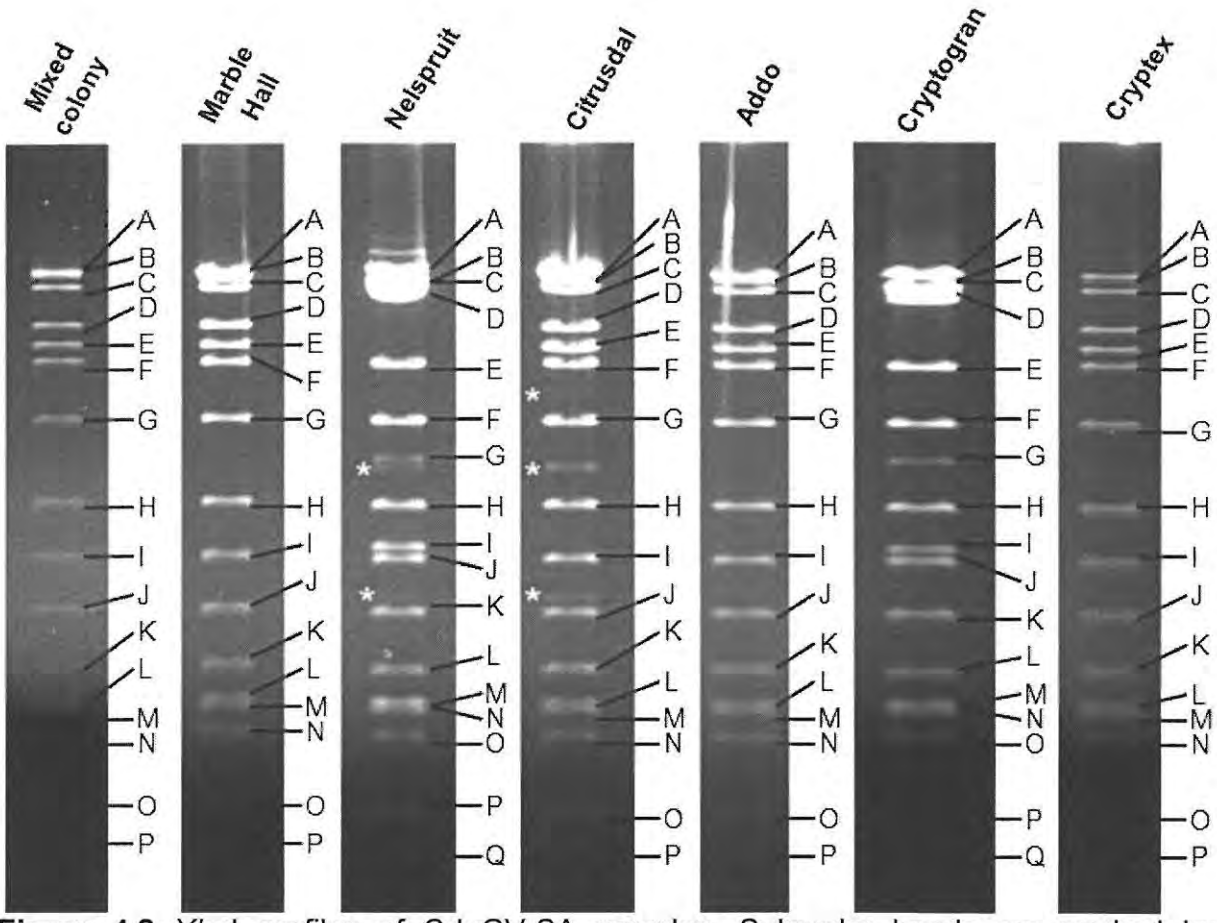

Figure 4.8 *Xba*I profiles of CrleGV-SA samples. Submolar bands are marked by asterisks (*).

Table 4.7 DNA restriction fragment sizes (kb) of CrleGV-SA samples after restriction endonuclease digestion with *Xba*1

	Commercial isolate	New CrleGV-SA isolates					Commercial isolate
Fragment	Cryptex isolate	Addo isolate	Mixed Colony isolate	Citrusdal isolate	Marble Hall isolate	Nelspruit isolate	Cryptogran isolate
A	19.86	19.86	19.86	19.86	19.86	20.60	20.60
B	19.16	19.16	19.16	19.16	19.16	18.89	18.89
C	16.76	16.76	16.76	16.76	16.76	15.76	15.76
D	12.61	12.61	12.61	12.61	12.61	14.85	14.85
E	10.41	10.41	10.41	10.41	10.41	**9.14**	**9.14**
F	**9.14**	**9.14**	**9.14**	**9.14**	**9.14**	**6.30**	**6.30**
G	**6.30**	**6.30**	**6.30**	**6.30**	**6.30**	5.19	5.19
H	**3.99**	**3.99**	**3.99**	**3.99**	**3.99**	**3.99**	**3.99**
I	**3.12**	**3.12**	**3.12**	**3.12**	**3.12**	3.38	3.38
J	**2.47**	**2.47**	**2.47**	**2.47**	**2.47**	**3.12**	**3.12**
K	**1.91**	**1.91**	**1.91**	**1.91**	**1.91**	**2.47**	**2.47**
L	**1.68**	**1.68**	**1.68**	**1.68**	**1.68**	**1.91**	**1.91**
M	**1.60**	**1.60**	**1.60**	**1.60**	**1.60**	**1.68**	**1.68**
N	**1.40**	**1.40**	**1.40**	**1.40**	**1.40**	**1.60**	**1.60**
O	**0.97**	**0.97**	**0.97**	**0.97**	**0.97**	**1.40**	**1.40**
P	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**	**0.97**	**0.97**
Q						**0.75**	**0.75**
Total	112.13	112.13	112.13	112.13	112.13	112.02	112.02

Fragments are labelled in order of decreasing size, "A" being the largest. Fragment sizes appearing in all samples are shown in bold.

4.2.5 CrleGV-SA genomic DNA digested with the restriction enzyme *Pst*1

*Pst*1 profiles for Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates generated 14 prominent bands (Fig. 4.9 & 4.10) which are indicated as fragments (A – N) (Table 4.8 & Fig. 4.11). Cryptogran and the Nelspruit colony isolate generated 15 prominent bands (Fig. 4.9 & 4.10) which are indicated as fragments (A – O) (Table 4.8 & Fig. 4.11). It was noted that, fragments B, C, D, E, L, M and N, which were shared by Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates were located as fragments A, B & C, E, F, M, N and O in Cryptogran and the Nelspruit colony isolate. However, fragment C, J and K were located in the same position for all samples. *Pst*1 profile (Fig. 4.9 & 4.10) and fragment sizes (Table 4.8 & Fig. 4.11) generated two

distinct CrleGV-SA genome types. The genome size for Group one (Cryptex, and Addo, Citrusdal, Marble Hall and Mixed colony isolates) and two CrleGV-SA (Cryptogran and the Nelspruit colony isolate) were estimated to be 112.15 and 112.57 kbp respectively.

One submolar band was observed in Cryptex, Cryptogran and the Nelspruit and Marble Hall colony isolates. These individual submolar bands were located between fragments D and E for Cryptex and the Marble Hall colony isolate and between fragments E and F for Cryptogran and the Nelspruit colony isolate. The submolar bands are marked with asterisks (Fig. 4.9, 4.10 & 4.11). This indicates that these four samples each consist of more than one genotype of CrleGV-SA.

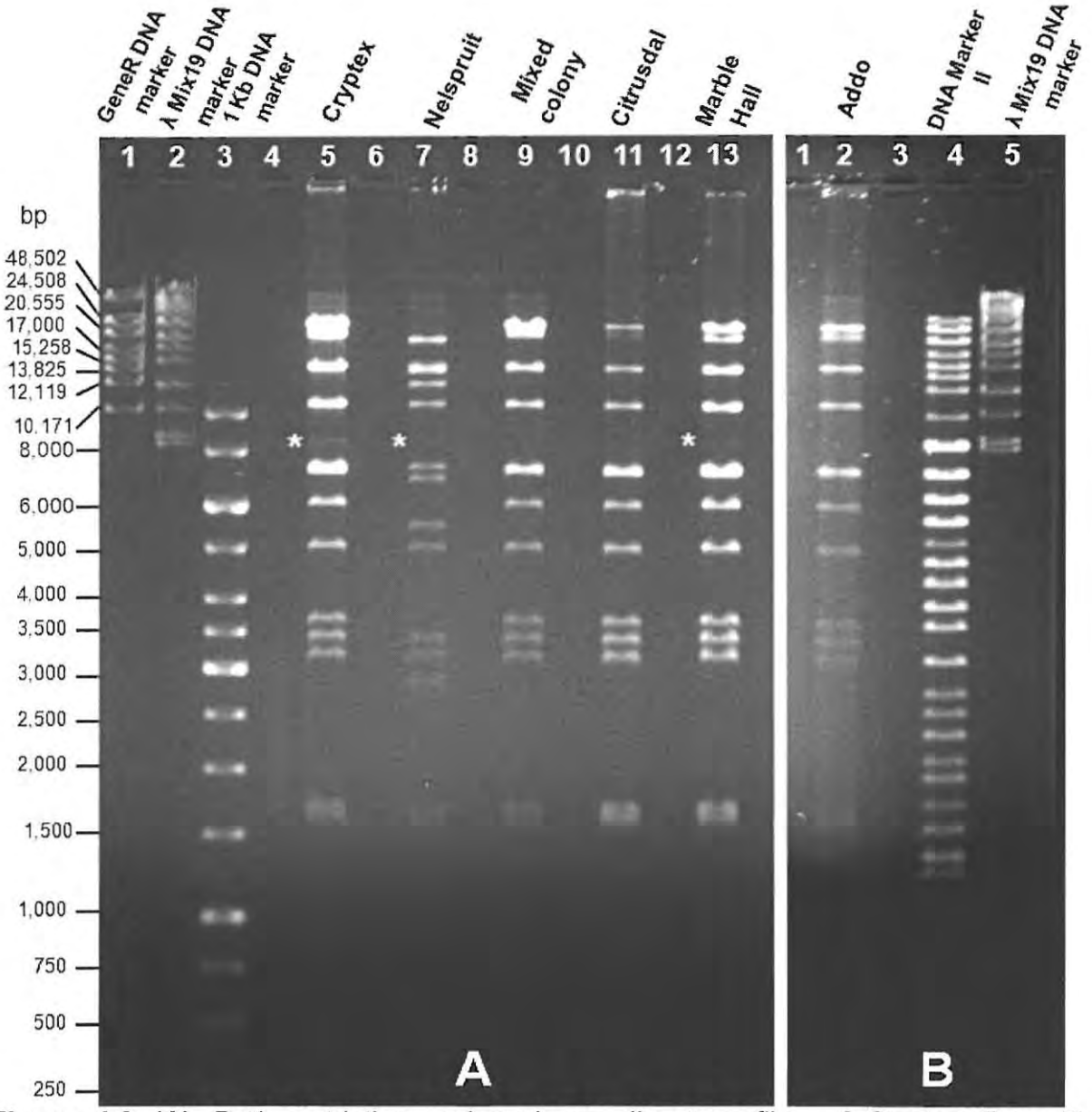

Figure 4.9 (A) *Pst*1 restriction endonuclease digest profiles of Cryptex (lane 5), Nelspruit colony isolate (lane 7), Mixed colony isolate (lane 9), Citrusdal colony isolate (lane 11) and Marble Hall colony isolate (lane 13) samples. **(B)** *Pst*1 profiles of the Addo colony isolate (lane 2). All samples analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: GeneR marker (lane 1); λ Mix 19 marker (lanes 2 and 5) and 1 Kb DNA marker (lane 3) were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

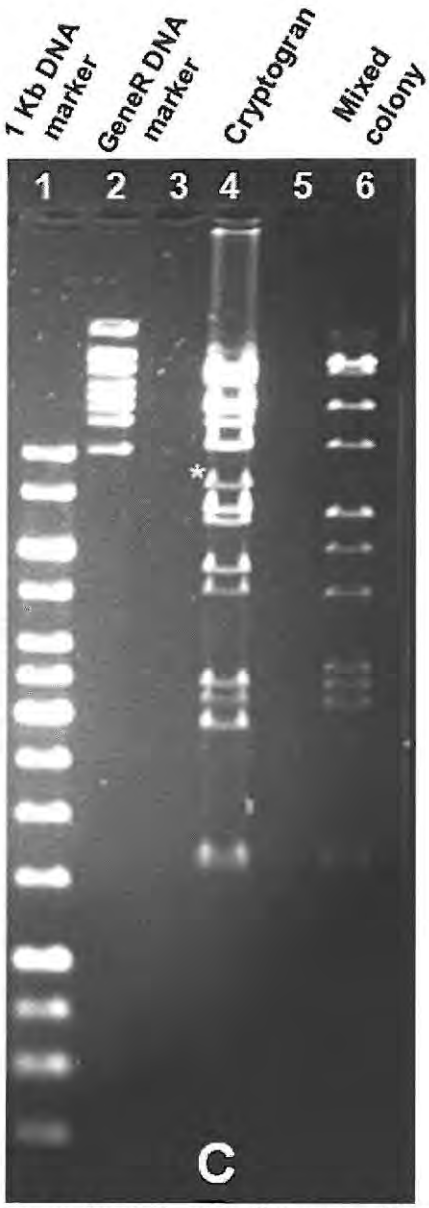

Figure 4.10 (C) *Pst*1 restriction endonuclease digest profiles of Cryptogran (lane 4) and the Mixed colony isolate DNA (lane 6) analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: 1 Kb DNA marker (lane 1) and GeneR marker (lane 2) were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

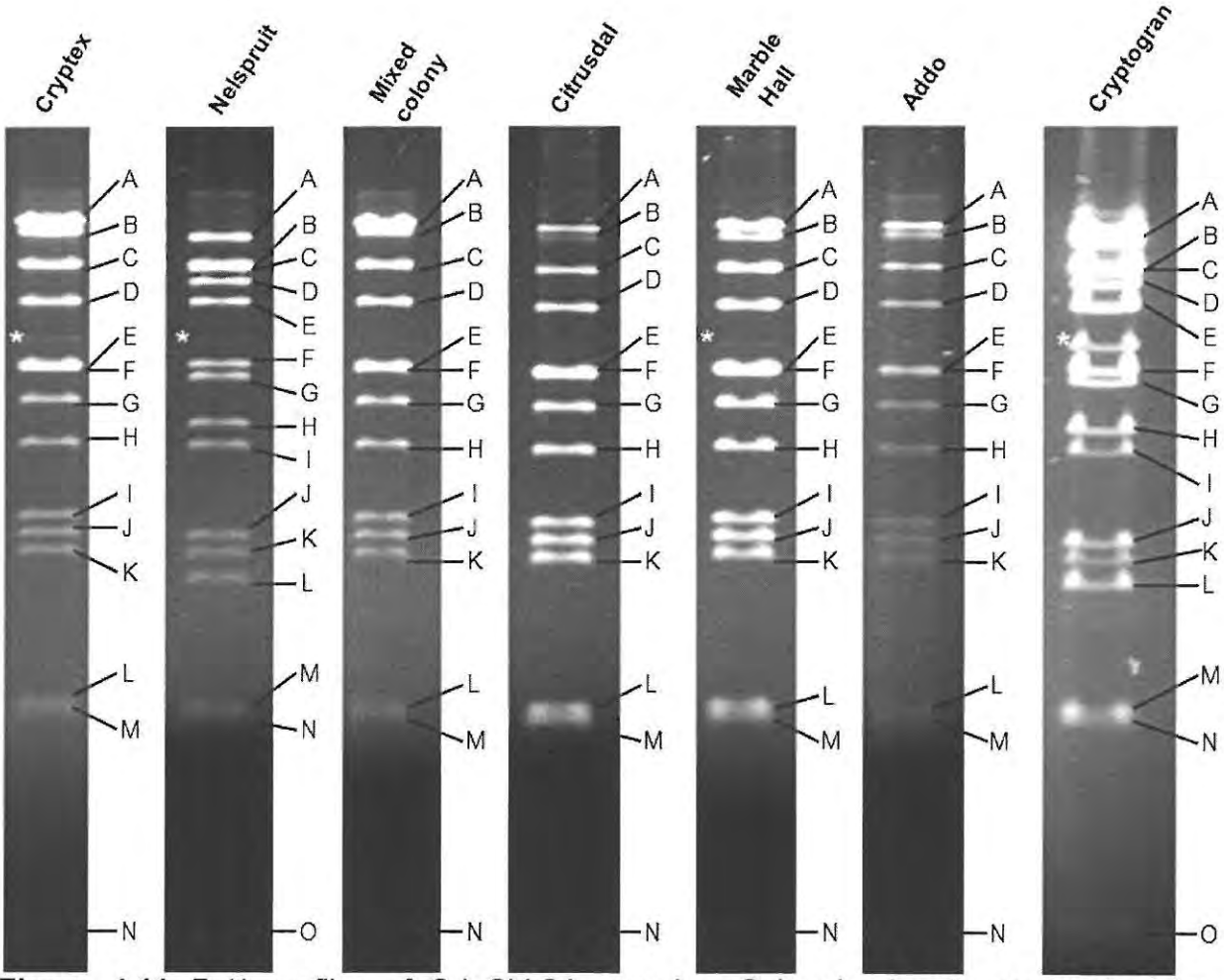

Figure 4.11 *Pst*1 profiles of CrleGV-SA samples. Submolar bands are marked by asterisks (*).

Table 4.8 DNA restriction fragment sizes (kb) of CrleGV-SA samples after restriction endonuclease digestion with *Pst*1

	Commercial isolate	New CrleGV-SA isolates					Commercial isolate
Fragment	Cryptex Isolate	Addo isolate	Mixed Colony isolate	Citrusdal isolate	Marble Hall isolate	Nelspruit Isolate	Cryptogran isolate
A	24.37	24.37	24.37	24.37	24.37	**21.92**	**21.92**
B	**21.92**	**21.92**	**21.92**	**21.92**	**21.92**	***13.98**	***13.98**
C	**13.98**	**13.98**	**13.98**	**13.98**	**13.98**	***13.98**	***13.98**
D	**11.45**	**11.45**	**11.45**	**11.45**	**11.45**	12.75	12.75
E	**7.44**	**7.44**	**7.44**	**7.44**	**7.44**	**11.45**	**11.45**
F	7.32	7.32	7.32	7.32	7.32	**7.44**	**7.44**
G	6.11	6.11	6.11	6.11	6.11	7.10	7.10
H	5.18	5.18	5.18	5.18	5.18	5.62	5.62
I	3.65	3.65	3.65	3.65	3.65	4.91	4.91
J	**3.48**	**3.48**	**3.48**	**3.48**	**3.48**	**3.48**	**3.48**
K	**3.24**	**3.24**	**3.24**	**3.24**	**3.24**	**3.24**	**3.24**
L	**1.77**	**1.77**	**1.77**	**1.77**	**1.77**	2.70	2.70
M	**1.73**	**1.73**	**1.73**	**1.73**	**1.73**	**1.77**	**1.77**
N	**0.52**	**0.52**	**0.52**	**0.52**	**0.52**	**1.73**	**1.73**
O						**0.52**	**0.52**
Total	112.15	112.15	112.15	112.15	112.15	112.57	112.57

Fragments are labelled in order of decreasing size, "A" being the largest. Fragment sizes appearing in all samples are shown in bold. Asterisks (*) indicates fragment repeats.

4.2.6 CrleGV-SA genomic DNA digested with the restriction enzyme *Xho*1

*Xho*1 profiles for Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates generated 6 prominent bands (Fig. 12) which are indicated as fragments (A – F) (Table 4.9 & Fig. 4.13). Both Cryptogran and the Nelspruit colony isolate generated 7 prominent bands (Fig. 12) which are indicated as fragments (A – G) (Table 4.9, Fig. 4.13). It was noted that, fragment F which were shared by Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates was located as double fragments F and G, in Cryptogran and the Nelspruit colony isolate. However, fragments D and E were both located in the same position as double fragments for all samples.

*Xho*1 profiles (Fig. 4.12 & 4.13) and fragments (Table 4.9, Fig. 4.13) generated two distinct CrleGV-SA genome types. The genome size of both the Group one (Cryptex, and the Addo, Citrusdal, Marble Hall and Mixed colony isolates) and two (Cryptogran and the Nelspruit colony isolate) CrleGV-SA were estimated to be 112.32 and 112.36 kbp respectively. No submolar band was observed in any sample (Fig. 4.12 & 4.13).

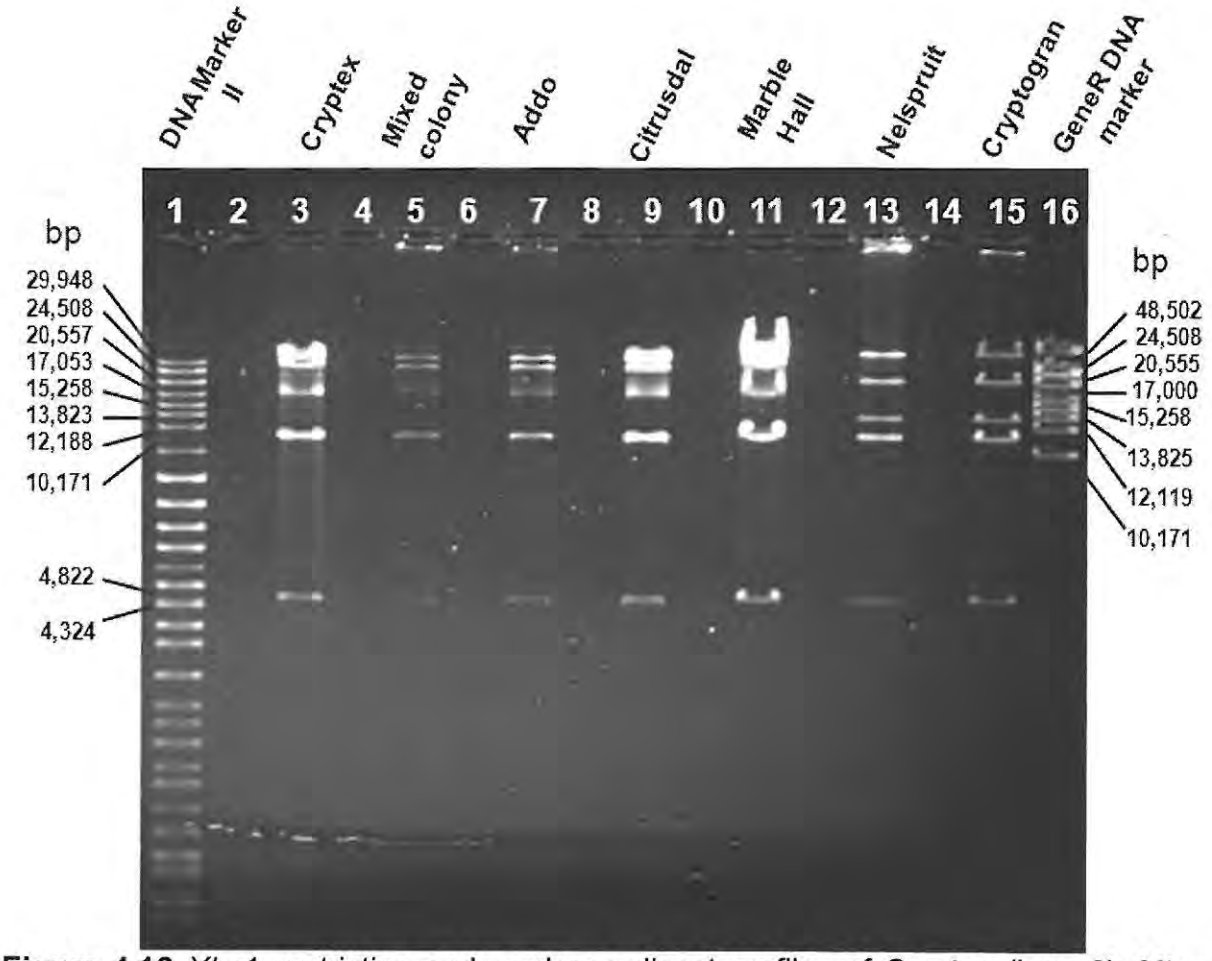

Figure 4.12 *Xho*1 restriction endonuclease digest profiles of Cryptex (lane 3), Mixed colony isolate (lane 5), Addo colony isolate (lane 7), Citrusdal colony isolate (lane 9), Marble Hall colony isolate (lane 11), Nelspruit colony isolate (lane 13) and Cryptogran (lane 15) analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: DNA marker II (lane 1) and GeneR DNA marker (lane 16) were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

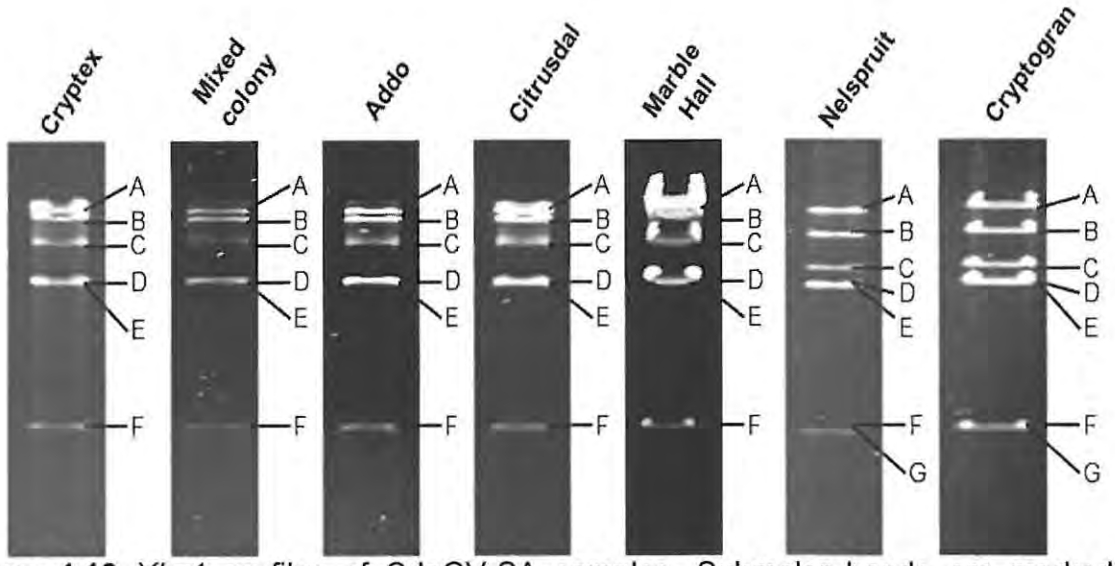

Figure 4.13 *Xho*1 profiles of CrleGV-SA samples. Submolar bands are marked by asterisks (*).

