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Elucidating Novel Biopesticide Modes of Action in Insects: Physiological, Cellular and Molecular Approaches

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The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution. I certify that I have complied in all other aspects with the rules, requirements, procedures and policies relating to the award of this degree at Western Sydney University.

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[redacted]

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Preface

Some of the components presented in this thesis were derived from work carried out as part of a larger Cotton Research and Development Corporation (CRDC)-funded foundation coded UWS1401, "Novel insecticides and synergists from endemic and exotic flora", and were, therefore, performed by others.

- The extract library was prepared by members of the foundation project, Dr. Karren Beattie, Ms. Christine Murray, and Ms. Beatrice Venkataya National Institute of Complementary Medicine (NICM) at Western Sydney University's Campbelltown Campus, NSW, Australia.
- Initial screening of 414 samples on *Tetranychus urticae*, *Aphis gossypii* and *Helicoverpa armigera*, was carried out by Dr. Albert Basta and Dr. Duong Nguyen, with the assistance of Mrs. Geraldine Tilden and myself at Western Sydney University's entomology laboratory, Hawkesbury campus, Richmond, NSW, Australia.
- HPLC fractionations of several active compounds from *Podolepis jaceoides* were performed by Dr. Karren Beattie assisted by Ms. Christine Murray and are presented in this thesis as fraction 42 (68.Fr42) and fraction 44 (68.Fr44). Likewise, the purification of podopyrones, which led to the subsequent identification of fraction 42 as 10'-oxopodopyrone and fraction 44 as 10'-oxo-8-methyl podopyrone is the sole work of Dr. Karren Beattie.
- Additional methanol extract of *P. jaceoides* used in bioassays to compare response of *A. gossypii* vs *Drosophila melanogaster* and related molecular work, as well as some cytotoxic assays was prepared by Dr. Duong Nguyen in the entomology laboratory, at Hawkesbury campus.

This PhD study was also supported by the CRDC and coded UWS1601, with the originally title "Electrophysiological and molecular identification of novel biopesticides".

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Several researchers have also been instrumental in performing tasks either directly or indirectly contributing to this body of work, have offered keen insights and challenged my thinking, as well as encouraged my progress and taught me valuable techniques. To Dr. Albert Basta, Dr. Duong Nguyen, Dr. Alexie Papanicolaou, Mr. David Randall, Mrs. Geraldine Tilden and Ms. Rhoda O'Higgins, thank you for everything you have taught me and for every challenge you have helped me overcome. Special thanks goes to the very talented Dr. Guang Chen and Dr. Chenchen Zhao for help with my RNA sequencing analysis, and to Dr. Duong Nguyen for producing the additional *P. jaceoides* methanol extracts.

I would also like to thank all the technical staff at Western Sydney University, who have often gone above and beyond to offer me technical and ordering assistance, encouragement and friendship, with special mention to Sharleen Hamersma, Rosemary Freeman, Michael Duncan, Sumedha Dharmaratne, Liz Kabanoff, Linda Westmoreland and Jennie Nelson.

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your professional pursuits and I wish you every happiness for the future. I hope, now that I am finished, we can see each other, outside of University, on the weekends!

And last, but definitely not least, I dedicate this work to my two beautiful daughters, Xenedra Mak and Pierenii Mak, who have walked this long journey with me every step of the way. Thank you for all your patience, for being my angels when I was so busy I didn't know what day of the week it was, and for being my strength and reason to carry on when I was exhausted. You have both spent a substantial time of your childhood watching your mother study and struggle and have grown up to be kind, gracious, patient, self-sacrificing, determined and beautiful individuals. I truly believe that you have taught me more that I have taught you and I could not be prouder of you both.

> "*Impactful research is what drives change and academics should always be asking themselves 'How will Australia benefit by knowing this research?'*" Dr. Ian Wright.

Table of Contents

List of Tables

List of Figures

Key Words

Aphis gossypii, bioassay, confocal microscopy, *Drosophila melanogaster*, insect cell lines, ion flux, ion channel, microelectrodes, phytochemistry, reactive oxygen species, spectrometry, *Spodoptera frugiperda*, transcriptomics.

Published Work

Due to potential intellectual property (IPs) issues associated with this work, there is only one, non-confidential method paper, approved for publication. A patent application is currently being prepared to cover the major findings of my thesis work. The publication is presented herein as Chapter 3, which was submitted on 17 April 2020 to the following peer-reviewed journal:

Mak M, Beattie K.D, Basta A, Randall D, Chen Z-H, Spooner-Hart R. **2020**. Triangulation of methods using insect cell lines to investigate insecticidal mode of action. ***Pest Management Science*** Impact Factor: 3.255 (Accepted article published online 19 August 2020)

In addition, I worked on several collaborations that helped hone my MIFE, molecular, analytical and writing skills, which have benefited this research project and resulted in a number of publications on high quality journals during my PhD candidature.

Mak M, Zhang M, Randall D, Holford P, Milham P, Wu F, Zhang G, Chen ZH. **2019**. Chloride transport at plant-soil interface modulates barley cd tolerance. ***Plant and Soil*** 441:409-421. Impact Factor: 3.259 Citations: 0

Liu X, Fan Y, **Mak M,** Babla M, Holford P, Wang F, Chen G, Scott G, Wang G, Shabala S, Zhou M. **2017**. QTLs for stomatal and photosynthetic traits related to salinity tolerance in barley. ***BMC Genomics*** 18:1-9. Impact Factor: 3.501 Citations: 20

Liu X, Cai S, Wang G, Wang F, Dong F, **Mak M,** Holford P, Ji J, Salih A, Zhou M, Shabala S. Chen ZH. **2017**. Halophytic NHXs confer salt tolerance by altering cytosolic and vacuolar K^+ and Na^+ in *Arabidopsis* root cell. ***Plant Growth Regulation*** 82(2):333-51. Impact Factor: 2.473 Citations: 14

Chen ZH, Wang Y, Wang JW, Babla M, Zhao C, García-Mata C, Sani E, Differ C, **Mak M,** Hills A, Amtmann A. Blatt MR. **2016**. Nitrate reductase mutation alters potassium nutrition as well as nitric oxide-mediated control of guard cell ion channels in *Arabidopsis*. ***New Phytologist*** 209(4):1456-69. Impact Factor: 7.299 Citations: 42

Abstract

This PhD project forms a significant, embedded part of the project, "Novel insecticides and synergists from endemic and exotic flora", funded by the Cotton Research and Development Corporation (CRDC), 2015-2018. I aimed to identify and develop new tools for integrated pest management (IPM) in cotton (*Gossypium hirsutum*). While adoption of transgenic cotton has resulted in reduced synthetic insecticide use against the cotton bollworms *Helicoverpa* spp., secondary pests such as two-spotted spider mite, cotton aphid, green mirid, and silverleaf whitefly continue to be of concern. Thus, there is an urgent need to investigate and develop novel options, such as biopesticides and semiochemicals for insect pest management.

My proof-of-concept study employed two insect cell lines and evaluated three pyrethroid insecticides by combining three *in vitro* methods, absorbance spectrometry, confocal scanning laser microscopy (CSLM) and microelectrode ion flux estimation (MIFE) to assist in elucidating possible mode of action, which could be adopted to evaluate insecticidal activity of complex, unknown, or multi-constituent formulations. I observed that the two cell lines produced distinctly different responses. *Drosophila melanogaster* D.mel-S2 cell line was a useful model to monitor ion flux changes, resulting from insecticides with neural toxicity; however, it was less useful to determine some metabolic pathway indicators of toxic stress. Conversely, the *Spodoptera frugiperda* Sf9 cell line produced acute reactive oxygen species (ROS) in response to insecticide treatments, but was not highly responsive in electrophysiological experiments. I also showed that the natural, multi-constituent botanical extract of pyrethrum elicited different Na^+, Cl^- and Ca^{2+} ion fluxes than its synthetic, single constituent analogues, *α*-cypermethrin and esfenvalerate. These two methods used in combination with absorbance spectrometry measuring cell growth inhibition plus cell mortality assays shed some light on cytotoxic responses in differing model cell lines. The study highlights the importance of utilising multiple cell types and interdisciplinary methods to provide a better insight into mode of insecticidal action. This is especially pertinent to novel biopesticide discovery, as the underlying mechanisms for toxicity in initial screening processes are likely to be unknown.

A laboratory direct application insect bioassay utilising a Potter precision spray tower was employed during the initial foundation project to screen over 400 plant extracts for their efficacy on a selection of cotton insects, after which I identified 20 extracts for further experimentation. I have investigated the insecticidal mode/s of action from a physiological, cellular and genetic standpoint with a focus primarily on three major cotton pests, one model species and two insect cell lines. A combination of insect bioassays, absorbance spectrometry, CSLM, and MIFE have been employed to provide insight into potential target sites for these novel botanical pesticides. Ion channels are the major target sites of action in many insecticides. I found novel physiological responses of two model insect cell lines, D.mel-S2 and Sf9 and a selection of insects to some of the novel botanical extracts using cytotoxicity assay, cell-stress response and ion flux, which provide greater insight for understanding multiple toxicity responses. Moreover, a combination of insect bioassays, RT-qPCR and RNA-sequencing were employed to provide insight into potential molecular targets for these novel botanical pesticides. I identified a large number of differentially expressed genes (DEGs) especially in the categories of membrane transport and oxidative stress that are relevant for the understanding of mode of action (MOA) of these novel extracts on cotton insects. Moreover, I found that, in comparison to the susceptibility of *Aphis gossypii* to the novel botanical extract 68N.M, the DEGs encoding such as chemoreceptors and Ca^{2+} channels may equip *D. melanogaster* with superior capacity to sense and tolerate 68N.M. Many of these key DEGs should be considered for more detailed functional analysis in both *D. melanogaster* and *A. gossypii* to elucidate the gene function in insecticidal MOA in the future.

In summary, by targeting ion transport, ROS production, chemoreceptors and their associated genes in insect cells and insects, there is a great potential to develop reliable laboratory screening methods to identify novel and environmental friendly botanical pesticides for Australian and global agricultural industry in the future.

Chapter 1: Introduction and Literature Review

1.1. General introduction

In 2013, Australian farmers spent AUD$1.4 billion on chemical inputs other than fertilisers; of that AUD$500 million is estimated to have been spent on insecticides (ABARES 2014). Australia requires new pest management products, which have been slow in meeting market demand (Gregg et al. 2010). Only three novel products, MOOV™, Magnet™, and Sero-X® have been developed and commercialized within Australia according to the Australian Pesticide and Veterinary Medicines Authority (APVMA) (2020) registration records. Therefore, there has been a paucity in research and development of new active ingredients as commercially available products for an innovation-thirsty market (Gregg et al. 2010). While the advancement in genetically modified (*Bt*) cotton crops has delivered a great windfall for the Australian cotton industry, the rate of development of resistance by *Helicoverpa armigera,* in particular, and *Helicoverpa punctigera* are of great concern. In a recent review of *Bt* resistance in Australia, Downes et al. (2016) identified a lack of data to predict the development of field-evolved resistance and cautioned against the assumption that resistance alleles in key pests are low, until proven otherwise. With this in mind, the Australian cotton industry is once again looking for novel compounds with new or complex modes of action that can support both transgenic technology and conventional cotton, and integrated pest management (IPM) strategies.

Insecticides produced from plant extracts and essential oils work on a wide range of target pests due to their lipophilic properties, which allow them to act as toxins, fumigants, repellents and anti-feedants and anti-oviposition/ovicides. Although many essential oils have been identified as effective biopesticides for both domestic and agricultural application, they have not been widely adopted, with preference for synthetic products (Isman 2006). Several reasons have been proposed as to why commercially available botanically-derived pesticides are very limited. These issues include but are not restricted to: the difficulties in obtaining sufficient and sustainable quantities of raw material that provide a reliable chemical signature in order to produce standardised products on a commercial scale; regulatory and patent

constraints; slower mode of action (MOA) effects and a consumer familiarity with fast knockdown synthetics; and lastly, limited residual action leading to more frequent application rates (Isman 2006; Koul et al. 2008).

In the past, the obstacles of production and commercialization have seemed overwhelming, even in the face of scientific evidence supporting the effectiveness of naturally derived compounds. However, with increasing consumer preference for organically grown crops and other protein sources, challenges of pest resistance to synthetic pesticides and genetically modified crops, and an increasing global focus on environmental protection, there is renewed interest in looking to nature for either direct pesticide sources or nature-equivalent solutions (Gregg et al. 2010; Isman 2006). Yamamoto (1970) suggests that interest in natural insecticides is warranted because the shorter environmental persistence may account for the rarer occurrence of resistance than for synthetic pesticides, and also they are often selective in their toxicity, citing "nicotine, rotenone and pyrethrins interact with different target sites in different ways". For instance, pyrethrins act on nerve axons found in both the central and peripheral nervous systems of insects, targeting the sodium channels to disrupt signal transduction but do not affect muscle respiration (Gammon et al. 1981; Yamamoto 1970). Nicotine acts on the central nervous system synapses, targeting the nicotinic acetylcholine receptor (NAChR) by mimicking acetylcholine at the nerve-muscle junction (Rattan 2010; Shivanandappa et al. 2014). Rotenone acts on the mitochondrial respiratory chain by inhibiting electron transport, NADH2 oxidation and the reduction of cytochrome-b, predominantly in tissues of high energy needs such as muscles and flight apparatus (Hollingworth et al. 1994; O'Brien 1966; Rattan 2010). Unfortunately, this biochemical target site is shared with mammals, resulting in a high toxicity and limiting its use in agriculture (Casida et al. 2013; Walia et al. 2017). Azadirachtin, derived from the neem tree, *Azadirachta indica* Juss (Meliaceae) is a good example of a commercial botanical insecticide, which has been favoured because of its relative safety to mammals. Since its identification in 1968, azadirachtin has been used because of its widely published insect anti-feedant and growth regulation activities. However, in recent years it has also been reported to cause nervous system disruption and calcium channel blocking (Qiao et al. 2014), and currently azadirachtin's MOA is still classified by the Insecticide Resistance Action Committee as

unknown/uncertain (IRAC). This highlights the need to thoroughly investigate target sites and MOAs of new insecticides, preferably prior to their commercialisation (Veitch et al. 2008). Moreover, by combining advances in physiological, genetics and genomic techniques, evidence of specific ion channels and receptors as target sites for insecticides can be shown (Raymond-Delpech et al. 2005). Insects are useful model organisms to study many genetic, biochemical, and molecular responses. *Drosophila melanogaster* in particular has a number of well-known membrane channels and transporters, and also a widely studied and easily manipulated genome (Byerly et al. 1988).

My study continues from some earlier and concurrent biopesticide and semiochemical discovery work undertaken through the Cotton Research and Development Corporation (CRDC) funded project "Novel insecticides and synergists from endemic and exotic flora" (UWS1401). During that project, 358 plant extracts were assessed for their toxicity against three key arthropod species that pose significant pest challenges in Australian cotton. The objective of my study was to use a selection of botanical extracts that displayed high efficacy during initial bioassays and investigate the cellular responses they elicit in insect pest and model organisms, to elucidate their possible MOA at the physiological, cellular and molecular level. Particular attention was paid to the physiological and molecular regulation of ion transporters and channels, in order to offer insight into the potential insecticide-resistance challenges of the compounds tested.

1.1.1. Correlation of toxicity between whole organisms and cell lines

For insecticides to be effective they must enter an organism through the cuticular, respiratory or gut membrane barriers before reaching their target site of action. To evaluate the selected novel botanical extracts, comparative studies between whole insects and their model cell lines were performed, see Table 7-4, Appendix 3 for selected extracts and their efficacy against the three whole organisms. Comparing between responses of whole organisms and their representative cell lines was considered likely to provide a direct indication of loss of toxicity as a result of the insects' external barriers. This also provided a proof of concept for the methods that were employed, linking cellular analysis of model organisms back to whole organism responses.

Therefore, my first hypothesis was:

- Insect cell line cytotoxicity responses to the active compounds in the novel plant extracts are comparable to whole organism mortality from bioassays, provided there is no significant whole organism barriers or metabolic detoxification.

1.1.2. Elucidation of the target site and mode of action

In order to identify the possible target sites and mode(s) of action (MOA), several techniques were used. CSLM was used to measure ROS fluorescence as an indicator of intracellular stress response to each toxicant. This may help identify the molecular pathways involved in cellular defence. I then measured the ion flux response of insect cells to the same concentration of extracts in order to identify which ion channels were involved in toxicity responses and how cell homeostasis was modified. For example, if sodium channels showed an extended activation of current or a lack of steady-state inactivation, the mode of action would be similar to pyrethrins, which cause a prolonged opening of sodium channels and a loss of sensitivity to membrane potentials.

Two hypotheses were postulated to investigate the underlying mode(s) of action:

- In model insect cell lines, the over-production of ROS indicates a high level of cellular stress-response to individual extracts, while an under-production in ROS indicates extracts which are less toxic to insect cells or have antioxidant properties.
- In model insect cell lines, the increase of ion flux indicates a high level of cellular toxicity resulting from novel extracts with nerve-poisoning-type modes of action.

1.1.3. Changes in gene expression in *Drosophila melanogaster* and *Aphis gossypii*

When developing an insecticide for a particular crop, such as cotton, with known insecticide-resistant pests, such as *Helicoverpa armigera*, *Helicoverpa punctigera, Tetranychus urticae* and *A. gossypii*, understanding genetic regulation provides two benefits. First, correlations between electrophysiological ion flux studies and ion channel gene expression may lead to MOA discovery. Second, differential gene expression of previously identified insecticide-

resistance genes may provide valuable insight for the industry in making future decisions relating to integrated resistance management program development and future targeted research endeavours.

Therefore, my last hypotheses were:

- The differentially expressed genes (DEGs) of dead insects of one pest species and one model species following exposure to novel plant extracts indicate key genes controlling the insecticidal modes of action.
- The DEGs of surviving insects exposed to novel plant extracts elucidate key genes in the detoxification pathways.

1.2. Review of literature

1.2.1. Sustainable agriculture and the Australian cotton industry

Australian agriculture faces heightened challenges in the form of climate mitigation, resource management and pest and disease mitigation. This is due to the long history of successive droughts and floods, low-nutrient soils, limited water resources, and mild winters in the major Australian crop growing regions that provide almost continual pest pressures (Cotton Australia 2019). Despite this, the cotton industry has consistently improved its sustainability over the last few decades. From 1992 to 2019 the average bale now takes 48% less water, 34% less land and 97% less insecticides to produce (Cotton Australia 2019). The majority of cotton is grown by family owned and operated farms (80%) in the states of NSW (66%) and Queensland (33%) on an average of 195,000 ha (ABARES 2019; Cotton Australia 2020). Over the last decade, Australian cotton growers produce an average of 3 million bales or 0.7 million tonnes of cotton lint and 1.0 million tonnes of seed making them major contributors to the Australian economy, exporting an average AUD$2.0 billion annually, which sees Australia ranked as the third largest exporter of cotton in the world (ABARES 2019). On average, each farm produces 11.8 bales per hectare, and spends AUD$129 per hectare on chemical inputs. With cotton prices relatively static and input costs increasing, the increasing profit gains are due to improving efficiencies and yield (Sellars et al. 2019). The industry as a whole has achieved this through its world-leading research, development and extension practices, which are joint funded by growers and government (Cotton Australia 2019). Therefore, grower engagement and innovation adoption levels are high and result in improved yield, quality, efficiencies, and resource management. Reductions in insecticide use against *Helicoverpa* spp. have come primarily from the innovation and adoption of *Bacillus thuringiensis Bt*-cotton (Cattaneo et al. 2006; Fitt 2008; Fitt et al. 2005; Lu et al. 2012) and strong farmer uptake of IPM through extension and engagement strategies, as well as the conservation and use of beneficial insects (Mensah 2002). However, improvements are still needed to manage sucking pests which are not affected by Bt-toxins in addition to protecting pollinators and beneficial insects , as well as novel chemicals to broaden pesticide rotation

options that support pest resistance management strategies (Fitt 2008; Mensah et al. 2013; Walia et al. 2017).

During the post green-revolution, agriculture saw an increase in intensification leading to an erosion of crop genetic diversity and a fragmentation of natural habitats, which had previously supported insect pest regulation by natural enemies. In order to improve the management of both primary and secondary pests while enhancing crop yields and limiting environmental impacts, a system-wide approach is needed (Brévault et al. 2014; El-Wakeil et al. 2012). One recent and major advancement in crop protection came from the introduction of transgenic plants such as potato, corn and cotton, which enabled the crops themselves to possess insecticidal action. These crops have been genetically enhanced to contain the delta-endotoxin crystal protein (Cry) producing genes from the entomopathogen *Bacillus thuringiensis* Berliner (*Bt*) and are commonly known as *Bt*-crops (Downes et al. 2010). *Bt*-crops have been quickly adopted by their respective industries, including cotton, significantly reducing reliance on insecticides (Ware 2000). In 2012, it was reported that the widespread adoption of *Bt*-cotton in China caused a reduction in insecticidal use, and aided the recovery of natural enemies, resulting in increased biocontrol (Lu et al. 2012). However, it has recently been reported that China's intensive planting of *Bt*-cotton has resulted in *Bt* resistance in target pests such as cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (An et al. 2015; Zhang et al. 2012). Furthermore, it is suggested that the insertion of *Bt*-toxin genes have caused changes in concentration of secondary metabolites such as terpenes, tannins and flavones, which act as natural defence compounds (Li et al. 2015). The Australian cotton industry has also experienced *Bt* resistance in *H. armigera* first-hand and has accepted that multidisciplinary research is needed to formulate innovative strategies to meet the current challenges (Baker et al. 2013; Brévault et al. 2014; Downes et al. 2016).

Prior to 1992 in the pre-IPM age of cotton in Australia, pests such as *Helicoverpa* spp. were managed by overreliance on broad-spectrum synthetic insecticides and, often, their indiscriminate use. This led to insecticide resistance, environmental pollution and the loss of beneficial predators and parasites, as well as increased input costs and the need for human and environmental risk management strategies (Brévault et al. 2014; Guedes et al. 2016;

Mensah 2002). Chewing insects such as *Helicoverpa* spp. cause significant tissue damage during feeding, especially to developing buds (squares), flowers and bolls, leading also to secondary infections by fungi or pathogens that cause damage or discolouration and in turn reduce the lint grade (Munro 1994). As agricultural industries continue to move away from inexpensive organophosphorus methyl carbamate and synthetic pyrethroid insecticides in favour of safer options that pose less risk to humans and the environment, there arises an urgent need for more natural, yet cost-effective alternatives. Generally speaking, the management of pests by insecticides is becoming more expensive and progressively more difficult as the issue of insecticidal resistance becomes more common. Studies show that IPM programs that rely on intercropping, supplementary food sprays and beneficial predators alone are unable to deliver yields comparable to insecticide management; hence, alternative strategies are needed (Casida 1980; Mensah 2002).

In 2015, approximately 90% of commercial cotton grown in Australia was *Bt* varieties, leaving approximately 10% of conventional cotton growers relying exclusively on chemical insecticides or organic techniques (Mensah et al. 2015). Transgenic *Bt*-cotton was first employed by Australian cotton growers in the mid-1990s with the introduction of Cry1Ac toxin expressing Ingard™ (Downes et al. 2012). The efficacy of Cry1Ac toxin is affected by plant part, plant age and environmental factors, which eventually lead to increased *Bt* resistance by *H. armigera*. This instigated the development of a double transgenic *Bt*-cotton (Bollgard II™) which expresses both Cry1Ac and Cry2Ab toxins (Downes et al. 2012; Olsen et al. 2005a; Olsen et al. 2005b). Globally, multiple *Cry1Ac* and three *Cry2Ab* genes and alleles have been identified in *H. armigera*, prompting the introduction of the third generation of *Bt*-cotton, Bollgard 3®, containing an additional *Bt* gene, *vip3A*. (Monstano n.d; Whitehouse et al. 2014). An additional problem associated with continuous use of *Bt*-cotton has been the resurgence of secondary pests, particularly sucking insects such as aphids, mirids and whiteflies (Wilson et al. 2018)

The key aim of the foundation project, and additionally the work presented in this thesis, was to identify novel botanical pesticides that could effectively manage *Bt*-resistant *Helicoverpa* spp. as well as secondary pests, without harming pollinators and natural enemies,

which play an important beneficial role in cotton IPM programs (Downes et al. 2012; Keogh et al. 2010; Mensah et al. 2013).

1.2.2. Cotton pests

The Australian cotton industry is no stranger to the challenges of pest control, with plants being vulnerable for their entire six-month growing period (Wilson et al. 2018). An extensive range of pests, including over 40 species of insects and 7 species of mites, cause damage that results in economic loss for the industry. These include chewing pests such as cotton bollworm *Helicoverpa armigera*, native budworm, *Helicoverpa punctigera* Wallengren, and locusts; sucking pests such as aphids *Aphis* spp. and *Myzus persicae* (Hemiptera: Aphididae), spider mites *Tetranychus* spp. (Acari: Tetranychidae), green and brown mirids *Creontiades* spp., yellow mirids *Campylomma liebknechti* (Hemiptera: Miridae), tobacco thrips *Thrips tabaci,* tomato and western flower thrips *Frankliniella* spp. (Thysanoptera: Thripidae), green vegetable bug *Nezara viridula* L. (Hemiptera: Pentatomidae) and pale cotton strainer *Dysdercus sidae* Montrouzier (Hemiptera: Pyrrhocoridae); honeydew-secreting pests such as silverleaf whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), and mealybug *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae); and soil pests such as wireworms (Coleoptera: Elateridae), earwigs (Dermaptera) and symphylans (Myriapoda: Symphyla) (Fitt 1994; Maas et al. 2016, 2020; Wilson et al. 2018). The three major pests examined in my study, *Helicoverpa armigera*, *Tetranychus urticae* and *Aphis gossypii,* are discussed below.

1.2.2.1. *Helicoverpa* spp. (cotton boll and budworm)

Helicoverpa spp. is a moth genus belonging to the family Noctuidae. In Australia two species, *H. armigera* and the native *H. punctigera*, are prolific polyphagous pests. *Helicoverpa armigera* is the most economically damaging pest of cotton worldwide, feeding on new growth and reproductive structures (Fitt 1989; Mensah et al. 2014). The facultative migration behaviour of *Helicoverpa* spp. based on environmental cues and diverse alternate host-plants adds to its high mobility and widespread distribution in Australia (Fitt 1989; Gunning et al. 1994). Additionally, some individuals overwinter in spent crops and volunteer seedlings after

harvest or move to nearby host-plants, emerging in the spring to re-establish populations (Downes et al. 2012). As of May 2020, *Helicoverpa armigera* is resistant to 48 insecticidal compounds but the cotton industry has managed this pest in recent decades by the adoption of *Bt*-cotton expressing up to three *Bt* toxin genes. However, *H. armigera* has also developed significant resistance to these plant-embedded toxins (Mota-Sanchez et al. 2020b; Sparks et al. 2015).

1.2.2.2. *Tetranychus urticae* (two-spotted spider mite)

Tetranychus urticae is one of the most polyphagous species of the family Tetranychidae, feeding on over 1,100 plant species from more than 150 different families including cotton, fruit trees, field and greenhouse vegetables, and ornamentals, and is recorded in over 120 countries (Herron 1994; Migeon et al. 2019). In the Australian cotton industry, *T. urticae* has been identified as the dominant mite pest since the early 1980s with a propensity for developing insecticide and acaricide resistance due to its extraordinary adaptability (Herron 2003). As of May 2020, *T. urticae* has developed resistance to 96 compounds (Mota-Sanchez et al. 2020c; Sparks et al. 2015)

1.2.2.3. *Aphis gossypii* (cotton aphid)

Aphis gossypii is a widely distributed and polyphagous species, and an identified high priority pest by the Australian cotton industry (Brévault et al. 2008; Maas et al. 2016). *A. gossypii* feeds on over 100 species of crops and many more weeds and is a vector for more than 50 plant viruses (Van Emden et al. 2017). In addition to its direct damage through feeding on plant sap, it can also cause indirect damage by contaminating cotton lint with honeydew and transmitting important plant viruses such as cucumber mosaic virus and cotton bunchy top virus (Badgery-Parker 2015; Deguine et al. 2007; Reddall et al. 2004). Reproduction is parthenogenetic and almost continuous in warmer climates like Australia (Chen et al. 2013). This, combined with previous high pesticide use in Australian cotton, have led to it developing resistance to many pesticides (Mota-Sanchez et al. 2020a; Van Emden et al. 2017). Additionally, it produces detoxification enzymes, such as peroxidases, in saliva which contribute to the breakdown of natural plant defence compounds (Ebert et al. 1997). As early

as 2001 in Australia, a survey revealed high levels of resistance in some *A. gossypii* populations to a selection of seven commonly used cotton insecticides from three different chemical groups (Herron et al. 2001). *A. gossypii* to date has shown resistance to 50 compounds (Mota-Sanchez et al. 2020a).

1.2.3. Botanical pesticides

Plants employ a wide range of chemical and physical mechanisms to defend themselves against pest and disease attack. They synthesize many secondary metabolites that are not involved with essential development, growth and reproduction processes. Secondary metabolites can be divided based on their biosynthetic origins into three major groups: terpenoids, the largest group consisting of over 25,000 compounds; alkaloids, which include compounds commonly used in human therapy such as atropine, morphine, nicotine and caffeine amongst 12,000 others; and phenolics, which include tannins and flavonoids that are commonly employed as insecticides (Bassman 2004). Secondary metabolites may act as ingestion toxins, repellents, feeding or oviposition deterrents, or growth and development inhibitors to enemies (Bassman 2004) while also acting as attractants to pests and beneficial insects such as parasitoids and predators (Khan et al. 2008). There are four major groups of botanically derived pesticides (pyrethrum **I** and **II**, rotenone **IV**, neem, essential oils) and three lesser used groups (nicotine **III**, ryania, sabadilla) (Casida 1980; Isman 2006).

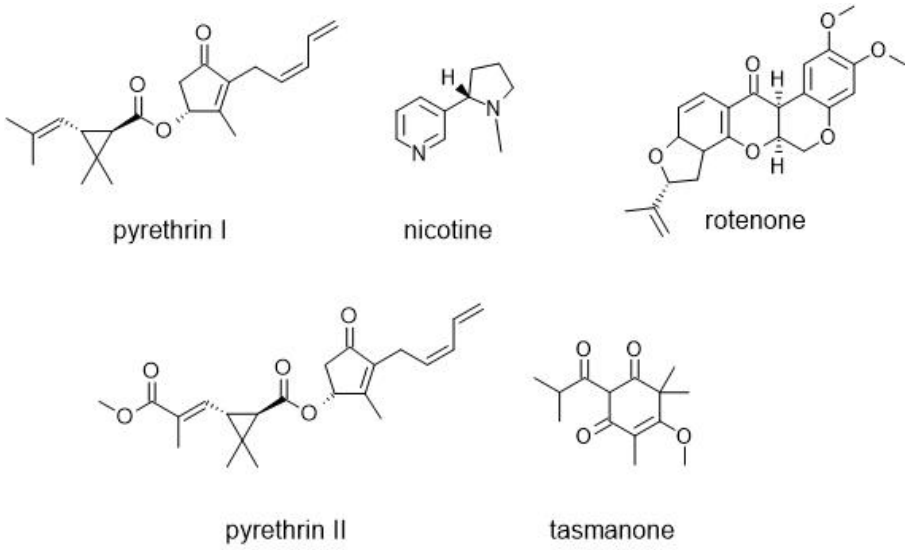


Figure 1-1 Some key botanical pesticide chemical structures.

Structures sourced from PubChem online database and redrawn in ChemDraw Professional v15.1

1.2.3.1. Pyrethrum

Pyrethrum is extracted from the dried flowers of *Chrysanthemum cinerariifolium* and *Chrysanthemum coccineum* of the Asteraceae family. These species are native to the Republic of Croatia and have been used as pesticides since the early 1800s. Australia now produces over half of the world's supply of pyrethrum (Casida 1980; O'Malley et al. 2015; Perry et al. 1998). The *Chrysanthemum* extract contains two active groups of ester compounds, "pyrethrin I" – the esters of chrysanthemic acid; pyrethrin I, cinerin I and jasmolin I, and "pyrethrin II" – the esters of pyrethric acid; pyrethrin II, cinerin II and jasmolin II (Bloomquist 1999; Casida 1980). Pyrethrins are neuroactive insecticides, targeting the voltage-gated sodium channels (Wakeling et al. 2012). Intoxication by pyrethrin I is characterized by hyper-excitation, whole body tremors, and convulsions. Intoxication by pyrethrin II is characterized by incoordination and general loss of body functions (Bloomquist 1999).

1.2.4. Insecticidal modes of action

Insecticides are generally intended to kill the selected organism; they do this by blocking usually one or occasionally multiple metabolic processes (Ware 2000). The most common

physiological target for insecticides is the nervous system, primarily because of its sensitivity and inability to recover from irreversible damage (Perry et al. 1998). Although the MOA at a molecular level is not known for all insecticides, there is a growing body of evidence that the disruption of neural signalling by axonic poisons can be monitored, explained and capitalized upon by the measurement of ionic movements across membranes (Yu 2008). Besides disrupting the electronic nerve impulse as it travels along the axon nerve membrane, some insecticides work by interfering with neurotransmitters. For example, compounds belonging to the organophosphate and carbamate subgroup inhibit the enzyme acetylcholinesterase, while neonicotinoids interfere with the acetylcholine binding site (Perry et al. 1998). There are at least 11 known target sites illuminating the MOA for neuroactive insecticides (Casida et al. 2013).

The Insecticide Resistance Action Committee (IRAC) has identified the MOAs of insecticides that are used globally to manage *H. armigera* (Table 1-1). The categories are: 1A & 1B (AChE inhibitors), 2A, (gaba-gated chloride channel blockers), 3 (sodium channel modulators), 5 (nAChR allosteric modulators), 6 (GluCl channel allosteric modulators), 13 (suspected uncouplers of oxidative phosphorylation via disruption of the proton gradient), 18 (ecdysone receptor agonists), 19 (octopamine receptor agonists), 22 (voltage-dependent sodium channel blockers), and 28 (ryanodine receptor modulators) work on nerves and muscles, while category 11 are microbial disruptors of the insect midgut membrane (IRAC 2015).

Specific assays have been developed to screen putative insecticides for a number of these MOAs. An electrophysiology study may use mutant cell lines such as the S2-RDLA^{302S} or use *Xenopus* oocytes to take patch-clamp recordings used to measure membrane currents and screen for GABA sensitivity linked to dieldrin-resistance (Buckingham et al. 1996). Those interested in signalling pathways resulting in detoxification of neonicotinoids may use reporter gene assays to identify a cytochrome P450's putative promoter CYP6CM1, linked to imidacloprid resistance (Yang et al. 2020). However, due to their specificity, they have restricted use for broader insecticidal studies, such as for biodiscovery.

Table 1-1 IRAC 2011 - Mode of action class for *H. armigera* insecticides

IRAC Cat #	Primary Site of Action	Insecticide Class	Insecticide Name (COUNTRY)
1A	Acetylcholinesterase (AChE) inhibitors	Carbamates	Methomyl USA,SPA,CAM,AUS, Thiodicarb USA,SPA,IND,AUS
1B	Acetylcholinesterase (AChE) inhibitors	Organophosphates	Chlorpyrifos SPA,IND,AUS Chlorpyrifos-methyl AUS Methidathion AUS Omethoate AUS Parathion-methyl AUS Profenofos CAM,AUS Thiometon AUS
2A	GABA-gated Chloride channel blockers	Cyclodiene organochlorines	Endosulfan SPA,CHI,CAM,IND
3	Sodium channel modulators	Pyrethroids and Pyrethrins	Alpha-cypemethrin AUS Bifenthrin USA,AUS Beta-cyfluthrin AUS Cypermethrin USA,CAM,IND,AUS Cyfluthrin USA,AUS Deltamethrin AUS Esfenvalerate USA,AUS Fenvalerate CHI,IND Gamma-cyhalothrin USA,AUS Lambda-cyhalothrin CHI,SPA,AUS
5	Nicotinic acetyl-choline receptor (nAChR) allosteric modulators	Spinosyn	Spinosad USA,CAM,AUS
6	Glutamate-gated Chloride channel (GluCl) allosteric modulators	Avermectin	Emamectin benzoate CHI,CAM,AUS
11	Microbial disruptors of insect midgut membranes	*Bacillus thuringiensis* and the insecticidal proteins produced	Dipel USA,AUS, *Bt*-cotton USA,CHI,AUS
13	Uncouplers of Oxidative phosphorylation via disruption of the proton gradient	Pyrroles Dinitrophenols Sulfluramid	Chlorfenapyr AUS
18	Ecdysone receptor agonists	Diacylhydrazines	Methoxyfenozide AUS
19	Octopamine receptor agonists	Triazapentadiene	Amitraz AUS
22	Voltage-dependent Sodium channel blockers	Oxadiazine	Indoxacarb USA,CAM,AUS
28	Ryanodine receptor modulators	Diamides	Chlorantraniliprole USA,AUS Cyantraniliprole AUS

Note: Chemical insecticides and *Bt* formulations used around the world to manage *H. zea* and *H. virescens* in USA, and *H. armigera* in China (CHI), Spain (SPA), India (IND), Cameroon (CAM) and Australia (AUS). (Avilla et al. 2010; Brévault et al. 2009; Chaturvedi 2007; IRAC 2015; Maas et al. 2015; USDA 2014; Yang et al. 2013b)

1.2.5. Ion channel modes of action and related genes

Understanding how insecticides affect ionic movements is critical to many MOA and target site studies. Ions move across cell membranes via either voltage-gated or chemically-gated ion channels, carrier molecules, or active pumps. Ion channels and carrier molecules move with electrochemical gradients, whilst pumps such as the Na^+- K^+ ATPase move against their concentration gradient at the cost of ATP energy generated from hydrolysis (Eldefrawi et al. 1985). In insects there are at least four known K^+ channels on nerves and muscles with a further 30 predicted K^+ channels, 9 voltage-gated Ca^{2+} channels, 4 voltage-gated Na^+ channels and 3 Cl^- channels (Eldefrawi et al. 1985; Towers et al. 2002).

At a physiological level, compound entry mechanisms, toxicology and MOA studies are the initial steps in evaluating pesticides at a whole organism level; however, model cell lines can also prove invaluable in the discovery of non-toxic and protective responses, such as enzymatic detoxification, shifts in metabolism and physiological trade-offs of growth and reproduction (Guedes et al. 2016). At the insect cellular level, experiments incorporating advanced electrophysiological techniques such as voltage and patch clamping, and ion flux studies can provide insights into several areas of toxin research, including insecticidal MOA (Breer et al. 1985; Raymond-Delpech et al. 2005). Insect ion channel research has been somewhat hindered by the difficulties in harvesting adequate quantities of tissue compared to mammalian subjects; cell cultures offer a convenient alternative when utilizing electrophysiological and biochemical studies to investigate ion channels and carriers at a whole cell level (Eldefrawi et al. 1985). At a subcellular level, molecular techniques and cloning have helped to understand pesticidal MOA sites as well as to identify and predict gene mutations associated with insecticide resistance. Although *Drosophila melanogaster* D.mel-S2 cells are widely used for transfected gene expression studies of ion channels, native ion channels have not been investigated in detail (Nakao et al. 2010; Parnas et al. 2007; Yeromin et al. 2004). Only one early study has successfully measured K^+ ,Na^+, and Ca^{2+} currents of *D. melanogaster* neuron in embryonic cultures (Byerly et al. 1988).

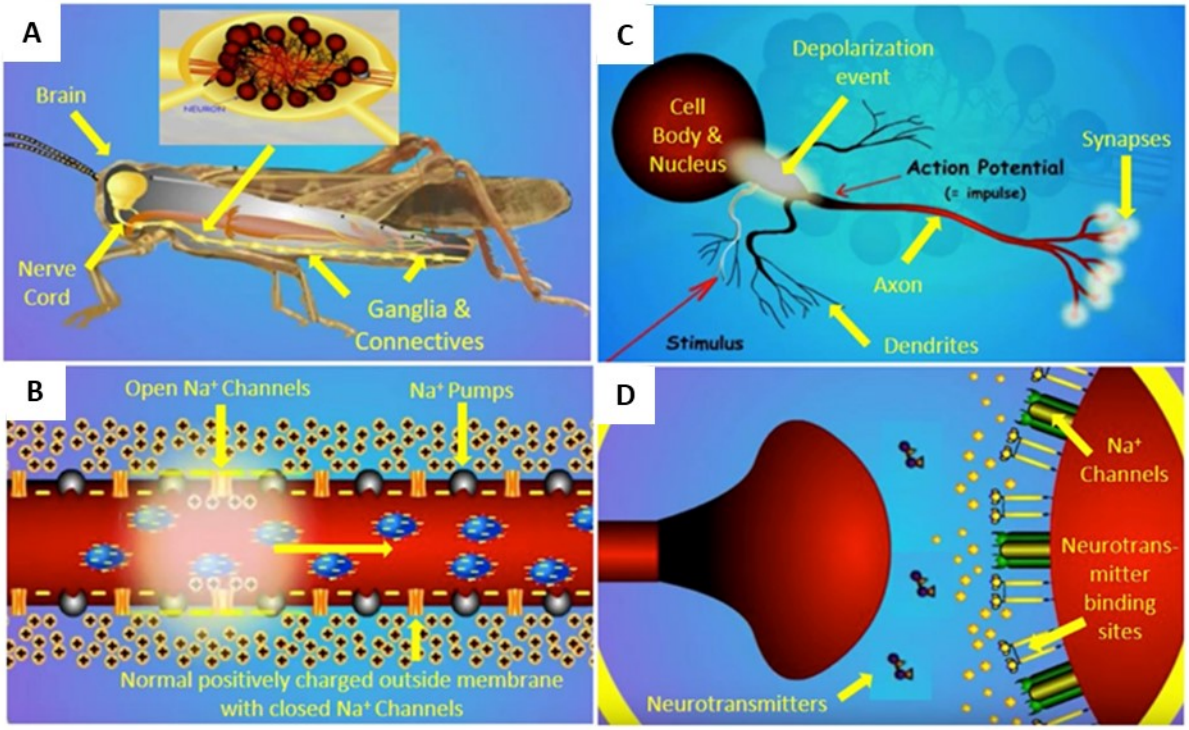


Figure 1-2 Schematic diagram of insect nervous system

(**A**) The structure of the insect nervous system showing the route from the brain to nerve cord. The nerve cord is made up of ganglia housing neuron clusters and connectives (Wigglesworth 2016). (**B**) The structure of an individual neuron showing the input stimulus picked up by the dendrites causing a depolarization event at the axon junction which then moves along the axon as an action potential to the synapses (Schmitt 1962). (**C**) The axon membrane showing the normal positively charged membrane at rest preceding and following the impulse as it moves along the axon in response to a change in membrane potential. Na^+ channels open in response to external depolarization (shown by yellow dashes). Ions flow into the neuron cell via the open ion channel until the channel closes. The electrical impulse then moves along the axon. Na^+ pumps open after the impulse has passed in order to pump ions back outside the cell, reinstating the normal resting potential (Ganetzky et al. 1986). Open Na^+ channels are the site of pyrethrin/pyrethroid mode of action which prohibits channel closure (Raymond-Delpech et al. 2005; Wakeling et al. 2012). (**D**) Impulses must 'jump' the synapse gap to move from one neuron to the next. Neurotransmitter binding sites open Na^+ channels to depolarize the dendrite of the next neuron. Enzymes degrade neurotransmitters to allow channels to close and the membrane potential of the dendrite to return to normal and await the next signal (Raymond-Delpech et al. 2005). Organophosphates and carbamates bind with enzyme sites, inhibiting the breakdown of neurotransmitters, and thereby over stimulating the channels to an open position. Neurotransmitter binding sites are affected by nicotine/neonicotinoid type insecticides (Casida et al. 2013). Underlay graphics credited to Professor Emeritus Dr. Larry Keeley, Department of Entomology, Texas A&M University, https://www.youtube.com/channel/UC4A4XQm-sjDJLaPZjcOCazA

1.2.5.1. Na^+ channels and their encoded genes

In insects, voltage-dependent sodium channels are essential in nervous and cellular system electrical signalling (Dong et al. 2015). Sodium channels have been identified through voltage-clamp studies and are a primary site for the MOA of many insecticides, including dichloro-diphenyl-trichloroethane (DDT), pyrethroids and dihydropyrazoles (Khambay et al. 2010). Pyrethroids, such as tetramethrin, cause voltage-gated sodium ion channels to close more slowly than normal, thereby prolonging the 'open' stage which allows sodium ions to enter the cell for an extended period, resulting in repetitive firing and depolarization of the membrane (Bloomquist 1996; Yamamoto et al. 1983). There is also strong evidence that pyrethroids bind two molecules simultaneously at two sites, thereby locking sodium channels into the open position (Silver et al. 2014). Pyrethroids may also inhibit the delayed outward rectifying K^+ channel at sub-micromolar concentrations (Eldefrawi et al. 1985). DDT's general MOA is similar to Pyrethrin I in that it also modulates sodium channels, causing hyper-excitation, convulsions and paralysis (Narahashi 2010). Sodium channels have, however, been shown to have differing characteristics within a species, dependent on their location. In cockroaches, the inward Na^+ current recorded from isolated giant axons takes four times longer to change from activation to inactivation, with an activation threshold 15 mV more positive than that recorded in dorsal unpaired median cells (Lapied et al. 1990). Several genes have been shown through *Drosophila* mutants to affect sodium channels. Paralysis locus (*Para*^{ts}) is located on chromosome X of *D. melanogaster* and codes for temperature-sensitive alleles, and above a restrictive temperature, of 29°C for adults (Suzuki et al. 1971) and 37°C for larvae (Siddiqi et al. 1976), results in a loss of action potentials needed for normal nervous system signalling. *Temperature Induced Paralytic E* (*Dmel**tipE*) is co-expressed with *Para*^{ts} and has been found at the temperature-induced paralysis locus. The *Para*^{ts} locus encodes the principle class of sodium channels which are expressed in both the central and peripheral nervous systems (Ganetzky et al. 1986; Hong et al. 1994). *No action potential locus* (*nap*) is also temperature sensitive, and causes defects in nerve-membrane excitability similar to *Para*^{ts} mutants. *No action potential* larval mutants show increased sensitivity to the potent neurotoxin tetrodotoxin (TTX), which binds to voltage-gated Na^+ channels, blocking Na^+ and inhibiting action potentials (Ganetzky et al. 1986). *Drosophila Sodium Channel 1* (*DSC1*),

originally named a sodium channel gene, was later found to code for a novel type of voltage-gated cation channel in insects which has no orthologues in vertebrates. The *DSC1* channel has been suggested to have some influence on pyrethrum toxicity as *DSC1* knock-out mutants are more susceptible to sodium channel activators (Dong et al. 2015). Furthermore, the identification of channels unique to insects, such as the *DSC1* family, provide opportunities to develop insecticides that target known MOA sites without adverse effects in vertebrates (Dong et al. 2015). It may be advantageous to investigate these genes in relation to the MOA for the novel extracts and pure compounds used in this study, along with the Na^+ flux recordings.

