Understanding and improving the residual efficacy of the *Cryptophlebia leucotreta* granulovirus (CRYPTOGRAN)

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Abstract

False codling moth (FCM), *Thaumatotibia* (=*Cryptophlebia*) *leucotreta* (Meyr) (Lepidoptera: Tortricidae), is one of the most important pests on citrus. The *Cryptophlebia leucotreta* granulovirus (CrleGV) has been developed into a successful biological control agent, registered under the name Cryptogran, and is currently the preferred product for the control of FCM on citrus in South Africa. A prerequisite to the continued success of Cryptogran as a means of controlling false codling moth is to understand the factors affecting field persistence of the virus, and to find ways to improve it. The aim of this study was to gain a clearer understanding of the product and the abiotic and biotic factors affecting its persistence in the field, and to investigate methods to improve this persistence. The effect of UV-irradiation on the virus was determined, and various products were tested as UV protectants in laboratory bioassays. Lignin was the most effective additive, and was tested in several field trials, where it also enhanced the efficacy of Cryptogran. Laboratory trials indicated that Cryptogran is rainfast. Cryptogran applications early in the season had a longer period of residual activity than sprays applied closer to harvest. Daytime applications were less effective that evening sprays. Sprays applied coinciding with peaks in pheromone moth trap catches were more effective than those applied between peaks. Biotic factors influencing persistence were investigated. Residual efficacy was longer when treatments were applied to blocks than as single tree treatments. Attempts were made to quantify the effect of the navel end of a navel orange on the field persistence of Cryptogran. Cryptogran was shown to be compatible with many agricultural chemicals used on citrus. Economic thresholds and various cost-benefit analyses are discussed. A list of practical recommendations to growers was drawn up, and possibilities for future research are presented.

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List of abbreviations

µm - micrometer

% - percent

ºC – degrees Celsius

AFLP - amplified fragment length polymorphism

AfMNPV - *Anagrapha falcifera* nuclear polyhedrovirus

Bt – *Bacillus thuringiensis*

BV – Baculovirus

CGA – Citrus Growers Association

cm – Centimetre

CpGV – *Cydia pomonella* granulovirus

CPV – Cytoplasmic polyhedrosis virus

CRI – Citrus Research International

CrleGV - *Cryptophlebia leucotreta* granulovirus

DIP – Delivered in port

DNA – deoxyribonucleic acid

ds – double standard

EPV – Entomopoxvirus

F1 – first generation offspring

Fam. – Family

FCM - False codling moth

g – grams

h – hour

ha - hectare

GV – Granulovirus

Gy - Gray

HzSNPV – *Helicoverpa zea* single capsid nucleopolyhedrovirus

Kr - Kilorads

l – litre

LC_{50} – medial lethal concentration

LD_{50} – medial lethal dose

LET_{50} – medial lethal exposure time

M – million

m – metre

min - minute

ml - millilitre

mm – millimetre

n – number of replicates

nm – nanometre

NPV – Nucleopolyhedrovirus

OB – occlusion body

OAR – original activity response

PIBs – Poly inclusion bodies

PoGV - *Phthorimaea operculella* granulovirus

R – South African Rand.

RNA – Ribonucleic acid

SD_{50} – survival dose 50

SDS – Sodium Dodecyl Sulphate

SeMNPV - *Spodoptera exigua* multicapsid nucleopolyhedrovirus

SIT – Sterile insect technique

SpliMNPV - *Spodoptera littoralis* nucleopolyhedrovirus

SRV – Sundays river Valley

ss – single standard

SUV – simulated sunlight ultraviolet

USA – United States of America

US$ - United States dollar

UV – ultraviolet

WAT – weeks after treatment

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Chapter 1: Review of Literature

1.1. Introduction

False codling moth (FCM), *Thaumatotibia* (=*Cryptophlebia*) *leucotreta* (Meyr) (Lepidoptera: Tortricidae), is one of the most important pests on citrus, causing an estimated annual loss of up to R100 million to the southern African citrus industry (Moore *et al*, 2004a; P. Hardman, CGA, personal communication). Chemical control of FCM is limited and problematic. Resistance to chitin synthesis inhibitors has developed (Hofmeyr *et al*, 1998), and stricter residue restrictions enforced by the overseas markets has caused a general shift away from the use of broad based insecticides (Moore *et al*, 2004a). *Cryptophlebia leucotreta* granulovirus (CrleGV) is a pathogen of FCM in sub-Saharan Africa. Larvae in a laboratory culture in Citrusdal spontaneously developed viral infection, and this virus showed potential for the control of FCM (Singh *et al*, 2003). The granulovirus has been developed into a successful microbial insecticide, registered under the name Cryptogran (Moore *et al*, 2004a).

1.2. The Host

1.2.1. Taxonomy and distribution

Taxonomically, FCM belongs to the order Lepidoptera and the family Tortricidae (Annecke & Moran, 1982). Fuller (1901) reported a species of the genus *Carpocapsa* as a pest on citrus in Kwa-Zulu Natal, naming it the Natal codling moth. In 1909 Howard referred to the 'orange codling moth'. Meyrick originally described the species *Argyroploce leucotreta* in 1913, and Clarke (1958) placed the insect under the genus *Cryptophlebia*, and the accepted classification was *C. leucotreta* (Meyr.) (Newton, 1998). The species name *leucotreta* was removed from the genus *Cryptophlebia* and placed in *Thaumatotibia* by Komai in 1999 (Venette *et al*, 2003).

Thaumatotibia (=*Cryptophlebia*) *leucotreta* is found throughout sub-Saharan Africa and the neighbouring islands of the Indian and Atlantic Ocean (Hill, 1975). Most recently, the moth has been reported as an agricultural pest in Israel, possibly due to an accidental

introduction (Wysoki, 1986). FCM overlaps in distribution and host range with two close relatives in southern Africa, the litchi moth, *Thaumatotibia (=Cryptophlebia) peltastica* (Meyrick) (Lepidoptera: Tortricidae) and the macadamia nut borer, *Thaumatotibia (=Cryptophlebia) batrachopa* (Meyrick) (Lepidoptera: Tortricidae) (Newton, 1998). Other members of the *Cryptophlebia* genus are *C. ombrodelta* (Lower) (Lepidoptera: Tortricidae), which is also known as the macadamia nut borer and the litchi fruit moth in Australia (Throne *et al*, 2003) and *C. illepedia* Butler (Lepidoptera: Tortricidae), the koa seedworm, native to Hawaii (Throne *et al*, 2003). It can also easily be confused with the codling moth, *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) because of appearance and damage, although *C. pomonella* attacks mainly apples and pears (Venette *et al*, 2003).

1.2.2. Host range of FCM

FCM has a catholic range of wild and cultivated host plants (Newton, 1998). It is highly polyphagous (Erichson & Schoeman, 1994) and has been recorded attacking a wide range of cultivated crops (Table 1.1) and wild hosts (Table 1.2), which makes it a difficult pest to control, as reinfeststion of citrus could arise from these hosts.

Table 1.1. Cultivated plants which have been recorded as hosts of FCM (Daiber, 1980; Newton, 1998; Pinhey, 1975; Vennette *et al*, 2003).

Common Name	**Species**
Avocado	*Persea americana*
Apricot	*Prunus armeniciata*
Banana	*Musa paradisiacal*
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 guyajava*
Litchi	*Litchi chinensis*
Loquat	*Eribotrya japonica*
Macadamia nut	*Macadamia ternifolia*
Mango	*Mangifera indica*
Olive	*Olea europaea* subsp. *Europaea*
Pepper/pimento	*Capsicum* spp.
Persimmon	*Diospyros* spp.
Plum	*Prunus* spp.
Pineapple	*Ananas comosus*
Pomegranate	*Punica granatum*
Sorghum	*Sorghum* spp.
Tea	*Camellia sinensis*

Table 1.2. Wild plants which have been recorded as hosts of FCM (Schwartz, 1981; Venette *et al*, 2003).

Common Name	**Species**
Bur weed	*Triumfeta spp.*
Bluebush	*Diospyros lycoides*
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	*Annona reticulata*

Elephant grass	*Pennisetum purpureum*
English Walnut	*Juglans regia*
Governors plum	*Flacourtia indica*
Indian mallow	*Abutilon hybridium*
Jakkalsbessie	*Diospyros mespiliformis*
Jujube	*Zizyphus jujuba*
Jute	*Abutilon* spp.
(Wild) Kaffir plum	*Harpephyllum caffum*
Kapok/copal	*Ceiba pentranda*
Kei apple	*Dovyalis caffra*
Khat	*Catha edulis*
Kudu-berry	*Psuedolachnostylis maprouneifolia*
Lima bean	*Phaseolus lunatus*
Mallow	*Hibiscus* spp.
Mangosteen	*Garcinia mangostana*
Marula	*Sclerocarya caffra, Sclerocarya birrea*
Monkey pod	*Cassia petersiana*
Oak	*Quercus* spp.
Okra	*Ablemoschus esculentus*
Peacock flower	*Caesalpinia pulcherrima*
Pride of De Kaap	*Bauhinia galpini*
Raasblaar	*Combretum zeyheri*
Red milkwood	*Mumisops zeyheri*
Rooibos / Bushwillow	*Combretum apiculatum*
Sida	*Sida* spp.
Snot apple	*Azanza garckeana*
Stamvrugte	*Chrysophyllum palismontanum*
Sodom apple	*Calotropis procera*
Soursop	*Annona 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, real	*Podocarpus latifolius*

Kirkman and Moore (2007) conducted a survey of possible alternative wild hosts. Three sites were selected in the Eastern Cape: in the Addo, Kirkwood and Uitenhage districts. Fruits, galls and fleshy plant components, which could possibly host FCM, were collected from plants in these areas at monthly intervals over a two-year period. A sub-sample of each plant or plant-part collected was inspected for the presence of FCM larvae. Neonate larvae were placed onto the remainder of plants and plant parts, which were inspected after two weeks for the presence of FCM. Larval infestation was recorded on *Schotia afra, Ricinnus communis, Crassula ovata, Opuntia ficus-indica, Passiflora caerulea, Asparagus crassicladus* and *Albuca* sp.

1.2.3. Life history of FCM on citrus

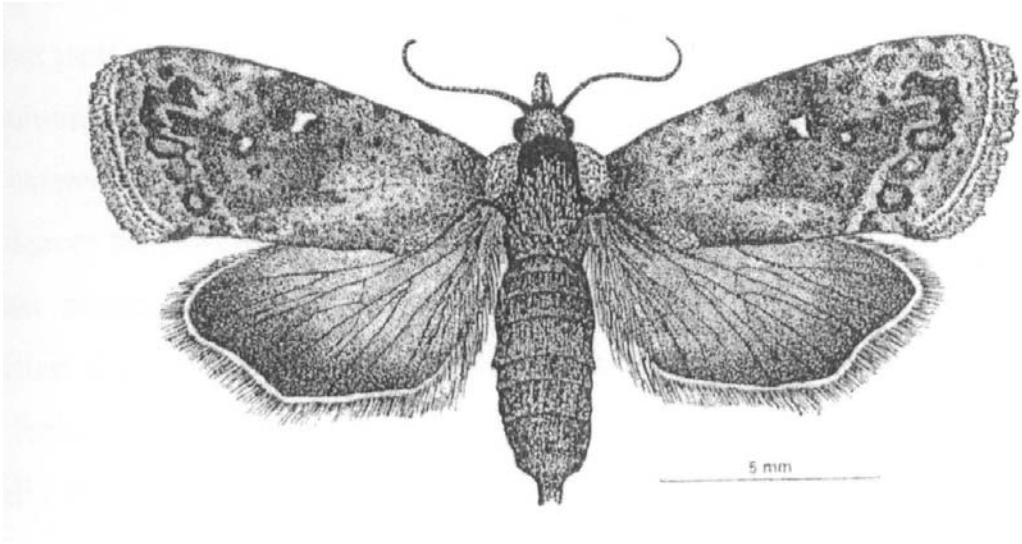

Fig 1.1. *Thaumatotibia leucotreta* moth (Pinhey, 1975).

Females (Fig 1.1) lay their eggs singly, sometimes in large numbers, on the rind of the fruit. The egg is a cream coloured, flat, oval-shaped disc with a granulated surface, measuring approximately 0.77 mm in length and 0.6 mm in width (Daiber, 1979a). Egg incubation varies between 9 and 12 days in winter, and 6 to 8 days in summer. The larvae normally feed on the inner rind of the fruit and on the juicy inner flesh (Pinhey, 1975). Larval development takes 35 to 67 days in winter and 25 to 35 days in summer (Newton, 1998). The neonate larva is white with a black head capsule (Stofberg, 1948). The larva bores into the fruit after a short period of time, usually within 24 hours (Kirkman, 2006, unpublished data).

Daiber (1979b) recorded five larval instars, determined by the width of the head capsule. He found that the average duration of the larval stages reared on artificial medium (maize meal porridge inoculated with a fungal spore suspension, which increases the nutritional value of the diet), was 11.6 days at 25ºC, 18.8 days at 20ºC and 45.6 days at 15ºC. Larval development in the field takes 25 to 67 days, dependant on temperature (Stofberg, 1948), and food quality (Daiber, 1979b). With age, the larva becomes pinkish to red in colour, with the mature larva being 15 to 20 mm long. The fifth instar larva exits the fruit either

before or after it has dropped off the tree (Moore, 2002). The larva then spins a cocoon from silk and soil particles. The pre-pupa moults into a pupa, which is at first soft skinned, until the chitin hardens and becomes dark brown (Daiber, 1979c). The pupal stage lasts 21 to 80 days, depending on temperature (Daiber, 1979c).

The adult moth is inconspicuous, with mottled grey forewings and paler, more evenly coloured hind wings, which are fringed (Moore, 2002). Stofberg (1948) found that female moths laid up to 300 eggs each, while Daiber (1979a) recorded 456 eggs per female moth. Egg laying appears to decline after 5 days under laboratory conditions (Moore, 2002). The total developmental period is therefore 1.5 to 2 months in summer, and 2.5 to 4 months in winter, and there are 5 to 6 generations per year (Newton, 1998).

1.2.4. Economic importance of FCM on citrus

FCM is responsible for annual losses of more than R100 million (US$ 14 million) to the southern African citrus industry, which makes it one of the most important pests on citrus (Moore *et al*, 2004a). There losses are caused by reduction in yield, due to infested fruit dropping onto the ground. Post-harvest decay, due to undetected infested fruit being packed and shipped, is also responsible for heavy losses. FCM is considered a phytosanitary pest due to it being endemic to sub-Saharan Africa. This can lead to an entire consignment of fruit being rejected due to the detection of a single pest (Moore, 2002). Also, certain markets, such as China, will only accept fruit from ‘guaranteed FCM-free’ orchards. It is difficult to ascertain the exact amount lost to FCM each year, as reports do not always specify the exact cause of rejections. Losses in 2004 were estimated at R70 million (Hardman, 2004). Losses due to decay and FCM infestation are not always separated. Other losses occur when fruit destined for lucrative, sensitive markets such as the USA are rejected due to FCM infestation. This fruit may then be re-routed to less sensitive markets for lower income (H. Bester, personal communication). These losses are not always calculated, and so the actual cost of FCM infestation could be higher.

1.2.4. Control of FCM

1.2.4.1. Inspection and monitoring

Inspecting citrus fruits for eggs is difficult, as they are small and transparent. Dropped fruit can be dissected and inspected for the presence of FCM larvae, but this only provides an indication of FCM levels a few weeks prior to the fruit dropping. This is therefore of limited use to monitor current levels of FCM (Hofmeyr, 2003). The most common means of monitoring FCM is the Lorelei trap, which serves as an early warning management tool, but it cannot be used for the control of FCM (Hofmeyr, 2003). The trap consists of a pheromone dispenser, which attracts male moths, and a polybutene based adhesive for ensnaring the moths. These components are housed in a beige PVC pipe. The traps are placed in the fourth or fifth row from the perimeter of the orchard, on the upwind side of the orchard (Hofmeyr, 2003). The threshold value for trap catches is currently fixed at 10 males per trap per week. When this threshold is exceeded for a few weeks consecutively, subsequent FCM infestation can cause pre-harvest damage that will economically justify the application of a control programme using a registered product (Hofmeyr, 2003). However, the traps and their thresholds have little relevance to potential post-harvest losses, which are more potentially greater (S. Moore, personal communication). For the first few years of monitoring with these traps, it is important to observe infestation, fruit drop and damage in relation to trap counts, and to record data for each orchard. This historical data will assist growers to decide whether to apply control measures (Hofmeyr, 2003).

1.2.4.2. Biological control

Effective suppression of FCM populations is provided by the naturally occurring egg parasitoid, *Trichogrammatoidea cryptophlebiae* (Nagaraja) (Hymenoptera: Trichogrammatidae). The parasitoid should be released repeatedly from as early as October. Four releases of 25 000 per hectare is usually adequate, except in the Western Cape, where a fifth release of 25 000 per hectare is required (Hofmeyr, 2003). This figure of 100 000 parasitoids per hectare was an arbitrary figure, and no studies had been conducted to prove that the programme was effective, until Moore and Fourie (1999)

showed that fruit infestation by FCM was reduced by 49% due to releases. Moore and Richards (2001) found that augmentative releases caused a reduction in infestation of 61%. These results proved that 100 000 parasitoids per hectare was an acceptable figure.

Other parasitoids of FCM recorded include the braconids *Apanteles* sp (Hymenoptera: Braconidae) and *Agathis bishopi* (Nixon) (Hymenoptera: Braconidae), and the ichneumonid *Apophua leucotreta* (Wilkinson) (Hymenoptera: Ichneumonidae) (Prinsloo, 1984). None of these parasitoids are commercially available, but researchers at Citrus Research International and Rhodes University are investigating the effectiveness of *A. bishopi* as a biological control agent for FCM (Gendall *et al*, 2006).

1.2.4.3. Cultural control

Orchard sanitation can contribute towards the control of FCM. Hepburn and Bishop (1954) recommended that all infested fruit, on the ground and in the tree, be collected at least once a week and destroyed. Moore and Kirkman (unpublished data) showed that when fruit is picked up weekly, 75% of FCM larvae are still in the collected fruit. Therefore by sanitising weekly, three-quarters of the inoculum of FCM can be removed. December was too late to start orchard sanitation (Schwartz, 1974), as higher numbers of pupae were recovered from orchards from late October to mid December than at any time of the season thereafter (Moore, 2002). Small, hard fruit should be buried at least 30 cm deep and covered with compacted soil, while more mature fruit should be pulped with a hammer mill. Out of season fruit should be removed each year (Hofmeyr, 2003).

1.2.4.4. Mating disruption

The mating disruption approach for the control of FCM relies on the prevention of mating, thereby reducing the number of viable eggs deposited on fruit. This is achieved in practice by applying synthetic female sex pheromone in such a way that the males become confused, repelled or habituated to such an extent that they are unable to find the females for mating (Hofmeyr, 2003). The mating disruption product, Isomate (Bioglobal Limited, Austria), is registered for FCM control. It consists of polyethylene ampoules containing female sex pheromone, which is released into the atmosphere. The synthetic

pheromones are made up of different ratios of (E)-7-dodecenyl acetate, (E)-8-Dodecenyl acetate and (Z)-8-Dodecenyl acetate. Mating disruption is used to control various other species, including the codling moth, *Cydia pomonella*, pink cotton bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) and the tomato pinworm, *Keiferia lycopersicella* (Walshingham) (Lepidoptera: Gelechiidae) (Pedigo & Rice, 2006).

1.2.4.5. Attract and kill

The attract- and -kill technique is similar to mating disruption, except that the males are attracted by the pheromone, make contact with the poison and are killed. The product, Last Call FCM (Insect Science, South Africa), is registered for the control of FCM. It consists of a synthetic pheromone and a pyrethroid incorporated into a transparent gel, which is applied in droplet form to trees. The product has been demonstrated to be less effective than contemporary mating disruption products and will probably work best against low FCM infestations (Hofmeyr, 2003). This is because the treatment is density dependant, i.e. if there are high numbers of FCM males present, there is a probability that some males may escape the control measure and find a female to mate with. If males are attracted, as is the case with FCM, a very high percentage needs to be removed to have an effect. For instance, if each male mates with 10 females, 90% of the males would have to be removed to make a significant difference (Pedigo & Rice, 2006).

1.2.4.6. Chemical control

Two chitin synthesis inhibitors (benzoyl urea group), Alsystin (Bayer, Germany) and Nomolt (Cyanamid, South Africa), are registered for FCM control. They are ovicides, which disrupt embryonic development of larvae in the eggs. They only work if eggs are laid on the treatment residue (Hofmeyr, 2003). These products have shown varying results. On the negative side, they can sterilise certain predatory beetles and occasionally result in citrus red mite and oriental mite population explosions (Hofmeyr 2003). They may also not be used on fruit destined to be exported to the United States of America (Hofmeyr 2003).