Table 4.9 DNA restriction fragment sizes (kb) of CrleGV-SA samples after restriction endonuclease digestion with *Xho*1

	Commercial isolate	New CrleGV-SA isolates					Commercial isolate
Fragment	Cryptex Isolate	Addo isolate	Mixed Colony isolate	Citrusdal isolate	Marble Hall isolate	Nelspruit isolate	Cryptogran isolate
A	40.22	40.22	40.22	40.22	40.22	45.11	45.11
B	25.11	25.11	25.11	25.11	25.11	19.48	19.48
C	18.64	18.64	18.64	18.64	18.64	14.77	14.77
D	***11.86**	***11.86**	***11.86**	***11.86**	***11.86**	***11.86**	***11.86**
E	***11.86**	***11.86**	***11.86**	***11.86**	***11.86**	***11.86**	***11.86**
F	**4.64**	**4.64**	**4.64**	**4.64**	**4.64**	***4.64**	***4.64**
G						***4.64**	***4.64**
Total	112.32	112.32	112.32	112.32	112.32	112.36	112.36

Fragments are labelled in order of decreasing size, "A" being the largest. Fragment sizes appearing in all samples are shown in bold. Asterisks (*) indicates fragment repeats.

4.2.7 CrleGV-SA genomic DNA digested with the restriction enzyme *Kpn*I

*Kpn*I profiles for Cryptex and the Addo, Mixed, Citrusdal and Marble Hall colony isolates generated 12 prominent bands (Fig. 4.14 & 4.15) which are indicated as fragments (A – L) (Table 4.10 & Fig. 4.15). Cryptogran and Nelspruit colony isolates generated 13 prominent bands (Fig. 4.14 & 4.15) which are indicated as fragments (A – M) (Table 4.10, Fig. 15). It was noted that, fragments C, E, H, I J, K and L which were shared by Cryptex and the Addo, Mixed, Citrusdal and Marble Hall colony isolates were located as fragments B, F, I, J, K, L and M in Cryptogran and the Nelspruit colony isolate. However, fragment A and G were located in the same position for all samples.

*Kpn*I profiles (Fig. 4.14 & 4.15) and fragments (Table 4.10, Fig. 4.15) generated two distinct CrleGV-SA genome types. The genome size of both the Group one (Cryptex, and the Addo, Citrusdal, Marble Hall and Mixed colony isolates) and two (Cryptogran and the Nelspruit colony isolate) CrleGV-SA were estimated to be 112.21 and 112.30 kbp respectively.

Several unique submolar bands were observed in Cryptogran, Cryptex and the Addo colony isolate, with each band being dissimilar in terms of banding position and number of fragments generated (Fig. 4.14 & 4.15). The submolar bands were located between fragments D and F for Cryptex and the Addo colony isolate and between fragments F and J for the Citrusdal colony isolate (Fig. 4.14 & Fig. 15). This indicates genetic differences between these three samples, with each sample consisting of more than one CrleGV-SA genotype.

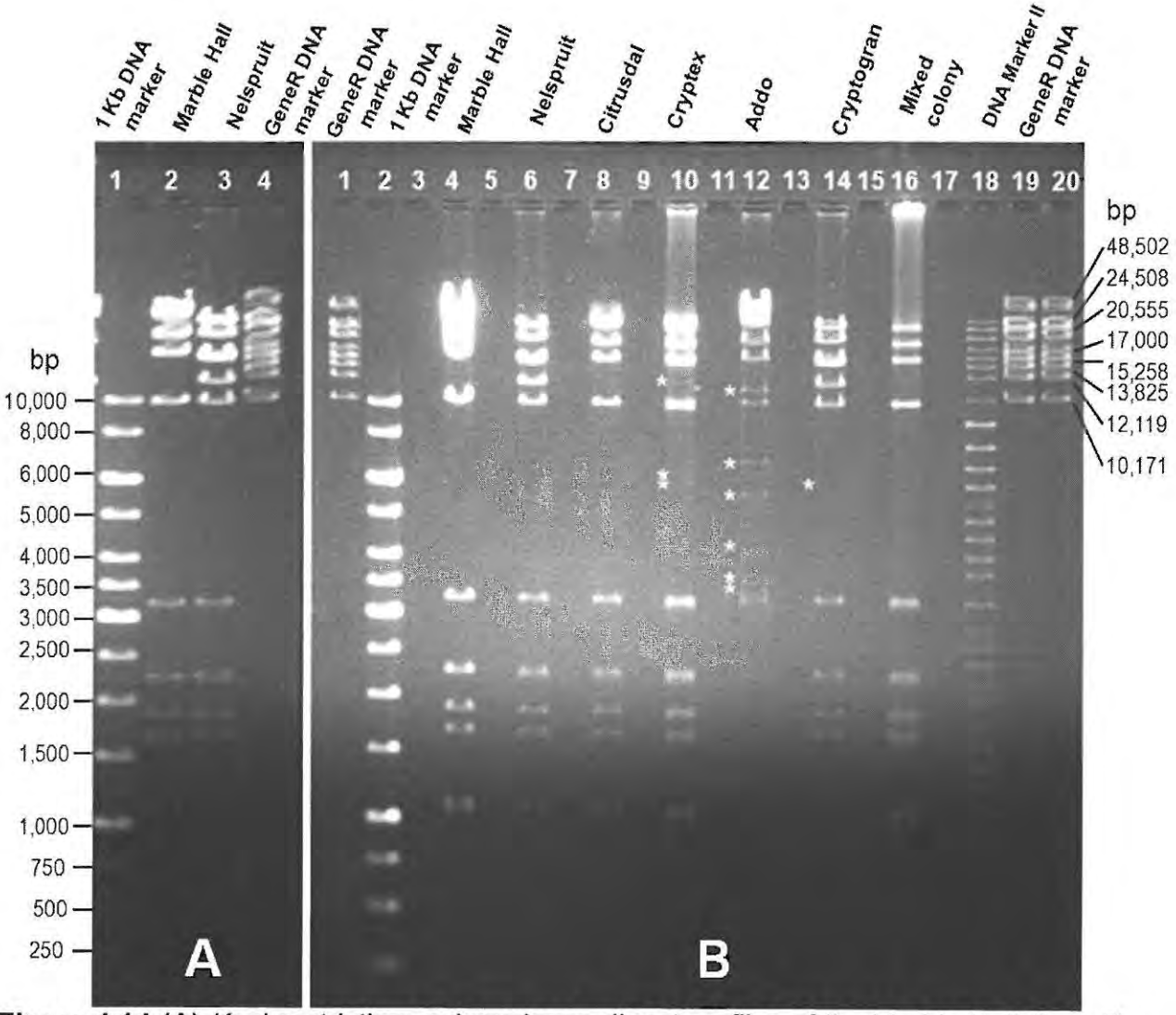

Figure 4.14 (A) *Kpn*I restriction endonuclease digest profiles of the Marble Hall (lane 2) and Nelspruit colony isolate (lane 3). **(B)** *Kpn*I profiles of Marble Hall colony isolate (lane 4), Nelspruit colony isolate (lane 6), Citrusdal colony isolate (lane 8), Cryptex (lane 10), Addo colony isolate (lane 12), Cryptogran (lane 14), and the Mixed colony isolate (lane 16). Samples analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: 1 Kb DNA marker (lane 1), GeneR marker (lane 4, 19 & 20) and GeneR DNA marker (lanes 18) were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

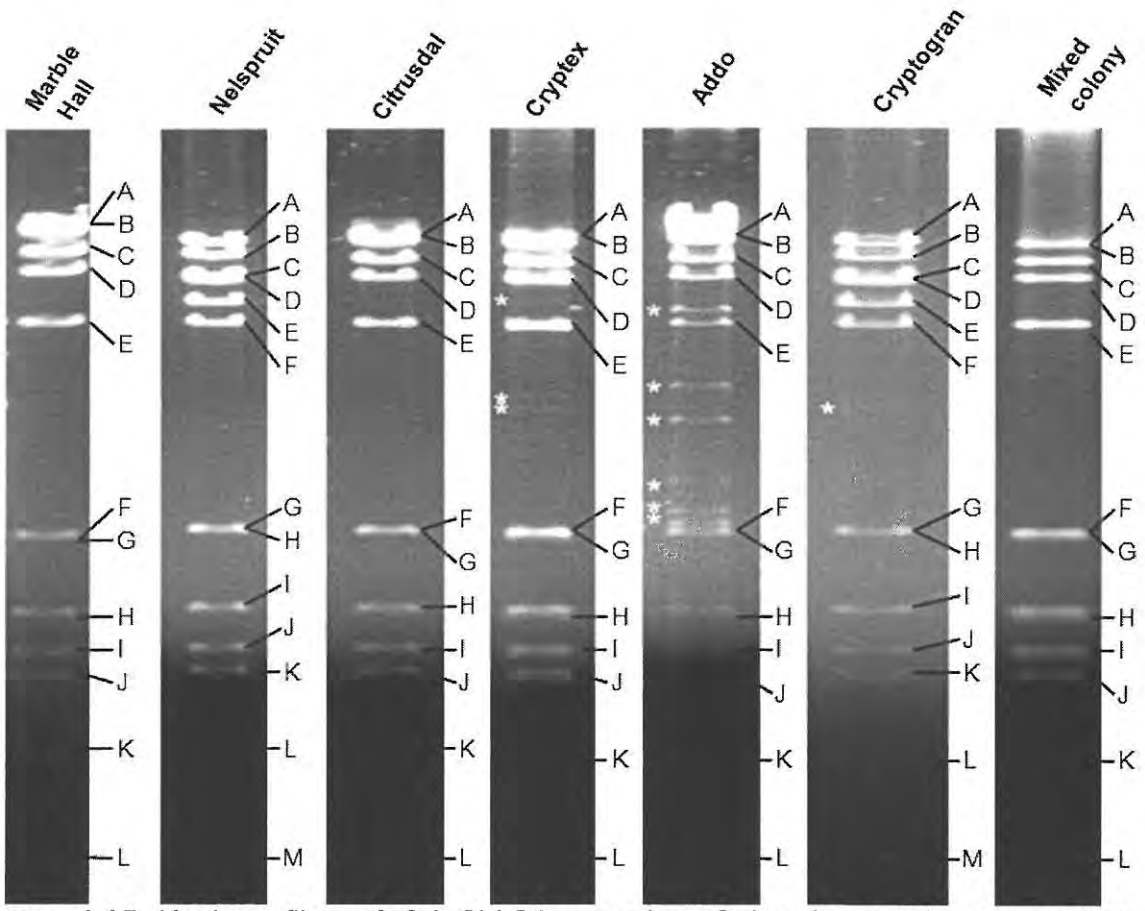

Figure 4.15 *Kpn*I profiles of CrleGV-SA samples. Submolar bands are marked by asterisks (*).

Table 4.10 DNA restriction fragment sizes (kb) of CrleGV-SA samples after restriction endonuclease digestion with *Kpn*I

	Commercial isolate	New CrleGV-SA isolates					Commercial isolate
Fragment	Cryptex Isolate	Addo isolate	Mixed Colony isolate	Citrusdal isolate	Marble Hall isolate	Nelspruit isolate	Cryptogran isolate
A	***25.74**	***25.74**	***25.74**	***25.74**	***25.74**	***25.74**	**25.74**
B	***25.74**	***25.74**	***25.74**	***25.74**	***25.74**	***20.64**	**20.64**
C	**20.64**	**20.64**	**20.64**	**20.64**	**20.64**	*15.25	*15.25
D	16.18	16.18	16.18	16.18	16.18	*15.25	*15.25
E	**10.17**	**10.17**	**10.17**	**10.17**	**10.17**	11.51	11.51
F	***3.27**	***3.27**	***3.27**	***3.27**	***3.27**	**10.17**	**10.17**
G	***3.27**	***3.27**	***3.27**	***3.27**	***3.27**	***3.27**	***3.27**
H	**2.21**	**2.21**	**2.21**	**2.21**	**2.21**	***3.27**	***3.27**
I	**1.72**	**1.72**	**1.72**	**1.72**	**1.72**	**2.21**	**2.21**
J	**1.57**	**1.57**	**1.57**	**1.57**	**1.57**	**1.72**	**1.72**
K	**1.10**	**1.10**	**1.10**	**1.10**	**1.10**	**1.57**	**1.57**
L	**0.61**	**0.61**	**0.61**	**0.61**	**0.61**	**1.10**	**1.10**
M						**0.61**	**0.61**
Total	112.21	112.21	112.21	112.21	112.21	112.30	112.30

Fragments are labelled in order of decreasing size, "A" being the largest. Fragment sizes appearing in all samples are shown in bold. Asterisks (*) indicates fragment repeats.

4.2.8 CrleGV-SA genomic DNA digested with the restriction enzyme *Hind*III

*Hind*III profiles for Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates generated 21 prominent bands (Fig. 4.16, 4.17 & 4.18) which are indicated as fragments (A – U) (Table 4.11 & Fig. 4.18). Cryptogran and the Nelspruit colony isolate generated 20 prominent bands (Fig. 4.16, 4.17 & 4.18) which are indicated as fragments (A – T) (Table 4.11 & Fig. 4.18). It was noted that, fragments B, H, J, K, L, M, N, O, P, S, T and U which were shared by Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates was located as fragments C, G, I, J, K, L, M, N, O, R, S and T in Cryptogran and the Nelspruit colony isolate. Again double fragment Q and R which was shared by the Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates was located as fragments P and Q in Cryptogran and the Nelspruit colony isolate.

*Hind*III profiles (Fig. 4.16, 4.17 & 4.18) and fragments (Table 4.11 & Fig. 4.18) generated two distinct CrleGV-SA genome types. The genome size of both the Group one (Cryptex, and the Addo, Citrusdal, Marble Hall and Mixed colony isolates) and two (Cryptogran and the Nelspruit colony isolate) CrleGV-SA were estimated to be 112.23 and 112.07 kbp respectively.

Several unique submolar bands were observed in Cryptex, Cryptogran and the Nelspruit and Citrusdal colony isolates. Their submolar bands were dissimilar in terms of their banding position and number of fragments generated (Fig. 4.16, 4.17 & 4.18). The submolar bands were located between fragments A and J for Cryptex and the Citrusdal colony isolate and between fragments A and N for Cryptogran and the Nelspruit colony isolate. This shows genetic differences between these four samples, with each sample consisting of more than one CrleGV-SA genotype.

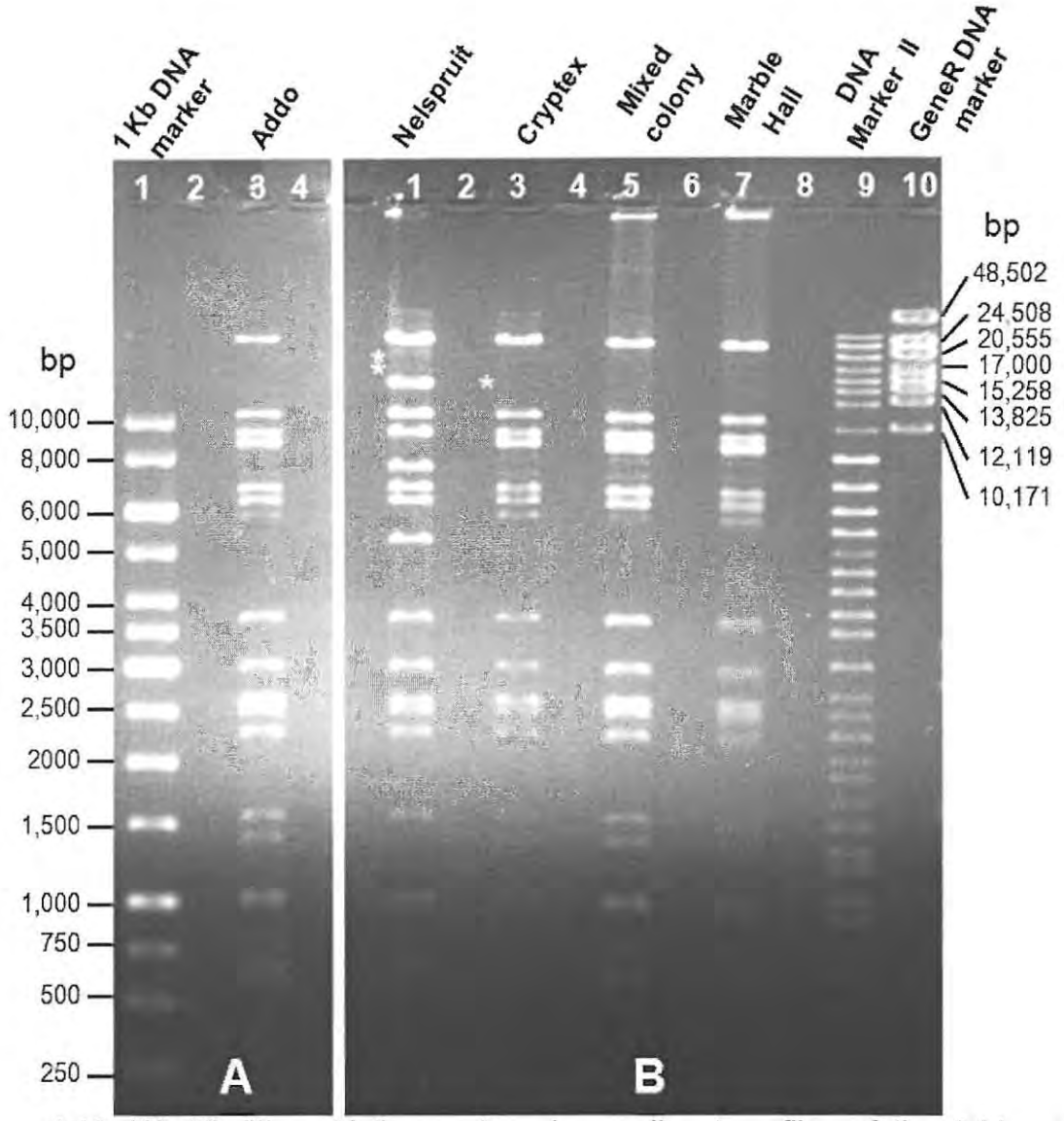

Figure 4.16 (A) *Hind*III restriction endonuclease digest profiles of the Addo colony isolate (lane 3). **(B)** *Hind*III profiles of the Nelspruit colony isolates (lane 1), Cryptex (lane 3), Mixed colony isolate (lane 5) and Marble Hall colony isolate (lane 7). Samples analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: 1 Kb DNA marker (lane 1), DNA marker II (lane 9) and GeneR DNA marker (lane 10) were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

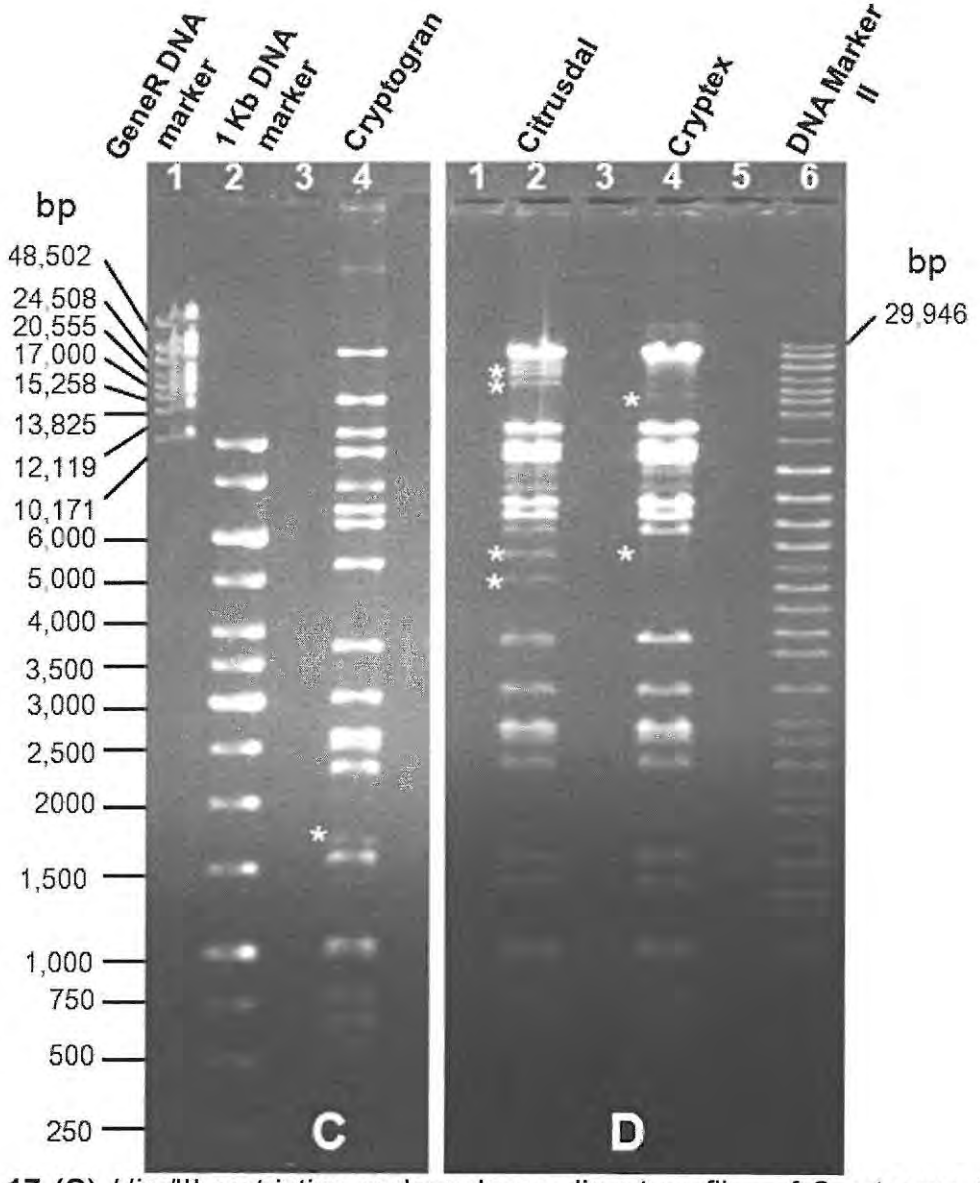

Figure 4.17 (C) *Hind*III restriction endonuclease digest profiles of Cryptogran (lane 4). **(D)** *Hind*III profiles of the Citrusdal colony isolate (lane 2) and Cryptex (lane 4). Samples were analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: GeneR DNA marker (lane 1), 1 Kb DNA marker (lane 2) and DNA marker II (lane 6) were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

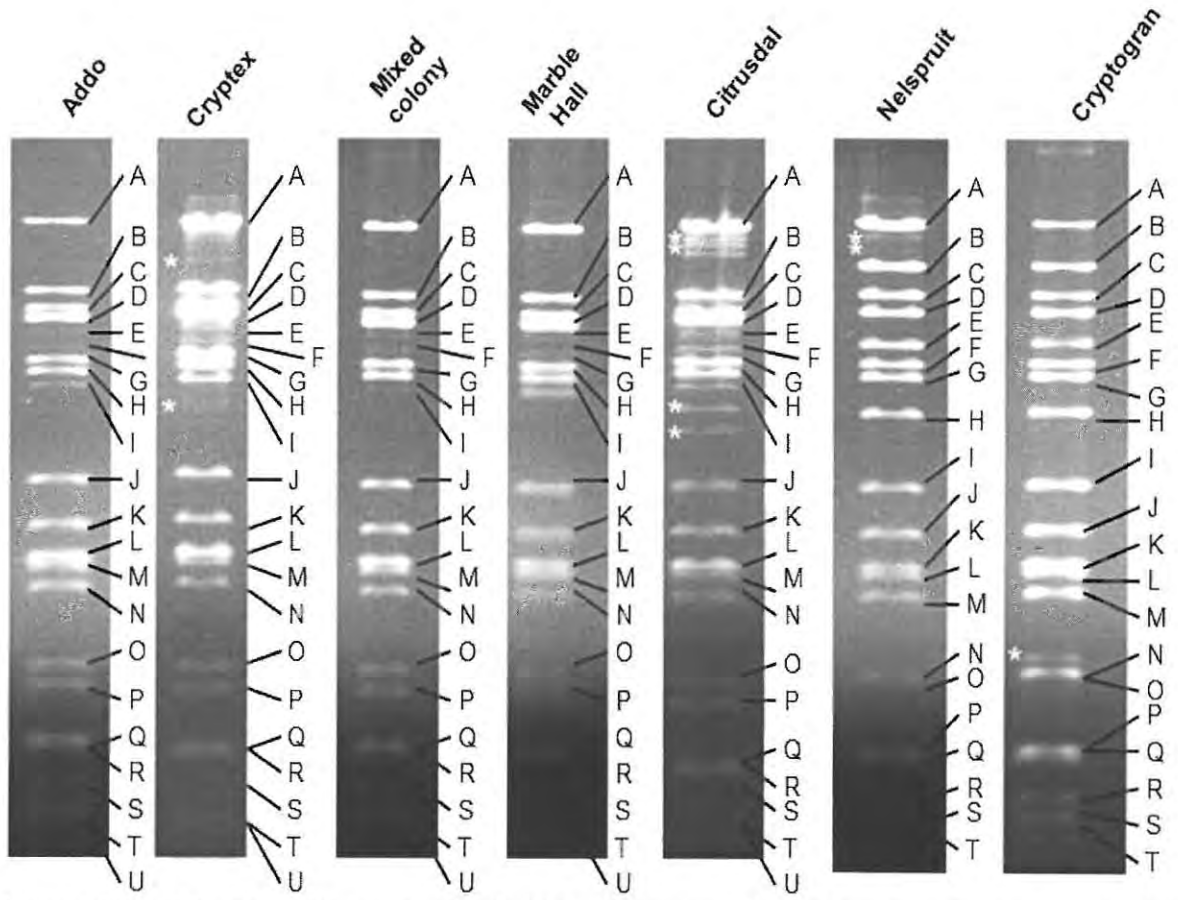

Figure 4.18 *Hind*III profiles of CrleGV-SA samples. Submolar bands are marked by asterisks (*).

