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An investigation into yeast-baculovirus synergism for the improved control of *Thaumatotibia leucotreta*, an economically important pest of citrus

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Abstract

A mutualistic association between *Cydia pomonella* and yeasts belonging to the genus *Metschnikowia* has previously been demonstrated. Larval feeding galleries inoculated with *M. andauensis*, reduced larval mortality and enhanced larval development. Additionally, adult *C. pomonella* female oviposition preference was also shown to be influenced by the volatiles produced by *M. andauensis*. This mutualistic relationship was manipulated for biological control purposes, by combining *M. pulcherrima* with the baculovirus Cydia pomonella granulovirus. The combination of *M. pulcherrima* with brown cane sugar and CpGV in laboratory assays and field trials resulted in a significant increase in larval mortality. A similar observation was made when *M. pulcherrima* was substituted for *Saccharomyces cerevisiae*. This indicates that yeasts harbour the potential for use in biological control, especially when combined with other well-established biocontrol methods.

Thaumatotibia leucotreta is a phytophagous insect endemic to southern Africa. It is highly significant to the South African citrus industry due to its classification as a phytosanitary pest by most international markets. An integrated pest management programme has been implemented to control *T. leucotreta*. The baculovirus Cryptophlebia leucotreta granulovirus forms one component of this programme and is highly effective. In this study, we proposed to determine which yeast species occur naturally in the gut of *T. leucotreta* larvae and to examine whether any of the isolated yeast species, when combined with the CrleGV-SA, enhance its effectiveness.

Firstly, Navel oranges infested with *T. leucotreta* larvae were collected from geographically distinct citrus-producing regions across South Africa. This led to the isolation and identification of six yeast species from the gut of *T. leucotreta* larvae via PCR amplification and sequencing of the internal transcribed spacer region and D1/D2 domain of the large subunit. Six yeast species were identified, *viz. Meyerozyma guilliermondii, Hanseniaspora uvarum, Clavispora lusitaniae, Kluyveromyces marxianus, Pichia kudriavzevii* and *Pichia kluyveri*. Additionally, *Saccharomyces cerevisiae* was included as a control in all trials due to its commercial availability and use in the artificial diet used to rear *T. leucotreta*.

Secondly, larval development and attraction assays were conducted with the isolated yeast species. *Thaumatotibia leucotreta* larvae that fed on Navel oranges inoculated with *M. guilliermondii, P. kluyveri, H. uvarum,* and *S. cerevisiae* had accelerated developmental periods and reduced

mortality rates. Additionally, it was demonstrated that *T. leucotreta* neonates were attracted to YPD broth cultures inoculated with *P. kluyveri*, *H. uvarum*, *P. kudriavzevii* and *K. marxianus* for feeding.

Thirdly, oviposition preference assays were conducted with adult *T. leucotreta* females to determine whether the isolated yeast species influence their egg-laying in two-choice and multiple-choice tests. Navel oranges were inoculated with a specific yeast isolate, and mated adult females were left to oviposit. *Meyerozyma guilliermondii*, *P. kudriavzevii* and *H. uvarum* were shown to influence adult *T. leucotreta* female oviposition preference in two-choice tests. However, multiple-choice tests using the aforementioned yeast species did not mimic these results.

Lastly, a series of detached fruit bioassays were performed to determine the optimal yeast:virus ratio, test all isolated yeast species in combination with CrleGV-SA and to further enhance yeast/virus formulation through the addition of an adjuvant and surfactant. CrleGV-SA was applied at a lethal concentration that would kill 50 % of *T. leucotreta* larvae. The optimal yeast concentration to use alongside CrleGV-SA was determined. *Pichia kluyveri, P. kudriavzevii, K. marxianus* and *S. cerevisiae* in combination with CrleGV-SA increased larval mortality compared to CrleGV-SA alone. The inclusion of molasses and BREAK-THRU® S 240 to *P. kudriavzevii* and *S. cerevisiae* plus CrleGV-SA formulations greatly enhanced their efficacy. Additionally, semi-field trials were initiated using *P. kudriavzevii* and *S. cerevisiae*, with promising preliminary results being obtained, although more replicates need to be performed. The experiments performed in this study provide a platform for further research into the application of a yeast/virus combination as a novel control and monitoring option for *T. leucotreta* in the field.

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List of Abbreviations

Units

bp	–	Base Pair
°C	–	Degrees Celsius
g	–	Gram
Hz	–	Hertz
LC_{50}	–	Lethal Concentration (50 %)
LC_{90}	–	Lethal Concentration (90 %)
LT_{50}	–	Lethal Time (50 %)
LT_{90}	–	Lethal Time (90 %)
L	–	Litre
µg	–	Microgram
µl	–	Microliter
µM	–	Micrometre
mL	–	Millilitre
mm	–	Millimetre
mM	–	Millimolar
M	–	Molar
ng	–	Nanogram
nm	–	Nanometre
N	–	Number
%	–	Percentage
P	–	P-value or calculated probability
×g	–	Times gravity
v/v	–	Volume per volume
V	–	Volts
w/v	–	Weight per volume

Abbreviations

AGE	–	Agarose gel electrophoresis
ANOVA	–	Analysis of variance

BLAST	–	Basic Local Alignment Search Tool
BV	–	Budded virions
CTAB	–	Cetrimonium bromide
CE	–	Controlled environment
DAFF	–	Department of Agriculture, Forestry, and Fisheries
DNA	–	Deoxyribonucleic acid
ddH_2O	–	Double-distilled H_2O
egt	–	*ecdysteroid uridine 5´–diphosphate–glucosyltransferase*
EPB	–	Entomopathogenic bacteria
EPF	–	Entomopathogenic fungi
EPN	–	Entomopathogenic nematode
EPV	–	Entomopathogenic virus
EDTA	–	Ethylenediaminetetraacetic acid
EU	–	European Union
FCM	–	False codling moth
GC-EAD		Gas chromatography electroantennographic detection
GCMS	–	Gas chromatography-mass spectrometry
gDNA	–	Genomic deoxyribonucleic acid
GDP	–	Gross domestic product
GMOs	–	Genetically modified organisms
GV	–	Granulovirus
ICTV	–	International Committee on Taxonomy of Viruses
IPM	–	Integrated pest management programme
ITS	–	Internal transcribed spacer
LSU	–	Large subunit
LC	–	Lethal concentration
LT	–	Lethal time
MEGA	–	Molecular Evolutionary Genetics Analysis
MNPV	–	Multiple nucleopolyhedrovirus
NTC	–	No template control
NPV	–	Nucleopolyhedrovirus

OB	–	Occlusion Body
ODV	–	Occlusion derived virion
Pen	–	Penicillin
PCR	–	Polymerase chain reaction
RH	–	Relative humidity
rpm	–	Revolutions per minute
rDNA	–	Ribosomal DNA
SNPV	–	Single nucleopolyhedrovirus
Na_2CO_3	–	Sodium carbonate
NaCl	–	Sodium chloride
SDS	–	Sodium dodecyl sulphate
SA	–	South Africa
ZAR	–	South African rand
SIT	–	Sterile insect technique
Strep	–	Streptomycin
TAE	–	Tris base, acetic acid and EDTA
USA	–	United States of America
UV	–	Ultraviolet radiation
YPD	–	Yeast extract Peptone Dextrose

Viruses

CpGV	–	Cydia pomonella granulovirus
CrleGV	–	Cryptophlebia leucotreta granulovirus
CrpeNPV	–	Cryptophlebia peltastica nucleopolyhedrovirus
HearSNPV	–	Helicoverpa armigera single nucleopolyhedrovirus
PhopGV	–	Phthorimaea operculella granulovirus

Research Outputs

Conferences:

International

van der Merwe, M., Knox, C.M., Hill, M.P and Moore, S.D., 2018. Yeast-baculovirus synergism: Investigating mixed infections for improved management of the false codling moth, *Thaumatotibia leucotreta*. Oral presentation at the International Congress of Invertebrate Pathology and Microbial Control and the 51st Annual Meeting of the Society for Invertebrate Pathology conference held at QT Gold Coast Hotel, Surfers Paradise, Queensland, Australia. 12 – 16 August 2018.

Local

van der Merwe, M., Knox, C.M., Hill, M.P., Moore, S.D., 2018. Yeast-baculovirus synergism: Investigating mixed infections for improved management of the false codling moth, *Thaumatotibia leucotreta*. Oral presentation at the 10th Citrus Research Symposium conference held at Champagne Sports Resort, Drakensberg, South Africa. 19 – 22 August 2018.

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Chapter One

General Introduction and Literature Review

1.1 Introduction

The agricultural sector in South Africa is of great importance as it contributes significantly to the country's gross domestic product (GDP). According to the latest data published by Statistics SA, the agricultural, forestry and fisheries sectors collectively contributed 2.4 % to the GDP and employed around 849 000 people in 2017 (DAFF, 2018). The horticulture sector's total gross value in 2017/18 was around 78 billion ZAR (5.3 billion US dollars). The citrus industry was the largest contributor, with 19 billion ZAR (1.3 billion US dollars) and employed more than 100 000 people (DAFF, 2019). The citrus industry is mainly export-orientated with nearly two million tons (135.2 million cartons) of citrus exported to more than 100 countries in 2018. The majority of the citrus industry's total income comes from exports, nearly 92 %, with a relatively small amount coming from local markets (CGA, 2019). Citrus is produced in Limpopo, Eastern Cape, Western Cape, Mpumalanga, KwaZulu-Natal, Northern Cape, North West and Free State (**Figure 1.1**). A total of 81 638 hectares was dedicated to citrus production in 2019, and that number is only expected to increase in the years to come (CGA, 2019). The industry's five main types of citrus are Valencias, Navels, Lemons, Grapefruit and Soft citrus. Valencia and Navel oranges make up 54 % of the total amount of fresh citrus fruit produced in South Africa. The economic value of the citrus industry in South Africa is clear, and thus the industry must be protected.

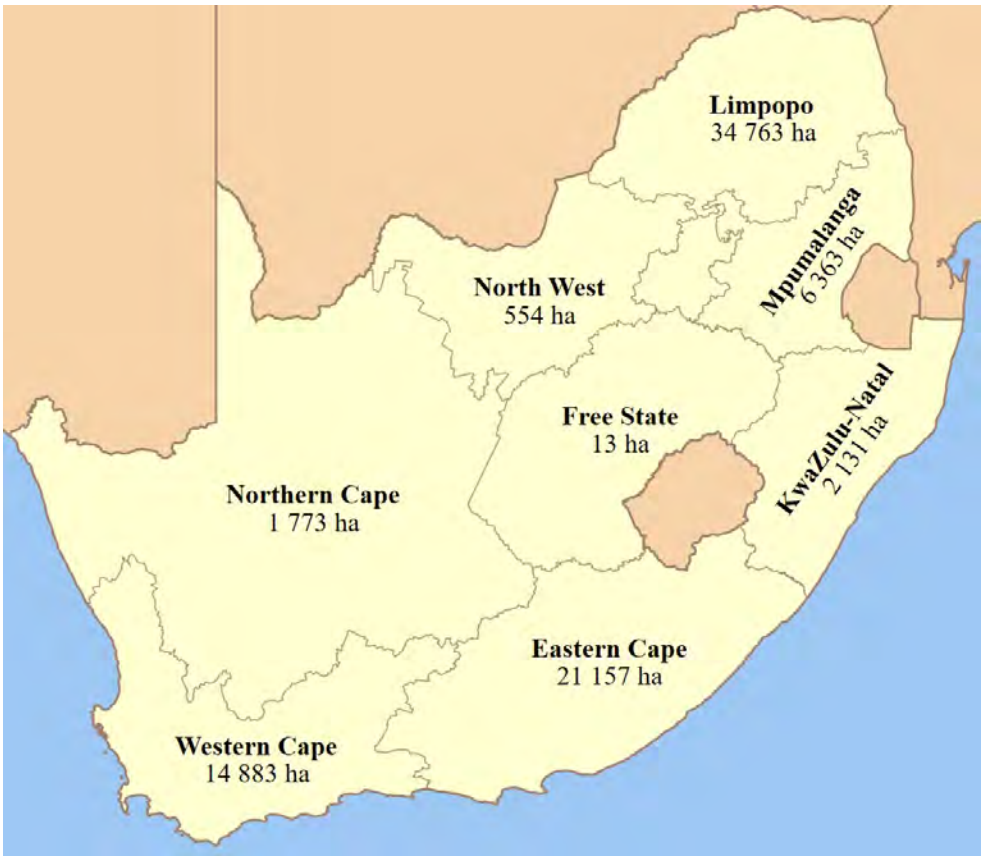

Figure 1.1: Number of hectares dedicated to citrus production in each province of South Africa (CGA, 2019).

Some of the main risks that the industry has identified include plant diseases and the loss of citrus fruit due to damage caused by insect pests. There are a large number of insect pests including locusts, aphids, scale insects, thrips, flies, ants, beetles and moths that affect the citrus industry in South Africa (Moore et al., 2008). One of the most detrimental pests is *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), or otherwise commonly known as the false codling moth (FCM). It is a major pest of citrus, which can cause substantial fruit damage throughout larval feeding (**Figure 1.2**) (Mkiga et al., 2019). When neonate larva bore into the fruit, it results in the rind's discolouration, and these penetrations sites are also subject to secondary infection by other microbial agents (Newton, 1998). The fruit's physiological state is also altered by larval penetration resulting in premature ripening, which leads to abscission (Newton, 1998). When larvae exit the fruit, they leave behind a distinctive trail of excrement or "frass" as it is commonly referred to. This affects the fruit's marketability, resulting in significant financial loss each year (Moore, 2002).

Additionally, and most importantly, *T. leucotreta* is classified as a phytosanitary pest in regions to which South Africa exports, such as the European Union (EU) and the United States of America (USA). *Thaumatotibia leucotreta* is endemic to Sub-Saharan Africa and poses a serious risk to South African citrus exports (Gilligan et al., 2011). The detection of a single larva within fruit destined to be exported can result in an entire shipment being rejected (Moore, 2002). The interception of infested fruit and its subsequent rejection has a significant economic impact on the citrus export industry. The challenge of controlling *T. leucotreta* is further exacerbated by the occurrence of resistance to chemical pesticides, public disapproval of their use and increased regulation placed on them (Ansell, 2008; Bielza et al., 2008).

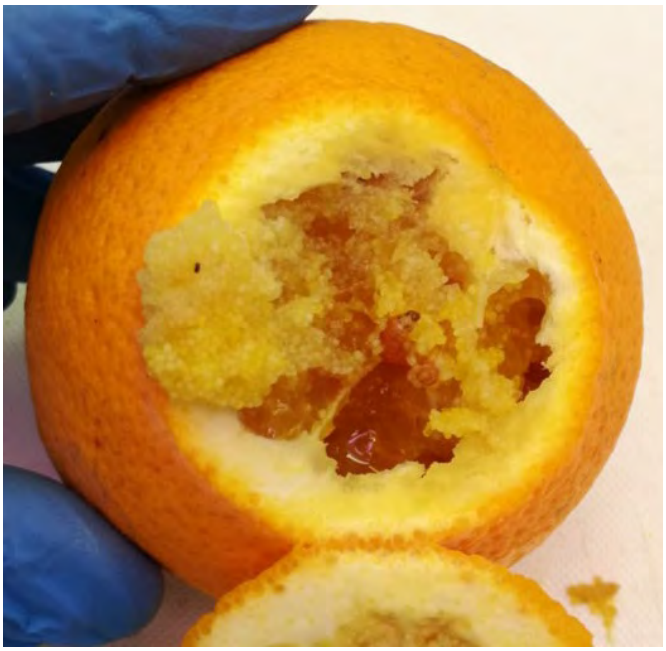

Figure 1.2: The damage caused to a Navel orange as a result of *Thaumatotibia leucotreta* infestation.

1.2 *Thaumatotibia leucotreta*

1.2.1 Taxonomy

Thaumatotibia leucotreta was originally described as *Argyroploce leucotreta* by Meyrick (1912), and its name was later revised to *Cryptophlebia leucotreta* by Clarke (1958). The species was known as *Cryptophlebia leucotreta* for an extended period before it was reclassified by Komai (1999), who transferred the species to its current genus *Thaumatotibia* (**Table 1.1**).

Table 1.1: The taxonomic classification of *Thaumatotibia leucotreta*, according to Komai (1999).

Taxa	Scientific classification
Phylum	Arthropoda
Class	Insecta
Order	Lepidoptera
Family	Tortricidae
Genus	*Thaumatotibia*
Species	*Thaumatotibia leucotreta*

1.2.2 Distribution

Thaumatotibia leucotreta is well established throughout Sub-Saharan Africa and is mostly confined to the subtropics and tropical regions (Karvonen, 1983). It has been reported in 31 countries throughout Africa as well as in Mauritius, Réunion and Saint Helena (**Figure 1.3**) (EPPO, 2014). It is important to note that the distribution of *T. leucotreta* does not include North America, Europe or Asia, and these regions are the main export markets of the South African citrus industry. However, *T. leucotreta* has occasionally been intercepted by border inspections in Denmark, Finland, Germany, Italy, Netherlands, Spain, Sweden and the United Kingdom, but these countries have remained pest-free (EPPO, 2014). This has resulted in *T. leucotreta* being classified as a serious phytosanitary risk in the EU (European Commission, 2017). The detection of a single *T. leucotreta* adult male in a pheromone trap in Ventura County, California, USA, in 2008 was cause for concern (Gilligan et al., 2011). However, no further detections of *T. leucotreta* were reported, and the finding was labelled as an isolated incident. North America was evaluated, and large regions were shown to be a suitable habitat for *T. leucotreta* establishment (Gilligan et al., 2011; Venette et al., 2003). The potential for future expansion of *T. leucotreta* in the USA has had it listed as a quarantine pest (NAPPO, 2016). In the 1980s, *T. leucotreta* was recorded in Israel as a pest of macadamias. However, this was thought to be an accidental introduction (Wysoki, 1986).

Figure 1.3: The geographic distribution of *Thaumatotibia leucotreta* throughout the African continent shown in green (EPPO, 2014).

1.2.3 Host range

Thaumatotibia leucotreta is considered to be polyphagous, with its host range, including more than 50 wild and cultivated plants (Rentel, 2013). Of these cultivated plant hosts, several are agriculturally and economically important in South Africa, and the most affected include macadamias, avocados, litchis and particularly citrus (Moore, 2002). The vast majority of citrus cultivars are susceptible to *T. leucotreta*, except for lemons and limes (Moore et al., 2015a). Navel oranges, and particularly certain specific Navel orange cultivars, have been demonstrated to be highly susceptible to *T. leucotreta* infestation (Love et al., 2014). Apart from these differences in host susceptibility, adult *T. leucotreta* females have been shown to have a greater preference for certain citrus cultivars (Love et al., 2014). Fruit that has been damaged mechanically or by other pests can attract *T. leucotreta* to oviposit on the injured fruit (Newton, 1989).

1.2.4 The life cycle of *Thaumatotibia leucotreta*

The life cycle of *T. leucotreta* begins with fertile female moths ovipositing on the bolls or fruits of host plants. Mated female moths prefer to oviposit in the depressions of the rind of the fruit at night (Moore, 2002). Eggs are oviposited singly or in small groupings of two to four. Female moths on average will produce between 87 – 456 eggs; however, individual fecundity can vary from 5 – 799 eggs (Daiber, 1980). *Thaumatotibia leucotreta* eggs are cream coloured oval-shaped discs when freshly oviposited. The development time of *T. leucotreta* eggs varies and is dependent on the environmental temperature and humidity. Generally, the development period is between 6 – 12 days (Daiber, 1979a). During this period, the eggs change from a cream colour when freshly oviposited to a reddish colour, and finally, they turn dark brown just before the neonate emerges (**Figure 1.4**). Once eggs are fully developed neonate *T. leucotreta* will emerge, feed for a short period on the fruit's surface before boring into the rind (Venette et al., 2003).

Figure 1.4: *Thaumatotibia leucotreta* eggs oviposited on wax paper in various stages of development.

Thaumatotibia leucotreta larvae progress through 5 stages during their development, known as instars, which can be determined by measuring the head capsule's width (Hofmeyr et al., 2016). The first instar larvae, also known as neonates, are extremely sensitive to humidity and temperature changes and usually have high mortality rates (Newton, 1998). Neonates will burrow into the fruit almost anywhere if the host plant has a soft rind such as citrus. The larval period lasts between 25 – 35 days in summer and 35 – 67 days in winter and is additionally dependent on food quality (Daiber, 1979b; Stofberg, 1954). The early instars feed near the fruit's surface (**Figure 1.5 A**)

whilst the later instars bore towards the centre (**Figure 1.5 B**). Mature 5th instars will exit the fruit, drop to the ground on silken threads, burrowing a few millimetres into the soil before spinning a cocoon and entering an inactive prepupal stage (Daiber, 1979c).

Figure 1.5: Early instar larva (A) and late instar larva (B) of *Thaumatotibia leucotreta*.

During the prepupal stage, the larva will stop feeding and spin a cocoon. They are soft-skinned and have a cream colour; once the chitin hardens, they enter the pupal stage and become dark brown (**Figure 1.6**) (Daiber, 1979c). The development period for female and male pupae differs, with males requiring more time to develop. Females require anywhere from 11 – 39 days to eclose and males from 13 – 47 days, depending on the season (Daiber, 1979c).

Figure 1.6: *Thaumatotibia leucotreta* pupae in varying degrees of maturity.

The *T. leucotreta* adults have mottled brown-grey forewings and pale-grey hind wings, which are fringed (**Figure 1.7**). They are strongly dimorphic, and males can easily be distinguished from females by their specialised hindwings, smaller size and heavily tufted hind tibia (Venette et al., 2003). Adult *T. leucotreta* have distinct morphological and molecular keys that make them easily distinguishable from other economically important Tortricidae in South Africa (Timm et al., 2007,

2008). They are nocturnal and rest in shaded spots throughout the day (Stofberg, 1954). Once eclosed, females tend to mate shortly afterwards and experience a pre-oviposition period, usually a day or less (Newton, 1998). Ovipositing appears to decline after five days under laboratory conditions (Moore, 2002).

Figure 1.7: An adult *Thaumatotibia leucotreta*. Images captured by David Taylor.

The total development period for *T. leucotreta* is between 1½ – 4 months, depending on the season, resulting in between six to eight generations per year (Daiber, 1980; Stofberg, 1954). Additionally, *T. leucotreta* does not experience a winter diapause making the pest prevalent year-round (Terblanche et al., 2014).

1.3 Control options for *Thaumatotibia leucotreta*

The most effective approach for controlling *T. leucotreta* in South Africa has been through utilising an integrated pest management (IPM) programme. Specific treatments are strategically combined to suppress *T. leucotreta* early on in the season, preventing the build-up of subsequent potentially harmful population numbers later on (Moore, 2019a, 2021; Moore and Hattingh, 2016). This includes, but is not limited to, the use of chemical pesticides, cultural practices (e.g., orchard sanitation), mating disruption, sterile insect technique and biopesticides. The aforementioned control options are not effective enough to be used as stand-alone treatments; therefore, the control of *T. leucotreta* must be multidisciplinary-based (Moore, 2019a). The programme relies on the intelligent selection and implementation of treatments to ensure favourable economic, ecological, and sociological outcomes (Sandler, 2010). Combining these control options into a single programme has been calculated to have a combined efficacy of around 97 % (Moore et al., 2015b).

1.3.1 Chemical pesticides

Generally, pests and diseases are controlled through the application of pesticides (Mkiga et al., 2019). Several chemical pesticides are registered for the control of *T. leucotreta* in South Africa, including insect growth regulators (Alsystin®, Nomolt® and Runner™), pyrethroids (Cypermethrin and Meothrin), a spinosad (Delegate™) and a diamide (Coragen ®), insect growth regulators and others (Moore, 2021; Moore and Hattingh, 2012). Chemical pesticides are highly effective in controlling *T. leucotreta*, but in recent years the number of available chemistries has dropped (Ansell, 2008; Bielza et al., 2008). There are several reasons as to why this has occurred. Firstly, *T. leucotreta* has developed resistance to some registered insecticides (Moore et al., 2004; Moore and Kirkman, 2009; Moore and Hattingh, 2012). Secondly, public perception of chemical pesticides has changed over the last few years, and the demand for pesticide-free fruit has increased within both international and local markets (Chandler et al., 2011; Moore, 2019a; Moore and Hattingh, 2012). For example, many markets will not tolerate certain insect growth regulators (IGR) residues on their fruit (Moore, 2019a). Chemical pesticides are for the most part non-target specific, resulting in detrimental effects on non-target organisms, natural enemies (predators and parasitoids) and pathogens (Catling and Aschenborn, 1978; Hofmeyr and Pringle, 1998; Moore, 2002). This works against an IPM strategy and could result in pest resurgences and compromise the ecosystem's health (Chandler et al., 2011; Lacey and Goettel, 1995). Lastly, chemical sprays cannot penetrate the rinds of oranges, making them ineffective against the larvae that have already bored into the fruit. However, there are newer products available that can control *T. leucotreta* and are compatible with an IPM strategy (Fullard and Hill, 2013; Moore, 2021; Moore and Hattingh, 2012). Nevertheless, alternative methods need to be developed for the efficient control of *T. leucotreta*.

1.3.2 Cultural control

Cultural practices form an integral part of any IPM programme. Orchard sanitation is regarded as the cornerstone of *T. leucotreta* control in South Africa and forms the foundation on which the efficacy of all other treatments are based (Moore, 2019a). This involves the regular removal and destruction of fallen fruit as well as all hanging fruit that is infected, damaged or decaying (Moore and Kirkman, 2009). Orchard sanitation generally serves three main purposes. Firstly, it removes *T. leucotreta* and *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Mediterranean fruit fly)

larvae from the orchard. Secondly, it removes injured fruit that would attract *T. leucotreta* oviposition (Newton, 1989). Lastly, it will reduce the contribution made by rotting fruit to postharvest decay and generally reduce the orchard's pathogenic fungal spore load (Moore, 2019a). It has been reported that by conducting weekly orchard sanitation, an average of up to 75 % of *T. leucotreta* larvae could be removed from an orchard (Moore and Kirkman, 2009).

