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A biologically-active pest deterrent: what is the mode-of-action and how does this affect pest behaviour?

Faye E. Mansfield
BSc (Hons) Biological Sciences

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Declaration

I declare that the contents in this thesis are my own work and have not been submitted in the same form for the award of higher degree at any other institution.

Faye E. Mansfield

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Abstract

The application of chemical pesticides to protect crops is standard practice worldwide, however, the detrimental repercussions of excessive use on human health and environmental degradation are becoming more evident. Integrated pest management strategies, providing non-chemical alternatives to pest control, have been prioritized by stakeholders in the United Kingdom and overseas. *Grazers Ltd* has set out to produce an environmentally sustainable feeding deterring product, which can be applied in a similar fashion as chemical pesticides, but without the harmful side effects.

The *Grazers Ltd* G3 product has a simple formulation, with calcium chloride acting as the active component. Before the product can be registered and utilized commercially in agriculture, the mode-of-action which alters the feeding behaviour of pests must be determined. Laboratory bioassays were performed to determine the insecticidal and antifeedant properties of the product on *Spodoptera littoralis*, when presented with treated semi-artificial wheatgerm-based diet cubes, *Triticum aestivum* leaves, and *Brassica napus ssp. Pabularia* leaves. Additionally, the ability of the Ca^{2+} ion from the active component to penetrate through the adaxial leaf surface was also tested. The product was analysed as a complete formulation ('Old'), with a new source of calcium chloride ('New'), and when broken down into each individual component.

The complete formulations exhibited a deterrent effect when applied to plants but not diet, indicating a plant-mediated effect in a two-choice setting. The 'Old' formulation appeared to alter oviposition behaviour significantly while

the ‘New’ formulation did not. Furthermore, the individual components did not show significant antifeedant abilities.

Despite a lack of research into calcium chloride as a feeding or oviposition deterrent, these findings suggest that the calcium chloride-based product exhibits a plant-mediated deterring effect on the phytophagous pest.

Chapter 1: General Introduction

1.0. Abstract

Global food security is under attack and if changes are not made, the consequences will be felt worldwide. With the expected population rising to 9.7 billion by 2050, a staggering 70% increase in food production is required to meet demands. Crop domestication began tens of thousands of years ago, and since its origin, pests and pathogens have caused starvation, spread of diseases and social upheaval. Chemical pesticides have been used to control pests, to reduce damage and spreading of diseases, however the negative implications on health and the environment have since become apparent. As a result, integrated pest management schemes have been implemented to control insect pests without the use of chemical pesticides. To produce effective strategies to combat damage to plants and crops, understanding the interactions between the host and the pest have been greatly researched.

1.1. Plant-pest interactions

Plants and their insect pests have been in an 'evolutionary arms race' since the dawn of both organisms, co-evolving to overcome defence mechanisms and developing new strategies to increase fitness at the expense of the opposition. Some plants live in mutualistic relationships with specific insects, exchanging food and shelter for pollination and protection from herbivores (Mello & Silva-Filho, 2002). On the contrary, some herbivorous insects may feed upon the plant matter or dine on the nutritious sap (VanDoorn & Vos, 2013). With any host-pest relationship, if the scale tips in favour of the pest, the damage to the host can be fatal.

Host plants and their herbivorous insect pests interact in a dynamic environment, within an ever-changing climate which pressurizes their survival continuously. In order to maintain their position ahead of their co-evolving companions, plants have acquired a range of defence mechanisms (War et al. 2018). Morphological barriers aim to physically impact the success of the herbivorous feeding. These include: thorns and trichomes (which can become more densely spread with evolution), a waxy cuticle with cell-wall lignification and silicon deposition below the cuticle (Alhoosan & Greger, 2018), and unpalatable sclerophyll leaf production (Mitchell et al. 2016). Additionally, some plants produce latex and resins to kill herbivorous insects or newly-emerged young through immobilisation and smothering of their mouthparts (Agrawal & Konno, 2009). Plants also produce a range of secondary metabolites, which do not aid in their primary functions. Instead, these have been recognised to trigger an immune response: through detection of herbivorous damage or elicitors. Upon recognition, biochemical pathways are activated through the production of phytohormones, and their signals are transduced and propagated throughout the plant (Belete, 2018). Different phytohormones activate a variety of pathways and can work either synergistically or antagonistically with each other, depending upon the stimulus (Figure 1.1).

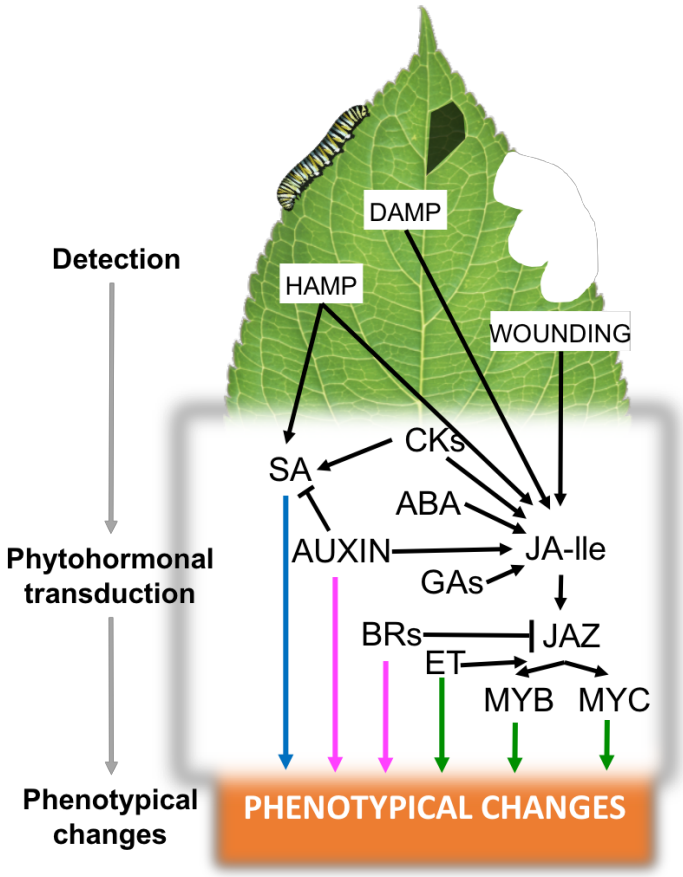

Figure 1.1: The phytohormone-regulated defence pathway in response to detection of herbivore-associated molecular patterns (HAMPs), damage-associated molecular patterns (DAMPs), and wounding. Jasmonic acid (JA) dependent (green arrows) and independent (pink and blue arrow) pathways are activated upon perception of injury. Signal transduction and propagation of induced hormones involves synergism and antagonism of complex pathways. Hormones involved: cytokinins (CK), abscisic acid (ABA), salicylic acid (SA), ethylene (ET), auxin, gibberellins (GA), brassinosteriods (BR). Jasmonoyl-L-isoleucine (JA-lle) accumulates and stimulates the activation of JAZ proteins, further promoting the interaction with MYB/MYC transcription factors. The phenotypical changes which occur following phytohormone activation and transduction can be specific to the stimulus (e.g. latex production) as well as general responses (e.g. production of herbivore-induced plant volatiles). Based upon illustration from Erb et al. (2012).

1.2. Chemical pesticides

Pesticides are a means to control pests, both macro- and microscopic, which directly or indirectly cause suffering to humans. Most pesticides are chemical agents that kills or immobilises the target pests.

Natural chemical pesticide use was first recorded over 4000 years ago in ancient Sumer (modern-day Southern Iraq) when elemental sulphur was spread over crops for protection against disease. In the centuries that followed, a range of naturally-occurring chemicals were extracted and utilised for their pesticidal qualities, such as pyrethrum and rotenone from plants. In 1939, synthetic chemical pesticides erupted into the global market when dichlorodiphenyltrichloroethane (DDT) was discovered. With a variety of uses, production and application of DDT, for agriculture, disease control, landscaping and other areas, skyrocketed worldwide (Goel & Aggarwal, 2007). However, the threat to biodiversity and the long-term negative effects on human health soon became apparent. This triggered the formation of DDT opposition programs worldwide, such as the 1967 Environment Defence Fund (EDF) in the United States of America (Conis, 2010), and the 2004 Stockholm Convention of Persistent Organic Pollutants (Magulova & Priceputu, 2016) to name a few. Nevertheless, the use of DDT in disease control against vector-borne diseases, such as malaria and visceral leishmaniasis (van den Berg, 2009), in lower income countries is still occurring (Eskenazi et al. 2018).
Countries like India and the People's Democratic Republic of Korea rely heavily on the pesticide industry for their economy, disease control strategies and food security. Others have banned the use of certain pesticides, such as DDT, for agricultural use due to the negative implications on health and the environment (Eskenazi et al. 2009), but still use pesticides for other

commercial practices, such as maintaining golfing greens and cleaning boat bottoms. The advantages and disadvantages of pesticide production and use are further discussed.

1.2.1 Beneficial uses

With the expected population rising to 9.7 billion by 2050, a staggering 70% increase in food production is required to meet demands (Nicolopoulou-Stamati et al. 2016). The agricultural industry relies heavily on pesticide use, largely in the form of herbicides, insecticides and fungicides, to maintain crop production quotas which are ever-increasing due to demand. The increase in pesticide use since the commercialisation of synthetic products has amplified successful crop harvests (Popp et al. 2013). Without the use of pesticides, food supply would undoubtedly fail to meet demand, resulting in rising food prices (Hossard et al. 2014), an increase in malnutrition and the preventable yet neglected diseases which accompany it (Jenson et al. 2010).

Another benefit of pesticide use is the reduction of area required to meet a specific yield of crops. Using Pakistan as a case study, as agriculture significantly impacts the country's economic stability (Khan et al. 2012), wheat is cultivated across 9 million hectares. The average loss of yield pre-harvest due to direct aphid damage without pesticide application is 35-40% (37.5% average) (Asghar et al. 2018). Without the use of pesticides, an estimated 3.375 million hectares of wheat may be lost. To maintain the yield required, an expansion of land to compensate would increase the total land use to 12.375 million hectares. As Pakistan relies heavily upon agriculture, it can be assumed that an expansion of land would not be possible as suitable land would already be utilised. Moreover, less land required for agriculture results in less fertilizer

use, lower labour costs, and less environmental impact (Rahman & Chima, 2018).

In addition to agricultural use, insecticides are largely used in control of infectious diseases (Table 1.1). Since the introduction of insecticides for vector and reservoir control, many case studies have shown a significant reduction in incidence rates in many neglected tropical diseases. Oguttu et al. (2017) recorded a reduction from 130 per 1000 cases of malaria to 45 cases per 1000 in children under 5, when long-lasting impregnanted nets (LLIN) and indoor residual spraying (IRS) were utilised in Tororo District, Uganda. Similarly, Hladish et al. (2018) modelled the use of IRS pre-season to *Aedes* mosquito emergence would reduce the incidence rate of symptomatic dengue fever infections by up to 89.7% if 75% of households were treated in the Yucatán state of Mexico.

Table 1.1: Vector-borne disease and their control with insecticides. IRS = Indoor residual spraying. LLIN = Long-lasting impregnated nets. ITN = insecticide-treated nets.

Vector	Pathogen	Disease	Insecticide	Application method	Study
***Aedes* sp.**	Yellow fever virus, dengue virus, zika virus, chikungunya virus	Yellow fever, dengue fever, zika, chikungunya.	Organophosphates, pyrethroids	IRS, LLIN, ITN	Reiter (2010)
***Anopheles* sp.**	*Plasmodium* sp.	Malaria	DDT, pyrethroids (lambda-cyhalothrin), organochlorines, organophosphates, carbamates	IRS, LLIN, ITN,	WHO (2016)
***Phlebotomus* sp.**	*Leishmania* sp.	Leichmaniasis	Permethrin, DDT	IRS, LLIN, ITN	Stockdale & Newton (2013)
***Triatoma* sp.**	*Trypanosoma cruzi*	Chagas disease	Triatomines, pyrethroids, DDT	IRS, IILN, ITN	Zerba (1999), Asale et al. (2014)
***Glossina* sp.**	*Trypanosoma brucei*	African trypanosomiasis	Deltamethrin (synthetic pyrethroid)	Treated cattle, IILN, ITN	Kotlyar (2010)

To summarise, without the use of pesticides for crop protection and disease control, the human race would certainly suffer. Insecticide use in agriculture depends largely on the season, climate and propagation of the pests, but the effect on yield can be controlled and is far more predictable.

1.2.2. Damaging effects on health

Despite the positive effects, rigorous measures and regulations are imposed on chemical pesticide use in agriculture, to ensure minimal risk to the environment and human health. Pesticides have been found in drinking water, surface soils and on contaminated food, and have been found to cause both acute and long-term health implications in humans (Thundiyil et al. 2008).

Those who are exposed to pesticides due to their occupation, environment, and residents have been found to have higher rates of morbidity (Mrema et al. 2017; Tsimbri et al. 2015; Lekei et al. 2014).

Focusing on insecticides, the effects on human health have found global attention as the consequences of chronic and sub-lethal exposure are now coming to light (Table 1.2).

Table 1.2: Diseases linked to insecticide poisoning and their incidence. ADHD = attention deficit hyperactive disorder, ALS = amyotrophic lateral sclerosis/motor neurone disease.

Disease type	**% contributing to total effect on health***	**Examples**	**Insecticide**	**Study**
Carcinogenicity	35	Childhood and adult brain tumours	Tetrachlorvinphos (carcinogen and recorded transfer from mother to foetus, during pregnancy of child sufferer).	Shim et al. (2009)
Metabolic toxicity	23	Diabetes and inflammation of insulin-responsive tissues	Organochlorines.	Mostafalou (2016)
Reproductive toxicity	17	Low sperm count and birth defects	DDT and derivatives.	Pant et al. (2007)
Developmental toxicity	10	Autism and ADHD	Dimethylphosphates	Yu et al. (2016)
Pulmonotoxicity	8	Chronic bronchitis and asthma	Heptachlor	Hoppin et al. (2007) Ndlouv et al. (2014)
Neurotoxicity	7	Parkinson's and ALS	Organophosphates	Oskarsson et al. (2016)

*statistics from Mostafalou & Abdollahi (2017).

It is estimated that over 30,000 deaths per year are due to acute, unintentional pesticide poisonings (vanderWulp, 2017; Pretty & Bharucha, 2015; Chatterjee & Riaz, 2013), with lower income countries being disproportionately impacted (Rahman & Chima, 2018). This is due to the high numbers of agricultural workers, lack of knowledge and training for pesticide application, and poorly controlled decontamination strategies for the crops. However, the morbidity rate from insecticide poisoning is predicted to be much higher, with the associated costs of treatment and loss of labour associated with long-term conditions (Gangemi et al. 2016).

1.2.3. Greenhouse emissions

The production of pesticides contributes to the greenhouse effect and other environmental ramifications (West & Marland., 2002). Emissions for most pesticides are not readily available, which makes monitoring and reducing a more challenging task. In addition, the amount of pesticide applied per application, and the number of applications per season, depend upon the crop type and the climate.

The weighted average of pesticide production (energies per unit mass), as determined by Audsley et al (2009), was 1364MJ kg^{-1} per hectare of arable crop; giving total emissions of 94kg CO_2e. In 2017, there were 17.5 million viable hectares across the United Kingdom (Department of Environment, Food and Rural Affairs., 2017), resulting is a predicted 1.645 million tCO_2e produced. This does not include emissions produced from transport and application of pesticides. As climate change substantially influences the lifecycles and distribution of pests across the planet, as well as the development and likelihood of crop success (Taylor et al. 2018), it is plausible that agricultural

sustainability could be negatively impacted by the carbon footprint of pesticide use.

Overall, pesticide production contributes to the greenhouse effect, and as global warming worsens, the need for pesticides will increase. This vicious circle of carbon emissions, global warming, fluctuating food security, and damage to the environment and human health, provides a need for innovative and novel alternatives to the damaging chemical pesticides.

1.2.4. Pesticide resistance

Another important issue of pesticide use comprises of target pests conferring resistance. Regarding malaria prevalence in Africa, DDT is an effective insecticide against the *Anopheles* sp. vectors. However, when applied in high concentrations and over large areas, DDT acts as a selection pressure, which allows pests which naturally exhibit resistance to breed and propagate (Ibrahim et al. 2018). Due to the short lifecycle of the mosquitoes, resistance to DDT is becoming apparent after only a handful of generations (Nkya et al. 2014). Furthermore, the mode-of-action (MOA) which DDT acts upon, interfering with the neurological pathways resulting in spontaneous neuron firings and eventual death, is similar to that of pyrethroid insecticides (Corbel et al. 2007). DDT-resistant mosquitos are becoming resistant to pyrethroids despite not coming into contact with them, compromising the most effective vector control strategies: pyrethroid-treated mosquito nets and indoor-residual spraying of DDT (Bhatt et al. 2015). As a result, cross-resistance of insecticides against mosquitos has and is continuously occurring. Multiple resistance can also occur when insect populations confer resistance to

insecticides with a differing MOA (Djouaka et al. 2016). This is less common than cross-resistance, but the consequences are far more extreme with the pests decimating areas of crops or causing high incidence rates due to lack of effective control. As with vector control, resistance to pesticides is a problem in the agricultural sector. The main impact of conferred resistance is a loss of yield. This results in a loss of profit, loss of available crops for cattle and humans, and a loss of produce for industries such as cotton. Moreover, agricultural workers irrationally respond to the loss of viable crops by increasing pesticide application concentration and rate (Sternberg & Thomas, 2018), further exposing harmful chemicals to the environment, produce and themselves. A lack of education and training further exacerbates this issue (Rijal et al. 2018).