Table 4.11 DNA restriction fragment sizes (kb) of CrleGV-SA samples after restriction endonuclease digestion with *Hind*III

	Commercial isolate	**New CrleGV-SA isolates**					**Commercial isolate**
Fragment	**Cryptex Isolate**	**Addo isolate**	**Mixed Colony isolate**	**Citrusdal isolate**	**Marble Hall isolate**	**Nelspruit isolate**	**Cryptogran isolate**
A	26.25	26.25	26.25	26.25	26.25	27.90	27.90
B	**11.05**	**11.05**	**11.05**	**11.05**	**11.05**	14.69	14.69
C	9.58	9.58	9.58	9.58	9.58	**11.05**	**11.05**
D	9.04	9.04	9.04	9.04	9.04	9.77	9.77
E	8.11	8.11	8.11	8.11	8.11	7.83	7.83
F	7.57	7.57	7.57	7.57	7.57	7.32	7.32
G	6.86	6.86	6.86	6.86	6.86	**6.25**	**6.25**
H	**6.25**	**6.25**	**6.25**	**6.25**	**6.25**	5.72	5.72
I	5.98	5.98	5.98	5.98	5.98	**3.75**	**3.75**
J	**3.75**	**3.75**	**3.75**	**3.75**	**3.75**	**3.09**	**3.09**
K	**3.09**	**3.09**	**3.09**	**3.09**	**3.09**	**2.63**	**2.63**
L	**2.63**	**2.63**	**2.63**	**2.63**	**2.63**	**2.52**	**2.52**
M	**2.52**	**2.52**	**2.52**	**2.52**	**2.52**	**2.30**	**2.30**
N	**2.30**	**2.30**	**2.30**	**2.30**	**2.30**	**1.54**	**1.54**
O	**1.54**	**1.54**	**1.54**	**1.54**	**1.54**	**1.41**	**1.41**
P	**1.41**	**1.41**	**1.41**	**1.41**	**1.41**	***1.11**	***1.11**
Q	***1.11**	***1.11**	***1.11**	***1.11**	***1.11**	***1.11**	***1.11**
R	***1.11**	***1.11**	***1.11**	***1.11**	***1.11**	**0.82**	**0.82**
S	**0.82**	**0.82**	**0.82**	**0.82**	**0.82**	**0.67**	**0.67**
T	**0.67**	**0.67**	**0.67**	**0.67**	**0.67**	**0.58**	**0.58**
U	**0.58**	**0.58**	**0.58**	**0.58**	**0.58**		
Total	112.23	112.23	112.23	112.23	112.23	112.07	112.07

Fragments are labelled in order of decreasing size, "A" being the largest. Fragment sizes appearing in all samples are shown in bold. Asterisks (*) indicates fragment repeats.

4.2.9 CrleGV-SA genomic DNA digested with the restriction enzyme *Eco*R1

*Eco*RI profiles for Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates generated 22 prominent bands (Fig. 4.19, 4.20 & 4.21) which are indicated as fragments (A - V) (Table 4.12 & Fig. 4.21). Cryptogran and the Nelspruit colony isolate generated 25 prominent bands (Fig. 4.19, 4.20 & 4.21) which are indicated as fragments (A - Y) (Table 4.12 & Fig. 4.21). It was noted that, fragments L, M, N, O, P, Q,

R, S, T, U and V which were shared by Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates were located as fragments N, O, P, Q, R, S, T, V, W, X and Z in Cryptogran and the Nelspruit colony isolate. Again, double fragments C, D, E, F, G, H, I and J which were shared by Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates were located as double fragments B, C, D, E, F, G, H and I in Cryptogran and the Nelspruit colony isolate.

*Eco*RI profiles (Fig. 4.19, 4.20 & 4.21) and fragments (Table 4.12 & Fig. 4.12) generated two distinct CrleGV-SA genome types. The genome size of both the Group one (Cryptex, and the Addo, Citrusdal, Marble Hall and Mixed colony isolates) and two (Cryptogran and the Nelspruit colony isolate) CrleGV-SA were estimated to be 112.43 and 112.15 kbp respectively.

A few submolar REN bands were observed in Cryptex and the Citrusdal, Marble Hall, Addo and Mixed colony isolates. However, with the exception of Cryptex, the submolar bands generated for the other four isolates were similar in terms of their banding position and number of fragments generated (Fig. 4.19, 4.20 & 4.21). These submolar bands were located between fragments J and K for Cryptex and between fragments B and K for the other four isolates (Fig. 4.21). This indicates that these five samples each consist of multiple genotypes of CrleGV-SA.

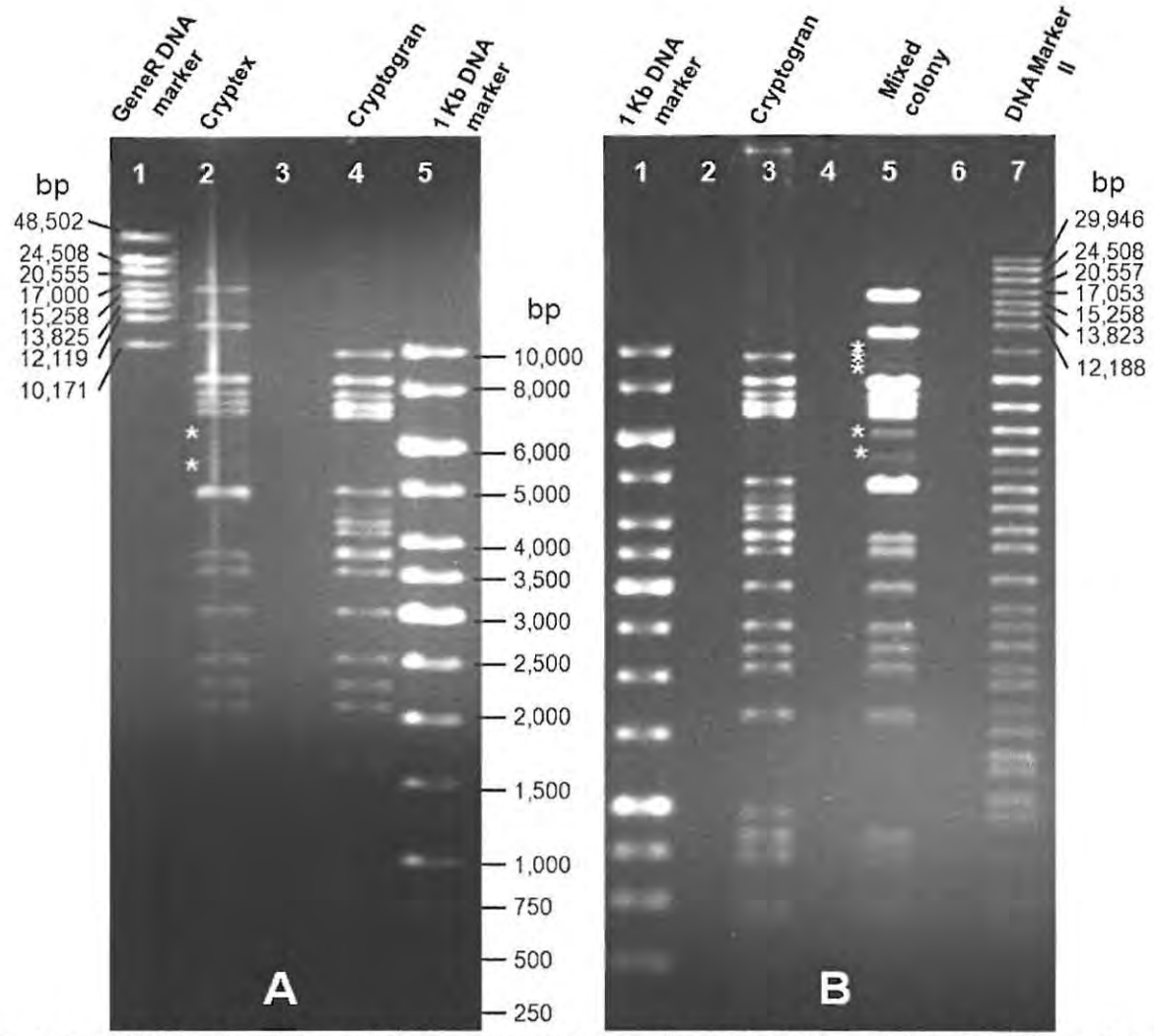

Figure 4.19 (A) *Eco*R1 restriction endonuclease digests profiles of Cryptex (lane 2) and Cryptogran (lane 4). **(B)** *Eco*R1profiles of Cryptogran (lane 3) and the Mixed colony isolate (lane 5). Samples were analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: GeneR DNA marker (lane 1), 1 Kb DNA marker (lane 5 & 1) and DNA marker II (lane 7) were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

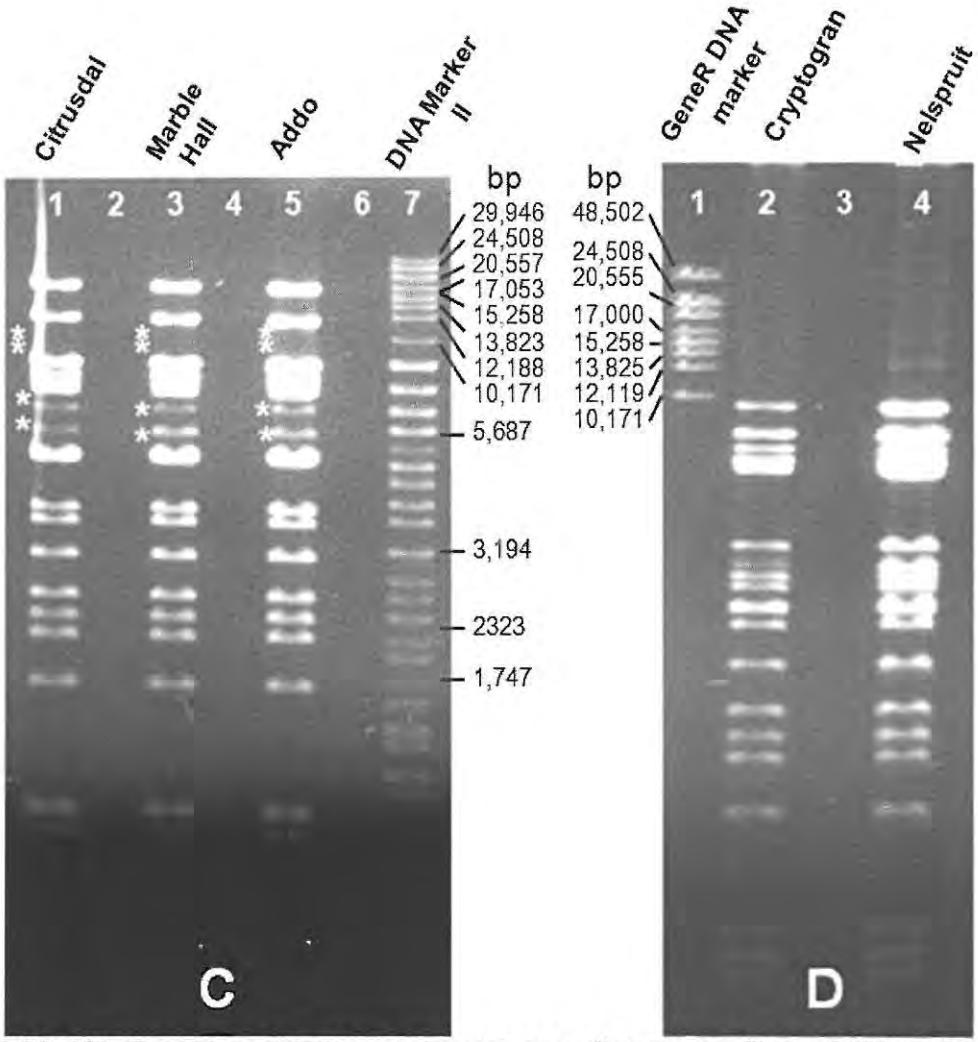

Figure 4.20 (C) *Eco*R1 restriction endonuclease digests profiles of Citrusdal (lane 1), Marble Hall (lane 3) and Addo (lane 5) colony isolates. **(D)** *Eco*R1profiles of Cryptogran (lane 2) and the Nelspruit colony isolate (lane 4). Samples were analysed by 0.6% AGE at 30 V for 16 hours. DNA markers: DNA marker II (lane 7) and GeneR DNA marker (lane 1) were run along the outside lanes of gels. Asterisks (*) indicate submolar bands.

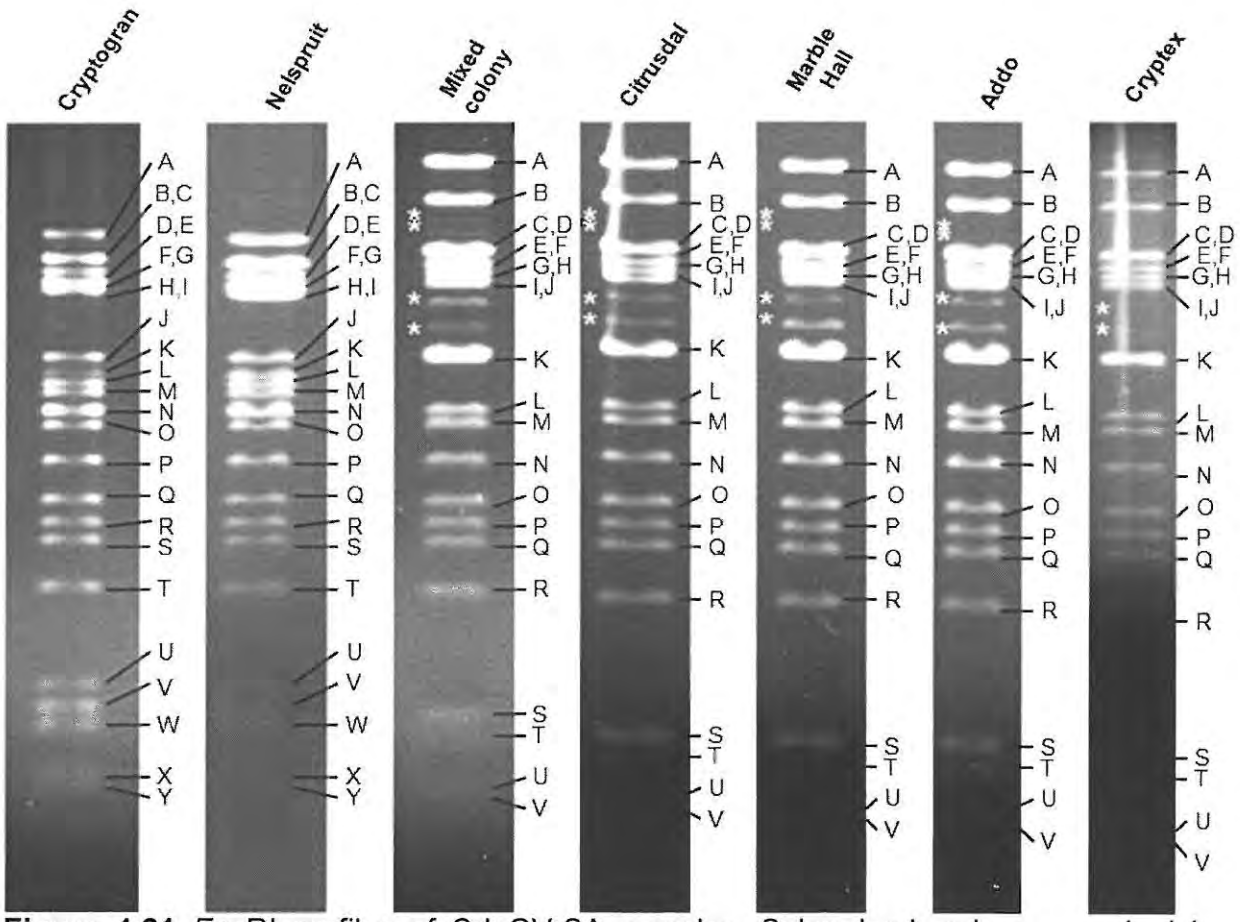

Figure 4.21 *Eco*RI profiles of CrleGV-SA samples. Submolar bands are marked by asterisks (*).

Table 4.12 DNA restriction fragment sizes (kb) of CrleGV-SA samples after restriction endonuclease digestion with *Eco*RI

	Commercial isolate	**New CrleGV-SA isolates**					**Commercial isolate**
Fragment	**Cryptex Isolate**	**Addo isolate**	**Mixed Colony isolate**	**Citrusdal isolate**	**Marble Hall isolate**	**Nelspruit isolate**	**Cryptogran isolate**
A	14.33	14.33	14.33	14.33	14.33	**10.7**	**10.7**
B	**10.97**	**10.97**	**10.97**	**10.97**	**10.97**	***8.53**	***8.53**
C	***8.53**	***8.53**	***8.53**	***8.53**	**8.53**	***8.53**	***8.53**
D	***8.53**	***8.53**	***8.53**	***8.53**	**8.53**	***7.75**	***7.75**
E	***7.75**	***7.75**	***7.75**	***7.75**	***7.75**	***7.75**	***7.75**
F	***7.75**	***7.75**	***7.75**	***7.75**	***7.75**	***7.07**	***7.07**
G	***7.07**	***7.07**	***7.07**	***7.07**	***7.07**	***7.07**	***7.07**
H	***7.07**	***7.07**	***7.07**	***7.07**	***7.07**	***6.87**	***6.87**
I	***6.87**	***6.87**	***6.87**	***6.87**	***6.87**	***6.87**	***6.87**
J	***6.87**	***6.87**	***6.87**	***6.87**	***6.87**	4.97	4.97
K	4.79	4.79	4.79	4.79	4.79	4.62	4.62
L	**3.74**	**3.74**	**3.74**	**3.74**	**3.74**	4.40	4.40
M	**3.65**	**3.65**	**3.65**	**3.65**	**3.65**	4.19	4.19
N	**3.13**	**3.13**	**3.13**	**3.13**	**3.13**	**3.74**	**3.74**
O	**2.53**	**2.53**	**2.53**	**2.53**	**2.53**	**3.65**	**3.65**
P	**2.38**	**2.38**	**2.38**	**2.38**	**2.38**	**3.13**	**3.13**
Q	**2.19**	**2.19**	**2.19**	**2.19**	**2.19**	**2.53**	**2.53**
R	**1.76**	**1.76**	**1.76**	**1.76**	**1.76**	**2.38**	**2.38**
S	**0.87**	**0.87**	**0.87**	**0.87**	**0.87**	**2.19**	**2.19**
T	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**	**1.76**	**1.76**
U	**0.46**	**0.46**	**0.46**	**0.46**	**0.46**	0.93	0.93
V	**0.44**	**0.44**	**0.44**	**0.44**	**0.44**	**0.87**	**0.87**
W						**0.75**	**0.75**
X						**0.46**	**0.46**
Y						**0.44**	**0.44**
Total	112.43	112.43	112.43	112.43	112.43	112.15	112.15

Fragments are labelled in order of decreasing size, "A" being the largest. Fragment sizes appearing in all samples are shown in bold. Asterisks (*) indicates fragment repeats.

4.2.10 Grouping of CrleGV-SA samples based on their RE and fragment profiles

Based on the similarities in the REN profiles of all seven CrleGV-SA samples digested with *Bam*H1, *Sal*1, *Xba*1, *Pst*1, *Xho*1, *Kpn*1, *Hind*III and *Eco*R1, the samples were placed into two distinct CrleGV-SA genome groups. Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates were placed as members of the Group one CrleGV-SA, while Cryptogran and the Nelspruit colony isolate were placed into Group two CrleGV-SA. The terms Group one and two CrleGV-SA would therefore be used through out.

4.2.11 Comparative analysis of the Group one and two CrleGV-SA samples against other documented CrleGV isolates

Profiles generated for both the Group one and two CrleGV-SA for *Bam*H1, *Xho*1, *Kpn*I and *Eco*RI were compared to those obtained for CrleGV-SA isolate reported by Singh *et al.* (2003). REN profiles of the Groups one and two CrleGV-SA were compared to those of other CrleGV-SA, CrleGV-CV and CrleGV-IC isolates reported by Fritsch (1989). Further comparisons were made with the RE profiles of a CrleGV-CV3 (a genotype of the CrleGV-CV) isolate reported by Jehle *et al.* (1992). For comparative purposes, DNA profiles of the CrleGV isolates reported by Fritsch (1989) and Jehle *et al.* (1992) are shown in figures 4.22 and 4.23 respectively.

Using *Eco*R1, *Hind*III and *Bam*H1, Fritsch (1989) previously demonstrated that CrleGV-SA, CrleGV-CV and CrleGV-IC isolates were genetically different (Fig. 4.22). It was therefore important that Fritsch's and Jehle's CrleGV isolates be compared against the Group one and two CrleGV-SA, in order to establish any differences or similarities between them.

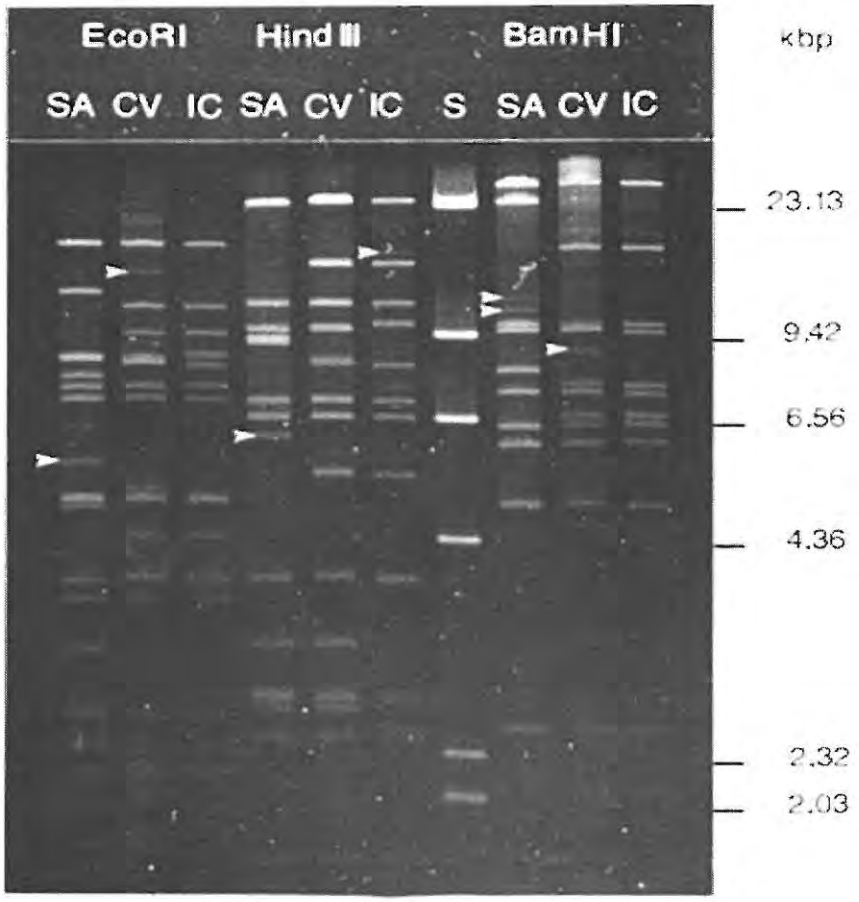

Figure 4.22 DNA profiles of a South African (SA), Cape Verde (CV) and Ivory Coast (IC) isolate of the CrleGV digested with the restriction enzymes *Eco*R1, *Hind*III and *Bam*H1. *Submolar REN bands are indicated by arrows (Source: Fritsch, 1989).

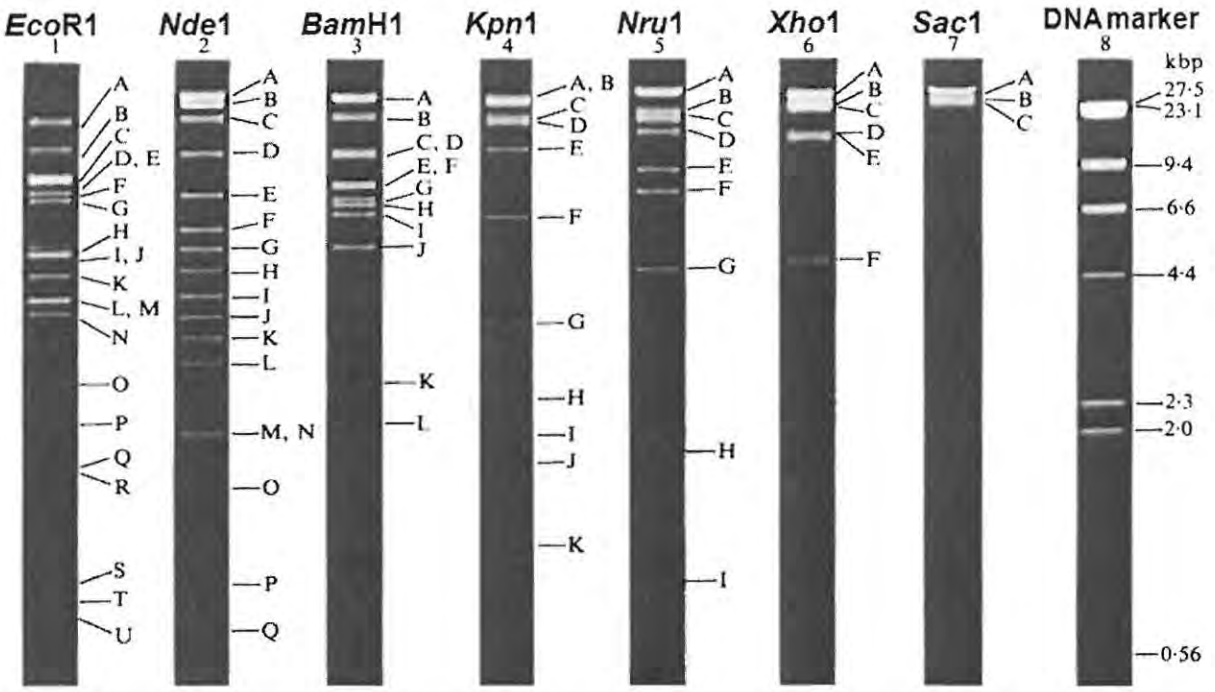

Figure 4.23 DNA profiles of a Cape Verde isolate, CrleGV-CV3 digested with the restriction enzymes *Eco*R1, *Nde*1, *Bam*H1, *Kpn*1, *Nru*1, *Xho*1 and *Sac*1. Fragments were separated on 0.7% agarose gels. A *Hind*III digest of lambda DNA was used as a ladder or DNA marker (Source: Jehle *et al.*, 1992).