Penncap (methyl parathion) (Elf Atochem, Netherlands) is an organophosphate insecticide which kills larvae on contact. However, it can only be applied no later than 50% petal drop due to international residue requirements, which eliminates it as a control option for FCM. Two pyrethroids, Meothrin (fenpropathrin) (Sanachem, South Africa) and cypermethrin (Agropharm, South Africa) are registered for FCM control. They are potentially toxic to a wide range of natural enemies, and their effectiveness is varied (Hofmeyr, 2003; Moore *et al*, 2004b). In one study conducted in the Eastern Cape, FCM infestation was higher in the Meothrin treatment than the untreated control. This may be due to the detrimental effect of Meothrin on natural enemies of FCM (Moore *et al*, 2004b). Chemical sprays, as opposed to attract-and-kill products, are generally not density dependant, because, assuming good spray coverage, all the surface area of the fruit or plant will be covered with the required concentration of active ingredient.

1.2.4.7. Sterile Insect Technique

Sterile Insect Technique (SIT) has been successfully used in Canada to control codling moth, *Cydia pomonella* (Bloem & Bloem, 2000). This technique has been investigated for the control of FCM in the Citrusdal area in the Western Cape (Hofmeyr *et al*, 2004). Laboratory research was initiated in 2002 to investigate the possibility that inherited F1-sterility can be induced in FCM with gamma irradiation. Results indicated that fecundity and fertility of irradiated moths were progressively reduced with increasing doses of gamma irradiation in the range 50 Gy to 350 Gy. The F1 population was smaller and consisted mostly of males. When F1 males were mated with F1 females, eggs were completely sterile and almost completely sterile where F1 females were mated with normal males, where exposure took place in 150 Gy to 200 Gy range (Hofmeyr *et al*, 2004). A pilot trial on 35 ha resulted in a 94.4% reduction in infestation (Hofmeyr & Hofmeyr, 2006). A company, Xcit, has been formed to apply the technique commercially on 6500 ha in Citrusdal in 2007, with the first phase of 1500 ha currently under treatment. This technique has also been used to eradicated and suppress American screwworm *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) in the United States and Mexico. SIT has also been used against tropical fruit flies (Diptera:

Tephritidae) in many countries, tsetse flies (*Glossina* spp.) in Zanzibar, horn fly *Haematobia irritans* (Linnaeus) (Diptera: Muscidae) on cattle in Texas, and very successfully against pink bollworm on cotton in California (Pedigo & Rice, 2006).

1.2.4.8. Post-harvest control

Cold sterilisation is used for post-harvest control of FCM. Myburgh (1963a) found that keeping larvae on artificial diet at –0.5ºC for 21 days led to 100% mortality of FCM larvae. Further trial results have lead to citrus fruits destined for certain markets being sterilised in this manner in transit. However, this is a very expensive process, and is only used for fruit destined for more lucrative markets such as the United States, Japan and China.

Nuclear irradiation has also been tested for post-harvest control of FCM. Myburgh (1963b) also found that exposures to gamma rays between 10 and 120 Kr inhibited development of moths from immature stages, but this process has not yet been used commercially in the citrus industry (Moore, 2002). Irradiation at 200 Gy resulted in zero survival of FCM larvae in artificial diet (Hofmeyr & Hofmeyr, 2005). The United States of America currently approve a generic dose of 400 Gy for post-harvest disinfestation. An irradiation dose ratio of 3:1 exists from the outside to inside of a full pallet of fruit, which implies that the approved dose would have to be 3 times higher than the effective dose to ensure no FCM survival (Hofmeyr & Hofmeyr, 2005). This would mean that the pallet would have to be exposed to a rate of 1200 Gy to ensure efficient radiation to the middle of the pallet, but this would negatively affect the quality of the fruit (Hofmeyr & Hofmeyr, 2005).

1.3. The Pathogen

Microbial control agents, including bacteria, fungi, nematodes, protozoa and viruses provide a more environmentally acceptable form of pest management than chemical insecticides, but require specialist knowledge and conditions for successful use (Lacey & Kaya, 2000). Viruses have a very narrow host range, which minimises their impact on the environment (Table 1.3) (Evans, 2000). Bacteria, such as *Bacillus thuringiensis,* are used

to control moths, mosquitoes and beetles (Pedigo & Rice, 2006). Many viruses are used to control Lepidoptera such as *Pieris brassicae* (Linnaeus) (Lepidoptera: Pieridae) (David, 1965) and *Cydia pomonella* (Dickler & Huber, 1988).

Table 1.3. Host ranges of some baculoviruses, illustrating the number of families, genera and species affected (Evans, 2000).

Original host	Order/Family	Baculovirus	Families	Genera	Species
Gilpinia hercyniae	Hymenoptera/ Dipronidae	NPV	1	1	1
Neodipirion sertifer	Hymenoptera/ Dipronidae	NPV	1	1	3
Pieris rapae	Lepidoptera/ Pieridae	GV	1	1	3
Cydia pomonella	Lepidoptera/ Tortricidae	GV	1	5	8
Choristoneura fumiferana	Lepidoptera/ Tortricidae	NPV	3	3	3
Helicoverpa zea	Lepidoptera/ Noctuidae	NPV	1	2	7
Anticarsia gemmatalis	Lepidoptera/ Noctuidae	NPV	2	6	9
Anagrapha falcifera	Lepidoptera/ Noctuidae	NPV	10	29	31
Autographa californica	Lepidoptera/ Noctuidae	NPV	12	46	56

1.3.1. Insect Viruses as control agents for pests

Insect viruses belong to many different families (Table 1.4) (Evans, 2000). Some of these viruses occur exclusively in arthropods and some include representatives that occur in vertebrates and/or plants. Many insect viruses are occluded, i.e. virions are embedded within a proteinaceous body. Occlusion bodies (OBs) are about 0.5 to 20 µm in diameter, and are visible under a light microscope (Hunter-Fujita *et al*, 1998).

Table 1.4. The main characteristics of the principal families of viruses that influence their use as possible microbial insecticides against arthropods (Evans, 2000).

Virus Family	**Genus**	**Nucleic acid type**	**Occlusion bodies**	**Hosts**	**Site of replication**	**Large scale use**
Ascoviridae	*Ascovirus*	ss* DNA	-	Lepidoptera: Noctuidae only	Nuclei: fat body, hypodermis, tracheal matrix	No
Baculoviridae	*Nucleopolyhedrovirus* (NPV),	ds* DNA	Polyhedral	Lepidoptera, Diptera, Hymenoptera	Nuclei: gut or systemic	Yes
	Granulovirus (GV)	ds DNA	Granular	Neuroptera, Siphonaptera, Thysanura, Trichoptera		Yes
Iridoviridae	*Iridovirus* and *Chlorirdovirus*	Ds DNA	-	Wide range of invertebrate families	Cytoplasm: fat body, haemocytes, sometimes systemic	No
Parvoviridae	*Densovirus*	ss DNA	-	Diptera: Blattoideae, Lepidoptera: Odonata, Orthoptera	Most tissues except midgut	No

Polydnaviridae	*Ichnovirus* *Bravovirus*	ds DNA ds DNA	- -	Hymenoptera: Ichneumonidae Hymenoptera: Braconidae	No effects on parasitoids	No No
Poxviridae	*Entomopoxvirus*	ds DNA	Spheroid	Coleoptera, Diptera, Hymenoptera, Lepidoptera, Orthoptera	Cytoplasm: mainly fat body but other organs can be infected	Minor
Unclassified	*Oryctes* virus	ds DNA	-	Coleoptera	Nucleus: gut in adults, systemic in larvae	Yes
Birnaviridae	*Birnavirus*	ds DNA		Diptera: *Drosophila*	No tissue symptoms: adults sensitive to CO_2	No
Caliciviridae	Chronic stunt virus	ss RNA		Lepidoptera: Noctuidae	Pathology poorly understood	No
Nodaviridae	*Nodavirus*	ss RNA		Diptera, Coleoptera, Lepidoptera	Cytoplasm: Gut and later systemic	No
Picornaviridae	*Picornavirus*	ss RNA		Diptera, Lepidoptera	Cytoplasm: gut and other organs	No

Reoviridae	*Cytoplasmic Polyhedrovirus*	ds RNA	Polyhedral	Diptera, Hymenoptera, Lepidoptera	Cytoplasm: gut only	Minor
Rhabdoviridae	*Sigma* virus	ss RNA	-	Diptera	No tissue symptoms: adults sensitive to CO_2	No
Tetraviridae	Tetravirus	ss RNA	-	Lepidoptera	Cytoplasm, chronic infection	No

*ss = single standard

** ds = double-standard

Occluded viruses belong to three virus families, namely baculoviruses (BVs, family Baculoviridae), cytoplasmic polyhedrosis viruses (CPVs, family Reoviridae), and entomopoxviruses (EPVs, family Entomopoxviridae) (Hunter-Fujita *et al*, 1998).

Baculoviridae comprises of two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Murphy *et al*, 1995). The OBs of GVs are smaller (0.3-0.5 µm in length) than those of NPVs (0.15-15 µm in diameter) and usually contain a single enveloped nucleocapsid (the virus particle), while the OBs of NPVs contain several hundred virus particles (Fig 1.2) (Hunter-Fujita *et al*, 1998).

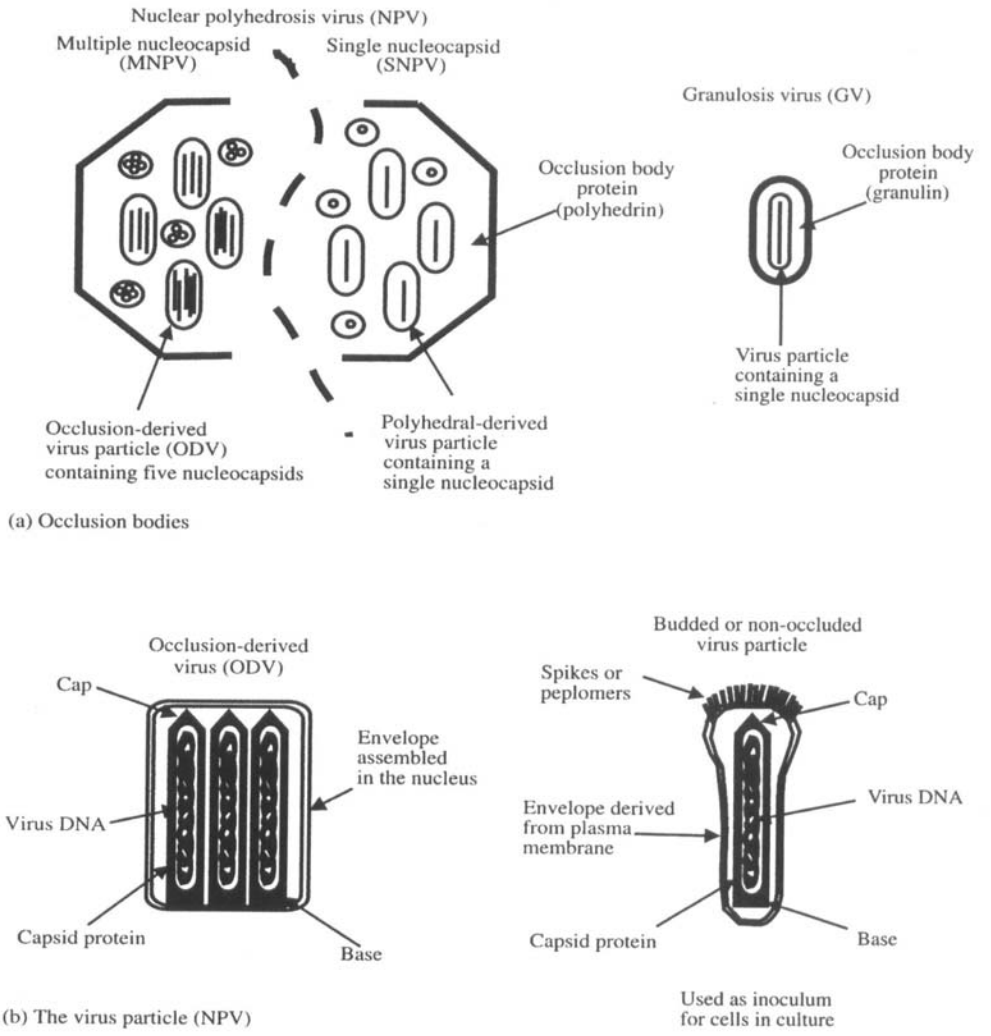

Fig 1.2. The morphology of members of the Baculoviridae family of insect pathogenic viruses (Hunter-Fujita *et al*, 1998).

The existence of baculoviruses has been known for hundreds of years, and accounts of baculovirus infections have been found in ancient Chinese literature in silkworm cultures (Millar, 1997). In the terrestrial environment baculoviruses have been found in hundreds of species of insects. These viruses are specially designed to survive outside their host and can persist in crevices and soil for years (Millar, 1997). They have the greatest potential for use as microbial insect pest control agents.

Paillot (1926) was the first to describe a granuloviral infection in the larva of the European cabbageworm, *Pieris brassicae* (Linnaeus) (Lepidoptera: Pieridae). The rod shaped viral particle was detected with an electron microscope by Bergold (1948) in capsules obtained from infected larvae of the pine shoot roller, *Choristoneura murinana* Hubner (Lepidoptera: Tortricidae). The infection was called granulosis because of the presence of minute granules (Steinhaus, 1949).

1.3.2. Pathology and pathogenesis of GVs

The first indication of infection in larvae is loss of appetite and a progressive colour change to pale and milky, especially on the ventral side (Huger, 1963). The whiteness is due to the abundance of capsules in the hypertrophied fat bodies. With the change in colour, the larva becomes progressively weaker, sluggish and flaccid (Tanada & Kaya, 1993). Most granuloses are confined to the fat body, while in some granuloses, mitotic proliferation of uninfected cells occurs in the fat body (Huger, 1963). This proliferation and hypertrophy of infected fat cells results in a bloated enlarged larva during the later stages of infection (Tanada & Kaya, 1993). In the case of systemic granulosis, the larva usually dies within a brief period, much shorter than an infection involving mainly the fat body. At death such larvae are smaller and are wilted (Tanada & Kaya, 1993).

1.3.3. Life Cycle

Infection with GVs normally takes place by ingestion of occlusion bodies (OBs) by larvae of the host insect, although transmission by parasites or transovarial transmission may sometimes be important (Crook, 1991). The ingested GV capsules are dissolved by the highly alkaline midgut juice (Fig 1.3) (Hunter-Fujita *et al*, 1998), and the liberated enveloped virions attach and fuse to the plasma membrane of the microvilli of the columnar midgut cells. The nucleocapsid enters a microvillus, migrates to the nucleus and attaches to the nucleic acid. Virogenesis begins in a nucleus with the formation of a virogenic stroma, followed by a brief eclipse period with only partial clearing of the nucleus, followed by the appearance of the virogenic stroma (Tanada & Kaya, 1993). Capsids appear in 6-12 hours and are incorporated in the viral nucleoprotein core (Consigli *et al*, 1986). Progeny nucleocapsids are formed, after which the nuclear

envelope breaks down and virogenesis continues in the nucleus and cytoplasm. Envelopment of the nucleocapsids and their inclusion in capsules can also occur in the nucleus and cytoplasm (Tanada & Kaya, 1993). After 24 hours the enveloped and unenveloped nucleocapsids may occur in rows in the intercellular spaces between midgut cells and near the basement membrane (Tanada & Kaya, 1993). Budded virions move from the midgut epithelium into the hemocoel, where secondary infection takes place. The infection of the fat body is by viropexis of nucleocapsids with peplomer envelopes. The occlusion of the virions occurs in the nuclear and cytoplasmic regions of the cell (Tanada & Kaya, 1993). OBs are released into the environment when the insect dies and disintegrates. Horizontal transmission (larva to larva) of the virus occurs, and the replication cycle continues (Moore, 2002).

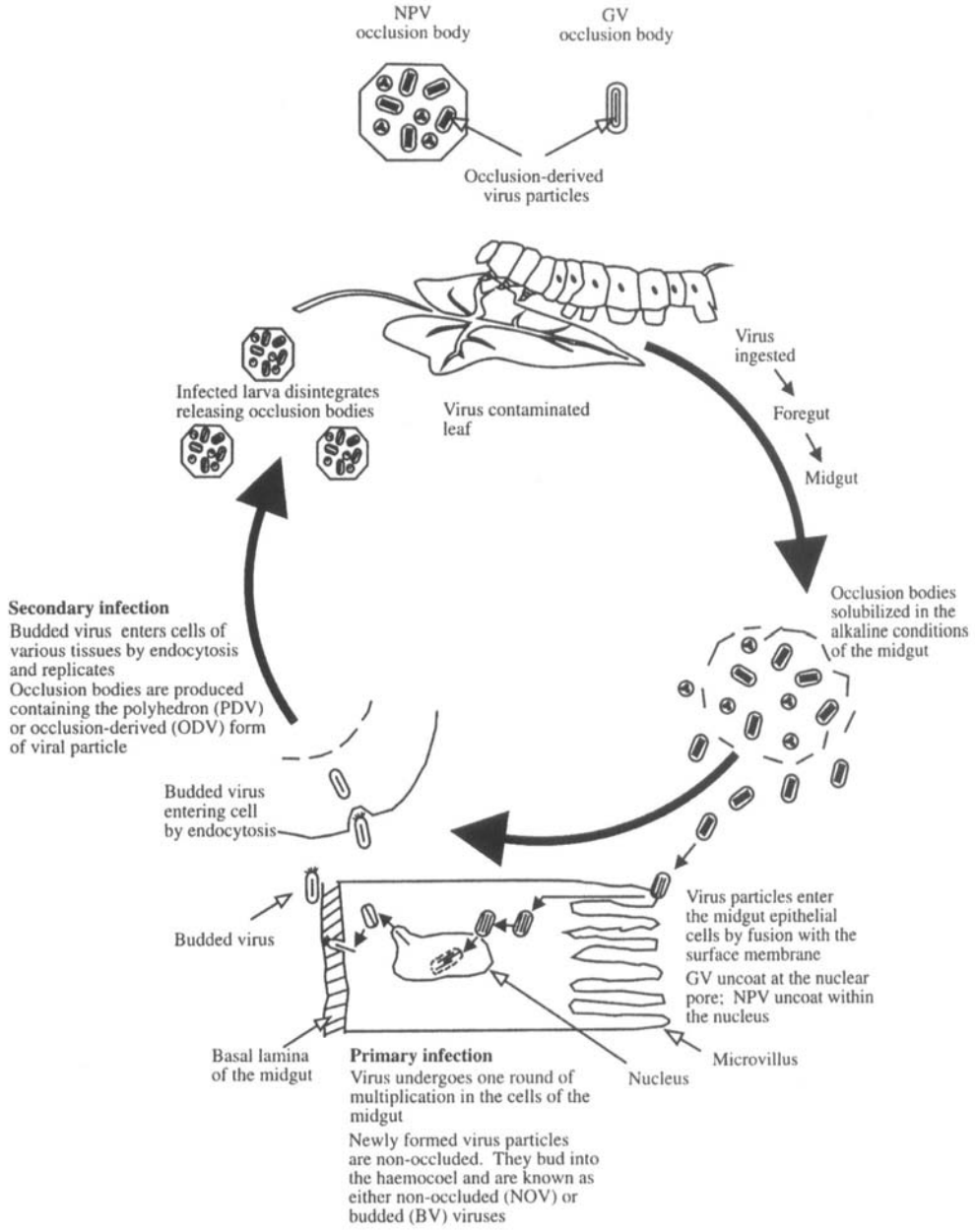

Fig 1.3. The main features of the biology of BVs (Hunter-Fujita *et al*, 1998).

1.3.4. *Cryptophlebia leucotreta* granulovirus (CrleGV)

CrleGV was first described by Angelini *et al* (1965), from an isolate obtained from infected FCM larvae from the Ivory Coast (Cote d'Ivoire). In 1997 a virus was discovered infecting FCM larvae in a laboratory culture in South Africa (Fig 1.4), and its

identity was confirmed to be the *Cryptophlebia leucotreta* granulovirus (CrleGV). It was demonstrated, with restriction endonuclease analysis with DNA extracted from the virus, that the virus was novel, as its DNA profile had not previously been documented (Moore *et al*, 2004a). The virus was designated the name CrleGV-SA (Singh *et al*, 2003). Moore (2002) demonstrated the potential of the virus as a biocontrol agent through laboratory bioassays, and the pathogenicity of the virus against neonate FCM larvae was calculated.