1.3. Alternative control methods

Overuse of pesticides has led to insecticide-resistant pests, contaminated food, long-term health issues for those exposed, and an environment inundated with toxic chemicals. Global strategies implemented by the United Nations and other governing bodies have pushed the need for research and adoption of alternative methods of pest control; contributing to the formation of the Integrated Pest Management (IPM) ecosystem (FAO, 2018). Following the production of pesticides after World War II, the problems associated with chemical pest control emerged, and focus was put on non-chemical crop protection strategies to maintain sustainable food security (Lamichhane et al. 2018). IPM was further implemented to monitor and provide alternatives to chemical pesticide use in areas other than agriculture, namely vector and disease control. IPM strategies can be split into five management types: biological agents, genetically modified agents, cultural practices, physical

techniques and mechanical systems. All of these must be considered thoroughly before the reimplementation of chemical pesticides; an absolute last resort if yield losses are too severe (Lamichhane et al. 2016).

1.3.1. Biological agents

Perhaps the most well-known example of biological pest control is the relationship between aphids and the predatory ladybird. Horticulturists and gardeners have been attracting ladybirds to the areas which aphids are rife through the use of companion plants with specific coloured flowers or volatiles for many years (Ben-Issa et al. 2017). This basic form of pest control has been manipulated and made available for commercialisation for use in agriculture. Biological agents, commercially known as biopesticides, include the use of microorganisms, natural predators and parasitoids, to control and reduce the effects of pest infestation.

Bacillus thuringiensis (*Bt*) is a soil-borne, spore-forming bacterium which is assimilated into a variety of biopesticide formulations, to effectively combat several lepidopteran pests worldwide. *Bt* Berliner, *Bt* var. *aizawai*, and *Bt* var. *kurstaki* (*Btk*) have been successfully used against the Diamondback Moth (*Plutella xylostella* L.) in South Africa (Legwaila et al. 2015), while *Bt* var. *kurstaki* has been used efficiently against *Spodoptera littoralis* in Egypt (Fetoh et al. 2015). Its effectiveness is due to α-endotoxins within the crystallised structure of the bacterium. Upon ingestion, the protoxin is metabolized to the toxic δ-endotoxin which disrupts the gut lining and neurological systems of the pest. The use of *Bt* products has been around for over three decades and is still an effective method to controlling herbivorous pests (Crickmore et al. 1998).

Additionally, the entomopathogenic fungus, *Metarhizium anisopliae*, is utilised for microbial biopesticide use due to the infectious conidia that germinate, penetrate and propagate within the pest, upon dermal contact (Rustiguel et al. 2018). Another fungus, *Beauveria bassiana*, has been shown to cause 80% mortality in the diamondback moth (at 76% relative humidity) (Masuda, 1998). An important aspect of fungal-based biopesticides is that mortality rate is dependent upon temperature and humidity (Bugeme et al. 2009).

Entomogenous viruses which are used for biopesticides, fall into one of two categories: inclusion viruses (IV) and non-inclusion viruses (NIV). IV produce inclusion bodies which are viral structures unique to each pathogen and the result of infection (Hoenen et al. 2012). These are further sub-categorized into polyhedron viruses (PV), depending upon areas of inhabitation: nuclear polyhedrosis viruses (NPV) or cytoplasmic polyhedrosis virus (CPV) (Kachhawa, 2017). Baculoviruses, which are NPVs, have been successfully used for *Spodoptera* sp. pest management in Africa and Asia, to protect crops including cotton, soybean and vegetables. *Spodoptera litura* nucleopolyhedrovirus (SpltNPV) has been seen as a potential candidate to control *S. litura* in Japan and China (Kamiya et al. 2004), while *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV) has been shown to control outbreaks of *S. exempta* in Tanzania (Mushobozi et al. 2005).

Natural predators and parasitoids of pests are often attracted to host plants, which have been infested with a certain pest, through the release of HIPVs. *Encarsia formosa*, a chalcid wasp, parasitize the sap-sucking whitefly which damage ornamental crops and vegetables. The wasp deposits eggs into the juvenile whiteflies, and the parasitic larvae feast upon the whitefly, killing it in

the process (Hoddle et al. 1998). This natural pest-parasitoid relationship has been manipulated for use since the early 20th century (van Lenteren, 1995). Furthermore, natural enemies to pests (parasitoids and predators) and biopesticides have been used together to combat the emerald ash borer, *Agrilus planipennis*, in North America (Haavik et al. 2015). The combination of biological control agents has been shown to have a synergetic effect on the control of pests.

However, there are limitations to using biopesticides and natural enemies of pests to help control them. The application of microbial biopesticides is most commonly through foliar application. This exposes the virus to ultraviolet radiation and fluctuations in environmental conditions which damages the virus and reduces its efficacy as a biopesticide (Ghosh et al. 2018). Also, biopesticides have a lower rate of control, require multiple applications before a positive effect is seen and have a limited compatibility with other crop enhancement agents (e.g. fertilizers) (Chandler et al. 2011). Also, introducing natural enemies of pests into new environments has resulted in the invasive organisms altering the balance of the ecosystem (Johnson & Boe, 2002). Despite these drawbacks, biological agents have the potential to provide sustainable crop protection but currently they are very specific and too dependent upon environmental conditions.

1.3.2. Genetic modifications

Genetically modified (GM) crops are an alternative IPM strategy which are used to prevent crops from being overly damaged by pests. GM crops are transformed with the *cry* gene from *B. thuringiensis* and produce the endotoxins which are effective against pests. GM maize was produced to

combat the European corn borer and the corn rootworm larvae (Hellmich & Hellmich, 2012), and over seven million cotton farmers in India (corresponding to over 26 million acres) had switched to *Bt* cotton varieties by 2017 (ISAAA, 2017). Additionally, some crops have been engineered to produce elevated levels of pheromones. *Triticum aestivum* (wheat crops) have been engineered to produce higher levels of *E*-β-farnesene, an alarm pheromone which repels the target pest, *Myzus persicae* (Bruce et al. 2015).

Like with most control strategies, it is possible for the pest to develop resistance against toxins produced by transgenic plants. It took approximately six years for *Helicoverpa zea* L. to confer resistance to Cry1Ac cotton plants in the United States of America (Pan et al. 2016), and only four years for *Spodoptera frugiperda* to become resistant to Cry1F maize crops in Puerto Rico (Storer et al. 2010). To combat this, 'pyramid crops' which express two or more transgenic *Bt* toxins with a different MOA have been produced (Ives et al. 2011). These 'pyramid crops' are often utilised in the 'high dose/refuge' strategy which reduces the development of resistant pests. The process works by cultivating 'high dose' crops and refuge (no *Bt* toxin) crops in the same area. Most pests will feed and breed within the refuge area, including those that are homozygous for resistance genes against the transgenic 'high dose' crops. The refuge area increases the probability of resistant moths mating with susceptible moths, producing heterozygous moths which are susceptible to the 'high dose' crops (Hellmich & Hellmich, 2012). This resistance strategy has been successful in North America over fifteen years of intense practice (Huang et al. 2011), but has had some failures in South Africa (although the strength of the 'high dose' transgenic crops has since some under scrutiny).

There are concerns regarding the production of GM crops as the effects on human health and environmental impact are not yet known in the long-term. Countries including the United States of America, India and Brazil GM crops have integrated into the normal diet, whereas countries in the European Union, including the United Kingdom, have a firm anti-GM media stance (Key et al. 2008). Most of the negative attention surrounding GM crops and the potential impact on human health has stemmed from inappropriate scientific sources; similarly, to the vaccine-autism scandal which still has movement despite the retraction of the controversial paper (Eggertson, 2010). Nevertheless, there has been little convincing evidence of a negative impact on human health.

Likewise, the negative effects of GM crops on environmental biodiversity has been raised. The sexual hybridisation of GM crops with wild crops was presented by Quist & Chapela (2001), highlighting the contamination of wild-type maize with GM maize. However, this work was disputed and later rejected as no transgenic crop was found within the wild crop (Key et al. 2008). Furthermore, studies debating if GM crops would reduce biodiversity within the local environment have failed to produce significant results, and in fact highlighting the positive effect GM crops have had on biodiversity through the reduction of pesticides (Carpenter, 2011).

Overall, the GM crops are a new and improving strategy which have successfully reduced the amount of pesticides applied. However, difficulties result from the cost of research and production, and negative publicity from the media.

1.3.3. Other alternative strategies

There are alternative strategies to chemical pesticides which do not involve GM crops or utilising biological agents which are simpler and cheaper (excluding robotic weeding) to enforce (Table 1.3).

Table 1.3: Examples of alternative strategies to chemical pesticides for pest control.

Practice type	Example	Pests	Study
Cultural	Crop rotation	Microorganisms	Curl (1963)
	Polyculture Trap cropping	Specialist, low mobility insects	Smith & McSorely (2000)
Mechanical	Robotic weeding	Weeds	Chauman et al. (2017)
Physical	Light and colour traps	Highly mobile insects	Jørs et al. (2017)
	Barriers	Insects (excluding root dwellers)	Boiteau & Vernon (2001)

These require constant surveillance and are often implemented on a small scale due to costs endured if they are unsuccessful. However, the lack of biological or chemical agents means registration of materials is not an issue and many of these strategies can be implemented using a variety of materials.

In summary, there are a collection of alternatives to chemical pesticides for crop protection and their other uses, but these can be expensive and require high levels of skill to successfully implement the strategies. However, the positive outcomes which have been recorded in areas which have switched from chemical pesticide use to a combination of IPM strategies clearly indicate

a positive step. Continuous research is underway to discover and produce more alternatives to harmful chemical pesticides.

1.4. Antifeedants

An antifeedant, as defined by Isman (2002), '*is a behaviour-altering substance that deters feeding through a direct action on the peripheral sensilla (taste organ) in insects'.* Many of these substances are plant extracts, with over 900 having been identified. The antifeedant characteristics of these extracts reduce the damage caused by herbivorous insects, maintaining a balanced trade-off of resources between growth and immunity (Züst & Agrawal, 2017). Some plant extracts have been isolated and tested for effectiveness as an antifeedant. For example, Oswau et al. (2007) determined that methanol extracts from *Zanthoxylum xanthoxyloides (lam.)* (candlewood) provided a strong antifeedant activity against the cowpea weevil (*Callosobruchus maculatus)*. Vattikonda & Sagnam (2016) determined that azadirachtin, isolated from the *Azadirachta indica* plant, was effective at deterring *Papilio demoleus* larvae in an increasing dose-response manner. Abdel-Rahman & Al-Mozini (2007) assessed the antifeendant activity of *Rhazya stricta* exacts against *S. littoralis*. They determined the strong deterring effect was due to inhibitory effects on biological functions of the host, but not critically to result in mortality.

Overall, it appears that many, if not most, plant-based deterring products result in a sub-toxic effect on the pest, resulting in an indirect change of behaviour.

Other possible sources of deterrent products are mostly derived from organic substances, rather than inorganic salts or compounds. There has been a lack of

interest in the effect of calcium-based salts, specifically calcium chloride, on the feeding behaviour of phytophagous insects.

1.5. The importance of calcium signalling

Calcium is a key central regulator for biochemical and physiological processes in plants, and plays an integral part in growth and development, cell division, photosynthesis and intra-cellular signalling through the production of phytohormones. Plant cells are able to transduce signals of specific stimuli through cytosolic calcium concentration increase. For example, upon detection of herbivore oral secretions, Ca^{2+} influxes into the cytosol from stores. The specific calcium signatures (specific rises in cytosolic calcium from specific stimuli) allows for rapid communication for a particular response. Calcium-dependent proteins, such as calmodulin-like proteins and calcium-dependent kinases, are activated (Fürstenberg-Hägg et al. 2013) and downstream cascades through phosphorylation of kinases and grouping of transcription inhibitors stimulate the production of jasmonates. Jasmonates, an essential phytohormone involved in response to tissue wounding, work by redirecting metabolic efforts from growth and development to an immune response and repairing damage (Vadassery et al. 2012). The importance of calcium signalling in plant defence responses has been studied using Ca^{2+}-chelating protein EGTA (Mohanta et al. 2012) and bioluminescence-based aequorin technology (Xiong et al. 2014). Furthermore, Vincent (2016) used a florescent calcium sensor (GCAMP3) to significantly determine the involvement of calcium signalling in *Arabidopsis* upon attack by aphids. Huang et al. (2017) have provided an insight into the use of exogenous calcium into reduce abiotic stresses in plants, specifically surrounding phytotoxicity caused by excessive uptake of cadmium. They suggest that Ca may be used to ameliorate Cd toxicity in plants and as a

phytoremediator for Cd-contaminated soils. This provides an interesting basis of exogenous calcium application directly affecting plant processes.

1.5.1. Crosstalk

Crosstalk of the phytohormones JA and salicylic acid (a phytohormone produced in response to bacterial infection) is an antagonistic interaction. Some pests, such as the Colorado potato beetle, utilise bacteria in a mutualistic relationship. Upon wounding tomato plant, the beetle secretes *Pseudomonas* sp. which triggers the SA-dependent pathway, resulting in negative crosstalk and suppression of the JA-dependent defences. Through manipulation of the phytohormone pathways, the beetle encounters fewer defences which would hinder their feeding and fitness (Chung et al. 2013). Likewise, the activation of SA signalling in *Arabidopsis,* through *tomato-spotted wilt virus* infection, suppressed JA-dependent defence pathway, making the plant more attractive and susceptible to the viral vector, *Frankliniella accidentalis* (western flower thrip) (Maris et al. 2004). This has been replicated with JA-attenuated strains of rice plants (Li et al. 2013; Zhou et al. 2009).
In response to the substantial amount of evidence provided, it has been hypothesised that manipulation of the calcium signalling in plants may provide a path to stress-tolerant crops (Aldon et al. 2018).

1.6. Calcium Chloride

Calcium chloride has been applied through foliar application to crops both pre- and post-harvest, reducing the severity of disease in both cases. Dry beans infected with white mould (*Sclerotinia sp.*) were treated with a $CaCl_2$-$CaSiO_3$ solution 45 days after disease emergence, resulting in a decrease in disease

incidence but not an increase in yield (Júnior et al. 2009). Likewise, Wang et al (2010) found that 0.5-2% $CaCl_2$ worked well at controlling black rot in cherry tomatoes post-harvest when treated with the marine antagonist, *Rhodosporidium paludigenum*. While the correlation between calcium administration and parasitic damage reduction in the host plant is clear, and the advantageous effects of calcium being applied exogenously have been reviewed, the mode-of-action to which calcium application may trigger a defence response to herbivory has not yet been determined.

1.7. Study system

The cotton leaf worm (*Spodoptera littoralis*) is a notorious pest across Africa, Asia and parts of Europe, with the capability of long-range dispersal (Salama & Shoukry, 1974). It is a generalist pest with the capacity to target over forty different plant families of economically crucial crops (Ladhari et al. 2013), including tomato, maize, cotton and soybean (Jeschke et al. 2017). The larvae may consume several different tissue types on a single plant, including the buds, leaves and foliage. This leaves the remainder of the plant as completely unsuitable for human consumption or use (Sukirno et al., 2018). The polyphagous nature, voracity and short generation time of the *S. littoralis* larvae makes them a hugely destructive pest but also an ideal model organism for integrated pest management studies.

Grazers Ltd, a Cumbrian family business, have developed a product which allegedly protects plants and crops against herbivores, without the harming the pests or contributing to environmental degradation. The product is characterised as ‘people, pet and planet friendly’. The G3 product contains

calcium chloride (10% calcium oxide, 7.2% w/w calcium) as the active ingredient, with a surfactant (UN65), dispersant (Ultrazine) and water component (Figure 1.2).

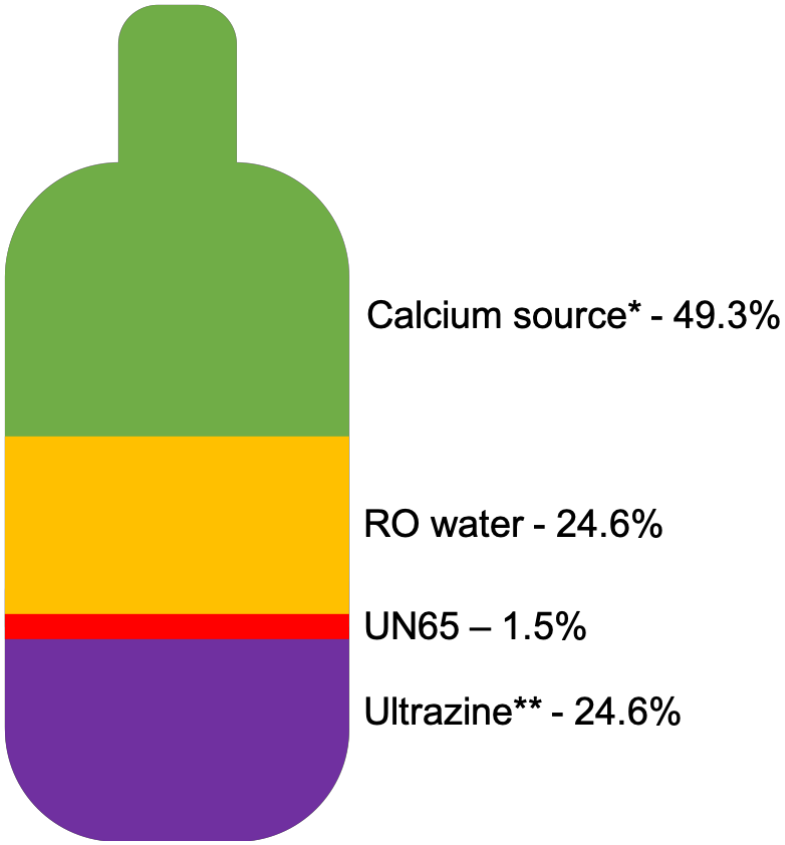

Figure 1.2: The composition of *Grazers Ltd* G3 product from concentrate. Formulations were made-up using this stock G3 product. *calcium source varies between the two formulations: 'Old' - *Stopit* stock, 'New' - new (unnamed) stock. **6.88g Ultrazine powder in 250mL RO water.