1.3.3 Sterile insect technique

Sterile insect technique (SIT) is an area-wide pest management tactic used to suppress a pest population down to negligible levels by flooding an orchard with fully or partially sterile insects (Dyck et al., 2021). This tactic has been developed and implemented in South Africa to reduce *T. leucotreta* infestation levels in the field (Hofmeyr et al., 2015, 2019). Mass-reared *T. leucotreta* moths are sterilised using gamma irradiation, and the moths are then aerially released over a target area (Bloem et al., 2007). Generally, a ratio of 10 sterile to 1 wild male moth is used resulting in the probability of a wild female moth mating with a sterile male moth being significantly greater than the probability of it mating with a wild male moth (Moore, 2019a). The wild females will then produce infertile eggs which in turn will decrease *T. leucotreta* field populations (Bloem et al., 2003). This technique can be extremely effective in suppressing *T. leucotreta* populations if applied over several years and is regarded as the most effective area-wide population suppression technique available (Moore, 2019a).

1.3.4 Mating disruption

Mating disruption programmes prevent *T. leucotreta* from copulating, reducing the number of viable eggs oviposited (Foster and Harris, 1997). This approach usually involves applying synthetic female sex pheromones to confuse or desensitise male *T. leucotreta* to such an extent that they cannot locate females for mating (Moore, 2019a). However, a large amount of synthetic pheromone is required, and its effect on male *T. leucotreta* is temporary. Attract and kill techniques operate similarly to mating disruption programmes, but instead of disrupting males from mating, they are killed, removing them from the orchard (Moore, 2021).

1.3.5 Biological control

For a biological control agent to achieve commercial significance, it has to have a close relationship with its target pest (Gupta and Dikshit, 2010; Kumar and Singh, 2015). The practice

involves exploiting the natural enemies of a pest to control the pest subsequently. There are several biological control agents available for use within South Africa (Hatting et al., 2019; Knox et al., 2015) and several of them are targeted against *T. leucotreta* (Coombes et al., 2015; Malan et al., 2018; Moore et al., 2004; Zimba et al., 2015). Biological control includes the use of entomopathogenic fungi (EPF), entomopathogenic viruses (EPV), entomopathogenic nematodes (EPN) and entomopathogenic bacteria (EPB) (**Table 1.2**). They are environmentally friendly, have no detrimental effects on consumers or applicators and are generally target specific (Knox et al., 2015).

Table 1.2: Biopesticides currently registered for the use against *Thaumatotibia leucotreta* in South Africa (Hatting et al., 2019).

Registration holder	Product name	Active
Fungi		
Plant Health Products	Eco-Bb	*Beauveria bassiana*
BASF	Broadband™	*Beauveria bassiana*
Viruses		
Madumbi	Cryptex®	Cryptophlebia leucotreta granulovirus
Chempac	Gratham®	Cryptophlebia leucotreta granulovirus
River Bioscience	Cryptogran™	Cryptophlebia leucotreta granulovirus
Bacteria		
BASF	BeTa Pro	*Bacillus thuringiensis* subsp. *kurstaki*
Nematodes		
River Bioscience	CryptoNem™	*Heterorhabditis bacteriophora*

EPFs and EPNs have shown great potential as biological control agents, particularly for their ability to target the soil associated and cryptic life stages of pests (Hatting et al., 2019; Malan et al., 2018). Several studies have been conducted to bioprospect and evaluate South African species of EPFs and EPNs against *T. leucotreta*. This led to the identification of several EPNs which were highly virulent against 5th instar larvae and pupae (Malan et al., 2011, 2018; Steyn et al., 2017). Currently, the only EPN-based product registered for use against *T. leucotreta* in South Africa is CryptoNem™ (River BioScience (Pty) Ltd, South Africa) which is formulated with *Heterorhabditis bacteriophora* (Hatting et al., 2019). Additionally, several EPFs were identified

and evaluated against *T. leucotreta,* both in laboratory and field settings (Coombes et al., 2013, 2015, 2016). These isolates have shown great potential against the soil-dwelling life stages and warrant further investigation and potential development into biological control agents. There are two EPF-based products registered in South Africa for the use against *T. leucotreta*. Both are formulated with *Beauveria bassiana* and are registered as Broadband™ (BASF, Germany) and Eco-Bb (Plant Health Products, South Africa). However, these are unfortunately only registered as foliar treatments, whereas the best results recorded with EPFs have been against the soil-dwelling life stages (Coombes et al., 2013, 2015, 2016; Goble et al., 2011).

Entomopathogenic viruses are one of the major biological control agents used in IPM programmes. Baculoviruses are the predominant EPV used against insect pests globally, particularly against Lepidoptera (Grzywacz, 2017; Moore et al., 2011; Szewczyk et al., 2006). Baculoviruses are perfect candidates for biological control agents, as they are species-specific, have a narrow host spectrum, are environmentally friendly and leave no residues on the fruit (Moore et al., 2004). Additionally, they can be used in conjunction with registered chemical pesticides and other biological control agents within IPM programmes. The drawbacks to their application include their relatively slow speed of kill, sensitivity to UV degradation, short field persistence and narrow host range (Rohrmann, 2019). Nevertheless, baculoviruses have formed an important part of IPM programmes in South Africa to control agriculturally important pests (Knox et al., 2015).

1.4 Baculoviruses

Baculoviruses are pathogens capable of infecting a diverse group of arthropods, many of which are agriculturally and economically important (Harrison and Hoover, 2012). They have a large circular double-stranded DNA genome of between 80 – 188 kilobase pairs packaged into a rod-shaped nucleocapsid. This nucleocapsid is surrounded by a membrane envelope which forms the occlusion derived virion (ODV). The ODV is encapsulated by a protective crystalline matrix, protecting them against ultraviolet (UV) degradation and mechanical stress, forming the occlusion body (OB). The crystalline matrix is either composed of the protein polyhedron in nucleopolyhedroviruses (NPVs) or granulin in granuloviruses (GVs) (Rohrmann, 2019). The baculovirus OB must be ingested by the host to cause infection, which typically is fatal.

1.4.1 Taxonomy and nomenclature

The Baculoviridae family is currently separated into four genera, based upon their host, morphological characteristics and genetic composition (ICTV, 2019; Jehle et al., 2006; Lauzon et al., 2004). The first genus is *Alphabaculovirus*, which is lepidopteran specific NPVs, followed by *Betabaculovirus*, which is lepidopteran specific GVs. The last two genera are *Gammabaculovirus* and *Deltabaculovirus*, which are comprised of the remaining NPVs, each of which infects hymenopteran and dipteran hosts, respectively (Rohrmann, 2019). The nomenclature of baculoviruses currently commands that new isolates be named after their host species and the type of OB they are correlated with. The first two letters of the host's genus and species combined with either NPV or GV depending on the virus type (Rohrmann, 2019). For example, the GV isolated from *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae), commonly referred to as potato tuber moth, was named Phthorimaea operculella granulovirus (PhopGV-SA). Although the insect species name is italicised, this is not the case when it becomes the virus species name.

1.4.2 Baculovirus classification and structure

Members of the Baculoviridae family are classified by the morphological characteristics of their OBs called polyhedra for NPVs and granules or capsules for GVs (Rohrmann, 2019). Polyhedra are about 0.6 – 2 µM in diameter, whereas granules are oval-shaped and about 0.2 – 0.4 µM in diameter (Ackermann and Smirnoff, 1983). Nucleopolyhedroviruses can either contain single (SNPV) or multiple (MNPV) nucleocapsids within their ODV (**Figure 1.8 A and B**), whereas a GV only contains a single nucleocapsid within its ODV. Nucleocapsids are rod-shaped particles about 230 – 385 nm in length and 40 – 60 nm in diameter, into which the viral genome is packaged (Federici, 1986). Lastly, NPVs can contain either single or multiple ODVs within their OB compared to a GV that only has a single ODV within its OB (**Figure 1.8 C and D**).

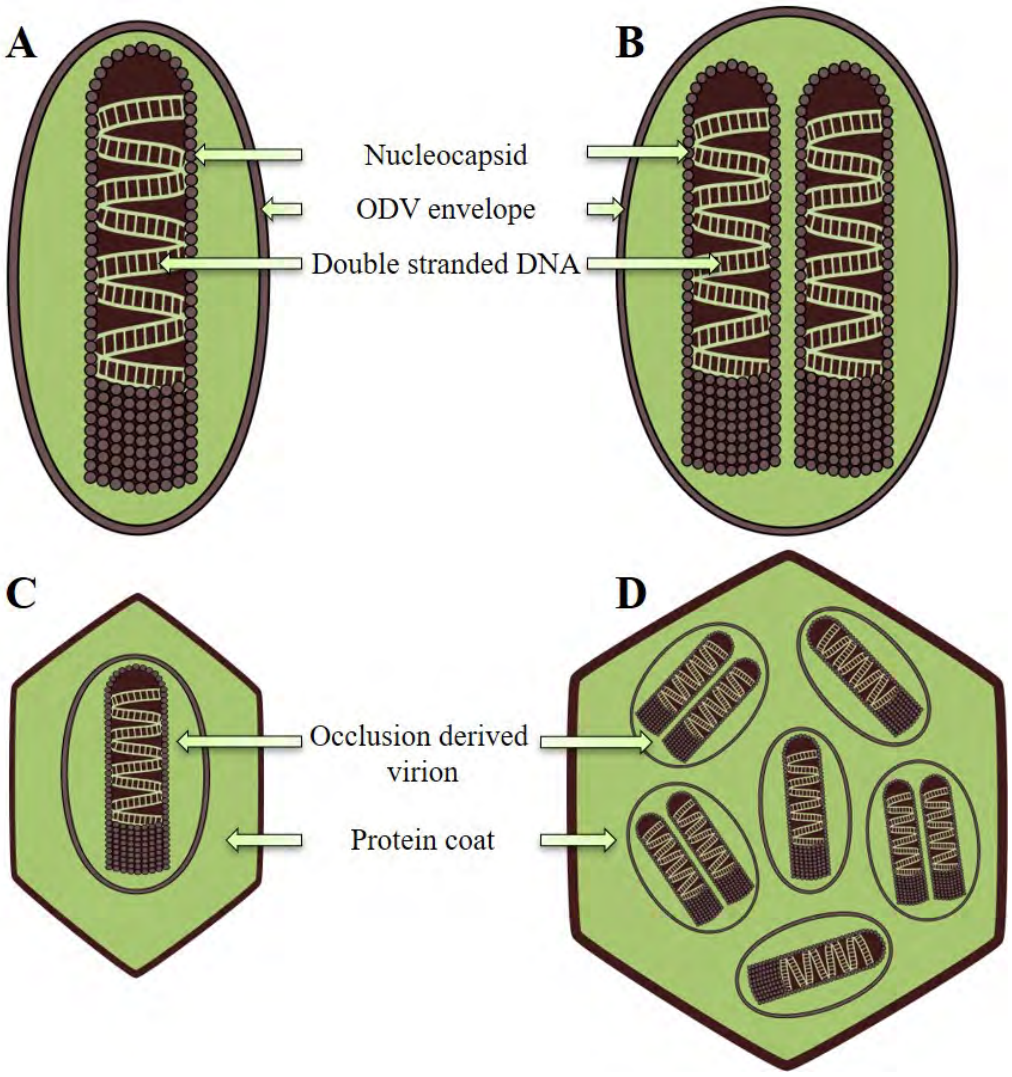

Figure 1.8: Baculovirus occlusion derived virion and occlusion body. A) occlusion derived virus with a single nucleocapsid. B) occlusion derived virus with multiple nucleocapsids. C) granulovirus occlusion body with a single ODV. D) nucleopolyhedrovirus occlusion body with multiple ODVs (Adapted from Rohrmann, 2019). Image created by Nicole Upfold.

1.4.3 Baculovirus life cycle

The baculovirus life cycle consists of two distinct stages: a primary and secondary infection caused by OBs and ODVs, respectively. Primary infection is initiated once a susceptible host ingests OBs, usually by feeding on a contaminated food source. Once ingested, OBs are dissolved by the high pH in the midgut lumen and are further degraded by OB associated proteinases that may also digest the peritrophic matrix. This releases the ODV, which then traverses the peritrophic membrane to

infect the midgut epithelial cells. The ODV envelope fuses with the host cell plasma membrane and results in the viral nucleocapsids release into the cytoplasm (**Figure 1.9 A**). The ODV nucleocapsid then migrates to the nucleus where it is uncoated, and viral DNA gene expression and replication occurs (**Figure 1.9 B**). Newly assembled nucleocapsids traverse the nuclear membrane and travel to the plasma membrane, which now contains viral proteins. They bud through the plasma membrane resulting in the formation of budded virions (BVs), which are responsible for cell-to-cell transmission of the virus. They bud into the haemocoel and are spread via the haemolymph to other tissues, causing a systemic infection. Budded virions enter these cells through endocytosis, after which their membrane fuses with the endosomal membrane, releasing the nucleocapsid into the cytoplasm (**Figure 1.9 C**). Replication occurs as previously described above.

Secondary infection is initiated when systemically infected cells begin to produce OBs. Nucleocapsids produced in these cells receive an envelope while in the nuclei, forming ODVs. The resulting ODVs are then occluded in their protective crystalline matrix of either granulin or polyhedrin, which is dependent on them being either NPVs or GVs, forming OBs (**Figure 1.9 D**). Occlusion bodies are responsible for the horizontal transmission of the virus (between insects). Host cell lysis occurs and eventually results in the insect's liquefaction within a few days, releasing the newly formed OBs back into the environment for the life cycle to be repeated (**Figure 1.9 E**).

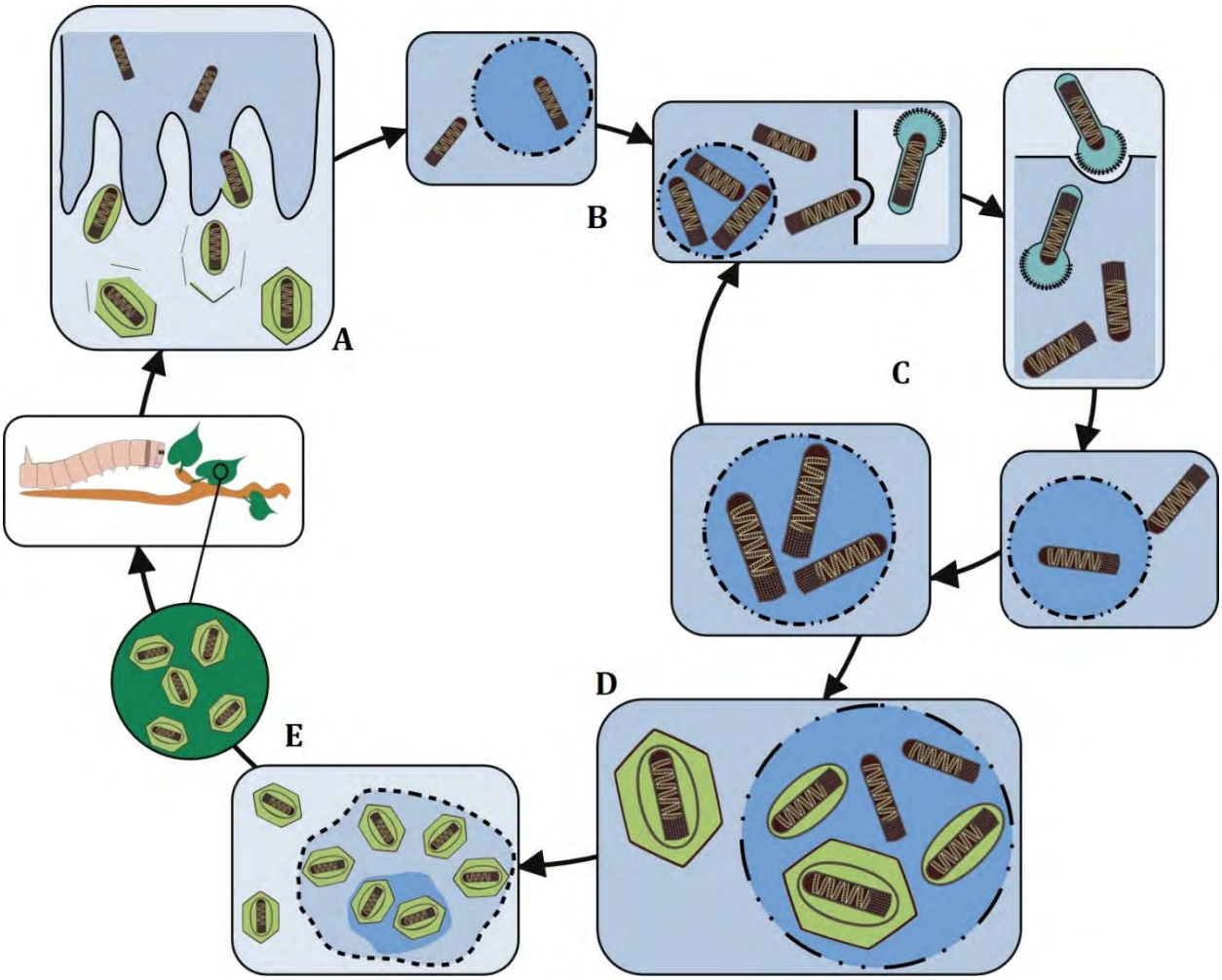

Figure 1.9: Baculovirus life cycle from ingestion to release. A) Occlusion bodies are ingested by the insect, which dissolve in the insect's alkaline midgut, resulting in the release of occlusion derived virions, which infect midgut epithelial cells. B) Nucleocapsids migrate to the nucleus, and replication occurs. C) Virions bud out of the infected cell in the basal direction, initiating the systemic infection; more budded virions are produced, spreading the infection. D) Nucleocapsids receive an envelope while in the nuclei, forming ODVs, which are then occluded in their protective crystalline matrix. E) Occlusion bodies are produced and released back into the environment upon insect death. (Rohrmann, 2019). Image created by Kate Bryan.

1.4.4 Cryptophlebia leucotreta granulovirus

There are three baculovirus biopesticides registered in South Africa for use against *T. leucotreta*, *viz*. Cryptogran™ (River Bioscience, South Africa), Cryptex® and Gratham® (Andermatt Biocontrol AG, Switzerland) (Hatting et al., 2019; Moore and Duncan, 2017). They are all

formulated with the betabaculovirus Cryptophlebia leucotreta granulovirus (CrleGV) (Moore and Jukes, 2019). Cryptogran™, however, is formulated with a naturally occurring endemic isolate of the virus (CrleGV-SA) which was discovered and developed into a biological control agent by Moore (2002). The baculovirus targets the early larval stages of *T. leucotreta*, allowing for the control of the life stage responsible for fruit damage (Moore et al., 2004). CrleGV-SA has been used commercially for more than 15 years in South Africa, with great success. The efficiency of CrleGV-SA has been reported to be comparable to that of chemical alternatives, reducing larval infestation by between 30 – 92 % (Moore et al., 2015b). The virus's virulence and genome stability were also accessed by comparing samples of CrleGV-SA collected in 2000 with samples collected in 2015. Over the 15 years, it was found that the genome was highly stable, and the biological activity had remained relatively consistent (Van der Merwe et al., 2017). CrleGV-SA remains a highly effective biological control agent used against *T. leucotreta* in South Africa when used as part of an IPM programme.

1.5 Improving the formula of CrleGV-SA

Baculoviruses make for effective biological control agents mainly because of their life cycle. In addition to killing their hosts, the baculovirus infection also results in the production of additional virus particles that are reintroduced back into the environment, leading to secondary infections. The baculovirus CrleGV-SA is an effective biological control agent against *T. leucotreta* (Moore et al., 2015b). However, there have been some challenges and limitations encountered with the use of baculoviruses such as UV susceptibility (Arthurs et al., 2008; Lacey and Arthurs, 2005), development of resistance in targeted hosts (Asser-Kaiser et al., 2007, 2011), and their virulence and slow speed of kill against a specific host (Rohrmann, 2019). These are only some of the challenges associated with baculovirus biopesticides. This highlights the importance of continuing research into developing innovative solutions to overcome both these and novel obstacles that may be encountered in the future.

1.5.1 Bioprospect for novel isolates

The occurrence of resistance to baculoviruses by insects was thought to be unlikely and had only been observed under laboratory conditions. However, it was reported in 2005 that several *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) populations had developed resistance to commercial products which had been formulated with the Mexican isolate of Cydia pomonella

granulovirus (CpGV-M) (Fritsch et al., 2006). There is currently only one baculovirus registered for use against *T. leucotreta* in South Africa, *viz*. CrleGV-SA. The discovery of *C. pomonella* populations becoming resistant to CpGV-M prompted bioprospecting for new CrleGV-SA isolates. Several novel isolates have been identified and characterised, with some isolates exhibiting higher virulence to host populations than others (Opoku-Debrah et al., 2013, 2016). The occurrence of resistance within *T. leucotreta* populations is always a concern. Still, with the continued evaluation of novel isolates and their development into new biopesticides, this possible problem can be managed, should it arise in South Africa.

A novel NPV was also recently isolated at Rhodes University (South Africa) from *Cryptophlebia peltastica* (Meyrick) (Lepidoptera: Tortricidae) and is capable of infecting *T. leucotreta* (Jukes, 2018; Marsberg, 2017; Marsberg et al., 2018). The development of CrpeNPV into a biological control agent against *T. leucotreta* will increase the number of control options against the pest and prevent the overuse of any option.

1.5.2 Enhance efficiency through coinfections.

Numerous studies have been conducted to investigate the types of interactions which can occur during baculovirus coinfections (Arrizubieta et al., 2015, 2016; Graillot et al., 2016; Lara Reyna et al., 2003). These interactions range from mutualistic, where both may have improved replication, to antagonistic, where improved replication of one virus occurs at the expense of the other (Cheng and Lynn, 2009). Interest, however, has been placed on overcoming resistance and improving virulence. *Cydia pomonella*, as previously mentioned, has become resistant to CpGV-M, while isolates such as CpGV-R5 are still able to establish infection. The combination of CpGV-M with CpGV-R5 resulted in both viruses infecting the resistant colony (Graillot et al., 2016). The mixture of various baculovirus genotypes has also been demonstrated in improving insecticidal activity. The relative potency of Helicoverpa armigera SNPV (HearSNPV) genotypes differed only slightly when used individually but by combining different variants an increase in the relative potency of up to 3.6-fold was recorded (Arrizubieta et al., 2016).

Additionally, studies have been conducted utilising NPVs in combination with GVs to investigate their interactions during coinfection. These coinfections have been shown to improve the virulence and speed of kill in laboratory bioassays (Biedma et al., 2015; Jukes, 2018; Lara Reyna et al., 2003). Yet, it remains to be seen if they could help prevent or control resistant insect populations.

1.5.3 Genetically modify baculoviruses.

The application of baculoviruses as commercial insecticides has been, to some extent, hampered by their slow speed of kill and UV sensitivity. Users were accustomed to the fast-killing action and broad host range of chemical pesticides and considered baculoviruses ineffective. A solution came with developing genetic engineering methods for baculoviruses to express foreign genes (Kroemer et al., 2015). Strides toward improving the speed of kill have been made by deleting selected genes and introducing others (Bonning et al., 2002). It has previously been demonstrated that deleting the baculovirus encoded *ecdysteroid uridine 5´–diphosphate–glucosyltransferase* (*egt*) gene responsible for preventing insect moulting, indirectly increases feeding activity, resulting in 30 % faster killing of larvae (O'Reilly and Miller, 1991). The modification of the baculovirus genome to express insecticidal peptides is far more popular than the deletion of selected genes to interference with host physiology (Kroemer et al., 2015). Larvae infected with recombinant viruses tend to die sooner than those infected with the wild-type, due to the toxicity of insecticidal peptides rather than the infection's pathology. The gene encoding for the AaIT toxin, originating from *Androctonus australis* (Linnaeus) (Scorpiones: Buthidae), has been one of the most promising insect-specific toxin genes used for the construction of baculovirus recombinants (Bonning et al., 2002). Recombinant baculoviruses constructed with the AaIT toxin gene have been reported to increase the speed of kill up to about 40 % and inadvertently reduced damage caused by feeding by about 40 % as well (Inceoglu et al., 2001). Additionally, the passage of a baculovirus through a heterologous host has also been shown to increase the speed of kill (Belda et al., 2019; Shapiro et al., 1982; Stairs, 1990). Improvements to the UV stability of baculoviruses have been made through genetic modification.

However, by changing the application times of baculovirus from during the day to evenings, similar results can be achieved (Kirkman, 2007). These genetically modified organisms (GMOs) have not been used in practice due to significant resistance by the public, and lawmakers to modified baculovirus genomes. However, through extensive research conducted over many years, these GMOs are considered safe (Szewczyk et al., 2009).

1.5.4 Combining baculoviruses with other microbial agents

Microbes are the most abundant living organisms on earth and can be found just about anywhere. Apart from within the general environment, they are also found within and on other living

organisms; insects, for example, are colonised by microorganisms. They are predominantly localised to the external cuticle and hindgut, as these regions offer the most favourable conditions for establishment (Douglas, 2015). Although the exoskeleton is recognised as an indispensable physical barrier against microorganisms, preventing them from entering the interior of insects, it is also a region that can be colonised by various microbes (Vallet-Gely et al., 2008). Some insects have even developed specialised structures on their bodies that harbour symbiotic fungi, and even bacteria in some instances (Douglas, 2015; Hulcr et al., 2012; Scott et al., 2008). Although the gut of insects might look like a hostile environment for microorganisms, due to the unfavourable physicochemical conditions, the secretion of digestive enzymes and immunological activity, they can survive and possibly reproduce there (Douglas, 2015; Stefanini, 2018). The greatest microbial populations are usually found within the hindgut of most insects, as it is a benign environment that lacks digestive enzymes and desiccation stress. The makeup of these microbial communities is for the most part comprised of bacteria, archaea, and eukaryotic microorganisms.