Fig 1.4. An FCM larva infected with CrleGV
(SD Moore)

A mass production technique for the host and *in vivo* production systems for the virus have been developed, and the product has been registered for use against FCM under the name Cryptogran.

Moore *et al* (2004a) suggested the two main reasons for applying Cryptogran as being to combat pre-harvest loss by applying a spray near the FCM peak in early December (in the Eastern Cape) and to combat post-harvest decay and avoid phytosanitary problems by applying the product approximately four weeks before harvest. In total, 31 field trials have been conducted with Cryptogran since 2000, in three provinces, mainly on navel oranges, but also on Satsuma Mandarins and Midknight Valencia oranges (Moore *et al*,

2004b). These trials have shown consistent and impressive efficacy against FCM on citrus (Moore *et al*, 2004a).

1.3.4.1. Combating pre-harvest loss

Various field trials have been conducted with Cryptogran. Moore *et al* (2003) conducted a trial on Robyn navel orange trees on Schoeman Boerdery in Mpumalanga. Two concentrations of Cryptogran (20 ml and 100 ml/100 *l* water Cryptogran (concentration 5 x 10^{10} Obs/ml) plus 0.5% molasses) were applied to single tree replicates in randomised block formation. After 9 weeks these treatments resulted in a 76.5% and 82.3% reduction in FCM infestation respectively. Eleven weeks after application there was still a reduction, but the trial was terminated at this point. The efficacy of Cryptogran against FCM was as impressive in many other trials (Moore *et al*, 2004a).

1.3.4.2. Combating post-harvest waste

Moore *et al* (2004b) conducted a series of trials where Cryptogran was applied shortly before harvest. The first was in an orchard of Autumn Gold navel orange trees on the farm Woodridge in the Sundays River Valley, Eastern Cape. A 1.3 ha orchard was divided into four blocks, each with about 500 trees. Cryptogran (10 ml/100 *l* + 0.5% molasses) was applied at 9.25 *l* / tree to two of the blocks using a tower mist blower. There was a 29% reduction in FCM infestation up to 6 weeks after application, and the overall reduction in fruit drop was 38% compared to the untreated control trees (because of rapid decay, a higher percentage of fruit might have been infested than was recorded). A further trial was conducted on Midknight Valencia orange trees on the Farm Brandwacht in the Sundays River Valley, Eastern Cape. Cryptogran (10 ml/100 *l* + 0.5% molasses) was applied to blocks of trees with three different machines: two oscillating tower mist blowers (Janisch and Malray) and a Janisch handgun machine were used. The Janisch mist blower, which gave a visibly better coverage at 18 *l*/tree, resulted in the greatest reduction in FCM infestation of 70%. The Malray mist blower and Janisch handgun machine delivered 28% less spray volume (13 *l* / tree) and only reduced FCM infestation by 45% and 55% respectively. These results highlighted the importance of spray coverage for effective control of FCM with Cryptogran.

A principle disadvantage of the use of baculoviruses in the field is their short residual activity due to their inactivation by UV irradiation (Huber, 1990; Shapiro, 1995). This shortcoming is such that the codling moth GV against codling moth on apples has to be applied every 7-14 days (Dickler & Huber, 1988). The *Cydia pomonella* GV was rapidly broken down on apples, with more than 50% of the activity lost in 2 days, although some activity remained after 10 days (Jacques *et al*, 1987).

Moore *et al* (2004b) speculated that there are four main reasons why FCM control persists for so long after one application of Cryptogran. Firstly, the citrus tree provides substantial shading and therefore UV protection of the virus. Secondly, it has been observed that during most of the growing season, the vast majority of FCM larvae enter the fruit through the navel end. A high density of virus could collect here and be protected here against UV-irradiation and possibly rainfall. Thirdly, FCM takes a long time to re-colonise an area, even once the residue of a spray might have expired. Lastly, Cryptogran would have little or no detrimental impact on the naturally occurring egg parasitoid, *Trichogrammatoidea cryptophlebiae* (Newton & Odendaal, 1990), and this biocontrol agent could aid in maintaining control of FCM once the virus is no longer effective.

It has also been shown in these trials that Cryptogran will not be as effective against very high levels of FCM, as the treatment is density dependant. The virus is applied in suspension and the insects need to ingest microbial particles in order to become infected. Some of the insects could survive due to feeding without ingesting the particles (S. Moore, personal communication). Spray coverage is of paramount importance. Good coverage is more difficult to achieve late in the season when the fruit are already big (Moore *et al*, 2004b).

Cryptogran has been registered, for the control of false codling moth on citrus, at 10 ml/100 *l* water with 0.5% molasses, and subsequently with 250 ml molasses and 18 ml Agral 90 (Syngenta, South Africa), or any other per 100 *l* water. Various trials have

shown that the efficacy of Cryptogran is enhanced and persists longer when sprayed with molasses (Moore *et al*, 2004b). The effect of molasses could be threefold (Moore *et al*, 2004a). Firstly, molasses acts as a sticker, preventing the virus from being washed off by rain or dews. Secondly, it could provide UV protection to the virus (this will be tested in the course of the study). Thirdly it acts as a feeding attractant to the FCM larvae, thus causing more larvae to ingest the virus and become infected. Another possible reason is that, due to the stickiness of the molasses, the likelihood of a larva inadvertently picking up and ingesting some virus particles increases (S. Moore, personal communication).

1.3.5. The effect of UV on baculoviruses

A principal disadvantage of the use of baculoviruses in the field is their short residual activity due to their inactivation by UV irradiation (Huber, 1990; Shapiro, 1995). This has also been demonstrated for CrleGV (Moore, 2002), the virus in Cryptogran. The strong germicidal effect of sunlight has long been recognised, mostly due to UV wavelengths which constitute about 0.1% of the energy of the sun (Table 1.5) (David, 1969). Radiation present in sunlight, ranging from 291.5 to 380 nanometres (nm), is less germicidal than shorter UV radiation from low pressure mercury lamps (253.7 nm) (David, 1969).

Table 1.5. Definitions of UV regions in the solar spectrum (David, 1969).

Definition	Wavelength (nm)
UVC	<280
UVB	280-320
UVA	320-400

Inactivation of entomopathogens by UV-B (280 – 320 nm) has been demonstrated (David, 1969), especially at wavelengths of 300 – 320 nm. Inactivation also occurs after exposure to UV-A (320 – 400 nm) (David, 1969), but at a slower rate. In general, exposure of virus at 280 – 320 nm, which is present in natural sunlight reaching the earth, is nearly as effective at breaking down virus activity, as exposure to shortwave UV (254 nm), which does not reach the earth (Shapiro *et al*, 1983).

A literature review revealed many attempts to extend the residual efficacy of entomopathogenic viruses by adding substances which could provide UV protection to the viruses. Substances can be effective as UV protectants for two reasons (Shapiro *et al*, 1983). Firstly, they can reflect irradiation due to their physico-chemical properties (e.g. Zinc-oxide, Titanium oxide, silicates and talcum). Bull *et al* (1976) successfully utilized *Helicoverpa zea* NPV – Titanium oxide microcapsules, which were quite resistant to solar or artificial UV irradiation. Secondly, they can selectively absorb UV-B rays, while transmitting UV-A and visible light, or additionally absorbing UV-A rays. Because most sunlight inactivation occurs in the UV-B region of the solar spectrum (David, 1969), this group may have potential as protectants for GMNPV (Shapiro *et al*, 1983).

The field persistence of the NPV, *Helicoverpa zea* NPV, of the bollworm *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), was extended by microencapsulation and the use of sunlight protection (Ignoffo & Batzer, 1971), and varying time of application (Young & Yearnian, 1974). Four known UV protectants, each of a different type, were encapsulated with starch (Ignoffo *et al*, 1991). These were a particulate, high porosity-activated carbon (Type RB), a fluorescent whitening agent (Tinopol CBS), a dye, Congo Red, and a polyflovanoid, catechin leuco-cyanidin copolymer (Shade). Starch-encapsulated formulations of *Helicoverpa zea* single capsid NPV (HzSNPV), with and without UV protectants, were exposed to simulated sunlight UV (SUV), and bioassayed against larvae of *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae). Starch – encapsulation improved UV protection. Carbon and Congo red provided the best UV protection. They concluded that increasing the concentration of corn oil (starch source) might reduce the particle size and increase the UV stability of HzSNPV formulated with carbon (Ignoffo *et al*, 1991). The addition of various dyes and stains, namely Direct Red 28, Direct Red 81, Disperse Blue 14, Alcian Blue 8GX, Methyl Orange, Disperse Orange 11, Mordant Brown 1 and Mordant Brown 33 to gypsy moth NPV suspensions increased UV protection five-fold (Podgewaite & Shapiro, 1986). Two lignosulphates, Raymix-L® and Orzan LS-50®, which are byproducts of the tree-pulping process, increased UV protection of the virus (Podgewaite & Shapiro, 1986).

Crude preparations of virus were shown to be more persistent than a purified virus of *Pieris brassicae* (David, 1965). This view is supported by Jones & McKinley (1996). This is as a result of physical UV protection given to the virus particles by fragments of insect body parts present in crude virus preparations. These formulations are prepared by mulching virus-infected larvae (Shapiro *et al*, 2002).

For UV studies, most researchers use virus concentrations which cause 90 – 100% mortality (Shapiro *et al*, 2002). Virus is then exposed to a radiation source for a single period (Ignoffo *et al*, 1991), or for different periods of time (David, 1969). In laboratory trials, Shapiro *et al* (2002) found that the higher the concentration of virus used, the longer the time required for inactivation. They also found that *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) was more UV tolerant than HzSNPV. In field trials they found similar trends. Where lower concentrations of virus were used, activity was shorter. The higher the concentration of the virus, the longer the field persistence. This indicated a positive relationship between virus rate and virus persistence. Most NPVs are applied at $10^{11} – 10^{12}$ OBs per hectare (Shapiro *et al,* 2002). Cryptogran is registered and recommended to be applied at 5×10^{13} OBs per hectare.

1.4. Aims of study

Between 2000 and 2007, 31 field trials have been conducted with Cryptogran. Varying levels of field persistence have been observed and recorded. A prerequisite to the success of Cryptogran as a means of controlling false codling moth is to understand the factors affecting field persistence of the virus, and to find ways to improve it. The aim of this study was to gain a clearer understanding of the product and the abiotic and biotic factors affecting its persistence and residual efficacy in the field, and to investigate methods to improve this persistence.

A study was conducted to determine the effect of UV on the virus using laboratory bioassays with FCM larvae. Additives will be tested with the aim of improving protection against UV. The most promising products/additives will then be tested in the field. The

rain-fastness of the product as registered (with molasses) will be determined. If necessary, additives/stickers will be tested in an attempt to improve the rain-fastness of the virus. The effect of timing of applications, i.e. time of the year and time of the day, on residual efficacy will be investigated.

Biotic factors influencing the residual efficacy of the virus will be investigated. Trials will be conducted to determine whether the navel-end of the fruit (navel orange) plays any role in protecting the virus and therefore improving its field persistence. The rate of dispersal will be investigated by comparing the efficacy and residual action of the product when applied to blocks and to single trees (Fig 1.5). Management recommendations will be listed on completion of the study.

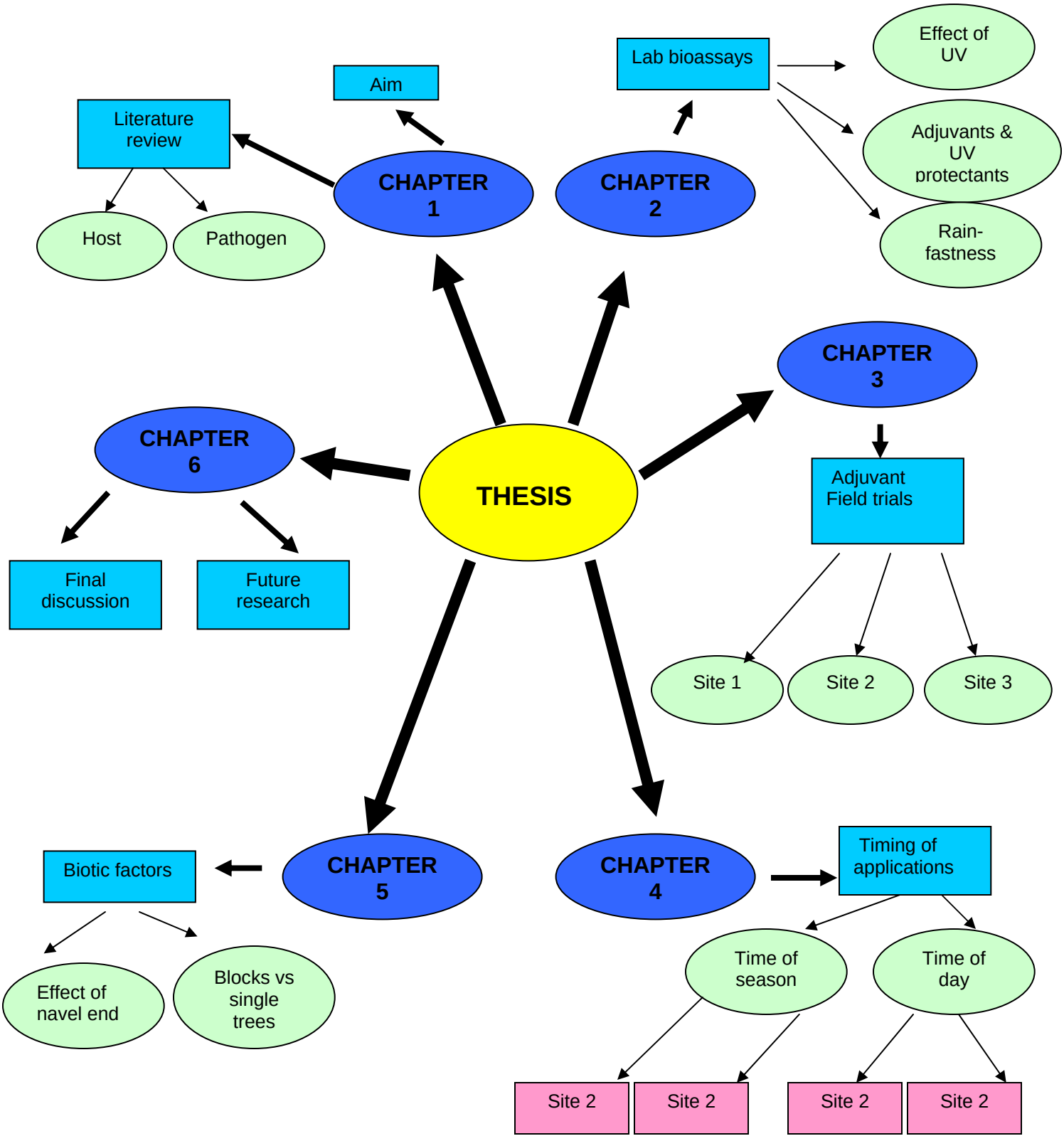

Fig 1.5. Aims of study for investigating and improving the field persistence of Cryptogran

Chapter 2: Laboratory bioassays to investigate the effect of the abiotic factors, UV and rainfall, on Cryptogran.

2.1. Introduction

A major disadvantage of the use of baculoviruses in the field is their short residual activity due to their inactivation by UV irradiation (Huber, 1990; Shapiro, 1995). Studies, which have been discussed in the previous chapter, have shown the breakdown rates for various viruses. In many cases, viral activity is greatly reduced within 24 – 48 hours due to UV irradiation (David *et al*, 1968). It is important to determine the effect of UV on Cryptogran in laboratory bioassays. Gendall (2005), who demonstrated that UV does have an effect on Cryptogran, was instrumental in developing the experimental protocols to be used in this study. The only previous study conducted on the effect of UV on Cryptogran, a field trial, revealed that Cryptogran broke down to 50% of its original activity in 5 days on the northern, sunny side of the tree (Moore *et al*, 2001).

The purpose of the study reported in this chapter was to determine the effect of UV on Cryptogran, using a germicidal UV lamp. Comparative bioassays were conducted with natural sunlight as a source of UV, to indicate whether the germicidal UV lamp had a similar effect as natural sunlight.

Another aim was to identify possible UV protectants which could be applied with Cryptogran. Products showing potential could then be tested in the field environment. Several adjuvants, which have been shown to add UV protection to viruses, were added to the virus to see if they showed any potential to improve efficacy or extend the residual efficacy of Cryptogran. Studies have shown that Cryptogran is more effective when applied with molasses (Moore *et al*, 2004b), but the reasons for the increased efficacy are not clear. Bioassays were conducted to determine if molasses provides UV protection to the virus.

It is extremely important to ascertain if the product is rainfast, and if so, to what extent. Growers need to know if rainfall will reduce the efficacy of Cryptogran, and whether reapplication is necessary. Laboratory bioassays were conducted to test the degree of rainfastness of Cryptogran.

2.2. Materials and methods

2.2.1. The effect of UV

2.2.1.1. Germicidal lamp

One hundred millilitres of a Cryptogran suspension was prepared at a concentration of 1.34×10^5 OBs/ml. This concentration, which is three times the LC_{80} value as determined by Moore (2002), was shown by Gendall (2005) to be a suitable concentration to measure the effect of UV on Cryptogran. Fifteen millilitres of this suspension were placed into each of 5 Petri dishes. The suspensions in these Petri dishes were then exposed to a germicidal UV lamp (254 nm) for periods ranging from 30 minutes to 240 minutes. After exposure the suspensions were inoculated onto artificial diet (Moore, 2002) in 25 cell bioassay trays, and bioassayed against neonate FCM larvae. Fifty microlitres of virus solution was inoculated onto the artificial diet in each cell, and when dry, one neonate FCM larva was placed into each cell (n = 25). An untreated control was retained, as well as one suspension of Cryptogran which was not exposed to UV. The trays were kept at 27ºC for 7 days, and then evaluated for larval survival/mortality (Moore, 2002). Probit analysis was conducted to determine the SD_{50} values.

2.2.1.2. Natural sunlight

A bioassay was conducted in a similar way, but the Cryptogran solutions in the Petri dishes were exposed to natural sunlight instead of the germicidal UV lamp. Where possible, a probit analysis was conducted to establish a dose-response relationship between the treatments and neonate FCM larvae. SD_{50} values were calculated by probit analysis.

2.2.2. Adjuvant and UV-Protectant bioassays

The first three bioassays described in this section were series dilution bioassays, conducted by inoculating 50 ml of each solution onto artificial diet in each cell of a 25 cell bioassay tray. Once dried, one neonate FCM larva was placed into each cell. The trays were kept at 27ºC for 7 days, and then evaluated for larval survival/mortality (Moore, 2002). Where possible, a probit analysis was conducted to establish a dose-response relationship between the treatments and neonate FCM larvae. LD_{50} values were calculated where probit analyses were conducted. The remaining bioassays (UV bioassays) were conducted according to the protocol described in section 2.1.1.1. for neonate FCM larvae.

2.2.2.1. The effect of Wetcit on neonate FCM larvae

The first bioassay was conducted with Wetcit (UAP, South Africa), a commercial wetter containing borax and citrus oil, distributed by Citrus Oil Products. Wetcit is reported to have insecticidal properties (Kirkman & Moore, 2006). In order to determine whether the product alone caused any FCM larval mortality, Wetcit was tested at the following concentrations (all per 100 *l* water): 25 ml, 50 ml, 100 ml, 200 ml and 400 ml. There were therefore five concentrations of Wetcit, prepared as a series of two-fold dilutions. A distilled water control was also applied.

2.2.2.2. The effect of boric acid on neonate FCM larvae

A second bioassay was conducted to test the effect of boric acid on FCM. It was initially intended to test borax, but this substance would not be dissolved in water, even when heated. Similar problems had been recorded during field trials, when the borax used had blocked the outlet pipes of the spray machine. Boric acid was bioassayed against neonate FCM larvae at the following concentrations (all per 100 *l* water): 0.25%, 0.5%, 1%, 2% and 4% - therefore, again a two-fold dilution series. A distilled water control was also applied.

2.2.2.3. Wetcit and Cryptogran

In a third bioassay, a combination of Wetcit and Cryptogran were tested against neonate FCM larvae. Cryptogran was prepared in a five-fold series dilution (Moore, 2002), while the Wetcit concentration was kept constant at 200 ml per 100 *l* water. This bioassay was replicated once.