1.2.5.2. Ca^{2+} channels and their encoded genes

Calcium channels, which are on nerve and muscle membranes, share a similar structure and mechanism with sodium channels, opening and closing with membrane depolarization caused by action potentials (Yu 2008). Ryanodine, chlorantraniliprole and flubendiamide have been shown to activate the calcium release channel in the sarcoplasmic reticulum, flooding protein filaments in insect muscles, and causing sustained contractions and paralysis (Bloomquist 1999). Whilst there are a smaller number of commercial insecticides showing a direct influence over Ca^{2+} channels, spider toxins such as *Plectreurys* toxin and *Holoena* toxin have a Ca^{2+} current blocking effect when applied to *D. melanogaster* neurons cultured from embryonic cells (Leung et al. 1989). Whole dissected *D. melanogaster* brains are also a comparable substitute for CNS neural functions when investigating sodium action potential-dependent and independent (using TTX sodium channel blocker) synaptic currents to better understand the nAChR-mediated fast synaptic transmissions. Whole brains have additionally been used to understand the role of Ca^{2+} channels in mediating miniature excitatory postsynaptic currents (mEPSC) (Gu et al. 2006; Qiao et al. 2014). Like Na^+ channels, Ca^{2+} channels have also been reported to elicit contractions in differing locations. Qiao et al. (2014) reported decreases in both Ca^{2+} current peak and sustained amplitude in *D. melanogaster* neurons associated with the botanically-derived insecticide azadirachtin.

1.2.5.3. Cl^- channels and their encoded genes

Insecticides that affect chloride channels, such as heptachlor, dieldrin and endrin, are rarely used due to their high mammalian residual activity and bioaccumulation, although it is worth noting their MOA is shared with the newer *meta*-diamide compounds. These insecticides target the inhibitory (suppressing) neurotransmitter, γ-aminobutyric acid (GABA), interfering with its binding to the receptor site or to chloride channels. Both actions have the same effect of restricting the moderating effects of GABA, which then leaves chloride channels open for extended influx of Cl^- ions, causing hyperpolarized membranes. Exposure to chloride channel blocking insecticides, therefore, results in hyper-excitation and convulsions, leading to death (Bloomquist 1999; Narahashi 2010). The first single ion channel recordings linked the commercially important phenylpyrazole insecticide fipronil (sodium channel open blocker) to the *Resistance to Dieldrin (Dmel\Rdl)* gene (Grolleau et al. 2000). *Dieldrin*-resistant *D. melanogaster* were then cloned to further identify the GABA receptor as the MOA associated with this dieldrin resistance-linked gene subunit (Nakao et al. 2016). Several pyrethroids of both type I and type II have been shown to affect Cl^- channels by decreasing the probability of channel opening in mammals, although little work seems to have been applied directly to insect cell lines (Wakeling et al. 2012). A recent study investigated the suitability of native (non-genetically modified) mosquito cells as a model for insecticide screening and corresponding ion currents with encouraging results. Jenson and co-workers (2016) focused primarily on voltage-sensitive chloride channels and showed that 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) and fenvalerate (type II pyrethroid) blocked most of the Cl^- current. *Dmel\Clic* is the protein coding gene related to intracellular chloride channels and is likely to elicit gene regulation in response to chloride current abnormalities.

1.2.6. Insecticide detoxification and related genes

Next-generation sequencing (NGS) technology can screen a large number of differentially expressed genes (DEGs) and identify multiple genes associated with insecticidal MOA and insecticide resistance potential by linking gene expression and insecticidal traits of interest. This methodology is suitable for the design of sustainable agricultural solutions that support insect resistance management (IRM) and IPM strategies (Poelchau et al. 2016). For instance,

glutathione transferases (GSTs) are a large family of enzymes that have been identified in metabolic detoxification of insecticides as well as providing protection against oxidative stress (Enayati et al. 2005; Li et al. 2007). Insects share four classes of GST genes with all eukaryotes with an additional two insect-specific cytosolic GSTs, *delta* (*GSTd*) and *epsilon* (*GSTe).* All major insecticides produce elevated levels of GST activity (Enayati et al. 2005; Saisawang et al. 2012). Since the number of genes associated with these enzymes is quite large and complex, single gene expression identification was beyond the scope of my study; however, the genome-wide transcriptomic analysis provided a better understanding of which genes, if any, are involved in the detoxification of the bioactive compounds. This study notwithstanding, future work in this area may prove invaluable in deciphering insect-resistance/tolerance due to detoxification and excretion.

Table 1-2 Candidate genes for botanical pesticide investigation

Gene/Gene Family	Name	Action	Chemicals
Dmel\Cad74A CG6445	Cadherin gene	Resistance to Cry1Ac in *H. armigera* also associated with Ca^{2+} binding	*Bt*-cotton Cry1 protein
Dmel\Clic CG10997	Cl^- intracellular channel coding	Chloride channel activity and calcium channel binding	
Dmel\NaCP60E (formally DmDSC1)	Na^+ channel protein 60E gene	Encodes Voltage-gated cation channel	
Dmel\GstE1 CG5164	Glutathione transferases enzymes	Enzymatic detoxification - response to oxidative stress	DDT
*Dmel**mle* (also *nap*) CG11680	*Drosophila* no action potential gene		
Dmel\Nos CG6713	Nitric oxide synthase	pro-inflammatory, anti-inflammatory and immunosuppressive agent	
Dmel\para CG9907	Paralytic gene (sodium channels)	Knock-down resistance and loss of nerve impulse	DDT, Pyrethrum
Dmel\Rdl CG10537	Resistance to Dieldrin	GABA receptor gated chloride channel modulation	Fipronil
Dmel\tipE CG1232	temperature-induced paralytic E	voltage-gated sodium channel regulator activity	

1.2.7. Cellular oxidative response to exogenous stressors, including toxins such as insecticides

1.2.7.1. Reactive oxygen species

Reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH^-$) are short-lived molecules generated by the partial reduction of oxygen, which results from normal metabolism (Hancock et al. 2001; Held 2014). The overproduction of ROS can damage and disrupt biochemical functions, and trigger programmed cell death and other deleterious downstream events. As such, ROS are often used as a good early indicator of cell stress (Helfand et al. 2003; Lambeth et al. 2014; Negre-Salvayre et al. 2002). ROS are known to break DNA strands and cause oxidative damage to base pairs, and it can also disrupt mitochondrial respiration, membrane permeability, and sodium channels as well as inhibit sodium/potassium ATPase and modify proteins. In the presence of pathogens ROS levels elevate and can sequester the signalling molecule nitric oxide (NO), which denies the cell of signals involved in physiological homeostasis. As a defence mechanism to microbes, microbial products, or inflammatory mediators, the sole role of the NADPH-oxidase family of enzymes is to produce ROS by reducing oxygen to superoxide and the subsequently binding of the two superoxide molecules (i.e. H_2O_2), which is accelerated in the presence of superoxide dismutase enzyme (SOD) (Lambeth et al. 2014). In phagocytic cells, a myeloperoxidase enzyme (MPO) uses H_2O_2 and cellular Cl^- to produce hypochlorous acid (HOCl), which then kills the engulfed microbes. ROS also plays a role in cell signalling and transcriptional regulation (Lambeth et al. 2014). Therefore, since ROS performs multiple roles such as, a mechanism for defence; as an early indicator of stress; and is involved in many different pathways including DNA and RNA transcription. I chose to measure the difference in ROS florescence because this may indicate toxicity or detoxification patterns between the plant extracts

1.2.7.2. Nitric oxide

Nitric oxide (NO) is both a signalling and toxic agent in mammals and insects (Coleman 2001). In situations favouring both oxidative and NO-mediated stress, high levels of NO react with

O_2- to form the strong oxidant $ONOO^-$. Depending on the cell type, NO can either induce necrosis in cells lacking energy or reduction equivalents, or may induce apoptosis through caspases activation (e.g. seen in lymphocytes) (Kröncke et al. 2001). When the pesticides chlorpyrifos (organophosphate) and carbendazin (carbamate) were applied to murine macrophage cells challenged with bacterial infection, NO production showed a dose dependent response, reducing immune defence as pesticide concentrations increased (Helali et al. 2016). A single dose of rotenone directly into rat's brains produced a positive dose dependent increase in NO measured after 30 days, suggesting that the inducible form of Nitric Oxide Synthase (NOS) was triggered giving rise to persistent elevated NO concentrations (Abdel-Salam et al. 2014). Malpighian tubules, which act as fluid transport and ion regulator organs in insects, have been studied in regard to stress tolerance and show that changes in Ca^{2+} activate NOS to produce NO which signals cell-specific response pathways to stress, such as the 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) pathways (Davies et al. 2014). Superoxide (O_2^-) reacts rapidly with NO to produce the highly reactive peroxynitrite ($ONOO^-$) which can produce secondary free radicals and cause molecular damage (Lambeth et al. 2014). Nitric oxide directly and indirectly influences mitochondrial functioning. It induces inhibitory influences over cell respiration as an adaptive response to stresses such as alcohol toxicity; whereas in response to pathogens, it stimulates mitochondrial biogenesis and serves a protective function (Nisoli et al. 2006; Venkatraman et al. 2004).

The NOS oxidizes L-arginine into nitric oxide and citrulline. *Drosophila* possess a single NOS gene (dNOS), which has comprehensive similarity to NOS-1 mammalian sequence, and which encodes up to ten transcripts. The constitutive forms of NOS-1 and NOS-3 respond rapidly and transiently to Ca^{2+} signals whilst NOS-2 responds to immunological signals, and once activated is continually expressed resulting in persistent elevated concentrations (Coleman 2001; Davies 2007; Regulski et al. 2004). Dissociated cells from locust antenna lobes, brain and thoracic ganglia show significantly increased NOS activity after Ca^{2+}/calmodulin (CaM) and acetylcholine (ACh) treatments, giving evidence that NOS release is stimulated by elevated cytoplasmic Ca^{2+} and excitatory neurotransmitters at a cellular level (Muller et al. 1994).

Although there is some understanding of the role ROS and NO play in the innate immune system of insects, there remains a knowledge gap to be explored in regard to the ROS and NO responses to exogenous chemical agents such as botanical pesticides. In this thesis, ROS response induced by the extracts was investigated by measuring ROS production in two cell lines using confocal laser scanning microscopy to record real-time changes and differentially regulated genes that may play a role in the biochemical metabolisms linked to ROS production was highlighted.

1.2.8. Objectives and structure of the thesis

The foundation CRDC project identified several novel plant extracts that showed selective toxicity against *H. armigera* without significant effects on cotton aphid or two-spotted spider mite; furthermore, other extracts showed high activity against aphids and spider mites but not *H. armigera*. These results are important for two reasons. First, selective insecticides can be used conjunction with biological pesticides such as botanical products in IPM programs whilst targeting specific pest outbreaks and minimising negative impacts on beneficial insects. Second, rotation of selective insecticides with broad-spectrum insecticides reduces the pressure for field-evolved resistance. The selection of two insect cell lines in my study from unrelated species aimed to provide a useful comparison for elucidating the MOA of these selective extracts and some of their fractions.

Continuing on from the foundation CRDC study where the toxicity of each novel extract was tested against three unrelated arthropod species in whole organism bioassays, my study used a small selection, 28 from 358, of these novel extracts, see Table 7-4, Appendix 3 and two HPLC fractions to elucidate the site(s) and MOAs using model organisms at the whole organism, cellular and molecular levels. The last stage of my investigation explored the genetic regulation of ion transporter and channel genes, and insecticide-resistance genes of *D. melanogaster* and *A. gossypii* in response to these extracts and compounds.

Objective 1: To evaluate a range (including novel) of methodologies to investigate activity of botanical pesticides.

Objective 2: To determine the responses and mortality of *Helicoverpa armigera* caterpillars and *Drosophila melanogaster* flies with selected botanical extracts and assess the correlation between the whole organism level and cellular level using *Spodoptera frugiperda* (Sf9) and Schneider's *Drosophila* 2 (D.mel-S2) cell lines, respectively.

Objective 3: To treat cell lines with potentially insecticidal extracts and measure the elicited stress responses by measuring ROS production of the two cell lines using confocal fluorescence microscopy.

Objective 4: To measure ion transport across membranes of the two cell lines using the MIFE technique to discover the target site and MOA of novel insecticidal compounds.

Objective 5: To measure changes in differential gene expression of *D. melanogaster* and *A. gossypii* after exposure to the novel botanical extracts.

The structure of this thesis is as follows:

Chapter 1 provides a brief introduction, a literature review, thesis objectives and broad hypotheses to situate the PhD study in the context of botanical pesticide discovery and MOA investigations.

Chapter 2 provides an understanding of the general materials and methods which are common to many of the experiments performed in these studies. In addition, this chapter explains the foundation project methods and its outcomes which led to selection of the extracts for my PhD study.

Chapter 3 serves as a proof of concept and explains the reasoning behind using a combination of physiological and cellular approaches to elucidate insecticidal MOA. This chapter was submitted for publication as an original research methodology article in *Pest Management Science*, and published online as of 19 August 2020, awaiting a print issue.

Chapter 4 explains the whole organism bioassays conducted independently from the foundation project which explored knockdown and dose-responses and attempted to elucidate the mode of entry and its influence on toxicity.

Chapter 5 uses the methodologies introduced in Chapter 3 to start the short-listed laboratory screening process, commencing with 20 extracts and ending with three of the most promising extracts for commercialisation. For one of these extracts, the two active components have been characterised by Dr. Karren Beattie and a patent is currently in the process of application.

Chapter 6 describes the molecular component of this study that focused on two of the final three extracts and seeks key differentially expressed genes (DEGs) to address resistance considerations for further study.

Chapter 7 concludes the thesis with 7.1 general discussion of the thesis, 7.2 summary of key findings, and 7.3 limitations of this study and suggestions for future research. This summary chapter will add value to the scientific community, especially in the area of semiochemical research in plant protection.

Chapter 2: General Materials and Methods

2.1. Invertebrates used in bioassays

The foundation project, from which the candidate compounds for my studies were selected, focused on three key pests: cotton bollworm, *H. armigera*, two-spotted spider mite, *T. urticae* and cotton aphid, *A. gossypii*. These key cotton pests have been described in detail in Chapter 1. This PhD project further focussed on *A. gossypii*, as well as the model organism *Drosophila melanogaster* and its counterpart D.mel-S2 cell line, and a model cell line *S. frugiperda* (Sf9) of similar lineage to *H. armigera.*

2.2. Target arthropod species

2.2.1. *Helicoverpa armigera* (cotton bollworm)

Fresh *H. armigera* eggs were purchased from AgBiTech Pty Ltd. (Glenvale, QLD) biweekly, and stored under domestic grade refrigeration at approximately 4°C until needed for bioassays. Each Friday afternoon, eggs were carefully moved to a 90-mm gauze-lidded petri dish containing Milli-Q moistened filter paper, sealed with parafilm, and placed under laboratory conditions of 24 ± 2°C and RH 65 ± 10% to expedite hatching. This resulted in freshly hatched neonates by the following Monday morning. As larvae are cannibalistic, for bioassays a maximum of two neonates were transferred per leaf disc to allow adequate space for herbivory and minimise cannibalism.

2.2.2. *Tetranychus urticae* (two-spotted spider mite)

Tetranychus urticae Koch (TSSM) [Acarina: Tetranychidae] mites selected for screening were obtained from a culture of an organophosphate-susceptible strain initially obtained from Dr Grant Herron, the Elizabeth Macarthur Research Institute (EMAI), NSW Department of Primary Industries, Menangle, NSW, Australia (Herron et al. 1998) but subsequently maintained at Western Sydney University (WSU) Hawkesbury campus, Richmond, NSW, Australia for 16 years. The culture was reared on potted dwarf French bean (*Phaseolus vulgaris* L. *cv*. Redland Pioneer) in an insecticide-free, secure insectary maintained at 25 ± 2°C,

65 ± 5% RH and 14:10h light/dark photoperiod using broad spectrum growth lamps (600W HPS, GE Lighting, Smithfield, NSW, Australia). *Tetranychus urticae* were allowed to populate the feeding plant for up to three weeks to ensure full life cycles were completed and adequate numbers were maintained. New plants were infested approximately fortnightly to avoid physiological changes associated with overcrowding, by transferring leaves from the previously infested plants.

2.2.3. *Aphis gossypii* (cotton aphid)

An insecticide susceptible strain of *Aphis gossypii* Glover [Hemiptera: Aphididae] provided by Dr Grant Herron, the Elizabeth Macarthur Research Institute (EMAI), NSW Department of Primary Industries, Menangle, NSW, Australia) was cultured at Western Sydney University (WSU) Hawkesbury campus, Richmond, NSW, Australia. Aphids were reared on insecticide-free, non-GMO, potted, cotton seedlings (*Gossypium hirsutum* L. cv. Sicot 620) in a glasshouse maintained at 27 ± 3°C, 55 ± 10% RH and 14:10h light/dark photoperiod.

2.3. Model organisms

Model organisms are commonly used for fundamental studies on many aspects of biology and genetics of living species. In insects, the most widely used model organism is the ferment-vinegar fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). In my studies I used *D. melanogaster* adults (flies) for whole organism studies and D.mel-S2 cell line (Cat. no. R69007, ThermoFisher Scientific) for the cellular studies. I also used a second model cell line from *Spodoptera frugiperda* cell line, Sf9; although *S. frugiperda* whole organisms were not used because they were unavailable in Australia, we used this cell line to compare against *H. armigera* neonates, because these noctuid moth species are closely related.

2.3.1.1. *Drosophila melanogaster* (ferment-vinegar flies)

Drosophila melanogaster is an excellent model organism for studying insecticide assays, ion channel electrophysiology and gene expression because it is easy to assay at an organism level, having a short life-cycle, a commercially available cell line (D.mel-S2) and a well-documented genome (Towers et al. 2002). *Drosophila melanogaster* has been one of the most

intensively studied organisms in biology. It has a fully sequenced small genome of approximately 180 Mb in size, encoding for approximately 13,600 genes which share many developmental and cellular processes with humans, affording it premier model organism status (Adams et al. 2000).

Live, wild type, *D. melanogaster* were purchased from Southern Biological (Knoxfield VIC., cat. no. L7.3) and reared on *Drosophila* Instant Culture Medium (cat. no. CM 4L, Carolina Biological) as per the manufacturer's directions. Adult flies were moved to new culture bottles every 1-2 weeks, tracking generations, laboratory conditions were 24±2°C, 50±15% RH. Reusable bottles were washed and autoclaved between each use and foam stoppers were washed, soaked in 100% ethanol and oven dried. For some studies, pre-sorting of sexed flies was necessary. Therefore, *D. melanogaster* adult flies were taken from multiple communities of the same generational time-frame and allowed to lay eggs into fresh *Drosophila* medium with additional yeast paste in order to evacuate old eggs and allow for fresh egg production (Figure 4-4B). After 1 d, flies were transferred to another set of flasks prepared with fresh medium where flies were allowed to oviposit for 2 h, then removed. The adult flies emerging from this generation were therefore a cohort of similar age and were able to be easily separated into unmated females immediately following emergence, often before their wings were uncurled (Figure 4-4C). Unmated females from assays could then be used for subsequent molecular analysis. It has also been shown that male and female *Drosophila* have differing feeding patterns, presumably to support egg development in females (Wong et al. 2009). By using a single sex in some investigations, I expected to reduce variation in feeding assays.

2.3.1.2. *Drosophila melanogaster* D.mel-S2 cell line

Schneider's Drosophila-S2 cell line, D.mel-S2, is the second of three *D. melanogaster* cell lines developed in the early 1970s from primary cultures of late-stage embryos. This second line is used extensively in genetic studies and has the advantages of more uniform morphology with a loose monolayer growth habit, and being able to survive indefinitely, provided the growth medium is adequate (Schneider 1972). It also contains circulating macrophages which make it an excellent model for the study of cell-mediated immunity, especially when paired with

gene expression techniques (Rämet et al. 2002). In addition, D.mel-S2 cells are one of the most popular model systems for cell biological and functional genomic studies; furthermore, it is well suited to high-resolution light and fluorescence microscopic assays (Rogers et al. 2008).

The D.mel-S2 line was purchased from Life Technologies Australia (Life Technologies, Australia, cat #R690-07), in cryogenically frozen form and cultured in fresh, sterile filtered, compete Schneider's *Drosophila* Medium (CSD). Complete medium was made by mixing Schneider's *Drosophila* Medium (Life Technologies, Australia, cat #21720-024), supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Australia, cat #10082-139), 0.1% Pluronic F-68 surfactant (Life Technologies, Australia, cat #24040-032), and 1% Penicillin-Streptomycin (Sigma-Aldrich, cat #P4458-100mL) to give a final concentration of 50 units/50µg per mL of Penicillin-Streptomycin, as per cell culture instructions (Thermo Fisher Scientific 2018). D.mel-S2 cells were passaged biweekly into Greiner disposable T-25 suspension treated flasks (Sigma-Aldrich, Australia, cat #C6731). According to Schneider (1972) the original creator of the second *D. melanogaster* cell line, cell media should be maintained close to 360 mOsm and pH maintained between 6.7-6.8.

2.3.1.3. *Spodoptera frugiperda*

The fall armyworm, *S. frugiperda* (J.E. Smith) is an important pest in southern USA, Argentina, Africa and the Indo-Pacific regions (European and Mediterranean Plant Protection Organization 2020), and has recently been reported in Australia (Du Plessis et al. 2020; Hort Innovation 2020). It is related to *Helicoverpa* spp., both genera being in the family Noctuidae. Cell lines of *S. frugiperda* have been developed, originally from ovarian tissue, and are commonly used for recombinant protein production using baculovirus (Vaughn et al. 1977). They are also readily available and easy to maintain (Altmann et al. 1999). As commercial cell lines of *Helicoverpa* spp. are not available, I chose *S. frugiperda* (Sf9) cell lines (ThermoFisher Scientific, Australia, Cat. #11496015) as a surrogate for the comparative cell assays, while using larvae of *H. armigera* for the whole organism assays. The Sf9 cell line provided a unique contrast to the unrelated D.mel-S2 cell line as they are likely to be more susceptible to plant extracts and *B. thuringiensis* endotoxins, providing an opportunity to compare different

responses between the two cell lines to insecticidal compounds. In previous studies, Kwa et al. (1998) found Sf9 cells to be the most sensitive to Cry1C toxicity and binding, while *D. melanogaster* (Dm1) cells were the least sensitive. Likewise, Rasikari et al. (2005) reported that Sf9 cells were more susceptible than D.mel-S2 cells to Lamiaceae plant extracts.

2.3.1.4. *Spodoptera frugiperda* Sf9 cell line (fall armyworm)

The *S. frugiperda* (fall armyworm) cell line, IPLB-SF21-AE, was originally developed from immature ovaries removed from *S. frugiperda* pupae (Vaughn et al. 1977). From this parental cell line, the clonal cell line Sf9 was developed (Pasumarthy et al. 1994). Sf9 cells can be grown as either an adherent monolayer in T-flask culture flasks, or as a suspension culture in shaker bottles. If grown as an adherent culture, as they were for our purposes, cells should be passaged when in mid-log phase of growth or at ~90% confluency (Invitrogen 2015). Cryogenically frozen, serum-free adapted Sf9 cells in Sf-900™ II SFM (Life Technologies Australia, cat #11496015) were purchased and cultured in Sf-900™ II SFM (Life Technologies Australia, cat #10902096) in 25 cm^2 Corning® tissue culture (TC) treated cell culture flasks (Sigma-Aldrich, Australia, cat #CL3056). Passaging took place on an approximate 10-d cycle.

2.4. Plant extract selection and preparation

A plant extract library was prepared by members of the foundation project, Dr Karren D. Beattie, Ms Christine Murray, and Ms Beatrice Venkataya from the National Institute of Complementary Medicine (NICM), Western Sydney University. Individual contributions were as follows: K.B. preparation of materials and methods, ~40% processing, extraction and preparation of extracts and fractions for screening; B.V. ~40% processing and extraction of plant samples, preparation of extracts for screening; C.M. ~20% preparation of extracts and fractions for screening.

Plant Material and Extraction

During the foundation project a total of 227 targeted plant species from 65 families were sourced from Mt Annan Botanic Gardens and Greening Australia and also from the wild, in the states of NSW, Qld and NT. Particular emphasis was focused on species in the families of Myrtaceae (68), Rutaceae (18), Asteraceae (16), Fabaceae (16) and Sapindaceae (12). The aerial parts of plant samples (approx. 200 g fresh weight) were collected from living plants and placed in labelled paper bags for transport to the NICM. All samples were dried in an oven at 40°C for 7d (≤18% moisture content), after which the material was ground and stored in appropriate containers at room temperature. Names and providence details were provided by Mt Annan Botanic Gardens for the specimens collected there, and the botanical identity of plant samples collected elsewhere was confirmed by botanists.

Approximately 25 g of each sample was added to a 1:1 mixture of methanol:chloroform (250 ml, ACS/HPLC grade, Chem-Supply Pty Ltd, Gilman, SA Australia). The mixtures were sonicated (1h) and macerated (24h), vacuum filtered, and the filtrate transferred into a separating funnel. Filtered extracts were separated to create a polar aqueous-methanol fraction and non-polar chloroform fraction in a separatory funnel by the addition of water (ca. 75 mL) until the two immiscible layers formed. These two layers were separated into pre-weighed round bottom flasks. The fractions were evaporated to dryness using a rotary evaporator (polar) or evaporation in a fume hood (non-polar). The subsequent dried weights of each extract were recorded. Each plant sample therefore yielded two extracts for screening: a polar (designated

P) fraction and a non-polar (N) fraction. Dried extracts were screened for insecticidal activity. A total of 414 extracts were prepared; 358 were provided to the foundation project (approx. 200 mg each) and screened for insecticidal activity, while a smaller amount of the 102 Mt Annan samples (Table 2-1) was provided for the PhD project (approx. 10 mg each).

Table 2-1 Mt Annan plant species and sample codes

Sample Code	Species	Sample Code	Species
1	*Leionema* sp. "Colo River"	52	*Enchylaena tomentosa*
2	*Eremophila maculata*	53	*Neolitsea dealbata*
3	*Elaeocarpus grandis*	54	*Prumnopitys ladei*
4	*Alyxia ruscifolia*	55	*Senna acclinis*
5	*Gmelina leichhardtii*	56	*Tristaniopsis exiliflora*
6	*Diospyros australis*	57	*Cinnamomum oliveri*
7	*Syzygium pseudofastigiatum*	58	*Backhousia myrtifolia*
8	*Glycosmis trifoliata*	59	*Thryptomene denticulatae*
9	*Hernandia bivalvis*	60	*Swainsona sejuncta*
10	*Harpullia pendula*	61	*Melicytus dentatus*
11	*Argophyllum nullumense*	62	*Lepidozamia peroffskyana*
12	*Eupomatia laurina*	63	*Cuttsia viburnea*
13	*Austromyrtus dulcis*	64	*Xanthostemon oppositifolius*
14	*Myrsine richmondensis*	65	*Psychotria poliostemma*
15	*Correa baeuerlenii*	66	*Homoranthus lunatus*
16	*Flindersia ifflana*	67	*Backhousia sciadophora*
17	*Austrosteensia blackii*	68	*Podolepis jaceoides*
18	*Geijera parviflora*	69	*Podocarpus elatus*
19	*Zieria tuberculate*	70	*Tristaniopsis laurina*
20	*Anetholea anisata*	71	*Pandorea jasminoides*
21	*Alstonia scholaris*	72	*Schoenia filifolia* subsp. *subulifolia*
22	*Flindersia acuminate*	73	*Chrysocephalum apiculatum*
23	*Melicope micrococci*	74	*Rhodanthe humboldtiana*
24	*Callistemon formosus*	75	*Rhodanthe chlorocephala* subsp. *rosea*
25	*Geissois benthamiana*	76	*Rhodanthe manglesii*
26	*Darwinia citriodora*	77	*Leucophyta brownii*
27	*Homoranthus flavenscens*	78	*Eremophila nivea*
28	*Peperomia blanda* var. *floribunda*	79	*Pycnosorus globosus*
29	*Choricarpia subargentea*	80	*Rulingia hermanniifolia*
30	*Commersonia bartramia*	81	*Thomasia triphylla*
31	*Prostranthera askania*	82	*Lechenaultia biloba* 'Big Blue'
32	*Commelina cyanea*	83	*Xerochrysum bracteatum*
33	*Aphanopetalum resinosum*	84	*Grevillea argyrophylla*
34	*Atractocarpus fitzalanii* subsp. *fitzalanii*	85	*Prostanthera striatiflora*
35	*Sannantha pluriflora*	86	*Castanospermum australe*
36	*Maesa dependens* var. *pubescens*	87	*Phaleria chermsideana*
37	*Doodia aspera*	88	*Antidesma bunius*

38	*Nothofagus moorei*	89	*Prunus turneriana*
39	*Pisonia umbellifera*	90	*Corynocarpus rupestris* subsp. *arborescens*
40	*Mentha satureioides*	91	*Bursaria spinosa*
41	*Kunzea ericoides*	92	*Acronychia laevis*
42	*Chamelaucium uncinatum* 'CWA Pink'	93	*Acronychia littoralis*
43	*Cordyline cannifolia*	94	*Acacia macradenia*
44	*Leptospermum spectabile*	95	*Polystichum proliferum*
45	*Diploglottis campbellii*	96	*Backhousia citriodora*
46	*Scolopia braunii*	97	*Xanthosetemon chrysanthus*
47	*Toona ciliatae*	98	*Melaleuca bracteata*
48	*Calotis lappulacea*	99	*Acronychia baeurlenii*
49	*Centhratherum riparium*	100	*Calytrix tetragona*
50	*Acronychia littoralis*	101	*Melaleuca macronychia* subsp. *macronychia*
51	*Olearia canescens*	102	*Melaleuca nodosa*

This PhD project focused on the efficacious novel botanical extracts identified from the foundation project, 'Novel Insecticides and Synergists from Endemic and Exotic Flora', see Table 7-4, Appendix 3. From those results, in conjunction with the foundation project's researchers, I selected several extracts based on either high efficacy against at least two target arthropod species (extracts 10N, 33N, 55N, 62N, 68N, 72N, 82N), or showing interesting behavioural responses (extracts 9P, 15N, 61P). In the earlier stages of method development, I also included several extracts as examples of moderate efficacy or single species toxicity (extracts 14P, 14N, 25N, 28N, 79P, 93N, 100N), as well as examples of low (78P, 79P) or no toxicity (36P, 54N) across all three species (Albert Basta pers. comm. 2015), as shown in Table 2-2 and Table 5-1. As a result of multiple methods of screening for efficacy, I found three highly efficacious candidates with no previously reported insecticidal activity (extracts 68N, 72N, 82N), for comparison I also investigated their polar counterparts (viz. 68P, 72P, 82P). One highly efficacious candidate, particularly against *A. gossypii*, extract 68N from *P. jaceoides,* and not previously identified as an insecticide, was selected for further fractionation and identification of its active constituents. These fractions were also included in my PhD studies.

Extract 61 *Melicytus dentatus* (Internal-plate standard)

Melicytus dentatus (Malpighiales: Violaceae), is an Australian native, originating in New South Wales but distributed north to southern Queensland and south to Southern Victoria. It is a medium shrub, growing up to 5m in height, in a range of habitats from open forest in the tablelands to altitudes of 1000m in the Blue Mountains, preferring sandy or sedimentary soils close to waterways. Sweet smelling, sparse, white to yellow, flowers are present from August to October, followed by blue-grey to mauve-grey, 8mm, ovoid fruits between December to March.

This species can be found under the synonyms *Hymenanthera angustifloria* and *Hymenanthera dentata* and also the common name, tree violet (James et al. 2017; Stajsic et al. 2015). To date, we have not found any research pertaining to insecticidal, pesticidal or medicinal uses either traditionally or otherwise.

Figure 2-1 *Melicytus dentatus* plant material using in this study. Image credit: Beatrice Venkataya (Western Sydney University)

Extract 68 *Podolepis jaceoides*

Podolepis jaceoides (Sims) Voss (Asterales: Asteraceae) is an Australian native herbaceous plant found in eastern and southern Australia from Queensland, through New South Wales, Victoria, South Australia and Tasmania. It can tolerate a wide range of habitats and soil conditions, from woodlands to grasslands and from heavy clay to sandy soils, thus resulting in its wide distribution. *Podolepis jaceoides* is commonly known as the Snowy Copper Wire Daisy. It grows up to 50cm in height with golden yellow flowers from early spring to late summer. Australian Aboriginal people utilize the edible roots. Zalucki et al. (1994) reported that field sampling found *H. armigera* adults and larva (n=71) living on *P. jaceoides* at three sample sites; however, only five of the 71 were reared to adulthood (Zalucki et al. 1994).

Figure 2-2 *Podolepis jaceodides* plant materials used in this study. Bottom image shows *P. jaceoides* in the foreground. Images credit: Karren Beattie (Western Sydney University)

Extract 72 *Schoenia filifolia* subsp. *subulifolia* (F.Muell.) Paul G. Wilson

Schoenia filifolia subsp. *subulifolia* (Asterales: Asteraceae) is an Australian species native to Western Australia. It was formerly known as *Helichrysum subulifolium* (Turcz.) F.Muell., *Xanthochrysum filifolium* Turcz. and commonly called yellow-paper daisy or Mingenew after the region where wild subpopulations are found. It is an annual daisy, with masses of single-headed yellow flowers from spring through to summer. It prefers well-drained soils in sunny positions and it is well suited to temperate climates. Propagation is by seed or more reliably by cuttings (Anpsa.org.au 2016; Western Australian Herbarium 1998). This subspecies has been classified by the Western Australian government as a threatened (rare) flora species, noting its declining distribution is due to land clearing, weed invasion, salinity and grazing pressure. It is estimated that its wild distribution may be less than 1 km^2 (Department of the Environment 2020). We found no reports of insecticidal properties associated with *S. filifolia*.

Figure 2-3 *Schoenia filifolia* subsp. *Subulifolia* (dried post-harvest) plant material used in this study. Images credit: Karren Beattie (Western Sydney University)

Extract 82 *Lechenaultia biloba* 'Big Blue'

Lechenaultia biloba Lindl. (Asterales: Goodeniaceae), commonly known as *Lechenaultia* blue (Jackson 2015), is an Australian native originating in Western Australia but more recently distributed for home gardeners due to its intense blue flowers. It is a small herbaceous shrub growing to 30 cm tall with five-petalled flowers in late winter to early spring (Anpsa.org.au 2007; Sage 2006). *Lechenaultia biloba* prefers loose, gravelly or sandy, well-drained soils. It is drought hardy and can withstand a warm climate in full sun and is also considered frost tolerant. *Lechenaultia*s are reported to have a low seed production, although they propagate well from cuttings. *Lechenaultia biloba* seeds can be collected in late summer (Sweedman et al. 2006).

Figure 2-4 *Lechenaultia biloba* 'Big Blue' plant material used in this study. Images credit: Karren Beattie (Western Sydney University)

Extract W11 *Eucalyptus cloeziana*

Eucalyptus cloeziana F.Muell (Myrtales: Myrtaceae) is a tall evergreen tree, endemic to QLD, Australia where it grows in open woodlands when soil is shallow or poor (10-25m) and forests where deeper, fertile soils allow it to become deep rooted (up to 55m tall). It is commonly known as Gympie messmate, yellow or Queensland messmate or dead finish. It has rough bark in a range of colours (grey-brown, yellow-grey, orange-brown), which is soft, flaky and fibrous depending on its age and location and dark green, thin, dull leaves, tapered at the basal end with lateral veins. It has small white or cream flower buds on 7-flowered umbrella like inflorescences (Euclid: Centre for Australian National Biodiversity Research 2020; Hill 2004). *Eucalyptus cloeziana* is grown as a plantation crop for use in maritime applications such as jetties and mooring poles due to its very straight, cylindrical shape and high marine borer resistance (Dupray et al. 2009; Wood Solutions International 2019). Spooner-Hart (2013) found that a rare chemovar of *E. cloeziana* had very high insecticidal activity against *Tetranychus urticae* and *Plutella xylostella* at 0.5 w/v and 1.0% w/v, respectively, due to its 95% single β-triketone constituent, tasmanone.

Extract W44 *Hakea microcarpa*

Hakea macrocarpa R.Br (Proteales: Proteaceae), commonly known as small-fruited hakea, is an Australian native shrub found along the lower eastern coast of Australia from Stanthorpe, Queensland, through New South Wales and Victoria, to most of Tasmania. It is a spreading evergreen shrub which grows to 2m tall, preferring poorly drained sub-alpine woodlands (Barker et al. 1999). The nectar of its flowers, which appear in spring and early summer, is especially attractive to birds. This plant is also recommended for post-bushfire revegetation purposes (Wakefield et al. 2013). *Hakea macrocarpa* can be found under the nomenclatural synonym: *Hakea microcarpa* R.Br. var. macrocarpa and the taxonomic synonyms: *Hakea patula* R.Br., *Hakea microcarpa* var. tasmannica Meisn., *Hakea bifrons* Meisn., *Hakea microcarpa* var. bathurstiana Meisn., *Hakea glabriflora* Gand., and *Hakea microcarpa* var. patula (R.Br.) Domin.

2.4.1. *Podolepis jaceoides* methanol extract

The foundation project identified *Podolepis jaceoides* (extract 68N) as a highly efficacious extract warranting further investigation. Although there is no literature published on insecticidal properties of *P. jaceoides*, the chemistry of some *Podolepis* spp. has been reported. Zdero et al. (1986) isolated nine compounds from aerial parts of *Podolepis hieraciodes* and identified the main constituents to be *γ*-pyrones. Although the absolute configuration was not identified for all nine compounds, structure **VI** (Figure 2-5) was proposed and named podopyrone. Jaensch et al. (1989) later investigated the constituents from other *Podolepis* species: *P. lessonii* (Cass) Benth, *P. longipedata* A. Cunn ex DC, *P. rugata* Labill. var. *rugata,* roots and aerial parts, and *P. capillaris* (Steetz) Diels. Again, γ-pyrones were identified along with sesamin, lignans, obliquins and sesquiterpenes. To date, the chemistry of *Podolepis jaceoides,* and its insecticidal activity identified in the foundation project, has not been investigated elsewhere.

In order to further test this extract during my PhD study, an additional methanol *P. jaceoides* extract (coded 68N.M) was prepared and was subsequently used in bioassays. This was extracted from plants grown at Western Sydney University's Hawkesbury campus. Approximately 20 g of dried, finely ground, whole aerial plant parts, comprising of stems and flowers, were extracted by maceration (24 h) using 200 ml technical grade methanol, followed by sonication (1 h). Sonication water was changed every 20 min to prevent heat from vaporising volatiles. The solution and solids were filtered through Whatman® Grade 1 filter paper and dried by evaporation inside a fume hood for as long as required, typically 24 h.

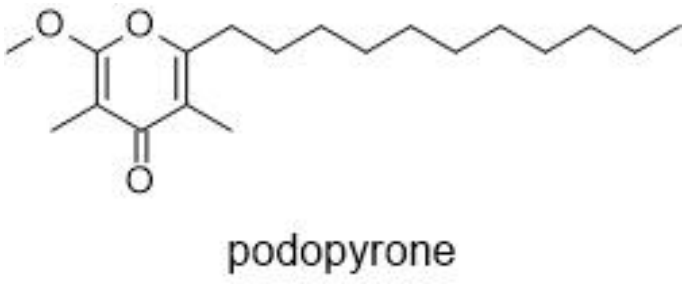


Figure 2-5 Podopyrone (Ui et al. 2006)

2.4.2. Botanical and synthetic pesticides as positive controls

During different sections of the PhD project, I used a selection of known insecticides as my positive controls, depending on the aim of the assay. Two botanical pesticides, one previously studied insecticidal steam-distilled essential oil from *Eucalyptus cloeziana* (containing >90% tasmanone V, Figure 1-1) from Western Sydney University (Spooner-Hart et al. 2017), and a pyrethrum extract (Pestanal®, CAS# 8003-34-7) were used, primarily to benchmark selected novel extracts. In addition, two synthetic single-compound commercially available type II pyrethroids, α-cypermethrin (Pestanal®, CAS# 67375-30-8) and esfenvalerate (Pestanal®, CAS# 66230-04-4), purchased from Sigma-Aldrich, Australia, were used to compare against natural pyrethrum. The comparative study is presented in Chapter 3 of this thesis. Alpha-cypermethrin and esfenvalerate were selected because they are registered in Australia for use against *Helicoverpa spp.* in cotton crops (apvma.gov.au 2020).