The term “symbiosis” describes a close and long-term interaction between two different biological organisms. The interaction can be beneficial to the one and detrimental to the other (parasitic), beneficial to one without being detrimental to the other (commensal), beneficial to both (mutualistic) or detrimental to both (competitive) (Cardoza et al., 2012; Dillon and Dillon, 2003). Additionally, symbionts can be housed inside (endosymbionts) or outside (ectosymbionts) of the insect’s body. If neither organism can live without the other, the association is considered primary (obligate), and secondary (facultative) if they can survive and reproduce without one another (Holland and Bronstein, 2008). In the past, microorganisms that had parasitic interactions with their insect host were studied, for their obvious use as biological control agents (Davis et al., 2013). However, microorganisms are far more often beneficial or even required by the host insect than overtly pathogenic (Douglas, 2015). Microorganisms can promote insect fitness by providing nutrition in the form of essential amino acids, B vitamins and sterols or by defending them against pathogens and natural enemies by producing specific toxins or modifying their immune system (Douglas, 2015; Gibson and Hunter, 2010). Additionally, they may produce specific chemical cues which invoke certain behavioural responses within their host insect (Ezenwa et al., 2012). Many insects are associated with specific microbes and understanding these specific insect-microbe interactions could lead to the development of novel species-specific pest management tactics (Douglas, 2007; Hamby and Becher, 2016).

The interactions between insects and bacteria have been well documented; however, yeasts represent a new avenue for exploration. Recently, studies conducted between yeasts and insects have highlighted their importance in food acquisition and the development and behaviour of insects (Stefanini, 2018). The diversity of yeast within the environment and their persistence in the gastrointestinal system of insects indicate that they possess great potential for use in biocontrol (Suh et al., 2005). Only a handful of studies have focused on the associations between yeast and insects of the order Lepidoptera. These insects are of great importance as they are well-known pests of economically important crops, such as citrus and cotton (Moore, 2002; Stefanini, 2018).

Yeasts are a phylogenetically diverse group of organisms belonging to the fungi kingdom and are split into two phyla, *viz*. ascomycetes and basidiomycetes, which forms the subkingdom Dikarya (Hibbett et al., 2007). They are further subdivided into budding yeasts, informally known as "true yeast", belonging to the Saccharomycotina subphylum, and yeast-like symbionts, which belong to the Pezizomycotina subphylum (Vega and Dowd, 2005). They are eukaryotic, single-celled microorganisms, and are generally characterised by budding or fission as their primary means of vegetative reproduction and have sexual states that are not enclosed in fruiting bodies (Kurtzman et al., 2011). Yeast cells exhibit great diversity concerning cell size, shape, and colour. Individual cells from a pure strain of a single species can display morphological heterogeneity (Feldmann, 2012). Yeast typically forms facultative associations with their insect host and can be recovered from faeces, ovipositors and specialised organs (Gibson and Hunter, 2010). The vast majority of symbiotic relationships between yeast and insects are formed with "true yeast", but yeast-like symbionts have been reported to form associations with insects as well (Gibson and Hunter, 2010).

Yeasts have been shown to form an essential part of the larval diet, and the volatiles that they produce elicit a strong response in the olfactory system of adult insects (Becher et al., 2012, 2018; Witzgall et al., 2012). The volatile signatures produced by yeasts have been found to mediate host finding and discrimination in *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) and *C. pomonella* (Becher et al., 2012; Witzgall et al., 2012). Additionally, insects are thought to employ two distinct chemosensory receptors, odorant receptors tuned to plant volatiles and ionotropic receptors that sense water-soluble compounds which include microbial volatiles (Benton et al., 2009; Croset et al., 2010). Volatiles produced by yeast represents a new source of attractants for pest monitoring and control.

Yeast from the genus *Metschnikowia* was shown to have a mutualistic association with *C. pomonella* (Witzgall et al., 2012). Knight and Witzgall (2013) manipulated this mutualistic relationship for biocontrol by combining the yeast with the baculovirus CpGV. It was demonstrated in laboratory assays and field trials, that combining *M. pulcherrima* with brown cane sugar and CpGV resulted in a significant increase in larval mortality and lowered the mean proportion of injured fruit. The same was observed when *M. pulcherrima* was substituted for *Saccharomyces cerevisiae* (Knight et al., 2015). Additionally, the numbers of stagnating larvae on trees were also significantly reduced. These results suggest that yeasts can enhance the effectiveness of an insect virus, in managing pest larvae. This indicates that yeasts harbour the potential for use in biocontrol, especially when combined with other well-established control methods.

1.6 Justification for this study

Thaumatotibia leucotreta is one of the major pests that plague the South African citrus industry. For the most part, *T. leucotreta* has been extensively studied. However, its mutualisms with fungi and especially yeast has, for the most part, remained unexplored. This could potentially represent a rich resource of new biological agents for the control of *T. leucotreta* and thus requires further investigation. It has been demonstrated that yeasts enhance the efficacy of baculovirus; this has only been reported in *C. pomonella* and not for *T. leucotreta*. Therefore, this study aimed to i) bioprospect for yeast from geographically distinct citrus producing regions, ii) investigate whether any of the isolated yeast species influence the behaviour of *T. leucotreta* neonates and adult females, and iii) examine whether any of these yeasts, when combined with CrleGV-SA, have a synergistic effect in increasing the mortality of *T. leucotreta* and improving its efficacy in the field on citrus.

1.7 Overview of Chapters

Chapter Two describes the isolation and identification of yeasts from the gut of *T. leucotreta* larvae. Yeast species would be purified using Pen/Strep YPD agar plates and identified via the PCR amplification and sequencing of the ITS region and D1/D2 domain of the LSU.

Chapter Three investigates the effect that the isolated yeast species could have on larval development and survival. Additionally, two-choice tests would be conducted to assess if the isolated yeast species influence the feeding behaviour of *T. leucotreta* neonates.

Chapter Four assesses how the isolated yeast species could affect adult *T. leucotreta* female oviposition preference through a series of two-choice and multiple-choice tests conducted in a laboratory setting. This, combined with the larval attraction assay described above, would act as a vetting technique to select the best yeast species for detached fruit bioassays and semi-field trials.

The efficacy of combining the isolated yeast species with the baculovirus CrleGV-SA would be assessed in **Chapter Five**. A series of detached fruit bioassays would be used to determine the optimal yeast:virus ratio, the effectiveness of each yeast species when combined with CrleGV-SA, and further improve the yeast/virus mixtures efficacy through the addition of an adjuvant (molasses) and surfactant (BREAK-THRU® S 240). Additionally, semi-field trials would be conducted with the overall best-performing yeast species and *S. cerevisiae*.

Lastly, **Chapter Six** is a general discussion of the significant results obtained from the previous chapters. Emphasis is put on their relevance to the management of *T. leucotreta* in South Africa with future perspectives and recommendations for improving the results obtained in this study.

Chapter Two

The isolation and identification of yeast associated with *Thaumatotibia leucotreta* larvae

2.1 Introduction

The relationships that exist between insects and microorganisms are complex and dynamic (Kaufman et al., 2000). They vary considerably and can range from accidental encounters to functional ones such as locating an attractive food source or providing essential nutrients missing from their diet (Stefanini, 2018). These interspecific interactions play a crucial role in the evolution of insects (Ganter, 2006; Herrera et al., 2010; Janson et al., 2008; Klepzig et al., 2009). Bacterial communities present within the guts of insects have been widely explored however only recent studies have started to focus on yeasts (Dillon and Dillon, 2003; Kwong and Moran, 2016; Yun et al., 2014). The gut of insects is generally considered as a hostile environment which prevents most microorganisms from establishing there, selecting against the sensitive and guaranteeing a less competitive environment for the resilient ones (Lemaitre and Miguel-Aliaga, 2013). Most yeasts associated with insects are found and housed within the gastrointestinal tract and typically form facultative associations with their hosts (Gibson and Hunter, 2010; Vega and Dowd, 2005). Studies conducted on yeasts and insects have highlighted their importance not only in food acquisition but also in the development and behaviour of insects. Yeasts benefit from these interactions by using their hosts as vectors, being carried between environments. Additionally, yeasts are also protected from the external environment and competitors. The gut of insects is also thought to provide an environment where yeasts may reproduce or generate sexual forms able to reproduce once reintroduced back into the external environment (Stefanini, 2018).

Very few of the studies conducted on interactions between yeast and insects have focused on Lepidoptera (Witzgall et al., 2012). There is growing interest in insects of this order as they are a well-known pest of many economically important crops. The search for yeasts in the Lepidoptera was fuelled by studies conducted on *D. melanogaster*. It was demonstrated that microbes were mainly responsible for influencing host locating and ovipositing, not plant cues as once thought (Becher et al., 2012). Witzgall et al., (2012) demonstrated that *C. pomonella* larvae were closely associated with yeast from the genus *Metschnikowia*. These yeasts were crucial in enhancing the

development of *C. pomonella* larvae by providing them with essential nutrients and protecting them from fungal infections. It was also demonstrated that the yeasts produced metabolites that contribute towards adult *C. pomonella* moths recognising and finding their host plant. The identification of this mutualistic association between *C. pomonella* larvae and yeasts from the genus *Metschnikowia* has led to the development of novel biocontrol strategies. By combining CpGV with yeast, a significant increase in neonate *C. pomonella* larval mortality was observed, both in laboratory assays and field trials, compared with CpGV being applied alone (Knight et al., 2015). This approach has only been demonstrated with *C. pomonella* and not in any other lepidopteran pests including *T. leucotreta*. Although CrleGV-SA is an effective biocontrol agent against *T. leucotreta* (Moore et al., 2015b), there is always a need to develop novel and innovative methods for the improved control of this pest. The combination of an insect pathogen with yeast represents a potentially new approach for the control of Lepidopteran pests.

Traditionally yeasts have been classified based upon their morphological, physiological and biochemical properties (Barnett et al., 1990; Walt and Yarrow, 1984; Zhao et al., 2018). However, these techniques are complex and time-consuming, which may result in ambiguous profiles leading to incorrect classifications (Deak, 2007; Neppelenbroek et al., 2014; Pincus et al., 2007). Molecular techniques have recently become the standard for identifying yeasts, as most of them are quick and simple (Zhao et al., 2018). The most widely used and accepted techniques include the sequencing of conserved DNA regions and restriction fragment length polymorphism (RFLP) of chromosomal and mitochondrial DNA (Pincus et al., 2007). The preferred molecular technique for yeast identification is the PCR amplification and sequencing of nuclear ribosomal DNA (rDNA) subunits, which are highly conserved but are separated by more variable domains that display species-specific sequences (Pincus et al., 2007; Zhao et al., 2018). The most targeted areas include the D1/D2 domain of the large subunit (LSU) and the internal transcribed spacer (ITS) region, which is comprised of the 5.8S rDNA gene and the two-variable flanking ITS1 and ITS2 regions (**Figure 2.1**). This allows for the reliable separation of a wide range of yeast species (Ferreira et al., 2010). The availability of universal primers and their relatively small size makes them appropriate targets for sequencing (Kwiatkowski et al., 2012; Zhao et al., 2018).

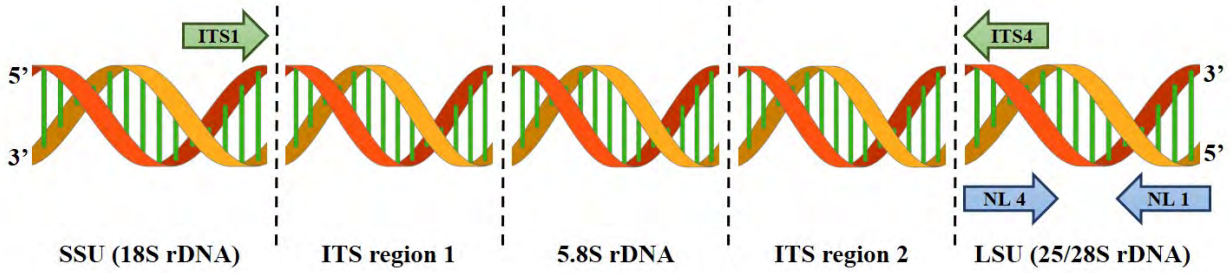

Figure 2.1: Schematic representation of the fungal ribosomal DNA regions showing the annealing positions of the universal oligonucleotide pairs ITS1/4 and NL 1/4 (Adapted from Pincus et al., 2007).

Some closely related yeast species are difficult to differentiate via sequencing alone, and RFLP has proven to be a useful tool (Esteve-Zarzoso et al., 1999; Guillamon et al., 1998; Pincus et al., 2007). The digestion of the ITS region and LSU may serve as potential taxonomic markers. The most commonly used restriction endonucleases to generate distinct profiles for the ITS region are *Cfo*I, *Hae*III and *Hinf* I (Guillamon et al., 1998; Pham et al., 2011).

This chapter aims to isolate and identify yeast species from the gut of *T. leucotreta* larvae collected from geographically distinct citrus-producing regions across South Africa. The first objective was to isolate yeast from the gut of *T. leucotreta* larvae and to obtain pure yeast cultures. The second objective was to identify these yeast species via the PCR amplification and sequencing of the ITS region and LSU.

2.2 Methods and Materials

2.2.1 Collection of *Thaumatotibia leucotreta* larvae

Three geographically distinct citrus-producing regions in South Africa were selected as sampling sites, *viz.* Stellenbosch (Western Cape), Addo (Eastern Cape) and Nelspruit (Mpumalanga) (**Figure 2.2**). Ten navel oranges (*Citrus sinensis* L. Osbeck) infested with *T. leucotreta* larvae were collected from each site between March and June of 2018. The larvae were extracted from the collected fruit using a sterile serrated knife and insect forceps. Extracted larvae were surface sterilised by rinsing in 10 % NaOCl, followed by a rinse with 70 % ethanol solution (v/v) and double-distilled H_2O (ddH_2O) before being placed into a sterile petri dish.

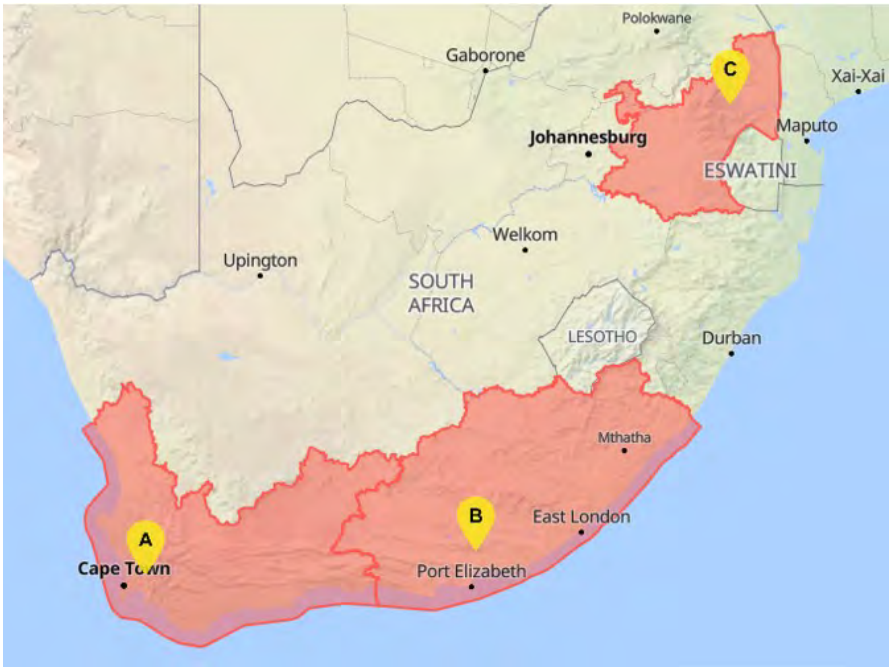

Figure 2.2: *Thaumatotibia leucotreta* larvae collection sites near citrus-producing regions of Stellenbosch in Western Cape (A), Addo in Eastern Cape (B) and Nelspruit in Mpumalanga (C).

2.2.2 Isolating yeast from *Thaumatotibia leucotreta* larvae

A modified version of the yeast isolation protocol described by Witzgall et al., (2012) was used to isolate yeast from the digestive tract of *T. leucotreta* larvae. The extracted larvae were stored at 4 ℃ for 2 hours to incapacitate them. Once immobilised larvae were dissected with a sterile scalpel in a fume hood. A sterilised inoculation loop was used to collect the guts of larvae which were then placed into a 1.5 ml tube and homogenised with a glass rod. The guts were then streaked onto a Yeast extract Peptone Dextrose (YPD) agar (Sigma-Aldrich, USA) plate containing 40 units/mL of penicillin (Pen) and 40 µg/mL of streptomycin (Strep) (Thermo Fisher Scientific, USA). The gut homogenate was then diluted with 200 µl of ddH_2O and filtered through muslin cloth, 100 µl of this filtrate was then spread plated onto Pen/Strep YPD agar plate. This process was repeated for each of the collected larvae. Plates were incubated at 27 ℃ for 48 hours under aerobic conditions with emerging colonies being selected and discontinuously streaked on Pen/Strep YPD agar plates. This process was repeated twice to obtain pure colonies.

2.2.3 Yeast genomic DNA extraction

Yeast colonies were selected from YPD agar plates and grown up in 5 ml of YPD medium containing 40 units/mL of Pen and 40 µg/mL of Strep for 24 hours at 27 ℃ while shaking. Thereafter, 100 µl of yeast culture was removed, and a 1:100 dilution was prepared with ddH_2O. Yeast cells were then enumerated using a Neubauer-improved counting chamber (Hirschmann Laborgeräte GmbH & Co. KG, Germany) with a depth of 0.1 mm and bright-field microscopy. Five central square quadrants were counted, more specifically top left, bottom left, top right, bottom right and a random centre square. The total number of small squares counted was 80 (16 per large square), resulting in a counted area of 0.2 mm^2. The yeast cultures concentration was then determined using **Equation 2.1** (Hirschmann Laborgeräte GmbH & Co. KG, Germany).

$$\text{cells per µl} = \frac{\text{Number of cells}}{\text{Counted area } (\text{mm}^2) \times \text{chamber depth (mm)} \times \text{dilution}}$$

Equation 2.1: Formula used to determine the number of cells per µl of yeast culture.

Genomic DNA (gDNA) was extracted using YeaStar™ Genomic DNA Kit (Zymo Research, USA) as per the manufacturer's instructions. Yeast cultures were diluted down to 3×10^7 $cells.ml^{-1}$ before extraction. Genomic DNA was eluted into 1.5 ml tubes with 60 µl of ddH_2O. The concentration of gDNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The extracted gDNA was visualised on 1 % agarose gel stained with ethidium bromide (0.5 $\mu g.ml^{-1}$) and separated at 90 V for 45 minutes in 1× TAE buffer (40 mM Tris-acetate, 20 mM acetic acid, 1 mM EDTA). Agarose gels were visualised using the ChemiDoc™ XRS+ System (Bio-Rad, USA) and images were captured with the Image Lab™ software (Bio-Rad, USA).

2.2.4 PCR amplification of ITS region and D1/D2 domain of LSU

The ITS region and D1/D2 domain of LSU were amplified using the oligonucleotides described in **Table 2.1** (Pincus et al., 2007). PCR reactions contained 1 µl of the forward oligonucleotide (10 µM) and 1 µl of the reverse oligonucleotide (10 µM), 12.5 µl Taq DNA Polymerase 2× Master Mix (Ampliqon, Denmark) and 50 ng of gDNA. Reactions were made up to 25 µl with ddH_2O, and a no template control (NTC) was also prepared for each set of primers. PCR amplification was carried out using a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, USA) under the

following conditions: an initial denaturation cycle of 94 ℃ for 5 minutes followed by 30 cycles of 95 ℃ for 30 seconds, 55 ℃ for 45 seconds, and 72 ℃ for 45 seconds. A final elongation cycle of 72 ℃ for 10 minutes was used (Witzgall et al., 2012).

Table 2.1: Oligonucleotides used to amplify and sequencing the internal transcribed spacer (ITS) region and D1/D2 domain of large subunit (LSU) on yeast genomes (Pincus et al., 2007).

Target	Name	Sequence (5' – 3')	Amplicon size (bp)
ITS region	ITS1	TCC GTC GGT GAA CCT GCG G	400 – 800
	ITS4	TCC TCC GCT TAT TGA TAT GC	
D1/D2 domain	NL 1	GCA TAT CAA TAA GCG GAG GAA AAG	600
	NL 4	GGT CCG TGT TTC AAG ACG G	

Amplicons were visualised on a 2 % agarose gel stained with ethidium bromide (0.5 μg.ml ^{1}) and separated at 80 V for 45 minutes in 1× TAE buffer. Gel images were captured, as mentioned previously.

2.2.5 Analysis of ITS and D1/D2 amplicons

Sequencing of the amplicons was carried out by Inqaba Biotechnical Industries (Pty) Ltd (South Africa). Yeast species with ITS amplicons greater than 600 bp were sequenced both in the forward and reverse direction. These sequences were assembled in MEGA X (Kumar et al., 2018). Sequences were analysed in Chromas (version 2.6.6), and any ambiguous nucleotides were corrected before the sequences were identified by comparison to GenBank database of non-redundant sequences using BLAST.

2.2.6 Yeast species culturing and storage.

Once a yeast species was identified, a monoculture was prepared by discontinuously streaking the yeast onto a Pen/Strep YPD agar plate, which was then incubated at 27 ℃ for 48 hours before being stored at 4 ℃ for up to 2 months. Glycerol stocks were also prepared for each yeast species by inoculating a single yeast colony into 5 ml of Pen/Strep YPD medium and incubating it at 27 ℃ while shaking for 48 hours. Thereafter, 2.5 ml of the yeast culture was added to 2.5 ml of 50 %

glycerol (v/v) solution in a clean 5 ml tube and stored at -80 ℃. Subsequent yeast cultures were always prepared from discontinuously streaked yeast on Pen/Strep YPD agar plates.

2.3 Results

2.3.1 Yeast isolation and purification

Six yeast species were successfully isolated from the gut of *T. leucotreta* larvae. Four unique yeast species were isolated from *T. leucotreta* larvae collected from Addo and Stellenbosch, respectively. The remaining two species were common in larvae collected from each region. Monocultures were prepared for each yeast species via discontinuous streaking, with single colonies being selected from these cultures to prepare 40 % glycerol stocks.

2.3.2 Analysis of yeast genomic DNA

Genomic DNA was successfully extracted from isolated yeast species (**Figure 2.3**). The extracted gDNA concentration ranged from 7 – 20 µg with A_{260}/A_{280} readings constantly being greater than 1.8.

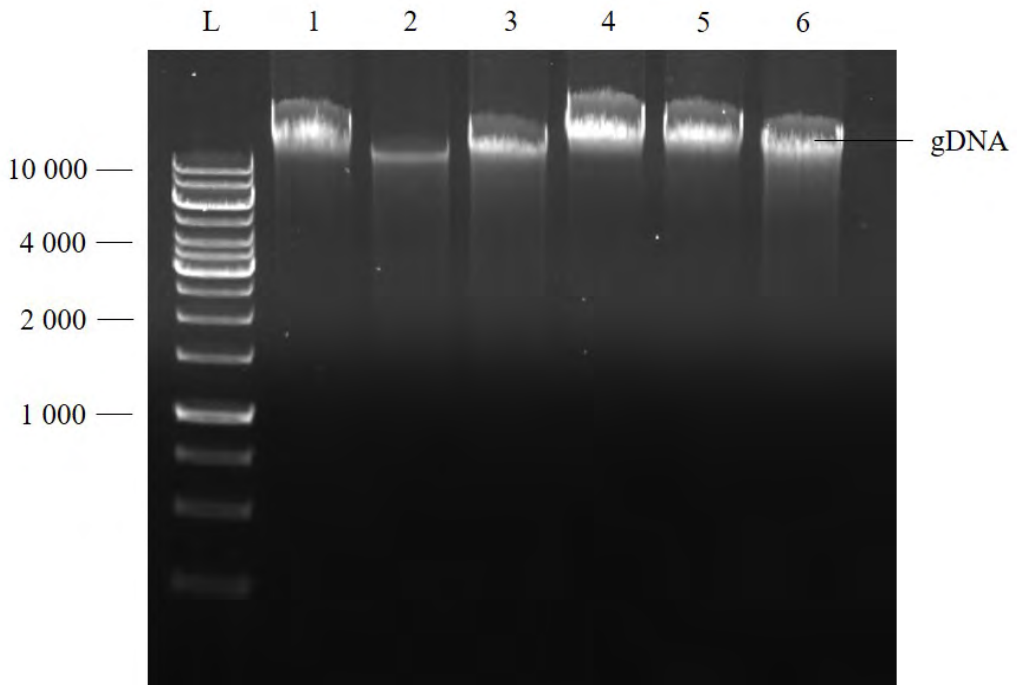

Figure 2.3: Genomic DNA extracted from yeasts isolated from the gut of *T. leucotreta* larvae. Lane L – GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, USA); Lane 1 to 6 – gDNA of isolated yeasts species.

2.3.3 PCR amplification of ITS region and D1/D2 domain

The ITS region and D1/D2 domain of LSU for each yeast species was successfully amplified via PCR amplification of extracted gDNA. The D1/D2 domain amplicons for each yeast species had an estimated size of 550 bp (**Figure 2.4**), whereas the ITS region amplicons for each yeast species ranged from 150 to 750 bp in size (**Figure 2.5**).

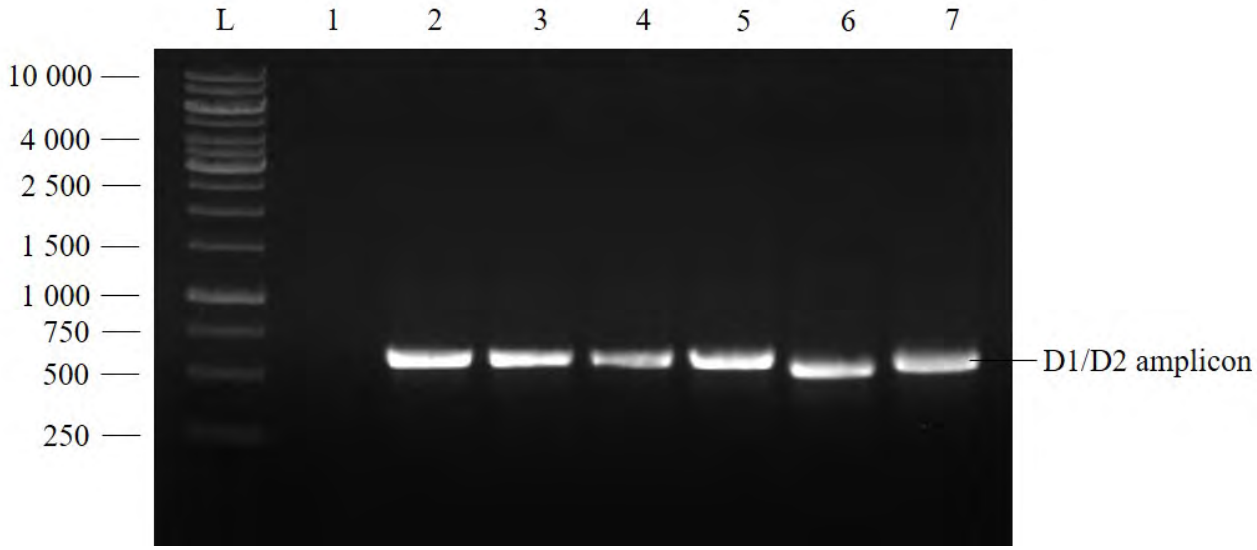

Figure 2.4: The D1/D2 domain amplicons produced for each of the isolated yeast species. Lane L – GeneRuler 1 kb DNA Ladder; Lane 1 – No template control; Lane 2 – yeast isolated from Addo "One"; Lane 3 – commonly isolated yeast "One"; Lane 4 – commonly isolated yeast "Two"; Lane 5 – yeast isolated from Addo "Two"; Lane 6 – yeast isolated from Addo "Three" and Lane 7 – yeast isolated from Stellenbosch.