***Bam*HI:** For *Bam*HI, Singh *et al.* (2003) reported 11 fragments for CrleGV-SA, in contrast to 9 fragments generated by the Group one and two CrleGV-SA in this study. Fragments A (32.04 kbp), B (24.95 kbp), C (11.55 kbp) and D (9.75 kbp) generated by the Group two isolates (Nelspruit isolate and Cryptogran) were similar to Singh's isolate. However, fragment B (24.95 kbp) was singly shared by only the Group one CrleGV-SA isolates (Cryptex, Addo, Mixed colony, Citrusdal and Marble Hall isolates) and Singh's isolate. This indicates that based on *Bam*HI fragments, the Group two isolates Cryptogran and the Nelspruit colony isolate, were more similar to Singh's South African isolate than the Group one CrleGV-SA. Jehle *et al.* (1992) reported 12 fragments for CrleGV-CV3, of which no fragments size similarities were observed between isolates.

By comparing the *Bam*H1 profiles of both Group one and two CrleGV-SA against CrleGV-SA, CrleGV-CV and CrleGV-IC isolates reported by Fritsch (1989) as well as the CrleGV-CV3 genotype reported by Jehle *et al.* (1992), the following was observed.

*Bam*H1 profiles from Fritsch's CrleGV-SA isolate were found to show closer similarities to Group one than the Group two CrleGV-SA. However, among the group one CrleGV-SA, the Addo colony isolate resembled Fritsch's CrleGV-SA isolate the most. *Bam*H1 profiles of Jehle's CrleGV-CV3 were dissimilar to both Group one and two CrleGV-SA. Their profile comparisons are indicated below in figure 4.24.

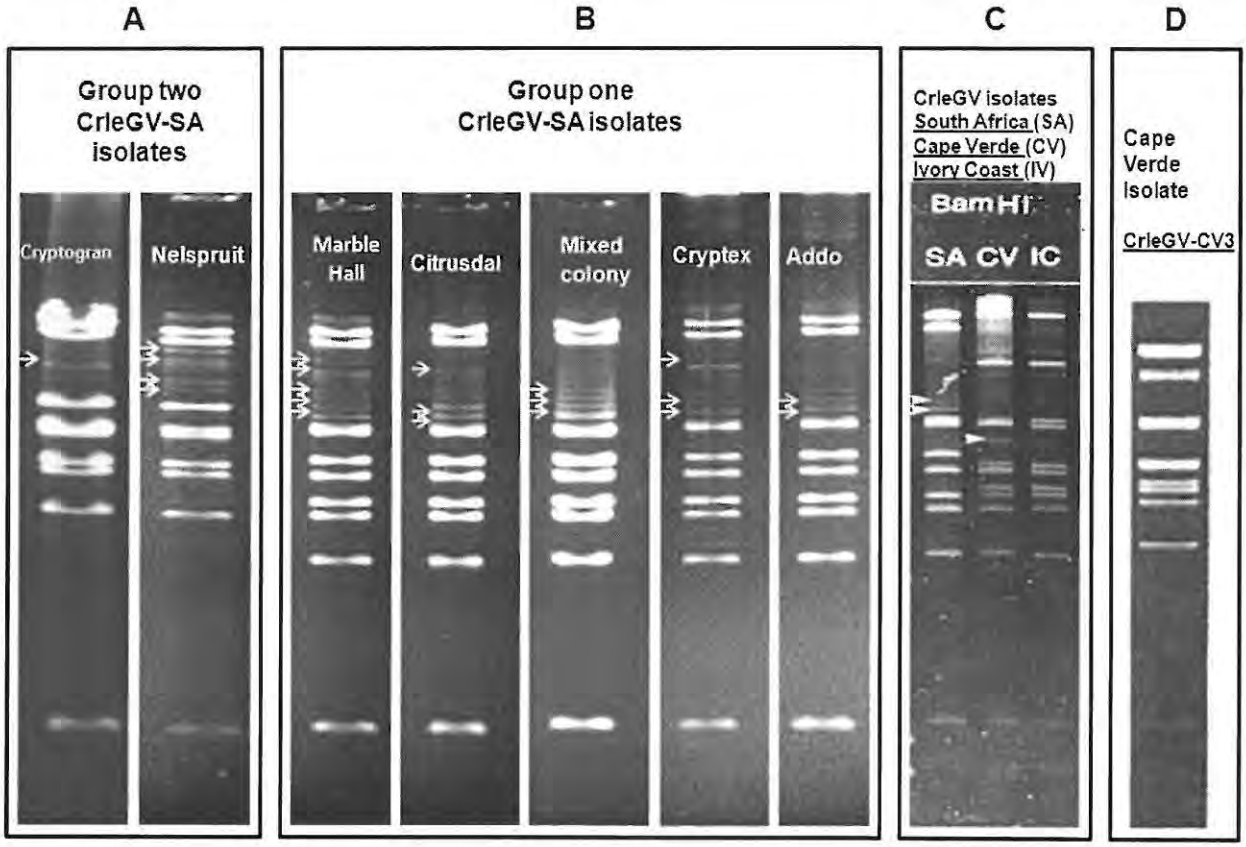

Figure 4.24 (A) *Bam*H1 profiles of Group two CrleGV-SA. **(B)** *Bam*H1 profiles of Group one CrleGV-SA. **(C)** *Bam*H1 profiles CrleGV-SA, CrleGV-CV and CrleGV-IC isolates reported by Fritsch (1989). **(D)** *Bam*H1 profile of a CrleGV-CV3 isolate reported by Jehle *et al.* (1992). *Submolar REN bands are indicated by arrows.

***Xho*1:** Using *Xho*1, Singh *et al.* (2003) reported 4 fragments for CrleGV-SA, in contrast to 6 and 7 fragments generated by the Group one and two CrleGV-SA in this study. Fragment F (4.64 kbp) of Singh's isolate was shared by both Group one and two CrleGV-SA. Jehle *et al.* (1992) also reported 4 fragments for CrleGV-CV3, and no profile similarities between it and the Group one and two CrleGV-SA was observed.

***Kpn*I:** Using *Kpn*I, Singh *et al.* (2003) reported 9 fragments for CrleGV-SA, in contrast to 12 and 13 fragments generated by both the Groups one and two CrleGV-SA respectively. Jehle *et al.* (1992) also reported 12 fragments for the CrleGV-CV3 isolate. Fragments C (16.20 kbp), F & E (10.0 kbp), G (3.30kbp) and K (1.10) reported by both Singh *et al.* (2003) and Jehle *et al.* (1992) was shared by both Group one and two CrleGV-SA. However, only fragment D (15.0 kbp) reported by both Singh *et al.* (2003) and Jehle *et al.* (1992) was shared by the Group two CrleGV-SA.

***Eco*RI:** Using *Eco*RI, Singh *et al.* (2003) reported 22 fragments for CrleGV-SA, in contrast to 22 and 25 fragments generated by both the Group one and two CrleGV-SA respectively. Fragments C (8.55 kbp) and D (7.76 kbp) of Singh's isolate was shared by the Group one and two CrleGV-SA. Jehle *et al.* (1992) reported 24 fragments for CrleGV-CV3 of which fragment C (8.50 kbp), F & G (7.60 kbp), U (0.75 kbp), W (0.46 kbp), X (0.44 kbp) were shared by both Group one and two CrleGV-SA. Again, *Eco*R1 profiles generated by Fritsch's CrleGV-SA isolate were observed to be more similar to the Group one than the Group two CrleGV-SA (Fig. 4.25).

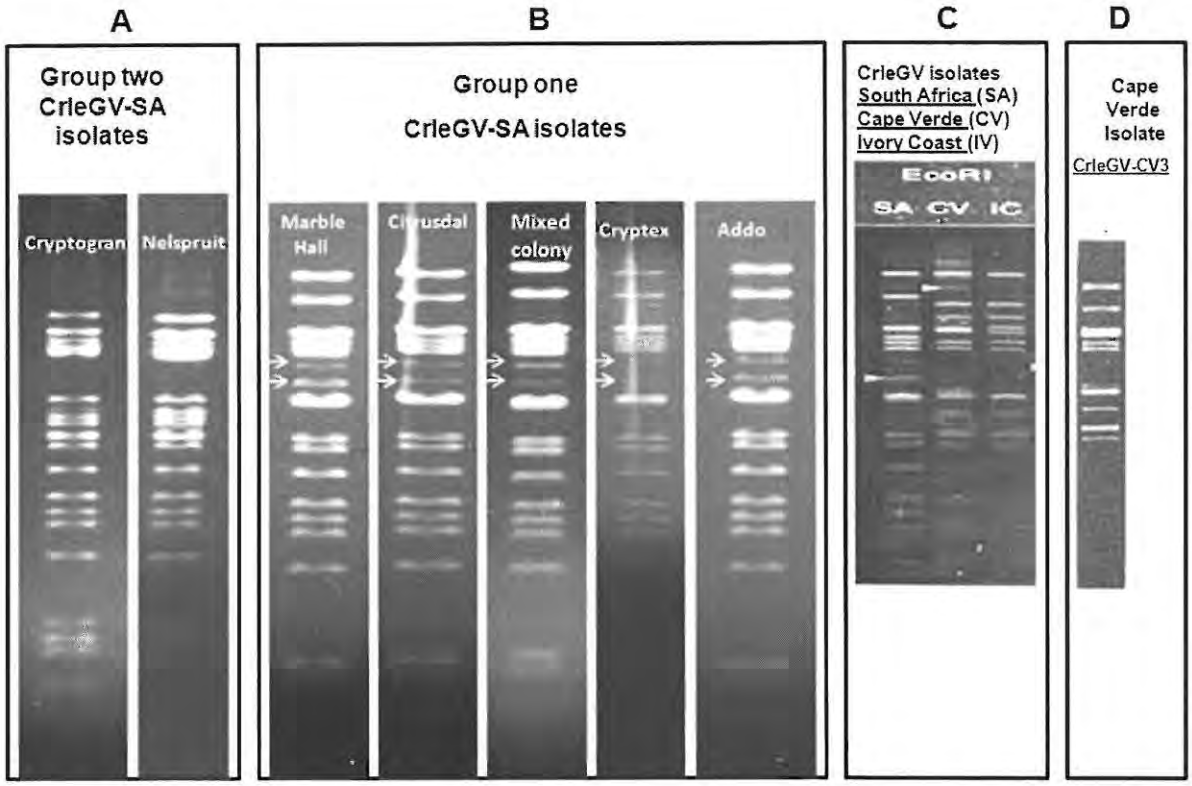

Figure 4.25 (A) *Eco*RI profiles of Group two CrleGV-SA. (B) *Eco*RI profiles of Group one CrleGV-SA. **(C)** *Eco*RI profiles of a CrleGV-SA, CrleGV-CV and CrleGV-IC isolate reported by Fritsch (1989). **(D)** *Eco*RI profile of a CrleGV-CV3 isolate reported by Jehle *et al.* (1992). *Submolar REN bands are indicated by arrows.

***Hind*III:** The *Hind*III profiles from Fritsch's CrleGV-SA isolate were more similar to the Group one than the Group two CrleGV-SA. On the other hand, the *Hind*III profiles from Jehle's CrleGV-CV3 isolate appeared to be more similar to the Group two than the Group one CrleGV-SA (Fig. 4.26).

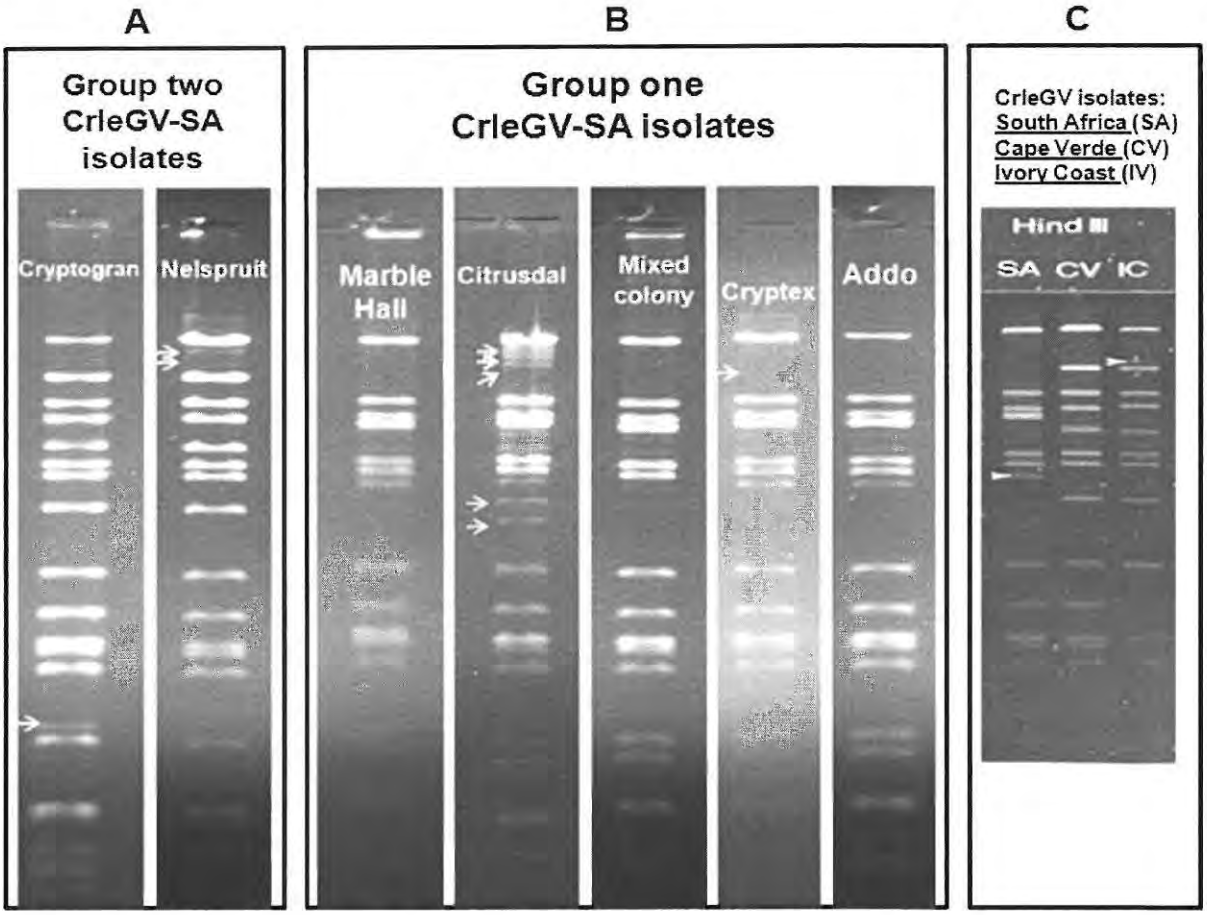

Figure 4.26 (A) *Hind*III profiles of Group two CrleGV-SA. **(B)** *Hind*III profiles of Group one CrleGV-SA. **(C)** *Hind*III profiles of a CrleGV-SA, CrleGV-CV and CrleGV-IC isolate reported by Fritsch (1989). *Submolar REN bands are indicated by arrows.

4.2.12 Restriction enzyme recognition sites in the nucleotide sequence of the *granulin* and *egt* genes of CrleGV-SA samples

Sequence data showed that *Eco*R1, *Bam*H1, *Kpn*1 and *Pst*1 recognition sites were present in the nucleotide sequence of the *granulin* gene of both the Group one and two CrleGV-SA (Table 4.13).

Table 4.13 Restriction enzyme recognition sites found in the *granulin* gene of both the Group one and two CrleGV-SA

Restriction enzyme	recognition sequence: forward (5'- 3')	Number of recognition sites found	Baculovirus isolates identified with this recognition sequence
*Eco*R1	GAATTC	1	group one and two CrleGV-SA
*Bam*H1	GGATCC	2	group one and two CrleGV-SA
*Hind*III	AAGCTT	0	NONE
*Kpn*1	GGTACC	0	NONE
*Pst*1	CTGCAG	2	group one CrleGV-SA
*Sal*1	GTCGAC	0	NONE
*Xba*1	TCTAGA	0	NONE
*Xho*1	CTCGAG	0	NONE

One *Xba*1 recognition site was found in the nucleotide sequence data of the *egt* gene of both the Group one and two CrleGV-SA (Table 4.14).

Table 4.14 Restriction enzyme recognition sites found in the *egt* gene of both the Group one and two CrleGV-SA

Restriction enzyme	Recognition sequence: forward & reverse prime (5'- 3' & 3' – 5')	Number of recognition sites found	CrleGV-SA samples identified with this recognition sequence
*Eco*R1	5'GAATTC 3' CTTAAG	0	NONE
*Bam*H1	5'GGATCC 3'CCTAGG	0	NONE
*Hind*III	5'AAGCTT 3'TTCGAA	0	NONE
*Kpn*1	5'GGTACC 3'CCATGG	0	NONE
*Pst*1	5'CTGCAG 3'GACGTC	0	NONE
*Sal*1	5'GTCGAC 3'CAGCTG	0	NONE
*Xba*1	5'TCTAGA 3'AGATCT	1	both group one and two CrleGV-SA
*Xho*1	5'CTCGAG 3'GAGCTC	0	NONE

4.2.13 Amplification of the *granulin* and *egt* genes from CrleGV-SA samples using polymerase chain reaction (PCR)

The *granulin* and *egt* genes were successfully amplified using gene specific primers (Table 4.1) and sequence data obtained from genomic DNA of both Group one and two CrleGV-SA, using PCR (Fig. 4.27). Using a 1Kb DNA marker, fragment sizes of the *granulin* and *egt* gene were estimated to be 750 bp and 1300 bp each, respectively.

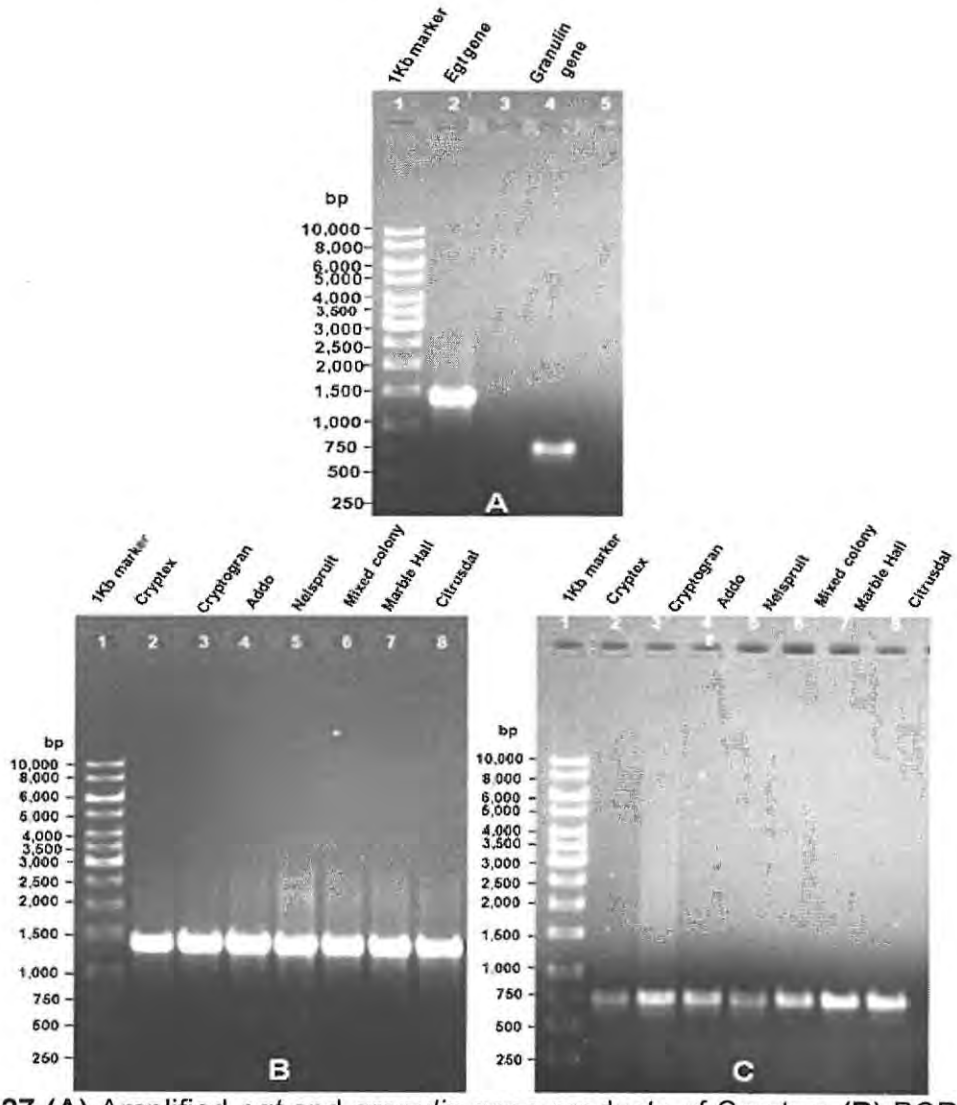

Figure 4.27 (A) Amplified *egt* and *granulin* gene products of Cryptex. **(B)** PCR products of the *egt* gene of Cryptex, Cryptogran and the Addo, Nelspruit, Mixed, Marble Hall and Citrusdal colony isolates. **(C)** PCR products of the *granulin* gene of Cryptex, Cryptogran and the Addo, Nelspruit, Mixed, Marble Hall and Citrusdal colony isolates. 0.6% AGE was performed at 30 V for 4 hrs, with a 1Kb DNA marker.

4.2.14 Comparative analysis of the nucleotide and amino acid sequences of the *granulin* gene of CrleGV-SA samples with other GV isolates

Granulin gene sequences of both Group one and two CrleGV-SA were amplified and aligned according to the methods described in section 4.1.6. Multiple alignments of the *granulin* gene sequence of both Group one and two CrleGV-SA revealed three single nucleotide polymorphisms (SNPs) between them. When *granulin* gene sequences of both Group one and two CrleGV-SA were subjected to BLAST analysis and compared with a CrleGV-SA (AY293731), CrleGV-CV3 (AY229987), CpGV (U53466) and PlscGV (AY706675) isolates *granulin* genes, SNPs were observed in some isolates.

SNPs in Group one CrleGV-SA: In multiple sequence alignments, three SNPs were observed in the *granulin* gene sequence of the Group one CrleGV-SA and that of CrleGV-SA (AY293731) isolate (Table 4.15). In a BLAST analysis, the *granulin* genes of Group one CrleGV-SA revealed a 99% nucleotide identity to CrleGV-SA (AY293731) isolate *granulin* gene. In a BLAST analysis, five SNPs were observed between the *granulin* genes of the Group one CrleGV-SA and that of the CrleGV-CV3 (AY229987) isolate, with a 99% nucleotide identity between them (Table 4.15). In a BLAST analysis, SNPs and percentage nucleotide identities between the Group one CrleGV-SA and that of the CpGV (U53466), and *Plathypena scabra* granulovirus, PlscGV (AY706675) isolate *granulin* genes, revealed 101 and 83 SNPs with 85% and 83% nucleotide identities, respectively (Table 4.15).

Table 4.15 BLAST comparison between the *granulin* gene sequence of Group one CrleGV-SA samples and some GVs on the NCBI data base

		CrleGV-SA	CrleGV-CV3	CpGV	PlscGV
Group one CrleGV-SA	% Maximum sequence identity	99%	99%	85%	83%
	number of SNPs	3	5	101	83

*South African isolate of *Cryptophlebia leucotreta* granulovirus, CrleGV-SA (accession #: AY293731). Cape Verde isolate of *Cryptophlebia leucotreta* granulovirus, CrleGV-CV3 (accession #: AY229987). *Cydia pomonella* granulovirus, CpGV (accession #: U53466). *Plathypena scabra* granulovirus, PlscGV-A25-6 (accession #: AY706675) (Source: www.ncbi.nlm.nih.gov/).

SNPs in Group two CrleGV-SA: In multiple sequence alignments with the *granulin* genes of Group two CrleGV-SA and CrleGV-SA (AY293731) isolate *granulin* gene, no SNPs were observed between them. In a BLAST analysis, the *granulin* genes of Group two CrleGV-SA revealed a 100% nucleotide identity to CrleGV-SA (AY293731) isolate *granulin* gene.

In a BLAST analysis, six SNPs were observed between the *granulin* genes of Group two CrleGV-SA and that of the *granulin* gene of the CrleGV-CV3 (AY229987) isolates. The Group two CrleGV-SA also showed a 99% nucleotide identity to the CrleGV-CV3 (AY229987) isolates *granulin* gene. SNPs and percentage nucleotide identities between the Group two CrleGV-SA and CpGV (U53466), PlscGV (AY706675) isolates *granulin* genes revealed 101 and 83 SNPs with 84% and 83% nucleotide identities, respectively (Table 4.16).

Table 4.16 BLAST comparison between the *granulin* gene of the Group two (Cryptogran and the Nelspruit colony isolate) CrleGV-SA and the *granulin* gene of some GVs on the NCBI data base

		CrleGV-SA	CrleGV-CV3	CpGV	PlscGV
Group two CrleGV-SA	%Maximum Sequence identity	100%	99%	84%	83%
	number of SNPs	0	6	101	83

*South African isolate of *Cryptophlebia leucotreta* granulovirus, CrleGV-SA (accession #: AY293731). Cape Verde isolate of *Cryptophlebia leucotreta* granulovirus, CrleGV-CV3 (accession #: AY229987). *Cydia pomonella* granulovirus, CpGV (accession #: U53466). *Plathypena scabra* granulovirus, PlscGV-A25-6 (accession #: AY706675) (Source: www.ncbi.nlm.nih.gov/).

Amino acid sequence of Group one and two CrleGV-SA: The amino acid sequences of the *granulin* genes of both Group one and two CrleGV-SA were subjected to a BLAST comparison with the *granulin* gene amino acid sequence of CrleGV-SA (AY293731) and CrleGV-CV3 (AY229987) isolates. This revealed no amino acid substitutions between them. A 100% amino acid identity was obtained from a BLAST query. This suggests that the *granulin* gene is highly conserved among CrleGV isolates.

On the other hand, five amino acid substitutions were observed between the *granulin* gene amino acid sequence of the Group one and two CrleGV-SA and the *granulin* gene amino acid of the CpGV (U53466) isolate (Table 4.17). This resulted in a 97% amino acid identity from a BLAST query. This suggests that the *granulin* gene is slightly variable among different GV species.