Small-scale field trials have been performed and the G3 product appears to have a deterrent effect against *Pieris rapae* larvae and other native (British) herbivorous pests against plants in the *Brassicaceae* family. However, this has yet to be replicated in a controlled laboratory environment. Additionally, *Grazers Ltd* have begun devising a new formulation of their G3 product. The

calcium chloride component in the ‘new’ formulation is obtained from a different supplier, and thus contains different contaminants.
There is evidence to support that calcium chloride may actively promote plant defence agianst microbial attack, however the effect of calcium chloride on defence responses against herbivory has not been considered.

1.8. Thesis aims

Grazers Ltd have taken advantage of the interactions between calcium chloride, the host plant and their pests. The result is a pest-deterrent product. Yet the mode-of-action of the product is not known.

The aims of this thesis were to determine if:
a. The product was toxic.
b. The product elicited a deterrent feeding behaviour in the pest.
c. Any response was through direct interaction or a plant-mediated effect.
d. The product affected behaviours other than feeding, specifically ovipositing.

In order to successfully answer the aims, the model organism, *S. littoralis*, was used. The tropical, polyphagous insect was ideal for this situation due to the short generation time from neonate to adult, large number of egg batches available from successful copulations, and large number of cultures available during the short time in which to perform the experimental segment of this thesis.

Chapter 2: Determination of the mode-of-action of the *Grazers Ltd* G3 product against generalist lepidopteran pest, *Spodoptera littoralis*.

2.0. Abstract

The present study was aimed to evaluate the mode-of-action of the proposed deterrent, *Grazers Ltd* G3 product, against the generalist pest, *Spodoptera littoralis*. Non-choice feeding tests were performed using semi-artificial wheat-germ diet cubes and wheat plants which were treated with increasing concentration of product via foliar application. There were no significant effects of the product on the survival or development of the larvae in either case. Next, two-choice feeding trials were performed with semi-artificial wheat-germ diet cubes, wheat leaves and salad rapeseed leaves. A significant antifeedant response was the product was applied to the wheat and salad rapeseed plants, but not the diet. This suggested a plant-mediated antifeedant response. When the product was broken down into the individual components, a single active component was not determined, indicating a possible synergistic effect. The product was also tested for dermal contact toxicity when applied to the surface in which the larvae resided, and a $LC_{50\ of}$ 6.91% was established. This highlighted the importance of correct application procedures.

Overall, the *Grazers Ltd* G3 product was found to have antifeedant effects on *S. littoralis* larvae in a two-choice setting, without having any significant implications on survival or development.

2.1. Introduction

The use of chemicals as a means to defend human livelihood has occurred for generations, with the production and application of artificially-made pesticides soring in over the past century (Romero, 2011). With this spike, the negative implications have also begun in arise. Some chemical insecticides have damaging effects on non-target organisms, including humans (Xavier et al. 2015). Moreover, the impact of global warming through carbon dioxide emissions from pesticide production, further aggravate this challenge (Wheeler & Braun, 2013); as the temperature of the planet increases, the stability of food systems will fluctuate, and the already-vulnerable areas for poverty, hunger and neglected diseases will be at risk of further exacerbation (Seligman et al. 2010). Climate change may also increase the possibility of successful establishment of pests in new, unsuitable regions (Bebber et al. 2013).

2.1.1. Chemical pesticides

Chemical pesticides, such as pyrethroids and organochlorines, are utilised worldwide (Costa, 2015). They are used on home-grown plants, commercial agriculture, and disease-eradication programmes. As such, exposure to pesticides and subsequent damage to human health is inevitable. Chronic effects as a result of exposure include abnormal hepatic functions, nervous system disorders (Hu et al. 2015), and hormone-related cancers (Alavanja, 2009). Chemical pesticides do not just damage human health, but the surrounding terrestrial and aquatic environments. Biodiversity has had a measured loss in areas where the non-specific pesticides are used. Beketov et al. (2013) found that the number of species declined with increasing pesticide exposure levels (Figure 2.1).

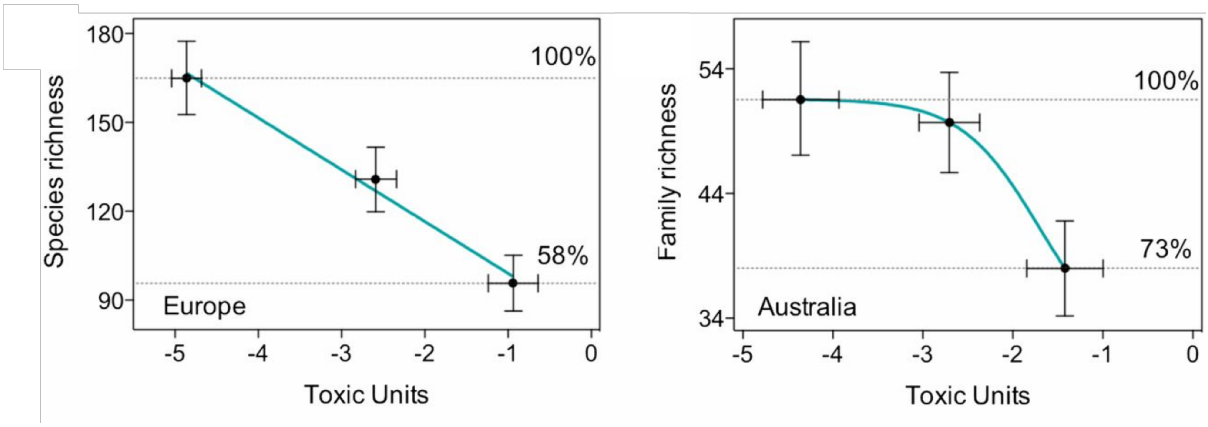

Figure 2.1: Species richness measure at European site, and family richness at an Australian site, after exposure to increasing levels of pesticide toxins. Linear regression (Europe) and log linear regression (Australia) was used to illustrate trend, while error bars are 95% confidence intervals. Dashed lines indicate minimum and maximum mean richness. Diagram from: Beketov et al. (2013).

2.1.2. Integrated pest management: Deterrents

As a strategy to reduce the amount of chemical pesticides utilized worldwide, IPM programs have been deployed to find suitable alternatives. One such possible solution involves the study of deterrents: chemicals that inhibit the normal behaviours, such as feeding or oviposition, of an organism at the site of application where such behaviours normally occur (Foster & Harris, 1997). Plant-based deterrents with consumption behaviour-changing qualities (also known as antifeedants) have been extensively studied at laboratory level, due to the well-documented literature surrounding their mode-of-action and bioactivity on host organisms. Antifeedants are secondary metabolites produced by plants but not used in the primary functions of growth, photosynthesis or propagation. Instead, it is hypothesized that secondary metabolites have evolved as a means to protect the immobile plant against pathogens and herbivores (Wink, 1988). However, how one insect species may respond to a specific plant allomone may have a completely different outcome

in a separate species. Furthermore, varying concentrations of an antifeedant have also been noted to have a different outcome on a single species (Koul, 2008). For example, János & Nádasy (2005) assessed the effect of 0.5% and 5% doses of an methanolic extract from *Matricana inodora* on the feeding behaviour of the Colorado potato beetle. Only the 5% dose resulted in an antifeedant effect and through behavioural observations and antifeedant indexes, it was suggested that *M. Inodora* acts as a sensory-mediated feeding deterrent rather than at a sub-toxic level. Alternatively, Yuan & Hu (2012) determined that an 0.212 mg/cm^2 dose of chloroform extract from dried *Lantana camara* leaves exhibited a toxic effect against the subterranean termite, *Reticulitermes flavipes*, with a mortality rate of over 90%, yet at a lower dosage of 0.106 mg/cm^2, a sub-toxic effect was recorded through a 40% feeding reduction. This highlights the number of different mechanisms involved in the feeding behaviour modification, and the difficulties of distinguishing between deterrent (inhibit normal behaviour at source) activity and repellent (active discouragement away from the source) activity (Maia & Moore, 2011).

Further propagation of antifeedant discovery in IPM systems has led to researching the chemical structures of naturally occurring antifeedant allomones. Many plant secondary metabolites, including the renowned neem compound from the *Azadirachta indica*, are complex, chiral-centred compounds made-up from large molecules and no direct structural link has been made to feeding deterrency (Pernak et al. 2013). Active synthetic compounds with simpler structures have also been studied in regard to antifeedants effects. Hilker et al. (2010) determined strong antifeedant effects of a naturally occurring dipeptide produced by the leaf beetle larvae

(Chrysomelidae). The larvae produce the dipeptide in defensive secretions to ward off predators. The secretions are stable, non-volatile, water soluble compounds. The progression of stable antifeedants has evolved over the years from from naturally-occurring compounds to synthetic products, with ionic liquids now also being considered. Pernak et al. (2013) tested seventeen different quaternary ammonium salts as antifeedants against *Trogoderma granarium* larvae and adults and determined that ionic liquids with larger cations appeared to have a stronger deterrent effect. The possibility that deterring compounds could be cheaply manufactured rather than expensively synthesised from plants has increased interest in their production (Pernak et al., 2012).

2.1.3. Calcium chloride

Most common and efficient antifeedants are extracts or oils from plants, rather than salt-based products. Therefore, there has been little research into the antifeedant effects of calcium chloride salts. There have been noted incidences of $CaCl_2$ application reducing infection and establishment of blister blight in tea (Chandra et al. 2014) through the induction of the plant defence response. Similarly, foliar application of $CaCl_2$ has been seen to reduce white mould intensity in dry beans (*Phaseolus vulgaris*), yet crop yield did not increase with $CaCl_2$ application, unlike crops treated with a fungicide (Trazilbo et al. 2009). Nevertheless, the efficacy of $CaCl_2$ as a pest antifeedant in plants has not been researched.

Calcium chloride contains two elements which, in the optimal concentrations, are a necessity to plant growth and survival. Calcium, typically in cationic form, is a critical player in plant cell division (through the formation of the mitotic

spindle) and membrane integrity. It is a macronutrient required at 123 mmol kg^{-1} (dry shoot weight) for adequate growth (George et al. 2007). If plants become calcium deficient, Ca is unavailable in new and developing tissues. This leads to conditions such as 'cracking' in fruit, 'bitter pit' in apples, and 'tip burn' in leafy vegetables. Conversely, excessive calcium leads to calcium toxicity which reduces plant growth, germination and increases accumulation in cell walls, leading to 'gold spotting' in fruit (White & Broadley, 2003).

Chlorine, typically in anionic form, is found at much lower concentrations of 3 mmol kg^{-1}, making it a micronutrient. Chloride ions are required in photosynthesis, for the water-splitting process in photosystem II, as well as maintaining turgor and osmoregulation (George et al. 2007). Plants which suffer from chloride deficiency display wilting, stunted fruit development, leaf burning and desiccation. Meanwhile plants suffering from chloride toxicity succumb to membrane instability and chlorosis, which stunts growth and results in leaf death (Tavakkoli et al. 2010).

Both calcium and chlorine are imperative to plant success, however the fine balance of their concentrations are continuously controlled to maintain equilibrium within the plant. The internal environment is influenced by the availability of these nutrients in the soil. Calcium ions in the soil aid in reducing heavy metal toxicity and high salinity through the absorbing of heavy metal elements to soil particles, subsequently increasing hardness of the water in the soil. On the other hand, chloride ions reduce soil permeability and fertility due to facilitating the release of these metal ions from soil particles and increasing salinity of the soil (White & Broadley, 2001).

Crop success is heavily dependent on salt tolerance, which soil salinity stress limiting productivity worldwide. As such, if foliar application of calcium chloride for antifeedant purposes occurs, one would need to consider the effects on other plant processes; reducing herbivory but also reduces crop yield would not be advantageous.

Calcium chloride application has been seen to reduce drought stress in zoysiagrass (Xu et al. 2013) and tea plants (Upadhyaya et al. 2011) when applied via foliar application. On the other hand, $CaCl_2$-based de-icers which are applied to roads via foliar application have been shown to cause tree die-back and browning on vegetation lining the highways due to vehicle salt spray (Bryson & Barker, 2006). Soil salinity is highly affected by foliar application of salts, therefore if considering this method of application for $CaCl_2$, the effect on the entire plant should be further studied.

2.1.4 Aims

The *Grazers Ltd* G3 product is a commercially-available $CaCl_2$ product, complete with an adjuvant and dispersant. The product has been previously used as a fertiliser yet a reduction in herbivorous damaged was noticed as. A side-effect.

The aim of this research was to determine the presence or otherwise of an antifeedant effect when the G3 product was applied, and if present, the mode-of-action which elicits the change in behaviour in the pest.

2.2. Materials and Methods

2.2.1. Insects

The *Spodoptera littoralis* (cotton leafworm) culture used was initially established in 2017 with egg batches collected near Alexandria, Egypt. High number have been maintained in culture, with efforts to reduce inbreeding. For this experiment, two blocks (experimental procedure repeated on two occasions to increase sample size) had larvae from a single mating pair. Larvae were then reared from egg stage to the necessary instar on wheatgerm-based semi-synthetic diet (Cotter, 2002) in 25mL plastic polypots, and then transferred to empty polypots 24 h prior to the experimental beginning and incubated at 27°C in a 16 h:8 h light:dark period. Insects were starved for this procedure to reduce the presence of non-experimental foodstuffs (wheatgerm from rearing) creating variability in the results.

2.2.2. Plants

Triticum aestivum (wheat) seeds were germinated in a moist filter paper-lined 90mm Petri dish, incubated for 3 days with a 22°C/14 h: 20°C/10 h day:night cycle. Upon germination, seeds were transferred to 20cm pots of M3 compost (Levington Horticulture, Suffolk, UK), watered every other day and incubated in a controlled environment room with a 22°C/16 h: 20°C/8 h day:night cycle. Plants were utilised at Stage 5 (leaf-sheathes strongly erected) of the Feekes scale (Large, 1954).

Brassica napus ssp. *Pabularia* (salad rape) seeds were used as a model plant in this system due to its fast growth, large leaf area and attraction to fertile female moths. Seeds were planted in plastic trays (5 x 5 x 5 cm), grown in M3

compost (Levington Horticulture, Suffolk, UK), and watered every other day. Plants were grown in a controlled environment incubator, with a 22°C/16 h: 20°C/8 h day:night cycle. Plants were grown until 2-weeks old and those with 3-4 fully expanded leaflets were chosen.

2.2.3. Measuring direct exposure effects on mortality

Treatments of the *Grazers Ltd* G3 product was made-up to concentrations in Table 2.1. Single 6th instar larvae were placed in a 60mm Petri dish containing 0.5mL of product evenly spread out on the inner surface. The larvae were incubated at 27°C in a 16h:8h light:dark cycle for 24 h. Surviving larvae were transferred to polypots of wheatgerm-based artificial diet for a further 3-days. Mortality and growth were recorded at 2 h, 24 h, 48 h and 72 h.

Table 2.1: Dermal contact assay set-up with 6th instar *S. littoralis* larvae and *Grazers Ltd* G3 product in a dose-response manner (n=5 per treatment).

Treatment (%)	G3 volume (mL)	RO water (mL)
0	0	5
1	0.05	4.95
2.5	0.125	4.875
5	0.25	4.75
6	0.3	4.7
10	0.5	4.5
25	1.25	3.75

2.2.4. Treated diet-plugs in non-choice feeding assay for toxicity.

A non-choice dose-response assay was conducted to determine if the product itself is toxic to the pest through direct ingestion. Product concentrations of 0.02% up to 19% were used, in addition to a control of RO water. To ensure the diet-plugs containing the product were ingested fully, without any deterrent

effects hindering the feeding behaviour, 0.05g sucrose was added to the product mix to increase palatability of the diet-plug to the larvae (see Table 2.2) (Salama et al, 1984).

Table 2.2: *Grazers Ltd* G3 treatments, made up using below concentrations or a 10-dilution series. These treatments were used in the wheat-germ diet cubes feeding trials.

Treatment	Dose (%)	G3 product (mL)	RO water (mL)	Sucrose (g)
Control	0.00	0	10	0.05
1	0.02	0.002	9.998	0.05
2	0.06	0.006	9.994	0.05
3	0.19	0.019	9.981	0.05
4	0.60	0.06	9.94	0.05
5	1.90	0.19	9.81	0.05
6	6.00	0.6	9.4	0.05
7	19.00	1.9	8.1	0.05

Diet plugs were made using smooth semi-artificial wheatgerm-based diet and a mould to give a constant size. Ten plugs were weighed to determine the average weight of 0.002g. Single diet plugs were placed in 96-well plates, treated with 1μL of product or the control, sugar-water only (n=30 per treatment). 3rd instar larvae (starved 24 h previously) were placed in each well with a diet plug, then the plate was sealed with parafilm. The plates were placed in a plastic bag, complete with a wet cloth to maintain humidity, and incubated at 27°C in a 16 h:8 h cycle for 24 h. After 24 h, those that had eaten the entirety of the diet plugs were transferred to untreated polypots containing wheatgerm-based diet; mortality, pupation and growth was recorded on days 1, 4, 8, 11 and 14.

To compare the overall mortality rate of each treatment, the following equation was used.

$$Mortality\ \% = \frac{Total\ number\ of\ deaths\ per\ treatment}{Total\ number\ of\ larvae\ per\ treatment} \times 100$$

Equation 1: Mortality percentage equation.