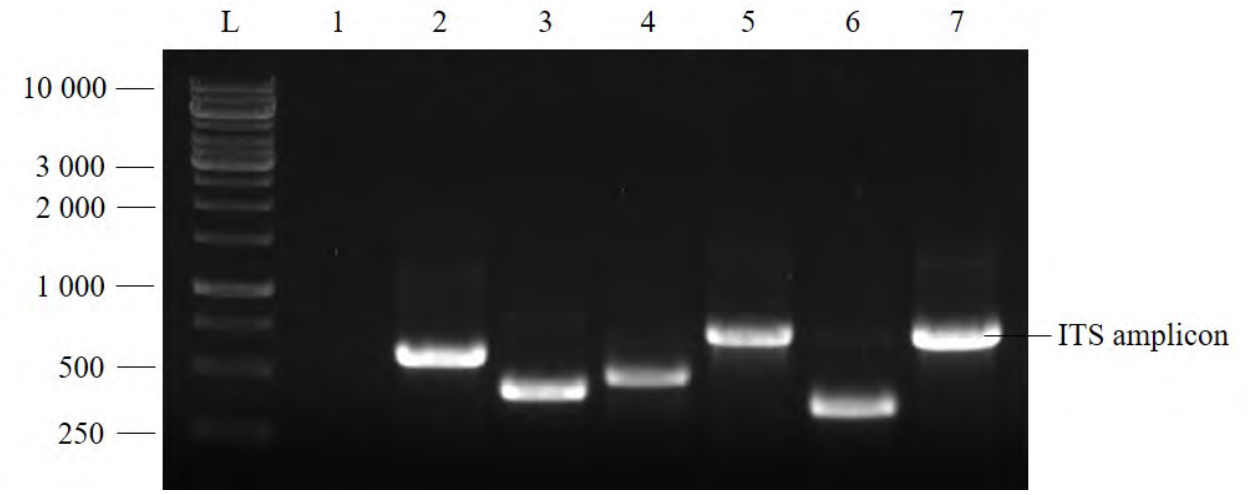

Figure 2.5: The ITS region amplicons produced for each of the isolated yeast species. Lane L – GeneRuler 1 kb DNA Ladder; Lane 1 – No template control; Lane 2 – yeast isolated from Addo "One" (±600 bp); Lane 3 – commonly isolated yeast "One" (±450 bp); Lane 4 – commonly isolated yeast "Two" (±500 bp); Lane 5 – yeast isolated from Addo "Two" (±750 bp); Lane 6 – yeast isolated from Addo "Three" (±300 bp) and Lane 7 – yeast isolated from Stellenbosch (±750 bp).

The amplicons produced for ITS region and D1/D2 domain of each yeast species corresponded with the expected sizes from the literature (**Table 2.1**). Sequencing of the produced amplicons was carried out by Inqaba Biotechnical Industries (Pty) Ltd (South Africa).

2.3.4 Analysis of ITS and D1/D2 domain sequences

The ITS region and D1/D2 domain sequences for each yeast species was analysed in Chromas (version 2.6.6) with any ambiguous nucleotides being corrected. Sequences were then assembled in MEGA X (version 10.1.8) and submitted for BLAST analysis. The analysis of the BLAST results revealed that six yeast species had been isolated from the gut of *T. leucotreta* larvae, *viz. Meyerozyma guilliermondii, Hanseniaspora uvarum, Clavispora lusitaniae, Kluyveromyces marxianus, Pichia kudriavzevii* and *Pichia kluyveri* (**Table 2.2**).

Table 2.2: The BLAST analysis of the ITS region and D1/D2 domain for each isolated yeast species.

Sample	Amplicon	Yeast species	Accession number	Identity
Addo "One"	ITS	*Meyerozyma guilliermondii*	MN537824.1	100 %
	D1/D2	*Meyerozyma guilliermondii*	EU285513.1	100 %
Addo "Two"	ITS	*Hanseniaspora uvarum*	MN371907.1	98.28 %
	D1/D2	*Hanseniaspora uvarum*	AY305681.1	99.64 %
Addo "Three"	ITS	*Clavispora lusitaniae*	KP765042.1	99.52 %
	D1/D2	*Clavispora lusitaniae*	MG871742.1	99.82 %
Stellenbosch	ITS	*Kluyveromyces marxianus*	KX376261.1	99.70 %
	D1/D2	*Kluyveromyces marxianus*	CP009307.2	100 %
Common "One"	ITS	*Pichia kluyveri*	KM982973.1	99.75 %
	D1/D2	*Pichia kluyveri*	MN464128.1	99.64 %
Common "Two"	ITS	*Pichia kudriavzevii*	LC389027.1	100 %
	D1/D2	*Pichia kudriavzevii*	MF461295.1	100 %

2.4 Discussion

The overall aim of this chapter was to isolate and identify yeast from the gut of *T. leucotreta* larvae via the PCR amplification and sequencing of the ITS region and LSU (D1/D2 domain) of nuclear rDNA. Navel oranges infested with *T. leucotreta* larvae were collected from three citrus-producing regions across South Africa. The guts of over 30 *T. leucotreta* larvae were screened for yeast, which resulted in the isolation and identification of six yeast species, viz. *M. guilliermondii, H. uvarum, C. lusitaniae, K. marxianus, P. kudriavzevii* and *P. kluyveri*.

Pichia kudriavzevii and *P. kluyveri* were commonly isolated from *T. leucotreta* larvae collected from each region. The remaining four yeast species were unique to *T. leucotreta* larvae from specific regions. Whether these yeast species were localised to a specific region or not has yet to be determined. *Pichia kudriavzevii, P. kluyveri* and *H. uvarum* are widely distributed in the environment and are often associated with fruits, including citrus (Las Heras-Vazquez et al., 2003; Vadkertiová et al., 2012). *Hanseniaspora uvarum* is known to be associated with *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), a major pest of soft summer fruits, and they are thought to have a mutualistic relationship (Bellamy et al., 2013; Hamby et al., 2012; Lee et al.,

2011). The use of *H. uvarum* as a live yeast bait for a more attractive and selective lure against *D. suzukii* has been suggested (Hamby and Becher, 2016). *Kluyveromyces marxianus* in combination with sodium bicarbonate ($NaHCO_3$) has been reported to be an effective biological control agent against *Penicillium digitatum*, a fungus which causes green mould in citrus fruit (Geng et al., 2011). Additionally, *H. uvarum* has also shown potential as a biological control agent against *P. digitatum* (Li et al., 2016). *Clavispora lusitaniae* is an opportunistic yeast which may cause disseminated candidiasis, but isolates have previously been isolated from environmental samples and orange juice concentrate (Deak and Beuchat, 1993; Lachance et al., 2003). *Meyerozyma guilliermondii* has often been associated with fermented foods (Hidalgo et al., 2012; Nova et al., 2009). It has also exhibited potential as a biological control agent against fungi responsible for postharvest spoilage of fruits and vegetables (Lima et al., 2013; Wilson and Chalutz, 1991). The isolated yeast species all belong to the "true yeast" subphylum Saccharomycotina. Generally, yeasts associated with insects are from "true yeast" genera, *viz. Candida, Metschnikowia* and *Pichia* (Urubschurov and Janczyk, 2011).

Conventional yeast identification mainly relied on phenotypic and biochemical methods (Romi et al., 2014). However, these methods are often unreliable and do not accurately differentiate between closely related yeast species (Neppelenbroek et al., 2014; Pincus et al., 2007). Sequencing allows for the rapid and accurate identification of yeast species. The ITS region and large subunit (D1/D2 domain) of rDNA have previously been described as targets for sequencing (Pincus et al., 2007). In particular, the ITS region provides a highly variable sequence allowing for the accurate identification of yeast species (Martin and Rygiewicz, 2005). The ITS 1/4 and NL 1/4 primers sets described in **Table 2.1** were selected as they are often used for fungal metabarcoding and RFLP fingerprinting (Esteve-Zarzoso et al., 1999; Fell, 1993). This allowed for the accurate identification and differentiation of yeast species in this study. The ITS amplicons exhibited extreme variability with sizes ranging from 150 to 750 bp, while the NL amplicons were a consistent 550 bp in size for all the isolates yeast species. The PCR amplification and sequencing of the ITS region and D1/D2 domain of LSU were effective in identifying yeast down to species level.

In conclusion, this chapter describes the successful isolation and identification of six yeast species from the guts of *T. leucotreta* larvae collected from three geographically distinct citrus-producing regions across South Africa. Sequencing of the ITS region and D1/D2 domain of LSU allowed for

the accurate identification of the isolated yeast species. The next chapter seeks to determine if any of the isolated yeast species affect the feeding behaviour of *T. leucotreta* neonates.

Chapter Three

The effect of yeast on the development and behaviour of *Thaumatotibia leucotreta* larvae

3.1 Introduction

Microbial communities play an essential role in insect-plant interactions and can favourably impact their ecology, evolution and behaviour (Barraclough, 2015; Chandler et al., 2011; McFall-Ngai et al., 2013; Murgier et al., 2019; Yun et al., 2014). Microbes affect the host plant's nutritional value, leading to positive, neutral or detrimental effects on the insect's fitness (Grunseich et al., 2020). Microbes ingested and hosted within the insect gut can strongly impact insect survival (Douglas, 2014; Martino et al., 2017), promoting insect fitness by providing nutrition in the form of organic nitrogen, essential amino acids, vitamins and lipids or by defending them against pathogens and natural enemies (Stefanini, 2018). Moreover, the metabolites produced by gut-associated microbes can affect the foraging and oviposition behaviour of insects (Grunseich et al., 2020). The relationship between fungi and insects has been less documented than their bacterial counterparts, despite their importance to insect fitness and behaviour (Stefanini, 2018).

Naturally occurring yeasts play an essential role in the life cycle and biology of numerous insects; they constitute a food source and produce volatile compounds that are attractive to their insect hosts (Ljunggren et al., 2019; Moore et al., 2014; Murgier et al., 2019; Witzgall et al., 2012). The presence, type and amount of yeast within an insect's diet can also affect their development and fitness (Bellutti et al., 2018; Murgier et al., 2019; Spitaler et al., 2020). Yeasts have proven to be an essential nutritional source for insect larval development and influence neonate larval feeding and behaviour (Grunseich et al., 2020). For example, the larval development of *C. pomonella* is strongly affected by *Metschnikowia andauensis* in its diet, which accelerates development and reduces mortality (Witzgall et al., 2012). Multi-cultures of yeasts have been shown to improve insect development (Stefanini, 2018). In Drosophila species, larval diets that contained mixed yeast cultures provide more significant benefits in terms of development speed and survivability compared to single cultures of yeast (Starmer and Aberdeen, 1990). The yeast diet of juvenile insects not only has a direct influence on their fitness and development but also indirectly affects

adult life traits. A limited yeast supply during larval development can negatively affect adult food preference, copulation, fecundity and longevity (Grangeteau et al., 2018; Murgier et al., 2019).

It is reported that insect larvae show a preference for specific yeast species during the early stages of development (Anagnostou et al., 2010; Lewis and Hamby, 2019). Ljunggren et al., (2019) demonstrated in larval feeding assays that *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae), commonly known as cotton leafworm, larvae preferred phyllosphere yeasts over yeasts associated with fruit and frugivorous insects. There is growing support that the vast majority of insects feed and benefit from yeast in their diet. Volatiles produced by yeasts can also elicit a strong response in neonates and adult insects (Ljunggren et al., 2019; Witzgall et al., 2012). In contrast to vision and contact, olfactory cues can be detected at a considerable distance from the source (Bruce et al., 2005; Tasin et al., 2011). Host finding and discrimination have been shown to be mediated by the volatile signatures produced by yeasts for *C. pomonella* (Witzgall et al., 2012).

Thaumatotibia leucotreta larval development in the field is completed between 25 to 35 days in summer and 35 to 67 days in winter (Newton, 1998). Fungi have been shown to influence *T. leucotreta* larval development on artificial diet (Moore et al., 2014). Initially, a maise meal diet was inoculated with a Rhizopus sp. fungus, which was thought to produce metabolites important to *T. leucotreta* larvae (Moore, 2002). However, *T. leucotreta* larvae are currently reared on an artificial diet containing *S. cerevisiae,* as it is a commercially available strain of yeast, and although pupation on the new diet was delayed by five days, this was not considered a problem for commercial purposes (Moore et al., 2014). It has been shown that adult *T. leucotreta* are attracted to both natural and synthetic volatiles (Love et al., 2014); however, no research has been done on neonate larval attraction.

Yeasts species isolated and identified from the digestive tract of *T. leucotreta* larvae were described in the previous chapter. This chapter aimed to investigate the effect that the isolated yeasts, *viz. M. guilliermondii*, *P. kluyveri*, *P. kudriavzevii*, *H. uvarum*, *C. lusitaniae* and *K. marxianus* have on larval development. Additionally, larval choice tests were used to determine the aforementioned yeasts' influence on neonate *T. leucotreta* behaviour and feeding preference.

3.2 Methods and Materials

3.2.1 *Thaumatotibia leucotreta* culture

Eggs and pupae were obtained from the heterogeneous *T. leucotreta* culture, known as "Mixed Colony", held at Rhodes University's Department of Zoology and Entomology, South Africa. Dr Sean Moore established the colony in 1996 using *T. leucotreta* collected from Citrusdal (Western Cape Province), Zebediela (Limpopo Province) and the Eastern Cape (Opoku-Debrah, 2012). The larvae were reared on an artificial diet consisting of maise meal, wheat germ, casein, Brewer's yeast, nipagin, sorbic acid and distilled water (Moore et al., 2014). The rearing protocols described by Moore et al., (2014) were used to maintain the colony.

3.2.2 Collection and storage of Navel oranges

Batches of Navel oranges were collected from orchards in the Sunday's River Valley, in the Eastern Cape Province of South Africa. No postharvest treatments had been applied to the oranges. The collected Navel oranges were stored in a 4 ℃ cold room to preserve the fruit until use. The oranges were checked weekly, with fruit showing any sign of disease or mould being discarded. Oranges were stored for a maximum period of 6 – 8 weeks.

3.2.3 *Thaumatotibia leucotreta* larval development assay

A modified version of the larval development assay described by Witzgall et al., (2012) was used to determine the effect that yeast had on *T. leucotreta* larval development. Seventy-two Navel oranges were removed from cold storage one day prior to being used in the larval development assay. These oranges were inspected for any sign of disease, mould, or damage, with any such fruit being removed, before being thoroughly washed in a 0.5 % bleach solution, rinsed twice in ddH_2O, and allowed to air dry in a controlled environment (CE) room at 27 ℃ overnight.

Single yeast colonies were selected from Pen/Strep YPD agar plates described in Chapter Two, section **2.2.2** and grown in 500 ml conical flasks containing 100 ml of Pen/Strep YPD medium. Yeast cultures were grown for 20 hours at 27 ℃ while shaking (180 rpm). Thereafter, a 1:100 dilution of the yeast culture was made with ddH_2O and counted as described in Chapter Two, section **2.2.3**. Cell counts were adjusted to 2×10^6 cells.ml^{-1} with ddH_2O. Nine sterilised Navel oranges were dipped three times into a specific yeast culture, placed on an egg carton tray and allowed to air dry in a 27 ℃ CE room. *Thaumatotibia leucotreta* eggs were obtained from the

culture and stored as previously described. Five newly hatched *T. leucotreta* neonates were then placed onto each orange using a fine paintbrush. The treated Navel oranges were then maintained in a 25 ℃ CE room for 35 days. Assays were conducted on four dates, with nine Navel oranges per treatment. After 10 days, cotton wool was placed between the Navel oranges to provide emerging 5th instar larvae with a pupation site. The cotton wool was checked daily thereafter, and the number of pupated *T. leucotreta* larvae recorded. After 35 days, each Navel orange was dissected to check for any remaining *T. leucotreta* larvae.

3.2.4 *Thaumatotibia leucotreta* larval feeding assay

A modified version of the assay described by Ljunggren et al., (2019) was used to investigate whether yeasts affect neonate *T. leucotreta* feeding and behaviour. Yeast cultures were prepared as previously described, and cell counts were adjusted to 1.5×10^7 cells.ml^{-1} with ddH_2O. A two-choice bioassay was conducted whereby two 50 µl drops of yeast culture and blank YPD medium were pipetted across from one another, ± 1 cm from the edge of a plastic petri dish. Red and blue colourants (Pioneer Foods (Pty) Ltd, South Africa) were used at 1:25 dilution to distinguish between neonate *T. leucotreta* that fed on yeast medium, blank medium or both for two hours.

Thaumatotibia leucotreta eggs laid on wax paper were collected from the culture described earlier and incubated in a petri dish at 25 ℃ with relative humidity (RH) of ± 30 – 60 % until hatching. Ten starved *T. leucotreta* neonates were collected 24 to 48 hours after hatching and placed in the centre of a petri dish using a fine paintbrush. The petri dish was then covered with a glass lid, to prevent the neonates from escaping, and was left for two hours for the larvae to feed. The colouration of the neonate's gut was then checked under a dissecting microscope. Twenty-two independent replicates with 10 *T. leucotreta* neonates were performed for each yeast.

3.2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.0.0 (GraphPad Holdings, LLC, United States). *Thaumatotibia leucotreta* larval development assays were analysed using a Fisher's exact test (Witzgall et al., 2012). Larval feeding of *T. leucotreta* on yeasts was compared using a Student t-test, with the level of significance set to $P = 0.05$ (Ljunggren et al., 2019).

3.3 Results

3.3.1 Influence of yeast on *Thaumatotibia leucotreta* larval development

Larval development bioassays were conducted using nine Navel oranges per yeast treatment, with five *T. leucotreta* neonates placed onto each orange. Four replicates were performed. Each bioassay was conducted at 27 ℃ with RH of ± 30 – 60 % for 35 days. Cotton wool was placed between the treated Navel oranges after 10 days. The number of pupated *T. leucotreta* larvae in cotton wool was recorded until the bioassay was completed.

It was found that Navel oranges treated with yeast significantly decreased larval development time (**Table 3.1**). *Pichia kluyveri* ($P = 0.0001$), *H. uvarum* ($P = 0.0092$) and *S. cerevisiae* ($P = 0.0024$) performed the best out of all the applied yeast treatments. Although *C. lusitaniae* ($P = 0.002$) also significantly decreased larval development time, the mortality of *T. leucotreta* was high at 76.67 %.

Table 3.1: Pupation rate of *T. leucotreta* larvae for 25 day detached fruit bioassays, where sterilised Navel oranges ($n = 36$) were inoculated with yeast or left untreated. * and ** indicate significant differences from the control according to a Fisher's exact test ($P < 0.05$ and $P < 0.01$, respectively).

Treatments	**Survived**	**Pupated**		**Percentage before 25 days**	**Significance**	***P*-value**
		Before 25 days	After 25 days			
Control (ddH_2O)	41	15	26	37 %		
M. guilliermondii	59	35	24	59 %	*	0.0414
P. kluyveri	78	58	20	74 %	**	0.0001
P. kudriavzevii	58	35	23	60 %	*	0.0253
H. uvarum	63	40	23	63 %	**	0.0092
C. lusitaniae	42	30	12	71 %	**	0.0020
K. marxianus	58	38	20	66 %	**	0.0075
S. cerevisiae	62	42	20	68 %	**	0.0024

Thaumatotibia leucotreta larval mortality was significantly lower on Navel oranges treated with *M. guilliermondii* ($P = 0.0452$), *P. kluyveri* ($P < 0.0001$), *H. uvarum* ($P = 0.0144$) and *S. cerevisiae*

(*P* = 0.0194) (**Figure 3.1**). Three yeast treatments, *viz. C. lusitaniae* (*P* > 0.9999), *P. kudriavzevii* (*P* = 0.0586) and *K. marxianus* (*P* = 0586), did not decrease larval mortality. Overall, *P. kluyveri* performed best, as it decreased larval mortality by 20.55 % and 74.00 % of larvae pupated before 25 days.

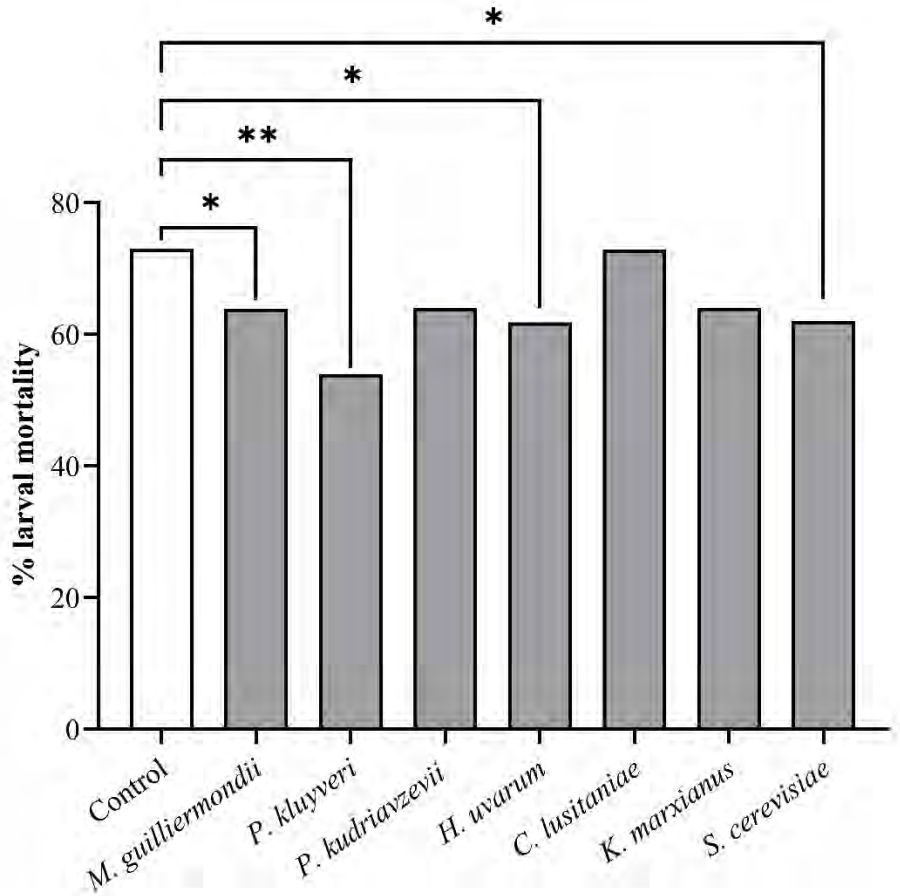

Figure 3.1: *Thaumatotibia leucotreta* larval mortality after 35 days on sterilised Navel oranges (*n* 36), inoculated with yeast or left untreated. * and ** indicate significant differences from the control according to a Fisher's exact test (*P* < 0.05 and *P* < 0.01, respectively).

3.3.2 Feeding preference of neonate *Thaumatotibia leucotreta* larvae

Preliminary tests did not show a bias in neonate *T. leucotreta* attraction to either the red or blue colourants (**Figure 3.2**) (*P* > 0.05; t = 0.2133; df = 27 [paired t-test]).

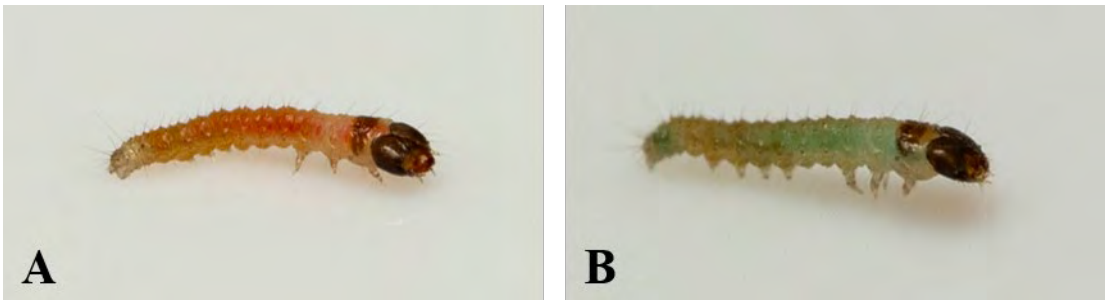

Figure 3.2: Neonate *T. leucotreta* larvae fed on YPD medium, containing either A) red or B) blue colourant. Images captured by David Taylor.

Three yeast species, *M. guilliermondii* (P = 0.0606), *C. lusitaniae* (P = 0.5907) and *S. cerevisiae* (P = 0.2579) had no significant effect on larval attraction and feeding. Neonate *T. leucotreta* behaviour was influenced by four yeasts, *viz. P. kluyveri* (P = 0.0043), *H. uvarum* (P = 0.0037), *P. kudriavzevii* (P = <0.0001) and *K. marxianus* (P = 0.0005) (**Figure 3.3**).