Table 4.17 Amino acid substitutions between the *granulin* genes of the Groups one and two CrleGV-SA and CpGV (U53466) isolate

GV isolate		Amino acid Position				
		13	100	126	177	188
CpGV (U53466) isolate		**N**	**M**	**G**	**R**	**Y**
Group one CrleGV-SA	Addo colony isolate	H	I	C	N	H
	Citrusdal colony isolate	H	I	C	N	H
	Cryptex	H	I	C	N	H
	Marble Hall colony isolate	H	I	C	N	H
	Mixed colony isolate	H	I	C	N	H
Group two CrleGV-SA	Cryptogran	H	I	C	N	H
	Nelspruit colony isolate	H	I	C	N	H

**Cydia pomonella* granulovirus, CpGV (accession #: U53466) (Source: www.ncbi.nlm.nih.gov/).

4.2.15 Comparative analysis of the nucleotide sequence of the *egt* genes of the Group one and two CrleGV-SA and that of the CrleGV-CV3 (AY229987) isolate

Multiple sequence alignments of the *egt* gene sequence for both the Group one and two CrleGV-SA revealed 16 SNPs between them. When both the Group one and two CrleGV-SA *egt* gene sequences and the CrleGV-CV3 (AY229987) isolate were subjected to a BLAST analysis, SNPs were observed in all isolates.

Twenty eight SNPs were observed between the *egt* genes of the Group one CrleGV-SA and that of the CrleGV-CV3 (AY229987) isolate *egt* gene sequence. The nucleotide identity recorded between these isolates was 98%. On the other hand, 23 SNPs were observed between the Group two CrleGV-SA and that of the CrleGV-CV3 (AY229987) isolate *egt* gene, with a 99% nucleotide identity observed between them. This suggests that, the *egt* genes of the Group two CrleGV-SA and that of the CrleGV-CV3 (AY229987) isolate *egt* gene are similar. Significantly, SNPs in the *egt* genes of these isolates resulted in some changes in their amino acid sequence.

4.2.16 Comparative analysis of the amino acid sequence of the *egt* genes of the Group one and two CrleGV-SA and that of the CrleGV-CV3 (AY229987) isolate

Four amino acid substitutions were observed between the *egt* gene sequences of Groups one and two CrleGV-SA (Table 4.18). The amino acids I (152), N (196), L (241) and R (310) in the Group one CrleGV-SA, were substituted as amino acids M (152), H (196), V (241) and G (310) for the Group two CrleGV-SA, respectively (Table 4.18). These findings suggest that, there is a high variability among the *egt* genes of geographically distinct CrleGV-SA isolates. The amino acids and their corresponding symbols are indicated in table 4.17 and table 4.18.

Seven amino acid substitutions were observed between the *egt* gene sequences of the Group one CrleGV-SA and that of the CrleGV-CV3 (AY229987) isolate (Table 4.18). However, only one amino acid, N (196), was shared between them. The Group one CrleGV-SA also showed a 98% amino acid identity to the CrleGV-CV3 (AY229987) isolate *egt* gene from a BLAST query.

Six amino acid substitutions were recorded between the alignments of the amino acid sequences of the *egt* gene of the Group two CrleGV-SA and that of the CrleGV-CV3 (AY229987) isolate (Table 4.18 & 4.19). However, two amino acids M (152) and V (241) were shared between them. The Group two CrleGV-SA had a unique amino acid G (310), which occurred in the same position as amino acid R (310) and K (310) for the Group one CrleGV-SA and the CrleGV-CV3 (AY229987) isolate respectively. The Group two CrleGV-SA also showed 99% amino acid identities to the CrleGV-CV3 (AY229987) isolate *egt* gene from a BLAST query. These findings may suggest that the Group two CrleGV-SA, Cryptogran and the Nelspruit colony isolate, share a more similar *egt* gene sequence with the CrleGV-CV3 (AY229987) isolate than the Group one CrleGV-SA.

Table 4.18 Amino acid substitutions between the *egt* genes of the Groups one and two CrleGV-SA and CrleGV-CV3 (AY229987) isolate

CrleGV isolate		Amino acids							
		Position							
		96	152	196	225	241	310	315	373
CrleGV-CV3 (AY229987) isolate		**V**	**M**	N	**R**	**V**	**K**	**I**	**M**
Group one CrleGV-SA	Addo colony isolate	A	I	N	Q	L	*R*	F	L
	Citrusdal colony isolate	A	I	N	Q	L	*R*	F	L
	Cryptex	A	I	N	Q	L	*R*	F	L
	Marble Hall colony isolate	A	I	N	Q	L	*R*	F	L
	Mixed colony isolate	A	I	N	Q	L	*R*	F	L
Group two CrleGV-SA	Cryptogran	A	**M**	**H**	Q	**V**	**G**	F	L
	Nelspruit colony isolate	A	**M**	**H**	Q	**V**	**G**	F	L

* Cape Verde isolate of *Cryptophlebia leucotreta* granulovirus, CrleGV-CV3 (accession #: AY229987) (Source: www.ncbi.nlm.nih.gov/).

Table 4.19 Amino acids and their corresponding symbols

Amino acid	symbol	Amino acid	symbol
Alanine	A	Arginine	R
Valine	V	Glutamine	Q
Methionine	M	Leucine	L
Isoleucine	I	Lysine	K
Asparagine	N	Glycine	G
Histidine	H	Phenylalanine	F

This analysis in combination with REN profiles supports the placing of the isolates into two genome types.

4.2.17 Comparative analysis of the *granulin* and *egt* genes of the Group one and two CrleGV-SA and that of other CrleGV isolates using molecular phylogenetic trees

Phylogenetic analysis of sequence data obtained from the *granulin* genes of both the Group one and two CrleGV-SA and that of CrleGV-SA (AY293731) isolate revealed the following. Both the Group one and two CrleGV-SA were placed into two groups based on their evolutionary relationship. Only the Group two CrleGV-SA were shown to be closely related to CrleGV-SA (AY293731) isolate (Fig. 4.28). These findings further support the grouping of CrleGV-SA samples, into two distinct groups.

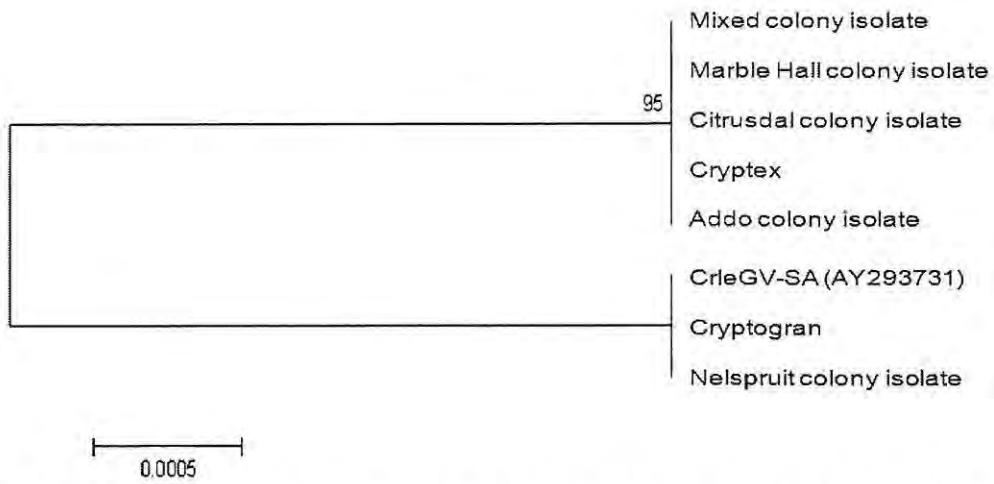

Figure 4.28 Molecular phylogenetic trees (using Minimum evolution, ME) showing relationship among *granulin* gene of Group one and two CrleGV-SA isolates and that of CrleGV-SA isolate (AY293731) reported by Singh *et al.* (2003). Numbers next to the branches indicate percent of support.

Phylogenetic analysis of sequence data obtained from the *egt* genes of both the Group one and two CrleGV-SA and that of the CrleGV-CV3 (AY229987) isolate revealed the following: Both the Group one and two CrleGV-SA were placed into two groups based on their evolutionary relationships. However, the Group one and two CrleGV-SA appeared to have originated from a common ancestor. The CrleGV-CV3 (AY229987) isolate, on the other hand, was shown not to be closely related to both the Group one and two CrleGV-SA (Fig. 4.29). Again, these findings also support the groupings of CrleGV-SA into two distinct groups, based on their REN profiles.

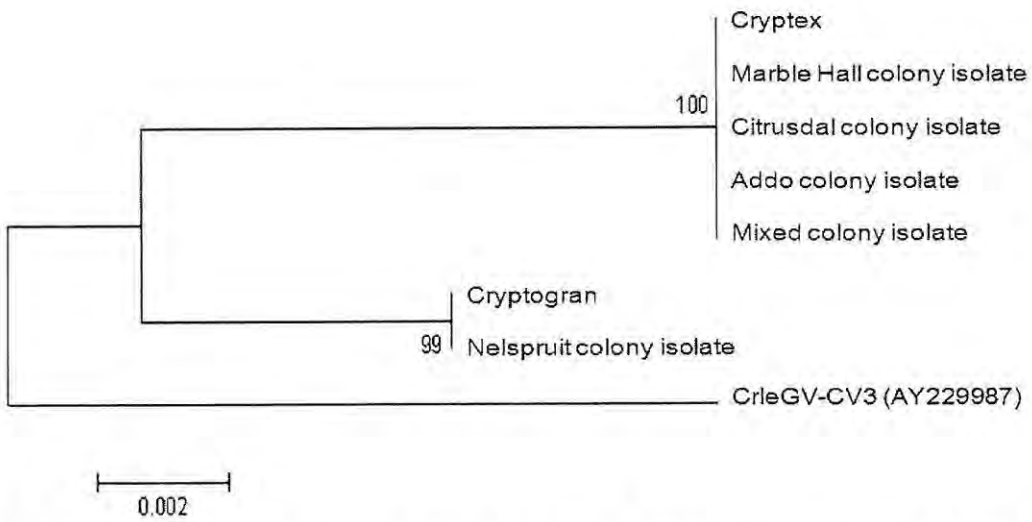

Figure 4.29 Molecular phylogenetic trees (using Minimum evolution, ME) showing relationship among *egt* gene of Group one and two CrleGV-SA isolates and that of the CrleGV-CV3 isolate (AY229987) reported by Lange & Jehle (2003). Numbers next to the branches indicate percent of support.

4.3 DISCUSSION

This chapter describes the genetic characterisation of five new CrleGV-SA isolates namely, the Addo, Citrusdal, Marble Hall, Nelspruit and Mixed colony isolates. CrleGV-SA isolates used to formulate Cryptogran (Moore *et al.*, 2004a & 2004b) and Cryptex (Kessler & Zingg, 2008) were used as a reference and were also characterised.

In this study, both the PC and CTAB DNA extraction methods proved successful in yielding genomic DNA from CrleGV-SA OBs. However, the CTAB DNA extraction method was superior to the PC method for REN analysis. Other authors have found these methods to be satisfactory for the extraction of genomic DNA from baculoviruses (Aspinall *et al.*, 2002; Parnell *et al.*, 2002; Hilton & Winstanley, 2008).

Single REN analysis of genomic DNA of all seven CrleGV-SA samples using *Bam*H1, *Sal*1, *Xba*1, *Pst*1, *Xho*1, *Kpn*1, *Hind*III and *Eco*R1 revealed genetic differences between them. This was confirmed by the presence of several unique submolar REN bands in all samples. Based on the DNA profiles generated by the RFLPs, two distinct CrleGV-SA genome types were observed for the CrleGV-SA isolate. Cryptex and the Addo,

Citrusdal, Marble Hall and Mixed colony isolates shared a similar genome type which was different from Cryptogran and the Nelspruit colony isolate. Cryptogran and the Nelspruit colony isolate shared a unique CrleGV-SA genome type. Therefore, Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates were placed as members of the Group one CrleGV-SA, while Cryptogran and the Nelspruit colony isolate were placed into the Group two CrleGV-SA.

Other authors have reported differences in genome types for NPV (Takatsuka *et al.*, 2003) and GV isolates (Eberle *et al.*, 2009). For example, quite recently, several CpGV genome types were found (Eberle *et al.*, 2009). Using REN analysis and partial sequences of the *lef*-8, *lef*-9, and *polh/gran* genes of six CpGV isolates from Iran (CpGV-I01, I07, I08, I12, I66 and I68; Rezapanah *et al.*, 2008) and two CpGV isolates from Georgia (CpGV-G01 and G02), and one *in vivo* cloned CpGV isolate from England (CpGV-E2; Crook *et al.*, 1985), Eberle *et al.* (2009) established that there are four CpGV genomes types that closely resemble other previously described CpGV isolates, including the Mexican (CpGV-M; Tanada, 1964), Russian (CpGV-R; Harvey & Volkman, 1983) and English (CpGV-E; Crook *et al.*, 1985) isolates. They subsequently placed CpGV into four distinct genome types namely, A (CpGV-M & I66), B (CpGV-E2), C (CpGV-R, I07, I68 & G01) and D (CpGV-I01, I08 & I12) (Eberle *et al.*, 2009). CpGV is also regarded as the best studied GV and currently serves as a model for other GVs (Eberle *et al.*, 2009).

The genome size for the CrleGV-SA isolate has been reported to be 112.36 kbp (Singh *et al.*, 2003) with the fully sequenced CrleGV-CV3 (AY229987) isolate genome being estimated to be 110,907 bp and is predicted to encode 129 open reading frames (ORFs), of which 124 are reported to be shared by other baculovirus ORFs (Lange & Jehle, 2003). In this study, however, due to the poor quality of some gels the DNA fragment sizes proposed for both Group one and two CrleGV-SA isolates may be prone to some degree of error. As such, the genome sizes reported for the seven CrleGV-SA isolates are only estimates. Nonetheless, since the purpose of this study was to establish genetic differences accurate mapping of the CrleGV-SA genome was not

considered important. In future double digests or preferably full genome sequencing would be a more favourable option.

Similarities in REN profiles were observed between both Group one and two CrleGV-SA and those of the CrleGV-SA isolate reported by Singh *et al.* (2003) and Fritsch (1989). For example it was observed that, the Group one CrleGV-SA shared a similar REN profile with Fritsch's isolate based on their *Eco*R1, *Hind*III and *Bam*H1 profiles. On the other hand, the Group two CrleGV-SA (Cryptogran and Nelspruit isolate) rather shared a similar profile with Singh's isolate, based on their *Bam*H1, *Xho*1, *Kpn*I and *Eco*RI fragment sizes. Again, REN profiles for both Group one and two CrleGV-SA were genetically different from the CrleGV-CV, CrleGV-IC and CrleGV-CV3 isolates reported by Fritsch (1989) and Jehle *et al.* (1992) respectively. These findings support previous studies by Fritsch (1989) and Jehle *et al.* (1992) which established the genetic heterogeneity between these CrleGV isolates.

Moreover, the presence of unique submolar REN bands, in all samples, for both the Group one and two CrleGV-SA rather indicates that each sample consists of multiple genotypes of CrleGV-SA, with each isolate differing genetically from each other. The appearance of submolar REN bands in restriction profiles of baculoviruses are commonly used to establish genetic heterogeneity between baculovirus isolates (Crook *et al.*, 1997) as well as indicate the presence of genotypes within isolates (Crook, 1986).

This genetic diversity among the five new CrleGV-SA isolates (Addo, Citrusdal, Marble Hall, Nelspruit and Mixed colony isolates) may have been influenced by several factors. Firstly, the establishment of several geographically distinct laboratory colonies of the host (FCM) may have played a significant role in obtaining a high genetically diverse gene pool of CrleGV-SA. Secondly, the methodology employed in the previous chapter for the induction and recovery of latent CrleGV-SA isolates, by pooling several insect larvae from each of the colonies, may have contributed to this increased genetic diversity (Cory & Myers, 2003; Erlandson, 2009). The origins of this genetic diversity in baculoviruses have been speculated as been caused by small mutations, sequence duplications and in some cases the acquisition of host DNA (Brown *et al.*, 1985).

It was observed that, the two commercial biopesticides, Cryptex (Group one CrleGV-SA) and Cryptogran (Group two CrleGV-SA) both belonged to either of the two CrleGV-SA genome types. This suggests that, these biopesticides may be formulated with several wild-type CrleGV-SA genotypes. Wild-type baculoviruses multiplied by passage and replication *in vivo*, in insect host systems may lead to an increased genetic variation (Possee & Rohrmann, 1997).

On a different note, it is possible that these multiple genotypes in Cryptogran and Cryptex may present some benefits in terms of improved virulence against FCM. Quite commonly wild-type isolates (mixtures of genotypes) appear to be more virulent than pure genotypes (Lopez- Ferber *et al.*, 2003).

Analysis of partial gene sequences of the *granulin* and *egt* genes for both the Group one and two CrleGV-SA revealed evolutionary relationships between them. For example, phylogenetic analysis based on the SNPs observed in their *granulin* and *egt* genes indicated the presence of two *granulin* and *egt* gene types. Analysis of the Group one CrleGV-SA showed that these isolates were closely related and possess a unique CrleGV-SA *granulin* and *egt* gene which was different to the Group two CrleGV-SA. These findings correlates well with the DNA profiles and support the grouping of the seven CrleGV-SA samples into two distinct genome types. More importantly, the amino acid substitutions in their *egt* genes may have significant implications as this could affect viral protein expression and possible differences in viral virulence. Based on the amino acid sequences for the *granulin* and *egt* genes, the *granulin* gene was found to be highly conserved among CrleGV isolates and slightly variable among different GV species. On the other hand, the *egt* gene showed more variability among the Group one and two CrleGV-SA isolates and a higher variability to the CrleGV-CV3 (AY229987) isolate.

A number of *Eco*R1, *Bam*H1 and *Pst*1 recognition sites were observed in the *granulin* gene of the Group one and two CrleGV-SA, indicating their potential for genetic manipulation. However, this gene may probably not be a suitable candidate for genetic manipulation as the *granulin* gene is highly conserved and an important gene for GVs

(O'Reilly *et al.*, 1992; Federici, 1997). The *granulin* gene codes for the *granulin* protein which protects the virus from adverse environmental conditions (O'Reilly *et al.*, 1992; Cory *et al.*, 1997). On the other hand, the presence of a single *Xba*1 recognition site in the nucleotide sequence of the *egt* gene may favour this gene's suitability for gene manipulation, as the *egt* gene is not considered to be an essential gene for the virus (O'Reillly *et al.*, 1992). The product of *egt*, ecdysteroid UDP-glucosyltransferase, has some significant advantages as it is thought to prolong the life span of the host by preventing infected larvae from molting or pupating, thereby enabling the virus to replicate much longer (Tumilasci *et al.*, 2003). Therefore, by modifying or deleting this gene the speed of kill of the virus can be greatly enhanced, as the host dies much quicker (O'Reillly *et al.*, 1992). This has also been the focus of other studies on genetically engineered baculoviruses (Black *et al.*, 1997).

In order to name the five new CrleGV-SA isolates (Addo, Citrusdal, Marble Hall, Nelspruit and Mixed colony isolates) recovered, several propositions were considered. Generally, the nomenclature of baculoviruses is considered to be highly problematic as there appears to be no accepted norm. The virus naming and abbreviation does not always follow the previous (Theilmann *et al.*, 2005) or current (Herniou *et al.*, 2011) taxonomy. However, the most commonly used methods are based on naming the virus directly after the host from which they are isolated, and secondly on the type of occlusion body (NPV or GV) associated with it (OECD, 2002; Bonsall, 2004; Fauquet & Fargette, 2005; Erlandson, 2009). For example, the baculovirus recovered from the false codling moth (FCM) *Thaumatotibia* (=*Cryptophlebia*) *leucotreta* is referred to as *Cryptophlebia leucotreta* granulovirus (Fristch, 1989; Jehle *et al.*, 1992; Singh *et al.*, 2003). Abbreviated versions of the first two letters of the genus and species name of the host are also commonly used methods, such as CrleGV denoting *Cryptophlebia leucotreta* granulovirus. To distinguish between baculoviruses recovered from the same host species from different countries, in some cases the country from which the virus is recovered may be added as an acronym or suffix, as in CrleGV-SA which denotes *Cryptophlebia leucotreta* granulovirus isolated from South Africa (SA) (Fristch, 1989; Singh *et al.*, 2003).

One problem with this system of nomenclature is that, if there is a taxonomic change in the name of the host, the naming of the virus is affected. For instance, even though the genus name of FCM has been changed from *Cryptophlebia* to *Thaumatotibia*, the virus name remains unchanged. More so, since several studies have shown that multiple baculovirus species can be derived from the same insect (Li *et al.*, 2002) or from different insect species (Goto *et al.*, 1992) as well as from different insect orders (Smith *et al.*, 1959; Sidor, 1960; Cory & Myers, 2003), these findings have therefore brought the naming of baculovirus species directly after their host into question.

Another problem with the nomenclature of baculoviruses, which relates to this study, has to do with the naming of geographic isolates or genotypic variants of baculovirus isolates recovered from the same host species. At present, there appears to be no consensus in distinguishing between geographic isolates of baculoviruses as different authors use different methods. For example, in one study, some geographic isolates of an Iranian CpGV isolate, CpGV-I were distinguished from each other using numbers (Rezapanah *et al.*, 2008). In another study, combinations of numbers and acronyms or acronyms alone were used. Two examples include the *Malacosoma distria* NPV geographic isolates, MadiNPV-A92 from Alberta, Canada and MadiNPV-Fred from Fredericton, Canada (Erlandson, 2009). Genetic variants and cloned genotypes of baculovirus isolates have also been distinguished from each other using numbers (Crook *et al.*, 1985; Lange & Jehle, 2003; Eberle *et al.*, 2009).

Considering all these different versions, two methods were adopted for the naming of the five new CrleGV-SA isolates. The method which involved using acronyms to denote the name of the geographic region from which an isolate is recovered was considered to be more ideal for the naming of the isolates. The Addo, Nelspruit, Marble Hall, Citrusdal and Mixed colony isolates were therefore referred to as CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates, respectively. The rationale for adopting this method is that, each isolate consists of more than one genotype, in future these genotypes can be distinguished from each other using numbers. *In vivo* and *in vitro* cloning techniques can be used to distinguish between the genotypes within each isolate (Crook, 1986; Erlandson, 2009).

CHAPTER FIVE

BIOASSAYS WITH CRLEGV-SA

5.0 INTRODUCTION

In the previous chapter, RFLP analysis of baculovirus DNA recovered from the same insect (FCM) species from different geographic regions revealed differences in genetic composition. The five novel CrleGV-SA isolates recovered were named as CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates. The new isolates as well as Cryptogran and Cryptex were placed into two genome groups. Cryptex and the Addo, Citrusdal, Marble Hall and Mixed colony isolates were placed as members of the Group one CrleGV-SA, while Cryptogran and the Nelspruit colony isolate were placed into the Group two CrleGV-SA.

Baculovirus isolates recovered from insect populations and varying in genotypes can display differences in virulence to the host (Parnell *et al.*, 2002; Takatsuka *et al.*, 2003; Eberle *et al.*, 2008 & Jehle *et al.*, 2008). This chapter investigated the comparative virulence of the new CrleGV-SA isolates against laboratory populations of FCM using bioassays. Bioassays are used to measure dose-response and time-response relationships, and are effective in assessing the biological activity of baculoviruses (Hughes & Shapiro, 1997; Jones, 2000). The different bioassay methods were described in Chapter one (section 1.3.2). For the purpose of this study, the droplet feeding bioassay technique as described by Hughes & Wood (1981) was used as it has significant advantages over the other methods. For example, due to the synchronous uptake of viral OBs, especially when conducted with neonate larvae that are uniform in weight and development, the droplet feeding bioassay method is considered to be highly sensitive and accurate for dosage-mortality and time-mortality bioassays (Ignoffo, 1966; Sheppard & Stairs, 1977; Boucias *et al.*, 1980). In addition, the ability of neonates to readily ingest and self-regulate the initial volume of OBs ingested has an added advantage as this allows large numbers of test insects to be treated with a relatively uniform amount of virus (Hughes & Wood, 1981). The accuracy of this bioassay method

for the determination of the volume ingested by a single larva also makes it suitable for the estimation of the lethal dosage (LD) (Hughes *et al.*, 1986; Jones, 2000).

The objective of this chapter was to investigate the comparative virulence of the five new CrleGV-SA isolates, as well as Cryptex and Cryptogran, against five FCM laboratory colonies using droplet feeding bioassays. This is important in order to shed more light on the susceptibility of FCM populations originating from different geographic regions in South Africa to genetically different CrleGV isolates. This information would also help facilitate decision making regarding the efficient application of CrleGV based biopesticides in South Africa.

5.1 MATERIALS AND METHODS

5.1.1 Droplet dose-response bioassays

The droplet feeding bioassay technique described by Jones *et al.* (1993) for the bioassay of neonate larvae was used. Newly laid FCM eggs on wax paper sheets were removed from the Addo, Nelspruit, Citrusdal, Marble Hall and Mixed colonies (Chapter Two) and placed in sterile preserve jars. The jars were covered with parafilm and secured with a lid. The eggs were left in the incubation chamber for hatching to take place. After five days, newly hatched neonates that had congregated on the parafilm were removed and droplet-fed with seven separate concentrations of inocula from the Group one and two CrleGV-SA, and assays conducted in the following manner.

Seven-fold serial dilutions (1:7 dilution factor) were carried out using the virus stock concentrations obtained from both the Group one and two CrleGV-SA (see Chapter three, Table 3.2). Their inocula were serially diluted in sterile distilled water, in sterile microfuge tubes, to obtain final concentrations of 6.07×10^2, 4.25×10^3, 2.97×10^4, 2.08×10^5, 1.46×10^6, 1.02×10^7 and 7.14×10^7 OBs/ml for each isolate. Thereafter, 50 µl of 1% (w/v) Brilliant blue R dye (USB Corporation, USA) was added to each tube in order to make a final volume of 650 µl. The resulting virus-dye suspension was administered to neonate larvae as numerous droplets of 2 µl, randomly distributed on the parafilm (Fig. 5.1 A). Control larvae were fed with a sterile distilled water-dye solution. Once

larvae had ingested a sufficient amount of virus-dye suspension (treatments) or water-dye solution (controls), larvae were removed and placed individually in each of the wells in the sterile 24-cell bioassay trays, containing diet (Fig. 5.1 B). Diets for the assays were prepared according to the methods described in Chapter two (section 2.1.2). The bioassay trays were then covered with sterile paper towels. Three replicate assays of 48 larvae per treatment and control were conducted for each colony and assays evaluated for larval mortality 7 days post inoculation. The function of the dye in the assays was to permit visual confirmation of ingestion as the gut of larvae that had ingested the virus appeared blue when viewed using a magnifying glass (Fig. 5.1 A). The hydrophobic nature of the parafilm ensured that the droplets were held in place and prevented the breaking of the surface tension of the virus-dye suspensions or water-dye droplets.