The effect of the treatments on developmental retardation was determined using pupation percentage (%) and relative growth rate (RGR) as below:

$$Pupation\ (\%) = \frac{Total\ number\ of\ successful\ pupations\ per\ treatment}{Total\ number\ of\ larvae\ per\ treatment} \times 100$$

Equation 2: Pupation percentage equation.

$$RGR = \frac{\ln Weight\ (\mathrm{g})_2 - \ln Weight\ (g)_1}{Time\ (days)_2 - Time\ (days)_1}$$

Equation 3: Relative growth rate (RGR) of *Spodoptera littoralis* after 14 days of feeding on a treated or control diet plug/ leaf. $_1$initial reading, $_2$final reading.

The LC_{50} (concentration at which 50% of the test group die) was also determined using the logit-dose model (Helps et al. 2017). Equation 4 displays the logit function using log dose data.

$$logit(m) = a + b\log(D)$$

Equation 4: Logit of mortality function where *m* is mortality over the time period, *D* is dose applied, *a* is intercept, *b* is the slope of the logit-dose curve.

2.2.5. Treated leaves in non-choice feeding assay for toxicity.

Twenty-four hours prior to the experiment, *Grazers Ltd* G3 product at the commercially recommended concentration (6%) was applied to the foliage of two winter wheat plants, via foliar spray application at a distance of 10 cm,

until just before run-off. One hour prior to the experiment, the product was applied to a different set of two plants in the same way. Also 1 h prior, two final plants were treated with RO water only to act as controls.

Wheat leaves (n=10) from each treatment were removed and fresh weight taken for each. The bottom of each leaf was placed in an Eppendorf tube with the lid removed, which was filled with distilled water and covered with several layers of parafilm, then placed in a 15cm centrifuge tube (Figure 2.2). Initial weight of the starved 3rd-instar larvae was taken, then each was introduced to a single wheat leaf. Tubes were incubated at 27°C in a 16 h:8 h cycle for 72 h. Fresh leaf weights, larval weights and mortality was recorded at 24 h intervals.

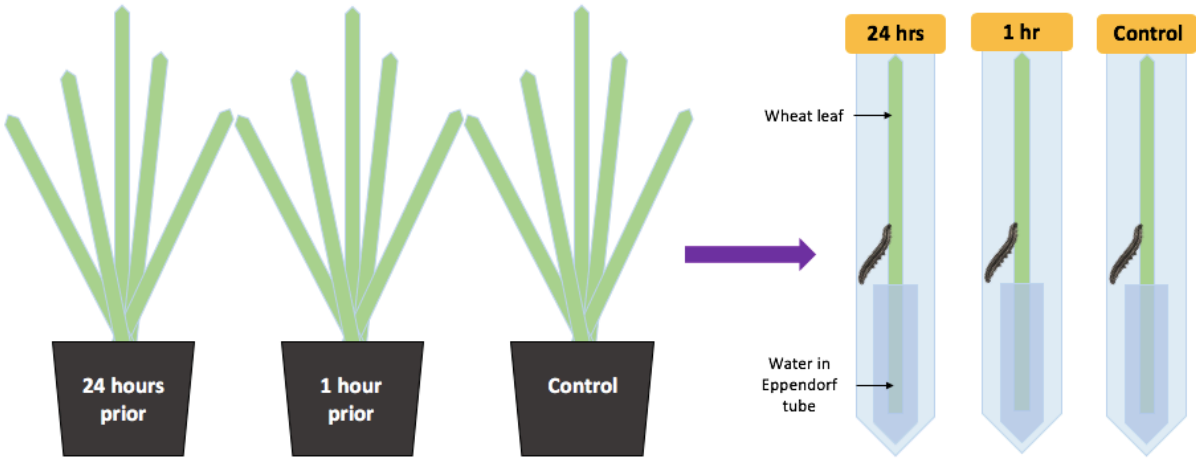

Figure 2.2: Set-up of non-choice test with winter wheat plants (stage 5, Feekes scale) and *Spodoptera littoralis* larvae (3rd instar).

As above, the mortality rate and relative growth rate were determined. In addition, relative consumption rate of the leaves was determined using equation 5.

$$RCR = \frac{\ln(Leaf\ fresh\ weight\ (g)_1) - (\ln Leaf\ fresh\ weight\ (g)_2)}{Time\ (days)_2 - Time\ (days)_1}$$

Equation 5: Relative consumption rate (RCR) of *Spodoptera littoralis* after 72 hours of feeding on a treated or control wheat leaf. $_1$initial reading, $_2$final reading. Calculated using equation from Lariviere et al. (2015).

2.2.6. Treated diet-plugs in a two-choice feeding assay for deterrency.

A two-choice dose-response assay to determine the antifeedant effect was performed using the treatments from Table 2.2, excluding the addition of sucrose. Two batches of smooth wheatgerm-based diet were produced, as per the method proposed by Cotter (2002), with the additional step of grinding the wheat-germ into a fine powder. This made the diet easier to cut to the correct 0.6 cm^3 size (1.00g $\pm$ 0.5g). In addition, blue food dye was added to the control batch and red food dye was added to the future treated batch; this allowed for easy identification of the diet cubes if the larvae moved them around the 60mm petri-dish (Figure 2.3).

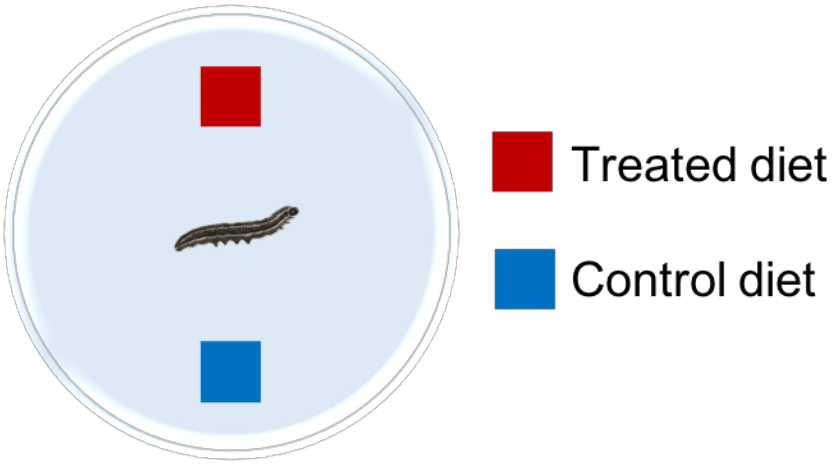

Figure 2.3: Set up of the two-choice feeding test with smooth semi-artificial wheatgerm-based diet cubes and *S. littoralis* (3rd instar).

50µL of the control or treatment solution was added to the corresponding cube, left for 15 minutes to absorb, then a starved (24 h prior) 3rd instar larvae was introduced. Larvae were left to feed for 3 days, with larval weight, and diet

plug weights were taken daily. Frass was removed every day to reduce contamination. The weight of the foodstuff was recorded and the antifeedant index (AFI) was determined as a measure of deterrency.

The per cent antifeedant index was calculated based upon the formulation of Arivoli & Tennyson (2013) as a measure of deterrency (Equation 6).

$$AFI = \frac{(Control\ diet - treated\ diet)}{(Control\ diet + treated\ diet)} \times 100$$

Equation 6: Per cent antifeedant index (AFI).

The effective dose (ED_{50}) was also calculated using the feeding inhibition rate (Equation 7) against the weight of diet consumed in a linear regression model (as described by Morimoto et al (2006)).

$$FI\ \% = (50 - AFI)\ \times\ 2$$

Equation 7: Feeding inhibition rate (%) of Grazers Ltd G3 product in a dose-response manner.

2.2.7. Treated leaves in a two-choice feeding assay for deterrency.

2.2.7.1. Wheat plants

Using the same treatment set-up as in Section 2.2.5., the post-treatment wheat leaves were removed and cut into 1cm-long cuttings and placed in a 60mm^2 Petri dish (Figure 2.4). A moistened piece of filter paper was also added to stop the leaves drying out.

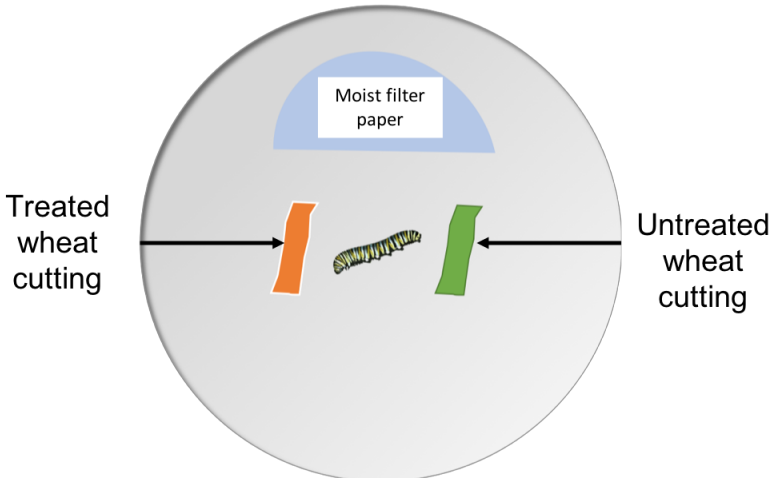

Figure 2.4: Set-up of two-choice feeding test with wheat cuttings (stage 5, Feekes scale) and *S. littoralis* (3rd instar).

3rd instar larvae were starved 24 h prior, weighed, added to the Petri dish, and left to feed for 6 h. Larvae and leaf cuttings were reweighed every 2 h. The antifeedant index was calculated as a measure of deterrency (Equation 6).

2.2.7.2. Salad rapeseed plants

Using a similar set-up to Section 2.2.7.1., salad rape leaves were treated with *Grazers Ltd* G3 product or the components (Table 2.3). Fully expanded leaves were then removed and cut into 1cm^2 leaf squares, then placed in a 60mm^2 Petri dish. A moistened piece of filter paper was added to stop the leaves drying out.

Table 2.3: Treatments used in two-choice test for deterrence with salad rape plants and *S. littoralis* pest. All concentrations made-up to commercial concentration of 6%, using RO water for dilutions.

	Label	Treatment	Concentration (%)	Comparison	Concentration (%)
A	Control	RO water	-	RO water	-
B	'Old' formulation	G3 Product ('Old' formulation)	6%	RO water	-
C	Surfactant	Surfactant	1.478%	G3 product ('Old' formulation)	6%
D	Dispersant	Dispersant	0.089%	G3 product ('Old' formulation)	6%
E	'New' vs. 'Old'	G3 Product ('New' formulation)	6%	G3 product ('Old' formulation)	6%
F	Calcium (1)	Calcium source	2.956%	G3 product ('Old' formulation)	6%

3rd instar larvae were starved 24 h prior, weighed, added to 60 mm^2 petri dishes and left to feed for 6 h. Leaves and larvae were reweighed every 2 h, and the antifeedant index was calculated as a measure of deterrency (Equation 6).

2.2.8. Statistical analysis

Statistical analysis performed wholly using 'R' (Version 3.5.0) and 'RStudio' (Version 1.1.453), using dplyr (Wickham et al. 2018), ggplot2 (Wickham, 2009) and ggfortify (Tang et al. 2016) packages. Data was presented using ggplot2.

Mortality was analysed using the general linear model with binomial regression if the variable was numeric (concentration of product). Data was transformed to meet normal distribution assumptions. For data which was categorical, mortality was determined using a Pearson's chi-squared test.

Development indices were analysed using a one-way analysis of variance (ANOVA) (if normally distributed) with post-hoc Tukey tests or the Kruskal-Wallis test (non-parametic) with Mann-Whitney pairwise comparisons.

Categorical data was analysed with a one-way analysis of variance (ANOVA) and post-hoc Tukey test (if normally distributed) or the Kruskal-Wallis test (non-parametic) with Mann-Whitney pairwise comparisons.

For the final analysis of product components, each component was analysed against the control in a student's independent t-test.

2.3. Results

Evaluation of the *Grazers Ltd* product on *S. littoralis* when ingested or applied to the abdomen of larvae was performed to determine its mode-of-action. In addition, the effectiveness of each component in the *Grazers Ltd* G3 product at altering the feeding behaviour of the larvae was tested to address the questions: Is the deterring effect the result of a single component or is it an additive or synergistic effect?

2.3.1. Dermal contact assay for mortality

In this experiment, the mortality of larvae increased with concentration of product (Figure 2.5). At concentrations above 2%, larvae started to die, leading to 100% mortality in the 25% product treatment group.

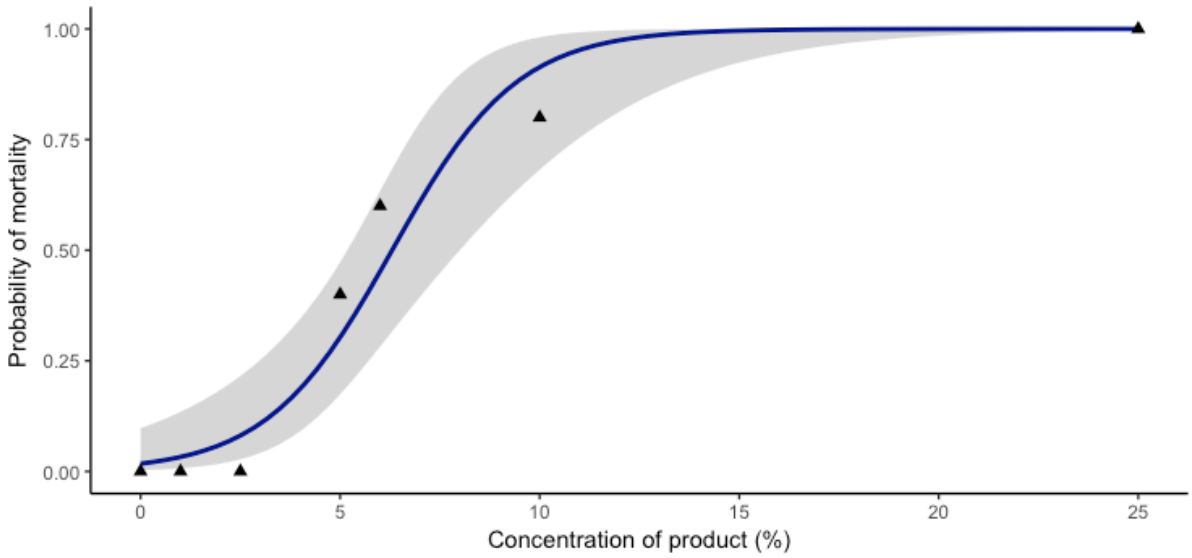

Figure 2.5: Effect of Grazers Ltd G3 product on mortality after 24-hours exposed to treatment via dermal contact. 6th instar larval death was recorded after 3-days (post-movement to fresh diet). Total sample size = 30 larvae per treatment, ▲mean percentage per concentration, shaded area illustrates 95% confidence intervals, solid line illustrates the best fitted model.

The resulting relationship was significant (z=2.684, df=33, p=0.007), indicating that with an increase in concentration by 1%, the probability of larval death increases by 9.99%. The LC_{50} recorded was 6.91% concentration.

2.3.2. Non-choice feeding assays for toxicity

The initial non-choice feeding assay to determine the direct effect of the product when applied to diet plugs and ingested was performed in a dose-response manner.

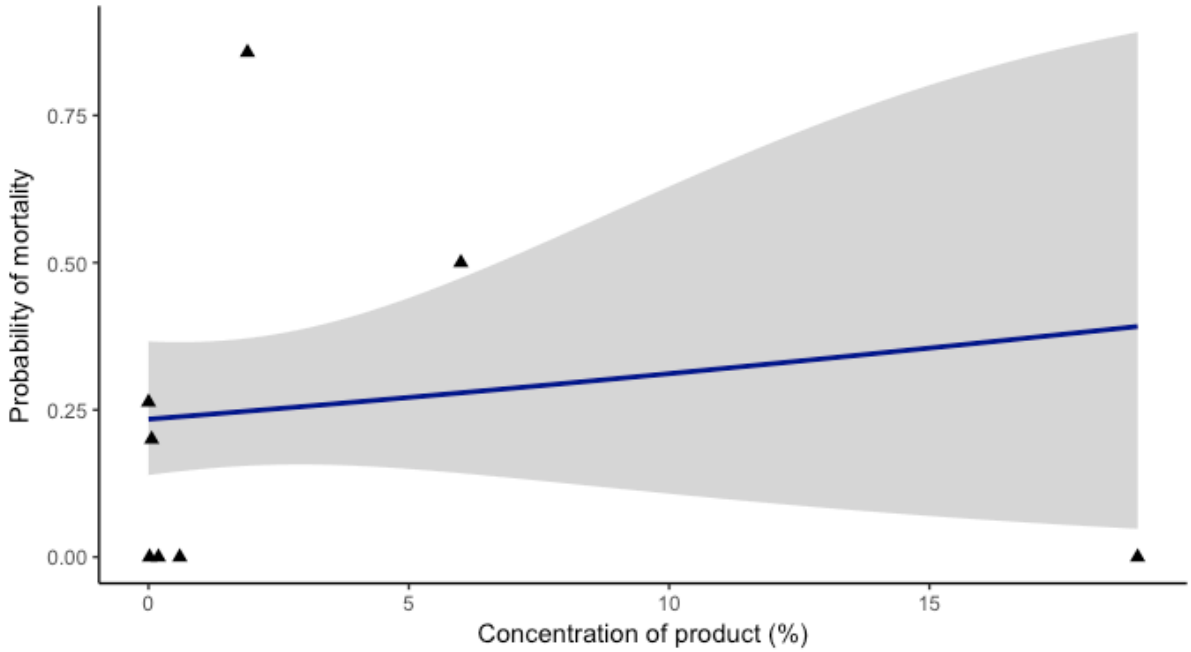

Figure 2.6: Effect of Grazers Ltd G3 product, in a dose-response manner, on 3rd instar *Spodoptera littoralis* larvae when ingested via a wheat-germ diet plug. Total sample size = 30 larvae per treatment, ▲mean percentage per concentration, shaded area illustrates 95% confidence intervals, solid line illustrates the best fitted model.