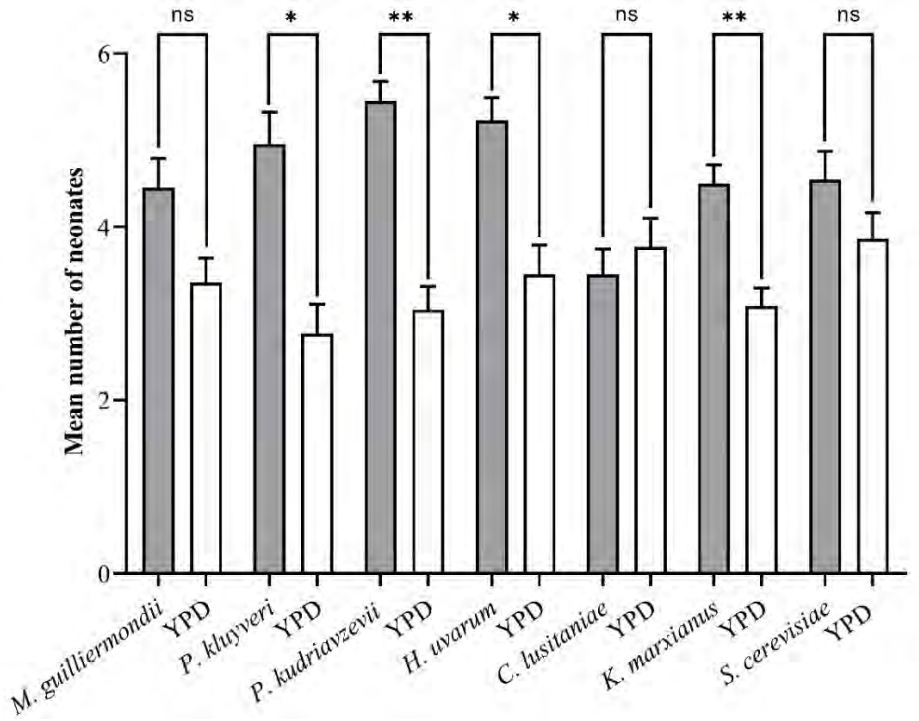

Figure 3.3: Neonate *T. leucotreta* larval attraction and feeding in response to seven yeasts species (n = 22). * and ** indicate significant differences from the control (YPD) according to a paired Student t-test (P < 0.05 and P < 0.01, respectively), ns indicates that differences were not significant.

3.4 Discussion

This chapter's overall aim was to investigate the effect the yeast species identified in **Chapter Two** had on *T. leucotreta* larval development and their influence on neonate feeding behaviour. Additionally, these experiments served as a screening procedure to select the yeast species most favoured by *T. leucotreta* larvae to use in further analyses. It is suspected that combining CrleGV-SA with a yeast preferred by *T. leucotreta* could lead to the improved control of the pest in the field, as it would increase larval ingestion of the virus. Prolonging larval feeding on the fruit surface would increase the larval exposure time to biological control agents such as CrleGV-SA.

An assay was successfully developed to analyse the influence that yeasts have on *T. leucotreta* larval development. This method proved to be effective as larvae pupated freely within the cotton wool, and fungal contamination was minimal. Previous attempts that made use of artificial diet were unsuccessful, due to the high levels of fungal contamination. *Saccharomyces cerevisiae* was included as a treatment, as it forms part of the artificial diet that *T. leucotreta* larvae are reared on (Moore et al., 2014). Additionally, *S. cerevisiae* is formulated into commercially available products. These larval development assays demonstrated that *M. guilliermondii*, *P. kluyveri*, *H. uvarum* and *S. cerevisiae* had a beneficial effect on *T. leucotreta* larvae, by decreasing developmental time and reducing mortality (**Table 3.1** and **Figure 3.1**). *Saccharomyces cerevisiae* has previously been shown to influence the development of *T. leucotreta* larvae (Moore et al., 2014). *Meyerozyma guilliermondii* and *H. uvarum* have been isolated from the intestines of Lepidoptera; however, there is no literature on their influence on larval development (Stefanini, 2018). *Hanseniaspora uvarum* has been proven to be an essential nutritional source for the development of *D. suzukii* larvae (Bellutti et al., 2018). *Pichia kluyveri* has been shown to influence the development and fitness of Carpophilus beetles, but its association and interaction with Lepidoptera has not been documented (Baig et al., 2020).

In this study, instances of detrimental fungal infestations were observed less frequently on Navel oranges treated with yeast than on the control. Fruit infested with *T. leucotreta* often does not rot, or rotting is delayed, particularly when compared to fruit fly infestations (Sean Moore, personal communication). This may indicate that *T. leucotreta* larvae feed on the secondary fungal infections, preventing, or delaying the fruit's decay. A similar observation has been found in *C. pomonella* larval feeding galleries, which rarely become infested with mould. Yeast inoculated

apples have also been found to have decreased mould incidences (Witzgall et al., 2012). The influence of yeasts on neonate *T. leucotreta* feeding and behaviour was analysed using a method described by Ljunggren et al., (2019). This method was effective in distinguishing between neonates that fed on either or both treatments. *Pichia kluyveri, H. uvarum, P. kudriavzevii* and *K. marxianus* were shown to influence neonate *T. leucotreta* feeding and behaviour (**Figure 3.3**).

Larval attraction assays have demonstrated that neonate *T. leucotreta* are attracted to and feed on yeast species isolated from their gut. Little research has been done on the attractive properties of yeast to neonate lepidopteran pests. *Pichia* and *Hanseniaspora* species have been shown to influence *Drosophila* flies and lepidopteran adults (Bellutti et al., 2018; Choi et al., 2011; Palanca et al., 2013). The volatile profiles produced by three *Metschnikowia* species and *Cryptococcus nemorosus* have been shown to influence cotton leafworm larval feeding and behaviour (Ljunggren et al., 2019). The attraction of insects, and particularly their larvae, to feed on yeasts, represents a new avenue that can be exploited for biological control purposes. As this is still a developing area of research, little is known about yeasts influence on neonate lepidopteran pests. The combination of a mutualistic yeast with an insect-specific virus could increase the efficacy of current and future biological control programmes.

In conclusion, a development assay determined that *M. guilliermondii, P. kluyveri, H. uvarum* and *S. cerevisiae* reduce *T. leucotreta* larval mortality and accelerate larval development. Larval feeding assays demonstrated that *P. kluyveri, H. uvarum, P. kudriavzevii* and *K. marxianus* attract neonate *T. leucotreta* for feeding. Although all the aforementioned yeast species show potential for further analysis, *H. uvarum* was the only yeast species shown to be beneficial in both instances. The following chapter will look at which isolated yeast species affects the oviposition preference of adult *T. leucotreta* females in a laboratory setting.

Chapter Four

Evaluating the oviposition preference of adult *Thaumatotibia leucotreta* females for gut-associated yeasts

4.1 Introduction

The two most widely produced citrus cultivars in South Africa are Valencia and Navel oranges (CGA, 2019). The most vulnerable citrus cultivar to *T. leucotreta* damage is Navel oranges, as they are highly susceptible to larval penetration (Love et al., 2014). An IPM programme is currently used to manage *T. leucotreta* infestations; however, chemical insecticides still form an integral part of many management programmes. Finding alternatives to long-standing chemicals is essential to fulfilling the stringent restrictions placed on exported fruit by foreign markets.

An alternative strategy that has been explored for *T. leucotreta* control is to take advantage of a pest's natural preferences for specific plant/microbial volatiles or sex pheromones and combining it with an existing control option, such as a microbial agent (Knight and Witzgall, 2013; Mkiga et al., 2019). The availability of attract and kill control options for *T. leucotreta* are few, with most pheromone-based trapping systems developed to provide a means to monitor male population levels (Hofmeyr and Burger, 1995; Levi-Zada et al., 2020). The selective attract and kill product Last Call™ F.C.M. (Insect Science (Pty)Ltd., Tzaneen, South Africa) is the only such product registered for use against *T. leucotreta* in South Africa (Malan et al., 2018). The product consists of a synthetic sex pheromone combined with permethrin (insecticide) and is incorporated into a transparent gel-like base material. Male *T. leucotreta* are attracted to and may attempt to mate with the droplet, resulting in exposure to a lethal dose of permethrin. However, Last Call™ F.C.M. is most effective when used against low levels of *T. leucotreta* early on in the season or when applied as a follow up to another product, which has already successfully suppressed *T. leucotreta* levels (Malan et al., 2018; Moore, 2019a). Recently, Mkiga et al., (2019) demonstrated that combining the entomopathogenic fungus *Metarhizium anisopliae* with Last Call™ F.C.M. significantly reduced *T. leucotreta* populations in orange orchards. Combining a mutualistic microbe with an existing biological control agent represents an ideal opportunity to develop a novel method for improved control of *T. leucotreta*.

Microorganisms form symbiotic relationships with a broad range of organisms (Margulis et al., 1991; Ruby et al., 2004), and insects are thought to be the largest group in which symbiotic microorganisms are universally found (Bourtzis and Miller, 2003). These symbiotic microorganisms are often harboured within the insect's gut, body cavities, or cells (Kikuchi et al., 2007). Although studies frequently report identifying microorganisms such as bacteria, fungi and protozoa from insect intestines, their importance regarding food acquisition, digestion and host health has only been demonstrated for a few species (Stefanini, 2018). Yeasts often form mutualistic relations with insects and play pivotal roles within their life cycle (Baig et al., 2020; Grangeteau et al., 2018; Ljunggren et al., 2019; Spitaler et al., 2020; Stefanini, 2018). These interactions include aiding in food localisation, contributing to food digestion, or providing essential nutrients, while insects facilitate yeast dispersal and growth on plant substrates (Stefanini, 2018). A chemical dialogue facilitates this interaction, but the volatile chemicals responsible for mediating the insect's response remains primarily unknown (Ljunggren et al., 2019).

Insects utilise sensory cues such as olfaction, contact chemoreception and vision for egg-laying and locating a suitable host (Chapman, 2003; Hilker and Meiners, 2008; Tasin et al., 2011). Female insects rely heavily on olfactory cues to locate and select oviposition sites; additionally, these signals may relay relevant information concerning the site's suitability (Davis et al., 2013). Compared to contact and vision, olfactory cues can be detected at a considerable distance away from the source (Bruce et al., 2005). Plants release volatile compounds in large quantities, but the production of volatiles by bacteria, fungi, and yeasts is thought to be just as prolific (Ljunggren et al., 2019). In addition to plant volatiles, yeast volatiles play a crucial role in the interface between insect herbivores and their food source (Baig et al., 2020; Becher et al., 2012; Davis et al., 2013; Spitaler et al., 2020; Witzgall et al., 2012). Insects possess olfactory receptors specifically for volatiles produced during yeast fermentation, signalling suitable substrates for adult and larval feeding (Arguello et al., 2013; Stensmyr et al., 2003).

Fermenting yeast species quickly dominate sugar-rich sources such as fruits, and in the presence of oxygen, produce ethanol and other volatile compounds (Cordente et al., 2012). Yeasts can also produce short to medium-chain alcohols (fusel alcohols) during the assimilation of plant-based amino acids which can be further oxidised to form organic acids (Günther and Goddard, 2019; Hazelwood et al., 2008). These volatile organic compounds can be converted into more complex

metabolites such as esters (Günther and Goddard, 2019). The olfactory recognition of these secondary metabolites is common in invertebrates feeding on fruit, and there is evidence indicating that they may act as insect attractants or repellents (Becher et al., 2012; Palanca et al., 2013; Scheidler et al., 2015). The formation of volatile esters and fusel-like aldehydes, alcohols and acids is not exclusive to yeasts and is common among fruits (El Hadi et al., 2013).

Volatiles produced during yeast fermentation have been shown to act as signals for larval host plant finding and may serve as signals for adult food source finding (El-Sayed et al., 2005; Ljunggren et al., 2019). Volatiles produced by yeasts are also antifungal and may directly, or indirectly through other plant microbiome members, impact host plant fitness (Droby et al., 2009; Sharma et al., 2009). Yeasts can also modify the composition and concentrations of fruit volatiles, thus producing a different chemical signature (Cordente et al., 2012). Volatiles produced by yeast escape the cell and rapidly diffuse through the air. These volatile compounds facilitate mutualistic interactions, and decoding this chemical dialogue is key to understanding the ecology of insect-yeast interactions (Babcock et al., 2019; Christiaens et al., 2014; Palanca et al., 2013).

This chapter aimed to determine which of the yeast species isolated and identified in **Chapter Two** affected adult *T. leucotreta* female oviposition preference on fruit by conducting laboratory two-choice and multiple-choice tests. Moreover, oviposition preference tests combined with larval attraction assays performed in **Chapter Three** would act as a vetting technique to select the best yeast species to be used in semi-field trials.

4.2 Methods and Materials

4.2.1 Collection and storage of Navel oranges

Batches of Navel oranges were collected from orchards in the Sunday's River Valley, Eastern Cape Province of South Africa. No postharvest treatments were applied to the collected fruit, which was also inspected for *T. leucotreta* eggs, with those containing being discarded. The storage and weekly inspection of Navel oranges remained the same as previously described in Chapter 3, section **3.2.2**.

4.2.2 Sexing *Thaumatotibia leucotreta* pupae

Thaumatotibia leucotreta larvae that had pupated in cotton wool were obtained from the "Mixed Colony" described in Chapter 3, section **3.2.1** and removed using dissecting forceps. Once

removed, *T. leucotreta* pupae were sexed using a dissecting microscope and insect forceps. Male pupae were identified by the presence of four articulated abdominal segments (**Figure 4.1 A**), whilst females only had three (**Figure 4.1 B**). Additionally, male *T. leucotreta* pupae could be identified by a genital aperture that females lack. Thereafter, individual pupae were placed into 25 ml glass vials stoppered with cotton wool, placed in a 25 ℃ CE room with RH of ± 30 – 60 % and allowed to eclose.

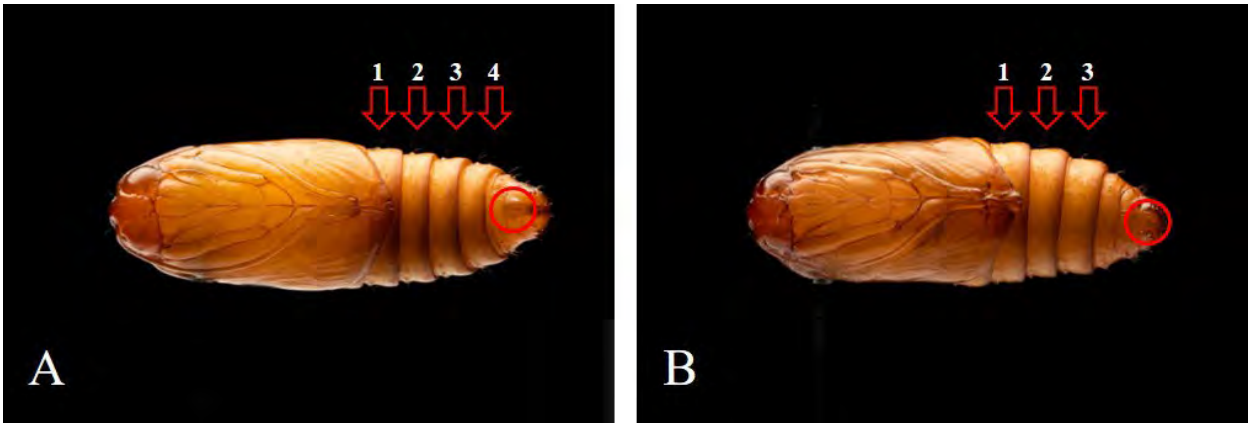

Figure 4.1: Sexing *T. leucotreta* pupae. A) male *T. leucotreta* pupal with four articulated abdominal segments (see arrows) and a genital aperture (see circle). B) female *T. leucotreta* pupal with three articulated abdominal segments (see arrows) and genital slit (see circle). Images captured by David Taylor.

4.2.3 Copulation of *Thaumatotibia leucotreta* adults

Once *T. leucotreta* pupae had eclosed, the adults were sexed to ensure they had been correctly identified when in the pupal stage. *Thaumatotibia leucotreta* adults were identified according to Love et al., (2014); males could be sexed by the presence of an anal tuft and dense tufts of scales on their hind tibia (**Figure 4.2 A**), both of which female moths lack (**Figure 4.2 B**).

Figure 4.2: Sexing *T. leucotreta* adults A) male *T. leucotreta* adult with an anal tuft (see arrow) and dense tufts of scales on the hind tibia (see circle). B) female *T. leucotreta* adult which lacks an anal tuft (see arrow) and dense tufts of scales on the hind tibia (see circle). Images captured by David Taylor.

Within 24 hours of eclosing, a virgin *T. leucotreta* female and male were paired together and allowed to copulate for 48 hours in a 25 ml glass vial, stoppered with cotton wool moistened in sugar water. Paired *T. leucotreta* moths were kept in a 25 ℃ CE room with RH of 30 – 60 % under light conditions before being used in the oviposition preference assay.

4.2.4 Oviposition preference of adult *Thaumatotibia leucotreta* females

A modified version of the ovipositional preference trial described by Love et al., (2014) was used to determine adult *T. leucotreta* females oviposition preference. Trials were divided into two-choice and multiple-choice tests. Ten two-choice tests were conducted per yeast species. Two Navel oranges were placed opposite one another in a container, one yeast inoculated and one control. To determine which yeast species influenced adult *T. leucotreta* female's oviposition preference. Thereafter, fifteen multiple-choice tests were performed using the best performing yeast species from two-choice tests. Multiple choice-tests consisted of 4 Navel oranges, 3 treated vs control. Navel oranges were placed in each corner of the container.

Trials were conducted in a 25 ℃ CE room with RH of 30 – 60 % under a day/night light cycle. Navel oranges were removed from cold storage one day prior to being used in the ovipositional preference trial, checked for *T. leucotreta* eggs before being thoroughly washed in a 0.5 % bleach solution, rinsed twice in ddH_2O, and allowed to air dry in a CE room at 25 ℃ overnight. Single

yeast colonies were selected from Pen/Strep YPD agar plates described in Chapter 2, section **2.2.2** and grown in 500 ml conical flasks containing 100 ml of Pen/Strep YPD medium. Yeast cultures were grown and counted as previously described with cell counts being adjusted to 2×10^6 cells.ml^{-1} with ddH_2O.

Sterilised Navel oranges were dipped three times into a specific treatment and allowed to air dry in a 25 ℃ CE room. Treated Navel oranges were then transferred onto 4 cm tall plastic stands randomly placed in a plastic container (60 × 40 × 40 cm) covered with muslin cloth. A single pair of mated adults were then released into the container, which was then sealed to prevent them from escaping. The mated pair of *T. leucotreta* adults were exposed to the treated Navel oranges for 48 hours. Once the choice test was completed, the Navel oranges were removed, and the number of eggs oviposited carefully counted. Navel oranges were checked under well-lit conditions for eggs and marked with a sharpie.

4.2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.0.0. The two-choice test was analysed using a Student t-test, with a significance level set to $P = 0.05$. The multiple-choice tests data were found not to be normal, and as a result, a nonparametric statistical test was used. A Kruskal–Wallis test was used to determine significance. A multiple comparison of mean ranks post hoc test was used to determine where the significant differences were (Fowler et al., 2013).

4.3 Results

4.3.1 Two-choice oviposition preference test

Ten replicates were performed for each two-choice test, with yeast treatments being applied at a concentration of 2×10^6 cells.ml^{-1}. Ovipositional preference trials were conducted at 25 ℃ with RH of 30 – 60 % under a natural light cycle for a duration of 48 hours. The number of eggs oviposited on Navel oranges was counted.

Significantly more eggs were oviposited on Navel oranges inoculated with *M. guilliermondii* ($P = 0.0090$), *P. kudriavzevii* ($P = 0.0471$) and *H. uvarum* ($P = 0.0013$) compared to the control during the two-choice tests (**Figure 4.3**). *Pichia kluyveri* ($P = 0.3768$), *C. lusitaniae* ($P = 0.5729$), *K. marxianus* ($P = 0.2838$) and *S. cerevisiae* ($P = 0.0517$) did not influence the oviposition preference of adult *T. leucotreta* females.

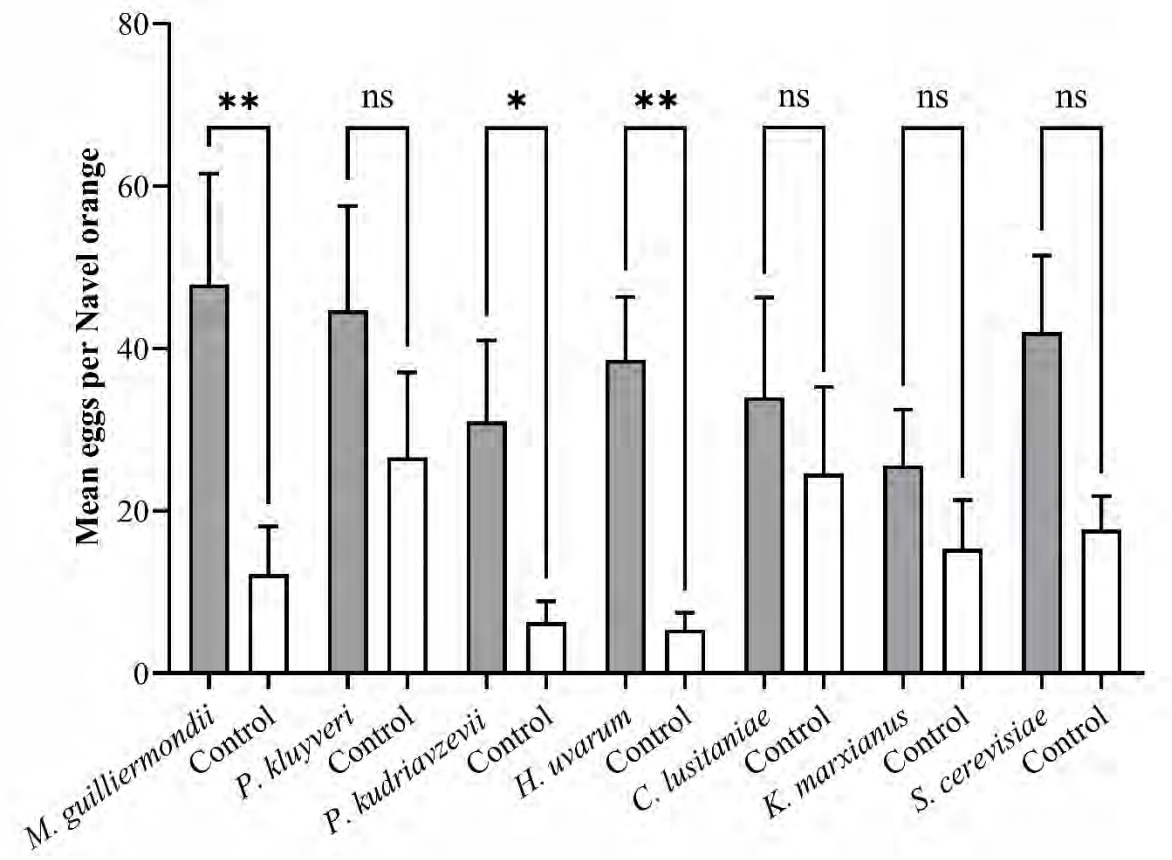

Figure 4.3: Oviposition preference of adult *T. leucotreta* females for different gut-associated yeasts species during two-choice tests (n = 10). * and ** indicate significant differences from the control according to a paired Student t-test ($P < 0.05$ and $P < 0.01$, respectively), ns indicates that differences were not significant.

4.3.2 Multiple-choice oviposition preference test

Meyerozyma guilliermondii, P. kudriavzevii and *H. uvarum* were shown to influence adult *T. leucotreta* female's oviposition preference during two-choice tests. These yeast species were selected for further analysis by multiple-choice tests. Fifteen replicates were performed for the multiple-choice test, with yeast treatments being applied at a concentration of 2×10^6 cells.ml^{-1}. Ovipositional preference trials were conducted and analysed as previously described.

Navel oranges inoculated with *M. guilliermondii, P. kudriavzevii,* and *H. uvarum* did not significantly increase the number of eggs oviposited on them. No significant differences were found between yeast and control treatments during multiple-choice tests (**Figure 4.4**).

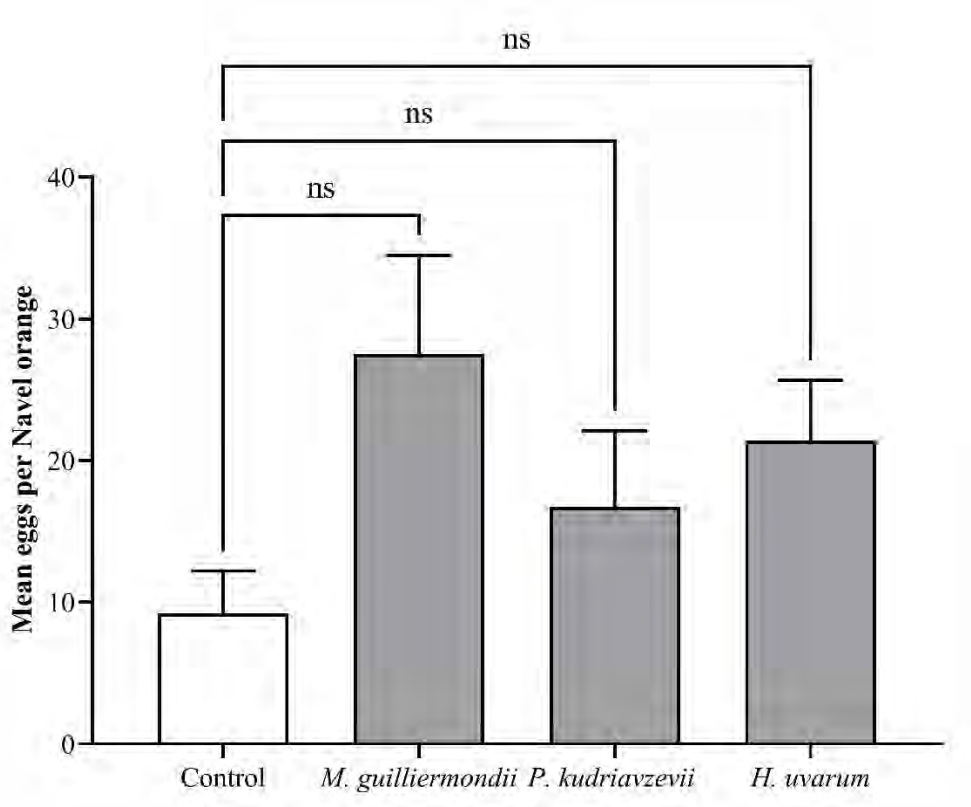

Figure 4.4: The oviposition preference of adult *T. leucotreta* females for Navel oranges inoculated with different treatments during multiple-choice tests (n = 15). A Kruskal–Wallis test was used to determine significance and multiple comparison of mean ranks post hoc test was used to determine where the significant differences occurred, ns indicating not significant.