2.4.2.1. *Eucalyptus cloeziana* essential oil

Eucalyptus cloeziana oil is steam distilled from a rare chemovar of this species that has the *β*-triketone tasmanone as its major primary component (84-96%) (Brophy et al. 1990). Tasmanone, along with some other *β*-triketones has been demonstrated to possess high insecticidal activity (Spooner-Hart et al. 2002), and the oil has subsequently been commercialised under the trademarked name Qcide™. *E. cloeziana* has an essential oil yield of 1.5-1.9% fresh weight leaves. Although large amounts of biomass must be distilled to provide commercial quantities of active compounds, *E. cloeziana* is still the preferred species for biopesticide development since almost the entire oil yield is tasmanone. The next highest sources of tasmanone are from *E. camfieldii* Maiden (40% of the oil w/w) (Hellyer et al. 1963) with only trace levels in other species. Although Qcide™ is not yet registered for agricultural use, laboratory testing performed at Western Sydney University and Southern Cross University demonstrated high efficacy against a range of insects, mites and snails whilst having minimal mammalian and bee toxicity (Brophy et al. 1990; Spooner-Hart et al. 2002; UWS Innovation). However, there is no literature reporting the MOA of tasmanone in insects. For the purposes of this study the essential oil Qcide™ was used in the earlier stages of

experiments, but I subsequently used a solvent extract of the high-tasmanone *E. cloeziana* (W11) (similarly prepared to the other studied extracts) in my more detailed investigations.

2.5. Whole organism bioassay techniques

2.5.1. Direct application via Potter Precision Spray Tower

The foundation project and the PhD project used the Potter Precision Spray Tower (Burkard, Rickmansworth, Herts, UK), an internationally accepted technology for laboratory insecticide bioassays, to deliver direct applications of non-polar and polar extracts. The methodology used in both situations occasionally varied, but where changes occurred in the PhD study, these have been described in the individual chapters. During the foundation study, extracts were tested for acute toxicity at 1.0% w/v concentrations against the main cotton pest*, H. armigera* (mixed sex neonates), and two important secondary pests: two-spotted spider mite, *T. urticae* (gravid females), and cotton aphid, *A. gossypii* (gravid females) (Table 7-4, Appendix 3).

2.5.2. Preparation of extracts for Potter spray tower bioassays

The surfactant Triton X-100™ (octyphenol ethylene oxide condensate, Union Carbide, Sigma, St. Louis, MO) was made up to 200 ppm concentration with Milli-Q water and mixed thoroughly for 30 min on a magnetic stirrer before being used as a background diluent for all extracts. Triton X-100 was selected because previous investigations had confirmed it was non-toxic to arthropods at these concentrations (Spooner-Hart et al. 2002). Since non-polar extracts can be more difficult to solubilize, all 200 mg extracts were initially dissolved in 1.0 ml acetone, diluted to 20 ml with water-Triton X-100™, giving a final treatment concentration of 1.0% w/v (max. acetone 5.0% v/v). Treatments were sonicated for 10 min in two to three bursts to reduce heating and therefore loss of volatile compounds whilst providing homogeneous solutions.

Three ml aliquots of each extract were applied to each Petri dish with a Potter spray tower as described by Herron et al. (1995), at 105 kPa (approx. 15 psi) inlet air pressure. The average weight of the solution sprayed on each dish was calculated to be 5.385 mg/cm^2 or 0.05385

mg/cm^2 active ingredient (a.i). Two true replicates (separate emulsions) were applied via the spray tower for each test extract. Following spray applications, chambers were monitored closely until almost air dried, at which time they were sealed with Parafilm M® and turned upside down to simulate the underside of leaf position. Replicates were maintained in the entomology laboratory at 24 ± 2°C and RH 65 ± 10%. In most cases, mortality, egg or offspring numbers, and faeces were monitored at 24 h and 48 h after treatment (HAT). Furthermore, the treated arthropods were regularly observed for signs of intoxication or other behavioural changes. For each batch of investigations, control sprays were conducted, with methodology identical to the treatments. The control solutions comprised 3 ml aliquots of 200 ppm Triton X-100 in distilled water and either acetone or hexane at the same background concentration as the treatment solutions (i.e., 5.0% v/v).

2.5.3. Direct application method

For *T. urticae*, screening of each sample (Table 7-4, Appendix 3) was conducted on young gravid female mites, which were evenly distributed on 1-3 French bean leaf discs contained in vented-lid 90 mm diam. Petri dishes (SPL Life Sciences, Korea). Fresh un-infested leaves were collected, washed with distilled water and dried before being stamped out into 25 mm diameter leaf discs. Leaves were placed lower side up with upper side pressed against wet absorbent cotton wool lining the base of the dish. Water was added to the dishes daily to prevent desiccation of the leaf discs and to stop the mites from escaping the leaf disc. Mites were transferred onto the disc by first tapping infested leaves onto white paper, then 20-40 mature, gravid, fast-moving females were selected for each bioassay replicate. Selected mites where carefully transferred onto prepared leaf disc with the aid of a moistened fine camel hair paint brush and checked for health under a stereomicroscope before proceeding. Mites that moved off the leaf disc prior to or post treatment were discarded from the counts. Death was recognized by the absence of movement when the test organisms were mechanically stimulated by prodding with a fine brush.

For *A. gossypii*, parthenogenetic adult female aphids were collected from the culture on cotton plants. Fifteen adults were transferred using a fine camel hair brush onto the lower side of a respective leaf disc 50 mm diameter mounted on 1.0% w/v agar in a plastic insect

dish measuring 50 mm diameter x 15 mm height (SPL Life Sciences, Korea). The dish had a lid with a hole (15 mm diameter) which was covered with fine mesh; the lid was removed just prior to spraying. Mortality counts were conducted at 24 and 48 HAT (Table 7-4, Appendix 3). Absence of aphid movement when prodded with a fine brush was taken as the criterion of death.

For *H. armigera*, neonate, 1st instar, larvae 4-12 h after hatching were transferred with a fine camel hair brush to a cotton plant leaf disc (90 mm diameter) mounted with its lower side uppermost on 1% agar in a vented-lid 90 mm diameter Petri dishes (SPL Life Sciences, Korea). Two neonates were transferred to each dish and sprayed with 3 ml aliquots using a Potter tower, as previously described. Thus, each replicate comprised 4, 1st instar, neonates. The number of dishes sprayed per sample was 4-10. Dishes were retained under laboratory conditions (24±2°C, 50±15% RH) for post-treatment observations. Mortality counts were conducted at 24 and 48 HAT (Table 7-4, Appendix 3). Absence of caterpillar movement when prodded with a fine brush was taken as the criterion of death.

The initial 358 extract screening results from project UWS1401 (for full list see Appendix 3), provided a short-list of 20 extracts with insecticidal properties that warranted further investigation (Table 2-2). I chose to start by initially testing cytotoxity against D.mel-S2 cells, and then reducing the number as the results of my (often run concurrently) bioassays directed the study towards the most efficacious and novel candidates. The list of the plant extracts I focused on are presented in Table 2-2, according to the rank I assigned them. My criterion was based on extracts having average efficacy across all three key cotton pests (Table 5-1): additional extracts of interest were selected either due to their interesting semiochemical effects (15N, 9P), or because of their very low efficacy/activity for comparison with the identified active extracts (78N, 79P, 78P, 36P, 54N).

As previously mentioned, the commercially produced Qcide™ steam-distilled essential oil, derived from the same plant as the solvent extract W11 – *E. cloeziana* was used as a positive control in my early experiments. However, two wild harvested plant material extracts, W11 (*E. cloeziana*) and W44 (*H. microcarpa)* were tested for their suitability as positive controls. Extract W44's selection was based on previous research in the foundation project (Spooner-

Hart et al. 2017) and its moderately efficacious insecticidal properties (Table 2.2). No other wild-harvested extracts were investigated further.

Table 2-2 Ranking of selected plant extracts from the whole organism bioassays.

Rank	Plant	Family	Fraction	Average efficacy
1	*Lechenaultia biloba* 'Big Blue'	Goodeniaceae	82 N	100.00
2	*Harpullia pendula*	Sapindaceae	10 N	95.06
3	*Aphanopetalum resinosum*	Aphanipetalaceae	33 N	93.18
4	*Podolepis jaceoides*	Asteraceae	68 N	85.56
5	*Schoenia filifolia* subsp. *subulifolia*	Asteraceae	72 N	76.19
6	*Peperomia blanda* var. *floribunda*	Peperomiaceae	28 N	72.35
7	*Lepidozamia peroffskyana*	Zamiaceae	62 N	61.24
8	*Senna acclinis*	Fabaceae	55 N	59.28
9	*Acronychia littoralis*	Rutaceae	93 N	56.52
10	*Xerochrysum bracteatum*	Asteraceae	83 N	53.61
11	*Geissois benthamiana*	Cunoniaceae	25 N	53.36
12	*Calytrix tetragona*	Myrtaceae	100 N	51.15
13	*Melicytus dentatus*	Violaceae	61 P	48.72
14	*Podolepis jaceoides*	Asteraceae	68 P	46.19
15	*Schoenia filifolia* subsp. *subulifolia*	Asteraceae	72 P	43.33
16	*Lechenaultia biloba* 'Big Blue'	Goodeniaceae	82 P	41.18
17	*Myrsine richmondensis*	Myrsinaceae	14 N	32.92
18	*Myrsine richmondensis*	Myrsinaceae	14 P	20.79
19	*Correa baeuerlenii*	Rutaceae	15 N	32.50
20	*Hernandia bivalvis*	Hernandiaceae	9 P	12.63
Low Efficacy	*Eremophila nivea*	Scrophulariaceae	78 N	16.88
Low Efficacy	*Pycnosorus globosus*	Asteraceae	79 P	13.03
No Efficacy	*Eremophila nivea*	Scrophulariaceae	78 P	6.67
No Efficacy	*Maesa dependens* var. *pubescens*	Myrsinaceae	36 P	5.03
No Efficacy	*Prumnopitys ladei*	Podocarpaceae	54 N	1.11
Control	*Eucalyptus cloeziana*	Myrtaceae	W11N	83.33
Control	*Hakea microcarpa*	Proteaceae	W44N	58.82

***Note*:** The ranking is according to either efficacy against multiple arthropods (1-13), single species specificity (14-18), non-fatal semiochemical effects (19 & 20), and additional low and non-efficacious benchmarking, and two positive controls.

2.6. Cell line bioassay techniques

This section presents the information relevant for the General Material and Methods. The detailed methods for cell line bioassay techniques are described in Chapters 3 and 5.

2.6.1. Cell counting

Cells were firstly viewed within their culture flasks using an inverted microscope to check for ~90% confluency. Cells were then slushed gently with a 1-ml pipette tip to remove them from the flask surface and release them into a homogeneous mixture; from this a 10 µL sample was removed and mixed on a coverslide with 10 µL of a cell viability stain (Trypan Blue, cat # T8154 Sigma-Aldrich, Australia). Cells were then manually counted using a haemocytometer under an upright microscope following Fuentes-Gari's (2015) protocol.

2.6.2. Cell growth and inhibition

Extracts were tested against both D.mel-S2 and Sf9 cell lines for growth and inhibition percentage by measuring the optical density change at 600 nm using a microplate reader (BMG Labtech, SpectroStar® Nano). Extracts were weighed into new 2 ml vials (Agilent Technologies, cat # 5182-0714) and dissolved with dimethyl sulfoxide (DMSO) to a final stock concentration of 1.0% w/v, then aliquoted into smaller volumes and frozen at -20°C for later use. Directly before each microplate run, stock concentrations of extracts were thawed at room temperature, diluted by adding 10 µl to 490 µl of the appropriate media for the cell line to give a working concentration of 0.02% w/v (2% v/v DMSO) and sonicated for 10 min. Extract 61P, known to have insecticidal activity, was used as an internal-plate control. Two positive controls were used, extract W11N, an oil extract containing >70% tasmanone and commercially purchased Pestanal® Pyrethrum extract (Sigma-Aldrich, Australia, cat # 33739-100mg). A pyrethrum stock solution was made by adding 1 µl to 49 µl of DMSO to give a concentration of 2% v/v pyrethrum (100% v/v DMSO) and frozen at -20°C for later use. Pyrethrum working solutions were freshly mixed for each run by diluting 5 µl of stock solution into 995 µl of 2% DMSO/media diluent, to maintain an equivalent DMSO background level as that found in the extracts, giving a final concentration of 0.01% v/v.

Once all treatments were made, 50 µl of each was pipetted into a flat-based, hydrophobic (treatment coated for suspension cells), 96-well plate (Sarstedt, Australia, cat #83.3924.500) and place with the lid on, inside an incubator set to 26°C to equalise to cell culture temperature. The external (border) lines of wells were not used for experimentation, to minimize any edge-effect bias due to evaporation (Figure 2-6). The cells were then selected according to their viability and log-phase growth, diluted to the required concentration with the appropriate cell media and poured into a multi-channel pipette reservoir. A multi-pipette was used to simultaneously deliver 50 µl of cells to each treatment well, then mixed gently to avoid creating air bubbles by pipetting up and down twice. The well plate was returned to the incubator for 1 h before commencing measurements. After incubation, the plate was housed in the SpectroStar Nano, pre-warmed to 26°C, and run on a kinetic growth program for 4 h, taking OD600 readings every 30 min. Plates were pre-programed to be shaken in a double orbital movement for 10 s at 100 rpm before each measurement in order to evenly disperse the cell suspension. Once the program had run its course, the data were exported into an Excel file for analysis and the plate was discarded.

	1	2	3	4	5	6	7	8	9	10	11	12
		-ve Control 1% DMSO	Internal 61P 0.01% 100µg/ml	14P 0.01% 100µg/ml	14N 0.01% 100µg/ml	20P 0.01% 100µg/ml	25N 0.01% 100µg/ml	28N 0.01% 100µg/ml	62N 0.01% 100µg/ml	W11N 0.01% 100µg/ml	+ve Control 0.005% Pyrethrum	
A	Empty Wells →											Empty Wells ←
B		50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% 61P	50µl Cells 50µl 0.02% 14P	50µl Cells 50µl 0.02% 14N	50µl Cells 50µl 0.02% 20P	50µl Cells 50µl 0.02% 25N	50µl Cells 50µl 0.02% 28N	50µl Cells 50µl 0.02% 62N	50µl Cells 50µl 0.02% W11N	50µl Cells 50µl 0.01% Pyrethrum	
C		50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% 61P	50µl Cells 50µl 0.02% 14P	50µl Cells 50µl 0.02% 14N	50µl Cells 50µl 0.02% 20P	50µl Cells 50µl 0.02% 25N	50µl Cells 50µl 0.02% 28N	50µl Cells 50µl 0.02% 62N	50µl Cells 50µl 0.02% W11N	50µl Cells 50µl 0.01% Pyrethrum	
D		50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% 61P	50µl Cells 50µl 0.02% 14P	50µl Cells 50µl 0.02% 14N	50µl Cells 50µl 0.02% 20P	50µl Cells 50µl 0.02% 25N	50µl Cells 50µl 0.02% 28N	50µl Cells 50µl 0.02% 62N	50µl Cells 50µl 0.02% W11N	50µl Cells 50µl 0.01% Pyrethrum	
E		50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% 61P	50µl Cells 50µl 0.02% 14P	50µl Cells 50µl 0.02% 14N	50µl Cells 50µl 0.02% 20P	50µl Cells 50µl 0.02% 25N	50µl Cells 50µl 0.02% 28N	50µl Cells 50µl 0.02% 62N	50µl Cells 50µl 0.02% W11N	50µl Cells 50µl 0.01% Pyrethrum	
F		50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% 61P	50µl Cells 50µl 0.02% 14P	50µl Cells 50µl 0.02% 14N	50µl Cells 50µl 0.02% 20P	50µl Cells 50µl 0.02% 25N	50µl Cells 50µl 0.02% 28N	50µl Cells 50µl 0.02% 62N	50µl Cells 50µl 0.02% W11N	50µl Cells 50µl 0.01% Pyrethrum	
G		50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% 61P	50µl Cells 50µl 0.02% 14P	50µl Cells 50µl 0.02% 14N	50µl Cells 50µl 0.02% 20P	50µl Cells 50µl 0.02% 25N	50µl Cells 50µl 0.02% 28N	50µl Cells 50µl 0.02% 62N	50µl Cells 50µl 0.02% W11N	50µl Cells 50µl 0.01% Pyrethrum	
H	Empty Wells →											Empty Wells ←

Figure 2-6 Absorbance spectrometry template example for cell growth bioassays.

2.6.3. Cytotoxic dose response

Serial dilutions of single extracts believed to be the most promising candidates for biopesticide activity were tested on cell lines to evoke a dose-dependent response. Extracts were dissolved in DMSO to give a final concentration of 5.0% w/v stock (100% DMSO), sonicated for 5 min then aliquoted into smaller volumes for freeze storage. Aliquots were later defrosted and mixed with cell media to give a final standard concentration of 0.1% w/v (2% DMSO v/v). Serial dilutions were then made using a 2.0% v/v DMSO/media diluent to maintain an equivalent DMSO background concentration. Half-strength serial concentrations of 0.0500%, 0.0250%, 0.0125%, 0.0062%, 0.0031% & 0.0015% w/v, called standards 2-6 respectively (Figure 2-7) were made, with positive control and internal-plate control, as previously described.

	1	2	3	4	5	6	7	8	9	10	11	12
		-ve Control Cells	-ve Control 1% DMSO	Standard 1 0.05% 68N.Fr42	Standard 2 0.025% 68N.Fr42	Standard 3 0.0125% 68N.Fr42	Standard 4 0.00625% 68N.Fr42	Standard 5 0.00312% 68N.Fr42	Standard 6 0.00156% 68N.Fr42	+ve Control 0.005% Pyrethrum	+ve Control 61P 0.01%	
A	Empty Wells →											Empty Wells ←
B	No Cells (CSD) →	50µl cells 50µl Media	50µl cells 50µl 2% DMSO	50µl cells 50µl 0.10% 68N.Fr42	50µl cells 50µl 0.05% 68N.Fr42	50µl cells 50µl 0.025% 68N.Fr42	50µl cells 50µl 0.0125% 68N.Fr42	50µl cells 50µl 0.00625% 68N.Fr42	50µl cells 50µl 0.00312% 68N.Fr42	50µl Cells 50µl 0.01% Pyrethrum	50µl cells 50µl 0.02% 61P	No Cells (CSD) ←
C		50µl cells 50µl Media	50µl cells 50µl 2% DMSO	50µl cells 50µl 0.10% 68N.Fr42	50µl cells 50µl 0.05% 68N.Fr42	50µl cells 50µl 0.025% 68N.Fr42	50µl cells 50µl 0.0125% 68N.Fr42	50µl cells 50µl 0.00625% 68N.Fr42	50µl cells 50µl 0.00312% 68N.Fr42	50µl Cells 50µl 0.01% Pyrethrum	50µl cells 50µl 0.02% 61P	
D		50µl cells 50µl Media	50µl cells 50µl 2% DMSO	50µl cells 50µl 0.10% 68N.Fr42	50µl cells 50µl 0.05% 68N.Fr42	50µl cells 50µl 0.025% 68N.Fr42	50µl cells 50µl 0.0125% 68N.Fr42	50µl cells 50µl 0.00625% 68N.Fr42	50µl cells 50µl 0.00312% 68N.Fr42	50µl Cells 50µl 0.01% Pyrethrum	50µl cells 50µl 0.02% 61P	
E		50µl cells 50µl Media	50µl cells 50µl 2% DMSO	50µl cells 50µl 0.10% 68N.Fr42	50µl cells 50µl 0.05% 68N.Fr42	50µl cells 50µl 0.025% 68N.Fr42	50µl cells 50µl 0.0125% 68N.Fr42	50µl cells 50µl 0.00625% 68N.Fr42	50µl cells 50µl 0.00312% 68N.Fr42	50µl Cells 50µl 0.01% Pyrethrum	50µl cells 50µl 0.02% 61P	
F		50µl cells 50µl Media	50µl cells 50µl 2% DMSO	50µl cells 50µl 0.10% 68N.Fr42	50µl cells 50µl 0.05% 68N.Fr42	50µl cells 50µl 0.025% 68N.Fr42	50µl cells 50µl 0.0125% 68N.Fr42	50µl cells 50µl 0.00625% 68N.Fr42	50µl cells 50µl 0.00312% 68N.Fr42	50µl Cells 50µl 0.01% Pyrethrum	50µl cells 50µl 0.02% 61P	
G	No Cells (CSD) →	50µl cells 50µl Media	50µl cells 50µl 2% DMSO	50µl cells 50µl 0.10% 68N.Fr42	50µl cells 50µl 0.05% 68N.Fr42	50µl cells 50µl 0.025% 68N.Fr42	50µl cells 50µl 0.0125% 68N.Fr42	50µl cells 50µl 0.00625% 68N.Fr42	50µl cells 50µl 0.00312% 68N.Fr42	50µl Cells 50µl 0.01% Pyrethrum	50µl cells 50µl 0.02% 61P	No Cells (CSD) ←
H	Empty Wells →											Empty Wells ←

Figure 2-7 Spectrometry template example for cell dose-response bioassay

Cell growth inhibition was calculated using the following formula, where 'control' is the negative control of DMSO at 1.0% v/v used at the same concentration in the background of each treatment dilution.

((Growth of Control – Growth of Treatment) / Growth of Control) *100

One-way ANOVA was conducted for the data, comparing results from treatments and controls using SPSS 22-25 (IBM, New York, USA). When ANOVA indicated there was a statistically significant difference ($p \leq 0.05$) differences between means were determined using the post-hoc Student's t test. Significance is presented as * p = 0.05-0.01, **p <0.01-0.001 and *** p< 0.001. All graphs were plotted in SigmaPlot 12.0 - 14.0 for Windows (Systat Software Inc., San Jose, CA, USA). All results are calculated and presented as means ± SE.

2.6.4. Potential threats and contaminations

Mycoplasma contamination is considered the most serious threat to cell line cultures and related data reliability; however, only 46% of Australian labs perform semi-annually testing, with many international papers citing contamination estimates of between 5-35% (Nikfarjam et al. 2012; Shannon et al. 2016). Mycoplasmas are a subclass of the simplest and smallest bacteria, lacking a rigid cell wall, rendering most antibiotics ineffective (Rottem et al. 1993). Mycoplasmas do not typically kill eukaryotic cells or cause changes in turbidity, can reach 10^8 cells per ml without apparent effect on cell growth, are undetectable visually under a microscope and are therefore easily overlooked. Standard sterile filtration methods are insufficient for mycoplasma exclusion and therefore require specialized 0.1 µm-pore low protein filters, to prevent contamination (Hay et al. 1989; Nikfarjam et al. 2012; Young et al. 2010). Sources of contamination include purchased animal serum products and media, laboratory personnel, inter- and intra- laboratory contamination, including contaminated liquid nitrogen, and aerosol particles (Nikfarjam et al. 2012). Mycoplasmas adversely affect cell cultures by depleting essential nutrients in media, altering downstream protein levels, metabolic functioning, DNA and RNA synthesis etc., and by their production of metabolites that act as toxins to eukaryotic cells (Uphoff et al. 2005).

Several detection methods exist, with pros and cons to each. Broth culturing and Agar plates, previously considered the “gold standard”, require keeping live, authenticated, reference strains for use as positive controls, thereby increasing the risk of accidental cross contamination. Furthermore, this process can take up to one month to identify contamination

with no guarantee that all mycoplasma species will grow and be detectable (European Pharmacopoeia 2005; Nikfarjam et al. 2012). DNA staining using Hoechst 33258 or DAPI for fluorescence detection is a fast and cost effective option but requires expertise to correctly identify mycoplasma DNA from other bacterial contamination or rouge DNA fragments floating in media. It is not sensitive to low contamination rates and has no speciation power. Furthermore, it may require the maintenance of a further host cell line such as Vero, adding to costs, storage and labour (Nikfarjam et al. 2012). Detection by PCR is the most sensitive and can be tailored to identify specific mycoplasmas, therefore allowing differentiation between the initial detection, a failed attempt to eradication and possible recontamination. Primers are designed from a conserved region of 16S rRNA gene and therefore will not give false positives for eukaryotic or bacterial DNA (Nikfarjam et al. 2012; Zhi et al. 2010).

For my purposes, prevention was a simpler, more time, labour and economically appealing option than designing multiple primer sets and routinely running PCR tests. It also seemed a much safer option than introducing a live mycoplasma reference line into the laboratory. I therefore opted to filter sterilise all media with 0.1 µm-pore low protein filters, maintain strict lab hygiene, work exclusively within a dedicated laminar flow cabinet and isolate my cultures into their own dedicated incubator. Hoechst 33258 stain was also used periodically during confocal scanning laser microscopy work to confirm that preventative methods were adequate.

Chapter 3: Triangulation of Methods Using Insect Cell Lines to Investigate Insecticidal Mode of Action

3.1. Introduction

The development of new pesticides is a key requirement for crop protection and resistance management in commercial agriculture. Globally, the discovery, development and registration of new crop protection products is either decreasing or becoming a lengthier and costlier process (Aliferis et al. 2011). Looking to nature to discover new compounds with pesticidal properties is experiencing a resurgence in interest due to the preference of consumers for natural alternatives to synthetic pesticides, reducing chemical residues in the food chain and a growing interest in reducing environmental impacts (Lima et al. 2015). This is especially important with regard to delaying the development of resistance, which is managed by rotating insecticides from differing MOA classifications (Sparks et al. 2015).

Pyrethrum is a multi-constituent extract from the daisy plant, *Chrysanthemum cinerariaefolium* (Asteraceae). Its active constituents are: Cinerin I, Jasmolin I, Pyrethrin I, categorised into the pyrethrins I group, and Cinerin II, Jasmolin II, and Pyrethrin II, categorised into the pyrethrins II group. There have been numerous reviews of pyrethrum extract and synthetic pyrethroids as insecticides (Casida 1980; Casida et al. 1995; Elliott et al. 1978; Gunasekara 2005; Wei et al. 2006). However, natural pyrethrum has limited uptake in agriculture due to its rapid degradation in sunlight, moisture and air; therefore, synthetic pyrethroids are often employed (Todd et al. 2003; Young et al. 2005). In general, the synthetic pyrethroids with the cyano group in the alpha position are considered type II pyrethroids and usually display choreoathetosis and salivation syndromes of toxicity (Todd et al. 2003). Moreover, it is generally accepted that natural pyrethrins and pyrethroids act as nerve toxins targeting voltage-gated sodium ion channels, causing either a slow activation of previously resting and closed channels (type I action); or alternatively, binding to activated sodium channels, causing delayed closing or permanently open states (type II action) (Casida et al. 2013). However, recent studies have also linked pyrethrum to non-nervous system responses and question if other cellular activities, such as autophagy, play a role in its insecticidal MOA

(Xu et al. 2017). Two commonly used synthetic type II pyrethroids, *α*-cypermethrin and esfenvalerate, were selected for assessment. In this study, we investigated new avenues of elucidating insect cell stress responses to pesticides, which could be incorporated into screening, discovery and investigation of novel compounds in insecticide research, as well as other applications.

Commercially available cell lines have been widely utilised for scientific advancement in both bio-medical and genetic investigations, as they provide many advantages including higher throughput of samples. However, the use of model insect cell lines in their innate form to investigate potential insecticides has been limited (Guo et al. 2020; Rasikari et al. 2005; Smagghe 2007). *Drosophila melanogaster* is a model insect species and its Schneider's second cell line (*D.mel*-S2) is a spontaneously immortalised, non-clonal cell line developed from late embryotic Oregon-R egg fragments (Adams et al. 2000; Mohr 2018; Rogers et al. 2008; Schneider 1972). The *Spodoptera frugiperda* cell line, IPLB-SF21-AE, was originally developed from immature ovaries removed from pupae to create the clonal *S. frugiperda* Sf9 cell line (Nandakumar et al. 2017; Pasumarthy et al. 1994; Vaughn et al. 1977). The Sf9 cell line is ideal for electrophysiological studies and this species is a serious, invasive pest; furthermore, it belongs to the same family (Noctuidae) as the multi insecticide-resistant cotton pest, *Helicoverpa armigera* (Carvalho et al. 2018; Tian et al. 2017).

Light-scattering spectrophotometry is routinely used to assess the cell density and growth phases of microbial cultures such as bacteria and yeasts for drug discovery and delivery, and protein expression (Stevenson et al. 2016). Whilst this is a universally acceptable technique in biomedical research, it requires some optimisation (Matlock 2017). Confocal scanning laser microscopy (CSLM) is employed in many fields of scientific discovery and diagnostics, using genetically encoded fluorescent probes such as green fluorescent protein for tracking proteins, monitoring promoter activity and labelling tissues and organelles for identification as well as other applications (Prescott et al. 2009). CSLM in combination with fluorescent indicators has been used extensively in plant cells to assess cellular changes in nitric oxide (NO), hydrogen peroxide (H_2O_2), reactive oxygen species (ROS), calcium (Ca^{2+}), sodium (Na^{+}) and potassium (K^{+}) in response to abiotic stresses (Chen et al. 2016; Liu et al. 2017; Wang et

al. 2016; Wu et al. 2015). However, CSLM technology has not been commonly employed to investigate ROS responses to insecticides in insect cell lines. Microelectrode Ion Flux Estimation (MIFE) is a non-invasive electrophysiological method to study ion distribution, movement and transport (Newman 2001). MIFE has been employed in research work in agricultural science, especially in abiotic stresses, such as root and leaf responses to salinity, (Chen et al. 2007; Shabala et al. 2005; Shabala et al. 2006b) drought, (Feng et al. 2016; Mak et al. 2014; Zhang et al. 2018) and heavy metals (Bose et al. 2010; Farrell et al. 2005; Mak et al. 2019; Ryan et al. 1992). Shabala et al. (2001) pioneered the use of MIFE to study membrane-transport processes in the bacterium *Escherichia coli* measuring net ion fluxes of H^+, Ca^{2+}, K^+ and NH^{4+}, as well as ion fluxes in *Listeria monocytogenes* (Shabala et al. 2002). Surprisingly, MIFE technology is yet to be applied for discovery of novel biopesticides using insect cell lines.

The aim of our research is to assess plant extracts for their potential as novel insecticides, using the combination of three reliable interdisciplinary methods to examine cellular physiological responses and putatively elucidate corresponding MOA and target sites. To the best of our knowledge, this the first report of the application of MIFE for MOA elucidation or stress response screening of insecticides. We show that a multifaceted approach is prudent in reducing the potential errors inherent in dealing with complex chemical extracts, experimental methodologies, and target insects.

3.2. Materials and methods

3.2.1. Insecticides and chemicals

Insecticides of analytical standard purity were used: pyrethrum extract 100 mg (Pestanal®, assayed at 49.2% pyrethrins Sigma-Aldrich), *α*-cypermethrin (**7**) 100 mg (Pestanal®, 99.8% purity, Sigma-Aldrich) and esfenvalerate (**8**) 100 mg (Pestanal®, 98.9% purity, Sigma-Aldrich). All other chemicals were purchased from Sigma-Aldrich, Castle Hill, NSW Australia unless otherwise stated.

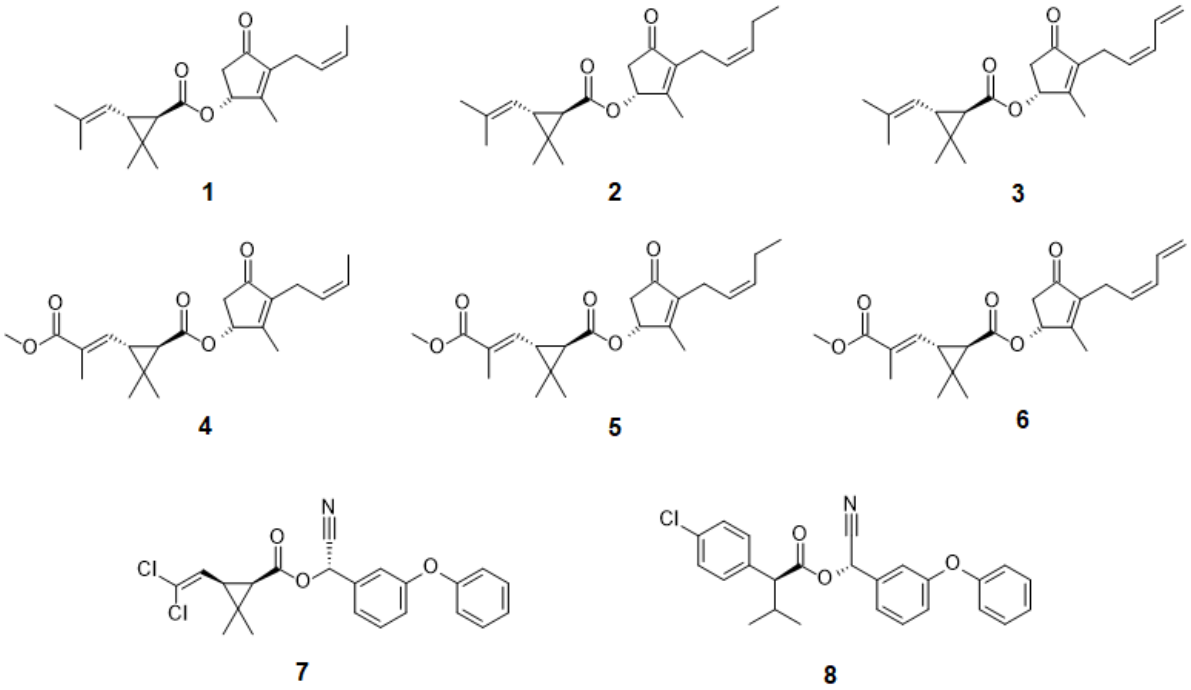


Figure 3-1 Chemical structures of the insecticides used in this experiment.

(**1**) cinerin I, (**2**) jasmolin I, (**3**) pyrethrin I, (**4**) cinerin II, (**5**) jasmolin II, (**6**) pyrethrin II, (**7**) α-cypermethrin, (**8**) esfenvalerate.

3.2.2. Cell culture techniques

Cryogenically frozen, serum-free adapted Sf9 cells (Life Technologies, Australia) were cultured in Sf-900™ II SFM media (Life Technologies, Australia) in 25 cm^2 cell culture flasks (Corning®, Sigma-Aldrich). Sf9 cells can be grown either as an adherent monolayer in T-flask culture flasks or as a suspension culture in shaker bottles. When grown as an adherent culture, as was the case during our experiments, cells were passaged when in mid-log phase of growth or at ~90% confluency (in an approximate 10d cycle) according to the manufacturer's manual. Cells were maintained at 26°C in a laboratory incubator (TEI-70G, Thermoline Scientific, Wetherill Park, NSW, Australia) without CO_2 amendment. Cells were initially treated with 5 mL/L and 5000U/5000 µg penicillin-streptomycin to eliminate any bacterial contamination, according to the manufacturer's manual. After the weaning period (approximately 7-10 passages), the cells were grown without the use of antimicrobials, and culture populations were maintained for approximately 30 passages before being discarded.

D.mel-S2 cells (Life Technologies, Australia) were cultured in fresh, sterile filtered, Compete Schneider's *Drosophila* media (CSD). CSD media was made by mixing Schneider's *Drosophila* Medium, supplemented with 10.0% v/v heat-inactivated fetal bovine serum and 0.1% v/v Pluronic F-68 surfactant (all media from Life Technologies) and 1.0% v/v (50 units or 50 µg/mL) penicillin-streptomycin D.mel-S2 cells were passaged bi-weekly into T-25 suspension treated flasks (Greiner, Sigma-Aldrich). CSD media was maintained at pH 6.7-6.8 and an osmolality of 360 mOsm/L (Schneider 1972), which is within range of osmotic pressure (342-378 mOsm/L) of hemolymph of 3rd instar larva of *D. melanogaster* (Echalier 1997). The osmolality of both cell lines was verified regularly using a vapour pressure osmometer (Vapro 5600, ELITechGroup, Logan, UT, USA).

3.2.3. Cell growth bioassays

Cell viability was first assessed by haemocytometer counts (Fuentes Gari 2015) in triplicate, and then averaged, using Trypan Blue live/dead stain. Cell communities with a viability >90% were then diluted in fresh media to a concentration of 1.0×10^6 cells/ml and re-incubated. This process was repeated for each replicate plate, using a new cell community each time, to ensure true biological replicates.

Under sterile conditions, daily stock solutions were made of dimethyl sulfoxide (DMSO) 2.0% v/v in media (diluent and negative control) and pyrethrum 2.0% v/v stock solution in DMSO (as treatment), and sonicated for 10 min. Working solutions of pyrethrum were made in concentrations of 0.02%, 0.01% and 0.005% v/v and sonicated for 10 min immediately prior to pipetting into a flat-based hydrophobic 96-well plate (Sarstedt, Nümbrecht, Germany). Treatment solutions were pipetted in 50 µl volumes according to the plate template (Appendix 2, Table 7-3), leaving columns 1 and 12 and rows A and H empty to remove any edge effect caused by potentially different evaporation rates over the combined incubating and scanning period of 5 h. Aliquots (50 µl) of homogenised cells were then pipetted over the top of the treatments using a multi-channel pipette (Eppendorf South Pacific, Macquarie Park, NSW, Australia) and gently aspirated and dispelled twice to mix. Final 96-well plate volumes were 100 µl, with final concentrations of pyrethrum at 0.01%, 0.005% and 0.0025% v/v whilst

maintaining a background concentration of DMSO equivalent to the negative control of 1.0% v/v.

The plate was then incubated for 1 h with the lid on, before being removed and placed on an absorbance spectrometer (SpectroStar® Nano, BMG Labtech, Mornington, VIC, Australia). The plate OD600 nm readings (Figure 3-2A & B) were taken every 30 min using a kinetic program function that was set to shake the plate in a double orbital rotation at 100 rpm for 10 s before each read cycle for 9 cycles over 4 h at 25°C. The resulting OD600 values were then exported from the MARS data analysis software (BMG Labtech, Mornington, VIC, Australia) for analysis. During selected replicate runs, two plates were made simultaneously; one was measured via the spectrometer while the other was housed within the incubator and sampled at 1 h and 3 h using Trypan Blue for viewing under the microscope. Thus, we confirmed that although OD600 increased due to some cell replication, a proportion of the cells have died. Inhibition of growth was calculated based on maximum growth in the negative control at 4 h: Inhibition (%) = [(normal activity-inhibited activity)/ (normal activity)] ×100%.

3.2.4. Confocal microscopy

Cells used for ROS detection were stained with 5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Thermo Fisher Scientific, Scoresby, VIC, Australia) and measured for changes in relative fluorescence, based on the first image taken at time point 0 min and the background without cells. The powdered stain was vortexed and dissolved in 100% DMSO to 5 mM, aliquoted into small volumes and frozen at -20°C. Immediately prior to experiments; it was diluted to 50 µM with the appropriate media for each type of cell line. The cells were initially diluted to approximately 1.5×10^6 cells ml^{-1}; 50 µl of the cells in media was then transferred into a 1 ml Eppendorf tube with 50 µl of 50 µM dye solution to give a final dye concentration of 25 µM. Each sample was then incubated for 20 min in the dark at 25°C. After incubation, the cells were centrifuged (Eppendorf® minispin® plus, Eppendorf AG, Germany) at the 800 rpm or 30 s, and carefully rinsed twice with the appropriate media to remove excess stain without causing disruption to the cell pellet. The pellet was then gently dispersed by pipetting, and 99 µl of cell solution was transferred to the bottom of a 35 mm glass bottom Petri dish (MatTek Corporation, Ashland,

MA, USA) and allowed the cells to settle for 5 min. Treatments were added as 1 µl of 1.0% v/v for consistency with other methods to give a final concentration of 0.01% v/v in a final volume of 100 µl. The two control treatments used (viz. 1.0% v/v DSMO) were calibrated to monitor the effects of light and mechanical stress only. All steps in this method were carried out in low light or dark conditions.

Fluorescence images were taken every 5 min over a 50 min interval using an inverted Leica TCS SP5 confocal scanning laser microscope controlled by LAS AF Software (Figure 3-2C) (Leica Microsystems, Germany). Lasers 488 nm and 633 nm were set to 10% and 1% for CM-H2DCFDA excitation and bright-field viewing, respectively. Emissions were collected between 510–550 nm based on a single peak verified by spectral analysis. Acquisition was made using ×64 water immersion objective, format: 1024×1024 pixels at 200 Hz with a pinhole of 111.46 µm, using a line average of three scans per image to improve resolution without adding high light stress.

3.2.5. Microelectrode ion flux measurements

Net ion fluxes of K^+, Na^+, Cl^- and Ca^{2+} were measured using the MIFE technique (Newman et al. 2012; Shabala et al. 1997). Custom-made MIFE chambers were constructed of 3 mm Perspex cut to give a triangular well with a side-notch removed, allowing the reference electrode to be located away from the measuring electrodes (Figure 3-2D). Cells in mid-log growth phase were collected, counted, and diluted to 1×10^6 cells ml^{-1}. One ml of the cell suspension was transferred to an Eppendorf tube and gently spun in a centrifuge for 30 s to create a loose pellet. Small rectangular pieces of microscopic cover glass were placed in the bottom of the MIFE chamber and covered with 376 µl of appropriate cell media. Into this, 20 µl of cell pellet was gently pipetted directly onto the glass in one fluid stream without dispersing the cells into the media. The cells naturally spread out and settled onto the glass stage to form a few cell layers that could then be used to focus the MIFE electrodes close to the cells (Figure 3-2E). Cells were left undisturbed for at least 30 min, in dark conditions within a sterile laminar flow cabinet to recover, prior to treatment. Four ion-selective electrodes were then aligned as per Mak et al. (2014) to measure net Na^+, Cl^-, K^+ and Ca^{2+} flux, respectively, for 15 min before adding 4 µl of treatment or negative control (DMSO 100%) to

give a final chamber volume of 400 µl. Changes in net ion flux due to treatments were measured for 25 min.

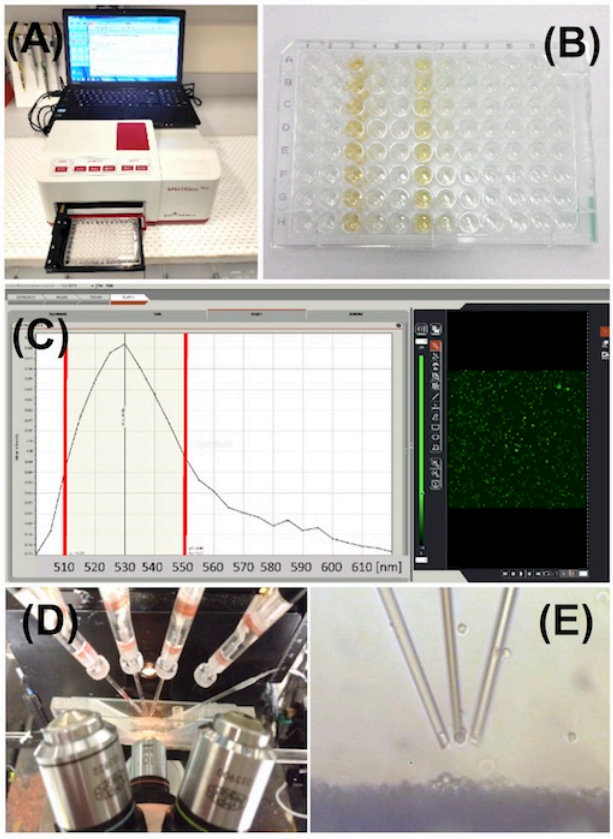


Figure 3-2 Essential equipment and configuration, including confocal spectral analysis.