2.2.2.4. Cryptogran with Wetcit as a UV protectant.

A fourth bioassay was conducted to determine the effectiveness of Wetcit as a UV protectant. A suspension of Cryptogran was again prepared at 1.34 x 10^5 OBs/ml and 15 ml was inoculated into each of four Petri-dishes. A similar Cryptogran suspension was prepared, with the addition of the Wetcit (200 ml/100 *l* water). This was inoculated identically into four Petri-dishes. These Petri-dishes were then exposed to a germicidal UV lamp for periods ranging from 30 minutes to 360 minutes. Immediately after exposure the suspensions were inoculated onto artificial diet and bioassayed against neonate FCM larvae. A distilled water treated control was retained, as well as a control of Wetcit (200 ml/100 *l* water). One control of each of Cryptogran and Cryptogran with Wetcit, which were not exposed to UV, were retained.

2.2.2.5. Cryptogran and lignin

A bioassay was conducted to determine the effectiveness of a lignin sulphate carrier, carrier 038A (Omnova Solutions, Greensboro) (200 ml/100 *l* water), as a UV protectant. This bioassay was conducted in a similar way to the previous bioassay with Wetcit. The Petri-dishes were exposed to a germicidal UV lamp for periods ranging from 30 minutes to 240 minutes. A distilled water treated control was retained, as well as a control of lignin carrier (200 ml/100 *l* water). One control of each of Cryptogran and Cryptogran with lignin, which were not exposed to UV, were retained. Two replicates of the bioassay were conducted.

Another bioassay was conducted, identical to the previous two, except that exposure times ranged from 60 minutes to 360 minutes. Two replicates of the bioassay were

conducted. A least squares regression of larval exposure on time of exposure to UV was conducted.

2.2.2.6. Cryptogran and other additives

Several other bioassays were conducted in a similar fashion, to determine the effectiveness of products as UV protectants. An optical brightener, Tinopol DMS-X (Ciba Specialty Chemicals, Switzerland) (1%), and silica (Nontox-Silica, marketed and distributed by Plant Bio Regulators), were tested. Cryptogran has been registered to be applied with molasses, due to the notable improvement which it gives to the efficacy of Cryptogran in field trials. Cryptogran (with and without molasses) was bioassayed against neonate larvae, after exposure to UV irradiation for varying periods. The molasses was added to the Cryptogran suspension at a rate of 250 ml/100 *l* water.

2.2.3. Rainfastness

Two bioassays were conducted to determine whether or to what extent Cryptogran is rainfast.

2.2.3.1. Fruit dip bioassay to determine the rainfastness of Cryptogran

In the first bioassay, 64 randomly chosen Valencia oranges were dipped in a Cryptogran (10 ml/100 *l*) and molasses (0.5%) treatment and allowed to dry. Once dried, half (32) of these fruit were dipped into clean water to simulate the effect of rainfall. As a control, 32 untreated oranges were dipped into distilled water and allowed to dry (Table 2.1). Once all the fruit had dried, they were each inoculated with 3 neonate FCM larvae, and kept at 27°C for two weeks. They were then dissected and inspected for penetration marks, decay and the presence of FCM larvae. The treatments were then compared using ANOVA and the Duncan's multiple range test.

Table 2.1. Treatments applied to Valencia oranges in a laboratory fruit dip bioassay in an effort to determine the rainfastness of Cryptogran.

Treatment	
1	Distilled water
2	Cryptogran (10 ml/100 *l*) + molasses (0.5%)
3	Cryptogran (10 ml/100 *l*) + molasses (0.5%) + 'rain' dip

2.2.3.2. Bioassay using a rain simulation machine

In a second bioassay, rainfall was simulated more accurately by using a rain simulation machine designed by researchers from CRI (Hattingh, 1998). Sixty randomly selected Valencia oranges were dipped in a Cryptogran, molasses and Agral 90 solution and allowed to dry. Half (30) of these fruit were then exposed to simulated rainfall at a rate of 36 mm in 5 minutes, which would be classified as a cloudburst (Aaron *et al*, 1986) (Table 2.2). Rain volume was determined by placing a rain gauge in the centre of the spray from the machine. Once all the fruit had dried, they were each inoculated with 4 neonate FCM larvae, and kept at 27°C for two weeks. They were then dissected and inspected for penetration marks, decay and the presence of FCM larvae. The treatments were then compared using ANOVA and the Bonferroni multiple range test.

Table 2.2. Treatments applied to Valencia oranges, with and without simulated rainfall, in a detached fruit bioassay to determine the rainfastness of Cryptogran.

Treatment	
1	Distilled water
2	Cryptogran (10 ml/100 *l*) + molasses (0.25%) + Agral 90 (18 ml/100 *l*)
3	Cryptogran (10 ml/100 *l*) + molasses (0.25%) + Agral 90 (18 ml /100 *l*) 'rain' @ 36mm per 5 minutes

2.3. Results

2.3.1. The effect of UV

2.3.1.1. Germicidal lamp

UV-irradiation generated by a germicidal lamp resulted in breakdown of the virus after 30 minutes of exposure (Table 2.3). A probit analysis delivered a SD_{50} value of 437 ± 1013 minutes. This survival dose value is the time of exposure of the virus to UV radiation, in minutes, that allowed 50% of the larvae to survive. This was an indication of the time it took for the virus to lose 50% of its original activity.

Table 2.3. Impact of UV (germicidal UV lamp) on Cryptogran (1.34×10^5 OBs/ml) measured by mortality of neonate FCM larvae in a dose-response bioassay (SD_{50} = 437 ± 1013 minutes) ($P<0.05$).

Treatment		Time of exposure to UV (min)	Mortality of neonate FCM larvae (%)
1	Distilled water	0	16
2	Cryptogran	0	68
3	Cryptogran	30	44
4	Cryptogran	60	48
5	Cryptogran	120	40
6	Cryptogran	240	36

2.3.1.2. Natural sunlight

When exposed to UV irradiation in sunlight, the virus again showed signs of reduced efficacy after 30 minutes of exposure to irradiation (Table 2.4). A probit analysis delivered a SD_{50} value of 463±417 minutes, which was indicative of time it took for the virus to lose 50% of its original activity. This SD_{50} value is similar to that of the previous bioassay where a germicidal lamp was used as a source of UV.

Table 2.4. Impact of UV (sunlight) on Cryptogran (1.34 x 10^5 OBs/ml) measured by mortality of neonate FCM larvae in a dose-response bioassay (SD_{50} =463±417 minutes) ($P < 0.05$).

Treatment		Time of exposure to UV (min)	Mortality of neonate FCM larvae (%)
1	Distilled water	0	12
2	Cryptogran	0	76
3	Cryptogran	30	72
4	Cryptogran	60	72
5	Cryptogran	120	52
6	Cryptogran	240	60

2.3.2. Adjuvant and UV-Protectant bioassays

2.3.2.1. The effect of Wetcit on neonate FCM larvae

Wetcit caused larval mortality of up to 28% (at a concentration of 200 ml/100 *l* water) (Table 2.5). However, there was no dose response, and it was not possible to conduct a probit analysis. Strangely, larval mortality was lowest with the highest concentration of Wetcit.

Table 2.5. The impact of a two-fold series dilution of Wetcit on the mortality of neonate FCM larvae in a dose-response bioassay.

Treatment (concentrations of Wetcit in ml/100 *l* water)		Larval mortality (%)*
1	Distilled water	-
2	25	20
3	50	24
4	100	16
5	200	28
6	400	8

* Larval mortality corrected for control mortality

2.3.2.2. The effect of boric acid on neonate FCM larvae

In the second bioassay, using boric acid, again no dose response was recorded, and no probit analysis was possible (Table 2.6). However, boric acid did cause a notably higher level of mortality than did Wetcit.

Table 2.6. The impact of a two-fold series dilution of boric acid on the mortality of neonate FCM larvae in a dose-response bioassay.

Treatment (concentrations of boric acid (%)		**Larval mortality (%)***
1	Distilled water	-
2	0.25	44
3	0.50	56
4	1.00	32
5	2.00	44
6	4.00	44

*Larval mortality corrected for control mortality

2.3.2.3. Wetcit and Cryptogran

In the third bioassay, mortality of larvae was relatively low (Table 2.7). Despite this, a dose-response relationship was noted for Cryptogran. The addition of Wetcit made no meaningful difference to the efficacy of Cryptogran, but it impeded the dose-response relationship.

Table 2.7. Mortality of neonate FCM larvae when bioassayed against a dilution series of Cryptogran, with and without the addition of Wetcit ($P < 0.05$).

Treatment	**Concentration of Cryptogran (OBs/ml)**	**Corrected larval mortality (%) with Wetcit (200 ml/100 *l*)**	**Corrected larval mortality (%) without Wetcit (200 ml/100 *l*)**
1	Control	-	-
2	1.22×10^2	12	0
3	6.10×10^2	16	4
4	3.05×10^3	0	8
5	1.53×10^4	8	12
6	7.63×10^4	24	24
LD_{50} value (OBs/ml)		1.25×10^9	1.56×10^4 *

* Where Wetcit was not added, larval mortality in the untreated control was decreased from 20% to 12% to make a probit analysis possible, as one of the treatments caused an equal level of mortality to the untreated control.

2.3.2.4. Cryptogran with Wetcit as a UV protectant.

Wetcit appeared to provide UV protection to the virus. This was reflected in the higher larval mortality after extended periods of exposure to UV, where Wetcit was added (table 2.8).

Table 2.8. Impact of UV-irradiation (germicidal lamp) on Cryptogran (1.34 x 10^5 OBs/ml), with and without Wetcit, measured by mortality of neonate FCM larvae in a dose-response bioassay.

Treatment		**Time of exposure to UV (min)**	**Mortality of neonate FCM larvae (%) (with Wetcit)**	**Mortality of neonate FCM larvae (%) (without Wetcit)**
1	Distilled water	0	28	16
2	Cryptogran	0	76	84
3	Cryptogran	60	60	52
4	Cryptogran	120	52	64
5	Cryptogran	240	68	64
6	Cryptogran	360	48	36

2.3.2.5. Cryptogran and lignin

The addition of lignin improved the residual efficacy of the virus, reflected by the higher larval mortality after longer periods of exposure where lignin was added. This was probably due to UV-protection provided by lignin, which reduced the rate of breakdown of the virus (Table 2.9). When plotted (Fig 2.1), the R^2 line (0.16) for the treatment where lignin was added is almost horizontal, which would indicate that exposure of the virus to UV had little effect on the survival of neonate FCM larvae exposed to Cryptogran. However, where no lignin was added, the R^2 value was higher (0.63), which showed a good correlation between exposure of the virus to UV and survival of neonate FCM larvae. There is thus a clear positive relationship between exposure time and larval survival where no lignin was added to the virus. Where lignin was added, survival was not affected by exposure of the virus to UV.

The next two bioassays, where the periods of exposure to UV were lengthened to 360 minutes, showed a similar result (Table 2.10) (Fig 2.2).

The bioassays revealed unexpectedly high mortality in the treatments no 6, where the virus was exposed to UV for the longest period. This could be due to the fact that some evaporation may have taken place from the Petri dishes, resulting in a lower volume, and thus a more concentrated virus suspension, which induced higher larval mortality. The temperature of the Petri dishes did not appear increase due to exposure to the UV lamp, so virus was not inactivated due to heat.

Table 2.9. Impact of UV-irradiation (germicidal lamp) on Cryptogran (1.34 x 10^5 OBs/ml), with and without lignin, measured by survival of neonate FCM larvae in a dose-response bioassay.

Treatment		Time of exposure to UV (min)	Replicate 1		Replicate 2		Total	
			Survival of neonate FCM larvae (n=25) (with lignin)	Survival of neonate FCM larvae (n=25) (no lignin)	Survival of neonate FCM larvae (n=25) (with lignin)	Survival of neonate FCM larvae (n=25) (no lignin)	Survival of neonate FCM larvae (n=50) (with lignin)	Survival of neonate FCM larvae (n=50) (no lignin)
1	Distilled water	0	18	19	20	21	38	40
2	Cryptogran	0	10	7	4	5	14	12
3	Cryptogran	30	8	7	4	5	12	12
4	Cryptogran	60	3	8	8	8	11	16
5	Cryptogran	120	8	11	2	8	10	19
6	Cryptogran	240	8	10	5	8	13	18

Table 2.10. Impact of UV-irradiation (germicidal lamp) on Cryptogran (1.34 x 10^5 OBs/ml), with and without lignin, measured by survival of neonate FCM larvae in a dose-response bioassay.

Treatment		Time of exposure to UV (min)	Replicate 1		Replicate 2		Total	
			Survival of neonate FCM larvae (n=25) (with lignin)	Survival of neonate FCM larvae (n=25) (no lignin)	Survival of neonate FCM larvae (n=25) (with lignin)	Survival of neonate FCM larvae (n=25) (no lignin)	Survival of neonate FCM larvae (n=50) (with lignin)	Survival of neonate FCM larvae (n=50) (no lignin)
1	Distilled water	0	21	23	21	21	42	44
2	Cryptogran	0	13	18	8	4	21	22
3	Cryptogran	60	11	14	9	12	20	26
4	Cryptogran	120	10	11	10	9	20	20
5	Cryptogran	240	12	14	9	9	21	23
6	Cryptogran	360	8	14	11	16	19	30

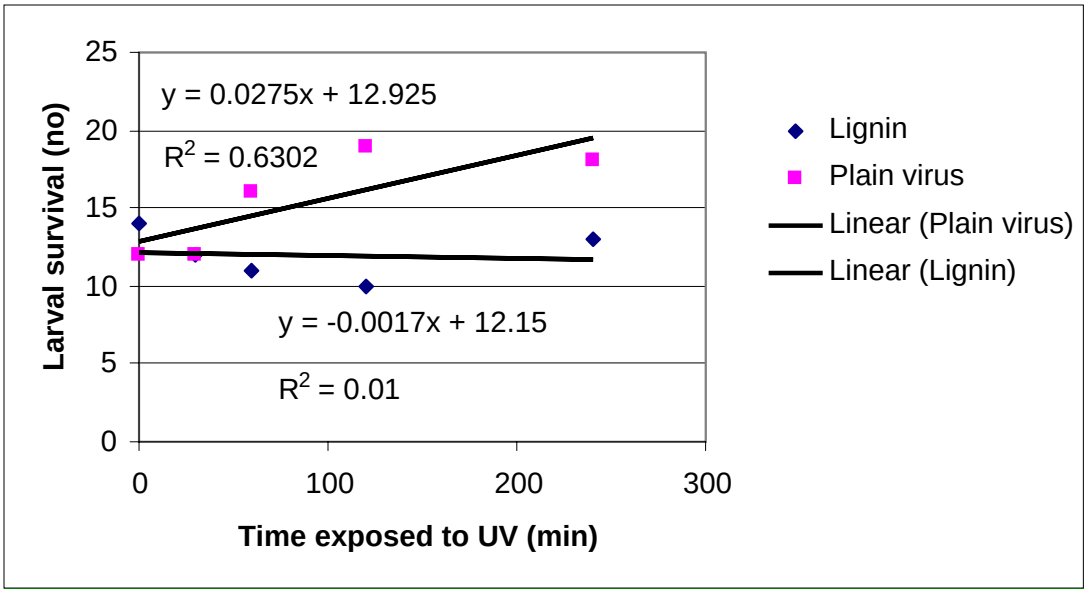

Fig 2.1. Survival of neonate FCM larvae exposed to Cryptogran, with and without lignin, exposed to a germicidal UV lamp for periods from 0 to 240 minutes, in laboratory bioassays.

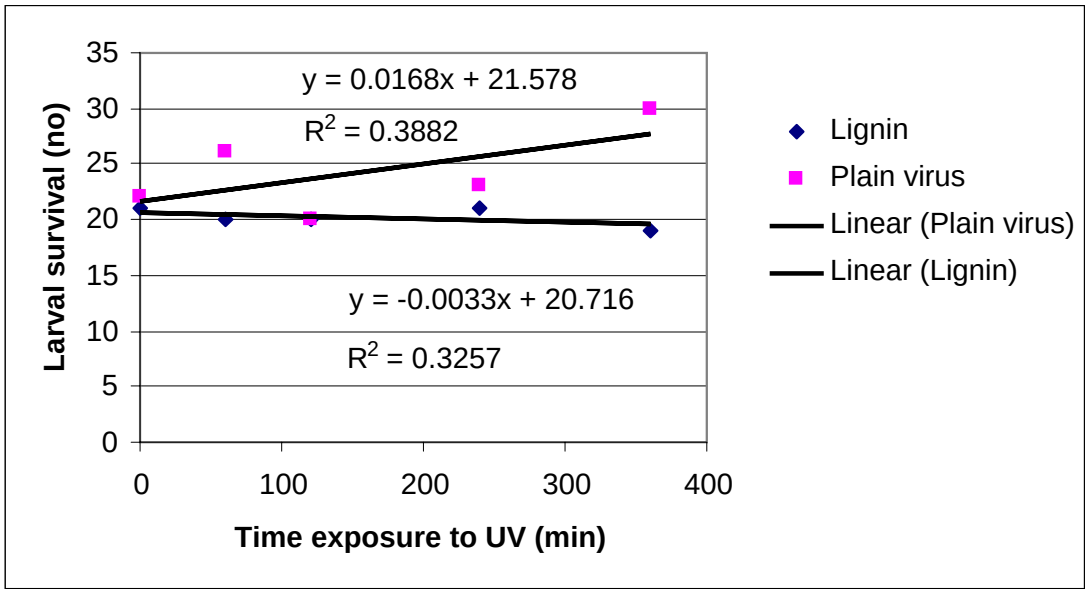

Fig 2.2. Survival of neonate FCM larvae exposed to Cryptogran, with and without lignin, exposed to a germicidal UV lamp for periods from 0 to 360 minutes, in laboratory bioassays.

2.3.2.6. Cryptogran and other additives

Control mortality in the Tinopol and Silica bioassays was unacceptably high (Table 2.11). In all three cases the suspension which was exposed to UV-irradiation for the longest period (360 minutes) caused higher mortality than most of the treatments which were exposed to UV-irradiation for shorter periods of time. This may have been due to evaporation from the Petri dishes. As previously explained, this would have resulted in a more concentrated virus suspension than that calculated, causing a higher than expected level of mortality.

Table 2.11. Impact of UV-irradiation (germicidal lamp) on Cryptogran (1.34 x 10^5 OBs/ml), with and without Tinopol and silica, measured by mortality of neonate FCM larvae in a dose-response bioassay.

Treatment		Time of exposure to UV (min)	Mortality of neonate FCM larvae (%) (without Tinopol or silica)	Mortality of neonate FCM larvae (%) (with Tinopol)	Mortality of neonate FCM larvae (%) (with silica)
1	Distilled water	0	32	56	52
2	Cryptogran	0	76	88	96
3	Cryptogran	30	92	76	92
4	Cryptogran	60	72	84	84
5	Cryptogran	120	56	64	72
6	Cryptogran	240	76	88	92

Even without molasses and after 240 minutes of exposure to the germicidal lamp, there was little breakdown of the virus (Table 2.12). This might have been due to evaporation of water from the Cryptogran suspension. Despite the low level of mortality, it was shown that molasses did not provide any UV protection for the virus (Table 2.12). Clearly there was no dose-response, so it was not possible to conduct a probit analysis. In field applications, the addition of molasses results in a substantial improvement in the

efficacy of Cryptogran (Moore *et al*, 2004b). This benefit may be from molasses being a feeding attractant and a sticker, rather than a UV-protectant.

Table 2.12. Impact of UV-irradiation (germicidal lamp) on Cryptogran (1.34 x 10^5 OBs/ml), with and without molasses (250 ml/100 *l* water), measured by mortality of neonate FCM larvae in a dose-response bioassay.

Treatment		**Time of exposure to UV (min)**	**Mortality of neonate FCM larvae (%) (with molasses)**	**Mortality of neonate FCM larvae (%) (without molasses)**
1	Distilled water	0	28	16
2	Cryptogran	0	80	68
3	Cryptogran	60	92	92
4	Cryptogran	120	88	84
5	Cryptogran	240	80	72

2.3.3. Rainfastness

2.3.3.1. Fruit dip bioassay to determine the rainfastness of Cryptogran

The first bioassay conducted indicated that Cryptogran was rainfast (Table 2.13). There was no significant difference between number of fruit penetrated and infested where Cryptogran treated fruit were exposed to 'rain' or not. Both Cryptogran treatments showed significantly less penetration marks and infestation rate than the untreated control.

Table 2.13. FCM damage and infestation of Valencia oranges in a detached fruit bioassay to test the rainfastness of Cryptogran

Treatment		Fruit decayed (%)	Fruit penetrated (%)	Mean penetration marks per fruit P = 0.0070 F = 5.23 df = 95	Fruit infested (%)	Mean larvae per fruit P = 0.0007 F = 7.87 df = 95
1	Distilled water	36.7	63.3	0.90±0.14**a**	66.7	0.90±0.14**a***
2	Cryptogran (10 ml/100 *l*) + molasses (0.5%)	10.0	33.3	0.33±0.31**b**	30.0	0.33±0.09**b**
3	Cryptogran (10 ml/100 *l*) + molasses (0.5%) + 'rain' dip	16.7	40.0	0.47±0.11**b**	26.7	0.30±0.09**b**

*Different letters in the same column denote significant differences (Duncan's multiple range test).