4.4 Discussion

Initially, the oviposition preference of adult *T. leucotreta* was assessed using a Y-tube bioassay apparatus. A Y-tube olfactometer is commonly used to evaluate the behavioural influences of microbes on insects. However, this proved unsuccessful as adult *T. leucotreta* females remained stationary during the test's duration, most likely due to the Y-tube's small diameter. Moreover, the exclusion of Navel oranges from the assay would be detrimental as yeasts have been shown to alter the chemical signatures of fruit (Cordente et al., 2012). The odour profiles of host plants are comprised of numerous volatile organic compounds (Knudsen et al., 1993). Microorganisms can modify the odour profiles produced by plants and the effect of plant-microorganism interactions on insects has not been extensively studied (Davis et al., 2013). An experimental setup that

incorporated both fruit and yeast in combination was selected to more accurately mimic field conditions.

The oviposition preference of adult *T. leucotreta* females was at first evaluated using a two-choice test. Navel oranges treated with ddH_2O were assessed against those inoculated with yeasts species isolated from the gut of *T. leucotreta* larvae. *Saccharomyces cerevisiae* was also included, as it forms part of the artificial diet on which *T. leucotreta* cultures are reared (Moore et al., 2014). Additionally, *S. cerevisiae* has been shown to attract a wide variety of insects (Meriggi et al., 2020). *Meyerozyma guilliermondii*, *P. kudriavzevii* and *H. uvarum* were identified as having a significant effect on the oviposition preference of adult *T. leucotreta* females during two-choice tests. The behavioural influences of *M. guilliermondii*, *P. kudriavzevii*, and *H. uvarum* on lepidopteran moths has to date, not been well documented. These yeasts are commonly associated with ripe tropical fruits such as oranges (Ganter et al., 2017; Vadkertiová et al., 2012), so their association with *T. leucotreta* is not unexpected. The behavioural influences of these yeasts on other agriculturally important pests, however, has been reported. *Hanseniaspora uvarum* has been shown to affect the ovipositional behaviour of drosophilid flies, acting as both an attractant and repellant (Hamby et al., 2012; Piper et al., 2017; Tasin et al., 2018). The volatiles produced by *P. kudriavzevii* modify the behaviour of both adult *Rhagoletis batava* (Hering) (Diptera: Tephritidae) females and males (Mozūraitis et al., 2020). It is speculated that *M. guilliermondii*, *P. kudriavzevii*, and *H. uvarum* may have similar behavioural influences on adult *T. leucotreta* females. The antennal response of *T. leucotreta* adults to volatiles produced by these yeasts could be assessed using gas chromatography-mass spectrometry (GCMS) combined with electroantennographic detection (GC-EAD) (Lindblom, 2012). The ability to detect minute but possibly behaviourally meaningful responses could be invaluable to understanding how adult *T. leucotreta* females locate a suitable host to oviposit on.

Subsequently, multiple-choice tests were conducted using Navel oranges inoculated with *M. guilliermondii*, *P. kudriavzevii*, *H. uvarum* and ddH_2O. Even though females preferred the treated oranges during the two-choice test, no significant differences were recorded between yeast inoculated fruit and the control when given a choice. Love et al., (2014) demonstrated that *T. leucotreta* prefers specific cultivars of Navel oranges over others, using similarly sized containers to those used in the ovipositional preferences trials. The volatile profiles of *M. guilliermondii*, *P.*

kudriavzevii and *H. uvarum* could overlap, which may explain adult *T. leucotreta* females behaviour during multiple-choice tests. Analysis of the volatile profiles produced by yeast species via GCMS may give further insight into the situation.

Laboratory two-choice and multiple-choice tests may indicate adult *T. leucotreta* female oviposition preference; however, this may differ during field trials. The volatile background produced by fruit in the field would be much higher than in a laboratory setting (Tasin et al., 2018). The *T. leucotreta* colony known as "Mixed Colony", used in ovipositional preference trials, was reared on an artificial diet containing *S. cerevisiae*. During larval development, the diet of insects has been shown to affect their adult life traits, including food preference and host locating (Grangeteau et al., 2018). Field populations of *T. leucotreta* may therefore have slightly different preferences due to their natural diet. Furthermore, during the laboratory trials conducted in this study, the quality of oranges was kept as consistent as possible, but some variation in host quality could have affected the odour profile they produced, which is known to influence insects searching for hosts (Agosta, 2006; Dötterl et al., 2009; Tasin et al., 2012).

The setup and execution of ovipositional preference trials described by Love et al., (2014) was successful, as *T. leucotreta* adults could be observed moving freely. Still, in some instances, adult *T. leucotreta* females would oviposit on the plastic container and muslin cloth instead of the treated Navel oranges. Furthermore, adult *T. leucotreta* would occasionally be observed copulating during the trial's duration, which is undesirable, as this negates or reduces the opportunity to measure oviposition behaviour and preference. Visual confirmation of adult *T. leucotreta* copulating over the 48 hours set aside for mating described in section **4.2.3** would rectify this; however, this is not always possible as *T. leucotreta* adults are nocturnal (Zagatti and Castel, 1987). Generally, adult *T. leucotreta* females copulate shortly after eclosing and only begin to oviposit after two days, reaching their peak at 3 to 5 days of age (Stotter, 2009), making the 48 hour copulation period sufficient.

Meyerozyma guilliermondii, *P. kudriavzevii* and *H. uvarum* were shown to influence adult *T. leucotreta* female's oviposition preference. Although individually specific yeast species did impact female behaviour, multiple-choice tests did not mimic these results. The following chapter will look at combining the aforementioned yeasts with CrleGV-SA in a laboratory setting to

determine whether a yeast-virus mixture is more effective in controlling *T. leucotreta* larvae than CrleGV-SA on its own.

Chapter Five

Evaluating the effectiveness of combining CrleGV-SA with yeast against *Thaumatotibia leucotreta*

5.1 Introduction

It has previously been demonstrated that the volatiles produced by yeasts species isolated from the gut of lepidopteran larvae contribute towards host finding behaviour in their neonates and adult females (Ljunggren et al., 2019; Witzgall et al., 2012). Therefore, it is conceivable that neonates and adult females detect the same volatiles to feed and oviposit, respectively. Knight and Witzgall (2013) developed a novel technique for controlling *C. pomonella*, whereby the mutualistic yeasts associated with the pest were combined with the granulovirus CpGV. Adult *C. pomonella* females would oviposit on fruit inoculated with *Metschnikowia pulcherrima* (Witzgall et al., 2012). Emerging neonates would then feed on *M. pulcherrima* covered with CpGV before burrowing into the fruit, increasing their exposure time and enhancing virus uptake (Knight and Witzgall, 2013). Additionally, the inclusion of *S. cerevisiae* plus brown cane sugar to CpGV also significantly enhanced its efficiency and reduced the number of fruit damaged by the pest (Knight et al., 2015).

The baculovirus CrleGV-SA is an effective biological control agent against *T. leucotreta* (Moore, 2021). The lethal concentration (LC) and the lethal time (LT) of CrleGV-SA against *T. leucotreta* have been well established through laboratory assays and field trials (Moore, 2002; Moore et al., 2011, 2015b; Opoku-Debrah et al., 2016). These values represent the concentration of virus required to achieve 50 and 90 % mortality (LC) and the time required to kill 50 % and 90 % (LT) of insects in a sample (Grzywacz et al., 2004). The LC_{50} and LC_{90} concentrations for CrleGV-SA during detached fruit bioassays were established to be 9.31×10^7 OBs.ml^{-1} and 1.515×10^9 OBs.ml^{-1}, respectively. The LT_{50} and LT_{90} were determined to be 4 days 22 h and 7 days 8 h with an LC_{90} dose (Moore et al., 2011). The discovery of resistant *C. pomonella* populations to CpGV prompted bioprospecting for additional CrleGV-SA isolates for potential use, should a similar situation arise within *T. leucotreta* (Eberle and Jehle, 2006; Opoku-Debrah et al., 2013). Bioassays using the newly identified isolates indicated that some isolates are more virulent to certain host populations than others (Opoku-Debrah et al., 2016), and the continued evaluation of novel isolates

and their development into new biopesticide formulations could lead to the improved control of *T. leucotreta* in South Africa.

Laboratory bioassays are crucial to developing new or improved biological control options. To obtain results in the laboratory representing what may be seen in the field, certain variables need to be controlled. The life stage used during laboratory assays should be the same stage targeted in the field. Neonate *T. leucotreta* larvae are the primary target of CrleGV-SA, leaving a small window of opportunity where they can be targeted before they penetrate the fruit. The application of treatments is also important and should mimic those used in the field. Virus formulations are typically applied using a spray system in the field; thus, surface inoculated bioassays are ideal for laboratory assays (Moore, 2002; Moore et al., 2015b; Opoku-Debrah et al., 2016). Detached fruit bioassays are more representative of field conditions than other assays, as there are often disparities between laboratory assays and field trials (Moore et al., 2011). The application period of baculoviruses in the field is also important due to their susceptibility to UV radiation (Petrik et al., 2003). Therefore, treatments should be applied late afternoon or after sunset to ensure the greatest possible number of viable OBs encounter the target pest before UV inactivation begins (Moore et al., 2015b). Additionally, it has been shown that OBs are more susceptible to UV inactivation when still wet, as UV can refract onto the OB within the droplet. In contrast, when dried, certain formulation ingredients will provide more protection (Grzywacz and Moore, 2017). The addition of adjuvants and surfactants such as molasses and BREAK-THRU® S 240 (Evonik Industries AG, Germany) to CrleGV-SA formulations have been shown to significantly improve the viruses' efficacy compared to when applied alone (Moore et al., 2015b). The addition of mutualistic microorganisms is also an important consideration that must be made to improve the efficacy of CrleGV-SA in the field.

This chapter aimed to evaluate the efficacy of combining gut-associated yeasts isolated from *T. leucotreta* larvae with CrleGV-SA through a series of detached fruit bioassays and semi-field trials. Detached fruit bioassays were used to determine the optimal yeast:virus ratio, test all isolated yeasts in combination with CrleGV-SA, and to further enhance yeast/virus formulation through the addition of an adjuvant and surfactant. Lastly, semi-field trials would be conducted using the best-performing yeast species isolated from gut *T. leucotreta* larvae. Additionally, *S. cerevisiae* would be included in the semi-field trials due to its commercial availability.

5.2 Methods and Materials

5.2.1 Purification of CrleGV-SA OBs from larval cadavers

A modified version of the 30 – 80 % (v/v) glycerol gradient purification protocol described by Moore (2002) was used to purify CrleGV-SA OBs from *T. leucotreta* larval cadavers infected with CrleGV-SA, which were obtained from Citrus Research International (Port Elizabeth, South Africa). A sterile pestle and mortar were used to macerate 1 g of frozen larval cadavers in 6 ml of 0.1 % (w/v) sodium dodecyl sulphate (SDS). The larval homogenate was then filtered through muslin cloth and sonicated at 60 Hz for 4 × 15 second intervals using a Vibra Cell™ sonicator (Sonics & Materials Inc, USA). The filtrate was then transferred into two JA–20 centrifuge tubes, filled with ddH_2O and centrifuged at 7 840 ×g for 30 minutes at 4 ℃ in a Beckman Coulter Avanti® J–E centrifuge (Beckman Coulter Inc, USA). The supernatant was discarded, and the pellets resuspended in ddH_2O and the centrifugation step repeated.

30 – 80 % glycerol gradients were prepared in two SW–28 ultracentrifuge tubes by layering glycerol solutions from most dense to least dense in 10 % increments and stored at 4 ℃. The pellets from the previous centrifugation step were resuspended in 1.5 ml of ddH_2O and transferred to glycerol gradients. The gradients were then centrifuged at 27 783 ×g for 15 minutes at 4 ℃ in a Beckman Coulter Optima™ L–90 K ultracentrifuge. Occlusion bodies form a whitish band at the gradient's centre were collected and transferred into two JA–20 centrifuge tubes. These tubes were then filled with ddH_2O and centrifuged at 7 840 ×g for 30 minutes at 4 ℃. The resulting pellets were resuspended in ddH_2O and combined into one JA–20 centrifuge tube before being centrifuged at 7 840 ×g for 30 minutes at 4 ℃. The resulting pellet was resuspended in ddH_2O, and the centrifugation step was repeated. Finally, the pellet was resuspended in 750 µl of ddH_2O and stored at -20 ℃.

5.2.2 CrleGV-SA genomic DNA extraction

The CTAB DNA extraction protocol described by Opoku-Debrah et al., (2013) was used to extract gDNA from purified CrleGV-SA OBs. In a 1.5 ml centrifuge tube, 200 µl of purified OBs was placed along with 90 µl of sodium carbonate (Na_2CO_3) (1 M) and incubated at 37 ℃ for 30 minutes. Next, 120 µl of Tris-HCl (1 M, pH 6.8), 90 µl of SDS (10 % w/v) and 50 µl of Proteinase K (25 mg/ml) (Sigma-Aldrich, USA) were added and further incubated at 37 ℃ for 30 minutes. Thereafter, 10 µl of RNAse A (10 mg/ml) was added and once again incubated at 37 ℃ for 30

minutes. The sample was then centrifuged at 12 100 ×g for 3 minutes at room temperature in a Super Mini Centrifuge (Hangzhou Allsheng Instruments Co., Ltd, China). The supernatant was transferred into a 2 ml centrifuge tube that contained 400 µl of CTAB buffer (54 mM CTAB, 0.1 M Tris-HCl pH 8.0, 20 mM Na_2EDTA, 1.4 M NaCl) that had been preheated to 70 ℃. The sample was then incubated at 70 ℃ for 45 minutes and inverted regularly. Thereafter, 400 µl of chloroform cooled to 4 ℃ was added to the sample, inverted several times, and centrifuged at 6 700 ×g for 10 minutes. The supernatant's upper aqueous phase was then collected and transferred into a new 1.5 ml tube containing 400 µl of ice-cold isopropanol (-25 ℃). The sample was then stored overnight at -25 ℃.

The sample was centrifuged at 12 100 ×g for 20 minutes the following morning, and the resulting supernatant was discarded. Thereafter, 1 ml of ice-cold ethanol (70 % v/v, -25 ℃) was added to the sample and centrifuged at 12 100 ×g for 5 minutes. The supernatant was discarded, and the pellet was allowed to air-dry at 37 ℃ to ensure that all the ethanol had evaporated. Once the pellet had dried, it was resuspended in 20 µl of ddH_2O for 30 minutes at room temperature and stored at -25 ℃. The gDNA concentration was determined using a NanoDrop 2000 spectrophotometer and was visualised on a 0.8 % agarose gel electrophoresis run at 90 V for 45 minutes in 1× TAE buffer stained with ethidium bromide. The gel image was captured as previously described in Chapter Two, section **2.2.3**.

5.2.3 PCR amplification of CrleGV-SA *gp41* gene

To ensure that CrleGV-SA OBs were obtained, a PCR amplification of the *gp41* gene was conducted. The gene was amplified using the CrleGV-SA specific oligonucleotides described in **Table 5.1** (Jukes, 2018). The reaction mixture contained 1 µl of the forward oligonucleotide (10 µM), 1 µl of the reverse oligonucleotide (10 µM), 12.5 µl of Taq DNA Polymerase 2× Master Mix (Ampliqon, Denmark) and 25 ng of gDNA. Reactions were made up to 25 µl with ddH_2O, and an NTC was also prepared. Amplification was carried out using a SimpliAmp™ Thermal Cycler under the following conditions: an initial denaturation cycle of 95 ℃ for 3 minutes followed by 30 cycles of 95 ℃ for 30 seconds, 55 ℃ for 30 seconds, and 72 ℃ for 30 seconds. A final elongation cycle of 72 ℃ for 1 minute was used (Jukes, 2018).

Table 5.1: The oligonucleotides used to amplify the *gp41* gene of CrleGV-SA (Jukes, 2018).

Target	Name	Sequence (5' – 3')	Amplicon
gp41 gene	ClGV-gp41F	TAGGGCCAACAATACAGCCA	197 bp
	ClGV-gp41R	CATGGGCCGATTCGTTGATT	

The amplicon was visualised on a 1.2 % agarose gel stained with ethidium bromide run at 90 V for 45 minutes in 1× TAE buffer. Gel images were captured, as mentioned previously.

5.2.4 Enumeration of CrleGV-SA OBs

Purified CrleGV-SA OBs were enumerated using a haemocytometer and dark field microscopy to determine the virus concentration. A 1:5 dilution of the purified OB suspension was prepared by mixing 20 µl of the virus with 80 µl of ddH_2O, which was homogenised by vortexing for 15 seconds. An additional 1:5 dilution of this suspension was made by mixing 400 µl of 0.07 % SDS (w/v) with 100 µl of the virus suspension resulting in a 1:25 dilution. The virus suspension was then sonicated at 60 Hz for 4 × 15 second intervals. A final 1:2000 dilution was prepared by mixing 10 µl of the sonicated virus suspension with 790 µl of ddH_2O and homogenised by vortexing for 15 seconds. A Neubauer-improved counting chamber with a depth of 0.1 mm was used to enumerate the virus. The counting chamber was cleaned with 70 % ethanol solution (v/v) between uses. Via capillary action, 5 µl of the 1:2000 virus suspension was loaded on to the counting chamber. Occlusion bodies were allowed to settle for 5 minutes before counting commenced. The counting chamber was viewed under a compound darkfield microscope, using phase-contrast at ×400 magnification (Taverner and Connor, 1992; van Beek and Davis, 2007). The number of OBs were counted as described in Chapter Two, section **2.2.3** and the concentration of purified CrleGV-SA OBs was calculated using **Equation 2.1** as previously described.

5.2.5 The collection and storage of Navel oranges

Batches of 150 Navel oranges were collected from orchards in the Sunday's River Valley, in the Eastern Cape Province of South Africa. The collected fruit had undergone no post-harvest treatments, and Cryptogran™ was the only preharvest spray applied. Navel oranges were inspected for signs of *T. leucotreta* infestation, with those showing symptoms being discarded. The collected fruit was stored as previously described in Chapter Three, section **3.2.2**.

5.2.6 *Thaumatotibia leucotreta* neonates

Thaumatotibia leucotreta eggs were obtained from the same "Mixed Colony" culture described in Chapter 3, section **3.2.1**. Eggs were stored in petri dishes sealed with parafilm in a 25 ℃ CE room with RH of 30 – 60 %. Once the eggs had turned dark brown, a piece of cotton wool moistened with ddH_2O was placed in the petri dish to ensure that emerging *T. leucotreta* neonates did not dehydrate.

5.2.7 Detached fruit bioassays.

Three sets of detached fruit bioassays were conducted to determine i) The optimal yeast concentration to use alongside CrleGV-SA; ii) Assess the effectiveness of combining each of the isolated yeasts with CrleGV-SA and iii) To further enhance the efficacy of the yeast/virus mixture through the addition of adjuvants such as molasses and BREAK-THRU® S 240.

All yeast treatments included CrleGV-SA at LC_{50} concentration of 9.31×10^7 OBs.ml^{-1}. The first set of bioassay treatments included ddH_2O alone, CrleGV-SA alone at 9.31×10^7 OBs.ml^{-1}, *P. kudriavzevii* at 2×10^8 cells.ml^{-1} plus CrleGV-SA, *P. kudriavzevii* at 2×10^6 cells.ml^{-1} plus CrleGV-SA and *P. kudriavzevii* at 2×10^4 cells.ml^{-1} plus CrleGV-SA. The second set of bioassay treatments included ddH_2O alone, CrleGV-SA alone and the remaining isolated yeasts at 2×10^6 cells.ml^{-1} plus CrleGV-SA. Finally, the third set of bioassay treatments included ddH_2O alone, CrleGV-SA, *P. kudriavzevii* and *S. cerevisiae* with molasses and BREAK-THRU® S 240 at 0.25 % and 0.005 %, respectively.

A modified version of the detached fruit bioassay described by Moore et al., (2011) was used to determine the effectiveness of combining CrleGV-SA with yeast against *T. leucotreta*. Navel oranges were removed from 4 ℃ cold storage one day prior to being used in a detached fruit bioassay. They were inspected for any sign of disease or mould, before being thoroughly washed in a 0.5 % bleach solution (v/v), rinsed twice in ddH_2O, and allowed to air dry in a 25 ℃ CE room overnight. The following morning, Navel oranges were placed onto a sterile metal rack and sprayed with a specific treatment until thoroughly covered. The treated oranges were then placed onto cardboard egg cartons, transported to a 25 ℃ CE room and allowed to dry for 30 – 45 minutes. Once dried, five *T. leucotreta* neonates were then added to each fruit using a fine paintbrush (size 000). A total of 30 Navel oranges were used per treatment, with each treatment replicated three

times. Detached fruit bioassays would run for 14 days after which Navel oranges were dissected with a serrated knife and inspected for the presence of live *T. leucotreta* larvae.

5.2.8 Yeast preparation for semi-field trials

Yeast colonies were selected from YPD agar plates described in Chapter Two, section **2.2.2** and grown up in 2 L of YPD medium containing 40 units/mL of Pen and 40 µg/mL of Strep for 72 hours at 25 ℃ while shaking. A 1:100 dilution of the yeast culture was then made with ddH_2O and counted as described in Chapter Two, section **2.2.3**. Yeast cultures were then transferred into two 1 L Duran® glass bottles and stored overnight at 4 ℃ before being transported to the field.

5.2.9 Semi-field trials

Semi-field trials were conducted in citrus orchards with a relatively low *T. leucotreta* presence on two separate occasions: the 7th of May 2020 and the 1st of July 2020. The first trial was conducted in an orchard at Pennyholme Farm (33°29’51.48 “S 25°40’25.18 “E) and the second at Tregoss Skinner Farm (33°25’19.06 “S 25°27’8.86 “E) in the Sunday’s River Valley, in the Eastern Cape Province of South Africa. All treatments were applied after sundown to avoid any immediate inactivation of biological agents due to UV radiation exposure. Molasses and BREAK-THRU® S 240 were used with most treatments at concentrations of 0.25 % and 0.005 %, respectively, per 100 L of water (**Table 5.2**).

Table 5.2: Treatments applied at the Pennyholme and Tregoss Skinner trial sites. Cryptogran™ was used at 5×10^{10} OBs.ml^{-1}, *P. kudriavzevii* and *S. cerevisiae* were applied at 2×10^{6} cells.ml^{-1}.

Trial sites	Treatments	Volume/per 100 L	Mean L/tree
Pennyholme and Tregoss Skinner farms	Cryptogran™*	10 ml	14.5
	Cryptogran™**	10 ml	
	Cryptogran™ + *S. cerevisiae**	10 ml + 2 L	
	Cryptogran™ + *S. cerevisiae***	10 ml + 2 L	
	Cryptogran™ + *P. kudriavzevii**	10 ml + 2 L	
	Cryptogran™ + *P. kudriavzevii***	10 ml + 2 L	

* and ** indicate treatments that included BREAK-THRU® S 240 and molasses plus BREAK-THRU® S 240, respectively

Treatments were prepared in a Janisch spray machine and applied as medium cover film sprays (until the point of run-off) using handheld spray guns with 2 mm nozzles at a pressure of 20 Bar (**Figure 5.1**). Eight to 10 adjacent trees were sprayed per treatment, and three unsprayed buffer trees were incorporated between each treatment.

Figure 5.1: Conducting semi-field trials in Sunday's River Valley A) The addition of treatments to Janisch spray machine. B) Application of treatments onto citrus trees using pressurised handheld spray guns. Images captured by David Taylor.

Thirty oranges were collected from both the northern and southern sides of trees to establish whether different sunlight exposure affects efficacy. Oranges were collected 1 day and 1, 2 and 4 weeks after application. Each orange was inoculated with four *T. leucotreta* neonates in the laboratory and maintained in a 25 ℃ CE room for 14 days. After 14 days oranges were dissected and inspected for live *T. leucotreta* larvae.

5.2.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.0.0. The number of dead larvae obtained from each detached fruit bioassays was summarised into a proportion. These proportions were transformed to arcsine (square root) prior to analysis of variance (ANOVA). Data were analysed with the Shapiro – Wilks test of normality. Data that could not be normalised with this transformation were analysed with the Kruskal-Wallis nonparametric test. Means were deemed to be significantly different at $P < 0.05$, Tukey's test.

5.3 Results

5.3.1 PCR amplification of CrleGV-SA *gp41* gene

Occlusion bodies were purified in three batches from *T. leucotreta* larval cadavers received from Citrus Research International (CRI) using a 30 – 80 % glycerol gradient. Genomic DNA was extracted from these OBs using the CTAB DNA extraction protocol and examined by PCR using the oligonucleotides *gp41*.