(**A**) SpectroStar® Nano absorbance microplate reader with attached computer for program driven spectrometry using MARS data analysis software. (**B**) Flat-bottom 96—well plate used for sample loading (Sarstedt). (**C**) Laser scanning confocal microscopy spectral analysis of ROS dyed cells to obtain emission range and check for autofluorescence or other interference artefacts. Single optimum peak range found between 510-550 nm. (**D**) Custom made MIFE chamber with four electrode holders and three microelectrodes and a reference electrode in place. (**E**) View of the same three microelectrodes through the microscope, with ion selective Liquid Ion Exchanger (LIX) in the tips. Distance from electrode tip to cell colony is approximately 40 µm.

3.2.6. Data analysis

Quantitative ROS fluorescence analysis of individual cells was achieved by selecting and numbering up to 26 similarly sized cells with healthy, regularly shaped membranes, starting with the initial image capture and repeating for subsequent images. Region of interest (RIO) tool for each of the ten images were processed using ImageJ software (National Institute of Health, Bethseda, MD, USA). Corrected total cell fluorescence (CTCF) was determined according to previous publications by Chen et. al. (2016) and McCloy et al. (2014). The mean and standard error of all cells in each image was then converted to a percentage value, giving the relative change in fluorescence compared to the initial zero time of 100%. The statistical significance ROS increase was evaluated as *** = $p<0.01$ using Student's t-test in SPSS 25.0 (SPSS Inc, Chicago, IL, USA).

MIFE flux data was processed using MIFEFLUX program (Shabala et al. 2013) to fix calibration files of the standards. Data were then exported to Microsoft Office Excel 2016 (Microsoft, USA) for bulk processing. Each graphed data point is an average of 5 points (approximately 25 s), then an average of all replicates at that time point. All graphs were plotted using SigmaPlot 14.0 for Windows (Systat Software Inc, San Jose, CA, USA).

3.3. Results

3.3.1. Growth inhibition of D.mel-S2 cells

The growth of D.mel-S2 cells was inhibited by pyrethrum in a dose-dependent manner (Figure 3-3A). Treatment concentrations of 0.01% v/v inhibited cell growth by 41.7%, 0.005% v/v by 28.4% and 0.0025% v/v by 13.0% (compared to the 1.0% v/v DSMO control). Although OD600 growth curves (Figure 3-3A) were still exponential in the initial term of 0–2 h at all concentrations and continued at the lowest concentration of 0.0025% v/v, haemocytometer counts of the mid-range concentration 0.005% measured at 1 and 3 h confirmed mortality in an increasing proportion of the cell community (Figure 3-3A B & C). This insecticide-induced growth inhibition was further validated by CSLM and MIFE techniques.

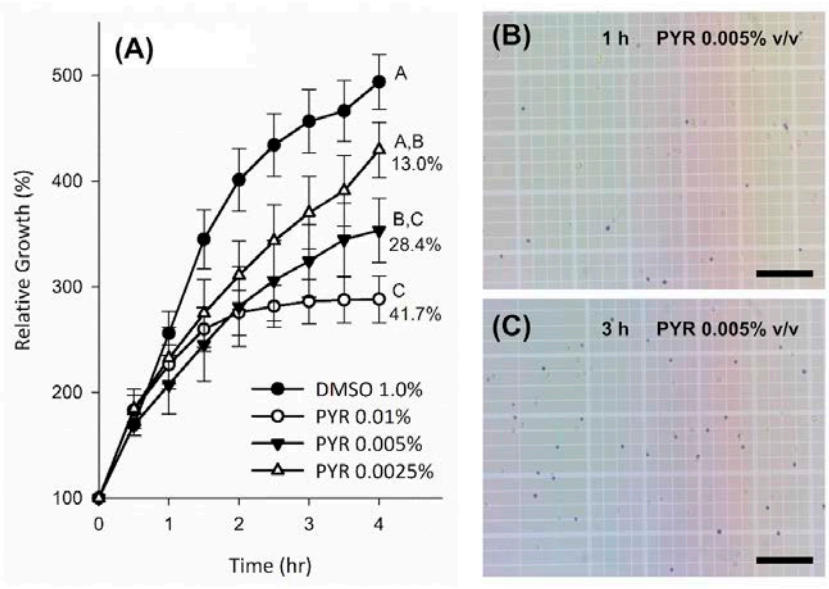


Figure 3-3 Drosophila Schneider D.mel-S2 cell response to pyrethrum

(**A**) Six technical replicates (between 5–15 plates) containing different culture batches were used to measure growth and assess inhibition against the background diluent (negative control) DMSO 1.0% v/v. Data is expressed as mean ± standard error of the mean (SEM). Statistical analysis performed using one-way ANOVA (F (3,27) = 11.366, p.0.001) and Welch's t-test, with Tukey and Games-Howell Post-hoc tests. Different letters indicate significantly different cell growth at 4 h between treatments at p>0.05. Percentages displayed beneath letters indicate the growth inhibition percentage. Similar results were seen when Sf9 cells were challenged (data not shown). Microscope haemocytometer images of *D.mel*-S2 cells using live/dead stain, trypan blue, (**B**) cells 1 h after treatment (1 HAT) and (**C**) 3 HAT with pyrethrum 0.005% v/v. Black solid scale bar represents 50 µm. Microscope magnification ×100.

3.3.2. Free radical production in Sf9 and D.mel-S2 cells

Confocal measurements from negative, calibration (CAL) and DMSO 1.0% v/v controls showed there were no substantial changes in ROS, confirming there were no adverse effects on the Sf9 cells from the laser scanning microscope or DMSO (Figure 3-4D). Similar results were also observed in D.mel-S2 cells (data not shown). ROS production was visualised qualitatively by comparing the cells in control conditions at 0 min (Figure 3-4A) and at the end of measurements at 50 min (Figure 3-4B). Sf9 cells responded strongly ($p<0.001$) in both the natural pyrethrum and the synthetic pyrethroids (α-cypermethrin and esfenvalerate), treatments at concentrations of 0.01% v/v (Figure 3-4D). However, *D.mel*-S2 cells showed

little change in fluorescence following exposure to natural pyrethrum, even at much higher concentration (viz. 1.0% v/v), or when images were taken at 1 min intervals to avoid missing a spontaneous burst in ROS (data not shown).

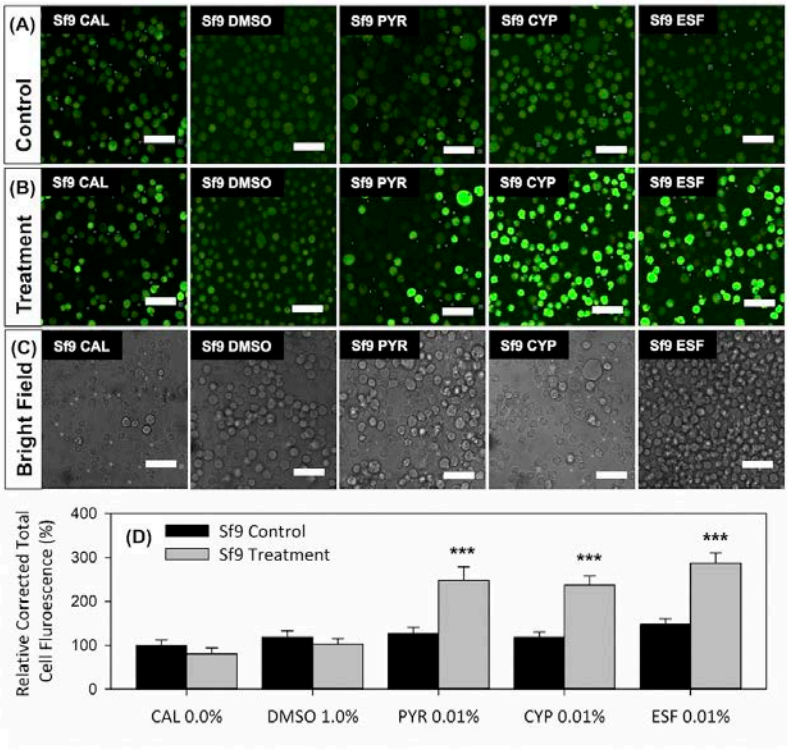


Figure 3-4 Confocal microscopy images and bar graph of ROS fluorescence from Sf9 cells under differing treatments.

CAL means calibration of cells only, that is; the cells natural reaction to light stress caused by the laser without any treatment was used to calibrate the cell change in florescence purely due to light stress. DMSO means cells were treated with the negative control of the diluent, to give a final concentration of 1.0% v/v DMSO. PYR represents 0.01% v/v final concentration of pyrethrum extract. CYP represents 0.01% v/v final concentration of *α*-cypermethrin. ESF represents 0.01% v/v final concentration of esfenvalerate. (**A**) Initial images at 0 min in control conditions for each treatment type. (**B**) Final images are 30 min post-treatment, that is; 50 min from the start of the experiment. (**C**) Bright field images, which are given to show representative cell numbers, however only cells in the same plane of focus, of similar size and healthy membrane wall structure are selected for measurements. White horizontal scale bars indicate a 50 µm distance. (**D**) Sf9 cellular ROS fluorescence. Control bars represent the mean florescence of the first 20 min in each case. The treatment bars represent the mean florescence from 20-50 min, which is the average change in ROS over a 30 min treatment time. Error bars represent ± SE, *** at $p<0.001$, n=4–6 replicates of 80–115 individually measured cells.

3.3.3. Ion flux responses of Sf9 and D.mel-S2 cell lines

Net Na^+ ion flux pre-treatment (Figure 3-5A-F) in both cell lines was a neutral to slight efflux in control conditions, although DMSO led to larger standard errors, possibly due to cell community variation or effect of DMSO on the ionophore's sensitivity to Na^+. The addition of pyrethrum 0.01% v/v to D.mel-S2 cells (Figure 3-5B) at 15 min resulted in a pronounced efflux of Na^+, which did not recover to pre-treatment values over the course of the transient measurement, indicating a potential depolarisation of membrane potential. This, however, was not observed in the Sf9 cells for any of the pyrethrum (Figure 3-5D), α-cypermethrin (Figure 3-5E) or esfenvalerate treatments (Figure 3-5F).

A small net Cl^- ion efflux was recorded pre-treatment in both cell lines (Figure 3-5G-L). In D.mel-S2 cells, the addition of pyrethrum 0.01% v/v induced an immediate, substantial and sustained net Cl^- influx (Figure 3-5H). In the corresponding Sf9 cell line (Figure 3-5J), however, the pyrethrum treatment was slow to register any change, resulting in a delayed and mild Cl^- influx, significantly less than that observed in D.mel-S2 cells (Figure 3-5H). A similar result was recorded in the α-cypermethrin treatment (Figure 3-5K). However, the esfenvalerate treatment (Figure 3-5L) resulted in an immediate Cl^- influx response that reduced in severity to almost pre-treatment values before slowly increasing in value, resulting in a moderate influx by the end of the measurement period.

In all treatments in both cell lines, the net K^+ flux changes from pre- to post-treatment were much smaller than the Na^+ and Cl^- fluxes. However, the addition of pyrethrum 0.01% v/v produced a mild efflux in D.mel-S2 cells (Figure 3-6B), which almost recovered by the end of the 25 min measurement period. This effect was not evident in the corresponding pyrethrum treatment of Sf9 cells (Figure 3-6D), nor in the two synthetic pyrethroid treatments (Figure 3-6E & F).

Net Ca^{2+} ion flux in the control treatments remained at near neutral flux in both cell types (Figure 3-6G-L), although for D.mel-S2 cells the addition of DMSO 1.0% v/v induced an initial, slight influx, which rapidly returned to pre-treatment neutrality (Figure 3-6G). However, D.mel-S2 cells responded to pyrethrum 0.01% v/v with a slow and mild influx over the course of the measurements (Figure 3-6H). In Sf9 cells, pyrethrum 0.01% v/v resulted in no change

in flux (Figure 3-6J); however, both α-cypermethrin 0.01% v/v and esfenvalerate 0.01% v/v recorded an initial efflux, which quickly returned to the control levels (Figure 3-6K & L).

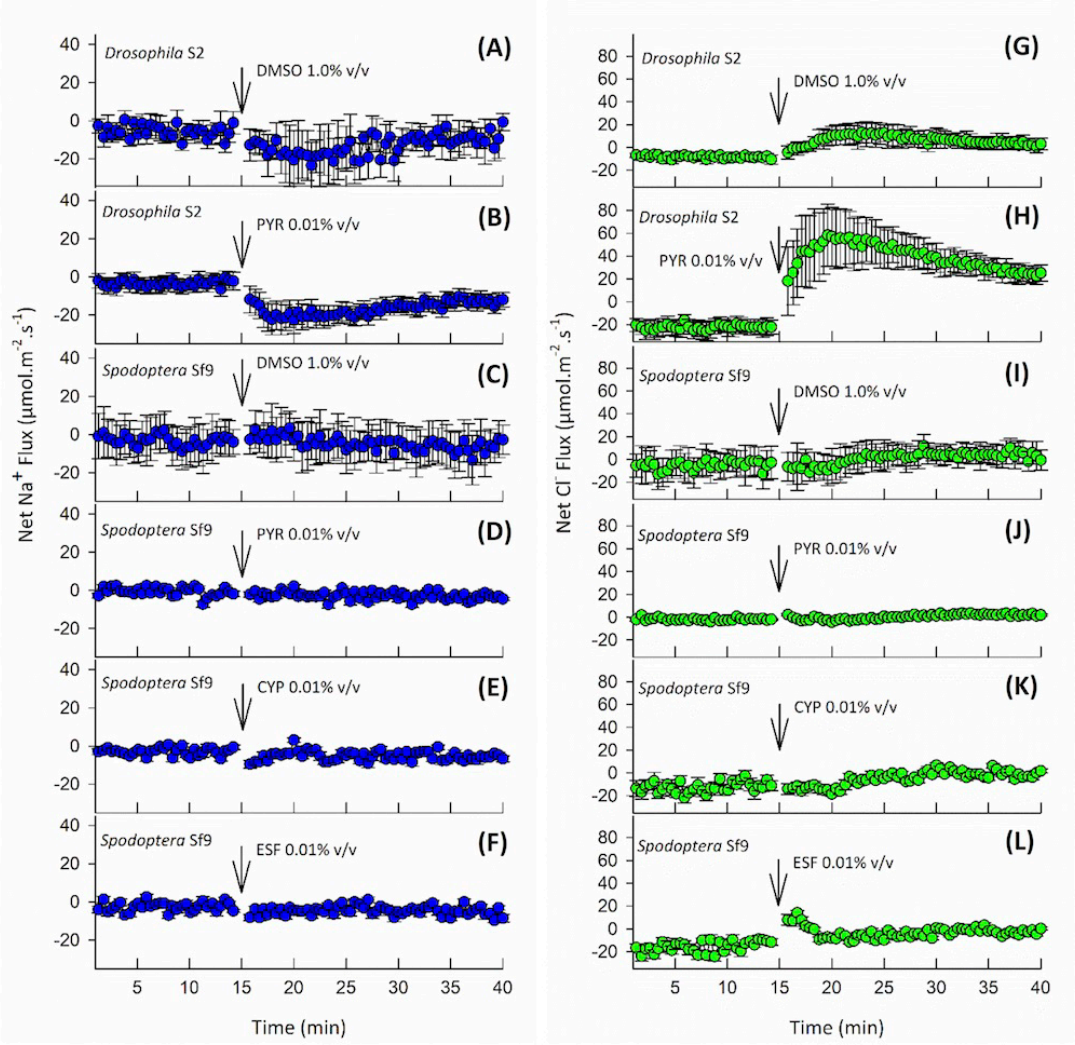


Figure 3-5 Net Na^+ and Cl^- flux of D.mel-S2 and Sf9 cells in control and treatment conditions.

Net Na^+ traces **(A** & **C)** and net Cl^- traces **(G** & **I)** show effects of the negative control, DMSO 1.0% v/v on both D.mel-S2 **(A** & **G)** and *Spodoptera* Sf9 **(C** & **I)** cell lines. The remaining traces show the treatment response of D.mel-S2 **(B** & **H)** and *Spodoptera* Sf9 **(D** & **J)** to pyrethrum at 0.01% v/v. Whilst, traces **(E** & **K)** and **(F** & **L)** show the treatment responses of Sf9 to *α*-cypermethrin and esfenvalerate at 0.01% v/v, for each ion, respectively. Each data point is an average of 5 points (25 s). Error bars represent ± SEM. n=3-14 replicates.

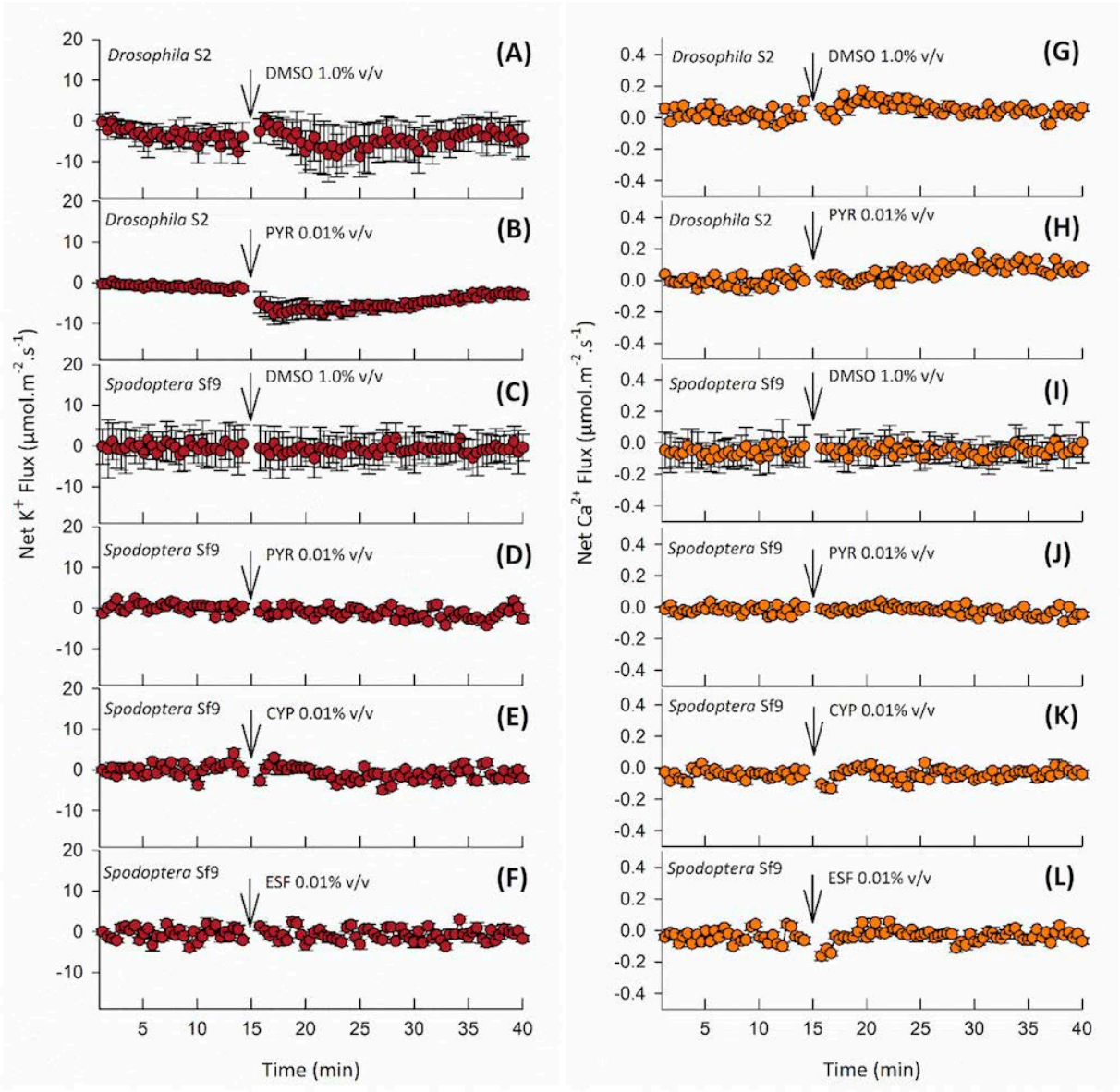


Figure 3-6 Net K^+ and Ca^{2+} flux of D.mel-S2 and Sf9 cells in control and treatment conditions.

Net K^+ traces (**A** & **C)** and net Ca^{2+} traces **(G** & **I)** show effects of the negative control, DMSO 1.0% v/v on both D.mel-S2 **(A** & **G)** and *Spodoptera* Sf9 **(C** & **I)** cell lines. The remaining traces show the treatment response of D.mel-S2 (**B** & **H)** and *Spodoptera* Sf9 **(D** & **J)** to pyrethrum at 0.01% v/v. Whilst, traces **(E** & **K)** and (**F** & **L)** show the treatment responses of Sf9 to *α*-cypermethrin and esfenvalerate at 0.01% v/v, for each ion, respectively. Each data point is an average of 5 points (25 s). Error bars represent ± SEM. n=3–14 replicates.

3.4. Discussion

3.4.1. The importance of cell proliferation when screening bio-pesticides

In cases where cell lines are used as a model for pesticide effects, as an alternative to whole organism bioassays, it is important to monitor cellular changes, such as cell viability, proliferation and membrane integrity before investigating molecular and cell biological changes (Tolosa et al. 2015). We found that using low densities of cell culture over short periods (<12 h) in 96-well plates allows for small sample volumes and higher throughput of samples and gives reliable quantitative measurements of cell growth and inhibition. It appears that avoiding the perimeter wells provided consistency, presumably by reducing evaporation influences (Van Meerloo et al. 2011). A further advantage to using this technique, besides it simplicity, is that cells grown as non-adhesive cultures can be assessed, which could otherwise pose issues for MTT assays which require repeated washing steps. This also allows for multiple cell lines to be compared using the same standard protocol, thereby reducing experimental variability inherent in alternative methods.

The closest report of insecticide screening on an insect model cell line that investigates multiple methods of assessment and aims to answer the question of cell line suitability is that of Decombel, Smagghe & Tirry (2004). Their study also used cell proliferation as a parameter to assess biological activity of their chosen adhesive cell line, *Spodoptera exigua*, under insecticidal pressures. To test for insecticidal efficacy correlation between cells and larvae of *S. exigua,* a range of neurotoxic insecticides with differing MOA were employed. Endosulfan, a GABA-gated chloride channel blocker, required the least concentration to reach IC_{50} in cells (1.53 ppm) but one of the highest concentrations to reach LC_{50} in larvae (3520 ppm). Abamectin, a glutamate-gated chloride channel modulator, resulted in moderate efficacy against cells (IC_{50} at 4.52 ppm) but required 7.5-fold higher concentration in larvae to achieve LC_{50} (34.2 ppm). Conversley, bifenthrin, a sodium channel modulator, required in excess of 12-fold higher concentrations to achieve IC_{50} in cells (14.7 ppm) compared to the LC_{50} in larvae (1.21 ppm) Other neurotoxins, such as parathion (acetylcholinesterase inhibitor), imidacloprid and spinosad (nicotinic acetylcholine receptor competitive modulators) showed non-activity in cells, regardless of varying LC_{50} values in larvae (217 ppm, >4,000 ppm and 8.64

ppm, respectively). They concluded that neuro-receptor-specific insecticides should be tested using electrophysiological and neurochemical approaches (Decombel et al. 2004).

It is not unexpected that different pyrethroids possess different potencies or even exhibit diverse MOA, owing to their variable side chain substituents. Lipophilicity, molecular weight, structural rigidity, and the number of hydrogen bond donors or acceptors can all influence the pharmacodynamics of a molecule. It has been noted from *in vivo* studies that not all structures with the same functional group display typical syndromes of intoxication that correspond with their pyrethroid type (Casida et al. 2013; Soderlund 2012). Given the complex chemical nature of pyrethrum (Figure 3-1), it is rational that this extract exhibited a different bioactivity profile to *α*-cypermethrin and esfenvalerate. It is also apparent that multi-component mixtures might have advantages over single compounds through synergistic insecticidal action. We suggest that, for mode/s-of-action studies of novel compounds, electrophysiological approaches should be conducted concurrently with metabolic and physiological ones.

3.4.2. Optimising operation of CSLM and selection of florescence probe minimise experimental artefacts

Confocal scanning laser microscopy is a reliable analytical tool for both qualitative and quantitative research methods once calibration and interference artefacts are taken into consideration. With regard to testing for chemically induced responses, such as insecticidal activity, each component of the sample should be individually tested for autofluorescence. Moreover, reducing light stress on both insect cell lines in this study was critical to achieving valid results, due to the speed of ROS production (Negre-Salvayre et al. 2002). We found that visual inspection of cell lines by microscope lights caused light stress. Therefore, we ensured that a sufficient proportion of cells could fall to rest against the glass-bottomed dish in a single focal plane. This enabled fast and efficient focusing with the absolute minimum of light by turning down the microscope light to its lowest intensity (Appendix 1, Figure 7-1). The use of the pinhole results in the elimination of “out-of-focus” fluorescence from above and below the samples’ focal plane being detected, reducing additional light contributions beyond the area of interest (Paddock 1999; Salih et al. 2011). Furthermore, acquisitions of each sample

were made using the identical photomultiplier tube (PMT) settings to prevent overexposure and related artefacts.

The importance of ROS as a critical mediator of cellular signalling pathways has been widely explored in plant responses to a range of stressors including mechanical injury, pathogen infection, hyperosmotic and abiotic stresses (Beffagna et al. 2005; Demidchik et al. 2003; Gilroy et al. 2014; Sandalio et al. 2008) and has been associated with a range of human metabolic signalling (Hamanaka et al. 2010). ROS detection by dichlorofluorescein fluorescence has been perceived to lack specificity in identifying one ROS molecule from another or intercellular ROS from extracellular leaks into surrounding media (Tarpey et al. 2004). However, we found the newer iterations of molecular probes, namely CM-H_2DCFDA, with its negatively charged characteristics once permeated into the cell, to be stable and with minimum leakage (Negre-Salvayre et al. 2002). We also report that variation in cell samples was reduced by taking kinetic measurements of the same cells before and after treatment and by deducting the background fluorescence in the data analysis.

3.4.3. Selection of appropriate cell lines for the discovery of specific insecticides

It has been well established though numerous electrophysiological studies (Buckingham et al. 1996; Chien et al. 2006; Grolleau et al. 2000; Narusuye et al. 2007; Towers et al. 2002; Yeromin et al. 2004) that embryonic *Drosophila* cells are an excellent model to study nervous system behaviours especially innate K^+, Na^+, and Ca^{2+} channel currents (Byerly et al. 1988). GABA receptor Cl^- channel complex has been shown to be blocked by pyrethroids (Bloomquist 1996). Our studies also identified the responsiveness of Cl^- flux to known insecticides such as pyrethrum (Figure 3-5G-L). Electrophysiology studies have been instrumental in identifying Na^+ channels as a primary target site for pyrethroids, whilst having a lesser effect on K^+ and Ca^{2+} channels (Narahashi 1992). We report similar results using the MIFE technology (Figure 3-5 & Figure 3-6). From our study, we propose that net ion fluxes from cell lines may be a good indication of the nerve toxicity potential of uncharacterised compounds.

The publication of a complete *Drosophila* genome sequence by Adams et al. (2000) nearly two decades ago, has resulted in an increase use of D.mel-S2 cell lines for multiple biological

applications. However, cell lines of well-known genetic libraries such as *D. melanogaster* may be inappropriate if the target pest is unrelated. In this study we used D.mel-S2 cell lines to assess the reliability of an additional cell line, Sf9, in response to known insecticides of both a natural and nature-equivalent, synthetic sources. In general, these insect cell lines have been shown to be useful in the elucidation of many insecticidal MOA, since they offer a direct access to target sites that may otherwise be difficult to access in whole insect assays, or for pesticides whose efficacy may be compromised by biological activities such as physical exclusion, excretion, metabolic detoxification or cellular degradation (Smagghe 2007).

We suggest a triangulation of methods, such as those reported here, is a useful approach to elucidating the activity of novel insecticides, as well as for other fields of cell biology research.

Chapter 4: Whole Organism Botanical Pesticide Investigations

4.1. Introduction

Synthetic pesticides are heavily relied upon for pest management in commercial agriculture. However, due to their perceived human health risks, environmental persistence, off-target toxicities and food residues, the development of alternative biopesticides is in high demand (Chaudhary et al. 2017; Walia et al. 2017). While biopesticides are experiencing compounding annual growth of 10-20%, they still only account for approximately 5% of the US$61.3 billion market and less of the European market, leaving synthetic pesticides the prevalent choice (Balog et al. 2017; Marrone 2019; Ndolo et al. 2019; Olson 2015). Furthermore, despite research efforts to find phytochemical biopesticides, or botanical pesticides as we refer to them, pesticides of this origin only account for less than 1% of the total pesticide use (Walia et al. 2017). Several practicalities in the development of novel botanical pesticides must first be overcome, namely: standardised extraction and purification, compound isolation and structural elucidation, reliable screening and bioassays, mode-of-entry and MOA identification, formulation, safety testing and regulatory acceptance (Walia et al. 2017). Nevertheless, looking to nature to provide novel biopesticides is experiencing a resurgence in interest due to the preferences of consumers towards natural alternatives to synthetic pesticides, reducing chemical residues in the food chain and a growing interest in reducing environmental impacts (Lima et al. 2015).

Navigating the pathway from discovery, screening, MOA identification and product registration can be confusing and complex. The US Environmental Protection Agency (EPA) defines biopesticides into three classes; 1.) biochemical pesticides that control pest by non-toxic, interference, deterrent or attractant mechanisms 2.) Microbial pesticides sourced from bacterium, fungus, virus or protozoan 3.) Plant incorporated protectants such as *Bt* transgenic mechanisms, accounting for 75% of all biopesticides (EPA 2016; Olson 2015). One may wonder then, where do botanical pesticides with toxic properties against insects sit? Therefore, it may not be surprising that there are still a limited number of botanical

biopesticides and even fewer botanical insecticides registered in the US or Australian markets. Most biopesticide discovery and commercialisation endeavours relate to microbially- sourced biofungicides and bioinsectides, with a paucity of botanically-derived chemical insecticides (Marrone 2019; Senthil-Nathan 2015)

Bioassays are used to quantify the effectiveness of a toxin on a target pest species or to estimate its safety to non-target organisms. The purpose of finding the quantal response to the toxin, that being the relationship between dose and efficacy, is to estimate the probability that the pest population in the field can be reduced below economically damaging levels (Robertson et al. 2017). I thus hypothesised that insect cell line cytotoxicity responses to the active compounds in the novel plant extracts are comparable to whole organism mortality from bioassays without encountering significant whole organism barriers or metabolic detoxification.

In this chapter I report the arthropod bioassays conducted to screen polar and non-polar plant extracts of unknown constituents for botanical pesticide activity and commercialisation potential (Table 2-2). From the foundation project, where 358 extracts were tested for their efficacy on the mortality of *T. urticae, A. gossypii* and *H. armigera* at 24 and 48 HAT (Table 7-4, Appendix 3) a sub-selection of extracts where chosen (Table 5-1). Based on those results, several extracts were selected for cytotoxicity against insect cells in Chapter 5: Cellular responses to novel plant extracts. Concurrently, the whole organism bioassays presented in this chapter assessed selected extracts based on the results of my experimental findings reported elsewhere in this thesis. During the whole organism bioassays, the following experimental extracts were investigated: 68N.M, 68N, 69P, 69N, 72N, 82N; and several known insecticides were also explored for their suitability as positive controls: pyrethrum, Qcide™ (a steam-distilled essential oil containing ˜90% of the β-triketone, tasmanone), tasmanone (both the natural and the nature-identical compound) and flavesone, another β-triketone reported to have insecticidal action (Miller et al. 2017). The purpose of reducing the number of extracts tested was to progress onto the discovery of MOA identification for only the novel and commercially viable candidates.

4.2. Direct application via Potter Precision Spray Tower

4.2.1. Methods

Direct application experiments using the Potter spray tower (see Chapter2) were carried out independently of the foundation project as part of my PhD study to focus on the effects of extract 68N on *D. melanogaster* and *A. gossypii* and determine their dose responses.

Using *Podolepis jaceoides* methanol extract 68N.M (see Chapter 2), a 200 mg sample of 68N.M was weighed into a 50 ml beaker and dissolved with 0.5 ml technical grade acetone (not more than 5% final conc.) and sonicated for 5 min. Triton X-100™/water at a concentration of 200 ppm (Union Carbide, Sigma, St. Louis, MO) was then added to provide a total volume of 10 ml, giving a final extract concentration of 2.0% w/v. Serial dilutions were then made to give treatments of 2.0%, 1.0%, 0.75%, 0.5%, 0.25%, 0.125% and 0.0625% w/v concentrations. A control solution was prepared in the same manner using the diluent Triton X-100™ without any extract.

Bioassays were conducted on both insect species, *A. gossypii* and *D. melanogaster,* at the same time in the following manner. Insect cages were as described in Chapter 2: General Materials and Methods, with the slight variation for the *D. melanogaster* where 50 mm filter paper discs were placed over the agar onto which a small amount of *Drosophila* Instant Culture Medium was added to provide a food source (Figure 4-4D). For the aphid bioassay, approximately 40 gravid *A. gossypii* were transferred onto the leaf disc and allowed to settle and commence feeding, with the dish lids closed but not sealed (Figure 4-4E). For the *Drosophila* bioassay, ten to fifteen pre-sorted, unmated *D. melanogaster* flies were anaesthetized with CO_2 and gently poured onto the filter paper lined dish immediately prior to spraying. Three ml of each extract concentration were applied to separate pairs of plates, commencing with the control and followed by increasing concentrations. The Potter tower was set at 15 psi inlet and calibrated to deliver extracts at 4.498 mg/cm^2; this equates to 0.0899 mg dried, ground extract per cm^2 when formulated at 2.0%. Six replicates were

conducted on separate days, using individually prepared extract emulsions for each day. For each bioassay replicate run, two dishes were sprayed simultaneously on the Potter tower stage, with one containing *D. melanogaster* and the other *A. gossypii.* Treated plates were retained in the WSU Entomology Laboratory at 24 ± 2°C and RH 65 ± 10% for assessment. Mortality, and for aphids the number of offspring, was recorded at 3 and 24 HAT. Absence of aphid movement when prodded with a fine brush was taken as the criterion of death. Mortality for flies was counted at 30 min, 3 and 24 HAT. Absence of appendage movement was taken as the criterion of death.

Probit analysis (Finney 1971) was used to estimate the major lethal concentrations (viz., LC_{50} and LC_{90}) and their confidence intervals. Heterogeneity of regressions was determined by Pearson's goodness of fit, chi-square characteristic using SPSS (Ver. 22 – 25, IBM, New York, USA).

4.2.2. Results

The Potter spray tower results showed a dose-dependent relationship between *A. gossypii* and extract 68N.M, however this was not reflected in results for *D. melanogaster.* Insecticidal effects of 68N.M on *A. gossypii*, were evident within 1 h, but are reported at the standard time of 24 HAT, for comparison with the foundation project results of 100% mortality of *A. gossypii* and 98.9% mortality of *T. urticae* at 24 HAT (Table 7-4, Appendix 3). *Aphis gossypii* recorded 90.88% mortality at 24 HAT in the 0.75% w/v treatment (Figure4-2A), whereas *D. melanogaster* recorded 2.0% mortality at 24 HAT in the 2.0% w/v treatment (Figure4-2A). Probit analysis calculated a LC_{50} of 2,103.7 ppm (95% CL 1,887.7–2,313.0 ppm), and LC_{95} of 10,869.8 ppm (9,139.7–13,558.4), n=1282 aphids from 6 biological replicates (Figure 4-1). This experiment also allowed a direct comparison between the target pest, *A. gossypii* and the model insect, *D. melanogaster* (Figure 4-2). However, because *A. gossypii* died very quickly, in order to extract viable RNA this bioassay was re-run allowing *A. gossypii* and *D. melanogaster* to be collected 1 h after treatment and used for molecular investigations in Chapter 6: Investigating insecticidal modes of action and resistance through molecular techniques.

Visual observations (Figure 4-3A-F) showed that aphids were rapidly affected by the 68 N.M treatment, initially trying to escape (possibly from hyperactivity or fumigant effects), then losing motor control, ceasing to feed, rolling onto their backs or haemorrhaging (Figure 4-3E & F). Commencing at the head in most cases (Figure 4-3C), treated aphids darkened (Figure 4-3D), followed by their death. In stark contrast to the control aphids at 24 HAT (Figure 4-3A) treated aphids appeared completely brown or black and dehydrated (Figure 4-3B). However, this extract did not cause any visual changes or interesting behavioural changes in *D. melanogaster*, nor was there significant mortality. In comparison to *A. gossypii*, *D. melanogaster* recorded a maximum corrected mortality of 1.8% at the highest concentration of 68N.M tested, which produced 100% mortality in *A. gossypii* (Figure 4-3A). Furthermore, there were no obvious visual or behavioural changes in *D. melanogaster*, indicating minimal toxic effects. Because *A. gossypii* died very quickly, and so that I could extract viable RNA for the molecular work reported in Chapter 6: Investigating insecticidal MOA and resistance through molecular techniques, this bioassay was re-run to enable treated *A. gossypii* and *D. melanogaster* to be collected at 1 HAT (Figure 6-3).

Parameter Estimates

Parameter		Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Dose conc.	2.306	.159	14.497	.000	1.994	2.618
	Intercept	-7.663	.555	-13.796	.000	-8.219	-7.108

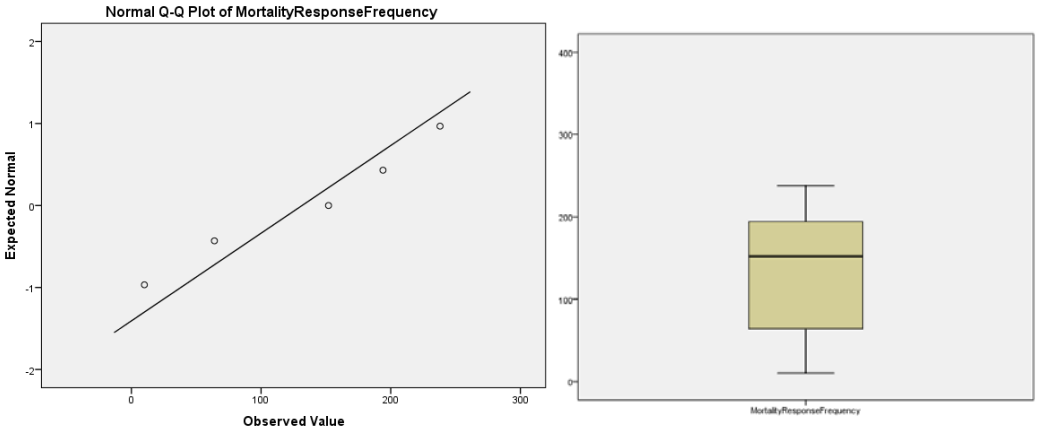
a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
MortalityResponseFrequency	.186	5	.200*	.956	5	.782

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



Chi-Square Tests

		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	1.658	2	.436[a]

a. Since the significance level is greater than .150, no heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based

Figure 4-1 Statistically analysis, Probit and tests for normality output.

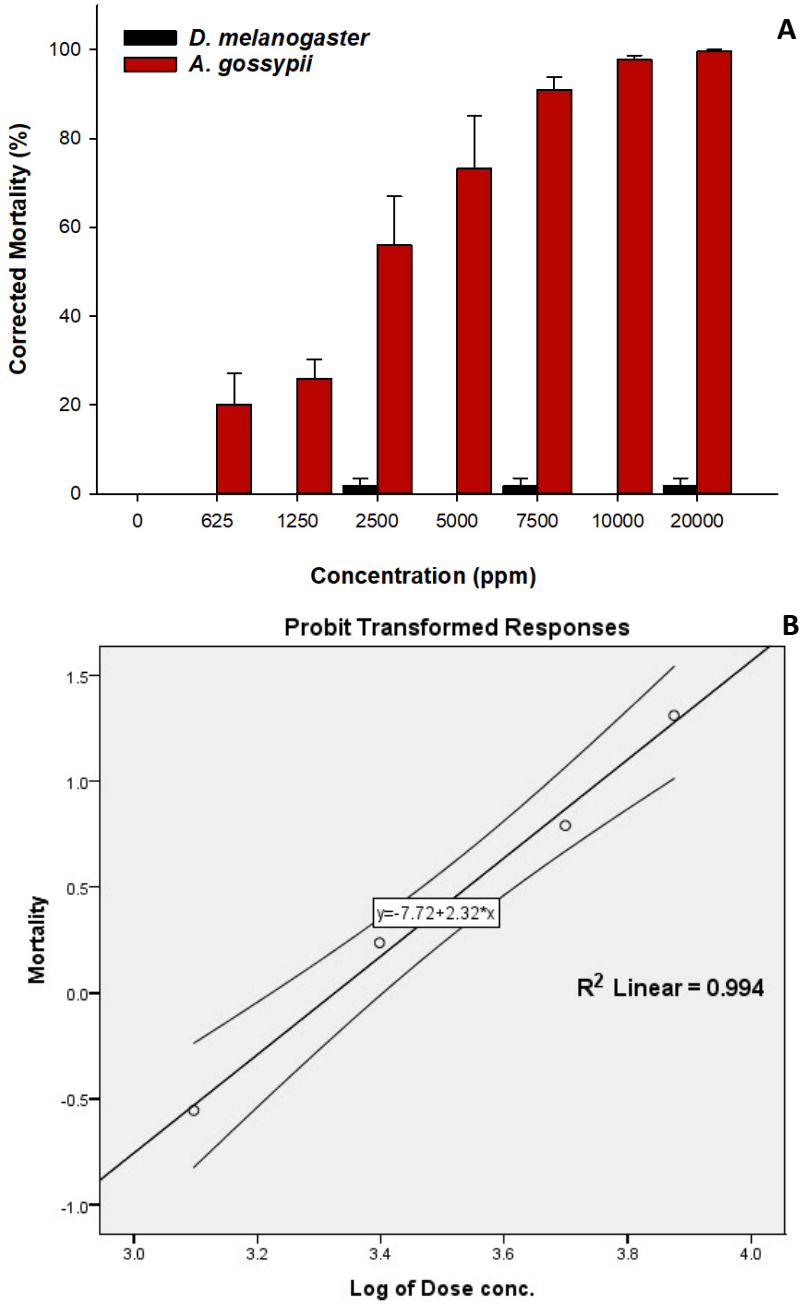


Figure 4-2 Mortality of *A.gossypii* and *D. melanogaster* in response to extract 68N.M. at 24HAT

(**A**) Dose-mortality response of *A. gossypii* and *D. melanogaster* to extract 68N.M at 24 HAT (**B**) Probit analysis graph and regression equation (with 95% CL) showing dose-mortality response of *A. gossypii* to extract 68N.M, n=1282 individuals.

Figure 4-3 Visual assessment of *A. gossypii* response to 68N.M.

(**A**) Control after spraying with 200 ppm Triton-X100™, 24 HAT (**B**) Brown, dehydrated aphids at 24 HAT with 68N.M 2.0% w/v (**C**) Changes in cuticle colour and texture, collapse of front legs at 1 HAT with 2.0% w/v 68N.M (**D**) Colour changes and mortality at 1 HAT with 2.0% w/v 68N.M (**E**) Haemorrhaging aphid at 1 HAT with 2.0% w/v 68N.M (**F**) the same aphid at 24 HAT.