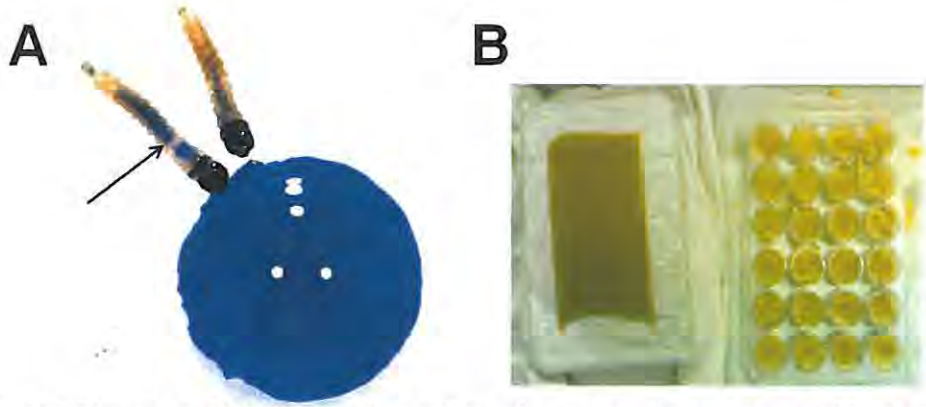

Figure 5.1 (A) Neonate FCM larva ingesting virus-dye suspension with their gut filled with blue (see arrow). **(B)** A 24-cell bioassay tray with artificial FCM diet inserted into each well.

5.1.2 Droplet time-response bioassays

Time-response bioassays were conducted in the following manner. Diets for the assays were prepared beforehand according to the methods as described in Chapter two (section 2.1.2). The cooked diet was cut into plugs using the lip of a 30 ml plastic polypot (Evron, South Africa), which had its base removed. The polypots were preferred over the 24-cell bioassays trays as Moore *et al.* (2011) found them to be suitable for time-response bioassays. For example, for larval observation, the polypots rendered themselves easily to opening and prevented against the risk of insects escaping unlike the 24-cell bioassays trays (Moore *et al.*, 2011).

The individual diet plugs were placed into each polypot and neonate larvae from each of the five colonies were selected and droplet-fed with both the Group one and two CrleGV-SA isolates at a single concentration of 1.0 x 10^8 OBs/ml. This single treatment concentration was selected on the basis of its ability to elicit 100% mortality in all test insects, from previous trials. For the controls, larvae were fed with a water-dye solution. Five neonates were selected per colony and placed into polypots. The polypots were then covered with multiple layers of sterile paper towel and secured with their lids. After 12 hours, both treatment and control polypots were inspected for larval mortality. Thereafter, polypots were inspected every 8 hours, three times a day, at 07:00, 15:00 and 23:00 until all larvae had died. Dead larvae were removed immediately after counting in order to prevent repetitive counting. For each of the treatments (Group one and two CrleGV-SA inocula), including the controls, 10 polypots were used with each polypot containing 5 larvae. Therefore, a total of 50 larvae were used per treatment and control. Bioassays were conducted in triplicates for each of the five colonies. It has been reported that when insects are placed in groups this exposes them to the effects of "density dependent prophylaxis," which could be problematic (Reynolds *et al.*, 2011). However, considering that a small number of neonates were used per very large polypot these effects were considered to be very minimal. Each larva therefore served as a replicate (Moore *et al.*, 2011). Again, the fact that each larva was initially independently droplet-fed with the virus-dye suspension and removed immediately post-mortality, also served as a good measure to minimise the probability of horizontal viral transmission.

5.1.3 Statistical analysis

Data from the droplet dose-response assays were analysed using PROBAN (Van Ark, 1995), a statistical software programme used for conducting probit analyses with bioassay data. PROBAN corrected the control mortality according to Abbott's formula (Abbott, 1925). Three replicates from the dose-response bioassays were pooled together and their LC_{50} and LC_{90} values determined. PROBAN transformed the doses to $\log_{10}$ and the percentage mortality response to empirical probits. Using this information, the fit of the probit (regression) lines were calculated as were the fiducial limits. Multiple comparisons of slopes of probit regression lines were conducted using the Bonferroni

method and significant differences between slopes were established at $P < 0.05$. If probit lines were parallel relative potency comparisons were conducted. Therefore, for each comparison one isolate was used as a standard and compared against the other. The LD values were estimated from LC values by multiplying each LC value by 0.0044, the volume (µl) ingested by a single neonate FCM larva (Pereira-da-Conceicoa, 2008), and the result divided by 1000 in order to obtain the number of OBs ingested per larva. Time-response relationships were determined by the Kaplan-Meier product limit estimator, using the GraphPad Prism software (version 5.04), which also takes into account control mortality. The median survival time (ST_{50}) was determined and expressed as the time at which 50% of the treated insects are still alive. Survival times of three replicates were pooled from each of the colonies and their means compared using the Log-rank (Mantel-Cox) and significant differences established at $P < 0.05$.

5.2 RESULTS

5.2.1 Dose-response bioassays with Group one and two CrleGV-SA against the Addo colony

In droplet feeding dose-response bioassay on Moore's FCM diet using both Group one and two CrleGV-SA isolates against neonate larvae from the Addo colony, a dose-response relationship was established. The seven CrleGV-SA inocula concentrations ranged between 6.07 $x10^2$ and 7.14x 10^7 OBs/ml. Control mortality ranged between 0% and 4% for all replicates. Mortality of larvae inoculated with the seven CrleGV-SA inocula ranged from 11.10% - 92.20% for the lowest and highest concentrations, respectively. Mortality of neonate larvae from the Addo colony was related to dosage for all seven CrleGV-SA inocula. The regression lines fitted to the corrected data for all replicates compared, the residual variances of the lines were determined to be homogenous and the slopes of the lines parallel and comparable. Using the Bonferroni method to compare the slopes of the regression lines (Fig. 5.2) between isolates, PROBAN established the slopes to be significantly different ($F_{6,41} = 4.288$; $P = 0.0020$). However, relative potency comparisons between isolates, did not establish significant difference.

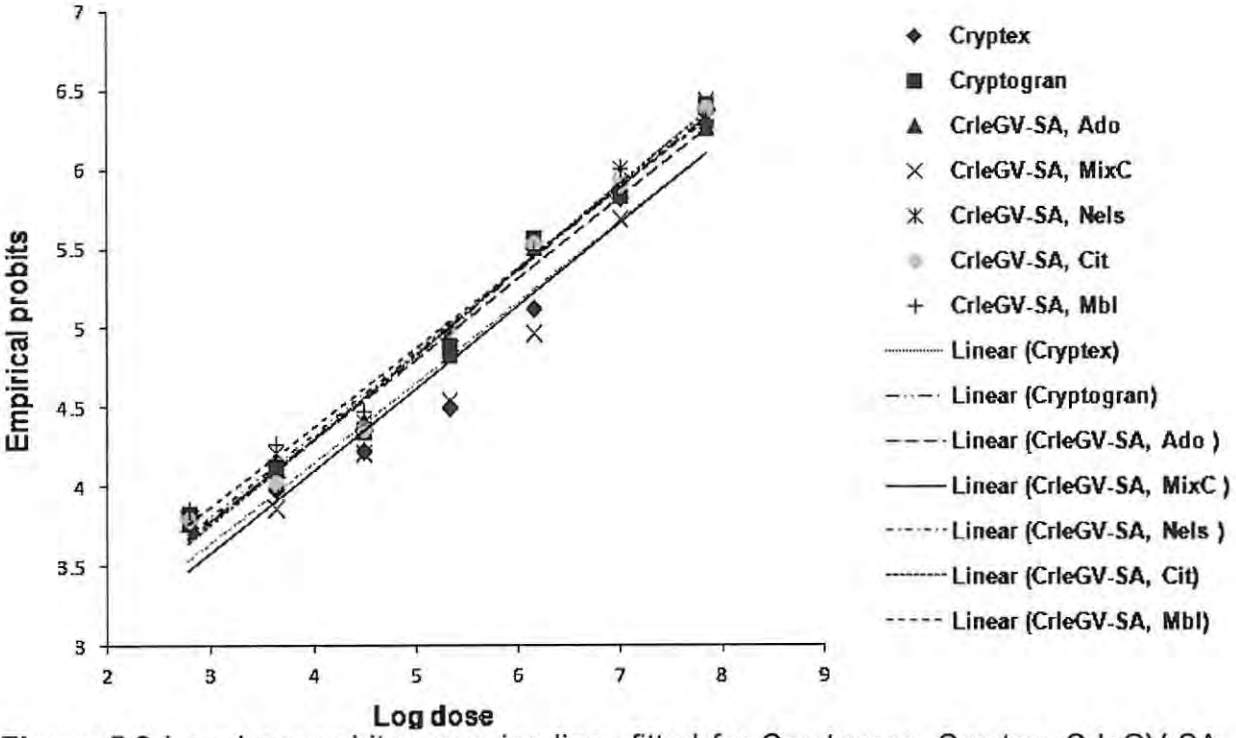

Figure 5.2 Log-dose-probit regression lines fitted for Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit against neonate larvae from the Addo colony.

The mean LC_{50} values of Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates ranged between 1.89×10^5 and 7.10×10^5 OBs/ml (Table 5.1). Their LD_{50} values ranged from 0.83 – 3.12 OBs per larva, with Cryptex and CrleGV-SA MixC both recording the highest LD_{50} values with viral OBs of 2.58 and 3.12 per larva, respectively (Table 5.1).

Table 5.1 Bioassay data and associated statistics for neonate FCM larvae from the Addo colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	LC_{50} (OBs/ml)	95% Fiducial limits		χ^2	df	*P*	LC_{90} (OBs/ml)	LD_{50} (OBs)	LD_{90} (OBs)
		Lower	Upper						
Cryptex	5.86×10^5	2.84×10^5	1.23×10^6	5.39	5	0.37	1.52×10^8	2.58	669.50
Cryptogran	2.31×10^5	1.17×10^5	4.57×10^5	2.36	5	0.79	6.20×10^7	1.02	272.86
CrleGV-SA Ado	2.60×10^5	1.30×10^5	5.24×10^5	1.511	5	0.91	8.16×10^7	1.14	358.89
CrleGV-SA MixC	7.10×10^5	3.54×10^5	1.47×10^6	6.66	5	0.25	1.76×10^8	3.12	773.32
CrleGV-SA Nels	2.05×10^5	1.03×10^5	4.05×10^5	2.13	5	0.83	5.68×10^7	0.90	250.11
CrleGV-SA Cit	2.21×10^5	1.10×10^5	4.37×10^5	1.39	5	0.92	4.96×10^7	0.97	218.04
CrleGV-SA Mbl	1.89×10^5	9.32×10^4	3.82×10^5	1.79	5	0.87	6.57×10^7	0.83	289.25

NB: χ^2: Chi-square goodness of fit. df: degrees of freedom for chi-square. *P*: Probability of a greater chi-square

5.2.2 Dose-response bioassays with Group one and two CrleGV-SA against neonates from the Citrusdal colony

In droplet feeding dose-response bioassays on Moore's FCM diet using Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates against neonate larvae from the Citrusdal colony, a dose-response relationship was established. The seven CrleGV-SA inocula concentrations ranged between 6.07 x 10^2 and 7.14x 10^7 OBs/ml. Control mortality was recorded to be 0% for all replicates. Mortality of larvae inoculated with the seven CrleGV-SA inocula ranged from 10.42% - 95.15% for the lowest and highest concentrations, respectively.

Mortality of neonate larvae from the Citrusdal colony was related to dosage for all seven CrleGV-SA inocula. The regression lines fitted to the corrected data for all replicates compared, the residual variances of the lines were determined to be homogenous and the slopes of the lines parallel and comparable. The regression lines (Fig. 5.3) of Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates did not differ significantly ($F_{6,41} = 0.453$; $P = 0.839$) from each other.

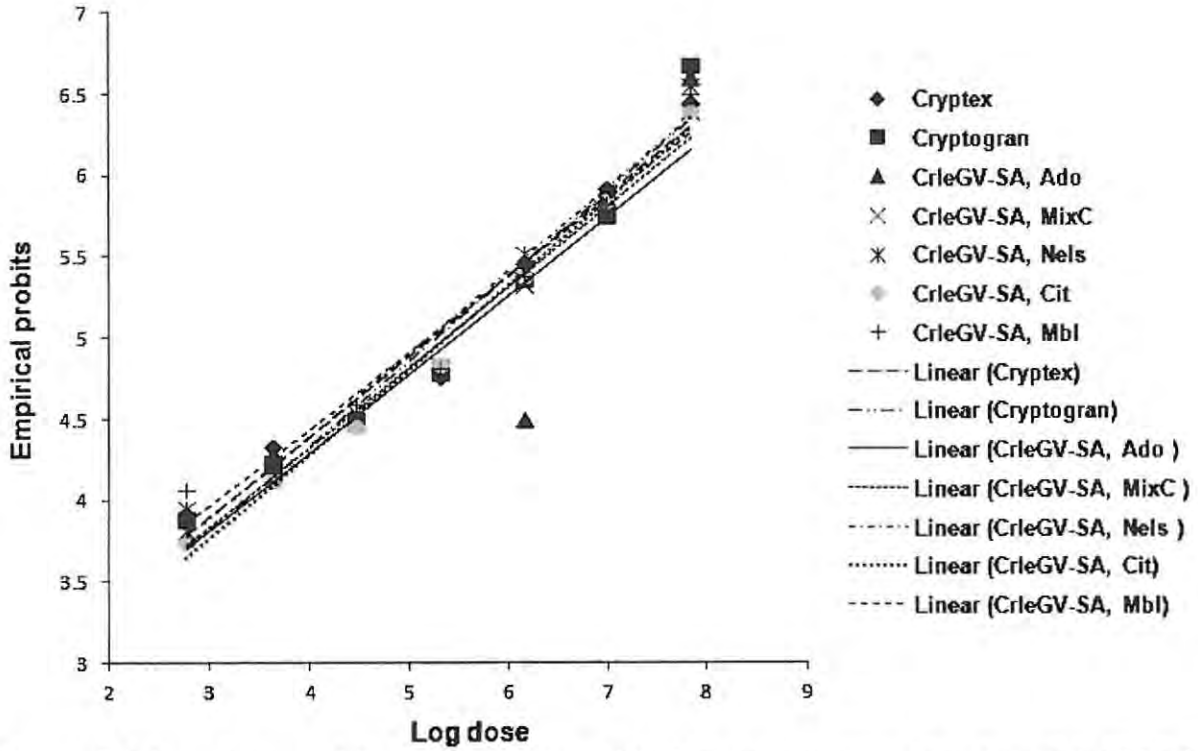

Figure 5.3 Log-dose-probit regression lines fitted for Cryptogran, Cryptex, CrleGV-SA, Nels; CrleGV-SA, MixC; CrleGV-SA, Ado; CrleGV-SA, Mbl and CrleGV-SA, Cit against neonate larvae from the Citrusdal colony.

The mean LC_{50} values of Cryptex, Cryptogran and the CrleGV-SA, Ado; CrleGV-SA, Nels; CrleGV-SA, Mbl; CrleGV-SA, Cit and CrleGV-SA, MixC isolates ranged between 1.45×10^5 and 2.64×10^5 OBs/ml (Table 5.2). Their LD_{50} values ranged from 0.80 – 1.18 OBs per larva (Table 5.2).

Table 5.2 Bioassay data and associated statistics for neonate FCM larvae from the Citrusdal colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	LC_{50} (OBs/ml)	95% Fiducial limits		χ^2	df	*P*	LC_{90} (OBs/ml)	LD_{50} (OBs)	LD_{90} (OBs)
		Lower	Upper						
Cryptex	1.94×10^5	9.43×10^4	3.99×10^5	3.82	5	0.58	8.03×10^7	0.86	353.38
Cryptogran	2.34×10^5	1.15×10^5	4.78×10^5	3.71	5	0.59	8.92×10^7	1.03	392.56
CrleGV-SA Ado	1.82×10^5	9.06×10^4	3.65×10^5	3.60	5	0.61	5.78×10^7	0.80	254.46
CrleGV-SA MixC	2.64×10^5	1.28×10^5	5.51×10^5	1.89	5	0.86	1.16×10^8	1.16	510.04
CrleGV-SA Nels	1.84×10^5	9.03×10^4	3.74×10^5	2.88	5	0.72	6.70×10^7	0.81	294.63
CrleGV-SA Cit	1.45×10^5	1.34×10^5	5.36×10^5	1.05	5	0.96	7.87×10^7	1.18	346.17
CrleGV-SA Mbl	1.85×10^5	8.65×10^4	3.93×10^5	3.48	5	0.63	1.09×10^8	0.81	479.6

NB: χ^2: Chi-square goodness of fit. df: degrees of freedom for chi-square. *P*: Probability of a greater chi-square

5.2.3 Dose-response bioassays with Group one and two CrleGV-SA against the Marble Hall colony

In droplet feeding dose-response bioassays on Moore's FCM diet using Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates against neonate larvae from the Marble Hall colony, a dose-response relationship was established. The seven CrleGV-SA inocula concentrations ranged between 6.07 $x10^2$ and 7.14x 10^7 OBs/ml. Control mortality ranged between 0% and 2% for all replicates. Mortality of larvae inoculated with the seven CrleGV-SA inocula ranged from 10.01% - 90.98% for the lowest and highest concentrations, respectively.

Mortality of neonate larvae from the Marble Hall colony was related to dosage for all seven CrleGV-SA inocula. The regression lines fitted to the corrected data for all replicates compared, the residual variances of the lines were determined to be homogenous and the slopes of the lines parallel and comparable. The regression lines (Fig. 5.4) of Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates did not differ significantly ($F_{6,41}$ = 0.398; P = 0.876) from each other.

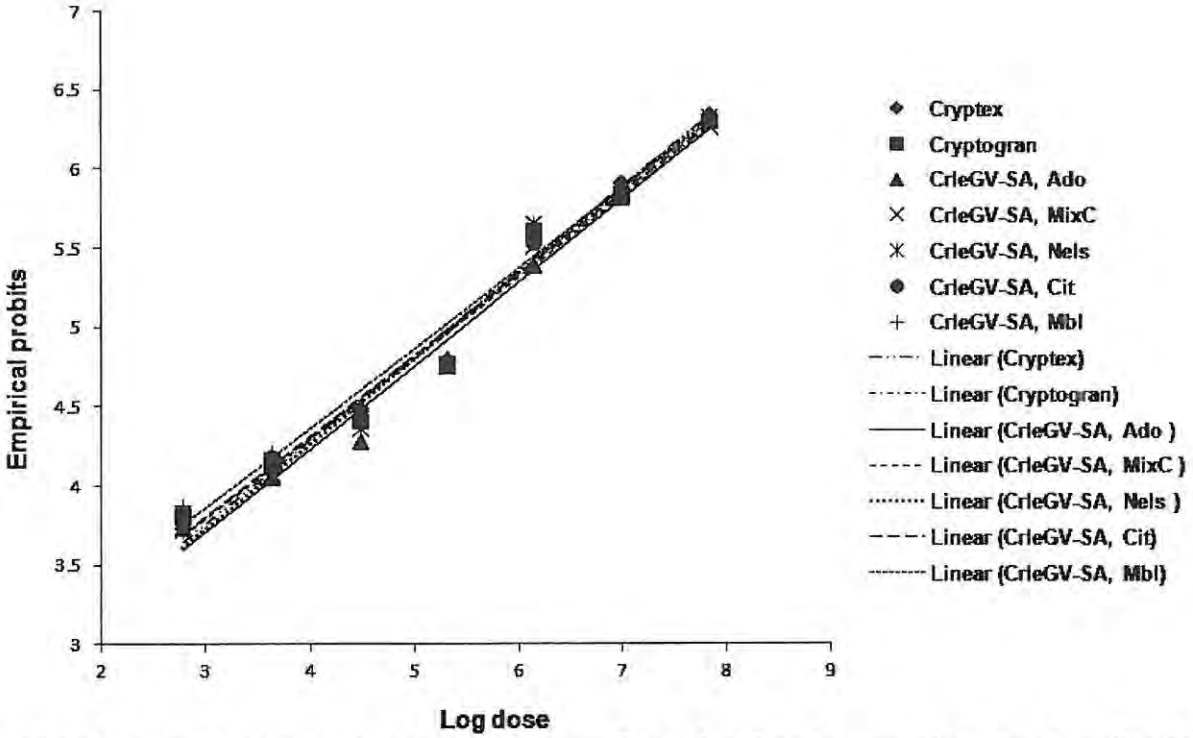

Figure 5.4 Log-dose-probit regression lines fitted for Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit against neonate larvae from the Marble Hall colony.

The mean LC_{50} values of Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates ranged between $2.03 \times 10^5 - 2.59 \times 10^5$ OBs/ml (Table 5.3). Their LD_{50} values ranged from 0.89 – 1.43 OBs per larva (Table 5.3).

Table 5.3 Bioassay data and associated statistics for neonate FCM larvae from the Marble Hall colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	LC_{50} (OBs/ml)	95% Fiducial limits		χ^2	df	*P*	LC_{90} (OBs/ml)	LD_{50} (OBs)	LD_{90} (OBs)
		Lower	Upper						
Cryptex	2.32×10^5	1.18×10^5	4.55×10^5	2.11	5	0.84	5.79×10^7	1.02	254.89
Cryptogran	2.47×10^5	1.23×10^5	4.96×10^5	3.37	5	0.65	7.67×10^7	1.09	337.70
CrleGV-SA Ado	3.25×10^5	1.65×10^5	6.48×10^5	1.77	5	0.88	8.60×10^7	1.43	378.49
CrleGV-SA MixC	2.59×10^5	1.29×10^5	5.24×10^5	2.26	5	0.81	8.51×10^7	1.14	374.62
CrleGV-SA Nels	2.55×10^5	1.28×10^5	5.03×10^5	3.07	5	0.69	5.57×10^7	1.12	244.86
CrleGV-SA Cit	2.40×10^5	1.19×10^5	4.82×10^5	2.11	5	0.83	7.63×10^7	1.05	335.72
CrleGV-SA Mbl	2.03×10^5	1.00×10^5	4.09×10^5	2.69	5	0.75	6.97×10^7	0.89	306.63

NB: χ^2: Chi-square goodness of fit, df: degrees of freedom for chi-square, *P*: Probability of a greater chi-square

5.2.4 Dose-response bioassays with Group one and two CrleGV-SA against neonates from the Nelspruit colony

In droplet feeding dose-response bioassays on Moore's FCM diet using Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates against neonate larvae from the Nelspruit colony, a dose-response relationship was established. The seven CrleGV-SA inocula concentrations ranged between 6.07 $x10^2$ and 7.14x 10^7 OBs/ml. Control mortality ranged between 0% and 3% for all replicates. Mortality of larvae inoculated with the seven CrleGV-SA inocula ranged from 10.0% - 91.41% for the lowest and highest concentrations, respectively.

Mortality of neonate larvae from the Nelspruit colony was related to dosage for all seven CrleGV-SA inocula. The regression lines fitted to the corrected data for all replicates compared, the residual variances of the lines were determined to be homogenous and the slopes of the lines parallel and comparable. The regression lines (Fig. 5.5) of Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates did not differ significantly ($F_{6,41} = 0.191$; $P = 0.978$) from each other.

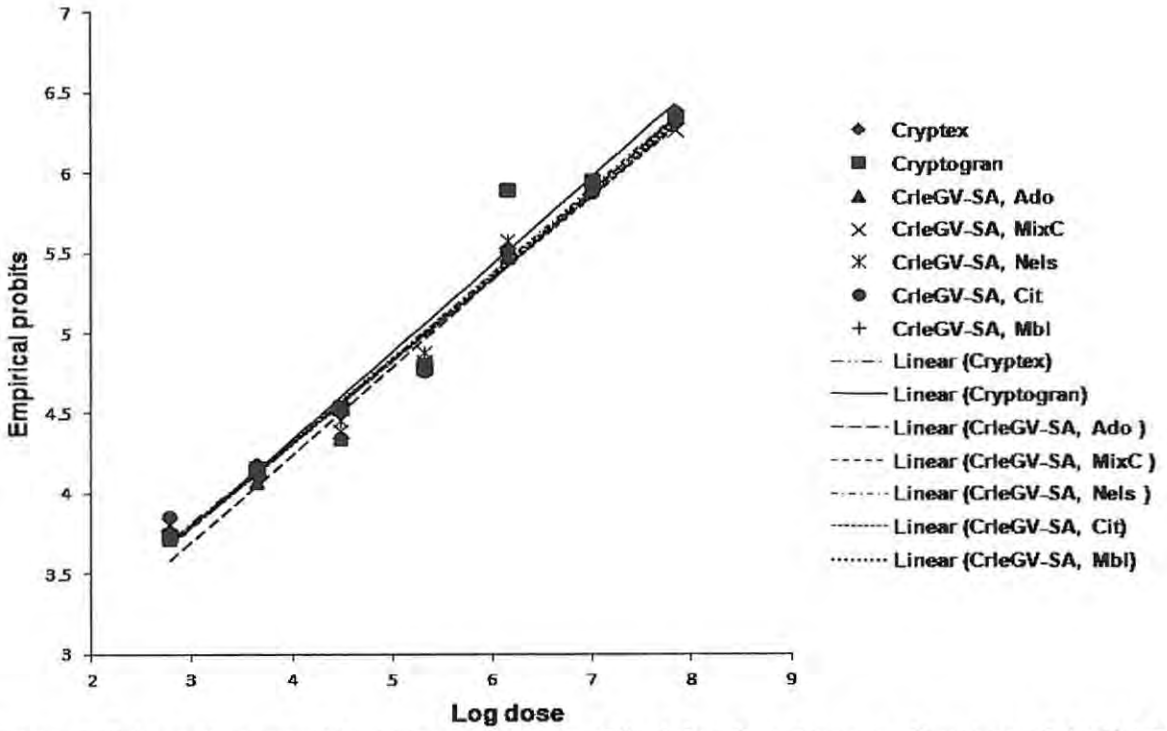

Figure 5.5 Log-dose-probit regression lines fitted for Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit against neonate larvae from the Nelspruit colony.