Mortality rate was highest in the 1.9% treatment group, while the 19% treatment resulted in no mortality recorded (Figure 2.6). 26.3% of the control group died within the 14-day period from natural causes. Overall, there is no significant correlation with an increasing concentration and mortality (z=1.848, df=59, p=0.06, generalised linear model with binomial distribution) and the experimental conditions may have contributed to a slightly higher mortality rate than expected.

In addition to mortality, total larvae growth, pupation probability, and RGR were all calculated (Table 2.4) in order to determine whether the product caused developmental retardation through subtoxic effects.

Table 2.4: Effect of *Grazers Ltd* G3 product on larvae in a dose-response manner via diet plug consumption in a non-choice manner. Mean result ± SE. Statistical significance determined by one-way ANOVA.

Treatment (%)	N	Total Larvae growth (g)	Pupation (%)	Relative growth rate (g day^{-1})
0	30	0.55±0.03	73.68±10.38	0.07±0.01
0.02	30	0.41±0.06	90.00±10.00	0.10±0.004
0.06	30	0.56±0.07	80.00±20.00	0.07±0.02
0.19	30	0.52±0.06	100.00±0	0.09±0.02
0.6	30	0.43±0.47	100.00±0	0.12±0.02
1.9	30	0.58±0.01	14.29±14.30	0.07±0.003
6	30	0.40±0.02	100.00±0	0.11±0.01
19	30	0.54±0.08	100.00±0	0.09±0.02
Statistical significance		NS	NS	NS

Overall, there was no significant effect of product concentration on either of the three measures of development ($p>0.05$). This indicates that energy required for growth was not diverted to the immune system as the product was non-toxic.

Table 2.5 shows the effect of *Grazers Ltd* G3 product at the commercial (6%) concentration on 3rd instar *S. littoralis* larvae when applied to wheat plants either 24 h or 1 h prior to feeding.

Table 2.5: Effect of *Grazers Ltd* G3 product on *S. littoralis* larvae when administered via treated wheat leaves over a 72-h period, in a non-choice setting. Mean result ± SE. Statistical significance determined using °one-way ANOVA or *Kruskal-Wallis non-parametric test. NS - non-significant.

Treatment	Total fresh leaf eaten (g)	Larval growth (g)	Relative growth rate (g day^{-1})	Relative consumption rate (g day^{-1})	RGR:RCR
Control	0.08±0.01	0.04±0.01	0.24±0.03	0.64±0.07	0.38±0.05
1 hour	0.07±0.01	0.06±0.004	0.34±0.02	0.71±0.07	0.49±0.04
24 hours	0.08±0.01	0.06±0.01	0.34±0.06	0.79±0.09	0.44±0.07
Statistical significance	NS°	NS°	NS°	NS*	NS°

No significant relationships between the different treatment groups and the nutritional indices were found. This indicates that there was no effect of treatment on possible developmental retardation over the 3-day period. A higher result indicates that most of the energy obtained from feeding is used for growth, whereas a lower result would indicate a trade-off of energy from growth to an immune response.

In a similar fashion to the non-choice assay with diet plugs, the significance of treatment on larval survival (measured as mortality) was assessed (Figure 2.7). The resulting relationship between treatment and survival was non-significant ($\chi 2 = 0.74$ df=2, p=0.69; Pearson's chi-squared test), indicating that treatment type does not affect survival.

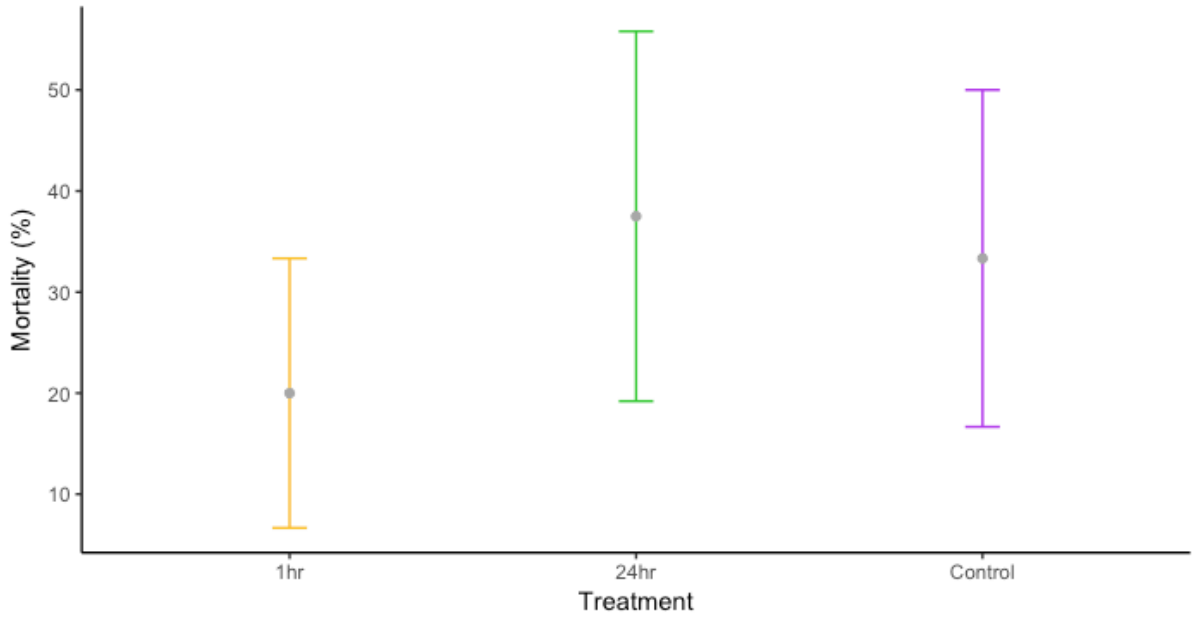

Figure 2.7: Effect of Grazers Ltd G3 product at commercial dose (6%) applied via foliar application either 1 h or 24 h prior to feeding by *Spodoptera littoralis* in a non-choice setting. Toxicity of each treatment was determined through mortality (%) after 72 h of feeding. Results are mean±SE.

2.3.3. Choice feeding assays for deterrence

After determining the lack of toxic effects of *Grazers Ltd* G3 product on 3rd instar *S. littoralis* larvae, two-choice feeding assays were performed using control and treated smooth wheat-germ diet cubes, wheat leaves and salad rape leaves. The weight of the foodstuff was recorded and the antifeedant index (AFI) was determined as a measure of deterrency.

There was no significance to suggest that *Grazers Ltd* G3 product had an antifeedant effect on the larvae when applied to diet cubes ($F=2.28$, $df=1,76$, $p=0.14$) (Figure 2.8). The control group had an AFI reading of 9.52±9.01 (mean±SE), indicating that there was an effect on feeding behaviour without

the product, most likely as a result of the unnatural experimental set-up or the small sample size (n=5) being unable to account for random variation.

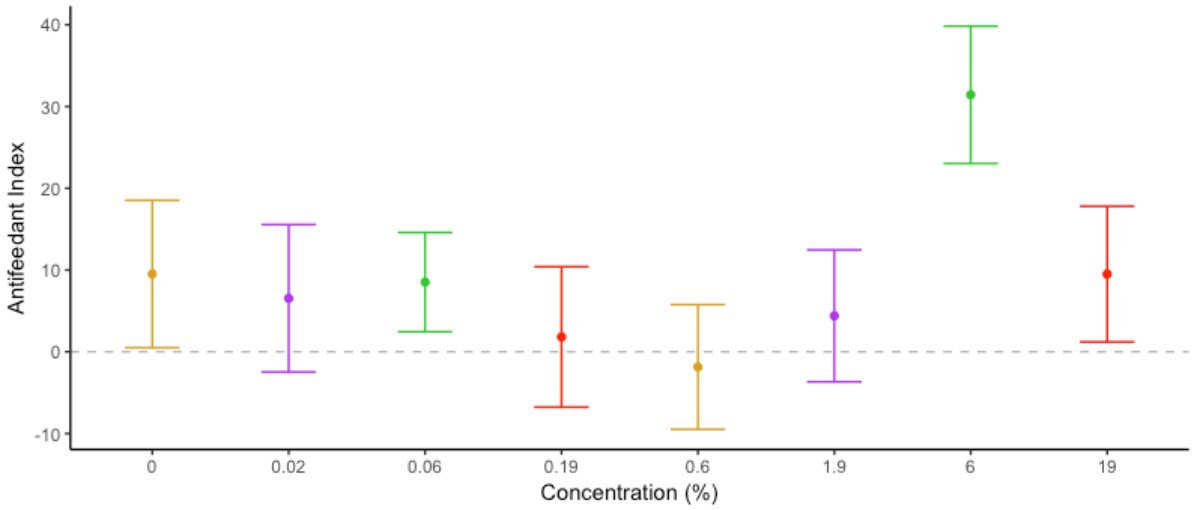

Figure 2.8: Effects of Grazers Ltd G3 product in a dose-response manner (n=5 per treatment) applied to smooth wheatgerm-based diet plugs and fed to *Spodoptera littoralis* in a two-choice setting. The antifeedant index (AFI) was determined as a measure of deterrency. Results are mean±SE.

However, there was no clear trend that an increase in product concentration elicited a response. Therefore, it be accepted here that the presence of the product applied to diet cubes did not alter feeding behaviour in *S. littoralis*.

Following the diet cube two-choice assay to determine any direct effect of feeding behaviour, two-choice plant feeding assays were performed using wheat plants and salad rape plants. Figure 2.9 displays the final AFI results for wheat plants treated with the commercial 6% dose either 1-hour or 24-hours prior to feeding. *Grazers Ltd* G3 product provoked a strong antifeedant effect in larvae when applied to wheat leaves ($F=7.2747$, $df=2, 57$, $p=0.0015$). Post-hoc comparisons using the Tukey HSD test indicated that the mean AFI for the control group (10.35±15.90) was significantly different than the 1 h group

(52.44±8.13, p=0.056) and the 24 h group (75.23±11.31, p=0.001), however the latter treatments were not significantly different from each other (p=0.39).

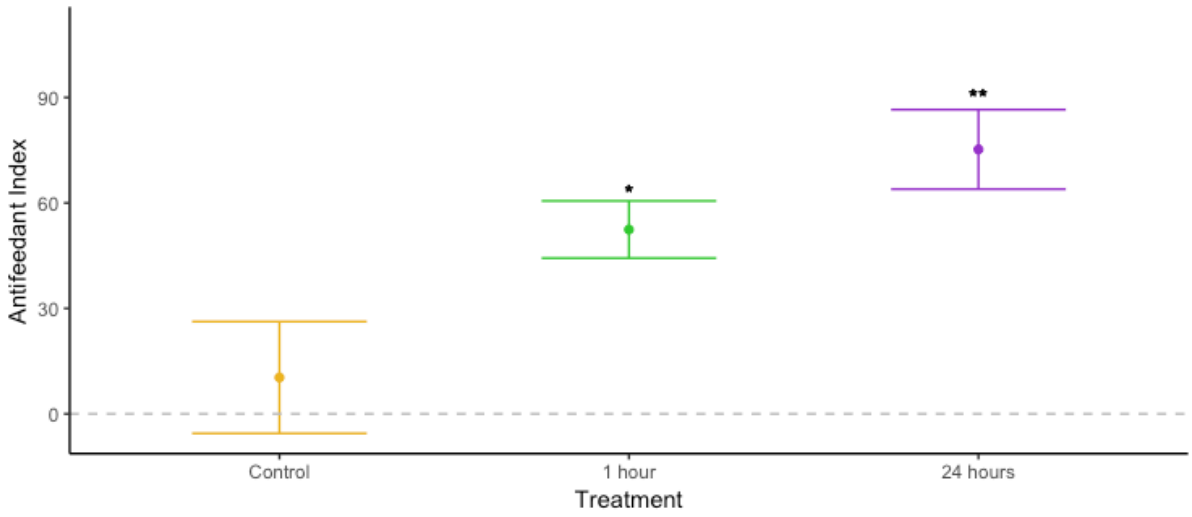

Figure 2.9: Effects of Grazers Ltd G3 product at commercial (6%) dose when applied to wheat plants (n=20 per treatment) via foliar application on *Spodoptera littoralis* in a two-choice setting. The antifeedant index was determined as a measure of deterrency. Results are mean±SE. *significant to p<0.05, **significant to p<0.01, as determined with post-hoc Tukey Test against the control group.

Ultimately, the presence of *Grazers Ltd* G3 product at commercial (6%) dose reduces the feeding behaviour of *S. littoralis*, when applied via foliar application to wheat plants either 1-hour or 24-hours prior to feeding.

To further determine the mode-of-action of the product, the two-choice assay was repeated using salad rape plants (Figure 2.10).

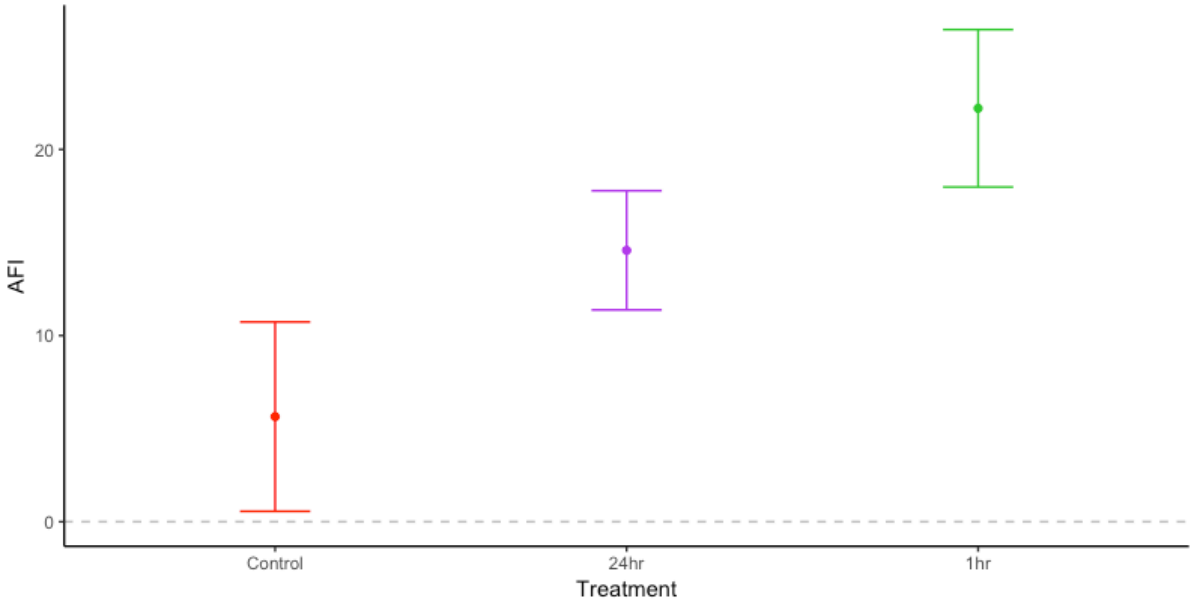

Figure 2.10: Effects of Grazers Ltd G3 product at commercial (6%) dose when applied to salad rapeseed plants via foliar application on *Spodoptera littoralis* in a two-choice setting. The antifeedant index was determined as a measure of deterrency. Results are mean±SE.

A significant response was determined between treatment type and AFI ($F=3.814$, $df=2,82$, $p=0.026$) yet when a post-hoc Tukey test performed, only the 1-hour treated gave a significant result ($p=0.019$). Different plant types used result in a different antifeedant response in the pest.

In order to determine which component of the product was the active ingredient, the full formulation was broken into each component: surfactant, dispersant and the calcium source. These components were tested against the full formulation to see if the antifeedant effect of the full product was matched by any individual component. Additionally, a potential new formulation (with a new source of calcium) was tested against the full product to determine if the antifeedant activity was just as strong (Table 2.6).

Table 2.6: Effect of treatment on the antifeedant index as a measure of antifeedancy against pest *S. littoralis* when applied to salad rapeseed leaves via foliar application. Full formations, original product components and a potential new formulation were tested against a water control or the full formulation (with antifeedant effect).

Treatment	Against	AFI	Significance°
Control	Control	5.6±5.1	-
24-hour°°	Water	14.6±3.2	ns
1-hour°°	Water	22.2±3.2	*
Surfactant	Old Product	-24.2±4.2	ns
Dispersant	Old Product	-31.7±14.3	ns
Calcium source	Old Product	-8.9±7.1	ns
New Product	Old Product	-3.5±10.4	ns

°t-test against the against control treatment.

°°Time between foliar application and experimental start.

Despite the full formulation exhibiting an antifeedant behavioural response in the pest during the two-choice feeding set-up, when the product is broken into the three components, a similar effect is not seen. Each component was paired with the full product and if it exhibited an antifeedant response, the AFI would have been 0 (if equal to the full product) or 0< (if stronger than the full product). As the recorded AFI were negative, this implies the full product elicited an antifeedant response over the individual components. As such, no individual active component was determined which suggests the product may work through synergism, in which the combination of components results in a stronger antifeedant effect than the components individually.

Furthermore, the AFI of the new formulation against the old formulation not significant, suggesting no difference in antifeedancy between the two formulations.