2.2.3.2. Bioassays using a rain simulation machine

Where the rainfall simulator was used, once again the simulated rainfall had no effect on the efficacy of Cryptogran (Table 2.14), as shown by the mean number of larvae per fruit. The difference between the two Cryptogran treatments and the untreated control, although apparent, was not significant.

Table 2.14. FCM damage and infestation of Valencia oranges in a detached fruit bioassay to test the rainfastness of Cryptogran using a rain simulation machine

Treatment		Mean penetration marks per fruit **P = 0.4318** **F = 0.85** **df = 89**	Mean larvae per fruit **P = 0.1948** **F = 1.67** **df = 89**
1	Distilled water	1.00±0.14**a**	0.93±0.14**a***
2	Cryptogran (10 ml/100 *l*) + molasses (0.25%) + Agral 90 (18 ml / 100 *l*)	0.77±0.12**a**	0.60±0.13**a**
3	Cryptogran (10 ml/100 *l*) + molasses (0.25%) + Agral 90 (18 ml / 100 *l*) 'rain' @ 36mm per 5 minutes	0.80±0.12**a**	0.67±0.13**a**

*Different letters in the same column denote significant differences (Bonferroni multiple range test).

2.4. Discussion

The effect of UV on Cryptogran was calculated, and it was determined that the germicidal lamp used caused a similar breakdown of the virus as normal sunlight. Unfortunately the standard error values were extremely high for both bioassays, indicating that the results did not simulate the predicted curve due to high variability. Although it is recognised that further replication is necessary, it was apparent that that the germicidal lamp was a good substitute for sunlight, under the conditions that the trial was conducted.

It is possible that in treatments where Cryptogran was exposed to UV-irradiation for longer periods, some evaporation of water from the Cryptogran suspension may have occurred from the Petri dish during the long period of exposure. This would result in the suspension being more concentrated than initially measured and hence the higher than expected larval mortality. Improved techniques to avoid this will be discussed in the final chapter.

Wetcit and boric acid appeared to cause a degree of mortality when bioassayed against neonate FCM larvae. As no higher than 28% mortality was recorded, it is unlikely that Wetcit will be adequately effective to be used as a stand-alone product for control of FCM. Wetcit, however, did not increase the efficacy of Cryptogran in a dose-response bioassay. The LD_{50} values indicate that the addition of Wetcit decreased the efficacy of the virus. This could be because some wetters contain detergents, such as Sodium Dodecyl Sulphate (SDS) which can be detrimental to baculoviruses (Lua *et al*, 2003). The addition of Wetcit to Cryptogran resulted in higher larval mortality after lengthy exposures to a UV lamp. This indicated that Wetcit provided some UV protection to the virus. A degree of the increased mortality could be attributed to the insecticidal properties of Wetcit, which is reflected in the higher control mortality where Wetcit was added.

Lignin consistently appeared to be the most effective UV protectant tested. There was very little difference in larval mortality between the distilled water control treatments, with and without lignin, in all of the bioassays. This would indicate that the higher larval mortality where lignin was added was not due to a synergistic effect between lignin and the virus, but due to UV-protection provided by lignin, which reduced the rate of breakdown of the virus. In related studies, the addition of lignin increased the half-life period of virus up to about 6.6 fold for a simulated sunlight system in Petri dishes when evaluating additives for the persistence of NPV of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) (SpliMNPV) in Egypt (Amin *et al*, 2004).

The bioassays with Tinopol (optical brightener) and Silica did not give clear indications of their effectiveness as UV protectants, and could be repeated in future trials. However, they did not appear to be as effective as lignin and Wetcit, and will not be tested further for the purpose of this study. Many studies have been conducted testing optical brighteners as UV protectants for viruses. Tamez-Guerra *et al* (2000) concluded that the inclusion of optical brighteners in AfMNPV (NPV of *Anagrapha falcifera* (Kirby) (Lepidoptera: Noctuidae), the celery looper) formulations did not improve initial activity of virus persistence, but a 1% tank mix addition significantly enhanced activity and

improved persistence. Optical brighteners improve the efficacy of viruses by providing UV protection and enhancing viral activity (Washburn *et al*, 1998). Larvae of certain species displayed both an increase in susceptibility to fatal infection and a reduction in time to death following ingestion of viruses in the presence of Tinopol (Washburn *et al*, 1998). The enhancement could be due to the ability of the optical brighteners to block sloughing of infected primary target cells within the midgut epithelium (Washburn *et al*, 1998). Tinopol enhanced the viral activity of the potato tuber moth (*Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae)) GV (PoGV) by factors ranging from 8.6 to 134, but higher mortalities as a result of irradiated PoGV with Tinopol in comparison to unprotected PoGV was attributed to enhancement of remaining viral activity rather than to increased viral persistence (Sporleder *et al*, 2004).

It has been shown by Moore *et al* (2004b) that molasses enhances and improves the efficacy of Cryptogran. The bioassays in this study would indicate that this is not due to UV protection. The benefit of molasses could be due to it acting as a sticker and/or a feeding attractant for the FCM larvae (S. Moore, personal communication). Ballard *et al* (2000) demonstrated that 15% cane molasses incorporated within a formulation of purified CpGV dramatically reduced the medial lethal exposure time (LET_{50}) for neonate codling moth larvae. They also found a greater reduction in codling moth damage in field trials where molasses was added to the virus.

Rainfall was noted in various field trials over the years, and has not appeared to have any detrimental effect on the efficacy of the virus (S. Moore, personal communication). This is consistent with the results of the fruit dip bioassay. The trial using the rain simulation machine indicated rainfastness, although the difference between the untreated control and the virus treatments was not significant. The intensity of the ‘rainfall’ applied was very high, and if Cryptogran can control FCM after this exposure, then it can be deemed rainfast for practical purposes. Jacques (1972) suggested that deposits of *Trichoplusia ni* NPV were not easily washed from foliage by rain, and sunlight was the main reason for loss of efficacy of the virus. The interim deduction would be that the product is rainfast, and does not need to be reapplied if rainfall occurs after the spray has dried on the trees.

Lignin and Wetcit were the most promising UV protectants in the laboratory bioassays, and need to be tested in field trials. This will be discussed in the next chapter (Chapter 3).

Chapter 3: Field trials to evaluate the effect of additives on the residual efficacy of Cryptogran

3.1. Introduction

Certain products tested in laboratory trials in the previous chapter have shown potential to increase the efficacy or residual efficacy of Cryptogran. Laboratory results are essential in assessing products, but translation of this information to field performance is difficult because so many biotic and abiotic variables have to be taken into account (Hunter-Fujita *et al*, 1998). Evans (1994) stressed the need for products to be tested under field, as well as laboratory conditions. Shapiro *et al* (2002) designed laboratory tests, using HzSNPV and SeMNPV, to determine the relationship between virus concentration and virus persistence, so as to serve as the basis for technology transfer in subsequent field trials to optimize field performance.

The specific aims of this chapter were to investigate if the addition of lignin and Wetcit to Cryptogran had similar effects on the residual efficacy of the product under field conditions, as in laboratory trials. Some individual components which make up Wetcit were tested to see if they increased the efficacy of the product. Certain products, which have been used in other trials found in literature studies, were also tested, although they were not tested in the laboratory during this study.

3.2. Materials and methods

3.2.1. Adjuvants field trial 1

The first trial was conducted to test the effect of various adjuvants when applied with Cryptogran. This trial was applied on an orchard of Lane Late navel oranges on Allandale farm in the Sundays River Valley (spacing 6 m x 3 m (rows x trees), planted 1998). The trial was laid out in a single-tree, random block formation, replicated 10 times. On 12, 13 and 14 December 2005, an average of 21 *l* of spray formulation (Table 3.1) was applied per tree. An untreated control was retained.

Fruit drop from data trees was evaluated from three weeks after application, until there was a substantial decline in efficacy. Dropped fruit from each tree was collected weekly, and analysed separately. Evaluations were done by inspecting and dissecting fruit in order to determine the cause of drop. FCM infestation was determined by the presence of the larva or its frass. Mean numbers of FCM infested fruit per tree per week were compared using ANOVA and the Bonferroni multiple range test, using Statgraphics Plus for Windows Version 2 (Statistical Graphics Corporation 1996). Reduction in FCM infestation was calculated by (A – B)/A x 100, where A = the total number of infested fruit for all replicates of untreated control, and B = the total number of infested fruit for all replicates of each treatment.

Table 3.1. Treatments applied on 12, 13 & 14 December 2005 for the control of FCM on Lane Late navel oranges at Allandale farm.

Treatment		**Dosage in 100 *l* water**
1	Untreated control	
2	Cryptogran + molasses + Raynox (Pace International, USA)	10 ml + 500 ml + 250 ml
3	Cryptogran + molasses + lignin carrier 038A	10 ml + 500 ml + 200 ml
4	Cryptogran + molasses + Wetcit	10 ml + 500 ml + 200 ml
5	Cryptogran + molasses + Nufilm 17 (Miller Chemical and Fertilizer Company, USA)	10 ml + 500 ml + 20 ml
6	Cryptogran + molasses	10 ml + 500 ml
7	Cryptogran + molasses + Agral 90 (Plaaskem, South Africa)	10 ml + 250 ml + 18 ml

3.2.2. Adjuvants field trial 2

A second field trial was conducted to test the effect of various other adjuvants when applied with Cryptogran. Borax and citrus oil are two chief components of Wetcit. Wetcit appeared to increase the efficacy of Cryptogran in the first trial, so it was decided to test these two products individually, in order to examine whether they would improve the

performance of Cryptogran. This trial was applied on an orchard of Autumn Gold navel orange trees on Carden farm in the Sundays River Valley (spacing 6 m x 2 m (rows x trees), planted in 1999). The trial was laid out in a double-tree, random block formation, replicated 10 times. On 29 and 30 March 2006, an average of 27 *l* of spray formulation (Table 3.2) was applied per double-tree replicate. An untreated control was retained.

As two trees were sprayed per replicate, fruit drop between the trunks of the two trees was evaluated from three weeks after application, until there was a substantial decline in efficacy. Dropped fruit was collected weekly, and analysed separately. Evaluations were done by inspecting and dissecting fruit in order to determine the cause of drop. FCM infestation was determined by the presence of the larva or its frass. Mean numbers of FCM infested fruit per tree per week were compared using ANOVA and the Bonferroni LSD multiple range test, using Statgraphics Plus for Windows Version 2 (Statistical Graphics Corporation 1996). Reduction in FCM infestation was calculated as described in 3.2.1.

Table 3.2. Treatments applied on 29 & 30 March 2006 for the control of FCM on Autumn Gold navel oranges on Carden farm.

Treatment		**Dosage in 100 *l* water**
1	Untreated control	
2	Cryptogran + molasses + Agral 90	10 ml + 250 ml + 18 ml
3	Cryptogran + molasses + Agral 90 + Acarol (Syngenta Crop Protection, Switzerland)	10 ml + 250 ml + 18 ml + 30 ml
4	Cryptogran + molasses + Agral 90 + Borax	10 ml + 250 ml + 18 ml 300 g
5	Cryptogran + molasses + Clementine oil	10 ml + 250 ml + 300 ml
6	Cryptogran + molasses +BP Medium spray oil (BP Southern Africa, South Africa)	10 ml + 250 ml + 300 ml
7	Cryptogran + molasses + Break-thru (Degussa, Germany)	10 ml + 250 ml + 5 ml

3.2.3. Adjuvants trial 3

A third trial was applied in an orchard of mature Owari Satsuma mandarin trees on Pennyholme farm in the Sundays River Valley. Literature studies revealed that milk powder (Pritchett *et al*, 1980; Ballard *et al*, 2000) and yeast (Jacques, 1971; 1972) had been tested in trials as UV protectants for insect viruses. These products were added to Cryptogran treatments to see if they would influence the efficacy of the treatments. Each treatment was applied on 03 February 2004, to 2 blocks of approximately 40 trees, using an oscillating tower mist-blower. An average of 9 *l* of spray mix was applied per tree. Two untreated blocks were retained (Table 3.3). Five data trees were selected in the centre of each block, and fruit drop was evaluated for FCM infestation as described in the previous trials. Reduction in FCM infestation was calculated as described in 3.2.1.

Table 3.3. Treatments of Cryptogran alone and in combination with various additives, applied to Satsuma mandarin trees at Pennyholme Farm on 2 February 2004.

Treatment		**Dose per 100 *l* water**
1	Untreated control	
2	Cryptogran	10 ml
3	Cryptogran + molasses	10 ml + 500 ml
4	Cryptogran + low fat milk powder	10 ml + 200 g
5	Cryptogran + Brewers yeast	10 ml + 200 g

3.3. Results

3.3.1. Adjuvants trial 1

In the first trial, the treatments were applied against a very high level of FCM, with almost 10 infested fruit per tree per week at the beginning, and averaging 3.9 infested fruit per tree per week over 11 weeks of evaluation in the untreated control. All of the treatments showed significantly less infested fruit than the untreated control, but there were no significant differences between the treatments (Table 3.4)

The addition of Wetcit appeared to cause a faster knock-down of FCM than the standard Cryptogran treatment (Cryptogran + molasses - treatment 7). As the evaluation progressed, it appeared that where lignin was added, the FCM infestation was lower than where the standard treatment was applied (Table 3.4). These differences were not statistically significant, but showed a trend, and this product warrants further investigation.

Table 3.4. FCM infestation of Lane Late navel oranges on Allandale Farm, subjected to various treatments (in combination with Cryptogran) applied 12-14 December 2005. (P<0.001, F = 6.98, df = 69).

Treatment	**Fruit from data trees infested with FCM**										**Reduction in infestation (%)**
	3 WAT**	**4 WAT**	**5 WAT**	**6 WAT**	**7 WAT**	**8 WAT**	**9 WAT**	**10 WAT**	**11 WAT**	**Mean/ Tree/ week**	
Untreated control	97	74	22	20	14	32	43	38	16	3.9±0.32**a***	
Cryptogran + molasses + Raynox	42	24	11	13	13	23	40	21	15	2.2±0.21**b**	43.3
Cryptogran + molasses + Lignin carrier	49	27	7	7	3	21	29	19	12	1.9±0.22**b**	51.1
Cryptogran + molasses + Wetcit	34	19	4	7	7	21	39	22	15	1.9±0.18**b**	52.8
Cryptogran + molasses + Nufilm 17	50	30	8	9	9	27	38	24	11	2.3±0.37**b**	42.1
Cryptogran + molasses	47	31	13	10	12	26	40	29	13	2.5±0.26**b**	37.9
Cryptogran + molasses + Agral 90	38	29	5	11	6	21	37	31	14	2.1±0.26**b**	46.1

*Different letters in the same column denote statistically significant differences (P<0.05, LSD multiple range test)

** Weeks after treatment

3.3.2 Adjuvants trial 2

In the second trial Cryptogran was tested separately with two known ingredients of Wetcit, i.e. borax and citrus oil, to determine if they had any individual positive effect on the efficacy of Cryptogran. However, neither significantly improved the efficacy of Cryptogran (Table 3.5).

Break-thru, an organo-silicon wetter, appeared to work as well as the registered alkylated phenol-ethylene oxide wetter. The addition of BP Medium spray oil to Cryptogran and molasses resulted in the greatest reduction in infestation of FCM. However, the reduction was not significantly different to the Cryptogran treatment without oil (Table 3.5).

Table 3.5. FCM infestation of Autumn Gold navel oranges on Carden Farm, subjected to various treatments (in combination with Cryptogran) applied 29-30 March 2006. (P = 0.0101, F = 3.10, df = 69).

Treatment	Fruit from data trees infested with FCM								Reduction in infestation (%)
	3 WAT**	4 WAT	5 WAT	6 WAT	7 WAT	8 WAT	9 WAT	Mean/ Tree/ Week	
Untreated control	8	13	8	7	9	3	3	0.76±0.14**a***	
Cryptogran + molasses + Agral 90	4	4	1	7	8	2	1	0.39±0.10**ab**	47.1
Cryptogran + molasses + Agral 90 + Acarol	3	5	4	5	3	1	1	0.31±0.08**b**	56.9
Cryptogran + molasses + Agral 90 + borax	4	3	5	3	9	0	5	0.41±0.05**ab**	43.1
Cryptogran + molasses + clementine oil	1	4	2	3	6	2	4	0.31±0.06**b**	56.9
Cryptogran + molasses + BP Medium spray oil	3	2	4	4	5	0	1	0.27±0.10**b**	62.7
Cryptogran + molasses + Break-Thru	3	2	5	6	6	0	1	0.33±0.09**b**	54.9

*Different letters in the same column denote significant differences between values (P<0.05, Bonferroni multiple range test)

** Weeks after treatment

3.3.3. Adjuvants trial 3

In the third trial on Satsuma mandarins, the addition of milk powder and yeast to Cryptogran caused a slightly higher reduction in infestation than Cryptogran alone (Table 3.6). However, neither additive was as effective as molasses, which resulted in a 59.6% reduction in FCM infestation when applied with Cryptogran. The trees were planted extremely close together, so it was difficult to determine which dropped fruit belonged to which tree. The fruit from the 5 data trees in each block were therefore evaluated collectively. The data could therefore unfortunately not be statistically analysed.

Table 3.6. FCM infestation of Satsuma mandarins at Pennyholme Farm in the Sundays River Valley, evaluated from three weeks after treatment on 3 February 2004, for a period of three weeks.

Treatment	Mean infested fruit per tree per week	% Reduction in infestation relative to untreated control
Untreated control	3.13	
Cryptogran (10 ml/100 *l*)	2.17	30.9
Cryptogran (10 ml/100 *l*) + molasses (500 ml/100 *l*)	1.27	59.6
Cryptogran (10 ml/100 *l*) + milk powder (200 g/100 *l*)	2.03	35.1
Cryptogran (10 ml/100 *l*) + Brewers yeast (200g /100 *l*)	1.70	45.7

3.4. Discussion

When Wetcit was added to Cryptogran, it appeared to cause a faster knockdown of FCM populations than Cryptogran alone. This is possibly due to the fact that Wetcit is claimed to have certain insecticidal properties (Kirkman & Moore, 2006), as the product is marketed by UAP as an insecticide. When citrus (clementine) oil and boric acid, which are components of Wetcit, were added to Cryptogran, they did not appear to enhance the

efficacy thereof against FCM. The addition of NuFilm-17 and Raynox (a sunburn protector) did not significantly increase the efficacy or persistence of Cryptogran. This is consistent with findings by Arthurs *et al* (2006). They tested various adjuvants with commercial formulations of CpGV in laboratory tests, and found that these two products did not provide solar protection for the virus. Lacey *et al* (2004) found that Raynox did not increase the residual activity of CpGV in apple orchard trials. Lignin appeared to increase the field persistence of Cryptogran, as reflected in lower FCM infestation in the treatment where lignin was added, several weeks after application. Lignin has previously been used as a UV protectant, and there are several formulations commercially available (Hunter-Fujita *et al,* 1998). The product was tested in further field trials, reported in the following chapter, and possibilities are discussed there.

In the second trial, the addition of a medium grade spray oil to Cryptogran resulted in the lowest FCM infestation. Certain pesticides are registered to be applied with oil, and at present the recommendation to growers is to add molasses at 0.5% when applying Cryptogran together with these pesticides, as there is no wetter added. The addition of a wetter is not recommended where oil is used, as the wetter may influence the viscosity of the oil, and thus reduce its beneficial properties (S. Moore, personal communication). Further trials could be conducted to see whether the molasses concentration can be reduced to 0.25% when oil is added (and a wetter is not used), as it appears from this trial that the addition of oil enhances the efficacy of Cryptogran. Where Agral 90 was replaced with Break-thru, results were comparable. This was encouraging, as there is a possibility that use of the nonylphenol polyethoxylate (NPE) wetters, including Agral 90, may be restricted by certain markets in years to come (Cox & Drys, 2003).

On Satsuma mandarins, of all the products added to Cryptogran, molasses gave the greatest reduction in infestation. This is consistent with results of many field trials conducted by Moore *et al* (2004b). The addition of milk powder and yeast also appeared to improve the efficacy of Cryptogran against FCM. Ballard *et al* (2000) showed that skimmed milk powder increased the efficacy of CpGV in laboratory and field trials. The addition of powdered milk to *Hyphantria cunea* NPV sprays increased the larval

mortality of *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) in field trials (Pritchett *et al*, 1980).