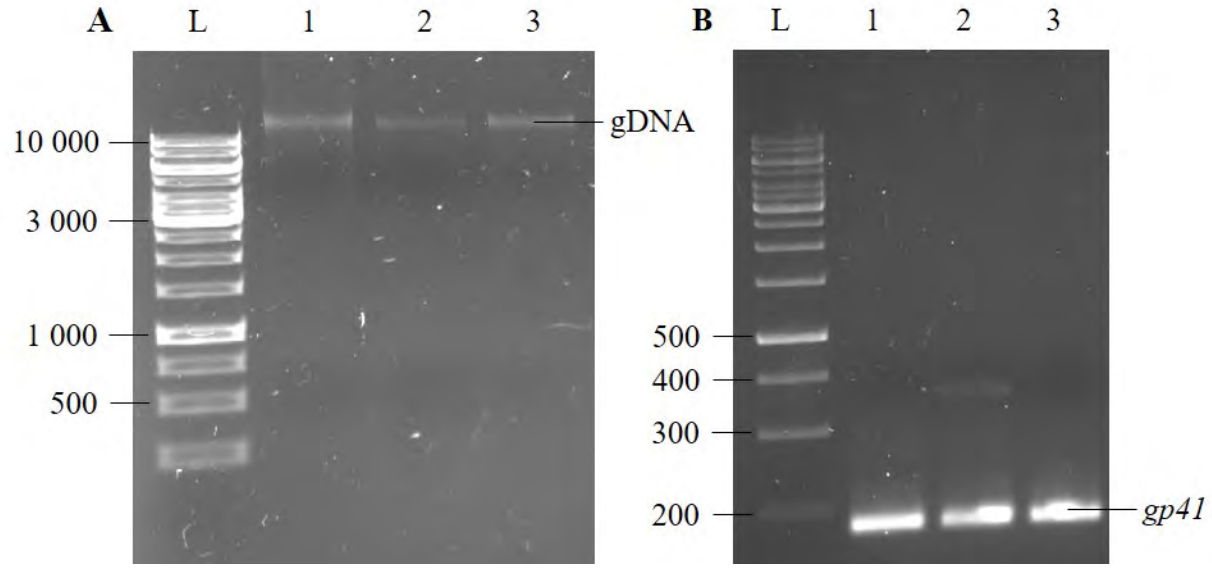

Figure 5.2: AGE images of A) gDNA extracts from *T. leucotreta* larval cadavers. Lane L – GeneRuler 1 kb DNA Ladder (Thermo Scientific, USA), Lane 1 – *T. leucotreta* homogenate 2018, Lane 2 – *T. leucotreta* homogenate 2019, Lane 3 – *T. leucotreta* homogenate 2020. B) PCR analysis of extracted gDNA. Lane L – GeneRuler 1 kb Plus DNA Ladder, Lane 1 – *T. leucotreta* homogenate 2018, Lane 2 – *T. leucotreta* homogenate 2019, Lane 3 – *T. leucotreta* homogenate 2020.

The presence of a single band, with an estimated size of ±190 bp (**Figure 5.2**), indicates that CrleGV-SA OBs were obtained from the batch of macerated larval cadavers, as the amplicon produced corresponded with the expected size of *gp41* gene referred to in **Table 5.1**.

5.3.2 Enumeration of CrleGV-SA OBs

Purified CrleGV-SA OBs were enumerated using a darkfield microscope under phase contrast with a Neubauer-improved counting chamber, and the concentration calculated using **Equation 2.1** (**Table 5.3**).

Table 5.3: Concentration of CrleGV-SA OBs extracted from *T. leucotreta* larval cadavers.

Batch	Mean number of OBs counted	Concentration
T. leucotreta homogenate 2018	123	1.23×10^{10} OBs.ml^{-1}
T. leucotreta homogenate 2019	370	3.7×10^{10} OBs.ml^{-1}
T. leucotreta homogenate 2020	340	3.4×10^{10} OBs.ml^{-1}

5.3.3 Detached fruit bioassays.

5.3.3.1 Determining the optimal yeast:virus ratio

The first set of detached fruit bioassays conducted were to determine the optimal yeast concentration to use alongside CrleGV-SA. *Pichia kudriavzevii* was selected as the yeast isolate to use during these bioassays due to its attractiveness to *T. leucotreta* neonates and adult females, demonstrated in **Chapter Three** and **Chapter Four**. No significant difference was recorded between CrleGV-SA and P. kudriavzevii applied at 2×10^8 cells.ml^{-1} ($P = 0.6372$). A significant difference was, however, recorded between *P. kudriavzevii* applied at 2×10^6 cells.ml^{-1} ($P = 0.0442$) and 2×10^4 cells.ml^{-1} ($P = 0.0415$), when compared to CrleGV-SA alone (**Figure 5.3**).

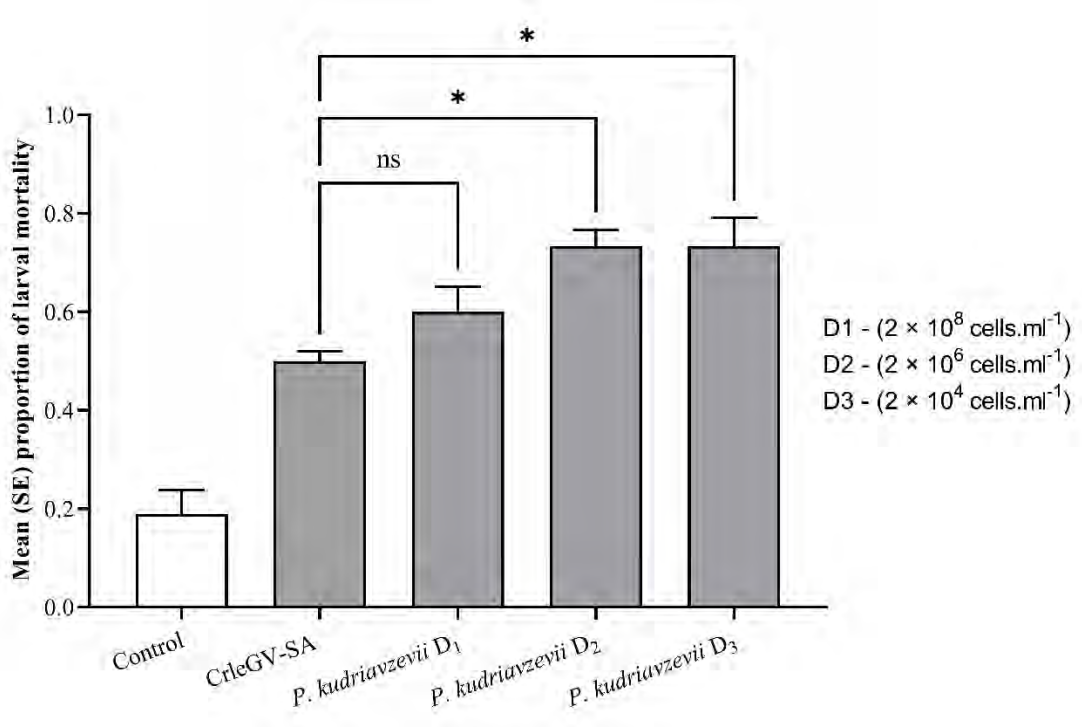

Figure 5.3: *Thaumatotibia leucotreta* larval mortality in 14-day detached fruit bioassays (n = 3) on Navel oranges treated with ddH_2O, CrleGV-SA at 9.31 × 10^7 OBs.ml^{-1}and *P. kudriavzevii* at varying concentrations ranging from 2 × 10^8 to 2 × 10^4 cells.ml^{-1} plus CrleGV-SA. * indicates that differences were significant, and ns indicates that differences were not significant, according to an analysis of variance (ANOVA) using Tukey's test ($P < 0.05$).

5.3.3.2 Combining yeasts with CrleGV-SA

The second set of detached fruit bioassays evaluated the efficacy of combining *M. guilliermondii*, *H. uvarum, K. marxianus, P. kluyveri* and *S. cerevisiae* with CrleGV-SA. Yeasts were applied at a concentration of 2 × 10^6 cells.ml^{-1} with CrleGV-SA at 9.31 × 10^7 OBs.ml^{-1}. A significant difference was recorded between Navel oranges treated with *P. kluyveri* (P = 0.0012), *K. marxianus* (P = 0.0005) and *S. cerevisiae* (P = 0.0041) in combination with CrleGV-SA compared to CrleGV-SA alone (**Figure 5.4**). *Meyerozyma* (P = 0.2784) and *H. uvarum* (P = 0.1443) did not enhance the efficacy of CrleGV when applied in combination.

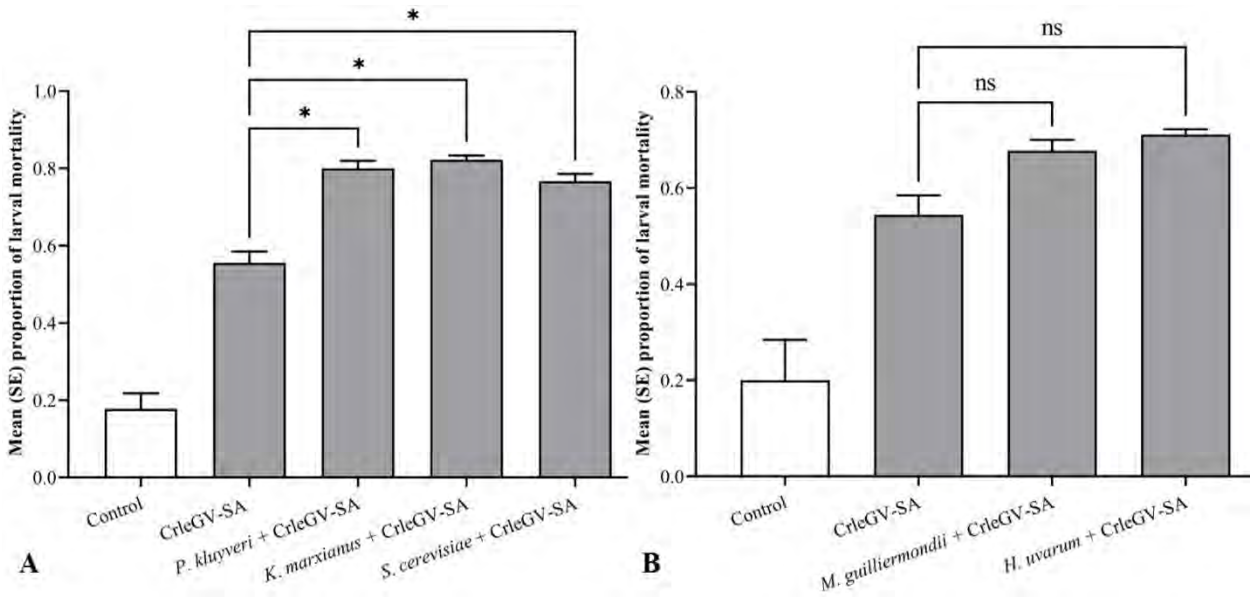

Figure 5.4: Larval mortality of *T. leucotreta* in 14-day detached fruit bioassays (n = 3). A) Navel oranges treated with *P. kluyveri, K. marxianus* and *S. cerevisiae* combined with CrleGV-SA. B) Navel oranges treated with *M. guilliermondii* and *H. uvarum* combined with CrleGV-SA. * indicates that differences were significant, and ns indicates that differences were not significant, according to an analysis of variance (ANOVA) using Tukey's test ($P < 0.01$, respectively).

5.3.3.3 Enhancing the efficacy of yeast:virus mixtures

Lastly, detached fruit bioassays were conducted to further increase the yeast/virus mixture's efficacy by adding molasses and BREAK-THRU® S 240. *Pichia kudriavzevii* was selected as it was shown to influence *T. leucotreta* neonates and adult females' behaviour. Additionally, *S. cerevisiae* was included, as it is a commercially available yeast strain. The addition of molasses and BREAK-THRU® S 240 significantly enhanced the efficacy of *P. kudriavzevii* (P = 0.0195) and *S. cerevisiae* (P = 0.0418) formulations compared to CrleGV-SA (**Figure 5.5**). *Pichia kudriavzevii* and *S. cerevisiae's* larval mortality increased by 12.71 % and 4.71 %, respectively, compared to treatments that excluded molasses and BREAK-THRU® S 240.

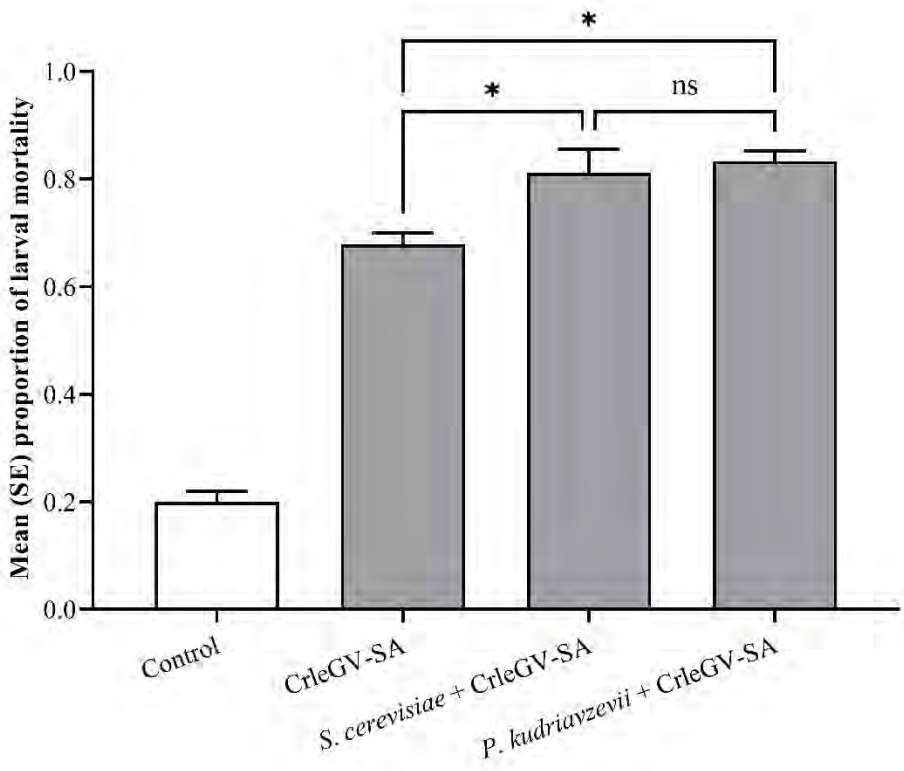

Figure 5.5: *Thaumatotibia leucotreta* larval mortality in 14-day detached fruit bioassays (n = 3) on Navel oranges treated with ddH_2O, CrleGV-SA at 9.31 × 10^7 OBs.ml^{-1}, *P. kudriavzevii* and *S. cerevisiae* at 2 × 10^6 cells.ml^{-1} plus CrleGV-SA. Except for the control, all treatments had the addition of molasses and BREAK-THRU® S 240 at 0.25 % and 0.005 %, respectively. * indicates that differences were significant, and ns indicates that differences were not significant, according to an analysis of variance (ANOVA) using Tukey's test ($P < 0.05$).

5.3.4 Analysis of semi-field trials

No significant differences (data not shown) were recorded between fruit collect from the North and South sides regarding the number of live larvae found, and thus data collected from each side were pooled (**Table 5.4**). The number of live *T. leucotreta* larvae found during the second semi-field trial was considerably higher than that of the first.

Table 5.4: Number of live *T. leucotreta* larvae found within the 60 Navel oranges collected from Pennyholme Farm (PF) and Tregoss Skinner Farm (TF) trial sites.

	PF	TF	PF	TF	PF	TF	PF	TF	PF	TF
Treatments	**Day 1**		**Week 1**		**Week 2**		**Week 4**		**% Reduction**	
Untreated control	16	28	16	89	8	86	26	62	0.00	0.00
Cryptogran™*	10	13	7	41	1	82	7	43	62.12	32.45
Cryptogran™**	11	24	11	71	11	64	13	69	30.30	13.96
Cryptogran™ + *S. cerevisiae**	12	8	13	53	5	62	15	49	31.82	35.09
Cryptogran™ + *S. cerevisiae***	6	5	7	46	6	55	9	36	57.58	46.42
Cryptogran™ + *P. kudriavzevii**	19	12	10	35	7	44	17	47	19.70	47.92
Cryptogran™ + *P. kudriavzevii***	19	4	14	38	6	45	15	47	18.18	49.43

* and ** indicate treatments that included BREAK-THRU® S 240 and molasses plus BREAK-THRU® S 240, respectively.

Cryptogran™ plus *P. kudriavzevii* with BREAK-THRU® S 240 and Cryptogran™ plus *P. kudriavzevii* with molasses and BREAK-THRU® S 240 were the least effective treatments during the first semi-field trial. They only reduced *T. leucotreta* larvae relative to the untreated control by 19.70 % and 18.18 %, respectively (**Table 5.4**). Cryptogran™ plus BREAK-THRU® S 240 saw an unusually high efficacy, outperforming Cryptogran™ plus molasses and BREAK-THRU® S 240, which is known to be the better formulation (Moore et al., 2015b). Cryptogran™ plus *S. cerevisiae* with BREAK-THRU® S 240 and Cryptogran™ plus *S. cerevisiae* with molasses and BREAK-THRU® S 240 performed as expected, with the addition of molasses plus BREAK-THRU® S 240 to the formulation drastically increasing its efficacy and outperforming Cryptogran™ plus molasses and BREAK-THRU® S 240. The residual efficacy of most treatments was only two weeks (post-application), with efficacy diminishing after that (**Figure 5.6**). The effect of Cryptogran™ plus molasses and BREAK-THRU® S 240 seemed notably diminished after two weeks, however, after four weeks seems to have recovered.

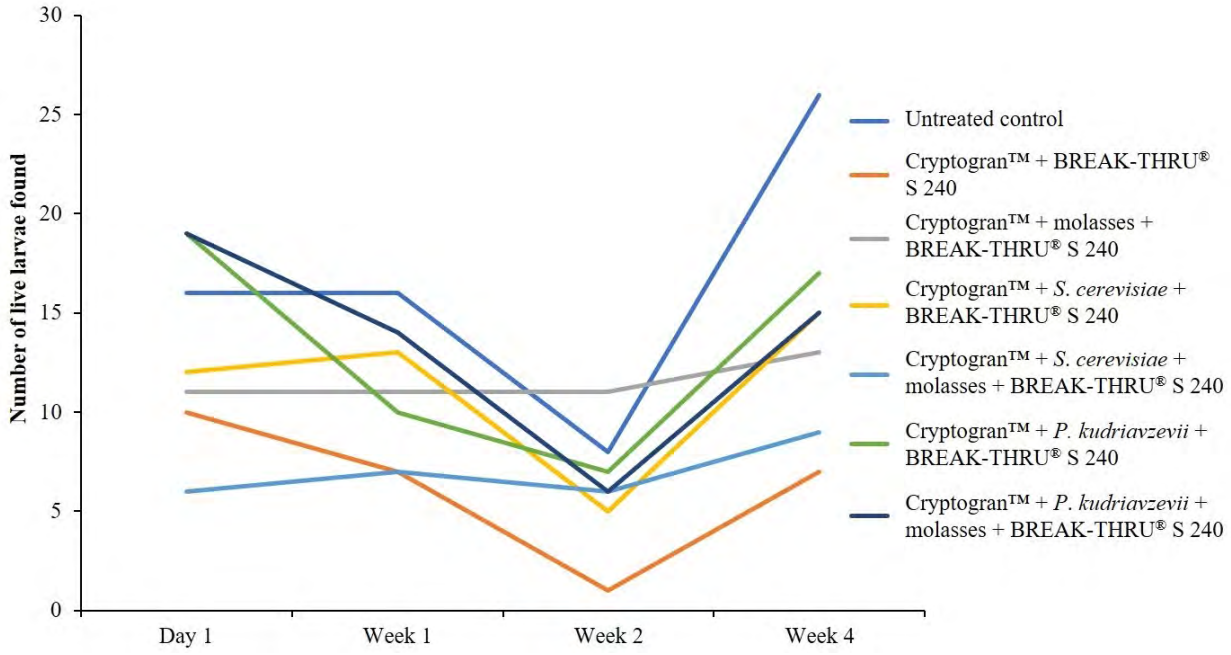

Figure 5.6: The approximate residual efficacy of each treatment applied at Pennyholme Farm trial site until four weeks post-application.

Cryptogran™ plus molasses and BREAK-THRU® S 240 efficacy was once again lower than expected and less than that of Cryptogran™ with BREAK-THRU® S 240. The Cryptogran™ plus *P. kudriavzevii* with BREAK-THRU® S 240 and Cryptogran™ plus *P. kudriavzevii* with molasses and BREAK-THRU® S 240 treatments saw a drastic increase in their efficacy, resulting in 47.92 % and 49.43 % reduction in *T. leucotreta* larvae relative to the untreated control, respectively (**Table 5.4**). The performance of Cryptogran™ plus *S. cerevisiae* with BREAK-THRU® S 240 and Cryptogran™ plus *S. cerevisiae* with molasses and BREAK-THRU® S 240 remained consistent with the latter, having a slight dip in its overall efficacy. Both Cryptogran™ plus BREAK-THRU® S 240 and Cryptogran™ plus molasses and BREAK-THRU® S 240 seemed to lose efficacy after two and four weeks, respectively (**Figure 5.7**).

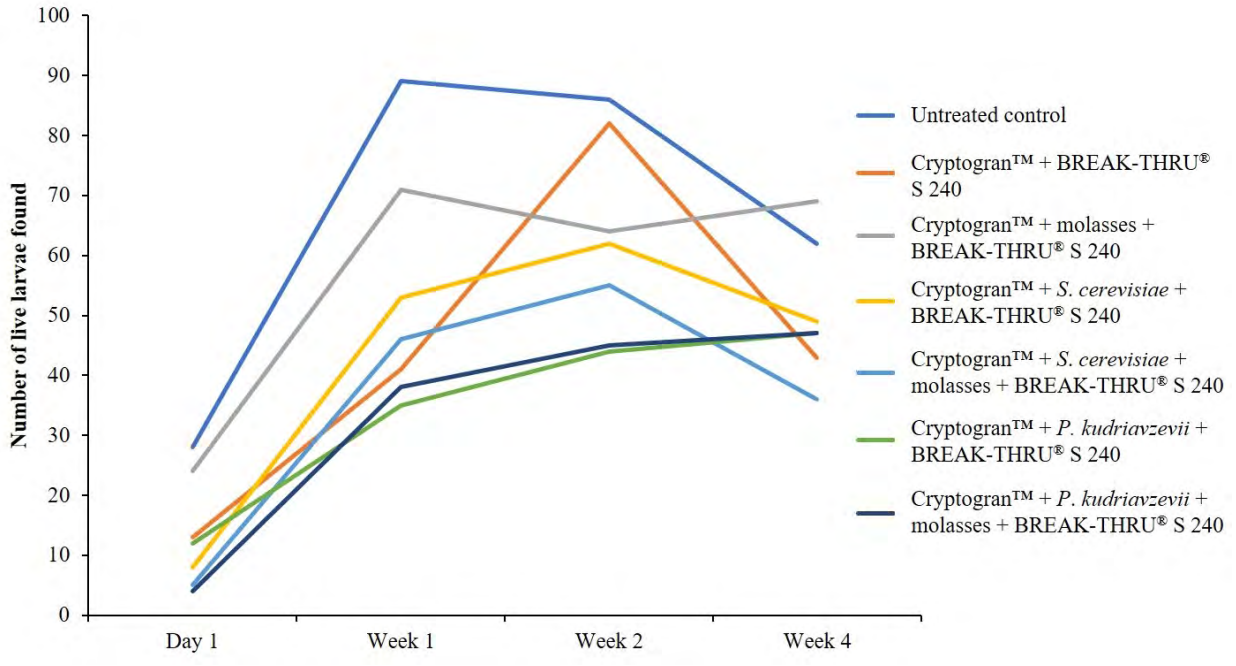

Figure 5.7: The approximate residual efficacy of each treatment applied at Tregoss Skinner trial site until four weeks post-application.

Due to the limited number of trials that could be performed due to the restricted time available before fruit harvesting, no statistical analyses were conducted on the collected data. Even though mean reduction in infestation, relative to the control, was determined with the data from the two sites. The residual efficacy of the applied treatments seemed to drop off after two weeks in the semi-field trials; thus, data from week four was excluded when determining the reduction in larval infestation. The data obtained does provide valuable insight into how *P. kudriavzevii* and *S. cerevisiae* perform in the field. Overall, the performance of *S. cerevisiae* was impressive and consistent, resulting in the least variability (**Figure 5.8**). The efficacy of *P. kudriavzevii* during the second semi-field trial was comparable to that of *S. cerevisiae*, but problems with its formulation during the first semi-field trial resulted in poor performance.

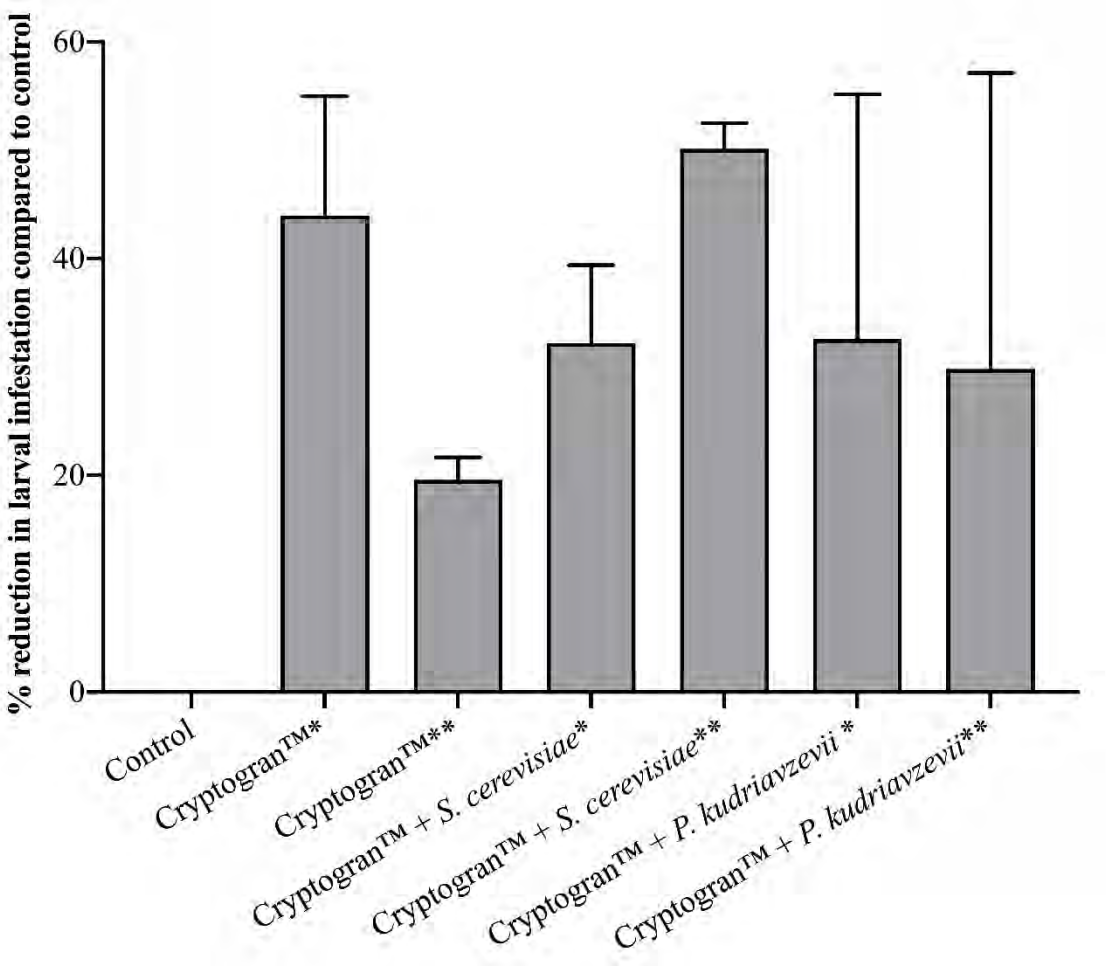

Figure 5.8: Mean reduction in larval infestation relative to each treatment's control during the first two weeks of the four-week period over which the two semi-field trials were conducted at Pennyholme and Tregoss Skinner farms. * and ** indicate treatments that included BREAK-THRU® S 240 and molasses plus BREAK-THRU® S 240, respectively.