The mean LC_{50} values of Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates ranged between 2.21×10^5 and 2.74×10^5 OBs/ml (Table 5.4). Their LD_{50} values ranged from 0.94 – 1.21 OBs per larva (Table 5.4).

Table 5.4 Bioassay data and associated statistics for neonate FCM larvae from the Nelspruit colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	LC_{50} (OBs/ml)	95% Fiducial limits		χ^2	df	*P*	LC_{90} (OBs/ml)	LD_{50} (OBs)	LD_{90} (OBs)
		Lower	Upper						
Cryptex	2.30×10^5	1.12×10^5	4.68×10^5	1.54	5	0.91	7.64×10^7	1.01	335.98
Cryptogran	2.25×10^5	1.13×10^5	4.45×10^5	1.53	5	0.90	6.18×10^7	0.99	272.06
CrleGV-SA Ado	2.74×10^5	1.38×10^5	5.42×10^5	1.93	5	0.86	5.85×10^7	1.21	257.54
CrleGV-SA MixC	2.41×10^5	1.20×10^5	4.82×10^5	2.10	5	0.84	7.43×10^7	1.06	327.13
CrleGV-SA Nels	2.13×10^5	1.08×10^5	4.17×10^5	1.22	5	0.94	5.44×10^7	0.94	239.38
CrleGV-SA Cit	2.53×10^5	1.22×10^5	5.16×10^5	3.24	5	0.66	7.06×10^7	1.11	310.85
CrleGV-SA Mbl	2.21×10^5	1.11×10^5	4.41×10^5	1.12	5	0.95	6.42×10^7	0.97	282.30

NB: χ^2: Chi-square goodness of fit. df: degrees of freedom for chi-square. *P*: Probability of a greater chi-square

5.2.5 Dose-response bioassays with Group one and two CrleGV-SA against neonates from the Mixed colony

In droplet feeding dose-response bioassays on Moore's FCM diet using Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates against neonate larvae from the Mixed colony, a dose-response relationship was established. The seven CrleGV-SA inocula concentrations ranged between 6.07 $x10^2$ and 7.14x 10^7 OBs/ml. Control mortality ranged between 0% and 2% for all replicates. Mortality of larvae inoculated with the seven CrleGV-SA inocula ranged from 10.71% - 93.06% for the lowest and highest concentrations, respectively.

Mortality of neonate larvae from the Mixed colony was related to dosage for all seven CrleGV-SA inocula. The regression lines fitted to the corrected data for all replicates compared, the residual variances of the lines were determined to be homogenous and the slopes of the lines parallel and comparable. Using the Bonferroni method to compare slopes of the regression lines (Fig. 5.6) between isolates, PROBAN established the slopes to be significantly different ($F_{6,41} = 2.642$; $P = 0.029$). However, relative potency comparisons between isolates, did not establish significant difference.

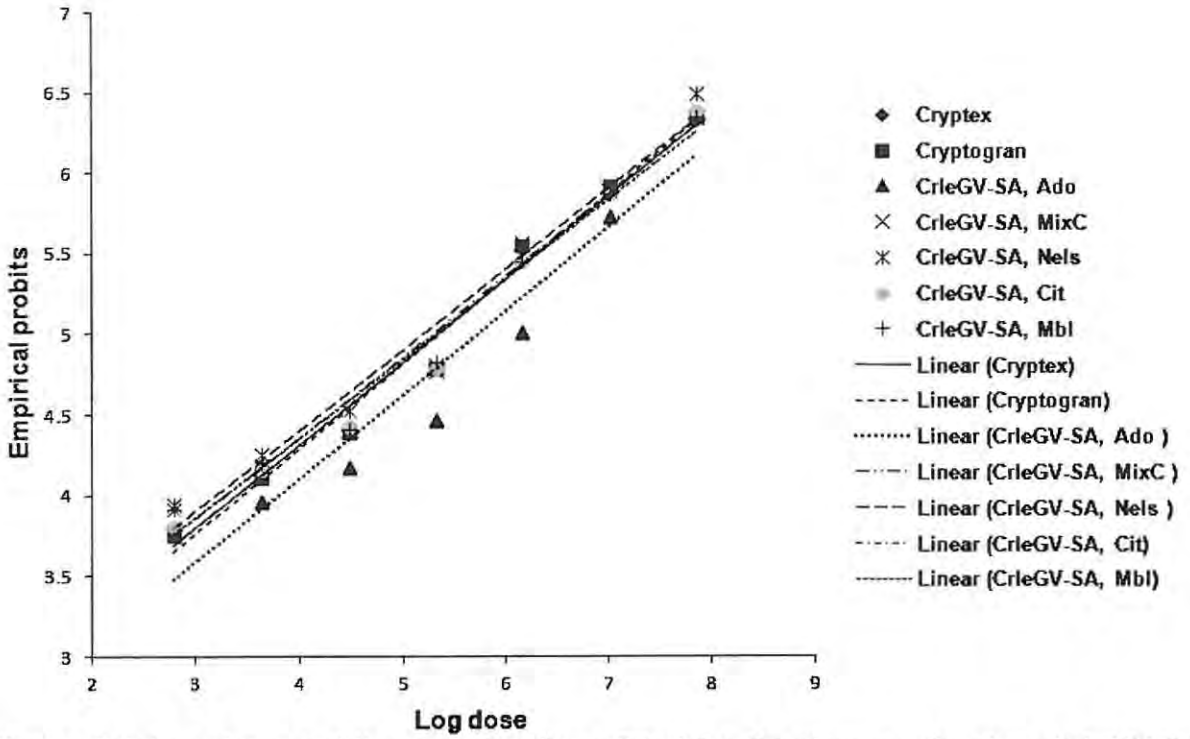

Figure 5.6 Log-dose-probit regression lines fitted for Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit against neonate larvae from the Mixed colony.

The mean LC_{50} values of Cryptex, Cryptogran and the CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates ranged between 2.15×10^5 and 6.86×10^5 OBs/ml (Table 5.5). Their LD_{50} values ranged from 0.79 – 3.02 OBs per larva. However, CrleGV-SA Ado recorded the highest LD_{50} value of viral 3.02 OBs per larva (Table 5.5).

Table 5.5 Bioassay data and associated statistics for neonate FCM larvae from the Mixed colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	LC_{50} (OBs/ml)	95% Fiducial limits		χ^2	df	*P*	LC_{90} (OBs/ml)	LD_{50} (OBs)	LD_{90} (OBs)
		Lower	Upper						
Cryptex	2.43×10^5	1.20×10^5	4.92×10^5	2.04	5	0.84	7.37×10^7	1.07	324.42
Cryptogran	2.41×10^5	1.23×10^5	4.75×10^5	2.45	5	0.78	6.15×10^7	1.06	270.70
CrleGV-SA Ado	6.86×10^5	3.42×10^5	1.42×10^6	6.48	5	0.26	1.71×10^8	3.02	754.57
CrleGV-SA MixC	2.15×10^5	1.06×10^5	4.35×10^6	3.76	5	0.58	7.52×10^7	0.95	331.02
CrleGV-SA Nels	1.80×10^5	8.78×10^5	3.68×10^5	2.92	5	0.71	6.99×10^7	0.79	307.49
CrleGV-SA Cit	2.44×10^5	1.22×10^5	4.91×10^5	2.00	5	0.85	7.56×10^7	1.08	332.67
CrleGV-SA Mbl	2.25×10^5	1.09×10^5	4.63×10^5	2.51	5	0.77	9.18×10^7	0.99	403.81

NB: χ^2: Chi-square goodness of fit. df: degrees of freedom for chi-square. *P*: Probability of a greater chi-square

5.2.6 Comparison of LC_{50} values from dose-response bioassays with Group one and two CrleGV-SA against neonates from five laboratory colonies

Dosage-mortality bioassays conducted with seven CrleGV-SA isolates against neonates from the Addo colony showed no significant differences in their LC_{50} values (Table 5.1). However, the LC_{50} values of Cryptex was 3.11-, 2.87-, 2.66-, 2.53-, and 2.26-fold lower than CrleGV-SA Mbl, CrleGV-SA Nels, CrleGV-SA Cit, Cryptogran and CrleGV-SA Ado, against neonates from the Addo colony respectively. The LC_{50} values of CrleGV-SA MixC isolate was also 3.76-, 3.47-, 3.22-, 3.06- and 2.74-fold lower than CrleGV-SA Mbl, CrleGV-SA Nels, CrleGV-SA Cit, Cryptogran and CrleGV-SA Ado, against neonates from the Addo colony respectively. The concentration required to elicit 50% mortality in neonates from the Addo colony with Cryptex and CrleGV-SA MixC was slightly higher than the other five CrleGV-SA. Again for each neonate larva from this colony inoculated with Cryptex or CrleGV-SA MixC, approximately 3 OB was required to elicit 50% mortality. By contrast, 1 OB from CrleGV-SA Mbl, CrleGV-SA Nels, CrleGV-SA Cit, Cryptogran and CrleGV-SA Ado was enough to cause the same effect.

For neonate larvae from the Citrusdal, Nelspruit and Marble Hall colonies inoculated with Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit there was no significant difference in the number of OBs required to elicit a 50% mortality (LD_{50}) in all test insects, as approximately 1 OB was sufficient to cause the desired effect (Table 5.2, 5.3 & 5.4). On the other hand, the LC_{50} values of CrleGV-SA Ado was found to be 3.82-, 3.18-, 3.05-, 2.85-, 2.82- and 2.79-fold lower than CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Mbl, Cryptogran, Cryptex and CrleGV-SA Cit, against neonates from the Mixed colony respectively (Table 5.5). Based on LC_{50} values, the concentration required by CrleGV-SA Ado isolate to elicit 50% mortality in neonates from the Mixed colony was slightly higher than the other six CrleGV-SA. Subsequently, a single neonate larva from this colony, inoculated with CrleGV-SA Ado, required an estimated 3 OB to cause 50% mortality in insects tested, in contrast to the approximately 1 OB required by insects fed with CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Mbl, Cryptogran, Cryptex and CrleGV-SA Cit.

5.2.7 Time-response bioassays with Group one and two CrleGV-SA against neonates from the Addo colony

The median survival time (ST_{50}) of neonates from the Addo colony inoculated with Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit ranged between 80 - 88 hours (Table 5.8).

Table 5.6 Bioassay data and associated statistics for neonate FCM larvae from the Addo colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	*n*	ST_{50} (hours)	95% CI Lower	Upper
CrleGV-SA Ado	135	88	87.00	88.99
Cryptogran	136	88	86.88	89.12
Cryptex	138	88	87.66	89.66
CrleGV-SA Nels	133	88	87.17	88.83
CrleGV-SA Mbl	141	84	83.13	84.87
CrleGV-SA Cit	136	80	79.17	80.84
CrleGV-SA MixC	132	88	86.99	89.01

Multiple comparisons of survival line of slopes from mortality-time data were conducted using Log-rank (Mantel-Cox) tests. There were no significant difference between the survival curves (Fig. 5.7) for AdoTex and AdoGra lines (χ^2 = 0.20; df = 1; P = 0.652), AdoTex and AdoNel lines (χ^2 = 0.60; df = 1; P = 0.438) and AdoTex and AdoMbl lines (χ^2 = 0.36; df = 1; P = 0.548), AdoTex and AdoAdo lines (χ^2 = 0.18; df = 1; P = 0.666), AdoTex and AdoCit lines (χ^2 = 0.53; df = 1; P = 0.467) and AdoTex and AdoMixC lines (χ^2 = 0.11; df = 1; P = 0.741). No significant differences were observed between the survival curves for AdoGra and AdoAdo lines (χ^2 = 0.24; df = 1; P = 0.62), AdoGra and AdoNel lines (χ^2 = 0.11; df = 1; P = 0.738), AdoGra and AdoCit lines (χ^2 = 0.08; df = 1; P = 0.778), AdoGra and AdoMbl lines (χ^2 = 0.04; df = 1; P = 0.846) and AdoGra and AdoMixC lines (χ^2 = 0.26; df = 1; P = 0.61). Percentage survival in the controls was 85.37% (Fig. 5.7).

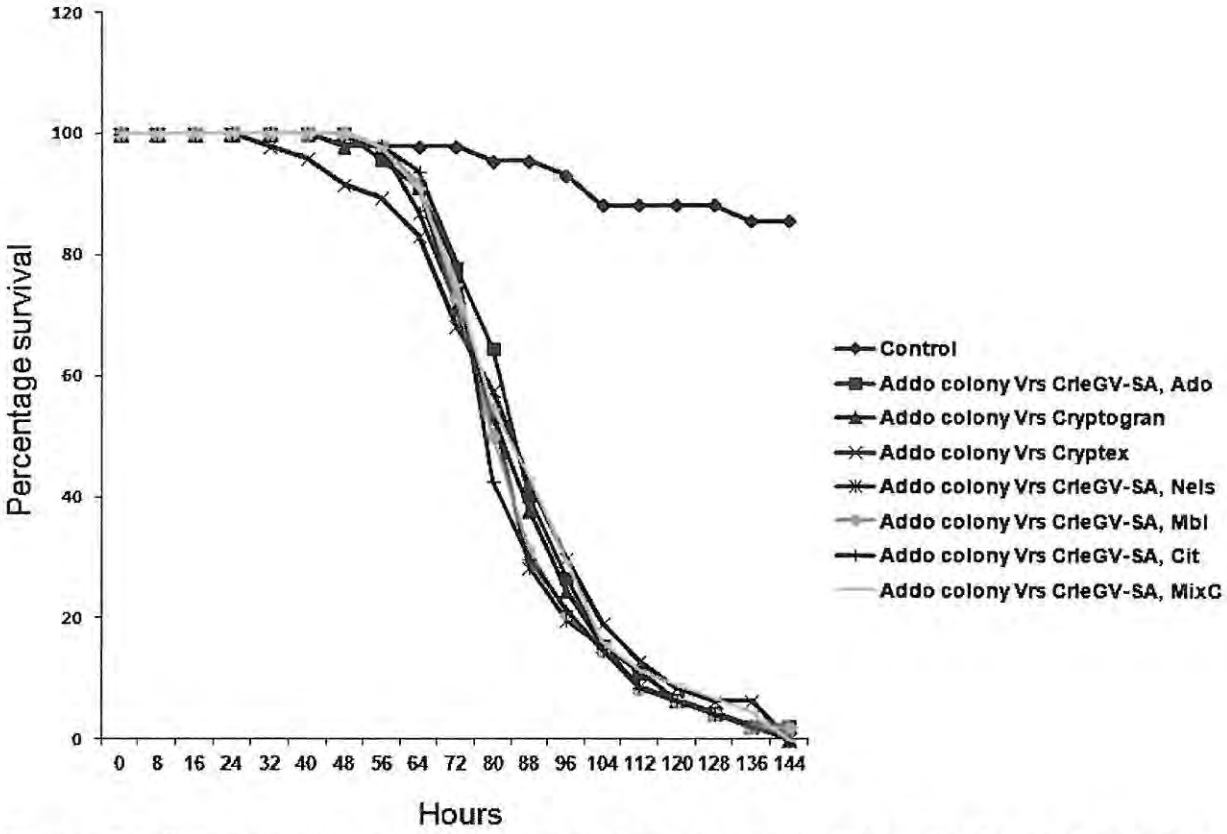

Figure 5.7 Survival curves for Cryptogran, Cryptex, and the CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit against neonate larvae from the Addo colony. Each death is shown as a drop in survival.

5.2.8 Time-response bioassays with Group one and two CrleGV-SA against neonates from the Citrusdal colony

The median survival time (ST_{50}) of neonates from the Citrusdal colony inoculated with Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit was determined to be 88 hours (Table 5.9).

Table 5.7 Bioassay data and associated statistics for neonate FCM larvae from the Citrusdal colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	*n*	ST_{50} (hours)	95% CI Lower	Upper
CrleGV-SA Ado	136	88	87.37	89.66
Cryptogran	133	88	87.35	89.71
Cryptex	130	88	87.65	89.65
CrleGV-SA Nels	138	88	87.66	89.66
CrleGV-SA Mbl	133	88	87.39	89.59
CrleGV-SA Cit	132	88	87.34	89.69
CrleGV-SA MixC	136	88	87.31	89.78

Multiple comparisons of survival line of slopes from mortality-time data were conducted using Log-rank (Mantel-Cox) tests. There were no significant differences between the survival curves (Fig. 5.8) for CitTex and CitAdo lines (χ^2 = 0.01; df = 1; *P* = 0.918), CitTex and CitGra lines (χ^2 = 0.04 df = 1; *P* = 0.836), CitTex and CitNel lines (χ^2 = 0.10; df = 1; *P* = 0.792), CitTex and CitMbl lines (χ^2 = 0.02; df = 1; *P* = 0.886), CitTex and CitCit lines (χ^2 = 0.04; df = 1; *P* = 0.850) and CitTex and CitMixC lines (χ^2 = 0.28; df = 1; *P* = 0.596). There were no significant differences between the survival curves for CitGra and CitAdo lines (χ^2 = 0.07; df = 1; *P* = 0.792), CitGra and CitNel lines (χ^2 = 0.02; df = 1; *P* = 0.889), CitGra and CitMbl lines (χ^2 = 0.19; df = 1; *P* = 0.681), CitGra and CitCit lines (χ^2 = 0.04; df = 1; *P* = 0.836) and CitGra and CitMixC lines (χ^2 = 0.16; df = 1; *P* = 0.687). Percentage survival in the controls was 78.58% (Fig. 5.8)..

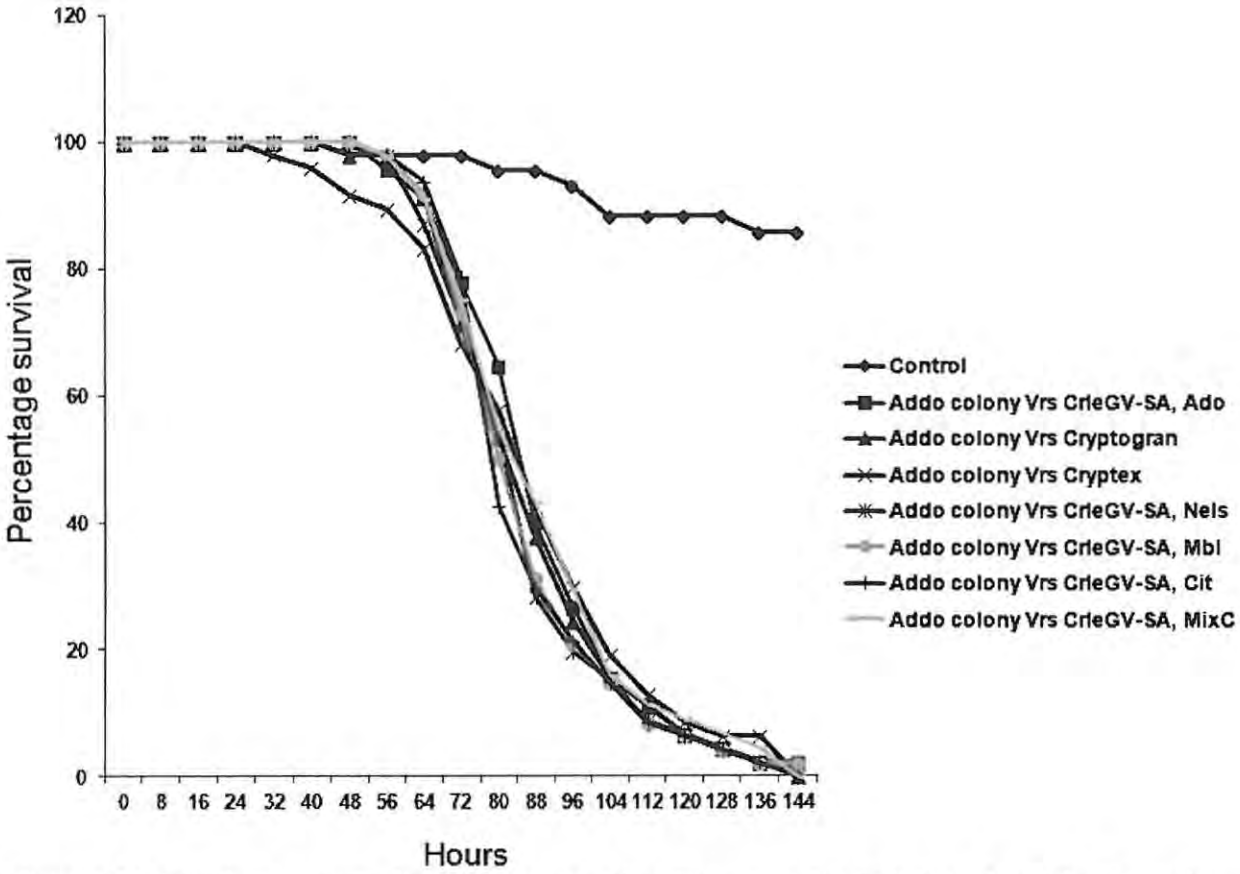

Figure 5.8 Survival curves for Cryptogran, Cryptex, and the CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit against neonate larvae from the Citrusdal colony. Each death is shown as a drop in survival.

5.2.9 Time-response bioassays with Group one and two CrleGV-SA against neonates from the Marble Hall colony

The median survival time (ST_{50}) of neonates from the Marble Hall colony inoculated with Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA Ado, CrleGV-SA MixC, CrleGV-SA Mbl and CrleGV-SA Cit was recorded to be 88 hours (Table 5.10).

Table 5.8 Bioassay data and associated statistics for neonate FCM larvae from the Marble Hall colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	*n*	ST_{50} (hours)	95% CI Lower	Upper
CrleGV-SA Ado	132	88	87.11	88.89
Cryptogran	131	88	86.97	89.03
Cryptex	134	88	87.66	89.65
CrleGV-SA Nels	137	88	86.86	89.14
CrleGV-SA Mbl	130	88	87.04	88.96
CrleGV-SA Cit	135	88	86.96	89.04
CrleGV-SA MixC	141	88	86.83	89.17

Multiple comparisons of survival line of slopes from mortality-time data were conducted using Log-rank (Mantel-Cox) tests. There were no significant differences between the survival curves (Fig. 5.9) for MblTex and MblAdo lines (χ^2 = 0.010; df = 1; *P* = 0.748), MblTex and MblGra lines (χ^2 = 0.19 df = 1; *P* = 0.662), MblTex and MblNel lines (χ^2 = 0.31 df = 1; *P* = 0.5784), MblTex and MblMbl lines (χ^2 = 0.02; df = 1; *P* = 0.875), MblTex and MblCit lines (χ^2 = 0.03; df = 1; *P* = 0.858) and MblTex and MblMixC lines (χ^2 = 0.43; df = 1; *P* = 0.512). There were no significant differences between the survival curves for MblGra and MblAdo lines (χ^2 = 0.01; df = 1; *P* = 0.909), MblGra and MblNel lines (χ^2 = 0.01; df = 1; *P* = 0.897), MblGra and MblMbl lines (χ^2 = 0.36; df = 1; *P* = 0.544), MblGra and MblCit lines (χ^2 = 0.05; df = 1; *P* = 0.816) and MblGra and MblMixC lines (χ^2 = 0.04; df = 1; *P* = 0.837). Percentage survival in the controls was 83.73% (Fig. 5.9).

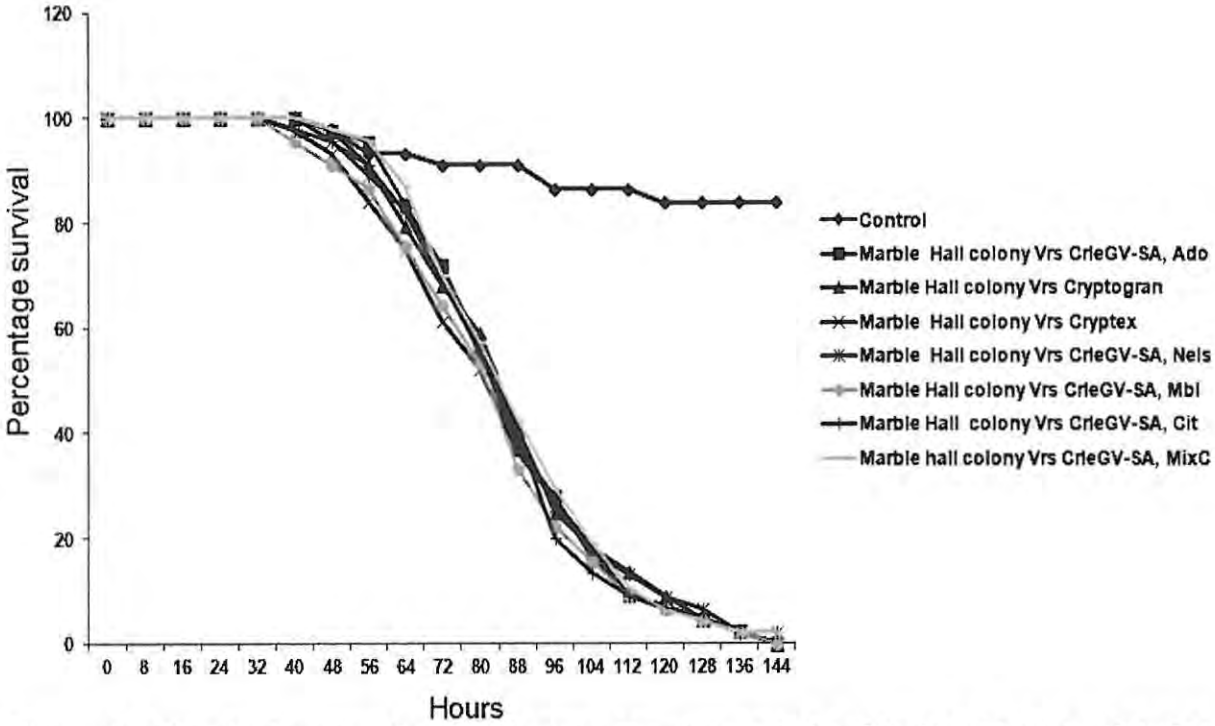

Figure 5.9 Survival curves for Cryptogran, Cryptex and the CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit against neonate larvae from the Marble Hall colony. Each death is shown as a drop in survival.

5.2.10 Time-response bioassays with Group one and two CrleGV-SA against neonates from the Mixed colony

The median survival time (ST_{50}) of neonates from the Mixed colony inoculated with Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA Ado, CrleGV-SA MixC, CrleGV-SA Mbl and CrleGV-SA Cit ranged between 80 - 88 hours (Table 5.11).