As mentioned, the addition of yeast also improved the efficacy of Cryptogran, but unfortunately the significance could not be shown statistically. Brewers yeast has been shown to prolong the activity of *Trichoplusia ni* NPV and *Pieris rapa*e GV on the leaves of collard plants (Jacques, 1972).

Although certain products increased the residual efficacy of Cryptogran, none that will radically enhance or extend the residual efficacy of Cryptogran have been revealed by this study. Each additive adds an extra cost to the application, and cost-benefit analyses need to be conducted to determine if the increase in residual activity of the product can justify the cost thereof. Certain other aspects, such as timing of spray applications, need to be investigated. These factors are discussed in the following chapter (Chapter 4).

Chapter 4: The effect of timing of applications on the residual efficacy of Cryptogran.

4.1. Introduction

The effects of additives on the residual efficacy of Cryptogran have been discussed in the previous two chapters. There are other factors which could affect residual efficacy, such as timing of applications. UV levels differ at various times of the year (Schulze *et al*, 1997), and this could have an effect on the residual efficacy of Cryptogran at various times of the year. Citrus fruits, particularly navel oranges, are susceptible to FCM infestation, resulting in losses for a period of up to 7 months of the year. It would not be financially viable to control FCM with Cryptogran throughout this entire period. Trials were therefore conducted to determine the best time of the year to apply Cryptogran, with regard to efficacy and the period of residual activity.

The Lorelei trap is currently the most effective monitoring system for FCM, as it releases pheromone at a constant rate (Hofmeyr, 2003). High trap catches indicate a male flight peak and the assumption is made that this corresponds with a high level of female moth activity. In an attempt to investigate the benefit of using trap catches to decide when to apply Cryptogran, trials were conducted to see whether Cryptogran applications were more effective when applied at (or shortly after) moth peaks or between moth peaks.

Degradation of insect viruses by UV irradiation has been well documented (David, 1969). Trials were conducted to test the efficacy of Cryptogran when applied at different times of the day, i.e. morning, midday and evening. Lignin has been shown in the previous two chapters to have an effect on the efficacy of Cryptogran. Lignin was once again added to the virus to see if it would provide UV protection, particularly when it was applied at midday when UV irradiation is at its highest (Schulze *et al*, 1997).

4.1.1. Time of the season

The physiology of the host fruit changes during the growing and ripening phase, and UV levels vary throughout the year. These factors could influence the residual efficacy of the virus. Because of UV degradation of viruses (David, 1969), and the resulting relatively short residual efficacy of virus insecticidal products, it would be impossible to protect the crop against FCM for the entire susceptible period. It would not be economically viable to apply sprays of Cryptogran throughout the season to protect the fruit from infestation by FCM. Therefore trials were conducted to investigate at which time of the season the sprays would be most effective, i.e. have the longest residual efficacy.

4.1.2. Time of day

UV has a detrimental effect on granuloviruses (Hunter-Fujita *et al,* 1998), and UV levels are at their highest around midday (Schulze *et al*, 1997). Many growers do not have enough spray machines to apply all the sprays required during the season. If they could apply Cryptogran during the day as well as the evening, it would enable them to utilise their machinery more optimally. The recommendation is to spray Cryptogran during the evening, when UV irradiation is at its lowest. The aim of trials conducted here was to investigate the effect of the time of day that Cryptogran was applied. The effect of the addition of lignin on the efficacy of these applications at, various times of the day, was also investigated.

4.2. Materials and methods

4.2.1. Time of the season

A field trial was conducted to determine the effect that the stage of the season has on the residual efficacy of Cryptogran. Two further field trials, one consisting of a Cryptogran application early in the season, and the other an application shortly before harvest, were conducted to replicate some of the treatments in the first trial.

4.2.1.1. Field trials

A field trial was applied to test the efficacy of Cryptogran when applied at various times of the year. The effect of applying Cryptogran coinciding with FCM trap catch peaks, as

well as between peaks, was also investigated. This trial was applied on an orchard of Lina navel orange trees on Junkyard Farm in the Sundays River Valley (spacing 6 m x 2 m (rows x trees), planted 1999). The trial was laid out in a semi-commercial block format, replicated twice. Each block consisted of about 60 trees. The treatments were applied using a tower blower, which was set to spray from only one side. Sprays were applied on 07 December 2005, 10 January 2006, 09 February 2006 and 14 March 2006. An average of 14 *l* of spray mix was applied per tree. Untreated control blocks were retained (Table 4.1). After application, the trial was evaluated in the following manner. Seven data trees were selected in the centre of each block (i.e. a total of 14 data trees per treatment). Fruit drop from the data trees was evaluated from three weeks after application, until there was a substantial decline in efficacy. Dropped fruit was collected weekly, and analysed separately. Evaluations were done by inspecting and dissecting fruit in order to determine the cause of drop. FCM infestation was determined by the presence of the larva or its frass. Mean numbers of FCM infested fruit per tree per week were compared using ANOVA and the Bonferroni multiple range test, using Statgraphics Plus for Windows Version 2 (Statistical Graphics Corporation 1996). Reduction in FCM infestation was calculated by (A – B)/A x 100, where A = the total number of infested fruit for all replicates of untreated control, and B = the total number of infested fruit for all replicates of each treatment.

Table 4.1. Cryptogran treatments applied to Lina navel orange trees on Junkyard Farm at various times of the year.

Treatment		Dosage in 100 *l* water	Date of application	Coinciding with trap peak?
1	Untreated control			
2	Cryptogran + molasses + Agral 90	10 ml + 250 ml + 18 ml	07/12/05	Yes
3	Cryptogran + molasses + Agral 90	10 ml + 250 ml + 18 ml	10/01/06	No
4	Cryptogran + molasses + Agral 90	10 ml + 250 ml + 18 ml	09/02/06	Yes
5	Cryptogran + molasses + Agral 90	10 ml + 250 ml + 18 ml	14/03/06	Yes

Another trial was conducted to investigate the efficacy of Cryptogran when applied early in the citrus growing season. The trial was conducted in an orchard of Lane Late navel orange trees on Allandale Farm in the Sundays River Valley (spacing 6 m x 3 m (rows x trees), planted 1998). The trial was laid out in a single-tree, random block formation, replicated 10 times. On 12 December 2005, 21 *l* of a suspension of Cryptogran (10 ml/100 *l)* + molasses (250 ml/100 *l*) + Agral 90 (18 ml/100 *l*) was applied per tree. An untreated control was retained.

Fruit drop from data trees was evaluated from three weeks after application, until there was a substantial decline in efficacy. Dropped fruit from each tree was collected weekly, and analysed separately. Evaluations were done by inspecting and dissecting fruit in order to determine the cause of drop. FCM infestation was determined by the presence of the larva or its frass. Mean numbers of FCM infested fruit per tree per week were compared using a t-test (Statgraphics Plus for Windows Version 2 (Statistical Graphics Corporation 1996)).

A further trial was conducted on Bernol Farm in the Sundays River Valley, in an orchard of 12 year old Palmer navel orange trees. The aim of the trial was to investigate the residual efficacy of Cryptogran later in the season i.e. shortly before harvest. A treatment of Cryptogran (10 ml/100 *l*), molasses (250 ml/100 *l*) and Agral 90 (18 ml/100 *l*) was applied on 22 March 2004, to single trees in a randomised block formation, replicated 10 times. A Janisch hand-gun applicator was used, applying an average of 19.5 *l* of spray mixture per tree, at a pressure of 20 bar. The trial was evaluated for 6 weeks in the same way as described in the previous trial. Mean numbers of FCM infested fruit per tree per week were compared using a t-test (Statgraphics Plus for Windows Version 2 (Statistical Graphics Corporation 1996)).

4.2.2. Time of the day

Two trials were conducted to determine whether the time of day at which Cryptogran was applied would affect the efficacy and field persistence of the product.

4.2.2.1. Time of the day – trial 1

The first trial was applied in an orchard of mature Owari Satsuma mandarins on Pennyholme Farm in the Sundays River Valley. One Cryptogran treatment was applied at midday and another in the evening. Each treatment was applied on 03 February 2004, to two blocks of approximately 40 trees each, using an oscillating tower mist-blower. An average of 9 *l* of spray mix was applied per tree. Two untreated blocks, of similar size, were retained (Table 4.2). Five data trees were selected in the centre of each block, and fruit drop was evaluated for FCM infestation as described in the previous trials. Reduction in FCM infestation was calculated as described in 4.2.1.1.

Table 4.2. Cryptogran treatments applied to Satsuma mandarin trees at Pennyholme farm, at midday and in the evening, on 2 February 2004.

Treatment		Dose per 100 *l* water	Time of application
1	Untreated control		
2	Cryptogran + molasses	10 ml + 500 ml	Midday (11h30 – 12h30)
3	Cryptogran + molasses	10 ml + 500 ml	Evening (17h00 – 19h00)

4.2.2.2. Time of the day – trial 2

A second trial was conducted in an orchard of mature Lane late navel oranges on Atmar farm in the Sundays River Valley. Cryptogran was applied in the morning (08h30), at midday (12h30) and in the evening (18h00). Additional applications were made at midday and in the evening, where a lignin sulphate carrier, carrier 038A (Omnova Solutions, Greensboro), was added to the Cryptogran suspension (Table 4.3). Treatments were applied on 31 January 2007, to single trees in a randomised block format, replicated 10 times, using a hand-gun applicator. An average of 22.8 *l* of spray mix was applied per tree. An untreated control was retained. Dropped fruit was collected and evaluated weekly for FCM infestation as described in the previous trials. Reduction in FCM infestation was calculated as described in 4.1.1.1

Table 4.3. Cryptogran treatments applied to Lane Late orange trees at Atmar farm at various times of the day on 31 January 2007.

Treatment		Dose per 100 *l* water	Time of application
1	Untreated control		
2	Cryptogran + molasses + Agral 90	10 ml + 250 ml + 18ml	Morning (08h30)
3	Cryptogran + molasses + Agral 90	10 ml + 250 ml + 18ml	Midday (12h30)
4	Cryptogran + molasses + Agral 90	10 ml + 250 ml + 18ml	Evening (18h00)
5	Cryptogran + molasses + Agral 90 + Lignin	10 ml + 250 ml + 18ml + 200 ml	Midday (12h30)
6	Cryptogran + molasses + Agral 90 + Lignin	10 ml + 250 ml + 18ml + 200 ml	Evening (18h00)

4.3. Results

4.3.1. Time of the season

4.3.1.1. Field trials

In the first trial, the December, February and March treatments were applied at or just after FCM male flight peaks, determined by the number of males caught in the Lorelei pheromone traps which had been placed in the trial orchard. The January treatment was applied between flight peaks, therefore possibly between FCM generations. FCM infestation in all treatments was evaluated until harvest on 27 April 2006 (Table 4.4).

Table 4.4. FCM infested fruit, per 14 data trees per week, of Lina navel oranges on Junkyard Farm, subjected to Cryptogran applied at various times of the year.

Date	Fruit from data trees infested with FCM				
	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**	**Treatment 5**
	Untreated control	**Applied 07/12/2005**	**Applied 10/01/2006**	**Applied 09/02/2006**	**Applied 14/03/2006**
04/01/06	40	15			
10/01/06	20	13			
18/01/06	14	2			
25/01/06	2	1			
02/02/06	2	1	2		
09/02/06	11	4	4		
17/02/06	10	5	5		
24/02/06	7	2	4		
03/03/06	2	1	2	0	
10/03/06	1	0	2	1	
17/03/06	3	2	3		
24/03/06	6	4	3	5	
30/03/06	5	2	5	5	
07/04/06	16	7	8	4	5
13/04/06	5	4	4	5	1
20/04/06	14				10

Treatments corresponding with flight peaks reduced infestation by 60.14% (December), 41.19% (February) and 45.76% (March), whereas the treatment applied between peaks reduced infestation by 39.89% (January) (Table 4.5). The December treatment resulted in the highest reduction in infestation. This control persisted for a period of 16 weeks, so it was more effective than the sprays applied later in the season (Table 4.5).

Table 4.5. Mean (±SE) FCM infestation per tree per week of Lina navel oranges on Junkyard Farm, per treatment, subjected to Cryptogran applied at various times of the season.

Treatment	**Date of Cryptogran application**			
	07/12/2005	**10/01/2006**	**09/02/2006**	**14/03/2006**
Mean fruit infested per week (control)	0.63±0.08	0.46±0.06	0.40±0.07	0.83±0.09
Mean fruit infested per week (Cryptogran)	0.25±0.02	0.28±0.05	0.24±0.05	0.45±0.09
Reduction in infestation (%)	60.14	39.89	41.19	45.76
Period of evaluation (weeks)	16	11	6	3

In the second trial, where Cryptogran was applied early in the season (12 December 2005), FCM levels were extremely high, with almost 10 infested fruit per tree per week at the beginning, and averaging 3.9±0.32 (mean ± SE) infested fruit per tree per week over 9 weeks of evaluation in the untreated control. Mean infested fruit per tree per week for the Cryptogran treated trees was 2.1±0.25 (mean ± SE), which equated to an average reduction in infestation of 46.1% over 9 weeks of evaluation (Fig 4.1). A t-test showed a significant difference between the two treatments ($t = 4.67385$, $P = 0.0002$). This was a good result, considering the high level of FCM infestation. This trial confirmed what was observed in the previous trial (Table 4.5), demonstrating a long period of residual activity (9 weeks) when Cryptogran was applied at this time of the season. Periods of efficacy of up to 17 weeks have been demonstrated in other trials where Cryptogran was applied early in the season, as is shown in Chapter 5.

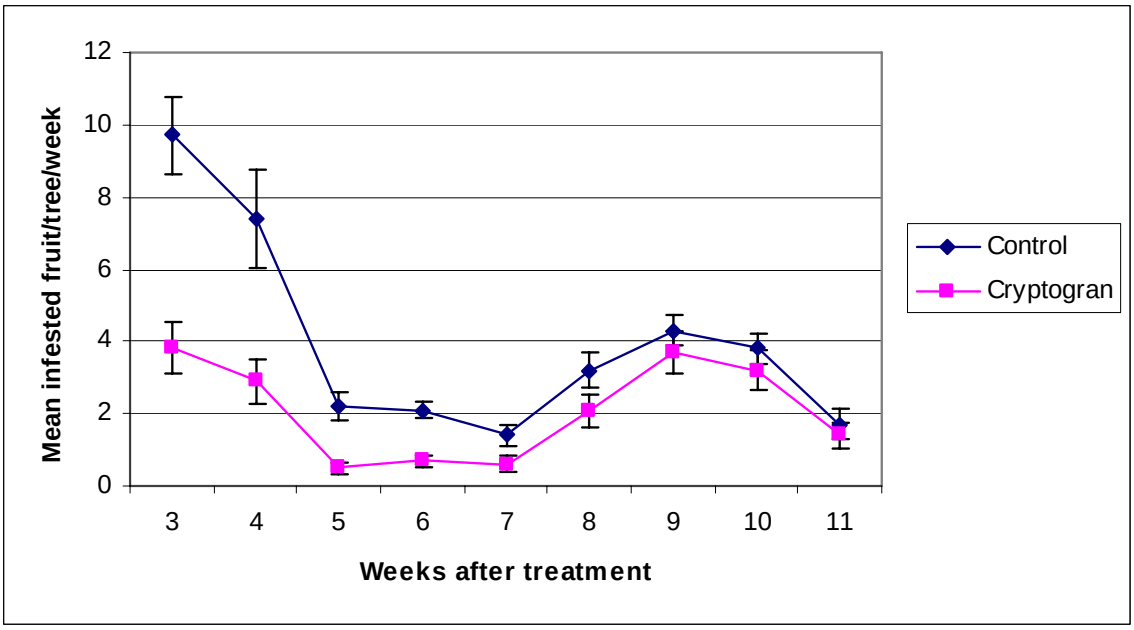

Fig 4.1: Weekly mean (±SE) FCM infestation for Cryptogran treatment and untreated control replicates at Allandale farm in the Sundays River Valley, applied on 12 December 2005.

In the third trial, Cryptogran application shortly before harvest resulted in a mean infestation (fruit per tree per week) of 0.27±0.06 (mean ± SE), compared with 0.6±0.12 (mean ± SE) infested fruit per tree per week in the untreated control. A t-test showed a significant difference between the two treatments ($t = 2.4246$, $P = 0.02607$). After six weeks of evaluation, there was no difference in FCM infestation between the treated and untreated trees i.e. there was no residual efficacy evident at this time (Fig 4.2). The Cryptogran treatment resulted in a 55% reduction in infestation over this period. The efficacy was as good as the treatments applied in earlier in the season (trial 1), but the residual working was a lot shorter at this time of the season. This confirmed observations in the first trial where Cryptogran was applied later in the season (Table 4.5).

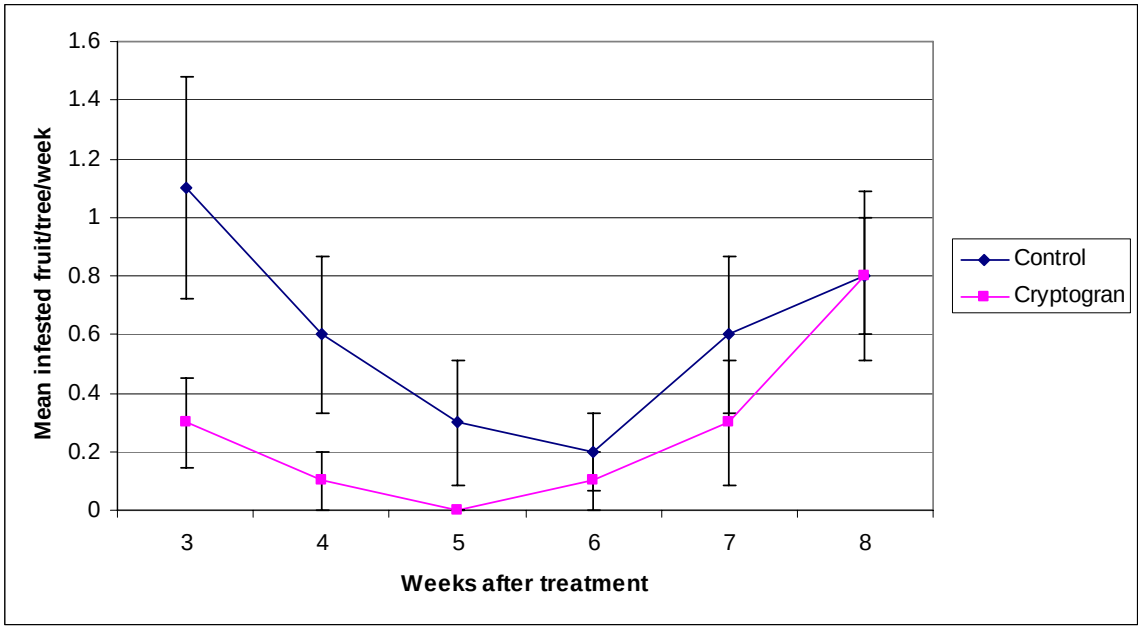

Fig 4.2. Weekly mean (±SE) FCM infestation for Cryptogran treatment and untreated control replicates at Bernol farm in the Sundays River Valley, applied on 22 March 2004.

4.3.2. Time of the day

4.3.2.1. Time of day – trial 1

In the first trial it was intended to evaluate each tree separately, but the trees were planted extremely close together, so it was difficult to determine which dropped fruit belonged to which tree. The fruit from the 5 data trees in each block were therefore evaluated collectively. The data could therefore not be statistically analysed, but it appears that the evening application was far more effective, resulting in a 59.6% reduction in FCM infestation, compared to a 41.5% reduction where the virus was applied at midday (Table 4.6).

Table 4.6. FCM infestation of Satsuma mandarins at Pennyholme Farm in the Sundays River Valley, evaluated for three weeks after treatment on 3 February 2004.

Treatment	Average infested fruit per tree per week	Reduction in infestation relative to untreated control (%)
Untreated control	3.13	
Cryptogran (10 ml / 100 *l*) + molasses (500 ml / 100 *l*) applied at Midday	1.83	59.6
Cryptogran (10 ml / 100 *l*) + molasses (500 ml / 100 *l*) applied in the evening	1.27	41.5

4.3.2.2. Time of day - trial 2

In the second trial, all three treatments resulted in a significant reduction in infestation of FCM. Where Cryptogran was applied in the evening, FCM infestation was significantly lower (0.39 fruit per tree per week) than for the morning (0.54 fruit per tree per week and noon (0.56 fruit per tree per week) applications (Table 4.7). This is consistent with the findings of the previous trial on Satsuma mandarins at Pennyholme Farm in 2004. There was no significant difference in infestation between the morning and midday treatments when analysed over the 7- week period of evaluation. However, it appeared that the morning treatment initially reduced FCM populations by more than the midday treatment. The addition of lignin resulted in a significant reduction in FCM infestation in both the midday (27% higher reduction in infestation) and evening applications (7% higher reduction in FCM infestation).