4.3. Self-contamination bioassays

4.3.1. Methods

Self-contamination bioassays, utilising dry, fresh residues on glass, were used to assess the MOAs of the selected extracts and known botanical insecticides in regard to their fumigant effects, and meta-tarsal mode of entry efficacy. Initially, 50 mL glass Schott bottles were selected for bioassays of extract 33P, 68N, 69N, 69P, due to the very small quantities of extract available from the foundation project. For each 50 ml bottle assay, extracts were weighed into clean vials and diluted with acetone to make 10.0% w/v stock solutions; 10 µl of each extract was then pipetted into each bottle with an additional 0.5 ml of acetone to aid distribution. The bottles were then sealed and rotated to evenly coat all surfaces. Once the internal surface of the lid had been coated many times, the lids were half-opened to allow the acetone to evaporate off. The bottles were then continuously rotated until all surfaces had thoroughly dried (for approx. 2 h) providing a uniform coating of the test extracts. The internal surface area of the bottle was calculated to be 84 cm^2, giving an a.i of 10 $\mu g/cm^2$ at 1.0% w/v. Due to the volume of extract needed to coat these bottles, only two genuinely independent replicates were able to be obtained.

The initial set of self-contamination bioassays utilised four known plant-derived insecticides and acted as a proof of concept for the bottle method. The insecticides chosen for this experiment were Qcide™, tasmanone (both the natural extract and synthetic nature identical) and flavesone at 1.0% v/v concentrations. I compared the results to an acetone only negative control.

In a second set of bioassays, I assessed the efficacy of non-polar and polar fractions of the most interesting candidate extract 69 (69N and 69P). These were assessed at concentrations of 1.0%, 0.1%, 0.01% and 0.001% w/v. In addition to the negative control, two positive controls were used; pyrethrum at 1.0% w/v, and Qcide at 0.1% w/v (this latter concentration was selected following the initial proof of concept bioassay results).

In a third set of bioassays, I compared the activity of three selected extracts 68N, 72N, 82N, that had shown high efficacy in the foundation project bioassays against at least two out of three invertebrates tested: *T. urticae*, *A. gossypii* and *H. armigera*. In addition to the acetone only negative control, I included Pyrethrum 1.0% v/v as a positive control. The purpose of this study was to assess the efficacy of these extracts against each other, and also their relative knockdown activity.

An additional self-contamination bioassay assessing 82N was conducted, when larger quantities of this extract was available in 2018. In this case, 250 ml Schott bottles were used; providing the flies a larger space for flight and a greater volume of air. The application of treatments was essentially the same as described for the smaller bottles, except that aliquots of the solid extract were measured out in 31.0 mg quantities into two clean beakers and dissolved completely in 1550 µl of ethyl acetate (cat # 319902 Sigma-Aldrich, Castle Hill, NSW) to give a final concentration of 2.0% w/v, or an active ingredient distribution of 0.1 mg/cm^2 over the internal surface area of 310 cm^2. Ethyl acetate is a commonly used pesticide solvent, equivalent to water-miscible solvents for both polar and non-polar compounds (Mol et al. 2003).

In all cases, used bottles were thoroughly washed, rinsed with tap water then double rinsed with acetone and dried overnight in a laboratory oven set to its highest setting prior to reuse. Once the bottles had cooled, 10 flies were introduced into three randomly selected, washed bottles, and assessed for 1 h for any signs of toxicity, to ensure that no residual contamination remained after the cleaning process.

As previously mentioned in Chapter 2: General Materials and Methods, *D. melanogaster* (mixed sex adults) were used for all self-contamination bioassays. Flies that had been pooled from six different cultures were introduced voluntarily to the bottles without the use of anaesthetics. The number of flies entering the test bottles before it was stoppered varied between 11-25 per bottle. The period of time of the experiments varied depending on rapidity of toxicity. For the first proof of concept study it was 1h, for the second study with 68N and 68P, it was 30 min; for the two other studies in was 24 h. For the latter two studies, flies were monitored continuously for the first 3 h, and then hourly for an additional 9 h, then once again

at 24 HAT. Results are primarily presented for 3, 12 and 24 HAT. From the fourth bioassay using extract 82N 2.0% w/v, flies were collected at 24 HAT for further RNA extraction and divided into categories based on either their healthy survival, visual sickness (e.g. spasms, intoxication) or mortality (see Chapter 6). Furthermore, the fourth assay described above used unsexed *D. melanogaster*, as I intended to extract their RNA is differentially regulated in response to mating (Mack et al. 2006). However, I conducted a short investigation to determine whether the sex of randomly selected flies may have impacted these outcomes, since male flies are slightly smaller than females. The methodology was the same as previously described.

Data from the bioassays in Chapter 4 were initially recorded, graphed and analysed for normal distribution in Microsoft® Excel® for Windows (Plus 16). Normal distribution was assessed using a Shapiro-Wilk's test ($p>0.05$) with skewness, as well as being visually determined using Q-Q plots. Corrected mortality was calculated according to Abbott's formula (Abbott 1925). Some figures were later graphed using SigmaPlot (Ver. 12.0 - 14.0, Systat Software Inc., San Jose, CA, USA).

4.3.2. Results

With the initial 50 ml bottle assays I found that all the known botanical insecticides tested were efficacious against adult *D. melanogaster* and that rapidity of knockdown was able to be tested using this method (Figure 4-5A). Although the error bars for Qcide™ are quite large, it appears that there is not a significant difference between the insecticides tested; therefore, for the following tests we continued with Qcide™ but at a reduced concentration of 0.1% v/v.

In the 50 ml bottle assay, serial dilutions of 1.0%, 0.1%, 0.01% and 0.001% w/v concentrations of extract 69N resulted in a dose-dependent fly mortality (Figure 4-5B) but there was no mortality recorded for any concentration of 69P (Figure 4-5C). Extract 69N 1.0% w/v was as efficacious as 1.0% w/v pyrethrum and 0.1% w/v Qcide™. Furthermore, 69N was rapid-acting, showing the greatest knockdown effect of any of the insecticides tested, with 100% mortality in the first 5 min. Due to the limited quantities available, several other potentially highly efficacious extracts could not be tested for this characteristic.

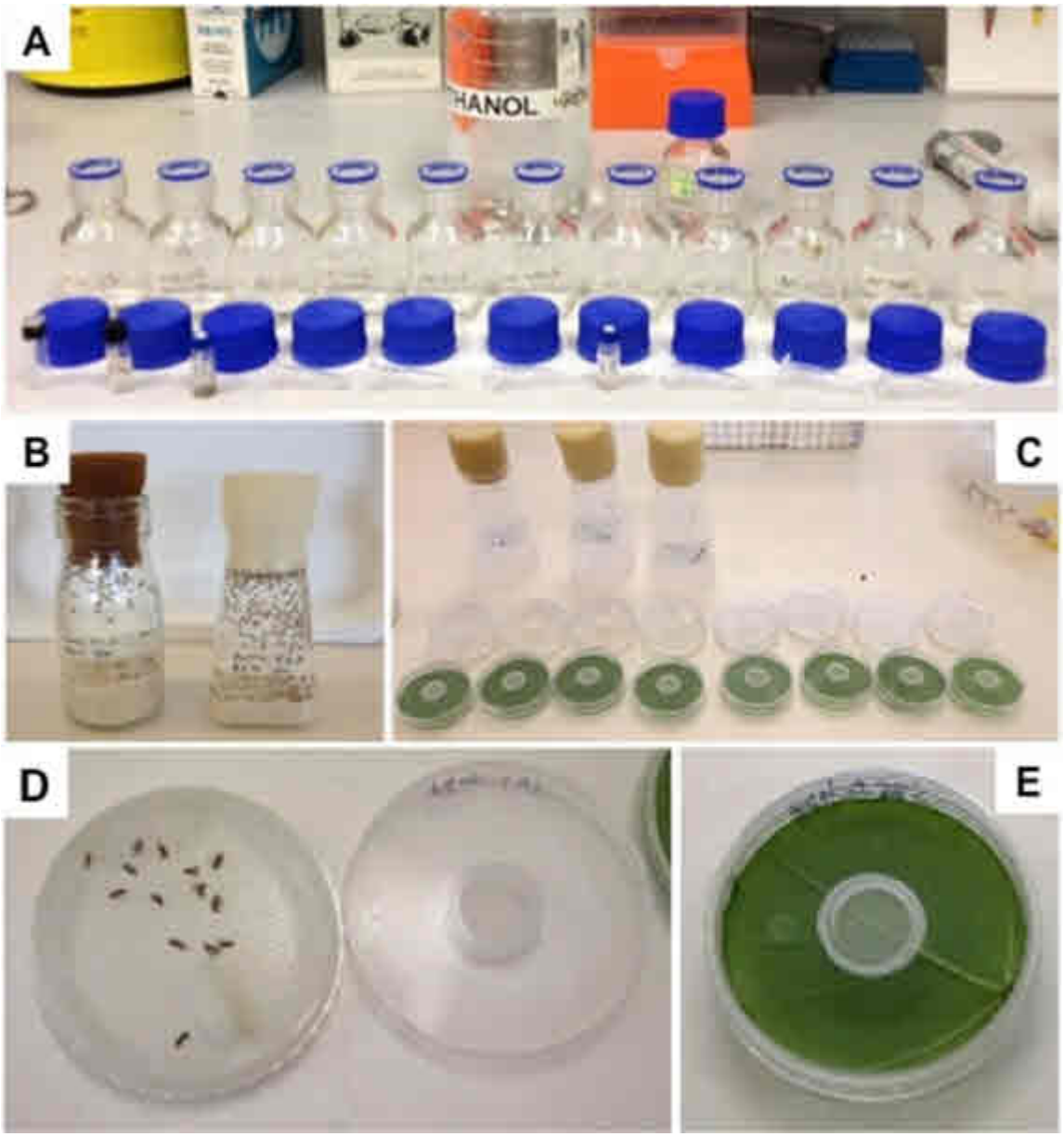


Figure 4-4 Arthropod bioassay setup.

(**A**) Self-contamination assays: Schott (50 ml) bottles and extracts (**B**) *D. melanogaster* egg evacuation of adult flies and (**C**) pre-sorted, newly emerged and unmated females *D. melanogaster* in culture bottles before Potter spray tower bioassay, *A. gossypii* bioassay chambers in foreground. (**D**) *D. melanogaster* flies immediately after treatment by Potter spray tower (seen with media pellet for food source) (**E**) *A. gossypii* chamber with insects transferred and feeding, ready for Potter spray tower treatment.

With regard to the other extracts tested, namely 68N, 72N and 82N, they did not display rapid mortality in comparison to the positive control pyrethrum 1.0% v/v (Figure 4-5D). Extract 82N 1.0% w/v showed the slowest effect, with no mortality recorded within the first 5 h and only 40% mortality at 24 HAT. Extract 68N 1.0% w/v also was relatively slow acting with the first mortality not recorded until 4 HAT, although the mortality by 24 HAT was 54.5%. Extract 72N 1.0% w/v was the fastest acting extract with some mortality within 2 HAT (8.3%) but only reached 33.3% mortality at 5 HAT; however, this extract did have the highest mortality (66.7%) by the end of the experiment (24 HAT).

The results from the 250 ml bottle bioassay showed that extract 82N at 2.0% w/v (Figure 4-5E) was not highly toxic to *D. melanogaster*. In fact, there was no mortality recorded at 3 HAT, 12% mortality at 12 HAT and only 58.9% at 24 HAT. This contrasted with the two positive controls (pyrethrum and Qcide at 2.0% v/v) that recorded 100% mortality at 3 HAT. While there was no clear knockdown activity in the 82N treatment, the level of mortality at 24 HAT and the fact that a number of survivors showed signs of intoxication, suggested this extract may be slow acting. As a result, apparently healthy, sick and dead individuals were collected for later gene expression analysis (see Chapter 6) to evaluate their resistance potential.

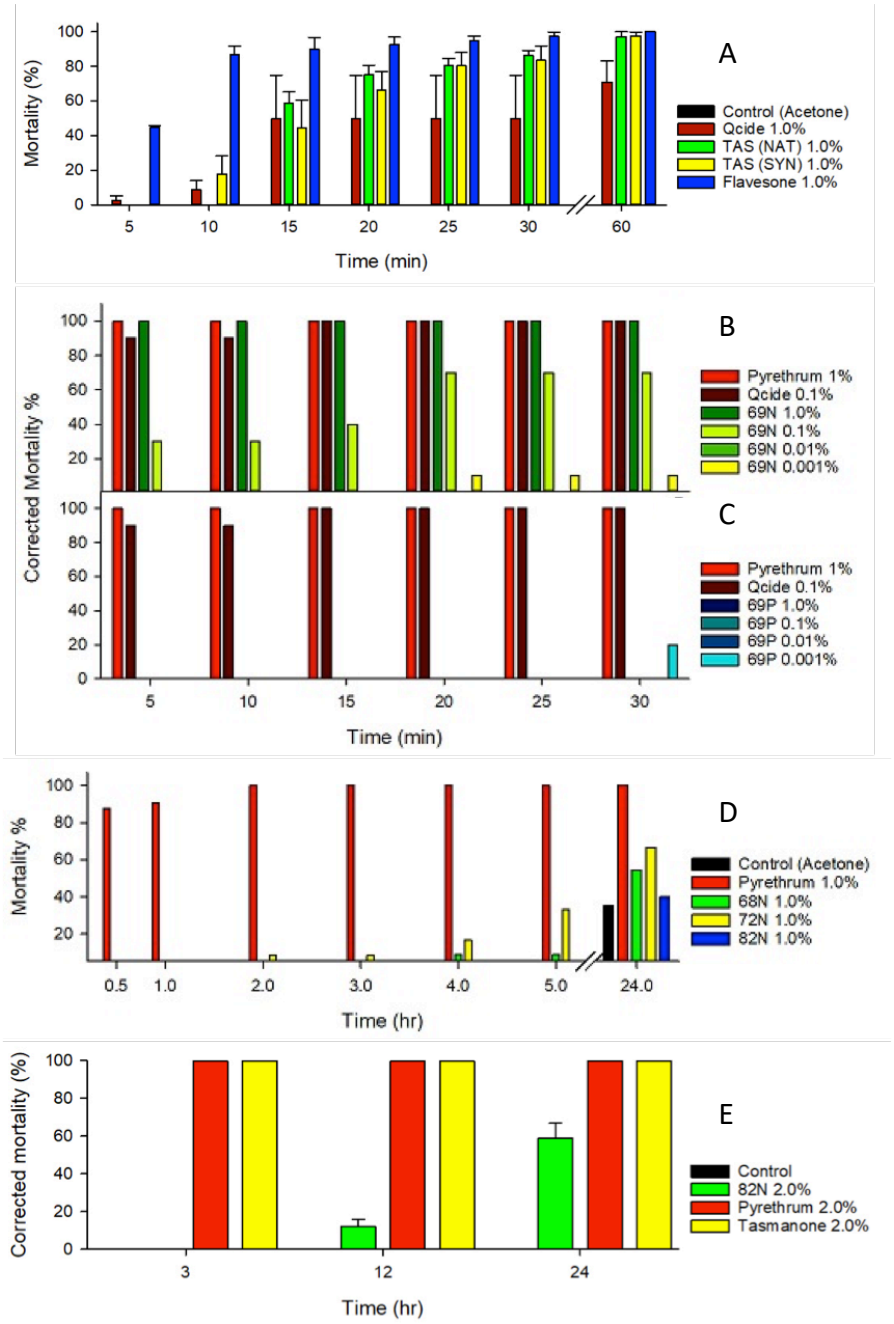


Figure 4-5 Self-contamination bioassay results for *D. melanogaster*

(**A**) *D. melanogaster* response to a selection of positive control insecticides via 50-ml self-contamination bioassay (**B**) *D. melanogaster* knock-down response to non-polar, 69N & (**C**) polar, 69P in serial dilutions (**D**) *D. melanogaster* knock-down response to superior extracts 68N, 72N and 82N (**E**) *D. melanogaster* response to 82N at 2.0% w/v via self-contamination bioassay compared to one negative control and two positive controls.

4.4. *D. melanogaster* development assays

4.4.1. Methods

An experiment was conducted to determine whether the selected plant extracts affected *D. melanogaster* egg hatching via their contact exposure to treated media, and if feeding on the contaminated media would affect subsequent emerging larval growth and development. Adult female *D. melanogaster* can retain eggs within their bodies if they are stressed due to factors such as lack of food, overcrowding or if they reject the laying substrate (Dubreuil et al. 1986; Qazi et al. 2010; Yang et al. 2008). For this reason, a culture of flies was transferred into new culture bottles with fresh medium for approximately 2 d prior to egg harvesting. The new medium was supplemented with additional yeast paste to "bulk-up" the females, encouraging them to produce more eggs. It is estimated that females reach peak egg laying approximated 72 h after the introduction of rich food (Piper et al. 2016). Flies were then released into clean medium-free bottles and inverted onto small pots containing fruit agar and a smear of yeast paste. The recipe used for fruit agar was modified from Featherstone et. al. (2009) and included 1 g agar in 50 ml of Milli-Q water, heated in a microwave until agar is dissolved and transparent, then add 2 teaspoons (approx. 10 g) of raspberry jam and 1 teaspoon (approx. 5 g) white sugar. Agar was poured into clean pots to set, then the agar was scored with a sharp instrument and a line of yeast paste was smeared perpendicular to the scorings. This provided additional food for the flies and a creviced humid surface for oviposition. After approximately 2 h the flies were removed and the eggs were collected with a wet, fine haired paintbrush. Twenty-five freshly laid eggs were transferred for washing in Insect Ringer's Solution before being placed onto treated *Drosophila* Instant Culture Medium in two, 12 well-plates, as housing. Treatments of 2 ml of extract 68N.M in serially diluted concentrations of, 1.0%, 0.5%, 0.25%, 0.125% and 0.0625% w/v were randomly applied to wells containing 0.5 g medium. All treatments were run in triplicate (i.e., 3 wells), with a negative milli-Q water control and 0.125% w/v pyrethrum as a positive control, and the plate lids were then sealed with breathable paper tape.

A second feeding bioassay was conducted following similar methodology as described above but using individual 30 ml glass McCartney bottles. Pre-weighed *Drosophila* Instant Culture

Medium was dosed with 2 ml of extracts 72P & 72N at 1.0% w/v, 82P at 0.125% w/v, 82N at 0.0625% w/v. These doses were chosen because the foundation project had reported high mortality in the test species at reduced concentrations, particularly of extract 82, in Potter spray tower bioassays. Triton-X 100 (200 ppm in water) was used as the negative control. Positive controls used were Qcide™ at 1.0% and 0.5% w/v and flavesone at 1.0% 0.5% and 0.125% w/v. Ten adult flies, 2 male and 8 female (to allow for normal mating and oviposition behaviour) were released into the bottles without anaesthetising. Flies were monitored continually for the first 2.5 h then recorded at 4, 6, 18 & 24 HAT. Due to limited quantities of the test extracts being available, only one replicate bottle of each treatment was run, and therefore there was no data analysis, although the results are presented in Table 4. 1.

4.4.2. Results

For the egg hatch and larval feeding investigation, the medium was checked after 2 d for egg hatch and larval activity. Numerous larvae were seen in every well, assuring that eggs were safely transferred but also were unaffected by contact with 68N.M. Larvae continued to grow and feed normally at each concentration, although fewer were initially observed in the pyrethrum 0.125% w/v treatment. By Day 14, no mortality was recorded in any 68N treatment concentration, with all eggs successfully hatching, and emerging larvae feeding normally, and pupating and emerging as normal, fertile adult flies. Therefore, the data are not graphed for presentation (although the results are included in Table 4.1) and flies were not harvested for further molecular work.

The results of the adult *D. melanogaster* feeding study are presented in Table 4.1. As previously stated, because of lack of replication, data were not analysed. Nevertheless, there are some clear trends from the results. The 72N extract showed similar, high toxicity to the two β-triketone positive controls, all resulting in 100% mortality within 24 HAT. While 72N extract initial knockdown activity appears to be quite rapid, the positive controls reached complete mortality much earlier than this extract. In comparison, extract 72P induced knockdown in up to 60% of flies, but by 24 HAT, all had recovered, suggesting they had the ability to detoxify the active constituents. There was no mortality recorded in the 82P and 82N treatments. However, these were applied at the lowest concentrations tested for any

treatment. The bioassay was unable to distinguish whether toxicity recorded was a result of stomach poisoning or metatarsal contact with the medium during feeding.

Table 4-1 *Drosophila melanogaster* feeding bioassay.

Time (h)/ Extract	Control 1	Control 2	72P 1.0%	72N 1.0%	82P 0.125%	82N 0.0625%	Qcide 1.0%	Qcide 0.5%	Flavesone 1.0%	Flavesone 0.5%	Flavesone 0.125%
1	0	0	1 Dead	4 Dead	0	0	2 Dead 2 Sick	0	4 Dead 1 Sick	0	0
1.5	0	0	2 Dead	5 Dead	0	0	7 Dead 3 Sick	3 Dead 7 Sick	10 Dead	5 Dead 3 Sick	0
2	0	0	2 Dead 3 Sick	5 Dead 3 Sick	0	0	7 Dead 3 Sick	6 Dead 4 Sick	10 Dead	6 Dead 2 Sick	1S
2.5	0	0	6 Dead	5 Dead 5 Sick	0	0	10 Dead	7 Dead 3 Sick	10 Dead	9 Dead 1 Sick	1 Dead 1 Sick
4	0	0	6 Dead	5 Dead 5 Sick	0	0	10 Dead	10 Dead	10 Dead	10 Dead	8 Dead 1 Sick
6	0	0	6 Dead	5 Dead 5 Sick	0	0	10 Dead	10 Dead	10 Dead	10 Dead	8 Dead 1 Sick
18	0	0	2 Dead	5 Dead 5 Sick	0	0	10 Dead	10 Dead	10 Dead	10 Dead	10 Dead
24	0	0	0	10	0	0	10 Dead	10 Dead	10 Dead	10 Dead	10 Dead

Note: Dead flies were assessed by lack of movement and sick flies were assessed when in unnatural positions but with wings or legs still moving.

4.5. Discussion

The work reported in this Chapter, found: 1). The non-polar fraction of *Podolepis jaceoides* (68N), which had not previously been reported to possess insecticidal activity, is a highly efficacious aphicide, but has no apparent detrimental effects on the fly *D. melanogaster.* This result suggests, along with the data generated from the foundation project (A. Basta, D. Nguyen, K. Beattie & R. Spooner-Hart, unpublished work) that this extract is not toxic to the lepidopteran *Helicoverpa armigera*, or the European honeybee *Apis mellifera* (Robert Spooner-Hart pers. comm., 2020), that this extract has a degree of specificity, sought after for use in IPM programs. 2.) Extracts identified as highly efficacious (namely 68N, 72N, 82N) when applied directly to arthropods via a Potter spray tower, did not necessarily produce similar results in self-contamination bottle bioassays with fresh dried residues. 3.) Extracts 68N, 72N and 82N, did not show rapid knockdown characteristics or fumigant activity in *D. melanogaster*. However, activity which results in slower mortality may be the result of more complex physiological changes in the target arthropod species, so should not be discounted. Interestingly, 68P showed knockdown in *D. melanogaster*, but 100% recovery, suggesting that the fruit flies may possess a detoxification mechanism for the active constituents in this extract.

My findings from the Potter spray tower investigations confirmed the foundation project's initial screening (A. Basta, D. Nguyen, K. Beattie & R. Spooner-Hart, unpublished work) that extract 68N was highly efficacious against *A. gossypii*. While I used the same Potter tower method for these bioassays, I used an additional extract (68N.M). The plant *P. jaceoides* was grown at the Hawkesbury campus of WSU, and a simple methanol extract was produced (instead of the more complex solvent extraction process for production of the foundation project's test N and P extracts; see Chapter 2. Since the reliability of the presence of stable, active compounds is a major consideration for consistency in botanical pesticide activity and may be affected by a multitude of factors such as plant maturity, environmental and/or harvest conditions and extraction processes, prior to my bioassays extract 68N.M was tested to confirm its high efficacy. In addition, my results confirmed that 68N.M is a selective

insecticide, with high mortality of *A. gossypii*, yet low mortality in *D. melanogaster* (>2.0% 24 HAT).

Taken together with the foundation project results of 100% mortality against *A. gossypii* and 98.9% mortality against *T. urticae* at 24 HAT (Table 7-4, Appendix 3), these results suggest that extract 68N may have a greater selectivity towards soft-bodied species, such as *A. gossypii* and *T. urticae* rather than to other species such as *D. melanogaster,* and therefore, possibly conserving pollinators and beneficial species. Additional bioassays carried out by Prof. Spooner-hart, not presented here, also resulted in no mortality against honey bee (*Apis mellifera*) following Potter spray tower applications of 68N 1.0% w/v at 36 HAT (Robert Spooner-Hart pers. comm., 2020).

The foundation project found that many more non-polar extracts were more efficacious insecticides than polar fractions. I chose to compare the insecticidal effects of these two fractions of extract 69P and 69N in self-contamination studies to see the knock-down effects of non-polar (Figure 4-5B) verses polar (Figure 4-5C). The dose-dependent results confirmed that the non-polar fraction of extract 69 (69N) was much more efficacious than its polar fraction (69P). The results showed that differences in efficacy between polar and non-polar fractions in the direct application bioassay were comparable to differences in efficacy in the self-contamination bioassay, even though a different species and technique was used. The non-polar fraction of extract 69 (*Podocarpus elatus*) was more efficacious against *D. melanogaster*, 100% mortality within 5 min at 1.0% v/v, than its polar counterpart, which elicited very little effect over the 30 min testing period.

Three of the most efficacious extracts were then tested against *D. melanogaster* flies using pyrethrum 1.0% v/v as a positive control via the 50 ml bottle self-contamination bioassay method (Figure 4-5D). The results showed that none of the extracts, 68N, 72N or 82N at 1.0% w/v possessed knockdown potential similar to pyrethrum at the same concentration. However, this is a very high concentration of pyrethrum (Bucur et al. 2014; Jensen et al. 2006) and it may also be that pyrethrum has fumigant effects that the three extracts do not possess. It is also possible that the three extracts tested are not as broad-spectrum as pyrethrum, so

weren't efficacious against *D. melanogaster*. This would be an advantage as selective botanical pesticides are needed to bolster IPM strategies.

Self-contamination bioassays assessing *D. melanogaster* response to extract 82N at the higher concentration of 2.0% w/v resulted in 58.9% mortality at 24 HAT This was different to the foundation project's 1.0 % w/v Potter spray tower mortality results of *T. urticae* 94.74%, *A. gossypii* 90.43% and *H. armigera* 100%. There are a number of reasons for this inconsistency. First, the target species was different. Second, the bioassay was different (direct spray application with Potter spray tower vs tarsal contact with fresh dry residues) Considering the reduction in surface area for toxin entry, extract 82N could still warrant further investigation for its potential as a broad-spectrum biopesticide; however, since this test employed a two-fold concentration compared to the Potter tower and resulted in approximately half the mortality, I conclude that poisoning by fumigant activity can be ruled out as a mode of entry for 82N.

Finally, my self-contamination bioassays used a range of known botanical insecticides; Qcide™ 1.0% v/v (a steam-distilled essential oil containing the β-triketone tasmanone at ~ 80%) tasmanone 1.0% v/v in both its natural extract and synthetic nature identical and flavesone 1.0% v/v a nature identical plant extract, as positive -controls. Qcide™ 1.0% v/v resulted in 49.9% and 70.9% fly mortality at 30 min and 60 min, respectively (Figure 4-5A). This gave a greater range to assess these extracts of unknown constituents against rather than the faster acting tasmanone (natural and synthetic) and flavesone. Qcide™ was chosen as my preferred second positive control in conjunction with the previously selected positive control, pyrethrum extract (see Chapter 3). In other comparative studies, I used the solvent extracts of *E. cloeziana* (W11) to provide a more direct comparison to the other tested plant extracts, rather than the distilled oil, which was used in cellular investigations (see Chapter 5).

In summary, the studies in Chapter 4 resulted in the selection of several novel extracts that may have desirable characteristics for their development into new insecticides, especially for application against high priority pest for the Australian cotton industry. In order to better understand the true potential of these extracts, cellular and molecular studies are needed to elucidate their possible MOAs. These studies are presented in subsequent chapters.

Chapter 5: Cellular Responses to Novel Plant Extracts

5.1. Introduction

Detecting the insecticidal effects of raw botanical extracts can be difficult as their multiple constituents may not be known. In Addition, the effects seen in bioassay of the whole organism may not shed light on the type of toxicity induced at a cellular level. Furthermore, the toxicity may come from a plethora of possible injuries and counteracting repair mechanisms (Stamenković-Radak et al. 2016). No single test can detect every possible cause or influence on mortality, therefore in this chapter I have explored several extracts using the triangulation of methods described in Chapter 3, namely cell growth bioassays to evaluate cytotoxicity and growth inhibition, confocal microscopy to measure ROS production and electrophysiology using microelectrode ion flux estimation (MIFE) to determine ion flux across cell membranes. In this chapter, I primarily used *Drosophila* D.mel-S2 cells, in keeping with the previous bioassay which focused primarily on *D. melanogaster* flies (see Chapter 4). However, during the later stages, I also employed *Spodoptera* Sf9 cells to address the question of how an alternative cell line, more closely related to the key cotton pest, *H. armigera* might respond under the same botanical extract challenges (Table 7-4, Appendix 3).

Drosophila melanogaster is an excellent model organism because studies can move easily from the molecular level to the organelle, cellular, tissue and whole organism levels to give insight into a range of biological responses resulting from stress, defence and toxicity (Ferrandon 2013). Additionally, using commercial cell lines and performing cytotoxicity studies is widely accepted for drug screening, especially in anti-cancer drug discovery where many tumour cell lines from different organ sources are available (Lindhagen et al. 2008; Podolak et al. 2010; Zhang et al. 2007). Often cytotoxicity is determined by an end-point measurement such as an MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) or resazurin reductase assay, after a relatively long incubation period (24-96 h) depending on the cell line, to assess dose-response relationships (Iqbal et al. 2019; Mbaveng et al. 2019; Van Meerloo et al. 2011; Zhou et al. 2019). In my preliminary methods tests, I found these types of assays to be unsuitable due to both the length of time needed and

because they did not seem to account for hyperactivity, a known effect of some neurotoxic pesticides. Alternatively, tests that used kinetic, non-destructive measurements, such as cell growth and inhibitory calculations over shorter periods of time were preferable. This is in keeping with the NCI-60 one-dose screening methodology of the National Cancer Institute in the developmental therapeutics program (National Cancer Institute 2020).

Likewise, confocal scanning laser microscopy (CSLM) is widely applied in many fields of scientific discovery and diagnostics from biomedical, life science and agricultural disciplines. The relative simplicity of sample preparation combined with the ability to collect high quality images which exclude out of focus light interference, time lapsed images to visualise change or Z-stacking spatial filtering to build a three dimensional representation, which have all led to its extensive application in multiple research disciplines (Fish et al. 2009; Salih et al. 2011). Fluorescence spectroscopy is an extremely sensitive technique that is detects low fluorophore concentrations. In practical terms this means less laser power is needed to excite the fluorophore within the sample, less heat is created within the sample and therefore; photobleaching is avoided and an emission can be reliably detected without causing undue stress to the living sample (Salih et al. 2011). I used CSLM to measure changes in ROS in response to selected novel botanical extracts. Reactive oxygen species are reactive molecules containing oxygen, which act as both beneficial cell signalling messengers and detrimental free-radicals (Apel et al. 2004; Held 2014). The overproduction on ROS is known as oxidative stress (Lambeth et al. 2014). ROS molecules include dioxygen (O_2), superoxide anion ($\cdot O_2^-$), peroxide ($\cdot O_2^{-2}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and hydroxyl ion (OH^-) (Apel et al. 2004; Held 2014). Most ROS are created during mitochondrial electron transport and are responsive to many environmental, biological, and chemical stimuli in almost all aerobic species. The challenge in dealing with this double-edged sword becomes a balancing act between creating cell signalling and immune defence responses when needed and then removing them before deleterious events occur.

The MIFE technology has been extensively used in stress biological studies in plant, microbe and animals to provide electrophysiological insights to the cell physiological mechanisms (Chen et al. 2005; Chung et al. 2010; Shabala et al. 2006a; Shabala et al. 2013). The MIFE

technique allows a non-invasive measurement of sample, in this case a cluster of cells, using a selective ionophore for each ion flux chosen (Newman 2001). Although there is great interest in membrane transport and a growing body of work that links ROS with responses to xenobiotics and pesticide metabolism (Chen et al. 2011; Felton et al. 1995; Ferré et al. 2002; Henkler et al. 2010; Kalyanaraman et al. 2016; Li et al. 2007; Sharma et al. 2016), the majority of insecticidal MOA's have been classified as toxic via effect on either neurotoxicity, ion channel modulation or neuro-signalling interference (IRAC ; Scharf et al. 2008). Therefore, this electrophysiological method to investigate the effect of the novel botanical extracts on D.mel-S2 and Sf9 cell lines from this aspect.

In this chapter, I report the cell growth (inhibitory or stimulatory) effects of 25 extracts on D.mel-S2 cells with some additional testing using Sf9 cells (Table 5-1). One of the superior extracts, 68N that was further fractioned and its two major constituents, 68N.Fr42 and 68N.Fr44, were tested against Sf9. I then investigated the relative change in ROS elicited by a selection of the extracts, 10N, 61P, 100N, 68N, 72N. Last, I used the superior extracts, 68N, 72N and 82N to measure the change in K^+, Na^+ and Cl^- ion flux using D.mel-S2 and Sf9 cells.

The two hypotheses explored in this chapter were:

- In model insect cell lines, the over-production of ROS indicates the high level of cellular stress-response to individual extracts, while an under-production in ROS indicate extracts which are less toxic to insect cells or have antioxidant properties.
- In model insect cells lines, the increase of ion flux indicates high level of cellular toxicity to individual extracts with novel modes of action.

The results presented in this comprehensive chapter seek to answer our Objectives 2, 3 and 4 (see Chapter 1).

Table 5-1 Novel extracts used to elucidate cellular responses.

Rank	Extract	*T. urticae*	*A. gossypii*	*H. armigera*	Average efficacy
1	82N	100.00***	100.00***	100.00***	100.00
2	10N	100.00***	100.00***	85.19***	95.06
3	33N	79.53**	100.00***	100.00***	93.18
4	68N	98.86***	100.00***	57.81**	85.56
N/A	QCIDE	100.00	100.00	50.00	83.33
5	72N	28.57	100.00***	100.00***	76.19
6	28N	100.00***	47.06	70.00**	72.35
7	62N	83.72***	100.00***	0.00	61.24
8	55N	96.63***	81.22***	0.00	59.28
9	93N	100.00***	69.57**	0.00	56.52
10	83N	66.67**	94.15***	0.00	53.61
11	25N*	88.96***	60.00**	11.11	53.36
12	100N	86.67***	52.50**	14.29	51.15
13	61P	89.66***	56.50**	0.00	48.72
14 - Single	68P	38.57	100.00***	0.00	46.19
15 - Single	72P	13.33	16.67	100.00***	43.33
16 - Single	82P	49.87	73.68**	0.00	41.18
17 - Single	14N	86.25***	0.00	12.51	32.92
18 - Single	14P	7.37	0.00	55.01**	20.79
19 - Semio	15N	45.00	20.00	32.51	32.50
20 - Semio	9P	17.90	0.00	20.00	12.63
Low Effect	78N	19.05	31.58	0.00	16.88
Low Effect	79P	11.11	27.98	0.00	13.03
Negligible	78P	20.00	0.00	0.00	6.67
Negligible	36P	2.60	0.00	12.50	5.03
Negligible	54N	0.00	3.33	0.00	1.11

Note: Rank and bioassay efficacy at 48 HAT against multiple cotton pests. Very high efficacy was determined as ≥80% mortality and denoted by ***, high efficacy was determined as ≥50% but <80% mortality and denoted by **, extracts showing interesting, semiochemical non-fatal behaviour are denoted by *, moderate efficacy was determined as ≥35% but <50 mortality, low effect was determined as ≥20% but <35% mortality and negligible effect was determined as ≤20% mortality.

5.2. Cell cytotoxicity assays using spectrophotometry

5.2.1. Methods

The first cytotoxicity assay was performed to develop absorbance spectrometry wavelength optimisation protocols for D.mel-S2 cells. Healthy, log-phase cells, with >98.5% average viability were selected. One 96-well plate (83.3924.500 - hydrophobic growth surface for suspension cells, Sarstedt, Nümbrecht, Germany) consisting of 200 µl from 5 serially diluted cell concentrations, standards 1-5, with a starting concentration of 14.4×10^6 cell per ml, (n=9 biological replicates) were used to measure absorbance value to determine seeding density and optimal OD (nm) wavelength measurement (Figure 5-2A). The results were then used to prepare subsequent assays to assess cell growth or inhibition elicited by the botanical extracts via absorbance range (scale 0-1).

The second cytotoxicity assay used the wild-collected botanical extract *Eucalyptus cloeziana*, denoted W11N, to determine the treatment concentration needed for a closer comparison between the novel plant extract treatments applied to the cells. Based on literature, patents and previous research work, *E. cloeziana* essential oil has shown potent insecticidal action (May 2016; Spooner-Hart 2013; Spooner-Hart et al. 2002). In Chapter 4, I tested the efficacy of the essential oil (Qcide™) at 1.0% v/v against *D. melanogaster* adults in the whole organism bioassays as a positive control; in the current chapter, I applied the botanical extract as a positive control against D.mel-S2 cells. The extraction and extract preparation are described in Chapter 2: General Materials and Methods, with the exception that starting concentration of 2.0% w/v was made to give serial dilutions at final concentrations of 0.02%, 0.01%, 0.005% & 0.0025% w/v.

A third bioassay was conducted to determine the most appropriate treatment concentration of the novel plant extracts. A selection of extracts, 55N, 61P, 78P, 83N & 100N, was measured over 24 h against D.mel-S2 cells at seeding concentration of 1.0×10^6 cells/ml. From the foundation project's Potter spray tower bioassay, both efficacious and non-efficacious (48 HAT) extracts from both non-polar and polar fractions were selected. In whole organism bioassays, extract 55N 1.0% w/v showed mortality of 96.6% in *T. urticae* and 81.2% in *A.*

gossypii, extract 61P 1.0% w/v (89.7% and 56.5%), and 100N 1.0% w/v showed 86.7% and 52.50 % mortality in *T. urticae* and *A. gossypii*, respectively. Extract 83N was efficacious but in favour of the alternative species, with mortality of 66.7% in *T. urticae* and 94.2% in *A. gossypii*. Extract 78P was considered non-efficacious, with only 20.0% mortality in *T. urticae* and no mortality in *A. gossypii.* None of these extracts displayed significant toxicity to *H. armigera* (0.0%, 0.0%, 14.3%, 0.0% and 0.0%, respectively) (Table 7-4, Appendix 3).

For this range-finding experiment 10 mg of each extract was dissolved in 100 µl DMSO to make a 10.0% w/v stock solution (100 mg/ml). These were added to half the plate (Columns 2-6, n=9) in 10 µl volumes with 90 µl of cells to give a final concentration of 1.0% w/v (10 mg/ml). A 100 x dilution was then made and administered to the second half of the plate (Columns 8-12, n=9) to give a final concentration of 0.01% w/v (100µg/ml). All other parts of the procedure are as given in Chapter 2 General Methods and Materials. The higher concentration correlated with the Potter tower bioassays, whilst the lower concentration is a more reasonable concentration for future end use prospects and is in keeping with published insect bioassay plant extract formulations (Rasikari et al. 2005). DMSO 1.0% v/v was used as a negative control in Columns 1 & 7, providing a physical division between both concentrations and a means of assessing maximum cell growth potential.

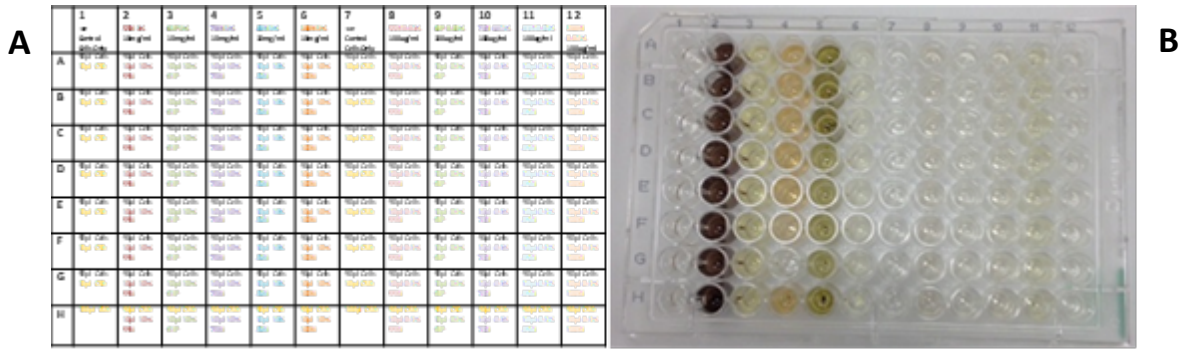


Figure 5-1 Extract concentration determination test.

(**A**) Dual concentration and multi-extract template for 96-well plate loading.
(**B**) 96-well plate showing the extract pigment considerations for absorbance measurements by spectrophotometers.

The fourth cytotoxicity bioassay test a range of novel extracts with varying efficacy (Table 5-1) using the established (from results in this next section) seeding concentration of 1.0×10^6 cells per ml at OD600 nm, detection wavelength and 0.01% w/v treatment concentrations on D.mel-S2 cells. Detailed methods and plating template is given in Chapter 2: Cell line bioassay techniques.

With the now established protocols, a fifth bioassay used the 3 premier extracts, 68N, 72N and 82N to compare non-polar verses polar cytotoxicity, following the established protocols, with the addition of extract W11N 0.01% w/v as the positive control.

A sixth bioassay was run to assess cytotoxic effects on Sf9 cells using a selection of extracts, 10N, 33N, 55N, 61P, 78N, 100N, W44N, using W11N 0.01% w/v and pyrethrum 0.005% v/v as two positive controls. This allowed comparison with D.mel-S2 cells, to determine if there was a significantly different effect on a model cell line of differing origin.

A seventh bioassay was run to investigate the unexpected results from the fifth assay, to explain the higher results of the polar fraction of extract 72 compared to the non-polar fractions I had been concentrating my effort on in other cellular investigations. This assay also incorporated serial dilutions of 72P to see if a dose-response could be established.

The eighth and last cytotoxicity bioassay used Sf9 cells to test the further fractions of extract 68N, which was separated into individual compounds by Dr. Karren Beattie, and of which several constituents were bioassayed via the Potter spray tower. Two fractions (coded 68N.Fr42 and 68N.Fr44) were found to be the most efficacious against cotton pests, and were subsequently tested for cytotoxicity using the previously described methods.