Table 5.9 Bioassay data and associated statistics for neonate FCM larvae from the Mixed colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	*n*	ST_{50} (hours)	95% CI Lower Upper
CrleGV-SA Ado	138	88	87.06 – 88.94
Cryptogran	130	88	86.88 – 89.12
Cryptex	133	88	87.17 – 88.83
CrleGV-SA Nels	133	88	87.12 – 88.88
CrleGV-SA Mbl	137	88	87.24 – 88.76
CrleGV-SA Cit	129	88	87.07 – 88.93
CrleGV-SA MixC	135	80	79.18 – 80.82

Multiple comparisons of survival line of slopes from mortality-time data were conducted using Log-rank (Mantel-Cox) tests. There were no significant differences between the survival curves (Fig. 5.10) for MixCTex and MixCAdo lines ($\chi^2 = 0.07$; df = 1; $P = 0.788$), MixCTex and MixCGra lines ($\chi^2 = 0.21$ df = 1; $P = 0.648$), MixCTex and MixCNel lines ($\chi^2 = 0.28$ df = 1; $P = 0.597$), MixCTex and MixCMbl lines ($\chi^2 = 0.1.17$; df = 1; $P = 0.279$), MixCTex and MixC MixC lines ($\chi^2 = 0.66$; df = 1; $P = 0.416$) and MixCTex and MixCCit lines ($\chi^2 = 0.09$; df = 1; $P = 0.753$). There were also no significant differences between the survival curves for MixCGra and MixCAdo lines ($\chi^2 = 0.57$; df = 1; $P = 0.447$), MixCGra and MblNel lines ($\chi^2 = 0.01$; df = 1; $P = 0.962$), MixCGra and MixCMbl lines ($\chi^2 = 0.40$; df = 1; $P = 0.526$), MixCGra and MixCCit lines ($\chi^2 = 0.02$; df = 1; $P = 0.892$) and MixCGra and MixCMixC lines ($\chi^2 = 0.15$; df = 1; $P = 0.700$). Percentage survival in the controls was 84.88% (Fig. 5.10).

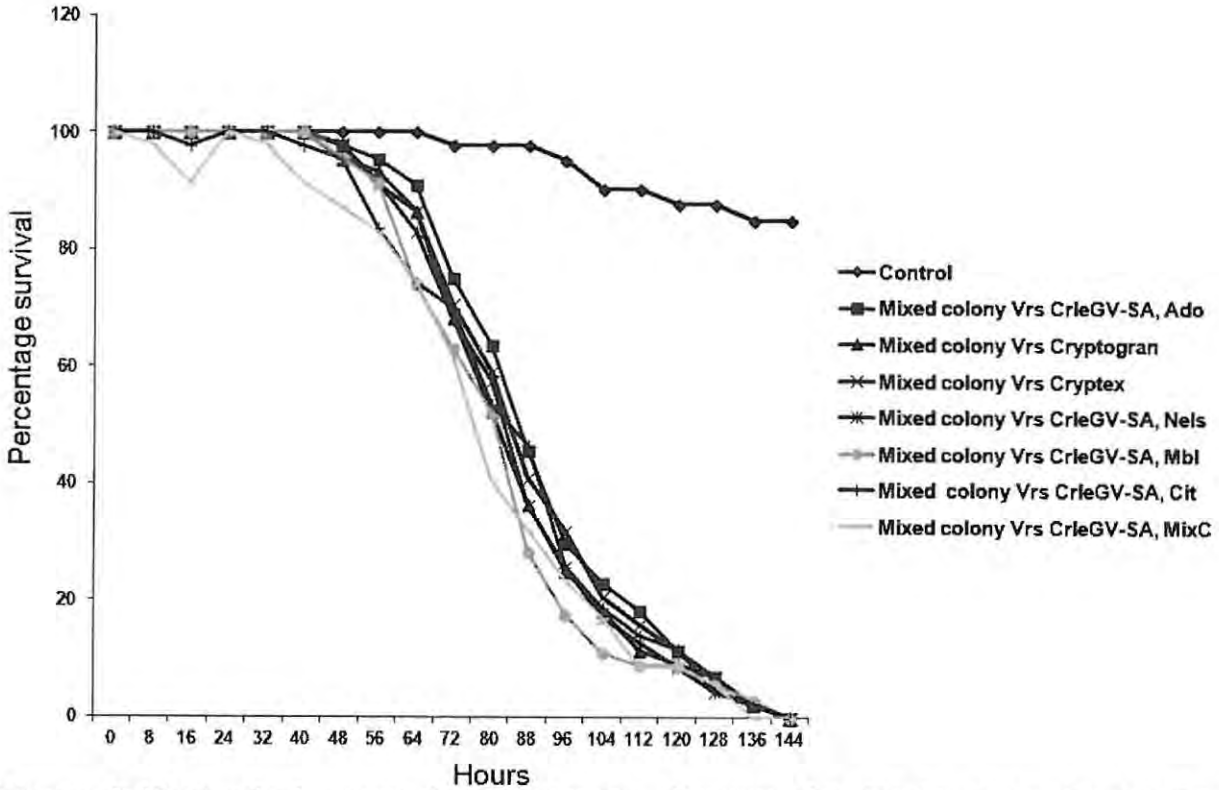

Figure 5.10 Survival curves for Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit against neonate larvae from the Mixed colony. Each death is shown as a drop in survival.

5.2.11 Time-response bioassays with Group one and two CrleGV-SA against neonates from the Nelspruit colony

The median survival time (ST_{50}) of neonates from the Nelspruit colony inoculated with Cryptogran, Cryptex, CrleGV-SA Nels, CrleGV-SA Ado, CrleGV-SA Cit, CrleGV-SA Mbl and CrleGV-SA MixC was recorded to be 88 hours (Table 5.12).

Table 5.10 Bioassay data and associated statistics for neonate FCM larvae from the Nelspruit colony in a dose-response bioassay with seven CrleGV-SA inocula

CrleGV-SA Inocula	*n*	ST_{50} (hours)	95% CI Lower	Upper
CrleGV-SA Ado	136	88	87.16	88.84
Cryptogran	130	88	87.09	88.91
Cryptex	133	88	87.10	88.90
CrleGV-SA Nels	139	88	86.79	89.21
CrleGV-SA Mbl	138	88	86.89	89.11
CrleGV-SA Cit	139	88	86.93	89.07
CrleGV-SA MixC	131	88	86.99	89.01

There were no significant differences between the survival curves (Fig. 5.11) for NelCTex and NelAdo lines (χ^2 = 0.48; df = 1; P = 0.486), NelTex and NelGra lines (χ^2 = 0.16 df = 1; P = 0.688), NelTex and NelNel lines (χ^2 = 0.61 df = 1; P = 0.435), NelTex and NelMbl lines (χ^2 = 0.19; df = 1; P = 0.658), NelTex and NelCit lines (χ^2 = 0.08; df = 1; P = 0.771) and NelTex and NelMixC lines (χ^2 = 0.03; df = 1; P = 0.854). There were no significant differences between the survival curves for NelGra and NelAdo lines (χ^2 = 0.08; df = 1; P = 0.779), NelGra and NelNel lines (χ^2 = 0.05; df = 1; P = 0.826), NelGra and NelMbl lines (χ^2 = 0.01; df = 1; P = 0.925), NelGra and NelCit lines (χ^2 = 0.06; df = 1; P = 0.811) and NelGra and NelMixC lines (χ^2 = 0.18; df = 1; P = 0.673). Percentage survival in the controls was 90.64% (Fig. 5.11).

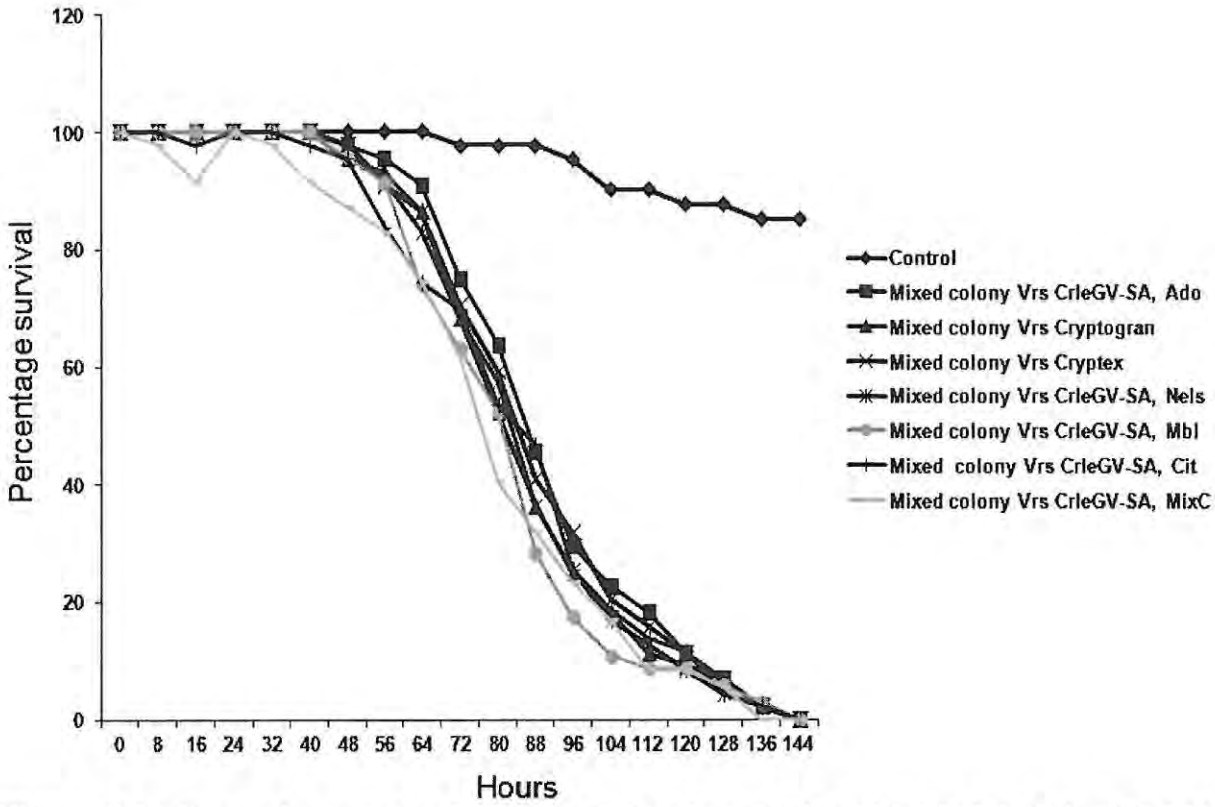

Figure 5.11 Survival curves for Cryptogran, Cryptex and the CrleGV-SA Nels, CrleGV-SA MixC, CrleGV-SA Ado, CrleGV-SA Mbl and CrleGV-SA Cit against neonate larvae from the Nelspruit colony. Each death is shown as a drop in survival.

5.3 DISCUSSION

This chapter describes an investigation of the comparative virulence of Group one and two CrleGV-SA against five laboratory populations of FCM. The modified version of the droplet feeding bioassay technique developed by Hughes & Wood (1981) and described by Jones *et al.* (1993) was used.

In surface inoculation dose-response bioassays, Moore *et al.* (2011) determined the LC_{50} for FCM neonates to be 7.10×10^3 OBs/ml. The LC_{50} values for the seven CrleGV-SA reported in this study, ranged between $1.45 \times 10^5 - 7.10 \times 10^5$ OBs/ml and were substantially higher than that reported by Moore *et al.* (2011). Interestingly, the LC_{50} values obtained in this study were similar to the LC_{90} value (1.185×10^5 OBs/ml) reported by Moore *et al.* (2011). Differences in the LD_{50} values for Group one and two

CrleGV-SA were observed, and were estimated to range from 0.80 - 3.12 OBs per larva. Similar variations in OB values have been reported by other researchers for CpGV. For example, Steineke (2004) reported the LD_{50} for CpGV as ranging between 0.6 – 1.3 OBs per larva. On the other hand, Sheppard & Stairs (1977) recorded an LD_{50} value for CpGV of 5 OBs per larva, while Crook *et al.* (1985) reported an LD_{50} value of 3 OBs per larva for CpGV.

The median survival times (ST_{50}) for neonate FCM larvae did not differ substantially from each other and was determined to range between 80 – 88 hours (3.33 – 3.67 days), for all five colonies. The ST_{50} values obtained in this study, were similar to that reported by Sciocco-cap *et al.* (2001) in droplet assays with EpapGV (*Epinotia aporema* GV) against neonate *Epinotia aporema* (Wals) which ranged between 85.6 – 94.4 hours (3.56 – 3.93 days). In another study, and using virus-diet incorporation bioassays, the LT_{50} for CpGV was estimated to be 3.3 days (Eastwell *et al.*, 1999). However, Moore *et al.* (2011) estimated the LT_{50} value for CrleGV-SA against FCM neonates to be 4 days 22 hours, using surface inoculation dose-response bioassays.

Differences in bioassay results between this study and that of Moore *et al.* (2011), for FCM neonates, may be attributed to a number of factors. For example, the different assay techniques employed between this study and that of Moore *et al.* (2011) may have contributed to the observed differences in assay results. In Moore's *et al.* (2011) study, the virus-suspension was inoculated on the surface of the diet as opposed to the virus being administered directly as droplet suspensions for larvae to ingest. Moore *et al.* (2011) argued that, since FCM is an internal feeder the method of administering virus on the surface of diets was more reflective of a typical field scenario where the virus is applied onto the surface of fruit. Therefore, the only chance of larvae coming into contact with the virus was restricted to the fruit surface.

Neonate larvae placed on the surface of CrleGV-SA inoculated diet may have wandered on the diet surface, ingesting bits and pieces of the virus-suspension before finally boring into the diet. This cumulative feeding rate may have rendered the LC_{50} values reported by Moore *et al.* (2011) sufficient to elicit 50% mortality in FCM neonates. The

droplet feeding bioassay method, however, prevents a cumulative feeding rate in larvae and has an added advantage of being able to estimate the dosage ingested by a single larva (Hughes *et al.*, 1986; Jones, 2000).

5.4 CONCLUSION

In this study, some level of variation in FCM larval susceptibility to different CrleGV-SA isolates was observed in two populations. However, these differences were not wide spread or substantial enough to support the view that different CrleGV-SA isolates vary in their virulence against different FCM populations.

CHAPTER SIX

GENERAL DISCUSSION

6.0 INTRODUCTION

The false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) is a serious pest of economic importance, which attacks a wide range of crops in sub-Saharan Africa. Two biopesticides, Cryptogran (Moore *et al.,* 2004a) and Cryptex (Kessler & Zingg, 2008) both formulated with the FCM baculovirus, *Cryptophlebia leucotreta* granulovirus (CrleGV) are used to control this pest in South Africa. Studies conducted for this thesis aimed to investigate whether there are meaningful differences in the susceptibility of FCM populations originating from different geographic regions to different CrleGV-SA isolates. The other aim was to bioprospect for more CrleGV-SA isolates to serve as substitute viral inocula in the event of FCM developing resistance to these biopesticides. The significant finding from this study was the recovery of five genetically different CrleGV-SA isolates from five geographic FCM populations, with some insect populations showing differences in susceptibility to the new CrleGV-SA isolates as well as the existing isolates (Cryptogran and Cryptex). This chapter intends to provide a summary of the significant findings and discuss their implications for baculovirus research, and the management of FCM.

6.1 INSECT BIOLOGY AND BIOLOGICAL PERFORMANCE

Insects are the most diverse species of organisms in nature and play an important role by serving as biological tools to conduct research (Cohen, 2001). To facilitate their study, insects are commonly reared in the laboratory using fresh host material or on an artificial diet. An artificial diet, such as that developed for the rearing of FCM (Moore, 2002) has facilitated the execution of studies aimed at understanding the biology of this insect (Moore, 2002; Sishuba, 2003; Gendall, 2007; Opoku-Debrah, 2008). The larval stages of FCM cause considerable damage to a wide range of cultivated and wild plants (Newton, 1998; Begemann & Schoeman, 1999; Moore, 2002; Hattingh, 2006). Identification of this pest at any life stage is therefore essential for control purposes. The

egg (Daiber, 1979a; Newton, 1998; Moore, 2002), larva (Fuller, 1901; Catling & Aschenborn, 1978; Daiber, 1979b; Newton, 1990; Moore, 2002), pupa (Daiber, 1979c; Timm *et al.*, 2007) and adult (Fuller, 1901; Catling & Aschenborn, 1978; Diaber, 1980; Annecke & Moran, 1982; Couilloud, 1988; Timm, 2005b) stages of FCM have been described, with some morphological descriptions and visual aids lacking for some life stages. The morphological descriptions and visual aids described in Chapter two of this thesis for the identification of all the various FCM life stages would therefore help augment other existing methods.

The ease in conducting scientific experiments with laboratory reared insects, as opposed to wild-types, has enabled the conducting of studies aimed at understanding the biology and behaviour of insects (Huho *et al.*, 2007). In order to understand the biological performance of insect populations originating from different geographic regions, studies described in Chapter two of this thesis were carried out. Using parameters such as pupal mass, female fecundity, egg hatch, pupal survival and adult eclosion to measure the biological performance of five geographic FCM populations, differences were observed in one case. Insects from the Citrusdal colony recorded the lowest pupal mass, female fecundity, egg hatch, pupal survival, adult eclosion and longest duration in larval and pupal development by comparison with the other colonies investigated. Some authors have attributed variations in insect pupal mass to differences in temperature (Gilbert, 1984) or nutrition (Scriber & Slansky, 1981), which in turn can affect insect biological performance. High female pupal mass has also been correlated with improved fecundity (Berger *et al.*, 2008). However, these arguments were not supported in this thesis, as all insects were subjected to similar rearing conditions.

It is suggested that the low biological performance observed in insects from the Citrusdal colony may have a genetic origin. This observation may be attributed to the fact that FCM wild populations sampled from the Citrusdal region (Western Cape) have been shown to be genetically heterogeneous with other wild-types from other geographic regions in South Africa (Timm, 2005b). It is also known that FCM is not indigenous to the Citrusdal area and may have been introduced from a very limited

gene pool from another region(s) (Sean Moore, pers. comm). Other authors have reported genetic factors as influencing insect biological performance (Rose, 1978; Mason *et al.*, 1987; Soldaat & Vrieling, 1992). However, one cannot just assume that the performance of an insect in the laboratory is entirely reflective of its performance in the field. Therefore, when differences in the biological performance of an insect population are observed in the laboratory, a follow up investigation in the field is essential.

6.2 LATENCY AND EPIZOOTICS OF BACULOVIRUS DISEASE IN INSECT POPULATIONS

When insects are reared in the laboratory, the incidence of baculovirus disease in their larval populations has been widely reported (Hukuhara & Aruga, 1959; Steinhaus & Dineen, 1960; Smith, 1967; Adams & McClintock, 1991; Cory *et al.*, 1997). The impact of these epizootics in field populations of insects is important for natural host population supression (Fuxa, 1989). Other studies have helped our understanding of the ecology of baculovirus-host interactions (Cory *et al.*, 1997; Cory & Myers, 2003). In the field, baculovirus epizootics have been observed with forest lepidopteran insects such as, the western tent caterpillar, *Malacosoma californicum* pluviale (Dyer) (Hoch *et al.*, 2001; Cory & Myers, 2009) and the gypsy moth, *Lymantria dispar* (L.) (Elkinton, 1990; Hoch *et al.*, 2001). In the laboratory, virus outbreaks may occur serendipitously (Murillo *et al.*, 2011), and, in some cases, may be induced when insects are subjected to stress conditions (Steinhaus & Dineen, 1960; Aruga *et al.*, 1963; Adams & McClintock, 1991; Boots *et al.*, 2003).

Evidence from Chapter three of this thesis indicates that, latent non-lethal baculovirus infections resident in five geographic populations of laboratory reared FCM can be activated into an overt lethal state by overcrowding. In the field, increased population density, most notably with lepidopteran insects, has been reported as being the source of several baculoviral disease outbreaks (Tanada & Fuxa, 1987; Richards *et al.*, 1999; Il'inykh *et al.*, 2004). The observations made in Chapter three of this thesis shows that

the virus which appears to be in a latent non-lethal state can be induced in the laboratory and transferred between generations via vertical transmission.

It was also observed that, when insects are subjected to stress conditions, the larval stages succumb to infection. For example, within the same jars that contained diseased larvae, it was not uncommon to find some larvae successfully pupating and emerging as adults while other insects succumbed to infection as larvae. Even though the source of this low susceptibility was not investigated, evidence from other studies has shown that some mechanisms favouring an age-dependent increased resistance in matured larval stages of insects may influence infection (Evans, 1983; Federici, 1997; Grove & Hoover, 2007). For instance, the thick peritrophic membrane commonly found in older larval stages of insects has been shown to serve as a protective barrier against baculovirus infection (Federici, 1997; Cory & Myers, 2003). In one study, mature larvae of *L. dispar* were shown to slough-off baculovirus infected midgut cells, as a way to evade infection (McNeil *et al.*, 2010). By extrapolating these observations of resistant individuals arising from artificially induced epizootics to a macro level (in the field), other researchers have proposed a disease defence hypothesis as an explanation for the selection for resistant individuals during field population explosions and associated baculovirus epizootics (Cory *et al.*, 1997; Cory & Myers, 2009).

6.3 GENETIC VARIATION IN BACULOVIRUS ISOLATES

Baculoviruses isolated from insects at one time and location, are commonly referred to as 'isolates' (Cory *et al.*, 1997). Several studies have shown that baculovirus isolates recovered from diseased insects collected from the field (Weitzman *et al.*, 1992; Li *et al.*, 2002; Rezapanah *et al.*, 2008) or in laboratory cultures (Eastwell *et al.*, 1999; Murillo *et al.*, 2011), exhibit high levels of genetic variation. In some cases baculoviruses recovered from the same insect host species from different geographic locations (Rezapanah *et al.*, 2008; Espinel-Correal *et al.*, 2010; Patel *et al.*, 2010) as well as from a single insect larva (Cory *et al.*, 2005) show high levels of genetic variation. Evidence for differences in genetic composition of baculovirus isolates recovered from the same

insect host species from different geographic regions of South Africa is presented in Chapter four of this thesis.

Significant findings confirmed this phenomenon leading to the recovery of five CrleGV-SA isolates (CrleGV-SA Ado, CrleGV-SA Nels, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC) from five geographic FCM laboratory populations. RFLP analysis of total genomic DNA from all five isolates revealed that they were genetically different from each other and consisted of multiple CrleGV-SA genotypes as shown by the presence of unique submolar DNA bands. This genetic diversity in baculoviruses has been shown to be caused by small mutations (Chapter Four), sequence duplications and in some cases the acquisition of host DNA (Brown *et al.*, 1985).

6.4 DIFFERENCES IN THE GENOME OF BACULOVIRUS ISOLATES

Genetically different baculovirus isolates can be placed into different genome groups based on the presence of prominent bands observed in their DNA profiles following RFLP analysis or by the presence of single nucleotide polymorphisms (SNPs) in the sequences of specific genes. This has been reported for NPV (Takatsuka *et al.*, 2003; Redman *et al.*, 2010) and recently for GV (Eberle *et al.,* 2009). These studies were able to link polymorphisms observed in some baculovirus genes, such as *lef*-8, *lef*-9, *chi*A and *polh/gran* genes, to differences in their DNA profiles, providing evidence for defining separate genome groups. Chapter four of this thesis provides a similar analysis, where DNA profiles and phylogenetic analysis based on SNPs observed in the *granulin* and *egt* genes of Cryptex, Cryptogran and CrleGV-SA Ado, CrleGV-SA Cit, CrleGV-SA Mbl, CrleGV-SA Nels and CrleGV-SA Mix isolates revealed the presence of two distinct CrleGV-SA genome types for the CrleGV-SA isolate. Subsequently, Cryptex and the CrleGV-SA Ado, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC isolates were placed as members of the Group one CrleGV-SA, while Cryptogran and CrleGV-SA Nels isolate were placed into the Group two CrleGV-SA. These findings have broad implications for our understanding of the genetic diversity and evolution of CrleGV and, in a broader context, the genome diversity of baculoviruses in general. To augment the

findings in this thesis, sequencing the entire genome of these CrleGV-SA isolates is required.

6.5 BIOLOGICAL ACTIVITY AND SUBLETHAL EFFECTS OF BACULOVIRUSES

Several studies have shown that different baculovirus isolates can show marked differences in biological activity to their host, with virulence being correlated to increased mortality of the host (Parnell *et al.*, 2002; Lopez- Ferber *et al.*, 2003; Takatsuka *et al.*, 2003; Barreto *et al.*, 2005; Jehle *et al.*, 2008). In other instances, differences in viral virulence have been shown to be correlated to different baculovirus isolate genome types. This was shown with CpGV where viruses belonging to CpGV genome type C appeared to have a lower virulence than both type A and D CpGV genome types against CM (Eberle *et al.*, 2009). Interestingly, these authors also found CpGV genome type C to be the ancestral genome of genome types A, B and D (Eberle *et al.*, 2009). In this thesis, however, although in a few instances some variation in host susceptibility to different CrleGV-SA isolates was observed, evidence to support the general view that different CrleGV-SA isolates or genome groups show marked differences in their virulence against different FCM populations could not be supported.

On a different note, some authors argue that defining the virulence of a virus by its ability to cause mortality alone is not sufficient as other non-lethal (sub-lethal) effects of baculovirus infections also play a significant role in virus potency (Sait *et al.*, 1994; Goulson & Cory, 1995; Cory *et al.*, 1997; Boots *et al.*, 2003). Sub-lethal effects of baculovirus infections such as reduced fecundity in females, altered host development, reduced egg viability and changes in sex ratio have been shown to contribute to suppression of insect populations in the field (Cory *et al.*, 1997; Cory & Myers, 2003). These non-lethal infections should therefore be considered as other important avenues for future research.

6.6 CONCLUSION

Considering the observations made in this thesis, it is suggested that the conventional norm of applying single genetic isolates of baculoviruses in commercial applications be

reviewed in order to safeguard the integrity of baculovirus based biopesticides. With regard to Cryptogran or Cryptex, it is recommended that in order to protect against resistance development by FCM to these biopesticides, it would be advisable for farmers to alternate between these two biopesticides during field applications. To be more proactive, the application of specific CrleGV-SA isolates against different field populations of the host, or a mixture of isolates against any population may be a useful alternative to prevent or slow the development of resistance to a particular isolate. The technology for formulation and field application of these biopesticides already exists. Therefore, further studies involving the execution of field trials with the five newly discovered CrleGV-SA isolates are required in order to ascertain the potential of these isolates as candidate biopesticides.

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