2.4. Discussion

Chemical pesticide use can cause damage to the environment, human health, and the climate if used in an uncontrolled and unmonitored manner. Therefore, it is important to find innovative pest management strategies which are both eco- and human-friendly. In the present study, *Grazers Ltd* G3 product was tested against 3rd instar larvae of *S. littoralis* and the antifeedant behaviour was determined. The results showed that the commercial (6%) dose was effective at eliciting an antifeedant behavioural response in *S.* littoralis when applied to either wheat and salad rape plants 1-hour prior to feeding, specifically in a two-choice setting. The change in behaviour is a plant-mediated response, rather than the direct action of the product. This was determined by the lack of deterrency in the artificial diet two-choice feeding assay. Additionally, *S. littoralis* showed no increase in mortality after ingestion of the G3 product up to 19%, nor any development retardation, in the no-choice feeding assays. This suggests that the product was not toxic to the larvae and the antifeedant behaviour later recorded was through a different mode-of-action.

To my knowledge, there are no reports of calcium chloride-based products which stimulates an antifeedant response in insect pests. However, there has been considerable research highlighting the effect of calcium chloride on fungal disease incidences. Chakraborty et al. (2017) noted an 84.24% incidence reduction of *Fusarium* wilt after foliar application of 0.5% calcium chloride on tomato seedlings. Foliar application of $CaCl_2$ led to an upregulation of plant defence genes, such as nitrate reductase and calmodulin. Gayed et al. (2017) recorded a significant reduction of fungal decay (%) in 'Easy Swell' peach fruits after pre-harvest applications of 2% $CaCl_2$, stating the role of Ca^{2+} ions in cell

wall strengthening attributes to reduced fungal infections. It appears calcium chloride application elicits defence responses in the treated plant, which induces resistance against fungal pests. This effect may offer an explanation to the behavioural alteration of *S. littoralis* larvae, through the activation of defence response proteins and allomones.

Most natural insect antifeedants have been isolated from plants, which produce deterring compounds as secondary metabolites (Gøkce et al. 2010). How these plant allomones are detected and processed by insect pests is distinctive of each species, through a variety of deterrent receptors and downstream processes. Despite olfactory and visual plant identifiers aiding in plant host selection, it is the chemical signals detected by gustatory receptors and structural signatures (contact chemoreception) which insects use for food-based decision-making (Mullin et al. 1994). Furthermore, no strong link has been detected between internal toxicity of plant allomones and feeding deterrence (Koul, 2008), therefore the detection of a deterrent, or lack of stimulant in the gustatory system of insects most likely results in feeding behaviour alteration (Montell, 2009). This may provide an explanation for the lack of toxicity detected in the non-choice diet assay and the non-choice leaf assay, yet an overall behavioural antifeedant effect was noted in choice leaf assays. To further determine the mechanism that triggers the behavioural alteration upon deterring chemical interaction, the sensitivity of the deterrent receptors in the gustatory system in the insect pests should be researched. High levels of mortality were recorded in the control group for the non-choice diet cube assay. Despite efforts to create optimal conditions through the performance of the pilot experiment, which highlighted the importance of balancing humidity to keep the diet moist without suffocating the larvae,

mortality due to external factors contributed to the irregular results. Thus, the presence and ingestion of the G3 product cannot be proven to cause mortality.

Contact insecticides are commonly used against parasitoids rather than insect pests due to the heightened susceptibility (Suthisut et al. 2011). As parasitoids offer a biological alternative to pesticides, it is not in the best interest to potentially damage the population (de Paiva et al. 2018). Thus, as *Grazers Ltd* G3 product is toxic to *S. littoralis* with an LC_{50} of 9.43% when applied in a large volume, this should not be detrimental to susceptible parasitoids if the commercial dose of 6% and foliar application instructions are followed.

In conclusion, *Grazers Ltd* G3 product at 6% elicits an antifeedant behaviour against *S. littoralis* in a choice setting, through plant-mediated effects. Calcium chloride has been shown to stimulate defence gene transcription against fungal pathogens, and other innate immune responses in plants. Unlike plant extract antifeedants, which have complex structures and specific receptor targets, calcium chloride appears to provoke a deterring effect through a direct interaction with the plant. The plant then produces specific non-volatile signals which insects recognise and change their behaviour accordingly.

Chapter 3: Evaluation of two different formulations of *Grazers Ltd* G3 product on ovipositing behaviour in adult *Spodoptera littoralis*.

3.0. Abstract

Integrated pest management strategies are an integral part to reduce the use of chemical pesticides worldwide. Deterrents which alter oviposition behaviour in insects is an innovative approach to reduce crop damage by insect pests. *Grazers Ltd* G3 product was applied to salad rapeseed plants via foliar application, and the effect on ovipositing behaviour was determined in adult *Spodoptera littoralis*. Two different G3 products were tested in a two-choice manner (control and treated plant in each enclosure) and the number of eggs and egg batches on each plant was recorded. The clutch size did not significantly differ between the control plant and the treated plant in each enclosure, nor between the treatment types as a whole. However, the 'Old' formulation significantly affected the ovipositing behaviour in female moths, yet the 'New' formulation (with a difference calcium source) did not. This confirms that the 'Old' formulation of *Grazers Ltd* G3 product may produce a deterrent response, altering the oviposition behaviour in *S. littoralis* adults, in a laboratory two-choice setting. Further analysis of the 'Old' formulation may highlight the active component of this deterring response.

3.1. Introduction

Oviposition preference is a form of pre-hatching maternal care and female choice which is exhibited by phytophagous insects, such as *Spodoptera littoralis* (Anderson & Alborn., 2013). In order to maximise offspring fitness and thus indirect fitness of the mother, female adults face a series of potential fitness trade-offs (Moon & Stirling., 2006). Trade-offs are a consequence of the finite amount of available resources to the mother and potential offspring, the limited energy expenditure the mother can commit to assess potential host plants, and the time constraints of the entire oviposition process. It is generally accepted that the ovipositing behaviour of adult females is tailored to minimise larval mortality and ultimately result in high fecundity (Karolewshi et al. 2017; Moreira et al. 2016; Zaluki et al. 2002). Gravid females are capable of discriminating between different host plants as well as plant parts, such as mature and young leaves (Azidah & Sofian-Azinn, 2006). This aids the gravid female to deposit the eggs of low mobility offspring at sites which will maximise development and survival, according to the preference-performance theory (Bonebrake et al. 2010). Clutch size, the number of eggs per group laid, is often used a measure of female choice to host suitability, alongside with overall egg number (Bergström et al. 2006).

Conflictingly, plants respond to herbivorous interaction through the release of herbivore-induced plant volatiles (HIPVs), oviposition-induced plant volatiles (OIPVs), and other volatile organic compounds (VOCs) (Holopainen & Blande, 2013). These chemicals deter further contact by conspecifics through direct production of elicitors, and indirectly by attracting predators and parasitoids of the damage-inducing herbivores (Aljbory & Chen., 2016; Wei et all., 2007; De Moraes et al. 1998). Plants are able to detect ovipositing behaviours of insects

due to Herbivore Associated-Molecular Patterns (HAMPs) present in the oviposition secretions (see Figure 3.1). HAMP detection after herbivore damage results in fluctuations of Ca^{2+} concentrations within the cytosol of plant cells (Arimura et al. 2011). The subsequent depolarisation or hyperpolarisation of plant membranes then transduces a variety of defence responses (Aldon et al. 2018). Plant defence responses include the production of reactive oxygen species (ROS), the alteration of the nutritional content to disfavour the herbivore (Sharma et al. 2017; Köpke et al. 2008), and hypersensitive responses (Griese et al. 2017). In addition, secondary metabolites, such as methyl-jasmonates which communicate the damage to conspecifics nearby, resins and latex which engulfs and effectively drown the insect pests, and terpenoids which attract natural predators of the insect pests are produced (War et al. 2012; Pashalidou et al. 2010).

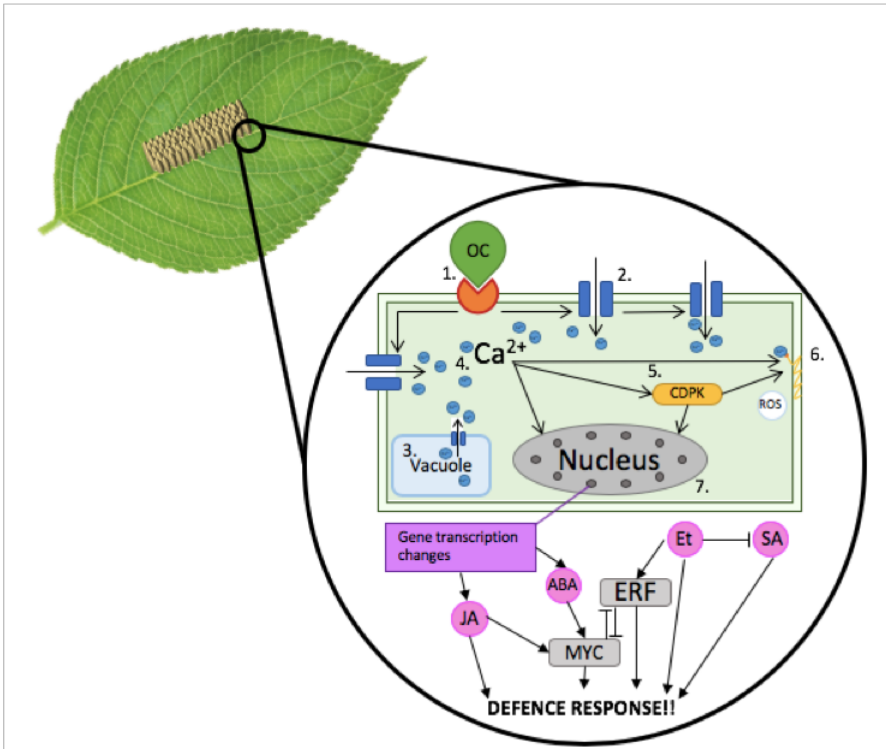

Figure 3.1: The plant defence response is triggered when HAMPs such as (1) oviposition fluid chemical elicitors (OC) are detected by membrane-bound receptors. Upon binding, membrane depolarisation triggers (2) Ca^{2+} ions influx into the plant cell from the extracellular space and (3) vacuole. Internal Ca^{2+} concentration spikes, (4) propagating and amplifying the signal via (5) calcium-dependent phosphokinases. Ca^{2+} binds to the (6) EF-hand on an NADPH oxidase, interacts with CDPKs, resulting in ROS production. Furthermore, (7) CDPK triggers gene transcription changes in the nucleus and initiates changes in phytohormone production. This triggers the defence response. Initials: CDPK = calcium-dependent phosphokinases, ROS = reactive oxygen species, JA = jasmonic acid, ABA = abscisic acid, Et = ethylene, SA = salicylic acid, ERF = ethylene response factor, MYC = transcription factors.

Detecting and preventing egg-laying reduces the number of neonatal larvae that will feed upon the plant in the future (Hilker & Fatouros, 2015). Hilker et al. (2002) determined the response in *Pinus sylvestris* plants to pine sawfly (*Diprion pini)* oviposition involves attracting *Chrysonotomyia ruforum*, an egg parasitoid, as well as modifying the surface chemistry of egg-laden leaves. It

was also established that systemic and local responses were mimicable using artificial jasmonic acid treatment. JA applications have been shown to reduce oviposition preference for the treated plant in *Brassica oleracea* against *Pieris brassicae* (Bruinsma et al. 2007). In addition, SA applications have been shown to reduce attractiveness of treated mango plants to the Oriental fruit fly (Damodaram et al. 2015). Optimising treatments for priming plant defences is an attractive alternative to non-specific and damaging chemical insecticides. Triggers of the internal plant defence system by exogenous substances, such as artificial JA and SA, may provide an innovative, non-damaging alternative management strategy to controlling herbivorous insect pests through oviposition behaviour alteration.

Both natural and artificial oviposition deterrents have been produced as alternatives to insecticides, with the aim of reducing herbivorous damage to agricultural crops or the spread of vector-bourse diseases (Uniyal et al. 2016). $CaCl_2$ has been shown to induce defence responses in plants upon infection with phytopathogenic fungi (Kumar et al. 2017; Seifu, 2017), however the mechanism in which it interacts with the plant systems has yet to be determined. The aims of this assay were to evaluate the effects of two formulations of *Grazers Ltd* G3 product, a calcium chloride-based product, on the ovipositing behaviour of *S. littoralis* females on plants treated via foliar application, under laboratory conditions. This is to determine the efficacy of the product as a deterrent for possible agricultural use.

3.2. Materials and Methods

3.2.1. Insects

Spodoptera littoralis, from a laboratory culture, was reared from egg stage to pupae on semi-artificial wheatgerm-based diet (Cotter, 2002) in 25mL plastic polypots, and then transferred to empty polypots until they had eclosed. Prior to the start of the experiment, *S. littoralis* were incubated at 27°C in a 16 h:8 h light:dark cycle. Adults were sexed after eclosing by comparing the markings on the wings.

3.2.2. Plants

Brassica napus ssp. *Pabularia* (salad rape) plants were utilised in this asssay due to its fast growth, large leaf area and attraction to fertile female moths. Seeds were grown in M3 compost (Levington Horticulture, Suffolk, UK), plants two per pot (2 x 2 x 2 cm) then watered every other day. Plants were grown in a controlled environment incubator, with a 22°C/16 h: 20°C/8 h day:night cycle. Plants were grown until 2-weeks old and those with 3-4 fully expanded leaflets were chosen.

3.2.3. Bioassay

Treatments of *Grazers Ltd* G3 'Old' and 'New' formulations were made up to 6% concentration as well as a RO water control (Table 3.1).

Table 3.1: Set-up for oviposition assay.

ID	Group	n	#females per pot	#males per pot
A	Control vs. Control	26	1	1
B	Control vs. 'Old' formulation	27	1	1
C	Control vs. 'New' formulation	27	1	1

Each treatment was applied 1 h prior to experiment via foliar application. Plant pots were labelled then transferred to the enclosure (Figure 3.2) which included a small shelter made from filter paper and cotton wool soaked in 5% sucrose for energy. The enclosure was made from two 250mL plastic mating pots, with added air holes, then secured with parafilm.

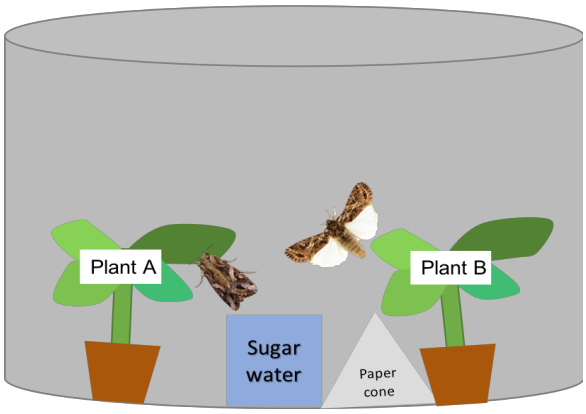

Figure 3.2: Experimental set-up of oviposition test. A single mating pair and two plants available to oviposit on. One plant is the control, in which the plant is sprayed with RO water, and the other has been treated with the 'Old' or 'New' formulation of *Grazers Ltd* G3 product at 6%.

Within 48 h of eclosing, a single male and female adult was added to the enclosure. After the initial 48 h, the number of eggs and egg batches were counted every 24 h until 96 h had passed. Plants were watered every 48 h with a syringe and the 5% sucrose was topped up every 24 h.

3.2.4. Statistical analysis

Statistical analysis performed wholly using 'R' (Version 3.5.0) and 'RStudio' (Version 1.1.453), using dplyr (Wickham et al. 2018), ggplot2 (Wickham, 2009) and ggfortify (Tang et al. 2016) packages. Data was presented using ggplot2.

The clutch size was determined for each treatment group, comparing the difference between each plant in the enclosures. A student's paired t-test was performed between plants within each treatment group, and a one-way analysis of variance (ANOVA) was performed to determine the relationship between overall treatment group and clutch size.

Equation 8: Clutch size per female laid on specific plant (control or treated plant) in each treatment group.

$$Clutch\ size = \frac{Total\ number\ of\ eggs\ on\ plant\ type}{Total\ number\ of\ egg\ batches\ on\ plant\ type}$$

Additionally, the ovipositing preference index (OPI) (Equation 9), the proportion of egg and egg batch count (Equation 10 and 11) was determined as a measure of deterrency and analysed accordingly.

Equation 9: Oviposition preference index (OPI).

$$OPI = number\ of\ eggs\ on\ control\ plant - number\ of\ eggs\ on\ treated\ plant$$

Equation 10: Percentage of eggs deposited upon the treated plant, per treatment group.

$$Percentage\ (\%) = \frac{Eggs\ count\ on\ treated\ plant}{Egg\ count\ on\ treated\ plant + egg\ count\ on\ control\ plant} \times 100$$

Equation 11: Percentage of egg batches deposited upon the treated plant, per treatment group.

$$Percentage\ (\%) = \frac{Egg\ batch\ count\ on\ treated\ plant}{Egg\ batch\ count\ on\ treated\ plant + egg\ batch\ count\ on\ control\ plant} \times 100$$

3.3. Results

Gravid females perform a choice regarding oviposition on two levels: the location of the eggs and the number of eggs per clutch. The two-choice oviposition assay used up to 27 mating pairs per treatment. The clutch size per plant in each treatment group (Figure 3.4), the OPI, and the proportion of egg and egg batch count deposited onto the treated plant, per treatment was determined (Table 3.2).

Table 3.2: The effect of product application on oviposition behaviour determined by analysing the oviposition preference index (OPI) and the final proportion of egg/egg batch count per treatment. EB = egg batches. Values with differing letters indicate significance to $p<0.01$. Kruskal-Wallis non-parametric test for overall significance and pairwise Mann-Witney tests between treatments were performed.