5.2.2. Results

During my procedures to determine experimental parameters and optimisation, the results indicated that at higher seeding concentrations there is a greater range between values captured at each OD wavelength (Figure 5-2A). This may be due to densities being at saturation point for the detector, or may be caused by cell morphology changes in relation to crowding; but in either case this would translate into less reliable data acquisition over time. However, as seeding density was reduced, so too, was variation between each OD wavelength. Concentrations of 1.6×10^6 cells/ml (Standard 3) and 0.5×10^6 cells/ml (Standard 4) resulted in similar consistency at each time point, between three different wavelengths, over the 48 h course of measurement. The least variation between nm at each time point, for example, 20 h at 450, 550 and 650 nm was seen in Standard 4. Starting OD values at 0 min for these two concentrations fell within 0.16 ± 0.013 SEM and 0.06 ± 0.005 SEM, giving a large range of OD values for potential increases without noteworthy variation between OD nm values. In standards 3 & 4 after 48 h growth, OD values still only reached 0.48 ± 0.015 SEM and 0.37 ± 0.008 SEM, respectively. From these results, it was decided that OD600 nm was a reliable detection wavelength and that a seeding density between 1.6×10^6 and 0.5×10^6 cells/ml would allow for detection of either stimulated or inhibited growth, therefore future plates were seeded at 1.0×10^6 cells/ml. Whilst a 48 h measurement duration was considered useful during the optimisation process, this extended duration was not needed to capture cellular changes. For the benefit of increasing throughput and reducing plate evaporation influences, shorter durations were used going forward.

The cytotoxicity of extract W11N to D.mel-S2 cells was determined by the cell growth inhibition compared to the negative control (DMSO 1.0% v/v), which was also used as the background diluent for each serial dilution. The final concentration of 0.02% w/v inhibited growth by 69.5% and caused 40% mortality as measured in Trypan blue live/dead cell counts at 4 h, 0.01% w/v inhibited growth by 35.7%, and 0.005% w/v inhibited growth by 10.3% (Figure 5-2B). The lowest concentration tested, 0.0025% w/v, did not inhibit growth and was therefore disregarded as a potential treatment concentration. This experiment also included a “cells only” negative control which showed the DMSO 1.0% v/v solvent-only negative control did not adversely affect cell growth (Figure 5-2B).

In order to determine the optimal extract concentration to be used, the third bioassay tested the relative growth percentage of D.mel-S2 cells when challenged with 5 extracts ranging in efficacy (Table 7-4, Appendix 3) 55N, 61P, 78P, 83N and 100N at two concentrations, 1.0% and 0.01% w/v. D.mel-S2 cell growth response at the higher concentration of 1.0% w/v (Figure 5-2C) resulted in a lack of differentiation between extracts, with the exception of 100N, Row 6, the lightest pigment fraction (Figure 5-1B). The internal control extract (61P) was also unreliable when dosed at 1.0% w/v, with absorbance at 24 HAT measured at -14% from its starting value. Extract 83N recorded no significant change (1% reduction) whilst extract 100N increased initially by 71% at 3 HAT; however, little change was detected over the proceeding hours (viz., 78% at 24 HAT). At the lower concentration of 0.01% w/v (Figure 5-2D) growth of extract-treated cells compared to the control resulted in significant inhibition, but with individualised responses. The internal control 61P this time performed as expected, reducing the growth by approximately 50%. Extracts 78P and 100N inhibited cells to a similar degree, 57.4% and 58.7% respectively, over 24 h but with different initial growth patterns in the first 0-6 h, at 64.0% and 50.0%, respectively. Extract 83N, previously undeterminable in at higher concentration, elicited the greatest inhibition at a steady rate over the course of the experiment, 63.5% at 6 h and 72.4% by 24 HAT. Since extract 100N had the least pigment (by visual assessment) and consistant growth inhibition over the 24 h period, I surmised that the unuseable 1.0% w/w results from the other extracts, 55N, 61P, 78P, 83N could be due to pigment artefacts. Therefore, an extract concentration of 0.01% w/v (100 µg/ml) was determined to be a more suitable test concentration going forward.

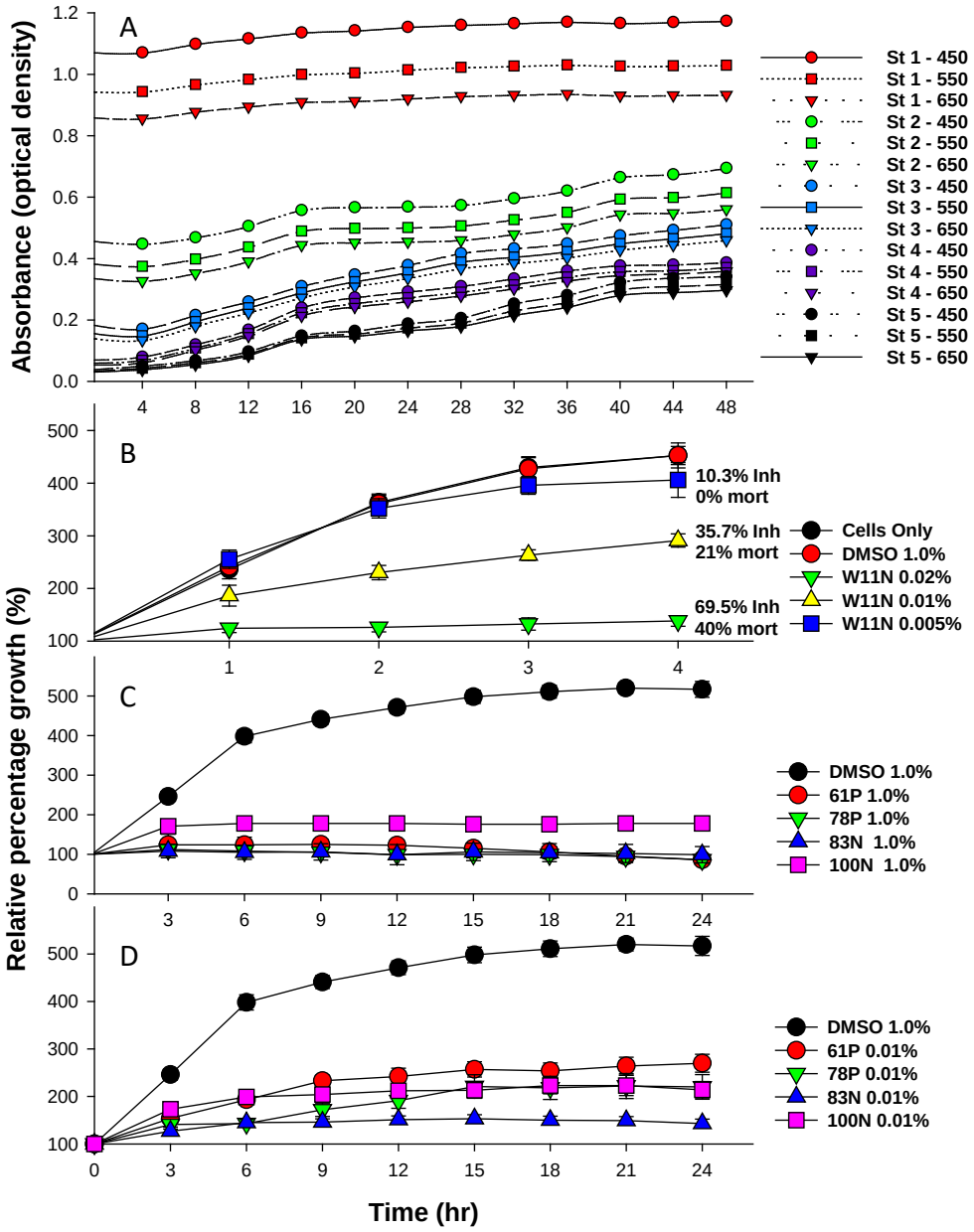


Figure 5-2 Experimental optimisations for absorbance spectrometry using D.mel-S2 cells.

(**A**) Standards 1 to 5 represent D.mel-S2 seeding concentrations in serial dilutions of 14.4, 4.8, 1.6, 0.5 and 0.1 × 10^6 cells/ml. Absorbance is plotted as the optical density output at 450 nm (circles), 550 nm (squares) and 650 nm (triangles) for each concentration. Despite data collection at 30 min intervals, 4-hourly data points have been plotted for ease of visualisation (**B**) D.mel-S2 growth curve responses to the positive control, W11N – *E. cloeziana* at a range of concentrations. Average cell mortality percentages as counted by haemocytometer are given along with average growth inhibition percentage in comparison to cell growth in the negative control. (**C**) D.mel-S2 relative growth compared to starting OD600 values, expressed as a percentage of growth over 24 h after treatment with a selection of extracts at 1.0% w/v and (**D**) 0.1% w/v determined the optimial extract treatment concentration.

The fourth cytotoxicity bioassay (with 20 mixed efficacy extracts, Table 5-1)) showed that some extracts with high to very high insecticidal activity against two to three of the target arthropod species resulted in moderate growth inhibition in D.mel-S2 cells; including extracts 10N (14.4%), 25N (31.2%), 28N (47.5%), 55N (23.3%), 62N (26.8%), 100N (17.9%) (Figure 5-3 A, B, E & F). Extract 14P which had a high efficacy against only *H. armigera* had essentially no effect on D.mel-S2 cells (6.1%); however, 14N which had very high efficacy against only *T. urticae* resulted in moderate growth inhibition (29.6%) (Figure 5-3E). Extracts 9P & 15N (Figure 5-3C) were selected because they induced interesting semiochemical-induced behavioural changes but without high mortality in the foundation project bioassays, stimulated growth of D.mel-S2 cells compared with the negative control in the cytotoxicity assays. Similarly, extracts 36P and 54N (with negligible mortality in bioassays) had higher cell growth than the negative control, especially in the early stages (0-2 h) (Figure 5-3D). The extract that did not follow this trend was 33N which showed high to very high efficacy in all three arthropods but essentially no inhibition (5.7%) of D.mel-S2 cells. Additionally, for two tests (Figure 5-3C & D), I reduced the concentration of pyrethrum to 0.0025% v/v, which increased absorbance in relation to increased cell growth and reduced inhibition compared to control, as expected. However, as this concentration did not suit the testing purposes aiming for mortality of approx. 50% to enable comparison with the novel extracts, I reverted to using pyrethrum 0.01% v/v for the rest of the D.mel-S2 assays.

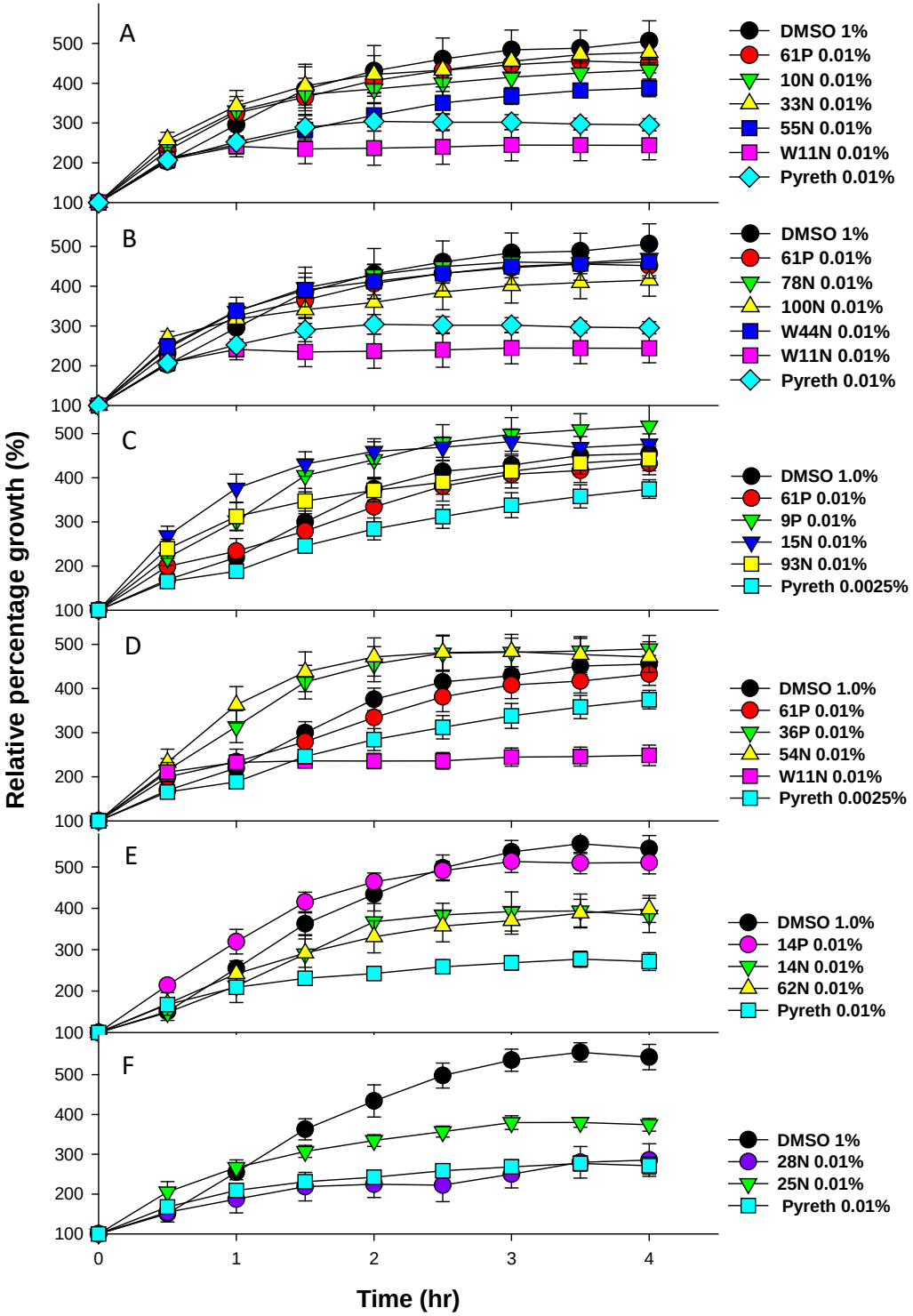


Figure 5-3 D.mel-S2 relative growth and cytotoxicity response to selected extracts.

Treatment effects on D.mel-S2 when challenged by extracts (**A**) 10N, 33N, 55N (**B**) 78N, 100N, W44N (**C**) 9P, 15P, 93N, (**D**) 36P, 54N. (**E**) 14P, 14N, 62N and (**F**) 25N, 28N, over a 4 h period. Extract 61P was used as an internal plate control. Extract W11N and/or pyrethrum were used as positive controls. Data points represent the mean ± SE bars (n=4, 36 wells).

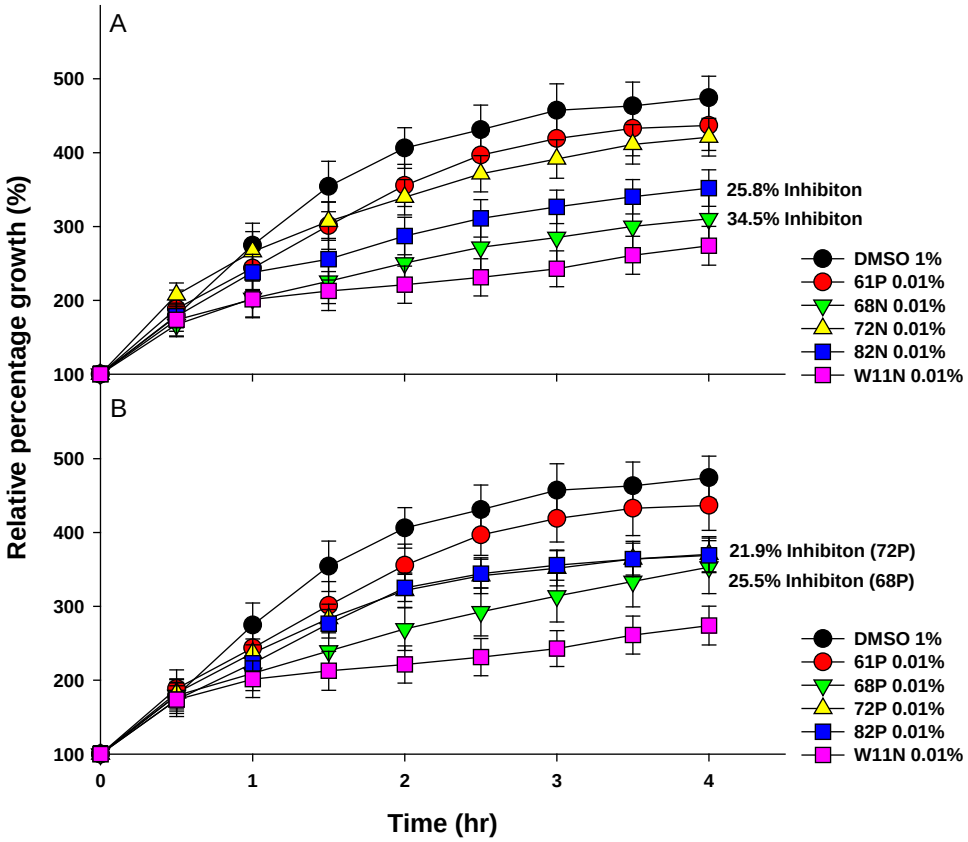


Figure 5-4 D.mel-S2 non-polar verses polar relative growth and cytotoxicity of top three candidates

Comparative non-polar and polar extract effects of the top three extracts on D.mel-S2 cells, (A) 68N, 72N & 82N (B) 68P, 72P, 82P. All measurements were recorded over 4 h against the negative control DMSO 1.0% v/v and the positive control W11N 0.01% w/v. Data points are means ± SE (n=3, 18 wells).

The fifth cytotoxicity bioassay of non-polar and polar fractions of the three premier extracts showed that 68N (34.5%) performed better than 68P (25.2%), consistent with the whole organism bioassays; 72N (11.2%) underperformed compared to its counterpart 72P (21.9%) in opposition to the whole organism bioassay; and extract 82 was quite similar (82N; 25.8%, 82P; 22.2%) (Figure 5-4). A major outcome was that the ranking of extracts from lowest (72N) to highest (68N) with regard to cell inhibition was the same for both non-polar and polar fractions.

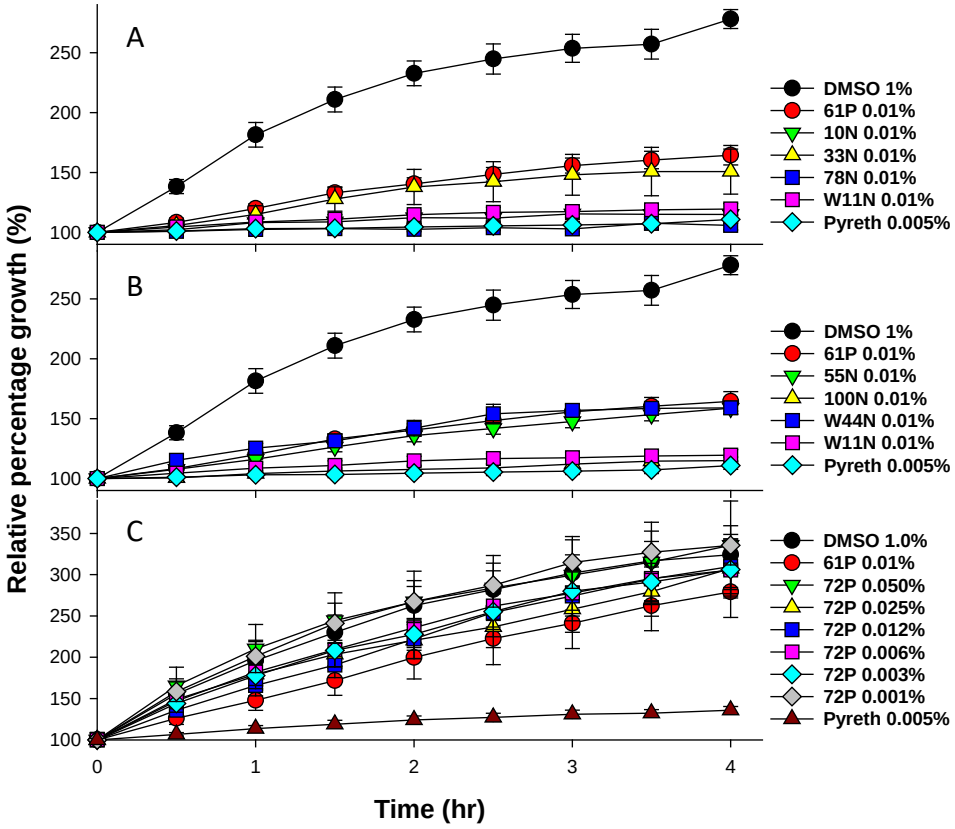


Figure 5-5 Sf9 relative growth and cytotoxicity response to selected extracts

Treatment effects on D.mel-S2 when challenged by extracts **(A)** 10N, 33N, 78N (**B**) 55N, 100N, W44N over a 4 h period. (**C**) Extract 72P did not cause any significant cytotoxicity with 4 h results averaging 4.3% inhibition compared to control. Replicates include a negative control, DMSO 1.0% v/v, an internal standard extract 61P 0.01% w/v and two positive controls, extract W11N 0.01% w/v and pyrethrum 0.005% v/v. Data points are means ± SE (n=18).

The sixth cytotoxicity bioassay using Sf9 cells showed that all selected extracts elicited high levels of growth inhibition compared to the control; with the results: Extract 10N (58.7%), 33N (45.8%), 55N (42.9%), 78N (61.9%), 100N (58.6%) and W44N (57.0%) over a 4 h period (Figure 5-5A & B). Extract 72P was also selected for further investigation using Sf9 cells. However, during the Sf9 cytotoxicity tests, it did not result in significant cell growth inhibition compared to control with an average response of 2.3% inhibition over the six concentrations tested.

The eighth bioassay results showed that both fractions caused substantial cell inhibition compared to controls. However, the dose-response was not highly correlated at two-fold dilutions. The growth inhibition of fraction 68N.Fr44, 10'-oxo-8-methyl podopyrone (Figure 5-6B), was more pronounced than fraction 68N.Fr42, 10'-oxopodopyrone (Figure 5-6A), with 68N.Fr44 recording no detectable dose-response due to high inhibition from all concentrations, ranging from 67.9 to 71.2% inhibition compared to the control (DMSO 1.0% v/v). This translates to an average growth rate from starting values of only 9.2% in response to 68N.Fr44 treatment.

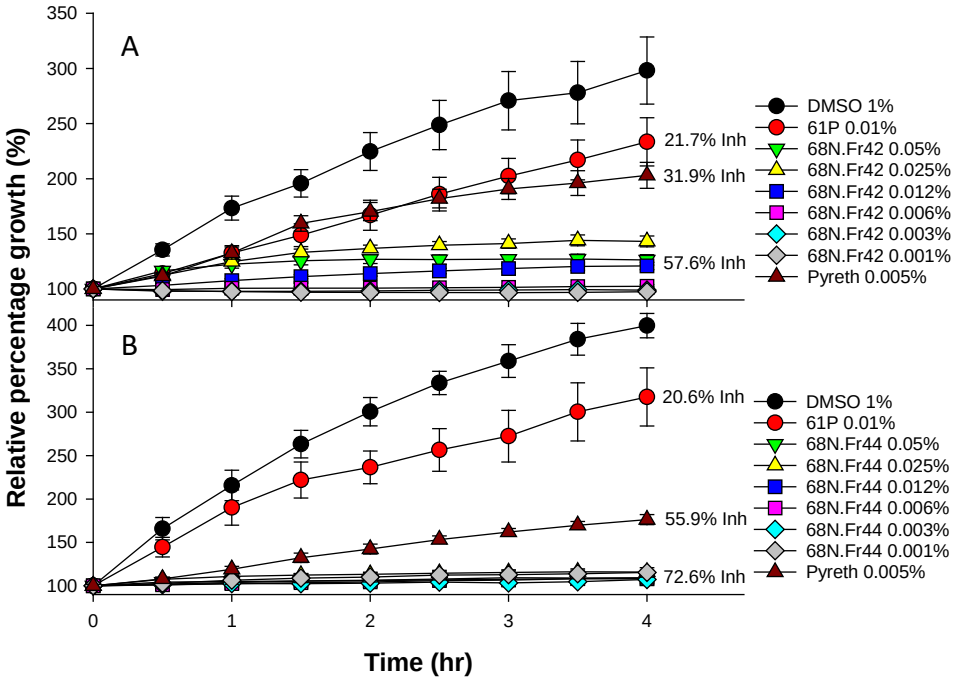


Figure 5-6 Sf9 relative growth and cytotoxicity response to single compounds identified from extract 68N

Dose-response growth and inhibition of Sf9 cells following exposure to (**A**) fraction 68N.Fr42 - 10'-oxopodopyrone and fraction 68N.Fr44 - 10'-oxo-8-methyl podopyrone (**B**) Data points are means ± SE (n=4, 24 wells).

5.3. ROS production as a stress response to novel extracts

5.3.1. Methods

The methods used for CSLM to measure changes in ROS production in response to extract challenges are described in detail in Chapter 3. The exception is in some cases where I have changed the concentration, and these can be found in the figure captions. Even though ROS production is a ubiquitous characteristic of cells under normal conditions, in D.mel-S2 cells, ROS production in all cases before treatment (0–15 min) was low and can be seen reducing slightly from the initial time (0 min) when focusing occurred. This is indicative of a sound methodology that reduced light stress from the onset and allowed light-stress artefacts to reduce rapidly. After treatment at 15 min, differences in ROS production in relation to starting values (0 min) can be seen over time.

The first cell fluorescence investigation used three extracts that showed high average insect mortality in Potter spray tower bioassays, 10N (95.0%), 68N (85.6%), 100N (51.2%), the internal plate control from the cell cytotoxicity bioassays 61P (48.72%), and one extract with negligible mortality 36P (5.0%). All extracts in this investigation were tested against D.mel-S2 cells at the higher concentration of 1.0% w/v. For details of methods, see Chapter 3. Briefly, cells from high viability communities were diluted to 1.5×10^6 cells ml^{-1} and stained with a ROS fluorescent dye (CM-H_2DCFDA). Using the confocal scanning laser technique, cells were photographed at 5 min intervals and images were processed using ImageJ (NIH, USA) to capture the relative ROS increase of individual cells. All extracts are graphed as fluorescence change relative to their own pre-treatment starting fluorescence and are also compared to cells exposed to the background DMSO diluent 1.0% v/v.

The second cell fluorescence investigation employed the same methods to test two of the prioritised extracts, 68N and 72N, and caffeic acid (SigmaAldrich, Australia), a surrogate analogue of the putative active constituent of 72N. However, I also used the Sf9 cell line and a lower final concentration of 0.01% w/v, consistent with other cellular bioassays.

5.3.2. Results

ROS fluorescence investigation using D.mel-S2 cells showed that each extract had a unique oxidation or free radical scavenging effect. Extract 10N 1.0% w/v showed a gradual increase in ROS production that represented a significant increase by 25 min post-treatment (Figure 5-7A). Extract 36P 1.0% w/v showed a sharp and immediate increase in ROS production which decreased after 15 min but remained elevated in comparison to the control (Figure 5-7B). Extracts 61P, 100N and 68N at 1.0% w/v (Figure 5-7C, D & E) did not elicit significant ROS responses; however, in 61P ROS fluorescence diminished over time, which was a unique trend compared to other extracts (Figure 5-7C).

Using Sf9 cells, extract 68N 0.01% w/v (Figure 5-8A) and extract 72N 0.01% w/v (Figure 5-8B) both showed a substantial, prolonged and significant ROS increase after treatment. In this investigation, I also tested a known antioxidant, caffeic acid 0.01% w/v (Figure 5-8C) because it was believed that it may have been an analogue of the active constituent of 72N. Although its influence on ROS fluorescence was not significantly different, a slight increase, followed by a sharp decrease is evident in the trend, and is in stark contrast to the novel extracts.

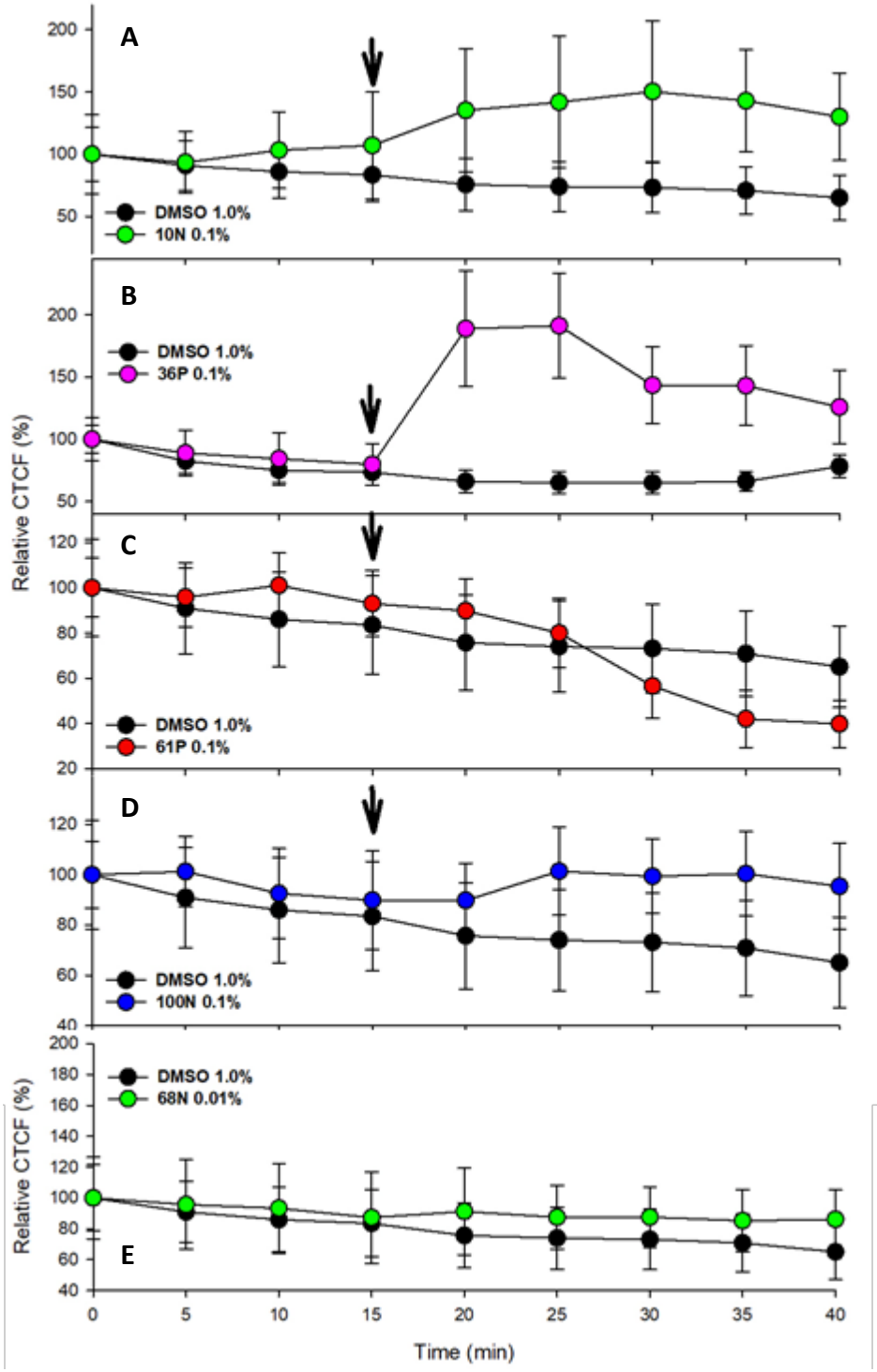


Figure 5-7 D.mel-S2 ROS response to 10N, 36P, 61P & 100N

Relative ROS fluorescence in D.mel-S2 cells from starting values (0 min), compared to control, DMSO 1.0% v/v, from (**A**) extract 10N (**B**) extract 36P (**C**) extract 61P, (**D**) extract 100N (**E**) extract 68N. All extracts are tested at 1.0% w/v. Data points are means of all cells ± SE. (n=12-25 cells per replicate with 2-4 biological replicates)

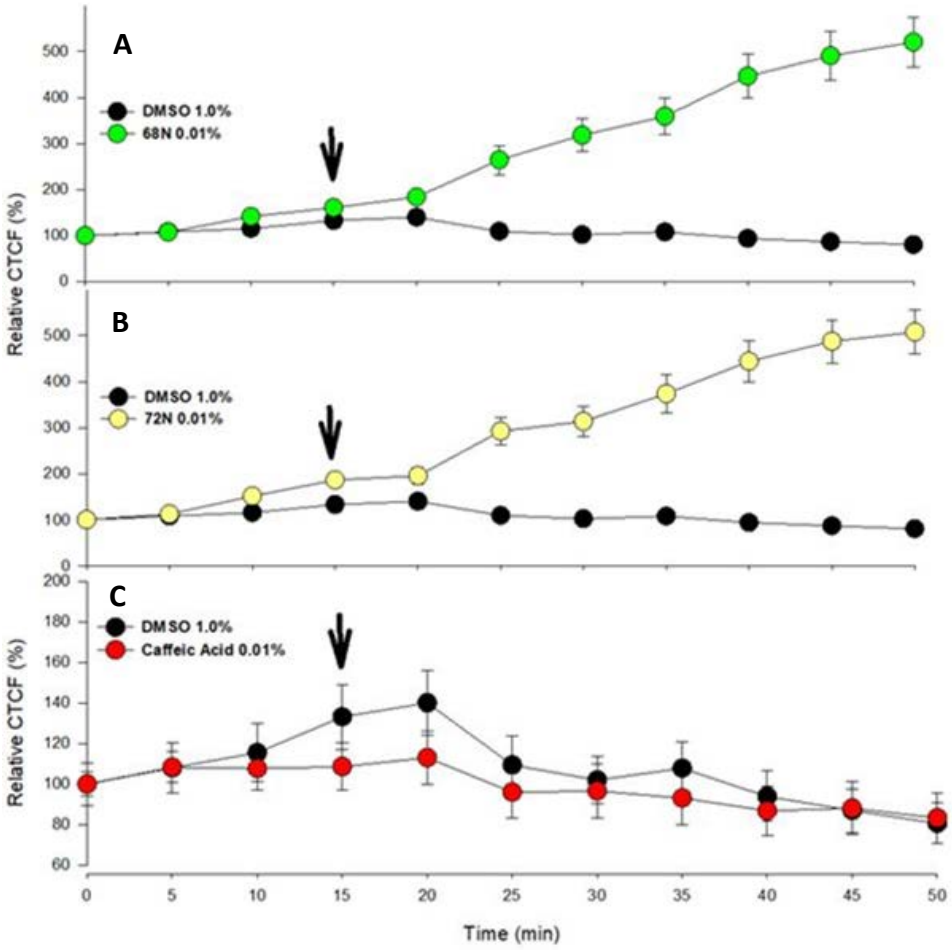


Figure 5-8 Sf9 ROS response to novel extracts, 68N & 72N and known antioxidant caffeic acid.

Relative ROS fluorescence in Sf9 cells from starting values (0 min), compared to control, DMSO 1.0% v/v, from (**A**) extract 68N (**B**) extract 72N (**C**) caffeic acid All extracts are tested at 0.01% w/v. Data points are means of all cells ± SE. (n=25 cells per replicate with 5-6 biological replicates)

5.4. Ion flux response in D.mel-S2 cells to novel extracts

Electrophysiological studies measure the ionic changes in organs, tissues or cells and are commonly used to elucidate the MOA of pesticides (Field et al. 2017; Kingan et al. 2012; Matsuda et al. 2001; Qiao et al. 2014; Zlotkin 1999), or to investigate responses to external stimuli in insects (Frazier et al. 1986; Hallem et al. 2006; Sakura et al. 2008). There are many techniques that can be used such as radiochemical transmitter-screening, single-unit extracellular recordings, voltage clamp, patch clamp, and MIFE. In this study, I chose to use the MIFE technique because it is non-invasive, flexible for use on multiple cells with the same uniform set-up and methodology, and recordings of a substantial time-frame can allow for both transient and steady-state ion flux responses.

In this study. I focused on the ion flux of K^+, Na^+ and Cl^- of the top three novel extracts that were prioritised from the original 20 extracts. In addition, two positive controls, pyrethrum and tasmanone, were used to compare ion flux in D.mel-S2. Pyrethrum was validated as an effective positive control for ion flux measurements in D.mel-S2 cells (see Chapter 3) and has been used throughout this PhD study. Tasmanone was selected because it has previously shown 100% mortality within 2 h in *D. melanogaster* bioassays conducted by Spooner-Hart (2013). However, tasmanone has neither been previously tested against D.mel-S2 cells nor investigated for its effect on ion flux using any electrophysiology techniques. Therefore, although this is not a crude plant extract, I thought it might bridge the gap between the available knowledge or at least provide a starting point, in terms of ion flux response.

5.4.1. Methods

The methods used for testing extracts were essentially as described in Chapter 3, where ion flux was measured when pyrethrins were used. Multiple replicates of each tested extract were combined to provide an average ion flux behaviour of the D.mel-S2 cells. Cells were selected randomly from the bank of cell populations and passages were grown over the course of the study and intentionally tested on different days. Net flux was measured in the control phase for approx. 14 min, then the treatment was administered and transient ion flux was measured for approx. 10 min (15.5-25 min). The period required to administration

treatments and stabilised readings (approx. 1.5 min) has been removed from MIFE traces and appears as a gap in the recordings. Following the transient phase, measurements were continued for a further 25 min to capture the stabilising phase. The data points (every 25 s) presented in each 40 min MIFE trace is an average of five data points (5 s each), error bars represent the average standard error (SE) of the five point's SE. Replicates of DMSO 1.0% v/v (K^+, n=4; Na^+, n=3; Cl^-, n=3), pyrethrum 0.01% v/v (all ions, n=14), tasmanone 0.01% v/v (all ions, n=7). Replicates of extracts are given in the figure captions. Where statistical significance is given, these have been analysed in Excel using the t-test: two-sample assuming unequal variances using the all raw MIFE data points (approx. 5 s intervals) for all replicated in the control, transient and stabilising phases. Significance is indicated by * $p \leq 0.05$. ** $p \leq 0.01$, *** $p \leq 0.001$.

5.4.2. Results

Extract 68N 0.01% w/v showed highly responsive flux changes in all ions, causing an immediate (within 5 min) and significant efflux of both K^+ (Figure 5-9A) and Na^+ (Figure 5-9C) in D.mel-S2 cells compared to the pre-treatment conditions, that although reduced over the stabilisation period (25-40 min) did not recover to pre-treatment values. This result is clearly seen in the relative response graphs (Figure 5-9B & D). Conversely, extract 68N had the opposite effect on Cl^- movement, which showed an immediate and significant influx in D.mel-S2 cells which was fatally non-recoverable, as seen by the levelling of movement (30-40 min) from the MIFE trace (Figure 5-9E). The long-lasting effect of Cl^- efflux are comparable to tasmanone 0.01 v/v and pyrethrum 0.01 v/v in the relative response graph (Figure 5-9F).

Extract 72N 0.01% w/v did not significantly affect the K^+ efflux (Figure 5-10A) in the transient response, however, in the stabilisation phase a slow leak was noted. The Na^+ efflux trace (Figure 5-10C) showed that there was a gradual move of Na^+ out of the cells in response to extract 72N, which was significantly different from pre-treatment conditions and was prolonged until the end of measurements (Figure 5-10D). The Cl^- efflux during measurements was reversed to an influx in all treatment cases including the negative control, suggesting that it was the DMSO diluent responsible for this movement. However, in the transient and

stabilising phases (Figure 5-10F), that influx was depressed by 72N 0.01% w/v in comparison to the relative percentage change of the controls.

Extract 82N at 0.01% w/v showed a highly responsive ion flux in D.mel-S2 cells. K^+ efflux (Figure 5-11A & B) that was significantly different in both the transient ($P<4.74E^{-75}$) and stabilisation ($P<5.44E^{-58}$) phases compared to the control, following treatment. There was also a significant reduction influx from the transient to the stabilisation phase, indicating that the cells were able to reduce significant K^+ losses, although not to a degree which would allow the cells to return the homeostasis of pre-treatment conditions, indicated by the significant difference between control and stabilisation ($P<5.44E^{-58}$). The Na^+ efflux (Figure 5-11C & D) was also significantly different in both transient ($P<6.83E^{-60}$) and stabilisation ($P<1.32E^{-89}$) phases compared to the control; however, they were not significantly different from each other, suggesting that the cells were unable to recover from the loss of Na^+ caused by 82N. Cl^- influx (Figure 5-11E & F) was significantly different between the control and both the transient ($P<2.54E^{-90}$) and stabilising ($P<7.95E^{-189}$) phases. There was a significant different between the transient and stabilisation phase ($P<0.002$), indicating that Cl^- influx slowed from the initial rapid and extreme influx in the transient phase, however this was still significantly different from the pre-treatment control ($P<5.88E^{-58}$), indicating that the cells may have experienced a fatal loss of Cl^- due to 82N.

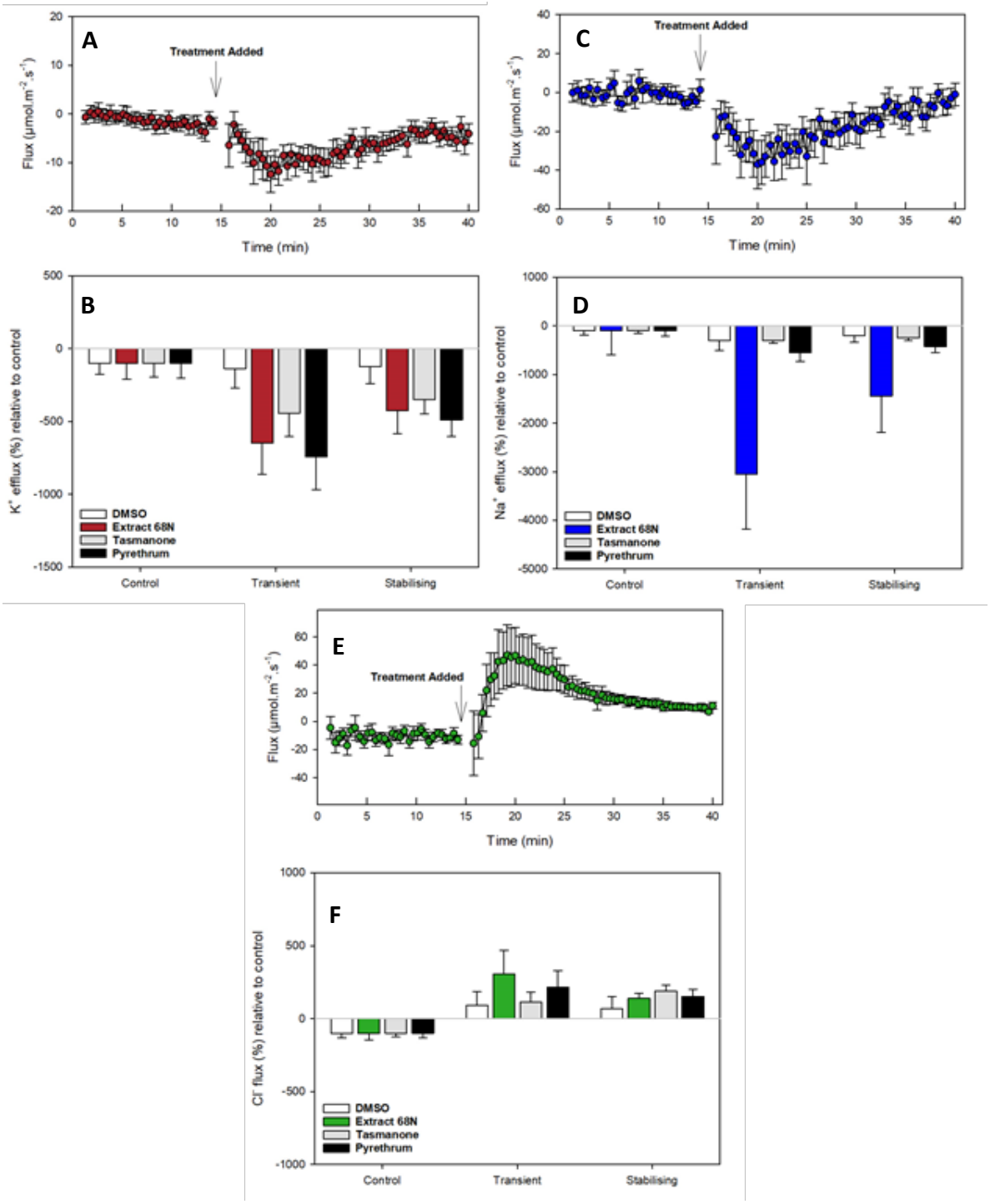


Figure 5-9 D.mel-S2 K^+ Flux response to 68N 0.01% w/v.