Table 4.7. FCM infestation of Lane Late navel oranges at Atmar Farm in the Sundays River Valley, evaluated from three weeks after treatment. (P = 0.0148, F = 3.13, df = 59)

Weeks after treatment /treatment	**Infested fruit per tree per week**							**Mean infested fruit/tree/week**	**Reduction in infestation (%)**
	3	4	5	6	7	8	9		
Untreated control	1.2	0.9	1.0	0.7	0.5	0.4	0.8	0.79±0.10**a***	
Cryptogran applied morning	0.8	0.5	1.1	0.4	0.2	0.1	0.7	0.54±0.07**ab**	31
Cryptogran applied midday	1.0	0.9	0.8	0.3	0.2	0.1	0.6	0.56±0.09**ab**	29
Cryptogran applied evening	0.8	0.6	0.4	0.2	0.1	0.2	0.4	0.39±0.12**b**	51
Cryptogran + lignin applied midday	0.5	0.8	0.2	0.1	0.3	0.1	0.4	0.34±0.10**c**	56
Cryptogran + lignin applied evening	0.4	0.5	0.6	0.3	0.1	0.1	0.3	0.33±0.09**c**	58

* Different letters in the same column denote significant differences between values (Duncan's multiple range test)

4.4. Discussion

Trials showed that early applications (December) resulted in the highest reduction in infestation, which also persisted for longer periods (16 weeks, (Table 4.5)). This was confirmed in the second trial (9 weeks of residual efficacy (Fig 4.1)) when applied early in the citrus growing season (December). When Cryptogran was applied later, shortly before harvest, the period of residual efficacy was shorter (6 weeks in the third trial (Fig 4.2)). The longer residual efficacy as a result of applications early in the season could be due to the effect of the navel end of the navel orange. This possibility will be discussed in detail in the following chapter (Chapter 5).

Trials also showed that treatments applied during or shortly after male flight peaks, were more effective that treatments applied between flight peaks (Table 4.5). This strongly indicated that there may be value in the theory that Cryptogran sprays should be applied shortly after flight peaks, as Cryptogran is only effective against the larvae and must be ingested by them. Assuming that male trap catch peaks coincide with female moth activity and egg laying, this could be as a result of the spray being applied at a time when many eggs are laid, and thus a concentrated amount of larvae will be hatching shortly thereafter. The virus will be freshly applied at this time, and will be at its most effective, before UV breakdown begins. This is therefore a very small window of opportunity, which occurs shortly after a peak in flight activity (Kirkman & Moore, 2006).

Treatments applied at midday were not as effective as those applied in the evening. It is speculated that this is due to faster UV breakdown while the virus is still in suspension. UV rays could be refracted within the water droplet, thus encountering more virus particles and causing greater inactivation of the virus, resulting in reduced efficacy of the product (S. Moore, personal communication). Smirnoff (1971) reported that the *Neodiprion swainei* NPV lost most of its activity after 150 hours of exposure to sunlight, and recommended that the virus be applied late in the day to reduce sunlight inactivation. Desiccation improves stability of viruses, with dry deposits of virus more stable than wet virus when exposed to a UV light (David, 1969). Jacques (1985) also suggests that surface moisture favours the inactivation of viral deposits exposed to sunlight.

The addition of lignin improved the efficacy of Cryptogran, especially when treatments were applied at midday. This reinforces the potential of lignin as a UV protectant, which was shown in the previous two chapters. Much has been published about lignin products adding UV protection, both in formulation and as spray additives. Arthurs *et al* (2006) found that lignin-based CpGV formulations provided solar protection at relatively high virus doses, but in season long orchard tests, lignin formulations did not significantly improve the control of codling moth on apples. Tamez-Guerra *et al* (2000) found that NPV isolated from the celery looper, *Anagrapha falcifera* (AfMNPV) had more insecticidal activity after exposure to natural sunlight when formulated with lignin.

McGuire *et al* (2001) found that virus formulations containing lignin retained activity significantly longer than unformulated *Anagrapha falcifera* and *Autographa californica* (Speyer) NPVs. Amin *et al* (2004) found that lignin products proved to more efficient than a fluorescent brightener (Tinopol LPW) as a UV protectant for baculoviruses. The aromatic chemistry of lignin makes it an excellent protective matrix for materials that are sensitive to degradation processes initiated by UV radiation. Lignin and lignin derivatives are also used as adjuvants to protect *Bacillus thuringiensis* crystal protein endotoxin from sunlight degradation (Tamez-Guerra *et al,* 2000). Lignin sulphate protected *Heliothis* NPV from UV inactivation on cotton, soybeans and tomatoes (Young *et al,* 1974).

Unfortunately the lignin product used for the trials is imported and expensive. The product is apparently easily washed off, and loses effect after significant rainfall (J. Podgewaite, personal communication). Cost-benefit analyses will be discussed in Chapter 6.

Chapter 5: Biotic factors affecting the residual efficacy of Cryptogran

5.1. Introduction

Various abiotic factors, such as UV-irradiation, rain, adjuvants and additives, which could affect the field persistence of Cryptogran, have been discussed in earlier chapters. There are various biotic factors which could also play a role in determining the period of residual efficacy of a microbial insecticide on citrus. As far as the insect host is concerned, these include factors such as position of egg laying, penetration points by the larvae, and migratory potential of the pest. Biotic factors pertaining to the plant host include time of the year of application, which has already been discussed in the previous chapter, shading of the citrus tree, susceptibility of different varieties of citrus to the pest, and the physical characteristics of the fruit (certain parts which could be more suitable for penetration by the pest (S. Moore, personal communication)).

Trials were conducted to determine the effect of the navel end of a navel orange of the residual efficacy of Cryptogran. The navel end is an invaginated area at the bottom of the fruit, formed by a supernumerary fruit. Virus within the navel end could be protected from UV irradiation and rain. In the evaluation of many field trials with Cryptogran, the place of penetration on the orange by FCM has been recorded at various times of the year. Early in the season, 95% of larvae chose to enter the fruit through the navel end (Fig 5.1) (Moore & Kirkman, unpublished data). There is therefore a high probability that a high percentage of larvae will encounter viable virus particles here, and thus the residual efficacy of the product would be extended. As the season progressed and the fruit developed, the behaviour of the pest changed and most of the larvae entered the fruit through the side, exactly where the virus was exposed to direct UV degradation and rainfall, resulting in a shorter period of residual activity.

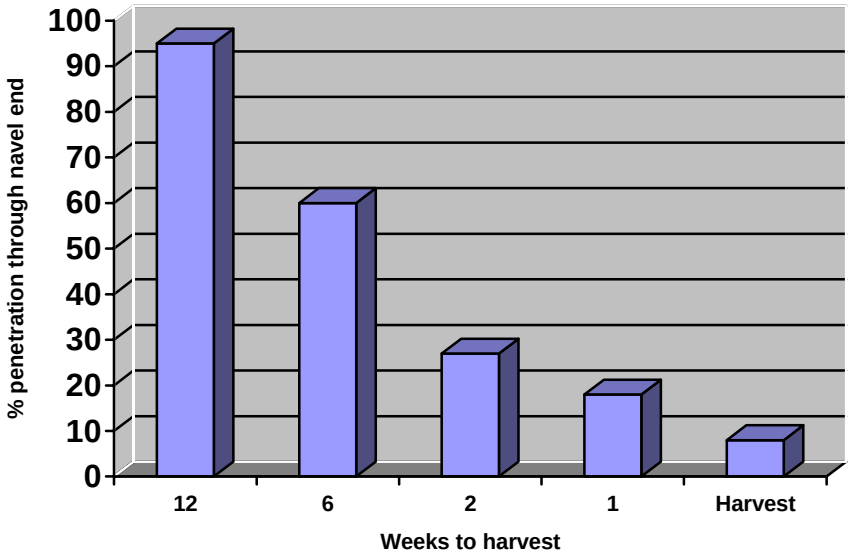

Fig 5.1. Percentage of larvae penetrating navel oranges through their navel end and various periods before harvest (Moore & Kirkman, unpublished data).

Cryptogran appears to be more effective when applied to larger areas of citrus trees (Moore *et al*, 2004a). Trials were also conducted in determine the effect of the size of treated areas on the residual efficacy of Cryptogran, by applying treatments to single trees and to blocks of trees.

5.2. Materials and methods

5.2.1. The effect of the navel end

A laboratory fruit-dip bioassay was conducted to determine the effect of the navel end of a navel orange on the residual efficacy of Cryptogran. It was speculated that the navel end sheltered virus from UV breakdown, as well as rain, in a place where most of the insects chose to penetrate the fruit in the early part of the season. This trial was designed to test if UV irradiation had a greater effect on virus on the sides of the fruit than virus in the navel end, in an attempt to prove the hypothesis. One hundred and eighty Autumn Gold navel oranges were harvested from the Citrus Foundation Block in the Uitenhage district. One hundred and twenty of these fruit were dipped in a solution of Cryptogran (10 ml / 100 *l* water) and molasses (0.5%). The rest (60) were dipped in distilled water as

a control. The Cryptogran treated fruit were then exposed to sunlight for 6 days. They were positioned similarly to their natural hanging position in a citrus tree (navel end facing down). In an earlier trial, arenas were made from polypropylene lids, attached to the fruit by a 50:50 Vaseline: wax mixture (Moore *et al*, 2004b). However, larvae managed to escape from these arenas as the seals were not effective. In this trial, Prestik, a white reusable adhesive, was used to attach the arenas (Fig 5.2), which successfully reduced escape. Half of the treated fruit (60) were then exposed to sunlight for a period of 6 days. Five neonate FCM larvae were put into the navel end of 30 Cryptogran treated fruit, which had been exposed to sunlight, and covered with arenas. Five neonate larvae were placed in arenas on the sides of the other 30 exposed, treated fruit. These larvae were placed on the side of the fruit that had been directly exposed to the sunlight. The process was repeated for 60 treated fruit which were not exposed to sunlight, as well as with the distilled water-treated fruit as a control (Table 5.1). Mean numbers of penetration marks per fruit, caused by FCM, and mean no of larvae per fruit were compared using ANOVA and the Duncan's multiple range test, using Statgraphics Plus for Windows Version 2.0.

Fig 5.2. Navel oranges with arenas attached to them to keep neonate FCM larvae in the area where they were placed.

Table 5.1. Cryptogran treatments applied to navel oranges in a detached fruit bioassay to determine the effect of the navel end of navel oranges on UV breakdown of Cryptogran.

	Treatment	**No of fruit per treatment**	**Position of larvae in arena**	**Exposure to sunlight (days)**
1	Distilled water	30	Navel	0
2	Distilled water	30	Side	0
3	Cryptogran (10 ml / 100 *l*) + molasses (0.5%)	30	Navel	0
4	Cryptogran (10 ml / 100 *l*) + molasses (0.5%)	30	Side	0
5	Cryptogran (10 ml / 100 *l*) + molasses (0.5%)	30	Navel	6
6	Cryptogran (10 ml / 100 *l*) + molasses (0.5%)	30	Side	6

5.2.2. Blocks vs single-trees

A trial was conducted in an eleven year old orchard of Palmer navel orange trees (555 trees per hectare), at Carden farm in the Sundays River Valley. One treatment was applied to single trees in a randomised block format, replicated 10 times. The treatment was applied with a Janisch hand gun applicator, at 20 bar pressure, which applied an average of 20.1 *l* of spray mixture per tree. An untreated control treatment was retained. A second treatment was applied to two blocks of approximately 68 trees each. These blocks were sprayed using the grower's tower-blower, applying an average of 15.3 *l* per tree. Both treatments were applied on 3 December 2003, commencing at 17h30 (Table 5.2). The treatments were evaluated by dissecting fruit that dropped under each tree, separately on a weekly basis, inspecting for FCM infestation. Infestation was identified by the presence of a FCM larva or its frass. In the blocks, five trees were selected in the centre of each block, totalling 10 data trees for the treatment. Blocks were sprayed to simulate a practical scenario. Due to orchard sizes it was not possible to spray more than two blocks, and so 5 data trees were selected per block. It is acknowledged that this could be perceived as pseudo-replication. Mean numbers of FCM infested fruit per tree per week for each treatment were compared using ANOVA and the Bonferroni multiple range test, using Statgraphics Plus for Windows Version 2.0 (Moore *et al*, 2004). Reduction in FCM infestation was calculated by (A – B)/A x 100, where A = the total number of infested fruit for all replicates of untreated control, and B = the total number of infested fruit for all replicates of each treatment.

Table 5.2. Cryptogran treatments applied to single trees and blocks of Palmer navel orange trees at Carden farm on 3 December 2003.

Treatment		**Dose per 100 *l* water**	**Trial Layout**	***l* / tree spray applied**
1	Untreated control		Single trees	
2	Cryptogran + molasses	10 ml + 500 ml	Single trees	20.1
3	Cryptogran + molasses + Agral 90	10 ml + 250 ml + 18 ml	Blocks	15.3

5.3. Results

5.3.1. The effect of the navel end

The use of Prestik did limit the escape of larvae from the arenas, but the results were still inconclusive (Table 5.3). There were no significant differences in infestation between the exposed and unexposed fruit, for both the side and navel end arenas on the fruit. The trials would need to be repeated to get an understanding of the effect of the navel end on UV degradation of the virus.

Table 5.3. Damage and FCM infestation of Autumn Gold navel oranges in a detached fruit bioassay to determine the effect of the navel end of the orange on UV breakdown of Cryptogran (n=30).

	Treatment	Position of larvae	Mean no of penetration marks per fruit P = 0.1146 F = 1.82 df = 129	Fruit penetrated (%)	Mean no of larvae per fruit P = 0.0634 F = 2.15 df = 129	Fruit infested (%)
1	Distilled water	Navel	0.55±0.16**ab***	40.91	0.55±0.16**ab**	40.91
2	Distilled water	Side	0.91±0.21**b**	59.09	0.73±0.18**b**	54.55
3	Cryptogran (10 ml / 100 *l*) + molasses (0.5%), unexposed	Navel	0.35±0.13**a**	30.00	0.25±0.12**a**	20.00
4	Cryptogran (10 ml / 100 *l*) + molasses (0.5%), unexposed	Side	0.78±0.18**ab**	60.87	0.39±0.15**a**	26.09
5	Cryptogran (10 ml / 100 *l*) + molasses (0.5%), exposed for 6 days	Navel	0.38±0.11**a**	38.10	0.24±0.10**a**	23.81
6	Cryptogran (10 ml / 100 *l*) + molasses (0.5%), exposed for 6 days	Side	0.50±0.11**ab**	22.73	0.23±0.09**a**	22.73

* Different letters in the same column denote significant differences between values (Duncan's multiple range test)

5.3.2. Blocks vs single trees

Evaluation of the treatments commenced three weeks after application. The single-tree treatment was evaluated for seven weeks, at which point there was little difference in

FCM infestation between it and the untreated control, i.e. Cryptogran no longer had any effect. The block treatments were evaluated for 18 weeks, when the treatment appeared to become ineffective. There was better control of FCM in the block treatments than in the single trees over the first seven week period of evaluation (Table 5.4) (Fig 5.3).

Table 5.4. FCM infestation of navel oranges at Carden Farm in the Sundays River Valley, until 10 weeks after treatment ($P < 0.001$, $F = 23.64$, $df = 29$).

Treatment	Mean infested fruit per tree per week*	% Reduction in infestation relative to untreated control
Untreated control	2.36±0.21**a**	
Cryptogran + molasses (500 ml / 100 *l*) applied to single trees	1.14±0.20**b**	51.5
Cryptogran + molasses (250 ml / 100 l)+ Agral 90 (18 ml / 100 l) applied to blocks	0.61±0.13**b**	73.9

* Different letters in the same column denote significant differences between values (Bonferroni multiple range test)

Control persisted in the block-treated trees for another eight weeks (Table 5.5) (Fig 5.3).

Table 5.5. FCM infestation of navel oranges at Carden Farm in the Sundays River Valley, until 18 weeks after block treatments ($P < 0.001$, $F = 46.97$, $df = 19$).

Treatment	Mean infested fruit per tree per week*	% Reduction in infestation relative to untreated control
Untreated control	1.76±0.14**a**	
Cryptogran + molasses (250 ml / 100 l)+ Agral 90 (18 ml / 100 l) applied to blocks	0.57±0.11**b**	67.6

* Different letters in the same column denote significant differences (Bonferroni multiple range test).

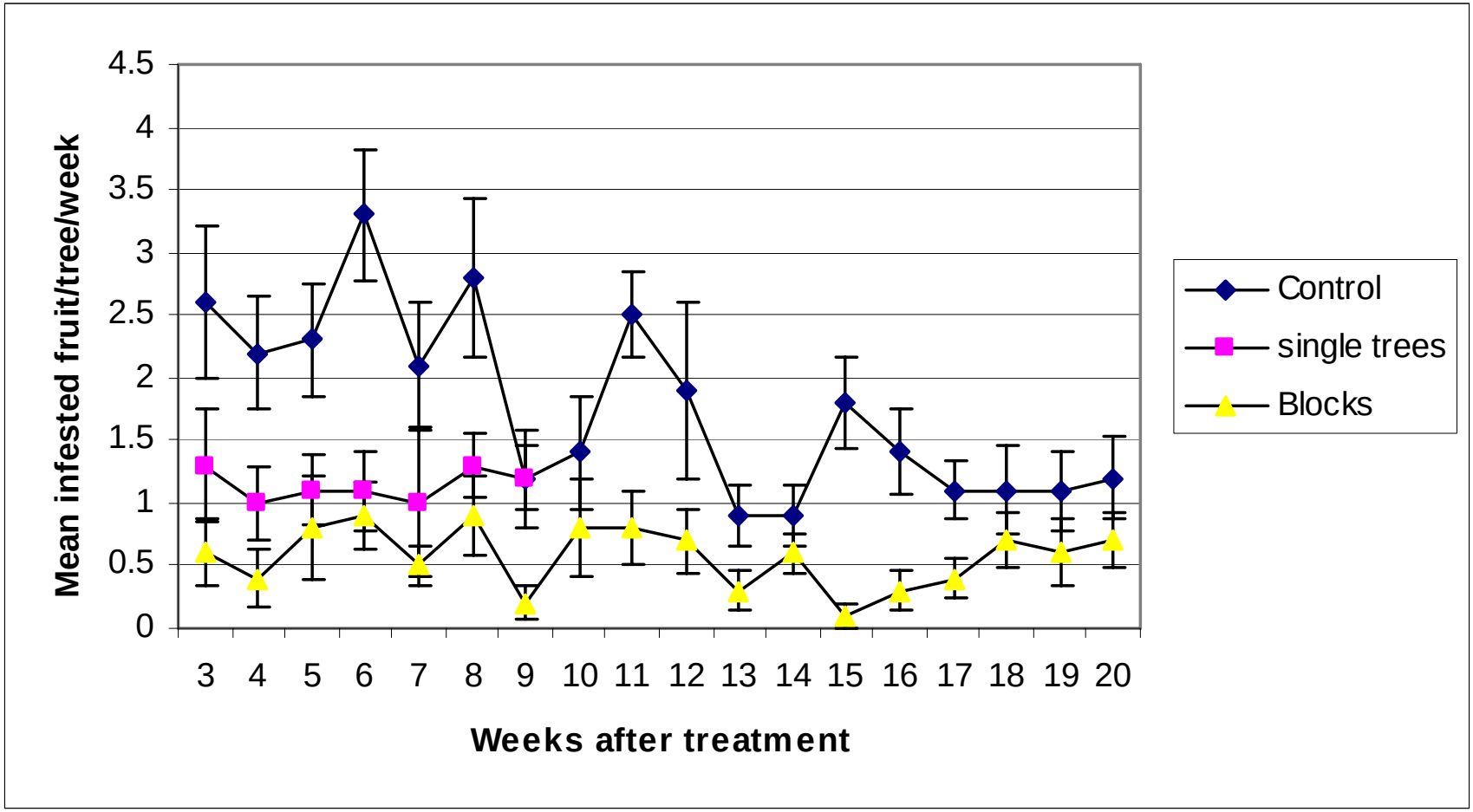

Fig 5.3. Weekly mean (±SE) FCM infestation for Cryptogran treatments and untreated control in single tree replicates and blocks, at Carden farm in the SRV.