Treatment	**OPI**		**Proportions (%)**	
	Eggs ± SE	EB ± SE	Eggs ± SE	EB ± SE
Control	-59.62 ± 114.29^{A}	-0.038 ± 0.257^{A}	48.59 ± 4.58^{A}	50.83 ± 2.97^{A}
'Old' formulation	235.56 ± 73.76^{B}	0.741 ± 0.189^{B}	31.08 ± 3.47^{B}	39.96 ± 2.18^{B}
'New' formulation	177.78 ± 103.27^{AB}	0.370 ± 0.214^{AB}	41.99 ± 4.35^{AB}	45.78 ± 2.56^{AB}
Significance	*	*	*	*

3.3.1. Location of eggs

OPI could not be transformed into a normally distributed data set, therefore non-parametric tests were performed (Table 3.2). The gravid females appeared to prefer the water-treated control plants rather than the formulation-treated plants, specifically the 'old' formulation. This is supported by the significant interaction of treatment on the overall proportion of eggs on each plant, with

the ‘old formulation’ resulting in a lower percentage of eggs being laid on the treated plant.

3.3.2. Clutch size

The effect of treatment type and plant treated on the clutch size per female (Figure 3.4).

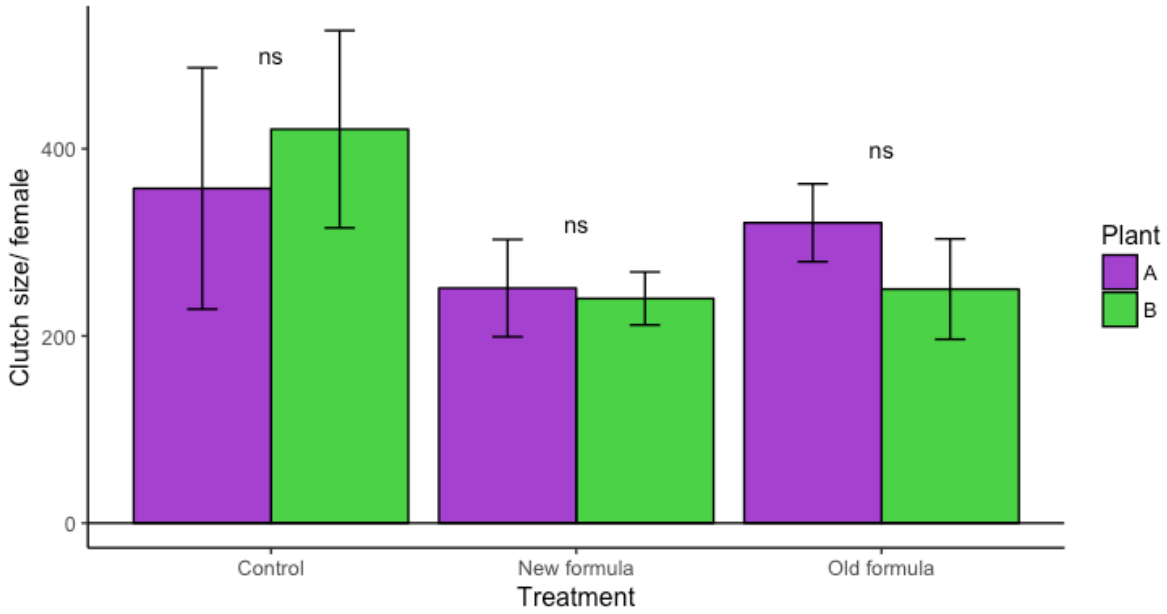

Figure 3.4: Effect of *Grazers Ltd* G3 product, ‘Old’ and ‘New’ formulations, on egg count per egg batch. Results are mean ± SE. A = control plant, B = treated plant or second control plant. ns = non-significant difference determined by Student’s paired t-test.

There was no significant difference recorded between the two plants in each enclosure across all treatment types (pairwise t-test results: Control treatment: p=0.71, New formula: p=0.88, Old formula: p=0.33), nor was there any significance of the treatment type on the overall clutch-size (F=0.57, df=2,13, p=0.58).

Overall, the treatment group does not affect the behaviour regulating the clutch size, but the ‘Old’ formulation does significantly affect the ovipositing

behaviour of the females; depositing less eggs and egg batches upon treated plants in a two-choice setting. The 'New' formulation appears to reduce the proportion of eggs laid upon treated plants over the 96-hour period but there is too much variation to reinforce a significant effect.

3.4. Discussion

There has been an abundance of research into the detection and production of oviposition deterrents, with the majority being derived from non-host plant extracts (Chantawee & Soonwera., 2018; Hossain & Khalequzzaman., 2018; Wagan et al. 2018), microbiological sources (Machtinger et al. 2016), or phenol-derived compounds from insect frass (Hashem et al. 2013). However, thorough investigation has concluded a lack of research into the effect of calcium chloride on the oviposition behaviour of gravid female phytophagous insects. This assay provides a first insight into the possibility of calcium chloride as a source of deterrency of ovipositing insects.

It is known that gravid females of *S. littoralis* respond to HIPVs and other defence volatiles to assess plant suitability (Zakir et al. 2013). This information is key to successful decision-making, especially important in an environment of high uncertainty in order to maintain fitness (Munoz & Blumstein, 2012). Host plants have been shown to release high concentrations of HIPVs upon mechanical damage or herbivore elicitor detection (Rojas et al. 2003) which are interpreted by insect pests as unsuitable sites for oviposition. Whether this is intentional by the host plant or eavesdropping of the herbivore is unclear, nevertheless the responses of gravid female preference to undamaged plants over damaged plants has been previously noted (Zakir et al. 2013b).

The application of *Grazers Ltd* G3 product to the adaxial leaf surfaces of salad rapeseed plants by foliar application may have triggered a mechanical defence response on possible two levels: through the physical act of the product hitting the leaves, and the absorption of the product initiating in the release of oviposition deterrent compounds. As the products and the water control were administered identically, the former could not be true in this system as a source of oviposition deterrence. Additionally, the deterring effect may mimic the oviposition deterrent pheromones produced after gravid females lay eggs to deter conspecifics and propagate spatial distribution, however due the composition of insect pheromones as organic compounds (Kumari & Kaushik, 2016), the $CaCl_2$-based product would unlikely stimulate the complimentary receptors.

The 'Old' formulation and 'New' formulation are identical except in terms of the source of the $CaCl_2$. This component appears to have a significant effect of the oviposition preference, as the 'Old' formulation elicited an apparent deterring effect. This supports the theory that $CaCl_2$ absorption into the leaf may stimulate a defence response in the host. However, the $CaCl_2$ source also contains an adjuvant and other unknown chemicals, therefore without testing an $CaCl_2$-only solution for oviposition deterrence, the overall causation of deterrence cannot be determined.

The results of this bioassay revealed that the plants treated with the 'Old' formulation of *Grazers Ltd* G3 product exhibited a significant deterrent effect to ovipositing by gravid female *S. littoralis,* when presented in two-choice setting against a control plant. The 'New' formulation did not significantly

deter ovipositing behaviour, although further replicates may offer a different result. Furthermore, if replicated, utilizing larger mating pots or cages should be considered with a mechanical air filtration system. This would improve conditions for insect mating as well as reduce saturation of the microenvironment with HIPVs, if the product initiates a plant-mediated response (Saveer et al. 2012). In addition, an odorant two-choice assay as described in Stelinski & Tiwari (2013) could be performed to determine if deterrency is reliant on volatile cues or non-volatile chemicals. These chemicals could be collected and analysed with gas chromatography mass spectrometry (Wang et al. 2018) and compared with pre-application volatiles to determine if the product promotes the production and release of HIPVs.

The 'New' and 'Old' formulations differ in a single component; the source of the calcium chloride component. The exact concentration of $CaCl_2$ and whether additional components, such as adjuvants, have been added is not known. Further analysis of the 'Old' formulation may highlight the active component or a combination of active components. This would provide the initial step into determining the mode-of-action of the *Grazers Ltd* G3 product as an oviposition deterrent against *S. littoralis*.

Chapter 4: Observation and analysis of calcium uptake through the adaxial surface of salad rapeseed plants following application of *Grazers Ltd* G3 product.

4.0 Abstract

Foliar application is a popular technique used to apply micro-nutrients and pesticides alike to crop surfaces in order to increase yield and quality. With population growth increasing exponentially, the need for successful agriculture and food production increases, and with this, new and sustainable products are a necessity.

Grazers Ltd have developed calcium chloride-based products to deter pests from feeding on crops through indirect means which are currently unknown. In order to determine the mode-of-action of these products, the penetrating abilities of the calcium ions have been tested.

Three calcium-chloride based treatments (an 'Old' *Grazers Ltd* formulation, the 'New' formulation, and a calcium chloride only source) were applied to banded areas on salad rapeseed leaves for a 48 h period and the calcium ion uptake across the adaxial surface was recorded using an Horiba LAQUAtwin compact Ca^{2+} B-751 electrode probe. The total calcium ion uptake (%), remaining calcium ion concentration (ppm), and the rate of penetration (% h^{-1}) were determined across all treatments. The effect of treatment type on these three variables were significant, with the full formulations containing the surfactant resulting in a higher uptake of calcium.

4.1. Introduction

Foliar application of nutrients has notably become an increasingly popular technique in commercial crop production across the globe (Alexander & Hursche, 2016). Calcium chloride is often applied to treat fruit disorders and deficiencies (Singh et al. 2013) and has shown to increase yield in wheat plants, as well as improve flower fertilization and overall growth, compared to untreated crops (Zoz et al. 2016). Calcium chloride application improved texture and flavour in papaya fruits, along with a reduction in disease incidence and Mg content (antagonistic effect with Ca) (Madani et al. 2015). Moreover, $CaCl_2$ applied preharvest to sweet bell peppers improved firmness retention and reduce postharvest decay (Toivonen & Bowen, 1999). Calcium ions assimilate well in young leaf and fruit tissues due to high cuticle permeability. This makes foliar application a more attractive choice to soil fertilisation, in which Ca^{2+} availability is lower. This is due to chelation by organic molecules in the soil humus and competition with Al^{3+}, Mn^{2+} and Fe^{2+} for uptake availability (Ochmian, 2012).

However, there are distinct disadvantages to using foliar application to deliver necessary nutrients to crops. Multiple applications, as many as eight, can be required to reduce disease incidence or increase Ca concentration in the leaves or fruits (Yamane, 2014) which may not be economically or environmentally sustainable. Leaf burn (Asad et al. 2003) and yellowing of leaf tips (Chang et al. 2004) occurs at higher salt concentrations of boron and calcium fertilizers. Furthermore, Singh et al. (2013) noted that some calcium chloride fertilizers have limited compatibility with other fertilizers, rendering them both useless if mixed.

Foliar application of hydrophilic solutes relies upon the mechanisms associated with the physical properties of the solution; complimentary characteristics with the optimal environmental conditions allows for spontaneous infiltration of the solute through the stomatal opening (Schrönherr & Bukovac, 1972). Features which need to be taken into consideration when designing foliar solutions are the point of deliquescence (POD), viscosity and wettability of the product (Yao et al. 2014), as well as the surface tension, structural barriers (such as trichomes), epicuticular waxes and roughness of real leaf surfaces (Burkhardt et al. 2012). These qualities determine how the solution droplets interact with the stomatal pore opening in specific environmental conditions (Fernández & Eichert, 2009). $CaCl_2$ is very hygroscopic, with a low POD of 32% relative humidity (Yamane, 2014), and with sources of water vapour coming from the air and through plant transpiration (Burkhardt et al. 2012), the salt maintains the solute form which is required for absorption. However, due to changing temperatures, light availability (Wójcik, 2004) and the presence of hydrophobic waxes on the leaf surface, surfactants are key for effective Ca^{2+} translocation (Pham et al. 2012). Surfactants are responsible for reducing the surface tension on plant leaves, allowing the solution to spread out across the surface (Schönherr, 2000). This increases the surface area to volume ratio, and subsequently reduces the contact angle size between the solution and the cuticle (Figure 4.1).

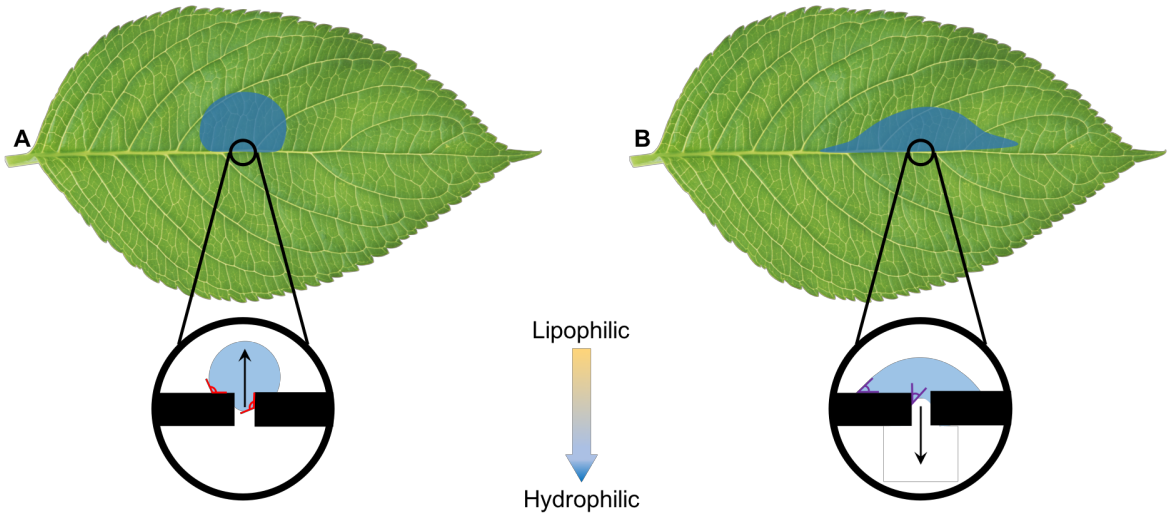

Figure 4.1: Simplified diagram of stomatal opening upon interaction with **(A)** droplet with no surfactant and **(B)** droplet with surfactant. Arrows depict direction of meniscus and the direction of the generated pressure. Spontaneous filtration into the capillary occurs when contact angle is θ < 90° (purple) but not θ > 90° (red). Based on: Fernández & Eichert, (2009) and Aryal et al. (2018).

The experiment in this chapter aims to establish the concentration of Ca^{2+} ions absorbed and the subsequent rate of penetrations of two different formulations of *Grazers* G3 product. The difference between the two formulations stems from the source of the calcium chloride, thus the effect of the source in complete formulation will be analysed. Additionally, the $CaCl_2$ source of the 'Old' formulation was also analysed without the addition of a surfactant, to determine the efficacy and efficiency of the surfactant in the full product. The optimal concentration at which the rate of penetration of Ca^{2+} becomes limited may also be determined.

4.2. Materials and Methods

4.2.1. Plants

As in previous chapters, *Brassica napus* ssp. *Pabularia* (salad rapeseed) was used as the model plant. Seeds were grown in trays of 5 cm^3 and filled with M3 compost (Levington Horticulture, Suffolk, UK), then watered every other day. Plants were grown in a controlled environment incubator, with a 22°C/16 h: 20°C/8 h day:night cycle. Plants were grown until 2-weeks old and those with 3-4 fully expanded leaflets were chosen.

4.2.2. Calcium probing

Grazers Ltd G3 product was diluted to 1.9 % as this dosage gave the highest recordable Ca^{2+} ion concentration using a LAQUAtwin compact Ca^{2+} B-751 electrode probe (Horiba Instruments Ltd, Northamptonshire, UK). The Ca^{2+} probe was calibrated using a 150 ppm calibrating solution of $CaCl_2$ then washed three times with RO water. Three treatment groups and a water control were analysed for Ca^{2+} concentration initially (0 h), and at 24 h and 48 h time periods (Table 4.1).

Table 4.1: Concentration of *Grazers Ltd* G3 product in each treatment.

Treatment	n	Concentration (%)
'Old' formulation	7	1.9
'New' formulation	7	1.9
$CaCl_2$ source	7	0.95*
Water	7	100

*The $CaCl_2$ source makes up 50% of the entire formulation, therefore concentration used mimics product without additional components.

50 μL of each treatment was applied to a banded circular area on one leaf per plant and incubated for 24 h in the conditions mentioned above. The banded area was produced from a *Subway* straw cut to 0.5cm in length and fixed to the plant with Vaseline gel. At 24 h, five leaves were randomly chosen per treatment. 50 μL of RO water was added to the banded area and the solution was aspirated off and analysed for Ca^{2+} concentration (ppm). This was repeated at 48 h in the same way (Figure 4.2). No leaves were removed during the study and contact to the plants was kept minimal.

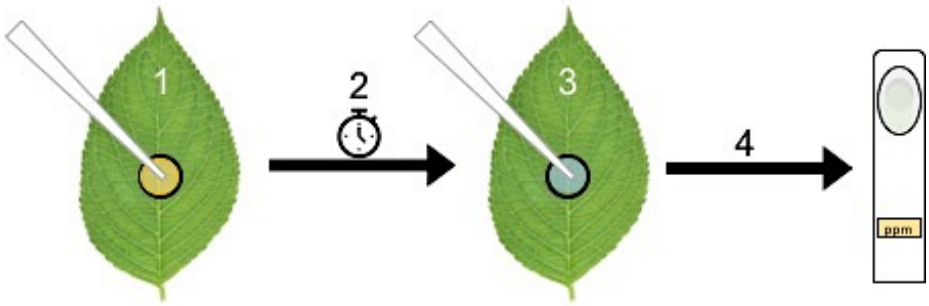

Figure 4.2: Experimental set-up of Ca^{2+} probing. **(1)** 50 μL treatment added to banded area (plastic tube secured with Vaseline) and left to dry for **(2)** 24 h. **(3)** 50 μL RO water added, and after 30 seconds the solution was aspirated off, **(4)** and analysed using probe.