MIFE trace of (**A**) K^+ flux, (**C**) Na^+ flux and (**E**) Cl^- flux changes after treatment with extract 68N 0.01% w/v (K^+ & Na^+ n=6, Cl^- n=5), and relative percentage response of (**B**) K^+ flux, (**D**) Na^+ flux and (**F**) Cl^- flux in comparisons to DMSO 1.0% v/v, tasmanone 0.01% v/v and pyrethrum 0.01% v/v.

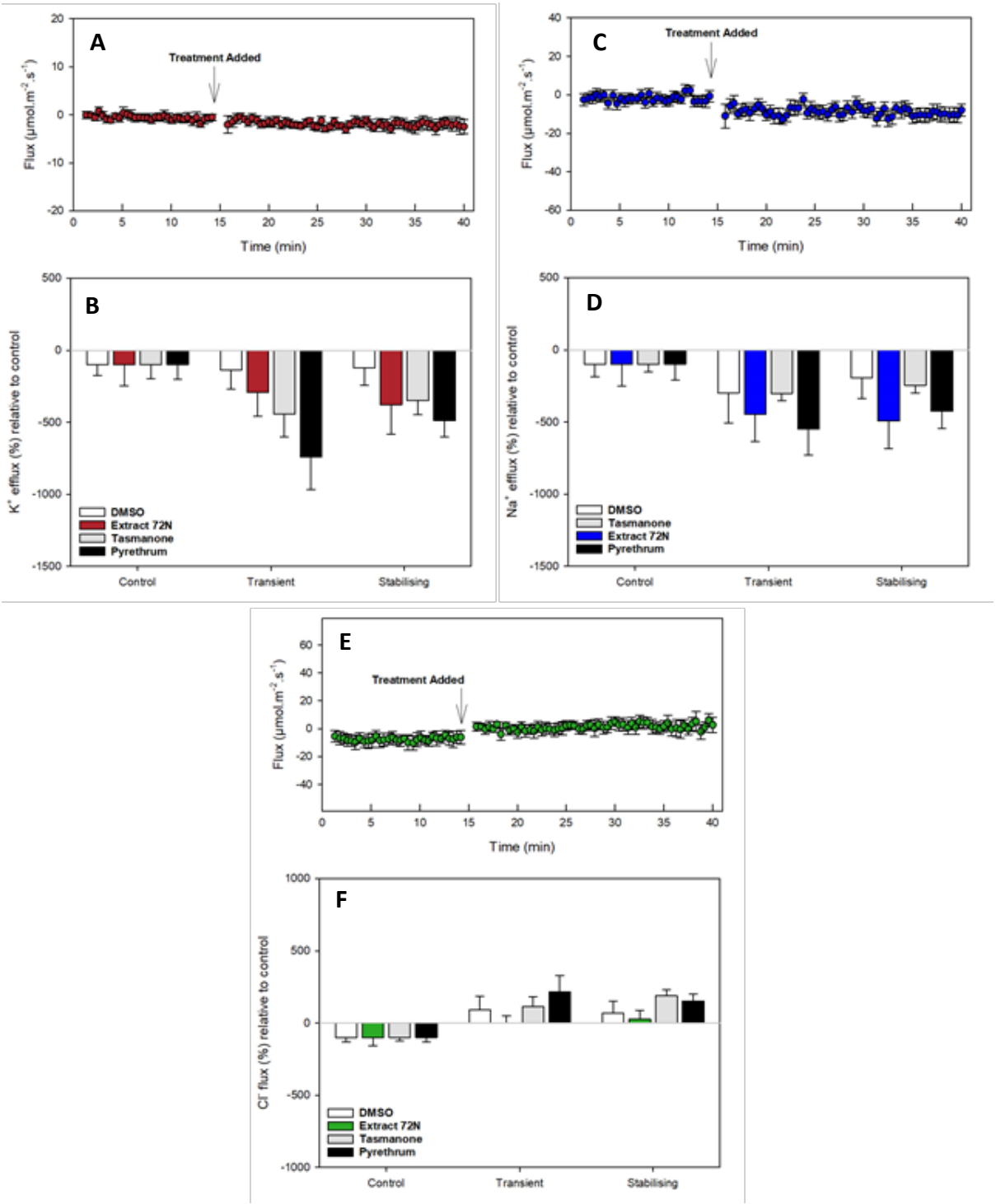


Figure 5-10 D.mel-S2 ion flux response to 72N 0.01% w/v.

MIFE trace of (**A**) K^+ flux, (**C**) Na^+ flux and (**E**) Cl^- flux changes after treatment with extract 72N 0.01% w/v (all ions n=10), and relative percentage response of (**B**) K^+ flux, (**D**) Na^+ flux and (**F**) Cl^- flux in comparisons to DMSO 1.0% v/v, tasmanone 0.01% v/v and pyrethrum 0.01% v/v.

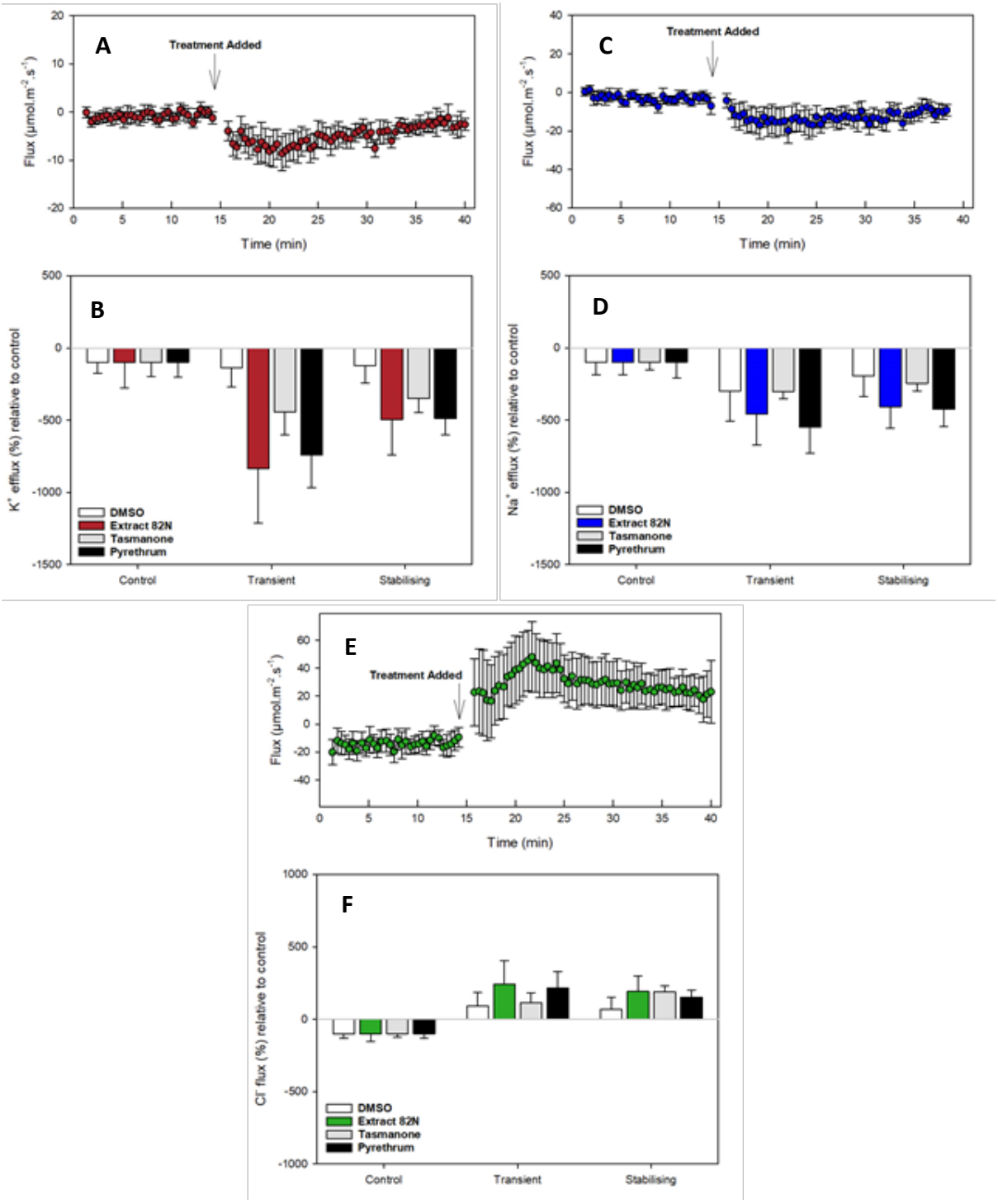


Figure 5-11 D.mel-S2 ion flux response to 82N 0.01% w/v.

MIFE trace of (**A**) K^+ flux, (**C**) Na^+ flux and (**E**) Cl^- flux changes after treatment with extract 82N 0.01% w/v (all ions n=7), and relative percentage response of (**B**) K^+ flux, (**D**) Na^+ flux and (**F**) Cl^- flux in comparisons to DMSO 1.0% v/v, tasmanone 0.01% v/v and pyrethrum 0.01% v/v.

5.5. Discussion

5.5.1. Cell spectrometry bioassay is a rapid method for identifying efficacy of novel botanical extracts

When working with a cell line, some growth parameters and test conditions need to be optimized in order to standardize replicates between different cell line passages and different extract treatments (Stevenson et al. 2016). Initial absorbance spectrometry optimization was carried out using the D.mel-S2 insect cell line at five culture concentrations, and three optical density (OD) wavelengths to assess the best seedling density and OD value. At the same time, I assessed the suitability of the suggested read time frame of 30 min and a 48 h duration of measurement (Figure 5-2A). I used a selection of extracts to evaluate the most appropriate treatment concentration. The results showed that the higher concentration used in the Potter spray tower bioassays (1.0% w/v) was either too cytotoxic to allow for discrimination between extracts or that the higher concentrations introduced excessive light scattering interference from extract pigments (Figure 5-1B). In terms of appropriate testing concentration, Rasikari et. al. (2005) reported high mortality (93%) of D.mel-S2 cells when tested against Lamiaceae plant extracts at 0.01% w/v (100 µg/ml) at 24 HAT, although mortality was measured by a different method and over a much longer time period. Rasikari et al. (2005) resolved to reduce the concentration to 0.001% w/v (10 µg/ml), which resulted in a modest 22% mortality at 24 HAT. From my cytotoxicty results using the absorbance spectrophotometry method, and in order to keep the higher cytotoxicity effects without losing essential, time sensitive data, I determined 0.01% w/v (100 µg/ml) to be an appropriate concentration based on 1.) the measurements being non-distructive and therefore allowing for cell counts to directly determine mortality rates, 2.) a higher concentration could allow for a shortened experimental time-frame, reducing the end point to 4 h rather than 24 h to improve efficiency, 3.) D.mel-S2 cell being identified from the literature as a more robust cell line than Sf9, therefore lower mortality rates were acceptable. My study comparing the same extracts assayed in both cell lines at the same concentration, confirmed, consistently higher cell growth inhibition in Sf9 than in D.mel-S2 cells (Figures 5-2, 5-3, 5-4, 5-5, and 5-6).

Interestingly, the particular extracts that were selected after eliciting non-fatal semiochemical behaviour in the Potter spray tower bioassay, actually stimulated D.mel-S2 cell growth. Since semiochemical behaviours are understood to be stimulated in response to olfactory detection (Ebrahim et al. 2015; Lin et al. 2007; Renou et al. 2000), it is curious that these extracts stimulated these cells. Further in-depth study will be required to test if the compounds in these extracts affect a greater range of target sites than currently thought. As expected, the two extracts with negligible efficacy in whole organism bioassays, had no significant effects on cells. One extract (33N) which had an average efficacy (Table 7-4, Appendix 3) across all three species tested in the whole organism bioassays of 93.2%, was ineffective on cells. Additionally, for two cytotoxicity tests, the concentration of pyrethrum was reduced to 0.0025% v/v. This reduction, as expected, increased absorbance in relation to increased cell growth and reduced inhibition compared to control. However, this did not suit our testing purposes as we aimed for a mortality rate of approx. 50% to allow for the broadest (stimulation of inhibition) comparison with novel active extracts and compounds. Therefore, I reverted to pyrethrum 0.01% for the rest of the D.mel-S2 assays, except for the more sensitive Sf9 cells, where 0.005% was used.

5.5.2. Cell lines influence the discovery of MOA of novel botanical extracts

Studies using *D. melanogaster* have been reported using a variety of plant extracts to explore a number of whole organism, cellular, metabolic and genetic responses including: chlorophyll inhibition of chromosomal mutations involving P450 enzymes (Negishi et al. 1997); dill, peppermint & pine essential oils' genotoxic effect on somatic mutation in *D. melanogaster* (Lazutka et al. 2001); whole grape extract antioxidant effect on locomotion and lifespan of Parkinson disease mutant flies (Long et al. 2009); and guava leaf essential oil toxicity by fumigation due to ROS production and other oxidative stress markers (Pinho et al. 2014). *Drosophila* flies and D.mel-S2 cells have also been employed to assess cytotoxicity, ROS production and DNA damage induced by the carbamate insecticide, methomyl (Guanggang et al. 2013) and the organophosphate pesticide, chlorpyrifos (Gupta et al. 2010). Although the model cell line D.mel-S2 had been chosen to align with the identification of novel differentially expressed genes (DEGs) and by extension, MOAs, a limited number of extracts were also

tested using the Sf9 cell line that is genetically closer to the *H. armigera* cotton pest, to assess the extracts and also to compare responses in different cell lines.

For instance, cytotoxicity assays were run using extract 72P twice; once in D.mel-S2 cells to compare non-polar vs polar extracts, then a second time using Sf9 cells to determine if the constituents in the polar phase were efficacious in a second cell line closely related to whole organism tested in Potter spray tower bioassays. In the D.mel-S2 non-polar vs polar cytotoxicity assessment, extract 72P inhibited growth by 21.9%, while extract 72N, caused a much lower cell inhibition (11.2%) even though in whole organism bioassays both caused very high mortality in *H. armigera*. However, when 79P was assayed using Sf9 cells, virtually no inhibition was measured. This was somewhat intriguing, since the previous investigations had shown non-polar fractions to be more efficacious than their polar counterparts in most cases. Furthermore, direct bioassays for 72P, had resulted in 100% mortality against *H. armigera* but limited activity against *T. urticae* and *A. gossypii*. In contrast, extract 72N was highly efficacious against both *H. armigera* (100%) and *A. gossypii* (100%). These results led me to propose that although the Sf9 cell line is from the same family as *H. armigera* (Rafaeli et al. 2007; Zhang et al. 2016), the insecticidal MOA of extract 72N may not be active at the cellular level The results were unclear in that cytotoxicity was not reproducible in a closely related cell line and there was no dose-response. What was generally clear was that the extracts, including the internal plate standard 61P 0.01% w/v, elicited greater inhibition in Sf9 cells than in D.mel-S2 cells at equivalent concentrations. This confirms the findings of Rasikari et al. (2005) that D.mel-S2 cells were more robust to plant extracts than Sf9 cells. In conclusion, since the non-polar fraction of extract 72 was highly efficacious against two arthropods, *H. armigera* (100% mortality) and *A. gossypii* (100% mortality), we continued our investigations using extract 72N. Therefore, the choice of two cell lines was suitable to detect the different cellular level MOAs of the different novel botanical extracts. Future research should focus on the molecular insights of these novel extracts using RNA-sequencing, cell lines with overexpressing or silencing of genes encoding potential insecticidal targets.

5.5.3. Unique patterns of ROS are produced in response to novel botanical extracts

The overproduction of ROS is known as oxidative stress (Lambeth et al. 2014). Many reports have showed that oxidative injury is one of the major cause of programmed cell death in insect cell lines (Wang et al. 2013; Yang et al. 2017; Yang et al. 2013a; Zhang et al. 2015). In this study, whilst not all treatment showed significant differences in D.mel-S2 cells, possibly due to a large variation in individual cell response, the trends are interestingly extract specific. Initially, extracts were tested at much higher concentrations (10 fold) for D.mel-S2 cells than was used in other cellular bioassays (absorbance spectrometry and MIFE) to assess the data collection time-frame in terms of intervals and treatment duration. Extract 36P, which did not show meaningful efficacy against any of the arthropods tested in Potter spray tower bioassays, was selected for its ROS fluorescence response to better understand if there are underlying metabolic changes, which may not result in mortality. At the higher concentration of 0.1% w/v, extract 36P showed a sharp increase in ROS production immediately following treatment. However, the decrease that followed suggested squelching by scavenging antioxidant enzymes, such as manganese superoxide dismutase (MnSOD) (Holley et al. 2011). This reduction in intracellular ROS may indicate that all three tested arthropod species in Potter spray tower bioassays survived extract 36P by presentation of entry or detoxification mechanisms at the whole-organism level. Extract 61P 0.1% w/v, my choice of internal-plate control which resulted in approx. 10% growth inhibition in D.mel-S2 cells and 20% inhibition in Sf9 cells, and is highly efficacious against *T. urticae* (89.6%) and efficacious against *A. gossypii* (56.5%), in the whole organism assays, resulted in reduced (but not significant) ROS production or ROS mitigation compared to the control cells.

Most interestingly, ROS production in Sf9 cells in response to extracts 68N and 72N at 0.01% w/v were far more pronounced that in D.mel-S2 cells. Extract 68N was tested in both cells at the same concentration with remarkably different results. I also tested the known antioxidant caffeic acid 0.01% w/v against Sf9 cells, with mixed results. Although there was some evidence of detoxification at this concentration, seen by the reduction in ROS below control levels, there was no statistically significant difference. This particular antioxidant was select because of its similarity of chemical structure to the main constituent in extract 72N. However, the

opposing ROS fluorescence clearly showed no correlation between the effects of 72N 0.01% w/v and caffeic acid 0.01% w/v. Thus, the high susceptibility of Sf9 cell lines to extracts 68N and 72N based on ROS fluorescence indicates that *Spodoptera frugiperda* and other related species may be potential targets for the development of commercial botanical pesticides from these novel extracts.

5.5.4. Ion fluxes are useful indicators of potential modes of action of cell lines in assessing high priority extracts

The MIFE technique allows for up to four ions to be measured simultaneously through system calibrations and ion selective ionophores (Shabala et al. 2012). Although numerous studies have been conducted with the MIFE technique for cell physiological research (Chen et al. 2005; Chung et al. 2010; Shabala et al. 2006a; Shabala et al. 2013), this is to my knowledge the first study, using the MIFE technique to discover potential biopesticides, along with other methods. The electrophysiology studies showed that all three extracts 68N, 72N and 82N affected all three ions: K^+, Na^+, Cl^- in some way. The exception was Ca^{2+}, which registered negligible flux changes in response to any of the three extracts. It is possible that D.mel-S2 cells are not necessarily the best model with active membrane transporters such as ion channels, pumps and co-transporters controlling these ions when more active ion channels are found in *Drosophila* neurons (Buckingham et al. 1996; Cao et al. 2013; Schulze et al. 1995).

From the results of extract 68N 0.01% w/v to D.mel-S2, the immediate efflux of K^+ and Na^+ suggests that cation channels may be one likely MOA target. However, since Cl^- influx, mediated by the major Cl^- channels in *D. melanogaster* (Asmild et al. 2000; Cabrero et al. 2014; Chien et al. 2006), was also significantly affected and particularly as two compounds have been identified from further fractionation of this extract it is unclear if each compound is affecting the membrane transport individually by targeting different channels or if they both work similarly or, indeed, synergistically. Extract 72N 0.01% w/v had a lesser effect on both K^+ or Na^+ flux in D.mel-S2 cells than did 68N. However, it is the suppression to near zero levels of Cl^- influx when compared to both the negative (DMSO) and positive (pyrethrum & tasmanone) controls that is particularly interesting. It is possible that this extract either blocks Na^+ channels or at least inhibits Na^+ movement, potentially through action to novel Na^+

channels (Loughney et al. 1989; Martin et al. 2000). Since all controls displayed a Na^+ flux change from efflux in pre-treatment conditions to influx post-treatment, it may be that the DMSO background diluent—common to all extracts— has some influence over Na^+ flux; however, the magnitude of this flux is not comparable to that seen in extract 68N 0.01% w/v. Extract 82N 0.01% w/v also had a similar effect on D.mel-S2 cells as extract 68N, but the magnitude was less and the action was slower. Both K^+ and Na^+ were similarly affected, with a significant efflux in the transient phase, which reduced a little in the stabilisation phase but was not recoverable. The Cl- flux again was by far the most affected, but I there is quite a bit of variability between the replicates which may indicate some cells have less sensitivity to this MOA than others.

In summary, from the top 20 extracts investigated in Chapters 4 and 5, three stood out as superior extracts, the non-polar fractions of *Podolepis jaceoides* (68N), *Schoenia filifolia* (72N) and *Lechenaultia biloba* (82N). In this chapter, I investigate their effect on cytotoxity and growth inhibition and compared the differences in two quite different insect cell lines. I measured the fluorescence of ROS to gauge cellular stress response to selected extracts and explored the MOA that may cause cell dead, apart from direct nerve poisoning. Last, I measured the changes in ion flux using different populations of D.mel-S2 to elucidate the possible ion channels and cotransporters that may be affected by each extract. From this study, I concluded that extracts 68N and 82N would be the most suitable candidates to take forward for molecular testing. Both are novel plant extracts not previously linked to insecticides; 68N has two major putatively insecticidally active compounds characterised, with the possibility that they work and have different MOAs, one affecting Na^+ channels and another affecting Cl^- together. Alternatively, they may work synergistically on a target site that is impacted by both of these ions. Extract 82N is a lethal toxin; however, self-contamination bioassays, cytotoxicity assays and ion flux measurements suggests it is slow acting. The potential MOAs of these novel botanical extracts, based on cytotoxicity, ROS signalling, and membrane transport studies using the two insect cell lines need to be further validated at the whole organism and molecular levels. This led to the study of qPCR and RNA-seq investigations on a major cotton pest *Aphis gossypii* and the model insect *Drosophila*

melanogaster, which are described in the following chapter. Chapter 6: *Investigating Insecticidal Modes of Action and Resistance Through Gene Expression Analysis.*

Chapter 6: Investigating Insecticidal Modes of Action and Resistance Through Gene Expression Analysis

6.1. Introduction

Aphis gossypii (cotton aphid) is one of the key cotton pests in Australia, alongside *Helicoverpa* spp. (cotton bollworm), *Tetranychus urticae* (two-spotted spider mite), *Thrips tabaci* (cotton/onion/tobacco thrips) and *Creontiades* spp. (mirids). In Australia, modern, intensive cotton production has been heavily reliant on pesticides since the 1960's through to the late 1990's (Fitt 1994). *Aphis gossypii* affects host plants in three ways: directly feeding on the plant via piercing and sucking mouthparts, reducing plant vigour, photosynthesis and consequentially, yield; by producing honeydew as a by-product of feeding and depositing honeydew onto cotton lint, which in turn reduces the commercial value and requires additional processing; and indirectly, by acting as vectors for plant pathogens, which result in diseases that reduce yields or may cause crop failure (Herron et al. 2017). One such disease is the cotton bunchy top disease, which presents with symptoms similar to those of magnesium deficiency. The concern with aphids acting as disease vectors is that their management requires earlier intervention to reduce infection, at the risk of increasing selection for pesticide resistance. Therefore, *A. gossypii* was selected for the work reported in this Chapter, to further our understanding of the molecular mechanisms of the novel extracts using gene expression analysis – both qPCR and RNA-sequencing, and because 68N was shown to be a potent aphicide in whole organism bioassays.

There are a few publicly available aphid genome assemblies, including *Acyrthosiphon pisum* (International Aphid Genomics Consortium 2010), *Diuraphis noxia* (Nicholson et al. 2015), *Aphis glycines* (Wenger et al. 2017), *Myzus persicae* (Mathers et al. 2017), *Myzus cerasi* (Thorpe et al. 2018), *Rhopalosiphum padi* (Thorpe et al. 2018) and *Rhopalosiphum maidis* (Chen et al. 2019b). An improved genome assembly of soybean aphid (*Aphis glycines*) has just been published (Mathers 2020). However, the draft genome of *Aphis gossypii* is still in progress (Quan et al. 2019) and this genome assembly was not ideal for transcriptome annotation due to its limited quality in a draft form. Therefore, it is likely that the annotation

of differentially expressed genes (DEGs) of the transcriptome profiling of *A. gossypii* will require genome information of the above assemblies of different aphid species. Despite the lack of a fully sequenced genome of *A. gossypii*, many transcriptome analyses have been conducted in response to different synthetic pesticides and plant allelochemicals with other insect species (Enders et al. 2020; Li et al. 2017; Li et al. 2013; Wu et al. 2018). However, to the best of my knowledge, there is no report of transcriptome profiling on the effects of biopesticides derived from novel botanical extracts against *A. gossypii,* which is likely to provide molecular insights into the gene function and MOA of novel biopesticides in *A. gossypii*.

Drosophila melanogaster is a species of vinegar fly, which is a common domestic pest in the family Drosophilidae. *Drosophila melanogaster* was an African fly species sharing a common origin with all the non-African lineages; and its geographic range includes all continents and islands (Baudry et al. 2004; Markow 2015). It is widely used for biological research due to its rapid life cycle, large number of offspring per generation, and relatively simple genetics (Adams et al. 2000; Sang 2001; St Johnston 2002). Therefore, *D. melanogaster* is a model insect species, which has been widely studied for genetics, physiology, genomics, microbial pathogenesis, and life history evolution, and discovery of pesticide (Adams et al. 2000; Lemaitre et al. 2007; Muñoz et al. 2013; St Johnston 2002). In agricultural pest management, *D. melanogaster* has been primarily used either directly as a model organism (Scharf et al. 2006) or in comparison with other major pests such as *Spodoptera frugiperda* (Gu et al. 2013; Muñoz et al. 2013). The deeply-sequenced and well-assembled genome (Adams et al., 2000) and extensively developed online tools and databases have advanced the process for genomic and transcriptomic studies in *D. melanogaster* (Attrill et al. 2016; Crosby et al. 2007; Drysdale et al. 2005; Robinson et al. 2013). Here, I also used *D. melanogaster* along with *A. gossypii* to test the gene expression of these insects in response to novel botanical extracts.

In the previous chapters, I have looked at different ways to assess the short-listed extracts found to be efficacious in the foundation project. Through direct application (Potter spray tower, Table 7-4, Appendix 3) bioassays, extract 82N showed the highest average efficacy across the three key target pests (100%), ranking 1st on my priority list. Extract 68N ranked 4th

with an average of 85.5%; however, achieving 100% mortality at low concentrations against *A. gossypii*, a pest with strains of high insecticide-resistance, in combination with its novelty deemed this extract also worthy of an in-depth investigation. Electrophysiology studies had shown that both these extracts affected the ion flux of K^+, Na^+ and Cl^-; however, the magnitude of flux was less so in extract 82N than 68N (see Chapter 5 for details).

Comparing these two extracts to the positive controls used, in 82N; K^+ influx was similar to pyrethrum but greater than tasmanone initially reducing to similar levels in the stabilise phase but remaining significantly greater than the negative control. Na^+ influx behaved similarly with pyrethrum showing greater influx than tasmanone, while Cl^- efflux was somewhat similar across all three, leading me to believe that extract 82N may have a similar MOA to pyrethrum. In regards to 68N, the K^+ influx results were almost the same as 82N but the magnitude was much greater. However, 68N caused far greater influx of Na^+ compared to either pyrethrum or tasmanone, notwithstanding pyrethrums MOA as a Na^+ channel modulator (ref). This would infer that if extract 68N's MOA is similar to that of pyrethrum, the potency of effect must be much greater, or perhaps it has influence over multiple channels and transporters regulating Na^+ flux. The effect of 68N on Cl- influx was similar to that of pyrethrum but greater than tasmanone in the transient phase, will all three being similar by the stabilising phase. In addition, self-contamination bioassays had shown that 82N was a slower acting toxin, allowing me to collect surviving, sick and dead specimens. On this basis, 82N was used for RT-qPCR investigations against a range of targeted genes, notably including the ion channel genes and metabolic detoxification genes. Subsequently, extract 68N, which had shown through bioassays to be a fast-acting and potent aphicide, was more suited for direct pest to model species transcriptomic comparison, in order to widely investigate both the MOA and resistance-potential.

Hence, the purpose of these molecular investigations was two-fold: first to investigate the differential expression elicited by one of highly efficacious extracts, 82N, by employing RT-qPCR using the model organism *D. melanogaster* (adult flies) as my test species and the self-contamination bottle method as the route of treatment. Second, by using high throughput next generation RNA sequencing I investigated the MOA of a second highly efficacious extract,

68N.M. In addition, this method should shed light on known insecticide-resistant genes, giving an indication of future resistance risks. *Aphis gossypii* and *D. melanogaster* adult flies were both used in this investigation to provide comparative insights, and employed direct application via the Potter tower as the route of treatment.

The hypotheses for Chapter 6 were:

- The differentially expressed genes (DEGs) of dead insects of one pest species and one model species following exposure to novel plant extracts indicate key genes controlling the insecticidal modes of action.
- The DEGs of surviving insects exposed to novel plant extracts elucidate key genes in the detoxification pathways.

6.2. Gene expression of *Drosophila melanogaster* after 82N extract treatment

6.2.1. Methods

Drosophila melanogaster mixed sex adult flies exposed to self-contamination bioassays (see Chapter 4 for details) were collected into three groups: dead, sick and surviving after 24 h exposure to extract 82N 2.0% w/v. Their gene expression was then compared to ethyl acetate (negative control) and two positive controls, pyrethrum 2.0% v/v and tasmanone 2.0% v/v from the same experiment. Samples were snap frozen in liquid nitrogen and stored at -80°C. RNA was isolated from two whole intact flies per sample using Tri Reagent® solution as per the manufacturer's instructions (Ambion 2010) with the exception of using a reduced rate of 0.5ml per sample to accommodate the small sample weight. Frozen fly samples stored in pre-autoclaved, RNA-free 1.5 ml Eppendorf tubes were homogenised using frozen metal knitting needles pre-soaked in 0.1% DECP water. We found metal knitting needles remained cold longer than other grinding tools, preventing RNA degradation through thawing, whilst also reducing the sample lost by adherence to grinding tool surfaces. They also fit perfectly into the conical end of Eppendorf tubes for efficient sample contact and grinding. Centrifugations were carried out in a precooled 4°C Eppendorf 5415R bench top centrifuge (Eppendorf South Pacific Pty Ltd, Macquarie Park, NSW, Australia) at recommended rpms for each step. Reagents were prepared as per instructions using ACS reagent grade chloroform and isopropanol, molecular grade ethanol 75% in nuclease-free water, and kept on ice in the sterilised and RNA-zapp treated laminar fume hood.

A 1-µl aliquot of each sample was used to determine the quantity and quality of the RNA after isolation and cDNA after synthesis using a NanoDrop ND-1000® spectrophotometer (Thermo Scientific, Waltham, MA, USA) by absorbance ratios $OD_{260/280}$ and $OD_{260/230}$. The total RNA concentration was calculated using the formula, 1200 ng/µL ÷ RNA concentration ng/µL, to create uniform template concentration for downstream applications and to account for the over estimation given by Nanodrop ND-1000® when using guanidine isothiocyanate based RNA isolation products such as TRI Reagent (Lee et al. 2014). One microgram of total RNA was

used to synthesise cDNA with the sensiFAST™ Kit (Bioline, Alexandria, NSW, Australia) following manufacturer's directions. First strand cNDA was then used in polymerase chain reaction (PCR) using GoTaq® Flexi DNA Polymerase kit (Promega, Alexandria, NSW, Australia) with gene-specific primers (Table 6-1) to validate RNA integrity and primer design by gel electrophoresis. cDNA synthase was also used in RT-qPCR experiments. Multiple primers were designed using FASTA from Flybase and several design tools, Premier Primer 5 (Premier Biosoft), NCBI (National Centre for Biotechnology Information) and IDT (Integrated DNA Technologies) and assessed for false-priming and cross-dimer probability, resulting in two primers being selected for some genes. In cases where two primers where designed for one gene, the gel image giving the strongest, single band was selected for downstream applications.

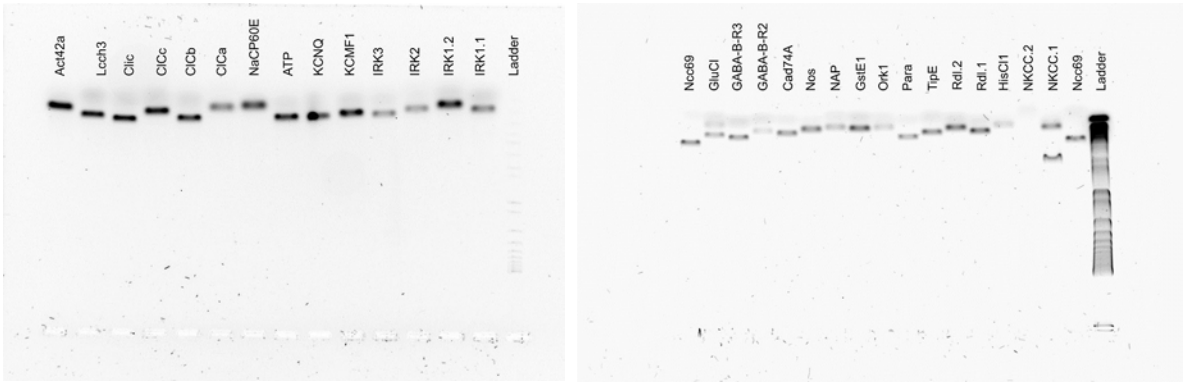


Figure 6-1 Gel electrophoresis used to determine primer efficiency

Table 6-1 Primers designed and selected for used for *D. melanogaster* 82N PCR and RT-PCR

Gene	Forward Primer	Reverse Primer
Dm.GstE1	GCACCTGAGCGAGGAATA	CACCAAGTAGGCGGCAAT
Dm.GluClα	GGAGCCTGGGTAGAACTG	GGGATGCGTATTGTGGAG
Dm.Kcmf1	GTTGCCAGATGTATGTGAGA	GCCGTATTTACTGAGGGTC
Dm.nap	TGTCCCGTATTGGAAGTA	AAGGCACAAAGTCTGTCATC
Dm.KCNQ	GGCACTTCCAGTCAACAC	TCCAGAAACTATCCACCC
Dm.NKCC.1	CGGCAGCCTAACTAAC	GGATGAGGAGGAGCAG
Dm.Ork1	ACAGGCTCTTTGGGTGAC	TGGTGCGAACTCTACTTACT
Dm.IRK1.1	AATAAGTCGCCAGTCTCCAAC	TGAGTAACCACAATACGCCC
Dm.IRK2	ACACGGAGACTTGGAAGA	GGAGAACAGGAAGCAGGA
Dm.IRK3	TAAGGCGGTGGTAAGTAA	GTTTGTCCACGATGATGT
Dm.Nos	CAAATAATCTACTCGCTACG	CTCCTTACTTTCACCCTCT
Dm.Act42A	GCGTCGGTCAATTCAATCTT	AAGCTGCAACCTCTTCGTCA

Note: Genes-specific primers were designed using Premier Primer 5. Where two sets of primers designed from different sections of the gene code are denoted by and additional "*.#*" at the end of the gene name, for example *Dm.NKCC.1.* All primers were tested by gel electrophoresis but not all were using in RT-qPCR. A full list of primers can be found in Appendix 4, Table 7-5.

Gene expression of the above genes was then assayed by RT-qPCR using SensiFAST™ SYBR No-ROX kit (Bioline, Alexandria NSW, Australia) as per manufacturer's instructions and the Rotor-Gene® Q6000 (QIAGEN, Hilden, Germany). Actin 42A (*Act42A*) was used for the reference gene in each individual RT-qPCR run based on its normalisation stability in RT-qPCR for *D. melanogaster* under different stressors (Ponton et al. 2011). For each RT-qPCR run, a 5-point standard for each gene was included by $1/5^{th}$ serial dilutions and later compared with CT values to cross-check results. I also included two technical replicates of each sample to pick up any pipetting anomalies. The cDNA template for each sample was uniformly diluted to 400 ng/µl. SYBR green signal data were acquired at end of the 3-step cycling protocol consisting of polymerase activation at 95°C for 2 min, 1.) 40 cycles of 5 s denaturation at 95°C, 2.) 10 s annealing at 63°C, 3.) 15 s extension at 72°C.

6.2.2. Results

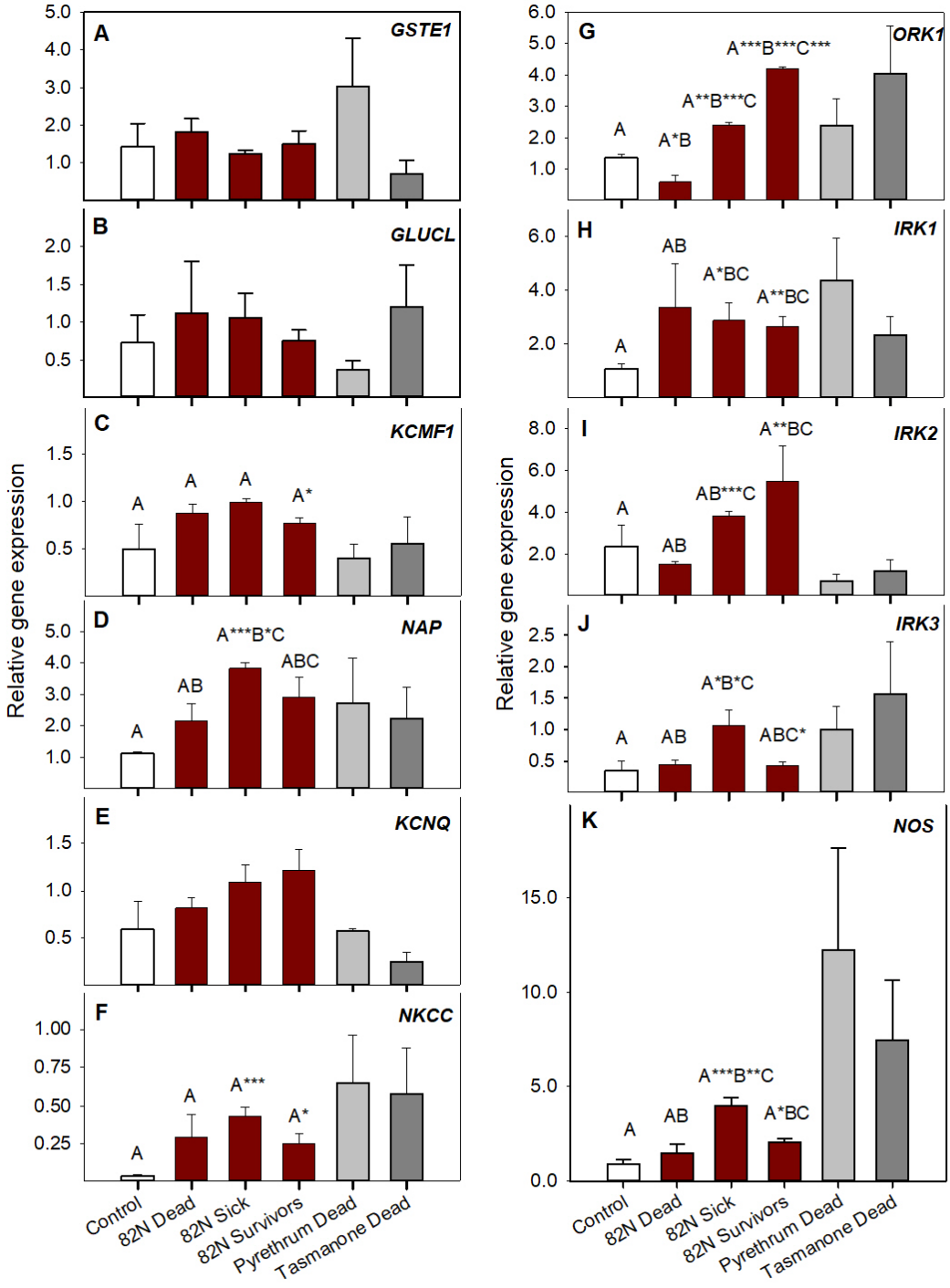


Figure 6-2 *D. melanogaster* flies differential gene expression to extract 82N.

(**A-K**) Target genes expressed in *D. melanogaster* after exposure to extract 82N 2.0% w/v. Data are mean ± SE. Extract 82N samples have been statistically compared to control and each other, where letter stand alone, there is no significance, where asterisks accompany letters they are statistically different from the same letter by significance levels of *, P<0.05; **, P<0.01; ***, P<0.001 using two-tailed Student's t-test against each group, excluding pyrethrum and tasmanone.

In the RT-qPCR experiment, gene expression of *glutathione-S-transferase epsilon 1* (*GSTE1*) (Figure 6-2A), *glutamate-gated chloride channel* (*GLUCL*) (Figure 6-2B) and *Potassium voltage-gated channel* (*KCNQ*) (Figure 6-2E) were not significantly different between the negative control and any groups of the *D. melanogaster* responses to 82N 2.0% w/v. *Potassium channel modulatory factor 1* (*KCMF1*) (Figure 6-2C) resulted in significant downregulation in the survivors compared to sick flies; however, there was no significant difference between the other 82N treated groups and/or the control, presumably due to the large variation in the control group. *No action potential* (*nap)* (Figure 6-2D) was significantly upregulated in dead, sick and surviving groups compared to control, with sick flies being statistically significantly different from the dead and survivors. The *sodium potassium chloride co-transporter* (*NKCC*) was significantly upregulated in both sick and survivor groups compared to control. The expression of *open rectifier K^+ channel 1* (*ORK1*) was the most significantly different from negative control than any other genes tested. *Inwardly rectifying potassium channel*s (*Irk1, Irk2 & Irk3*) also showed significant differential expression in response to 82N compared to negative control, *IRK1* upregulated in sick and surviving flies, *IRK2* upregulated in surviving flies, and *IRK3* upregulated in sick flies. *Nitric oxide synthase* (*NOS*) was significantly upregulated in both survivors and sick flies compared to the negative control; additionally, *NOS* expression of sick flies was significantly upregulated compared with dead flies (Figure 6-2). The qPCR results provided very useful information on the sensitivity and responsivity of these genes in response to a novel botanical extract. This also led to the exploration of the transcriptome profiling of both a model insect *D. melanogaster* and a major cotton pest *A. gossypii* in response to 68N.M - one of the most likely candidates of the CRDC project for future development of commercial botanical pesticide for the Australian cotton industry.

6.3. Transcriptomic analysis of *Aphis gossypii* and *Drosophila melanogaster* after extract 68N treatments

6.3.1. Methods

6.3.1.1. Insect material and treatments of novel botanical extracts

Ten to fifteen ferment flies, previously isolated and sexed, were anesthetised with a gentle flow of CO_2 gas for approximately 5-10 s before being gently poured onto the filter paper discs with a small amount of *Drosophila* media as a food source. Approximately 40 gravid female *A. gossypii* where transferred onto cotton leaf disks. See Chapter 2: Materials and Methods for insects used and Potter tower methods, and see Chapter 4: Direct application bioassay for detailed experimental design and images.