5.4 Discussion

This chapter aimed to determine whether the efficacy of CrleGV-SA could be increased through the addition of yeast. This was achieved through a series of detached fruit bioassays and semi-field trials. Detached fruit bioassays were used to determine the optimal yeast:virus ratio and to optimise the yeast/virus mixture through the addition of an adjuvant and surfactant. Semi-field trials were then conducted to try and validate the efficacy of the combination in the field.

The setup and execution of detached fruit bioassays described by Moore et al., (2011) and Knight and Witzgall (2013) was successful. The LC_{50} concentration was selected as this would result in a

50 % mortality of the *T. leucotreta* population, and thus when combined with a specific treatment, an increase or decrease in mortality can be observed. The mortality rates of *T. leucotreta* obtained during detached fruit bioassays using LC_{50} concentration of CrleGV-SA were similar to those previously reported by Moore et al., (2011). Bioassays were conducted in a CE room in order to limit environmental stresses against neonates, as unstable environmental conditions (such as temperature and humidity) can be lethal to the neonates (Boardman et al., 2012). Five neonates were added to each Navel orange for all treatments. This number of neonates was chosen as it would result in at least one larva infesting each fruit. This number has also previously led to an approximately 80 % infestation rate for the control treatments, allowing for comparison of each treatment (Sean Moore, CRI, personal communication). The utilisation of fresh fruit no older than three weeks in bioassays would further improve *T. leucotreta* infestation rates and reduce mould occurrence.

Previous studies conducted to enhance baculovirus efficacy using mutualistic yeast applied the treatments at a concentration of 6×10^8 cells.ml^{-1} (Knight et al., 2015; Knight and Witzgall, 2013). Initially, detached fruit bioassays utilising this concentration found no significant differences between *P. kudriavzevii* plus CrleGV-SA and CrleGV-SA alone (van der Merwe, 2018). This prompted setting up a detached fruit bioassay using a 100-fold serial dilution of *P. kudriavzevii*, ranging from 2×10^8 to 2×10^4 cells.ml^{-1} to determine the optimal yeast:virus ratio. Significant differences between *P. kudriavzevii* plus CrleGV-SA and CrleGV-SA alone were recorded using the lower yeast concentrations of 2×10^6 cells.ml^{-1} and 2×10^4 cells.ml^{-1}. The yeast concentration selected for use in subsequent bioassays was 2×10^6 cells.ml^{-1}, as similar dilutions were used in neonate behaviour assays, oviposition assays, and due to the suitability of the concentration for field trials (Ljunggren et al., 2019; Spitaler et al., 2020). Additionally, yeast overabundance may result in high alcohol concentrations, which could negatively affect insect physiology and behaviour (Madden et al., 2018). The efficacy of combining gut-associated yeasts with CrleGV-SA was also assessed. Although a significant increase in larval mortality was recorded with most gut-associated yeasts, *M. guilliermondii* and *H. uvarum* were the only isolates that did not enhance CrleGV-SA effectiveness. *Saccharomyces cerevisiae* was included as it has previously been demonstrated to improve the efficacy of CpGV against *C. pomonella* (Knight et al., 2015). *Thaumatotibia leucotreta* larvae are internal feeders, and once they penetrate the rinds of fruit, there is little to no chance of them encountering and ingesting further OBs (Moore et al., 2011).

Larval feeding stimulants, such as molasses, have improved baculoviruses effectiveness (Ballard et al., 2000a, 2000b; Moore et al., 2015b). However, the addition of beneficial microbes to current biological control agents to extend larval feeding has to date been limited (Knight et al., 2015; Knight and Witzgall, 2013; Usta, 2013). The inclusion of *P. kudriavzevii* and *S. cerevisiae* to CrleGV-SA treatments containing molasses increased larval mortality significantly. This indicates that the efficacy of CrleGV-SA can be further enhanced through the addition of yeast.

Two sets of semi-field trials were conducted to test whether laboratory observations were transferable into the field. *Pichia kudriavzevii* was selected as the most likely yeast isolate to improve CrleGV-SA efficacy in the field. It was demonstrated as having a significant effect on both *T. leucotreta* neonate (**Chapter Three**) and adult female (**Chapter Four**) behaviour. *Saccharomyces cerevisiae* was included as it is commercially available as Brewer's yeast and has been shown to increase baculovirus efficacy (Knight et al., 2015). Although no significant differences were observed between yeast treatments and those without, this was a key steppingstone to validating their efficacy in the field. The unusually high efficacy of the Cryptogran™ treatment containing both molasses and BREAK-THRU® S 240 during the first trial may have skewed the outcome. However, both *S. cerevisiae* treatments' consistent performance during the semi-field trials should be noted as this indicates that yeast's inclusion into Cryptogran™ formulation may increase its efficiency.

In conclusion, detached fruit bioassay determined that the optimal yeast concentration to use alongside CrleGV-SA was 2×10^6 cells.ml^{-1}. The inclusion of molasses and BREAK-THRU® S 240 to *P. kudriavzevii* and *S. cerevisiae* greatly enhanced its efficacy. Promising results were obtained from semi-field trials, although more replicates need to be performed. The next chapter discusses the significant results of this study and their relevance to the management of *T. leucotreta* in South Africa.

Chapter Six

General Discussion

6.1 Introduction

Thaumatotibia leucotreta is endemic to sub-Saharan Africa and is classified as a phytosanitary pest (Hattingh et al., 2020). Due to its classification, foreign markets have imposed a zero-tolerance stance for any infested fruit with live larvae in the market (Moore, 2021). Chemical insecticides were previously mainly used to suppress *T. leucotreta* infestations (Moore, 2019a; Moore and Hattingh, 2012). Although effective, the indiscriminate use of insecticides can result in the development of resistance, detrimental effects on non-target beneficial insects, and environmental contamination (Ha, 2014; Hofmeyr and Pringle, 1998; Kongtip et al., 2020; Kosamu et al., 2020; Lacey et al., 2015; Richardson et al., 2020). Chemical insecticides can also be costly compared to biological control counterparts (Moore, 2021). Environmentally friendly methods are needed due to the stricter regulations around the use of chemical insecticides and increased pressure to reduce their residues on food, particularly for exportation and in the environment (Lacey et al., 2015; Moscardi et al., 2011; Soloneski and Larramendy, 2012; Szewczyk et al., 2006). The use of biological control agents to manage *T. leucotreta* has received much attention in recent years (Moore, 2019a), and more biological products are currently registered for use against *T. leucotreta* than chemical insecticides (Moore, 2021).

The granulovirus CrleGV has been extensively used against *T. leucotreta* for almost two decades (Moore et al., 2004, 2015b; Moore and Jukes, 2019). Baculoviruses are ideal for IPM programmes, as they are highly host-specific, and the risk of affecting non-target beneficial insects is very low due to their high level of host specificity (Soloneski and Larramendy, 2012). They are considered environmentally safe and can easily undergo genetic manipulation, which allows for continuous work on improving their efficacy as a control method (Moscardi et al., 2011). However, their short field stability, due to their sensitivity to UV degradation and slow speed of kill, limits their effectiveness compared to chemical insecticides (Beas-Catena et al., 2014; Wilson et al., 2020). Additionally, OBs must be ingested by the host insect for primary infection to occur (Ikeda et al., 2015). The two most common approaches utilised to enhance baculoviruses are genetic modification or improving the formulation (Szewczyk et al., 2006). However, the public's negative perception of genetically modified organisms has limited their use (Moscardi et al., 2011).

6.2 Biocontrol applications of yeast

Biological pesticides are based on pathogenic microorganisms that are for the most part specific to a target pest and control them in an environmentally friendly manner (Glare et al., 2012; Gupta and Dikshit, 2010; Kumar and Singh, 2015). The most commonly used microbial pesticides are bacteria, fungi, viruses, nematodes and sometimes protozoa (Gupta and Dikshit, 2010). These microbes are used to suppress pest populations, including insects, pathogens, and weeds (Glare et al., 2012). Yeasts occur in all environments, and their potential use as biocontrol agents has not been sufficiently exploited. They have previously been described as potent antagonists of various citrus moulds, such as *Penicillium digitatum* (Green mould) and *P. italicum* (Blue mould) (Luo et al., 2012; Platania et al., 2012; Taqarort et al., 2008). However, they have not been demonstrated to have insecticidal properties, and as a result, their use as biocontrol agents has been limited. The vast majority of interactions between insects and microorganisms have been studied for their potential use as insect pathogens (Hajek et al., 2007). Insects are chronically colonised by microorganisms that are not overtly pathogenic and are often beneficial or even required by the insect host (Douglas, 2015; Ganter, 2006). Yeasts play an important role in nutrition physiology and host attraction of many insects (Stefanini, 2018). The volatile profiles produced by yeasts have been shown to elicit strong behavioural responses in both lepidopteran larvae and adults (Davis et al., 2013; Ljunggren et al., 2019; Witzgall et al., 2012). The potential value of manipulating the microbiota to control insect pests has only recently been explored and may lead to new pest management strategies.

Cydia pomonella larvae are closely associated with two mutualistic yeasts, *viz*. *Metschnikowia andauensis* and *M*. *pulcherrima* (Witzgall et al., 2012). The inclusion of these yeasts into the larval diet promoted their survival, accelerated their development, and reduced fungal infestations in apples. Additionally, the fermenting yeast volatile profiles were attractive to adult *C*. *pomonella* females and influenced their oviposition preference. Knight and Witzgall (2013) developed a novel method for controlling *C*. *pomonella* by combining mutualistic yeast associated with the insect pest and the baculovirus CpGV plus brown cane sugar. The combination significantly increased neonate *C*. *pomonella* larval mortality compared with CpGV alone, both in laboratory assays and field trials (Knight and Witzgall, 2013). Furthermore, it was established that similar results could be obtained through the use of *S*. *cerevisiae* (Knight et al., 2015). The enhanced efficacy of CpGV can be attributed to *C*. *pomonella* neonates feeding on yeast inoculated with the virus, thus

increasing its uptake. Yeast volatiles have been shown to influence neonate lepidopteran behaviour and feeding (Ljunggren et al., 2019). Current attract-and-kill techniques available for *T. leucotreta* are density-dependent and should not be used as a stand-alone treatment (Moore, 2021). This study's main focus was to identify a yeast species to use alongside CrleGV to enhance its efficacy against *T. leucotreta*.

Yeast species were isolated from the gut of *T. leucotreta* larvae and identified via PCR amplification and sequencing of select gDNA regions, as described in **Chapter Two**. The behavioural influences of the isolated yeasts on *T. leucotreta* neonates and adult females were assessed through neonate attraction (**Chapter Three**) and oviposition assays (**Chapter Four**). Finally, in **Chapter Five**, detached fruit bioassays and semi-field trials were conducted to evaluate the effectiveness of combining the isolated yeast species with CrleGV-SA to enhances its efficacy.

6.3 Influence of yeasts on *Thaumatotibia leucotreta* and their efficacy with CrleGV-SA

Navel oranges infested with *T. leucotreta* larvae were collected from geographically distinct citrus-producing regions across South Africa, *viz.* Addo, Nelspruit, and Stellenbosch. The microbiota composition of plants may vary across spatial and temporal scales (Rastogi et al., 2013; Wu et al., 2020). Yeast species were isolated from the gut of collected larvae, following the protocols described by Witzgall et al., (2012). Identifying potential yeast species among the other microbes present on the YPD agar plate was difficult. Additionally, selecting different isolates was also cumbersome due to the similarities in yeast morphology. The use of more selective plated media may aid in this process (Kurtzman et al., 2011).

The PCR amplification and sequencing of the ITS region and D1/D2 domain of the LSU were used to identify isolated yeast species (Pincus et al., 2007). A total of six yeast species were identified, *viz*. *M. guilliermondii*, *H. uvarum*, *C. lusitaniae*, *K. marxianus*, *P. kudriavzevii*, and *P. kluyveri*. Species from the genus *Pichia*, *Hanseniaspora*, and *Clavispora* are known to be associated with citrus (Arias et al., 2002; Las Heras-Vazquez et al., 2003). *Meyerozyma guilliermondii* is known to be associated with Coleoptera, and *K. marxianus* has previously been linked to drosophilids (Diptera) and Hymenoptera (Molnár et al., 2008; Rivera et al., 2009; Stefanini, 2018).

Thaumatotibia leucotreta neonate behavioural response to the isolated yeast species was assessed through a larval feeding assay described by Ljunggren et al., (2019). Initially, a camera setup was used to monitor the movement and penetration of neonate *T. leucotreta* on treated Navel oranges; however, this was inefficient and therefore not continued. The larval feeding assay was efficient, and neonates that had fed on specific treatments could be clearly observed. In some instances, neonates would escape the enclosure; to prevent them from escaping a tight seal between the petri dish and glass cover must be ensured. The behaviour of neonate *T. leucotreta* was influenced by *H. uvarum*, *P. kluyveri*, *P. kudriavzevii* and *K. marxianus*. The volatiles produced by yeasts is thought to stimulate larval feeding (Knight and Witzgall, 2013). Longer larval feeding time on yeast increases the probability that a simultaneously applied biopesticide will be ingested or that a greater amount of the biopesticide will be ingested (Knight et al., 2015; Knight and Witzgall, 2013).

A Y-tube olfactometer was initially used to assess the influence of the isolated yeast species on adult *T. leucotreta* female behaviour. However, this was unsuccessful, as moths would not move in the narrow space of the Y-tube. An oviposition preference assay described by Love et al., (2014) was adapted to evaluate the influence that the isolated yeast species have on adult *T. leucotreta* females. The concentration of yeast used to inoculate Navel oranges was 2×10^6 cells.ml^{-1}, similar to that used during the larval feeding assay (Ljunggren et al., 2019). *Hanseniaspora uvarum*, *P. kudriavzevii* and *M. guilliermondii* were shown to influence adult *T. leucotreta* female oviposition preference. However, when assessed against one another, no significant differences were found between them. The assay effectively evaluated the oviposition preference of adult *T. leucotreta* females; however, several improvements to the setup could be made. The use of larger cages would provide a greater area to adult moths to move more freely, eliminating chance encounters with Navel oranges. In some instances, *T. leucotreta* moths were observed copulating during the duration of the assay. This is undesirable, as it reduced the time that moths could be ovipositing. Including multiple pairs of mated *T. leucotreta* moths would increase the likelihood of at least one pair ovipositing on fruit. A visual confirmation that copulation had taken place would also ensure that only mated adult *T. leucotreta* females are used. Additionally, the assay duration could be extended to 72 hours as peak egg-laying only occurs 3 to 5 days after they eclose (Stotter, 2009). Both *H. uvarum* and *P. kudriavzevii* have now been shown to influence *T. leucotreta* neonate and

adult female behaviour. As previously mentioned, they are known to be associated with citrus; thus, their influence on *T. leucotreta* is not unexpected.

Detached fruit bioassays were used to evaluate the efficacy of combining CrleGV-SA with the isolated yeast species to enhance *T. leucotreta* larval mortality (Moore et al., 2011). The bioassay setup yielded consistent infestation rates in the untreated controls, indicating that the assays were effective. Three sets of bioassays were conducted, which determined that the optimal yeast concentration to use alongside CrleGV-SA is 2×10^6 cells.ml^{-1}. The combination of *P. kudriavzevii*, *P. kluyveri*, *K. marxianus* or *S. cerevisiae* with CrleGV-SA enhanced the efficacy of the virus. Finally, the inclusion of molasses plus BREAK-THRU® S 240 to *P. kudriavzevii* and *S. cerevisiae* formulations further enhanced CrleGV-SA efficacy compared to CrleGV-SA alone. *Pichai kudriavzevii* was selected for semi-field trials due to its behavioural influence on *T. leucotreta* and synergism with CrleGV-SA. *Saccharomyces cerevisiae* was selected and included in semi-field trials due to its commercial availability. Although only preliminary data were collected from the semi-field trials, the results obtained were similar to those reported by Knight et al., (2015) and Knight and Witzgall (2013). The inclusion of yeast seemed to enhance the efficacy of the baculovirus. The methodology used during the semi-field trials was a new technique, and adjustments still need to be made. It was adopted, as naturally occurring levels of *T. leucotreta* in commercial citrus orchards are now rarely at a level that facilitates reliable field results (Sean Moore, personal communication). The infestation rate of untreated fruit in semi-field trial bioassays was lower than expected. Several changes could be made to increase the infestation level of untreated fruit, such as conducting bioassays on riper fruit or placing more neonates onto the fruit.

6.4 Potential role of yeast in the IPM programme

IPM programmes successfully use both semiochemicals and pathogens to manage *T. leucotreta* populations in South Africa (Malan et al., 2018; Moore et al., 2015b; Moore, 2021). The combination of semiochemicals with pathogens in attract and kill strategies could significantly increase the efficacy of currently used biological control agents (El-Sayed et al., 2009; Gregg et al., 2018; Mkiga et al., 2021). The combination of a mutualistic yeast with CpGV enhanced *C. pomonella* larval control, both in the laboratory bioassays and semi-field trials (Knight et al., 2015; Knight and Witzgall, 2013). The occurrence of mould and the number of injured fruits was also

reduced. This study's ultimate objective was to identify potential yeast isolates that could be incorporated into the formulation of CrleGV-SA to enhance its efficacy and integrate it into the IPM programme for *T. leucotreta*. The validity of the concept has been demonstrated within a laboratory setting. However, before it can be incorporated into such a programme, field trials need to be conducted more extensively to assess the control agent's persistence and efficacy against the biotic and abiotic factors encountered in the field (Moore, 2002). The preliminary data obtained from *P. kudriavzevii* and *S. cerevisiae* semi-field trials demonstrate that they exhibit great potential for incorporation into CrleGV-SA formulation. The inclusion of yeast into virus formulations may have several additional benefits. The spray coverage of citrus trees could be reduced, as yeast is a significant larval feeding attractant (Ljunggren et al., 2019). Larvae will be drawn to the yeast to feed and as a result, will invest more virus increasing larval mortality. Alternatively, the concentration of virus applied could be reduced due to the greater feeding, thus reducing the cost of application. Furthermore, with more feeding taking place on the fruit's surface, attempted penetration by the larvae will be delayed. Larval mortality before penetration becomes more likely, either from the virus itself or by a multitude of other environmental factors that can affect the exposed fragile larva.

The utilisation of yeast in the IPM programme is not limited to its ability to enhance CrleGV-SA efficacy. Yeasts are also known to produce biofilms that enable the postharvest control of fungal diseases in fruit (Droby et al., 2009; Manso and Nunes, 2011; Sharma et al., 2009; Villa et al., 2017). The isolated yeast species have previously been shown to exhibit antifungal properties against citrus moulds (Geng et al., 2011; Hasan and Sikdar, 2019; Li et al., 2016; Lima et al., 2013; Wilson and Chalutz, 1991; Yan et al., 2018). Honeydew is a sugary excretion of carbohydrates, amino acids and water produced by various homopterans such as aphids, soft scales and mealybugs while feeding (Chomnunti et al., 2014; Styrsky and Eubanks, 2007). Ants are attracted to the sugary substance, and their presence can disturb natural enemies of other pests, decreasing parasitism levels (Yoo et al., 2013). Additionally, sooty moulds grow on the honeydew secreted by insects and produce a thin superficial network of dense, dark hyphae (Louise et al., 2002). Sooty mould cover inhibits the passage of sunlight to the leaves and negatively affects photosynthesis (Nelson, 2008). In severe situations, tree growth, yield, and fruit size and colour can be detrimentally affected, resulting in the fruit being downgraded for export (Moore, 2019b). It has been demonstrated that yeast can effectively reduce honeydew levels within the field (Dik et al.,

1991). The addition of yeast could aid in reducing honeydew levels present on citrus trees and combat sooty mould infestations.

Monitoring pest populations is also important to ensure optimal timing of treatments and to measure the effect that control options have on pests (Moore, 2021). It is also used to determine whether the infestation in an orchard is adequately low and suitable for export to a market for which *T. leucotreta* is a regulated phytosanitary organism (Hattingh et al., 2020). Insect attractants are mainly synthetic chemicals; however, microorganisms such as yeasts possess great potential for live insect attractants (Beck and Vannette, 2017; Davis et al., 2013; Spitaler et al., 2020; Witzgall et al., 2012). Pheromone traps are utilised to monitor *T. leucotreta* populations within citrus orchards in South Africa (Moore, 2019a; Moore and Hattingh, 2016). Only two pheromone monitoring systems are registered and commercially available for *T. leucotreta*, *viz.* F.C.M PheroLure® (Insect Science (Pty) Ltd, South Africa) and Chempac FCM Lure (Chempac (Pty) Ltd, South Africa) (Moore, 2019a). Current pheromone trapping systems use synthesized female pheromones to attract and trap male *T. leucotreta* adults. Three yeast isolates were shown to attract adult *T. leucotreta* females and could be developed into a novel trapping system. The ability to trap female *T. leucotreta* adults would provide a more accurate representation of damage potential than monitoring males as adult *T. leucotreta* females are responsible for laying the eggs that lead to infestation and damage. It has previously been shown that both male and female *C. pomonella* adults are attracted to the pear ester, ethyl (E, Z)-2,4-decadienoate (Light et al., 2001). Pear ester's effectiveness in capturing females can be improved by adding acetic acid and terpenoids (Knight, 2010; Knight et al., 2019). The ability to monitor females has improved phenology predictions and established a more precise action threshold for *C. pomonella* (Barros-Parada et al., 2015; Knight and Light, 2005). Additionally, other citrus pests, such as fruit flies, have also been shown to be attracted by volatiles produced by yeasts (Becher et al., 2012; Scheidler et al., 2015; Spitaler et al., 2020). Therefore, yeast could be used to monitor a wider range of citrus pests within an orchard compared to currently used species-specific traps.

The combination of yeast with pre-existing biological control agents is not limited to *T. leucotreta*. Several other lepidopteran pests could be targeted with a similar approach to enhance their control or improve their monitoring within the field. Additionally, it is not only lepidopteran pests that can be targeted, but also dipterans (Knight et al., 2016; Lewis and Hamby, 2019; Mori et al., 2017).

6.5 Conclusion and future prospects

Future work arising from this study would involve increasing the number of larvae sampled and expanding the bioprospecting process to other citrus-producing regions across South Africa, such as Limpopo Province. A small sample size of *T. leucotreta* larvae from each region was assessed (n = 10) for the presence of yeast and increasing the sample size may lead to the identification of novel isolates. The process whereby yeast species were isolated can be streamlined through use of more selective plated media to limit the growth of non-yeasts. Analysing the haemolymph and specialised cells such as fat bodies within *T. leucotreta* larvae may further indicate which of the isolated yeast species are mutualistic (Gibson and Hunter, 2010; Vega and Dowd, 2005). Interactions between insects and microbes with a mutualistic relationship are greater than those that interact by chance, as they have evolved together (Becher et al., 2018; Madden et al., 2018).

The results found during the ovipositional preference trials create various opportunities for future research. Performing ovipositional preference trials in much larger cages and conducting field trials may result in a more definite answer. Conducting field trials with the isolated yeast species may also inadvertently be attractive towards other citrus pests and lead to the development of a broader pest monitoring system. The volatile profiles of the isolated yeasts species should be purified via column chromatography and analysed via GCMS to identify the metabolites they produce. Analysing the volatile profiles produced by the isolated yeast species was initiated but could not be completed due to Covid-19 lockdown induced time constraints. However, this is currently still underway. Analysing adult *T. leucotreta* female flight response in wind tunnels without a visual cue could provide a better indication of their attractiveness to volatiles produced by the isolated yeast species (Witzgall et al., 2012). Additionally, analysing the antennal response of adult *T. leucotreta* females via GC-EAD may indicate the specific compound or bouquet of compounds that is/are responsible for eliciting an olfactory response (Gökçe et al., 2018). Identifying the unique odour profiles that yeasts produce and the isolation of these compounds' physiologically active constituents could potentially define the host's attraction (Scheidler et al., 2015).

A repeat of the semi-field trials would also be of considerable interest to determine whether the results obtained in this study can be replicated. However, adjustments to the detached fruit bioassay setup need to be made to improve the reliability of results. More neonates should be

placed onto oranges used in the assays, and bioassays should be conducted on riper fruit to facilitate a higher level of successful larval penetration and infestation. Additional replicates need to be performed to obtain data that can be statistically analysed. The trial duration should also be extended slightly to determine if the residual efficacy of *P. kudriavzevii* and *S. cerevisiae* extend beyond the four weeks tested. Once semi-field bioassays are completed, a high infestation field site should be sought where a more conventional replicated field trial can be conducted.

In conclusion, this study's primary aim was to identify a yeast species that would enhance the efficacy of CrleGV-SA against *T. leucotreta*. Six yeast species were successfully isolated and identified from *T. leucotreta* larvae collected from geographically distinct citrus-producing regions. Larval feeding and oviposition assays were conducted with the isolated yeast species to evaluate *T. leucotreta* neonate and adult female behavioural response. Detached fruit bioassays were then conducted to assess the efficacy of combining CrleGV-SA with the isolated yeast species. The mixture of *P. kudriavzevii* with CrleGV-SA significantly increased the efficacy of the virus in a laboratory setting. This led to the identification of *P. kudriavzevii* to use in semi-field trials. However, additional semi-field trials need to be conducted to confirm the formulation's efficacy in the field. Future research into the efficacy of *P. kudriavzevii* in combination with CrleGV-SA in the field is required to develop a novel biopesticide formulation.

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