5.3. Discussion

The effect of the navel end could not be fully quantified and explained. It is still speculated that the navel end could protect the virus from UV degradation and rainfall, at the time of the season when FCM choose to penetrate the fruit there. This would explain the longer field persistence during the early part of the citrus growing season. For the hypothesis to be true, an expected result would be a larger significant difference in infestation between exposed and unexposed fruit where larvae were placed on the sides of the fruit, due to UV degradation of the virus as a result of exposure to sunlight. A smaller difference in infestation between exposed and unexposed fruit, where larvae were placed on the navel end of the fruit, would have indicated that the virus was protected here from UV inactivation, and retained greater efficacy.

Results showed that the period of residual efficacy of Cryptogran was far longer in block-treated areas than where the virus was applied to single trees. Cryptogran performed superiorly, although not significantly so, when applied in blocks compared to single trees. By 10 weeks after application, there was no difference in FCM infestation between the single-tree treatments and the untreated control. In the block treated areas, FCM control persisted for a further eight weeks after that, and the reduction in FCM infestation was higher here. Also noted was the fact that superior control was achieved in the blocks despite a lower volume of spray mixture being applied. A lower rate of molasses was used in the blocks, and possibly the wetter, Agral 90, had some effect increasing control.

Timm (2005) collected FCM samples from three provinces, namely Mpumalanga, Western Cape and Eastern Cape. In all three provinces, highly distinct populations, with varied DNA profiles, were found within the provinces. In certain instances where populations were separated by less than 1 km, individuals could be ascribed to one or other population on the basis of their amplified fragment length polymorphism (AFLP) profiles. These results indicate that FCM is most likely a poorly dispersing species with little movement between orchards, and correlate well with results obtained for closely related species such as codling moth, *Cydia pomonella*, macadamia nut borer, *Thaumatotibia batrachopa*, and the litchi moth, *Thaumatotibia peltastica* (Timm, 2005).

This poor dispersal could explain the longer residual efficacy in the blocks (Table 5.4), compared to the single tree treatments. FCM populations were reduced in the area surrounding the data trees in the blocks, and with slow or little recolonisation of the area, infestation remained low for a longer time. This, combined with the effect of the navel end and the behaviour of the insect, could explain the longer persistence of the virus when applied in blocks, particularly during the early part of the season.

Chapter 6: General discussion

6.1. Introduction

The main aim of this thesis was to investigate and understand the factors which affect the field persistence of Cryptogran. Some of these factors, such as the effect of UV irradiation and UV protectants, are common with many insect viruses, and much literature is available on this topic (David, 1969; Hunter-Fujita *et al*, 1998). However, they still needed to be tested specifically for the *Cryptophlebia leucotreta* GV, its specific host and the citrus fruit.

6.2. Brief summary and achievement of aims

6.2.1. Laboratory bioassays

The main aims of this chapter were to measure the effect of UV-irradiation on the virus, and to test products which could enhance the efficacy or increase the residual efficacy of the product. The effect of UV-irradiation was measured, with a germicidal lamp and natural sunlight having similar effects on the virus. Lignin and Wetcit were found to be the most promising UV protectants and warranted further investigation. The known benefits of molasses are not UV protection, but possibly its action as a feeding attractant or a sticker (S. Moore, personal communication). Laboratory bioassays indicate that the product is rainfast.

6.2.2. Field trials

The aim of this chapter was to conduct field trials with products which showed promise in laboratory bioassays by improving residual efficacy in some way. The addition of Wetcit to Cryptogran resulted in a faster knockdown of FCM populations, but the addition of either of its primary components, i.e. boric acid and citrus oil, added no further benefit to Cryptogran. Lignin increased the residual efficacy of the product, although not significantly. Medium range spray oil, yeast and milk powder also increased the efficacy of Cryptogran.

6.2.3. Timing of applications

The aim of this chapter was to investigate how the timing of applications affected the efficacy of Cryptogran. It was shown that when Cryptogran was applied during the early part of the season (November/December), the residual efficacy was longer than when applied later (February - May). Applications which coincided with male pheromone trap catch peaks were more effective than applications between peaks. Treatments applied at midday were less effective in controlling FCM than sprays applied during the evening. The addition of lignin to Cryptogran improved the residual efficacy of the virus, particularly when it was added to sprays applied at midday.

6.2.4. Biotic factors affecting the residual efficacy of Cryptogran

The aim here was to investigate the effect of biotic factors, such as the size of the treated area and the effect of the navel end of the orange, on the residual efficacy of Cryptogran. Cryptogran performed better when applied to large blocks compared with single tree treatments. This is attributed to the fact that FCM does not appear to move large distances. Consequently, if a population is knocked down in an area, control can be quite persistent as recolonisation will be slow. The effect of the navel end was not satisfactorily explained, but the hypothesis still stands that it plays a significant role in the field persistence of the virus.

6.2.5. General

Lignin was the most promising additive in laboratory and field trials, but results were variable. This study did not reveal any UV protectants whose effect could be considered as outstanding. Possibly the best option for UV protection of viruses lies in crude preparation of the viruses. Cryptogran is produced *in vivo*, and is a crude virus formulation (S. Moore, personal communication). Studies have shown that crude, impure virus preparations withstood longer exposures to ultraviolet light than purified formulations (David, 1969). Impure Egyptian-produced *Spodoptera littoralis* NPV persisted appreciably longer than highly purified virus produced in the United Kingdom (Jones & McKinley, 1986). Crude preparations contain UV-absorbing homogenized host tissue and pigments, which could add UV protection (Shapiro *et al*, 2002). From a

practical viewpoint, crude preparations are widely used and will continue to be used until *in vitro* production of insect viruses becomes cost-effective (Shapiro *et al*, 2002). Many protectant products are also patented and expensive.

Trials have shown that timing of applications, both time of the day and time of the season, have a significant effect on the residual efficacy of the product. Correct management of the product, when applied with molasses and a wetter, as registered, is the most important factor that can influence the success and residual efficacy of Cryptogran.

6.3. Economic thresholds and cost: benefit analyses

The cost of any pesticide needs to be justified by a saving or a resultant increase in income. For this purpose a direct economic threshold was calculated.

One litre of Cryptogran costs R750.00. If applied according to recommendation, at a rate of 10 ml / 100 *l* water and at a volume of 10 000 *l* / ha, one litre of Cryptogran is sufficient to spray one ha of citrus. The cost of molasses amounts to R87.40 / ha, and the wetter (Agral 90) costs R45.10 / ha. The total material cost per hectare is therefore R882.60 (Table 6.1).

Table 6.1. Costs (ZAR) of materials to apply a Cryptogran spray to 1 hectare of citrus.

Product	**Cost per hectare (ZAR)**
Cryptogran	750.00
Molasses	87.40
Agral 90	45.10
TOTAL	**882.60**

The average value of a carton of fruit, delivered in port (DIP) to European Union (EU) markets, is approximately R45.00. Packing costs amount to R25.00 per carton. Therefore the value of a carton of fruit to the grower is R20.00 (H. Bester, personal communication). Assuming that the average size of navel oranges is count 64 (1 carton of oranges contains 64 fruit), the value of one orange is R0.22 (70% average packout

percentage estimated). When fruit is supplied to the USA market, the Delivered In Port (DIP) price per carton is R100.00, in which case one orange could be worth R0.82 (H. Bester, personal communication) (Table 6.2).

Table 6.2. Values and costs (ZAR) used to determine the value of one citrus fruit, for the EU and USA markets, for the purposes of determining economic thresholds and doing cost-benefit analyses.

	Value per carton (DIP)	**Packing costs**	**Nett Value per carton**	**Value of fruit**	**Packout Percentage**	**Nett value of 1 fruit**
EU	45.00	25.00	20.00	0.31	70	0.22
USA	100.00	25	75.00	1.17	70	0.82

Average reductions in infestation for all spray trials conducted at various times of the season, during the period 2000 to 2006, are as follows: November/December: 69%, January : 60%, February 47%, March : 57%, and April 63% (Moore, unpublished data). The average period of efficacy for trials conducted during these periods, the value of a fruit, and the cost of a Cryptogran application were used to calculate the economic thresholds, in terms of the number of infested fruit per tree per week which would economically have justified an application, for the different times of the year (Table 6.3). It is important to note these calculations only deliver a direct economic threshold, which is a break-even threshold. They do not take into consideration the knock-down effect, i.e. if FCM populations are knocked down early in the season, they are likely to remain lower throughout the season, due to slow recolonisation as discussed in section 5.3. **Phytosanitary implications, which are overridingly important (S. Moore, personal communication), have also not been considered, especially for the latest (April) applications**. The aim here is not only to save fruit, but to minimize the risk that live FCM larvae will be found in fruit after harvest. These thresholds should therefore be seen as no more than a guideline for FCM control practices. Some areas, such as the Western

Cape, which supplies the sensitive USA markets, are compelled to apply more treatments (S. Moore, personal communication). The values calculated for the different times of the year (Table 6.3) show whether a Cryptogran application was financially viable or not. They can unfortunately not be used as predictive thresholds, because the fruit has already been infested and dropped from the tree at the time the evaluations were done, and a spray would not be of immediate benefit. The information can be used to determine whether an application, coinciding with the next moth activity peak, could be necessary and beneficial. The calculations also help build a data base of information, which is important, as certain orchards are historically more prone to high levels of FCM infestation than others (S. Moore, personal communication). Therefore the data can be used to determine FCM control strategies for the following season.

A cost: benefit analysis was conducted to determine the nett financial gains which accrued as a result of Cryptogran applications for four of the field trials conducted in this study, discussed in the following chapters and sections: Allandale (section 4.3.1.1), Carden (section 5.3.1), Junkyard (section 4.3.1.3), and Bernol (section 4.3.1.2) (Table 6.4). Only the application at Bernol Farm produced no direct financial returns, i.e. the cost of application was greater than the value of the fruit saved as a result of the Cryptogran spray. However, the purpose of this application at that time of the year, shortly before harvest, was to minimize the risk of phytosanitary interceptions, and any fruit saved due to the Cryptogran application should be viewed as a bonus. Once again, the phytosanitary implications and requirements were not considered in the financial calculations.

Table 6.3. Values used to calculate the economic thresholds for Cryptogran applications at different times of the year (value of one fruit = R0.22, cost of Cryptogran application = R882.60 / ha).

Time of Application	Average reduction in infestation (%)	Average period of efficacy (weeks)	No of saved fruit required to justify application cost per ha (not adjusted for packout percentage)	No of saved fruit required to justify application cost per ha (adjusted for 70% export packout)	No of saved fruit per has per week required to justify treatment cost	No of saved fruit per tree per week required to justify treatment cost
Nov / Dec	69	13	4034.7	5847.3	449.8	0.8
Jan	60	8	4034.7	6724.4	840.6	1.5
Feb	47	7	4034.7	8584.4	1226.3	2.2
Mar	57	7	4034.7	7078.3	1011.2	1.8
Apr	63	3	4034.7	6404.2	2134.7	3.8

Table 6.4. Financial savings calculated as a result of Cryptogran applications, for the period of evaluation, for four trials discussed in this thesis (value of one fruit = R0.22, cost of Cryptogran application per ha = R882.60).

Farm	Infested fruit/tree/week in the untreated controls	Period evaluated (weeks)	Total no of fruit dropped per ha (untreated)	Reduction in infestation due to Cryptogran application (%)	No of fruit/ha saved due to Cryptogran application	Value of fruit/ha saved due to Cryptogran application	Nett financial gain as a result of Cryptogran application per ha
Allandale	3.96	9	19780	53	10484	R3276.10	R2393.51
Carden	2.09	17	19758	68	13435	R4198.58	R3315.99
Junkyard	0.62	15	7747	58	4493	R1404.13	R521.54
Bernol	0.6	6	1998	64	1277	R398.91	-R483.67

The addition of additives, such as UV protectants, may improve persistence of a product such as Cryptogran, but if it does not result in nett financial gains, it will not be used. Another cost: benefit analysis was conducted to determine whether it would be financially viable to add lignin to Cryptogran as a UV protectant (Table 6.5). The reductions in infestations for the various treatments for the trial applied at Atmar farm were used (section 4.3.2.2). For the purpose of this exercise, the infestation level used was 1.82 fruit per tree per week, which was the average infestation in the untreated control treatments for the 4 trials discussed in the previous paragraph. This was done for illustrative purposes, as the control infestation in the Atmar trial was very low.

For the evening applications, Cryptogran alone led to a saving of 4636 fruit per hectare (Table 6.5). Where lignin was added 5272 fruit were saved. The difference in fruit saved between these two treatments was 636, which could be attributed directly to the addition of lignin. If the value of one fruit for the traditional markets is R0.22 (Table 6.2), then R139.92 was saved. The cost of lignin used per hectare is approximately R500.00 (J. Podgewaite, personal communication), which outweighs the benefit derived from its use, so it is not financially justifiable in this case. For the midday applications, Cryptogran alone led to a saving of 2636 fruit per hectare (Table 6.5). Where lignin was added 5090 fruit were saved. The difference in fruit saved between these two treatments was 2454, which could be attributed directly to the addition of lignin. This would equate to savings of R539.88 per ha. This would appear to justify the cost of lignin. However, the evening application of Cryptogran without lignin saved 4636 fruit/ha (Table 6.5), which was 2000 more than the midday application. Therefore similar savings (R440.00) would have been effected if the spray was delayed by 5 hours (to the evening), and applied without lignin. The use of lignin is therefore not financially viable in areas supplying the traditional EU markets.

However, when the same calculations were done using the higher value of one fruit for the US market, i.e. R0.82 (Table 6.2), then the 636 fruit saved due to the addition of lignin to the evening application resulted in savings of R521.52 per ha, which would more than cover the cost of lignin. Most of the fruit supplied to the US market are from

the Western Cape, where solar radiation levels are higher than anywhere else in the country (Schulze *et al*, 1997). It is therefore speculated that lignin might play a greater role there. Also, the US markets have extremely strict phytosanitary requirements. The addition of lignin to Cryptogran could provide the added benefit of extra insurance against interceptions of FCM in fruit from the Western Cape.

Table 6.5. Values used for cost-benefit analyses calculations to determine the financial viability of adding lignin to Cryptogran, using the reduction in infestation shown in the trial conducted at Atmar Farm (section 4.3.3.2).

Treatment	Average Infested fruit/tree/week in untreated controls	Period evaluated (weeks)	Total no of fruit dropped per ha (untreated)	Reduction in infestation due to treatments applied (%)	No of fruit/ha saved due to treatment	No of fruit/ha saved directly due to the addition of lignin
Cryptogran applied midday	1.82	9	9091	29	2636	**Midday**
Cryptogran + lignin applied midday	1.82	9	9091	56	5090	2454
Cryptogran applied evening	1.82	9	9091	51	4636	**Evening**
Cryptogran + lignin applied evening	1.82	9	9091	58	5272	636

Another cost-benefit analysis was performed, using the results of a trial conducted in the Sundays River Valley, to test the effect on FCM reduction when applying Cryptogran with two different machines. This trial was not reported in detail in the previous chapters, but is of significance when it comes to drawing up a list of practical recommendations to the growers. A 'weaker', Malray machine applied 10 829 *l* of spray formulation per ha, which resulted in a 45% reduction in FCM infestation over a period of 7 weeks. The better Janish tower blower applied 14994 *l* per ha, which resulted in a 73% reduction in FCM infestation over the same period (Table 6.6). Better wind oscillation was observed with the Janisch machine, and this as well as the greater volume applied resulted in better penetration of spray formulation into the tree and better coverage overall. For the purpose of this calculation, the average infestation of 1.82 fruit per tree per week was used again for illustrative purposes. The calculations reveal that higher nett gains per ha (R371.30) as a result of the sprays were achieved where the Janisch machine was used, even though the application costs were higher due to the greater volume of products applied (Table 6.6).

Table 6.6. Cost-benefit analysis calculation to determine the financial viability of applying Cryptogran more effectively at greater volumes, using results of a trial conducted on Midknight Valencia orange trees on Brandwacht farm in the Sundays River Valley in 2004 (value of one fruit = R0.22; cost of Cryptogran application = R882.60).

Machine	**Volume of spray formulation applied per ha (*l*)**	**Average fruit/tree/week infested in control treatment**	**Period evaluated (weeks)**	**Reduction in infestation due to treatments applied (%)**	**Value of fruit saved per ha due to application**	**Cost of Cryptogran application**	**Nett saving as a result of Cryptogran application**
Malray	10829	1.82	7	45	R1044.66	R955.77	R88.89
Janisch	14994	1.82	7	73	R1694.67	R1323.37	R371.30

6.4. Compatibility with other products

Compatibility of Cryptogran with other products is another aspect which has been extensively researched in the course of this study, but the results have not been shown in the previous chapters as it does not have direct relevance on the field persistence of the virus. However, it does have a significant practical value. If Cryptogran was compatible with other products, and could be applied in combination with them, it would save the grower considerable expense. Growers in the Sundays River Valley estimate the mechanical application costs, i.e. diesel, wear-and tear on machinery, labour and overtime, to be between 8 and 10 cents per litre of spray formulation applied. It is recommended that Cryptogran be applied at a rate of approximately 10 000 *l* of spray formulation per hectare. This would equate to R800 to R1000 per application per hectare. If products can be applied together, these costs can be significantly diluted. In a series of trials, Cryptogran and molasses have been shown to be compatible with many chemicals, which represent the majority of the products most commonly used at the times at which Cryptogran is likely to be applied (Table 6.7).

Table 6.7. Agricultural chemicals commonly used in the citrus industry, which have been shown to be compatible with Cryptogran and molasses (Moore *et al*, 2004b; Kirkman & Moore, 2006; Kirkman & Moore, unpublished data).

Products	**Common trade name**
Pyriproxyfen + medium grade spray oil	Nemesis + H&R medium spray oil
Mancozeb + benomyl + medium grade spray oil	Dithane + Benlate + H&R medium spray oil
Abamectin + medium grade spray oil	Agrimec + H&R medium spray oil
Methidathion + wetter	Ultracide + Agral 90
Bromopropylate	Acarol
Methomyl	Lannate
Pyraclostrobin	Cabrio
Trifloxystrobin	Flint
Azoxystrobin	Ortiva
Didecyl dimethyl ammonium chloride	Sporekill

Copper oxychloride has been shown, in detached fruit laboratory bioassays, to be detrimental to Cryptogran (Kirkman & Moore, unpublished data). It is therefore recommended that Cryptogran should not be applied in combination with any copper products.

6.5. List of recommendations to growers

Academic results, as delivered in this thesis, are extremely important. However, the gap between theory and practice needs to be bridged. Applied entomology needs to be implementable. With this in mind, and using the findings of this study, a list of practical recommendations concerning field use of Cryptogran has been drawn up. The input of Sean Moore in is acknowledged in the drawing up of these recommendations:

- The product appears to be rainfast, so reapplication is not necessary after rainfall
- Cryptogran should be applied during the evening, not during the day
- Applications should coincide with male moth trap catch peaks

- Applications coinciding with the November/December trap peak are the most effective, and have the longest residual efficacy
- January/February applications are the least effective
- Applications 4 to 6 weeks before harvest are essential to reduce the risk of phytosanitary interceptions
- Economic thresholds can be used as a tool to decide whether or when to apply, but by far the most important factor to consider is phytosanitary risk. A November/December application will have a knock-down effect on FCM populations for the rest of the season, and will lead to lower population levels shortly before harvest. This will contribute to lower phytosanitary risk when a second spray should be applied 4 to 6 weeks before harvest.
- Cryptogran can be mixed in a spray tank with all the products listed in Table 6.6, but not with any copper products
- Lignin could be added to Cryptogran by growers in the Western Cape
- Effective spray coverage is essential for good FCM management. A tower mist-blower machine with good wind oscillation and the ability to apply 10000 *l* of spray formulation per hectare should be used.

6.6. Future research

Although it appears that crude preparation is the most effective and affordable way to protect viruses from UV radiation, lignin has shown that it does improve the residual efficacy of Cryptogran. When dealing with a microbial insecticide, such as Cryptogran, results are never as consistent and predictable as with a conventional pesticide, and this increases the need for replication in order to get reliable results. Laboratory bioassays need to be replicated to determine the exact effect of lignin, and the problem of evaporation from the Petri dishes must be addressed. More affordable, locally produced lignin formulations need to be sourced and tested.

The exact degree of rainfastness of Cryptogran needs to be established. The trial protocol is sound, but replication of the trial using the rain simulation machine needs to be conducted. Trials to determine the effect of the navel end also need to be repeated in an

effort to fully test the hypothesis that virus is protected from environmental elements within the navel end.

Trials are already in progress to determine the effect of earlier applications. The current recommendation is to apply the first spray coinciding with the Lorelei trap count peak in late November / early December. A small male flight peak has been observed during the latter half of October (Moore & Richards, 2002). It is possible that an early application at this point could reduce the size of the November/December peak, and thus have a knock-down effect for the rest of the season. There could also be benefit in having virus inoculum in the trees at an early stage before the population build-up occurs.

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