In order to determine the effect of the plant surface on Ca^{2+} absorption across the adaxial plant surface, a plastic petri dish was used as a control surface. This material control was performed identically to the plant surface experiment.

4.2.3 Statistical Analysis

A repeated measures ANOVA Analysis was performed to assess the effects of product concentration and surface type on calcium uptake over time. SPSS (IBM Corp. Released 2015. IBM SPSS Statistics for Mac, Version 23, Armonk, NY: IBM Corp.) was used for this analysis.

Furthermore, the effect of treatment type on total calcium uptake (ppm), total percentage of calcium ion uptake (%) and rate of penetration (% hr^{-1}) were all determined using the Kruskal Wallis non-parametic test as the data failed to meet assumptions for normally distributed data (sample size smaller than 10). Post-hoc analysis was performed using pairwise Mann-Whitney U-tests. R version 3.5.0 and RStudio 1.1.453, with the packages: dpylr (Wickham et al. 2018), rcompanion (Mangiafico, 2018), and multcompView (Graves et al. 2015). Figure was created using ggplot2 package (Wickham, 2009).

4.3. Results

The effect of each treatment on the total calcium ion concentration which penetrated into the plant via the adaxial surface following foliar application was analysed.

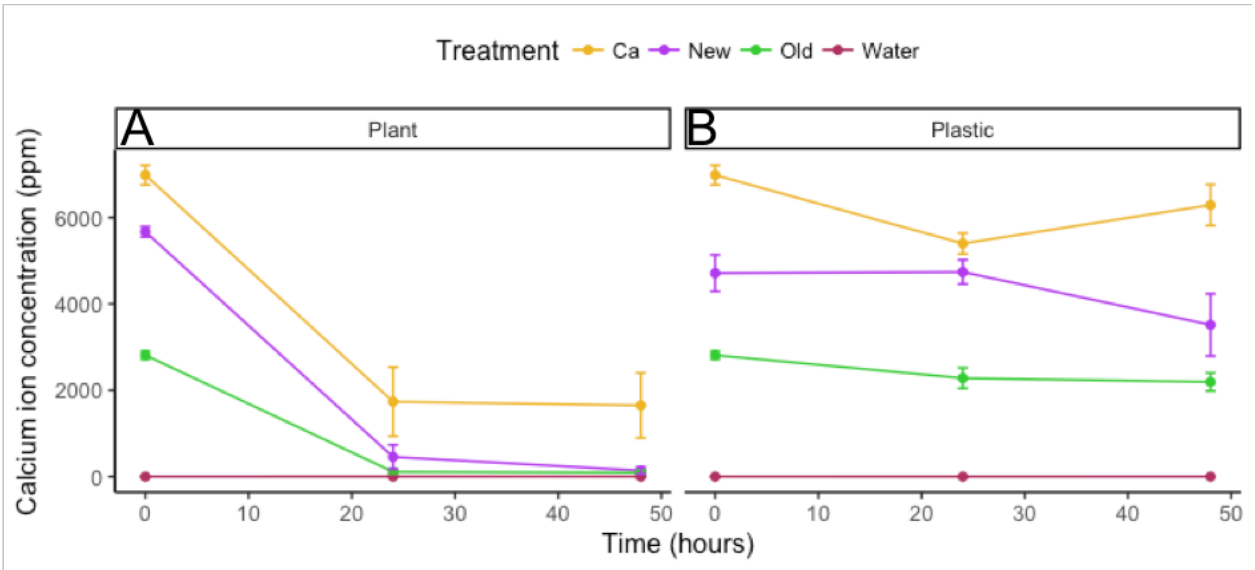

Figure 4.3: Calcium ion concentration (ppm) on **(A)** the adaxial surface of the salad rapeseed leaves, and **(B)** a plastic surface, across a 48 h time period, after treatment of the *Grazers Ltd* G3 product 'Old' formulation **(Old)**, 'New' formulation **(New)**, calcium chloride source **(Ca)** or water treatment **(Water)**. Results are mean ± SE.

For the repeated measures ANOVA, the data shown in figure 4.3 failed Mauchly's test of Sphericity (x^2 (2) = 11.747, p=0.003) so p values were adjusted using the Greenhouse-Geisser correction. Calcium ion uptake significantly changes over time (p<0.001) and substrate type affects how calcium reading changes over time (p<0.001), with calcium ion uptake increasing over time on leaf surface but not on plastic surfaces. The treatment type doesn't significantly affect calcium ion uptake over time (p=0.109). However, the overall interaction between treatment type, substrate type and calcium reading were significant (p=0.026). Tests of between subject effects confirm differences between treatment types (p<0.001) and post-hoc Tukey HSD tests confirm all three calcium treatments significantly from each other. The calcium chloride source had the highest concentration of Ca^{2+} ions initially of 6986 ± 22 ppm, despite also being the calcium chloride component in the

'Old' formulation, which had an initial concentration of 2814 ± 96 ppm. The reduction in Ca^{2+} ion concentration may be the result of other components reacting with the $CaCl_2$ source, taking free ions out of solution. The 'New' formulation had an initial concentration of 5671 ± 119ppm, which has over twice as much as the 'Old' formulation.

Due to the differing concentration of Ca^{2+} ions in each formulation, the total Ca^{2+} ion concentration (ppm) which penetrated into the plant is significantly lower in the 'Old' formulation compared to the 'New' formulation and the $CaCl_2$ source (Table 4.2).

Table 4.2: The rate of penetration of Ca^{2+} ions, and the total calcium concentration, which penetrated through the adaxial surface of salad rapeseed plants, following the application of three different $CaCl_2$ treatments. Results are mean ± SE. Values with differing letters indicate significance to $p<0.05$ (per column). n = 7 per treatment. ns = non-significant, *p<0.05, **p<0.01

Treatment	**Total Ca^{2+} ion concentration (ppm)**	**Calcium uptake (%)**	**Rate of penetration (% h^{-1})**	
			24 h	48 h
'Old' formulation	2631 ± 124^{A}	96.55 ± 1.04^{AB}	4.00 ± 0.07	2.01 ± 0.02^{AB}
'New' formulation	5400 ± 190^{B}	97.51 ± 1.40^{A}	3.83 ± 0.20	2.03 ± 0.03^{A}
$CaCl_2$ source	4540 ± 969^{AB}	76.83 ± 10.45^{B}	3.15 ± 0.45	1.60 ± 0.22^{B}
Statistical analysis	**	*	ns	*

The total uptake of Ca^{2+} ion was significant ($x^2=9.89$, df=2, p=0.007), with the 'Old' formulation having a considerably lower uptake of calcium ions, leading to significant differences between the other two treatments. As previously noted in Figure 4.3, the 'Old' formulation has a much lower stock calcium ion

concentration, therefore comparing the total uptake as a percentage rather than total concentration gave a better indication, removing the variability of the available calcium ions. The treatment type significantly affected the total uptake (%) (x^2 = 8.88, df=2, p=0.012), with the calcium chloride source having a much lower saturation point (76.8 ± 10.4%) than either of the surfactant-containing full formulations ('Old' 96.5 ± 1.0%, 'New' 97.5 ± 1.4%). This was reinforced by Kruskal-Wallis rank sum test showing significance difference between the full formulations and the calcium source (but no difference between the full formulations themselves).

The rate of calcium ion penetration (% h^{-1}) after the initial 24 h and after the total 48 h were determined. A significant effect of treatment type on calcium ion penetration rate was not found after the initial 24 hours (x^2=4.905, df=2, p=0.086), but was found after the entire 48 hours (x^2=9.89, df=2, p=0.007). Post-hoc pairwise comparisons were conducted with Holm adjustments which identified significant differences between the 'New' formulation and the calcium chloride source only.

4.4. Discussion

Following the application of the two formulations of *Grazers Ltd* G3 product, as well as the source of calcium, it is clear the final concentration of calcium ions penetrating through the leaf cuticle is dependent upon the presence of the surfactant (Figure 4.3 and Table 4.2). The rate of penetration is a fifth higher in the full formulations, than the calcium chloride source alone, with no difference between the 'Old' and 'New'. The rate of penetration of the calcium chloride source was rather spread compared to the full formulations, with random variation due to small sample sizes possibly explaining away this

effect. However, as the formulations with surfactant produced a higher level of precision with the same sample size, the high variability of the calcium chloride source is most likely a consequence of the lack of surfactant.

The surface properties of the epicuticular surface affect distribution and spreading of solute-containing droplets. The contact of the $CaCl_2$ with the cuticle surface is drastically reduced when surfactant is not present (Figure 1). Similar effects of surfactant presence, specifically non-ionic wetting agents, increasing Ca^{2+} ion penetration has been recorded by Schönhorr (2001), who determined the significant increase in reaction rate coefficient (k) when $Ca(NO_3)_2$ was applied with surfactant compared to distilled water when applied to pear (*Pyros communis* L.) leaves at 90% relative humidity. This further reinforces data suggesting the total penetration of hydrated $CaCl_2$ through the pear leaf cuticle would have readily increased if a wetting agent or surfactant was present (Schönhorr, 2000). Surfactant usage has been highlighted as a necessity when applying Ca^{2+} ions to apple trees pre-harvest. This is to ensure sufficient calcium content to reduce post-harvest disorders, such as bitter pit and internal breakdown (Roy et al. 1996; Harker & Ferguson, 1991; Lee & Dewey, 1981).

Limitations of this experimental design which should be considered if replicated includes using more replicates per treatment to reduce the random variation accompanied with a small sample size. In addition, the ability of each treatment solution to be successfully aspirated off the leaf surface following application of RO water needs to be re-considered. The addition surfactant increases the wettability of the formulations, therefore the calcium ions in

these solutions can be collected and analysed more easily than the $CaCl_2$ source solution.

Overall, the use of either *Grazers Ltd* G3 formulations increased the rate of penetration of calcium ions, and the final concentration of calcium ion uptake, into the plant compared to the $CaCl_2$ source only. The total concentration of calcium ions penetrating into the plants following the application of the 'New' formulation was 5400 ppm, compared to the 'Old' formulation of 2631 ppm. Therefore, if calcium penetration is imperative to the *Grazers Ltd* G3 product's mode-of-action, the 'New' formulation would be the most effective choice, due to the higher initial calcium ion concentration.

Chapter 5: General Discussion

Current strategies to combat the overuse of chemical pesticides have led to the research and development of feeding deterrent products. There has been a severe lack of interest in the effects of calcium chloride or calcium chloride-based products on the feeding behaviour of phytophagous insects when applied through foliar application. Furthermore, the effect of calcium ion penetration through the adaxial surface of leaves on the induction of defence responses against herbivorous pests has not, to my knowledge, been thoroughly investigated.

The *Grazers Ltd* G3 product does not exhibit a deterrent effect on the feeding behaviour of *S. littoralis*, when applied to semi-artificial wheatgerm-based diet cubes in a no-choice or two-choice environment, nor when applied to wheat plants in a no-choice setting. Conversely, the product significantly affected the feeding behaviour when applied to both wheat and salad rape plants and presented in a two-choice setting. The presence of a viable plant to record the presence of an antifeedant effect indicates a plant-mediated response which alters the feeding behaviour of the pest. Furthermore, the product was only effective when it was applied in the complete formulation, whether that be the 'Old' or 'New'. Breaking down the product into the individual components did not highlight calcium chloride as the active ingredient, nor did any of the other components appear to be responsible for the deterrent effect. The rate of penetration of the calcium chloride source, without the additional surfactant, was significantly lower compared to the complete formulation, yet the overall

concentration of ions which penetrated into the plant was significantly lower in the 'Old' formulation than the $CaCl_2$ source or 'New' formulation. The deterring effect of the product appears to be dependent upon the penetration rate of calcium ions, rather than the total concentration of ions, into the plant's adaxial leaf surface.

Additional analysis concluded the possible manipulation of ovipositing behaviour of the adult moth. 'Old' formulation-treated salad rape plants were found to house fewer eggs than untreated or 'New' formulation-treated plants. The clutch size did not differ across treatment types. This indicates that female choice occurred significantly at the initial, plant-choice level but not as the secondary, clutch size level. Gravid females will deposit fewer eggs on smaller or lower quality plants (Bergström et al. 2006), accounting for resource availability to future offspring (Anderson et al. 2011). It is not possible to determine whether the decision to deposit eggs onto a certain plant was taken pre- or post-contact with the plant.

The mode-of-action of the *Grazers Ltd* G3 product which elicits a deterring effect on feeding and oviposition behaviour of *S. littoralis* appears to rely upon a specific rate of penetration of calcium ions through the adaxial leaf surface, within the initial 48 h period following foliar application. The effects of foliar-applied Ca^{2+} ions have been investigated for disease incidence in plants (Arfaoui et al. 2018; Rab & Haq, 2012; Tzortzakis, 2009) but not for herbivore-induced damage. Madani et al. (2015) determined that the maximum uptake of foliar calcium in papaya plants was 180ppm, despite treatments of $CaCl_2$ ranging from 4000ppm to 5400ppm. The rate of penetration was not determined but the higher concentration of $CaCl_2$ applied resulted in a 100% disease reduction in papaya plants, whereas the lowest concentration gave a

91.2% reduction. Moreover, Deepa et al. (2015) determined that the calcium applied in nanoscale concentrations (up to 1000ppm) was more mobile within groundnut plants than bulk calcium sources, making it more suitable for foliar nutrition. This supports the results of my research which indicates that the overall rate of Ca^{2+} penetration into the plant is a determinant of inducing a defence response, rather than the total Ca^{2+} concentration.

Arfaoui et al. (2018) noted an increase in antioxidant defence enzyme (Apx1, Prx2b and tioredoxin) transcription in soybean plants after foliar application of a calcium-based formulation was used to reduce incidence of white mould. The effect of Ca^{2+} ions on ROS has also been analysed by Dolatabadian et al. (2013), determining that foliar calcium mitigates the effects of ROS, reducing the reallocation of essential plant nutrients for antioxidant production, and ultimately increasing the yield of spring wheat by reducing decay. It is an interesting concept that foliar calcium increases resistance of crops to necrotrophic pathogens. As herbivorous attack and necrotrophic pathogens both activate the JA pathway, this may provide insight into the mechanism behind calcium-induced defence.

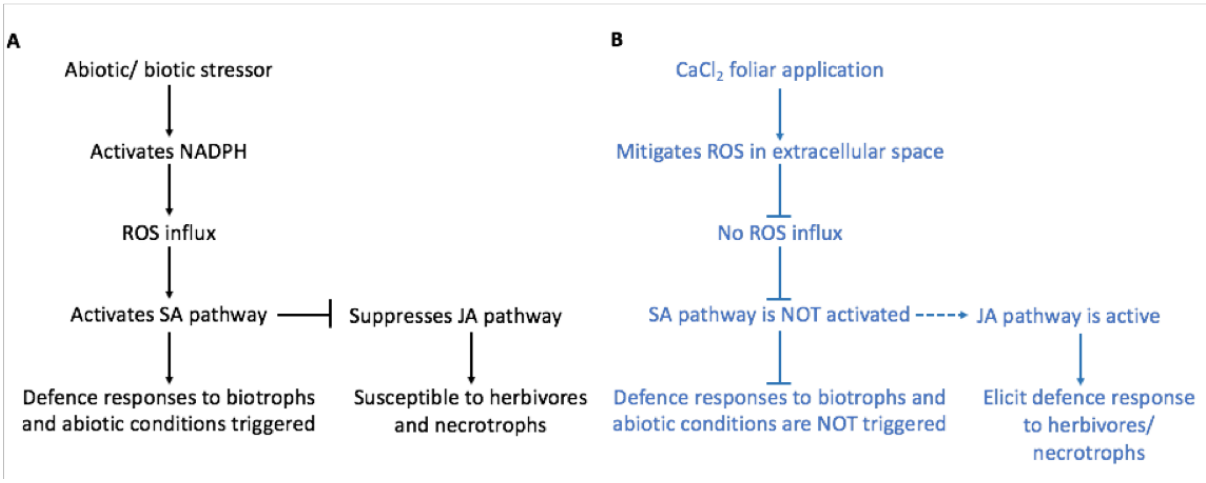

Figure 5.1: Simple representation depicting the possible effect of Ca foliar application on the signalling pathways involved in plant defence. **A** = without Ca foliar application, **B** = with Ca foliar application. NADPH = nicotinamide adenine dinucleotide phosphate hydrogen, ROS = reactive oxygen species, SA = salicylic acid, JA = jasmonic acid, $CaCl_2$ = calcium chloride.

ROS species interact synergistically with the SA pathway (Heera-Vásquez et al. 2015), which in turn acts antagonistically with the JA signalling pathway (He et al. 2017) (Figure 5.1).

To conclude, *Grazers Ltd* G3 product successfully deterred *S. littoralis* larvae from feeding on wheat and salad rape leaves treated with the commercially-recommended dose (6%) of the complete 'Old' formulation and "New' formulation, when presented in a two-choice setting. Adult *S. littoralis* female moths also altered their ovipositing behaviour to avoid salad rape plants treated with the 'Old' formulation when a control plant was also present. The deterring effect appears to be plant-mediated, possibly through interaction with the ROS defence system and JA/SA signalling. However, further investigation needs to be performed to certify this model.
Regarding the product as an IPM strategy, field trials should be conducted to assess the effect of environmental variables on the efficacy of the product and the possible implications on the surrounding environment. Additionally, the source of calcium chloride which differed between the 'Old' and 'New' formulations was insignificant regarding any alternation in feeding behaviour and rate of Ca^{2+} penetration. However, there was a difference in oviposition behaviour. As a result, further study into the effect of each formulation on adult female choice regarding egg deposition location and clutch size should also be performed. Lastly, the effects of the product on microbial disease incidence should be undertaken due to the level of evidence which suggests foliar application of $CaCl_2$ and other calcium salts increases plant resistance.

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