Extract 68N.M emulsions of 2.0%, 1.0%, 0.75%, 0.5%, 0.25%, 0.125% and 0.0625% v/v concentrations were prepared and 3 ml of each concentration was pipetted into the top of a Potter tower and delivered at a pressure of 15 psi, starting with the control and followed by increasing concentrations from lowest to highest (see Chapter 2). Bioassay results are presented in Chapter 4 (Figure 4-2). Since the *D. melanogaster* flies survived the treatment they were collected at the end of the 24 HAT count and snap frozen in liquid nitrogen for later RNA isolation. Because the *A. gossypii* died rather quickly, the direct application method was re-run with a further four replicates using exactly the same methanol extract stock and Potter tower protocols, except that spray timing was staggered by approximately 15 min to allow for collection of samples exactly 1 h after spraying for RNA isolation. At 1 HAT, *A. gossypii* were counted, classified as either alive or dead, collected into pre-labelled 1.5 ml eppendorf tubes and snap frozen in liquid nitrogen. Death was assessed by the absence of appendage movement when an individual aphid was prodded gently with a fine needle. Four biological replicates where run on 4 separate days, comprising of 2-4 petri-dishes each run, resulting in a total of 1982 individual aphids.

6.3.1.2. RNA extraction, library preparation, and transcriptome sequencing

Since aphids are quite small and RNA of a high purity was required for transcriptomic sequencing, *D. melanogaster* and *A. gossypii* RNA was isolated using the Isolate II RNA mini kit (Bioline, Alexandria, NSW, Australia) following the manufacturer's protocol which included an on-column DNA digestion step using DNase1 treatment (Bioline). Initial RNA quantity and quality was determined twice using a NanoDrop ND-1000® spectrophotometer (Thermo Scientific, Waltham, MA, USA) by absorbance ratios $OD_{260/280}$ and $OD_{260/230,}$ and Qubit® 2.0 Fluorometer (ThermoFisher Scientific, Vineyard, NSW, Australia). Samples with adequate RNA quantity but low $OD_{260/230}$ ratios were reprocessed using the same kit following the protocol. Samples were then further processed at Western Sydney University's next generation sequencing (NGS) facility where RNA quality was determined using an Agilent Bioanalyzer 2100 and an Agilent RNA 600 Pico Kit.

Library construction and sequencing were performed as described by Cao et al. (2014) and Chen et al. (2019a), using TruSeq™ mRNASeq using polyA selection and paired-end strand specific RNA-Seq Illumina. TruSeq™ RNA sample preparation kit (Illumina, San Diego, CA, USA) was used for library construction following the manufacturer's protocol. The raw data obtained from HiSeq 2500 platform (TruSeq SBS KIT-HS V3, Illumina) were tidied to remove empty reads and low quality bases (Q<30 and length <50bp) and adaptor sequences at the 3' end. RNA-Seq reads were assessed for quality control with FastQC v0.10.1 (Babraham Bioinformatics, Cambridge, UK).

6.3.1.3. Transcriptome dataset mapping and gene expression analysis

RNA-seq dataset of the *D. melanogaster* was mapped to the reference genome Drosophila_melanogaster.BDGP6 Release 6 (dos Santos et al. 2015). BWA (Burrows-Wheeler Aligner, software package, http://bio-bwa.sourceforge.net/bwa.shtml) was used to map clean reads following standard protocols (Li et al. 2009, 2010) to the genome reference of *D. melanogaster* (http://flybase.org/), and Bowtie (sequence analysis packages, http://bowtie-bio.sourceforge.net/index.shtml) for aligning short reads (Langmead 2010).

Despite the publication of a draft genome assembly (Quan et al. 2019), there is no complete reference genome available for mapping and annotating the *A. gossypii* RNA-seq dataset. Therefore, a reference genome was reconstructed using *de novo* RNA-sequencing fragments of raw dataset using the Trinity platform (Haas et al. 2013). The genes in other aphid genomes: *Acyrthosiphon pisum* (International Aphid Genomics Consortium 2010), *Diuraphis noxia* (Nicholson et al. 2015), *Aphis glycines* (Mathers 2020; Wenger et al. 2017), *Myzus persicae* (Mathers et al. 2017), *Myzus cerasi* (Thorpe et al. 2018), *Rhopalosiphum padi* (Thorpe et al. 2018) and *Rhopalosiphum maidis* (Chen et al. 2019b) was also used as references to annotate the *A. gossypii* DEGs.

The mapped reads assembling, abundance estimating and differentially expression genes (DEGs) were analysed by Cuffdiff of Cufflinks v2.1.1. Fragments per kilobase of exon per million fragments mapped reads (FPKM) and false discovery rate (FDR) methods were used to identified DEGs that are satisfied with the fold change of [FPKM(treatment)]/[FPKM(control)]>2 and FDR <0.05. FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels in many species including model insect such as *Drosophila melanogaster* (Lin et al. 2016). Differential expression analysis of treatments (dead or alive)/control) was performed, with three biological replicates for the control and treatments.

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the cluster Profiler R package (Yu et al. 2012). GO terms with corrected P value less than 0.05 were considered significantly enriched by differentially expressed genes. Cluster Profiler R package (Yu et al. 2012) was also used to test the statistical enrichment of differential expression genes in Kyoto encyclopaedia of genes and genomes (KEGG) pathways (http://www.genome.jp/kegg/). After mapping, GO annotation, Inter ProScan annotation and enzyme code annotation steps, the DEGs were distributed into three levels: Molecular Functions, Biological Processes and Cellular Components, and KEGG maps were generated for DEGs analysis. Volcano plots were generated with the Trinity platform (Haas et al. 2013).

Venn diagrams were designed online using Venny 2.1 (Oliveros 2007-2015). The scatter plots designed in R-studio using ggplot2, are the average of the fragments per kilo-base per million mapped reads (FPKM) values for the three replicates, then normalized using log_2 transformations. Gene expression values are given as $Log_2FC \leq -1$ for down regulated genes (green dots) and $Log_2FC \geq 1$ for up-regulated genes (red dots) (Wickham et al. 2016).

6.3.2. Results

Aphis gossypii, one of the key cotton pests, and *D. melanogaster*, the genome-sequenced model organism of choice showed very different susceptibility to the insecticidal properties of extracts from *Podolepis jaceoides* (tested as the methanol extract 68N.M). *Aphis gossypii* succumbed very rapidly to the toxic effects, often stagnating post-application, with a high percentage dying within 1 HAT. Probit analysis reported the LC_{50} 2807.3 ppm or 0.28% w/v (CL 1869.1-4037.8 ppm) and LC_{95} 12937.3 ppm or 1.29% w/v (CL 7339.6-65025.3 ppm) would be required for mortality within 1 h (Figures 6-3). The resulting aphid mortality was similar that of the initial 24 h bioassay (Figure 4-2). *Drosophila melanogaster*, on the other hand, recorded very low mortality, even at 24 HAT (Figure 4-2).

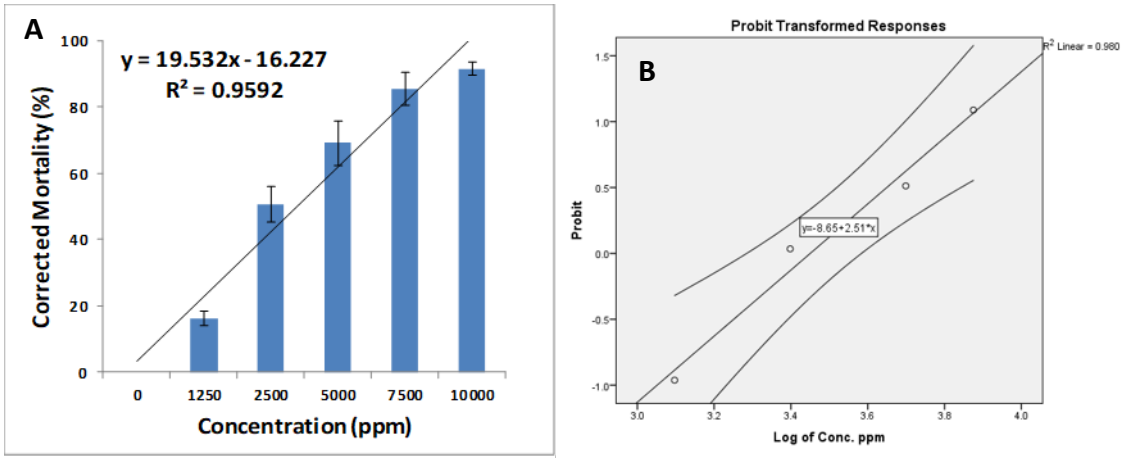


Figure 6-3 *Aphis gossypii* dose-response mortality at 1HAT, 68N.M

(**A**) Dose-response curve of *A. gossypii* at differing concentrations 1 HAT of 68N.M (n=4 biological replicates (separate mixtures) and up to 10 technical replicates (sprayed aphid cages) (**B**) Probit analysis showing the relationship between does and mortality, n=1982 individuals. Shapiro-Wilk test for normality ($p>0.05$) showed the data was normally distributed.

6.3.2.1. Gene expression analysis and identification of DEGs

Using RNA-seq, transcriptome data were generated to investigate the regulatory effects of extract 68N.M at two concentrations, 0.25% w/v and 1.0% w/v, on *A. gossypii* and *D. melanogaster* (Figures 6-4, 6-5, 6-6, 6-7, 6-8, and 6-9; Tables 6-2 and 6-3). Volcano plots and scatter plots allowing the visualisation of the overall distribution of DEG are presented for each individual group in *A. gossypii* and *D. melanogaster*. Volcano plots show up-regulated genes towards the right and down-regulated towards the left on the x-axis, with significantly different genes in each treatment comparison highlighted in red, and the greater the regulation the more towards the top. In addition, scatter plots allow easily visualisation of the change in clustering of genes between each group and allow easy comparison between the two species tested, *A. gossypii* (Figures 6-4 & 6-5) *and D. melanogaster* (Figure 6-6). The results showed that the distribution and level of the expression of DEGs of *A. gossypii* is much wider than *D. melanogaster*, indicating that the former were much more affected by 68N.M on a molecular level.

Using a $\log_2$ fold change <-1 or >1 threshold, 6,267 DEGs were identified in surviving *A. gossypii* compared with 391 DEGs in surviving *D. melanogaster* (68N.M at 0.25% w/v) in response to 68N.M at 0.25% w/v. This represents 14.1% of the total number of genes mapped for *A. gosspyii* and only 2.2% of the total number of genes mapped for *D. melanogaster*. However, 3,684 DEGs were differentially expressed in *A. gosspyii* that died at 0.25% w/v 68N.M. Only 248 out of the 3,436 DEGs in *A. gosspyii* that died at 0.25% w/v 68N.M were uniquely expressed in comparison to the surviving groups (Figures 6-4 and 6-5). Then, the threshold was increased to Log_2 fold change <-3 or >3 in *A. gossypii* to identify the highly expressed (regulation greater than 8 times between groups) DEGs for further investigation. This reduced the number of DEGs in the 0.25% w/v 68N.M-treated alive *A. gossypii* to 1,101 (Figure 6-9A purple circle), which are summarised in the heat map (Figure 6-8A). It also reduced the 0.25% w/v 68N.M-treated dead *A. gossypii* to 615 (Figure 6-9A yellow circle), which are summarised in the heat maps (Figure 6-8B). The Venn diagrams showed that the overlap of 57 DEGs (<-3 Log_2FC >3) between all three treatments (Figure 6-9A) and 39 high-confidence DEGs out of these 57 DEGs are summarised in Table 6-2.

Using the fold change of less than <-1 or >1 threshold, all flies survived the 68N.M challenge at 24 HAT in both 0.25% w/v and 1.0% w/v with the majority of genes of *D. melanogaster* not significantly differentially expressed (Figure 6-6A, C & E). The proportions of upregulated and downregulated genes across all three comparisons are very low as compared to those in *A. gossypii* (Figures 6-4 and 6-5). There were a few highly up-regulated genes in *D. melanogaster* treated with 1.0% w/v 68N.M compared to those of 0.25% w/v 68N.M treatment. Of the 391 DEG in *D. melanogaster* between 0.25% w/v and control, there was many more upregulated, see in the heatmap provided (Figure 6-8C), which is striking in comparison to the *A. gossypii* heatmap which shows 0.25 w/v alive compared to control were predominantly down-regulated DEGs (Figure 6-8A). Venn Diagram showed that 68N.M induced 188 shared DEGs between all three groups: 0.25% w/v and 1.0% w/v compared to control, and 1.0% w/v compared to 0.25% w/v (Figure 6-10A). A summary of the 51 high-confidence DEGs is given in Table 6-3.

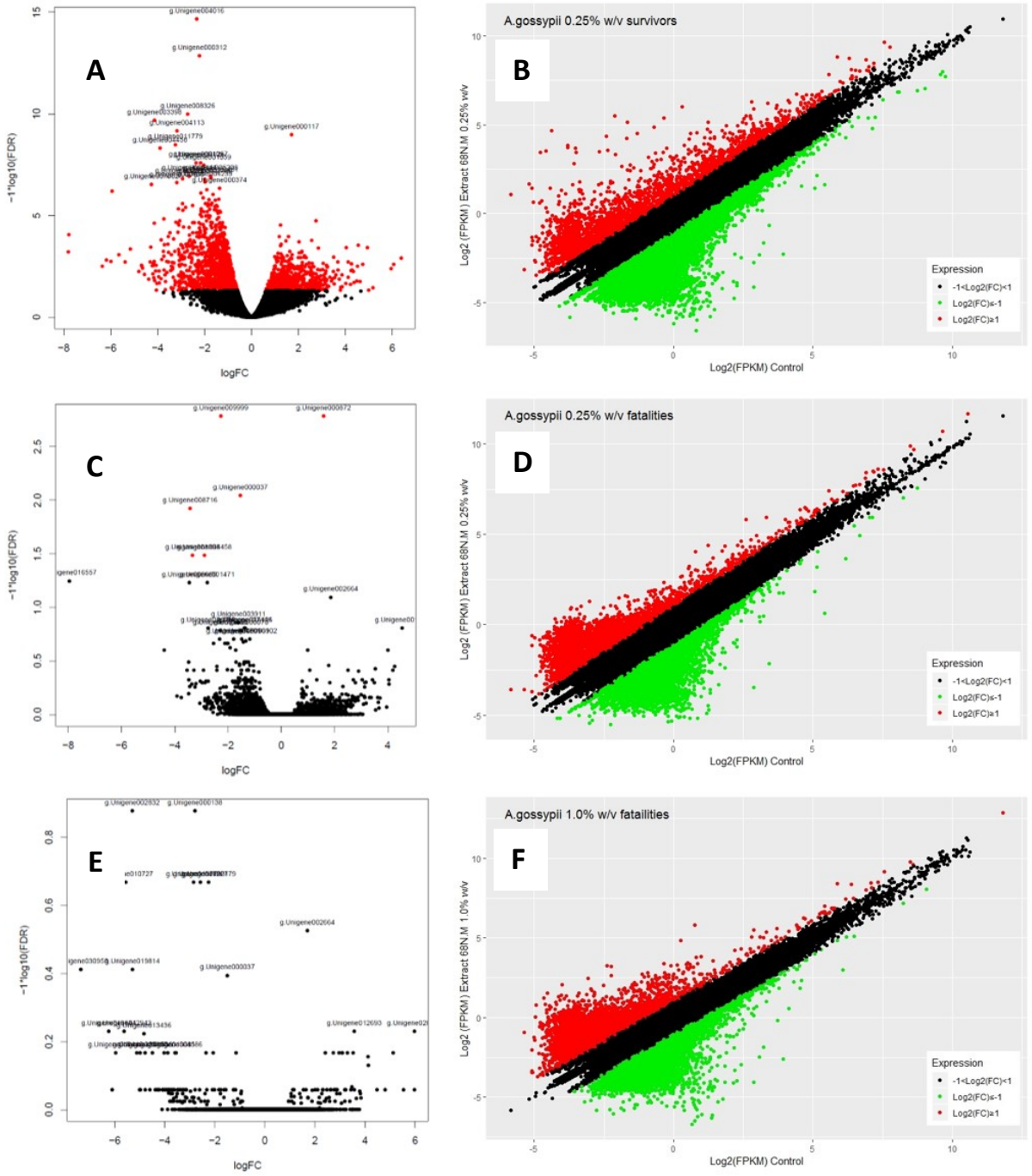


Figure 6-4 *A. gossypii* volcano and scatter plots of DEG after extract 68N.M 0.25% w/v

Volcano and scatter plots of 68N.M (**A** & **B**) 0.25% w/v alive compared to control, (**C** & **D**) 0.25% w/v dead compared to control, (**E** & **F**) 1.0% w/v dead compared control.

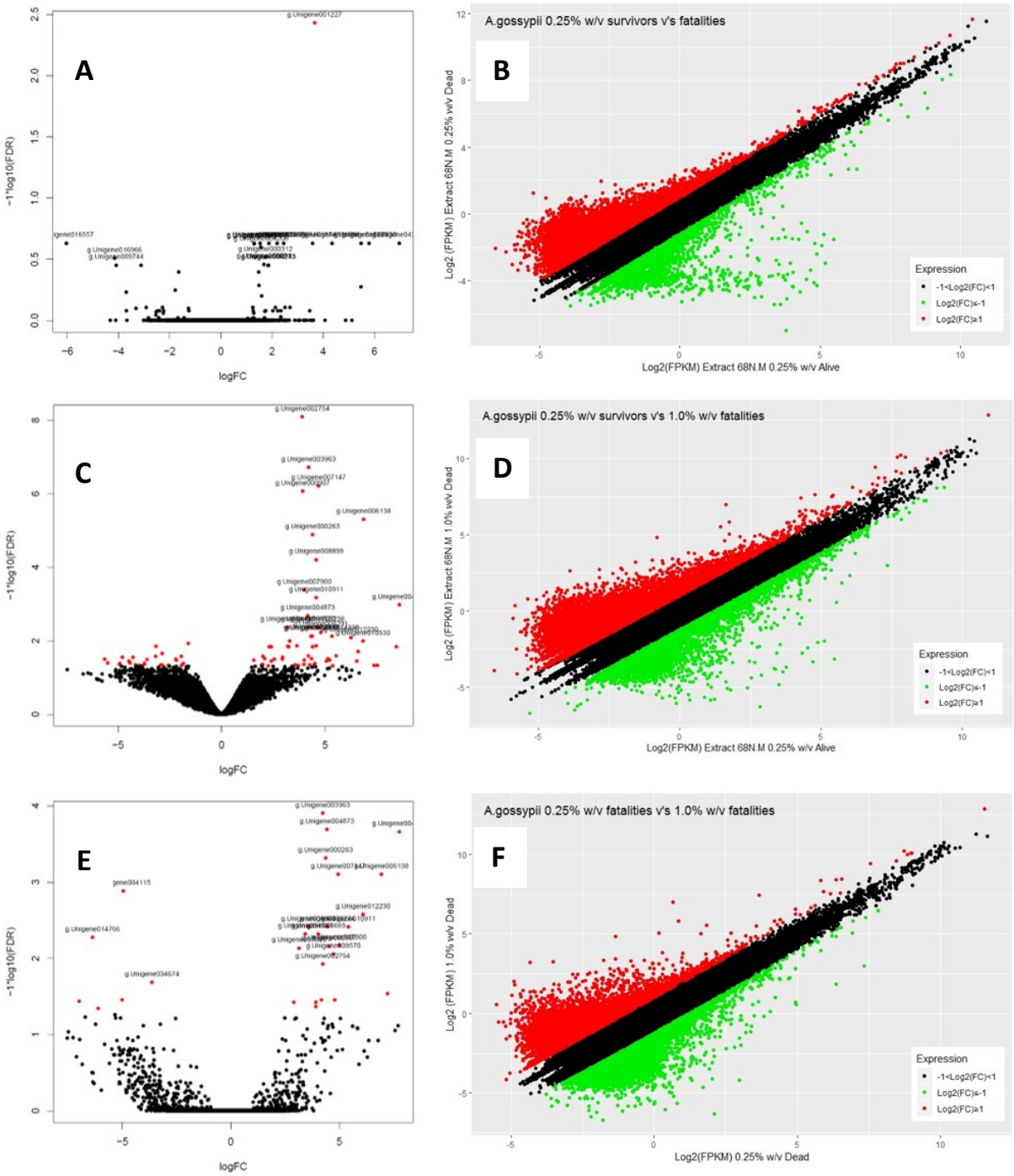


Figure 6-5 *A. gossypii* volcano and scatter plots of DEG after extract 68N.M 1.0% w/v

Volcano and scatter plots of 68N.M (**A** & **B**) 0.25% w/v alive compared to 0.25w/v dead, (**C** & **D**) 0.25% w/v alive compared to 1.0% w/v dead, (**E** & **F**) 0.25% w/v dead compared to 1.0% w/v dead.

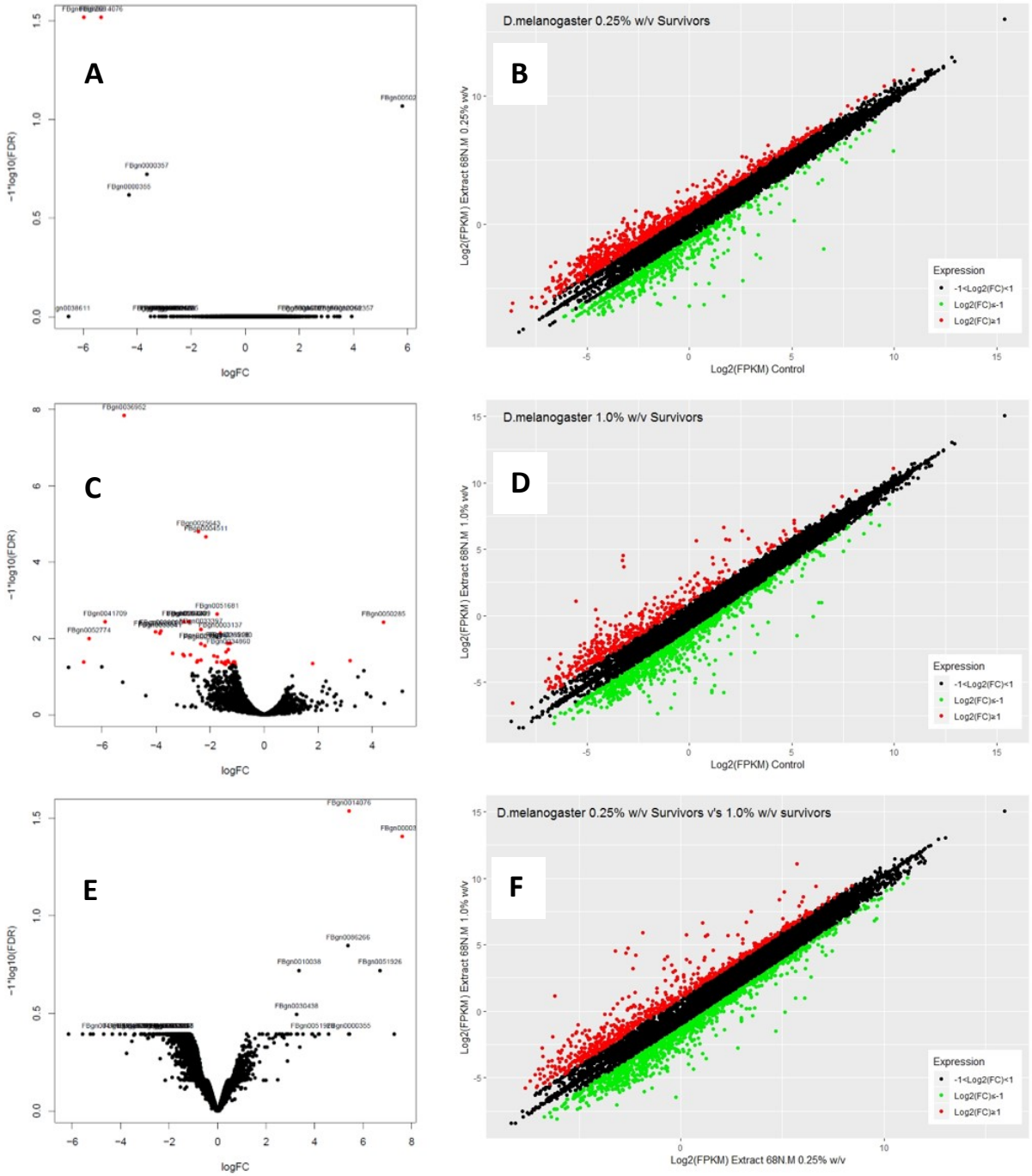


Figure 6-6 *D. melanogaster* volcano and scatter plots of DEG after extract 68N.M.

Volcano and scatter plots of (**A** & **B**) 68N.M 0.25% w/v compared to control, (**C** & **D**) 68N.M 1.0% w/v compared to control, (**E** & **F**) 68N.M 1.0% w/v compared to 0.25% w/v.

6.3.2.2. KEGG functional enrichment of DEGs

Then, the DEGs were used to perform Kyoto Encyclopedia for Genes and Genomes (KEGG) pathway classification and functional enrichment so as to gain further information on the expressed genes and their related functions (Figure 6-7). The KEGG pathway analysis of *A. gosspyii* showed that out of the 977 gene that were identified by GO numbers in the 0.25% w/v alive compared to control, 316 gene populated 9 key pathways (based on the log_2P-value and FDR<0.05). In comparison *D. melanogaster* showed that only 4 genes in 5 KEGG pathways were found in response to treatment of 1.0% w/v 68N.M (Figure 6-7). Moreover, there was only one common KEGG term (drug metabolism other enzymes) between *A. gosspyii* and *D. melanogaster* under the treatment of extract 68N.M. The key KEGG terms in 68N.M in response to extract 68N.M were steroid hormone biosynthesis, metabolism of xenobiotics, cytochrome P450, drug metabolism, ascorbate and aldarate metabolism. However, the potential pesticide related KEGGs in *Drosophila melanogaster* showed little in common to those in the *A. gosspyii* (Figure 6-7). These KEGG pathways provided useful information to further analyse the key DEGs in search for the potential molecular mechanisms for both *A. gosspyii* and *D. melanogaster* in response to the novel botanical extract 68N.M.

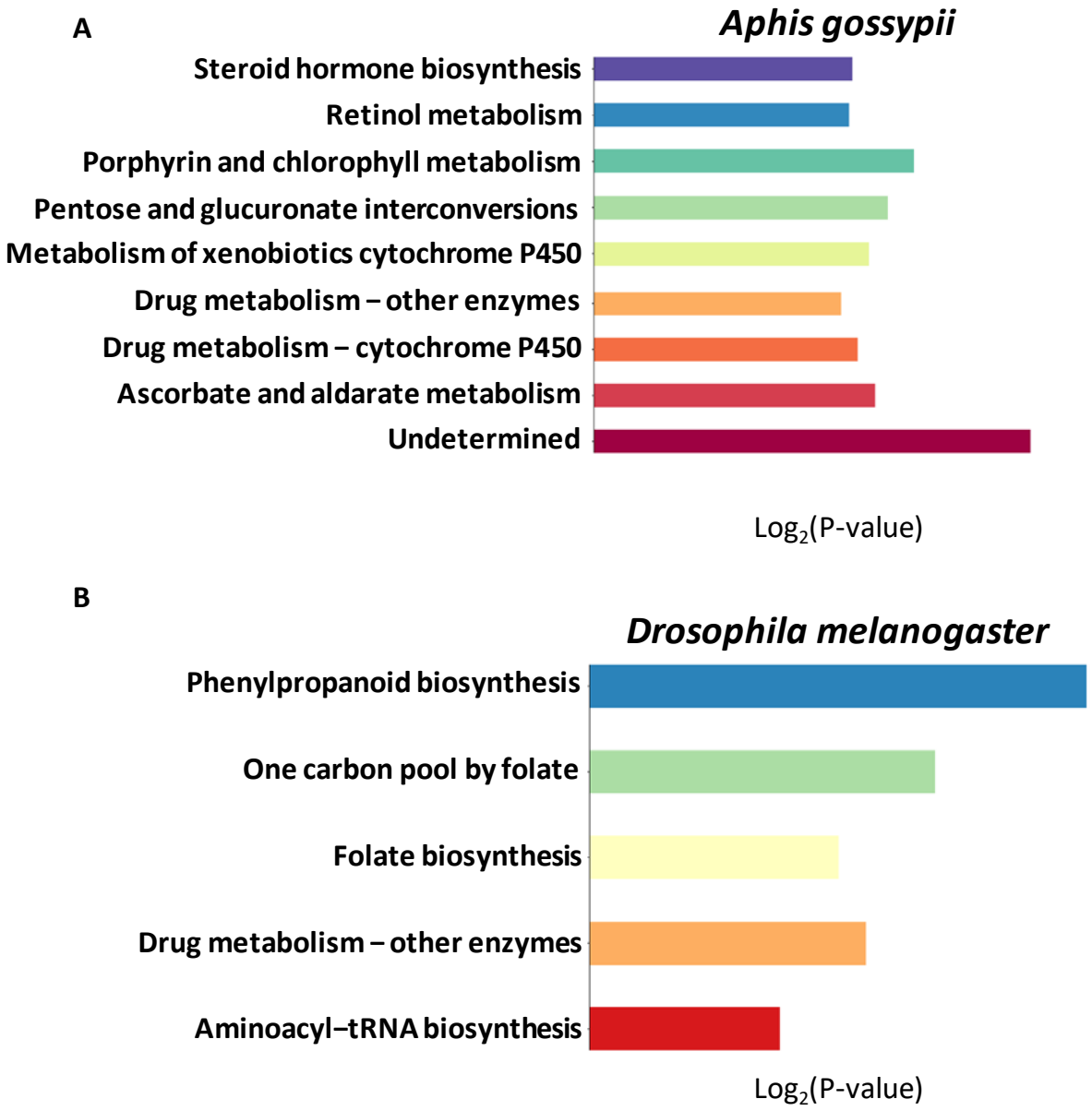


Figure 6-7 KEGG Pathways

Key KEGG Pathways identified after exposure to extract 68N.M in (**A**) *A. gossypii* 0.25% w/v alive compared to control, and (**B**) *D. melanogaster* 1.0% w/v alive compared to control. Statistically significant genes are represented as log_2(p-value), and colour coded to each KEGG description group. Log_2(p-value) and false discovery rate cut off was set to <0.05.

6.3.2.3. Key DEGs responsive to 68N.M

The majority of the key DEGs were downregulated by the novel botanical extract 68N.M in *A. gossypii*. There were 9 ribosomal proteins encoding genes all of which were down-regulated across all treatments, where 60S ribosomal protein L7 was the most down-regulated at -6.26 fold that of control. The most common DEGs of the treatment of 68N.M were 40S and 60S ribosomal proteins (g.Unigene020591, g.Unigene023028, g.Unigene024261, g.Unigene021125, g.Unigene021315, g.Unigene026424, g.Unigene019950, g.Unigene021141, g.Unigene018302), accounted for 23.1% of the total common DEGs (Table 6-2). The second highest group of DEGs were these encoding 3 heat shock proteins (g.Unigene013966, g.Unigene020992, and g.Unigene021183) that were downregulated by the 68N.M to up to -6.12-fold, and 3 cytochrome proteins (g.Unigene021742, g.Unigene014842, g.Unigene022900) which were significantly down-regulated with a -7.12 downregulation of *cytochrome c peroxidase* (Table 6-2). The only three upregulated genes were *neurogenic locus notch homolog protein 1-like* (g.Unigene017265), *protein lozenge-like isoform X1* (g.Unigene022901), and an uncharacterized gene (g.Unigene015960). Other relevant DEGs can be found in Table 6-3 and their detailed functions are available in the AphidBase (https://bipaa.genouest.org/is/aphidbase/).

The 68N.M-induced expression pattern of the 51 high confidence overlapping DEGs in *D. melanogaster* was very different from *A. gosspyii*. There was both upregulation and downregulation of novel DEGs in the two concentrations of 68N.M (Table 6-3). Four vitelline membrane genes (*Vm26Aa, Vm26Ab, Vm34Ca, Vml*) were highly downregulated by 0.25% w/v 68N.M but significantly upregulated by 1.0% w/v of 68N.M. There were three DEGs encoding the cuticular proteins (*Cpr11A, Cpr64Aa, Cpr92F*), mir stem loop (*mir-2a-1, mir-4985, mir-9378*), and small nuclear RNA U (*snRNA:U2:34Aba, snRNA:U2:38Aba, snRNA:U5:34A*). Other relevant DEGs can be found in Table 6-3 and their detailed functions are available in the FLYBASE (http://flybase.org).

6.3.2.1. Novel insecticidal activity-related DEGs

In *A. gossypii*, it was identified that *neurogenic locus notch homolog protein 1-like* (g.Unigene017265) is upregulated by 4.04 and 3.34 fold in response to 0.25% w/v and 1.0% w/v of 68N.M, respectively. Another commonly upregulated gene was *protein lozenge-like isoform X1* **(**g.Unigene022901), which was increased by 3.93-fold and 3.78-fold at the same two concentrations of 68N.M**.** Uncharacterised *protein isoform X1* (g.Unigene013979), showing the highest upregulation at 4.61-fold by 1.0% w/v 68N.M, was mapped to the green peach aphid genome, *Myzus persicae* and belongs to the FAS1 (fasciclin-like) domain (Table 6-2). In *D. melanogaster* fasciclin 1 is involved in cell adhesion and axonal guidance. For the downregulated DEGs, only one membrane transporter gene—*aquaporin AQPAe.a-like isoform X1*—was found to be significantly decreased by 68N.M, which may be linked to osmoregulation following the treatment of 68N.M on *A. gosspyii.* Three genes of the Cytochrome family relevant for drug metabolism in insects were downregulated by 68N.M in *A. gosspyii.* Moreover, *glutathione S-transferase 1* was found to be downregulated by -3.08-fold at 0.25% w/v and even more downregulated by -6.30-fold at 1.0% w/v of 68N.M (Table 6-2).

In *D. melanogaster*, there were quite a few DEGs encoding receptors (common targets of chemicals) induced by 68N.M. Two DEGS, *Gr89a* and *Gr98a*, encoding gustatory receptors were found to be significantly induced by 68N.M, whereas Gr89a was upregulated by 1.75-fold at 0.25% but downregulated by -3.02-fold at 1.0% w/v 68N.M. *Glutamate receptor IIB* (*GluRIIB*) was significantly downregulated by both 0.25% and 1.0% 68N.M (Table 6.3). Moreover, Ir56b (Ionotropic receptor 56b) of *D. melanogaster* showed significant downregulation in response to 68N.M. by up to -2.84-fold (Table 6.3). Leucine-rich repeat-containing G protein-coupled receptor 3 (Lgr3) was also significantly downregulated by 68N.M. Interestingly*, Odorant receptor 98a* (*Or98a*) was upregulated by 0.25% 68N.M, but significantly downregulated by 1.0%. Apart from the receptor genes, another novel DEG *brain-specific homeobox* (*bsh*)was upregulated by up to 3.46-fold. There were two membrane transporters, *Vacuolar H^+ ATPase AC39 subunit 2* (*VhaAC39-2*) and *Zinc transporter 33D* (*ZnT33D*), which were significantly upregulated by the lower concentration of 68N.M but downregulated by the higher concentration of 68N.M (Table 6.3). In both insect species, other

uncharacterized DEGs significantly regulated by 68N.M may also have novel roles in insecticidal response, which requires investigation in the future.

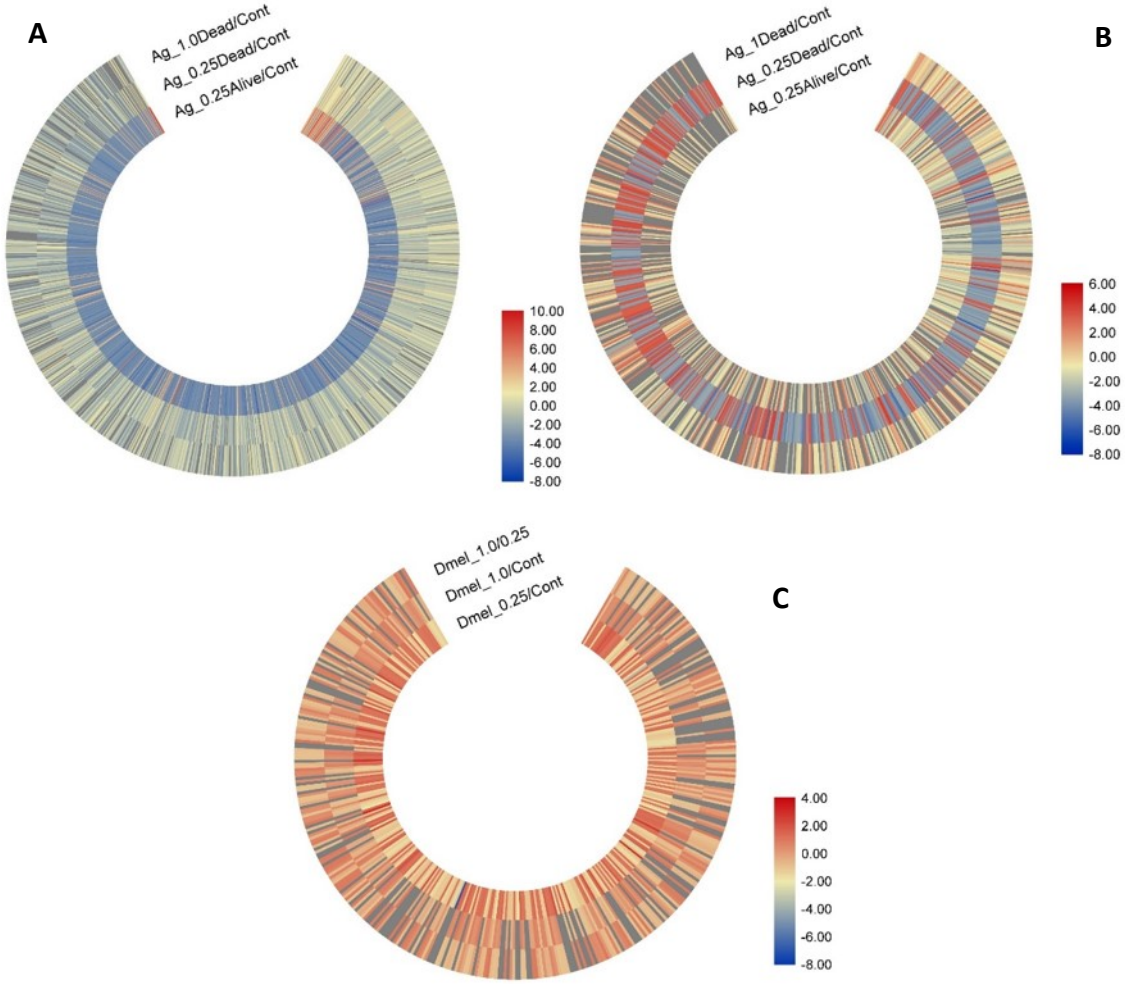


Figure 6-8 Heatmap overviews of differentially expressed genes after extract 68N.M treatment.

(**A**) Summary of 1101 DEG from *A. gossypii* alive 0.25% w/v compared to control <-3 Log_2FC >3 (inner-ring) and those same genes in the other two treatment scenarios. (**B**) Summary of 615 DEG from *A. gossypii* dead 0.25% w/v compared to control <-3 Log_2FC >3 (mid-ring) and those same genes in the other two treatment scenarios. (**C**) Summary of 391 DEG from *D. melanogaster* alive 0.25% w/v compared to control (inner-ring) and those same genes in the other two treatment scenarios. The bar on the right side of the heatmap represents relative expression level of DEGs. Heatmaps were generated by TBtools (v 0.6735). The DEGs were ascending ordered according to the gene name.

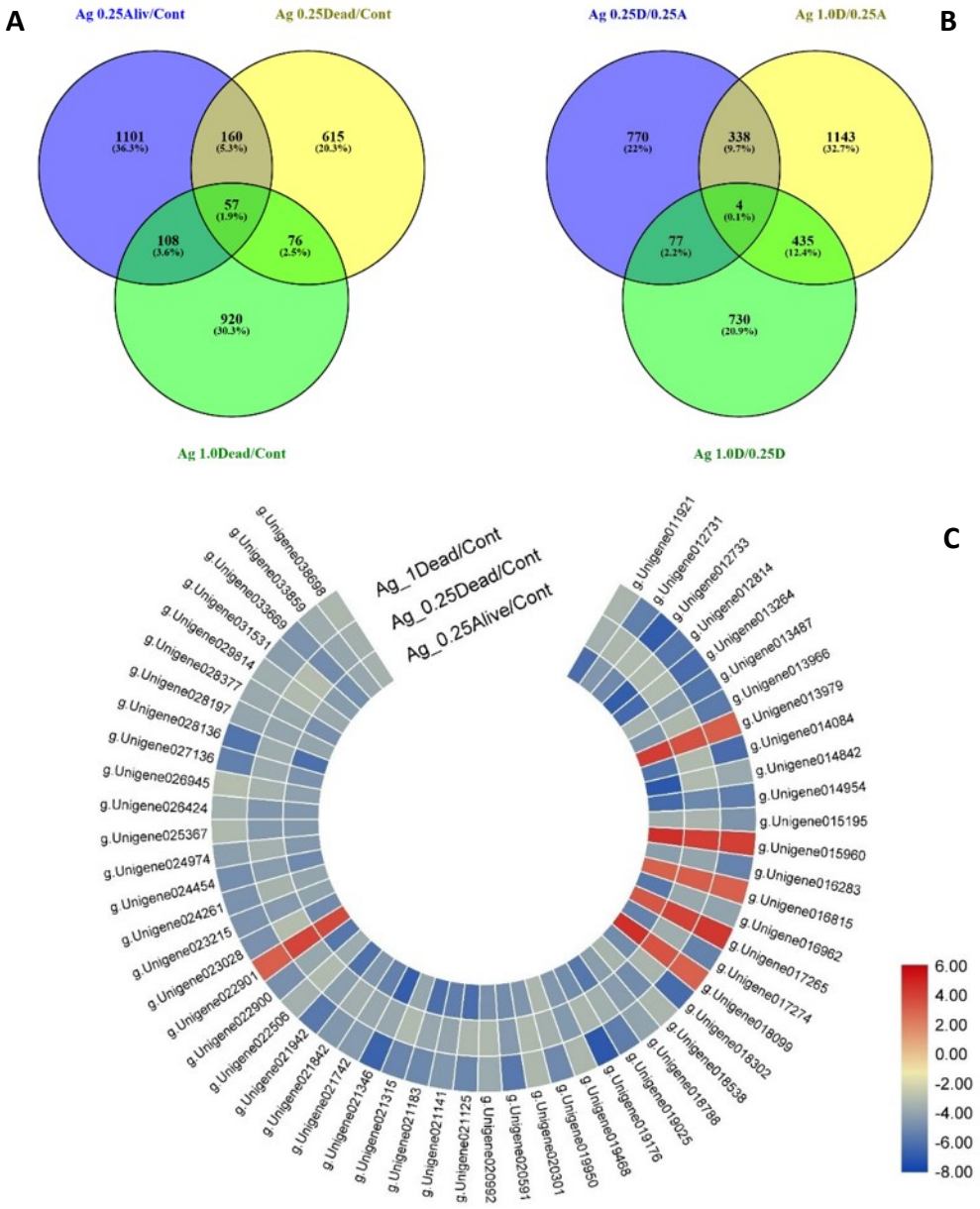


Figure 6-9 Venn diagrams and heatmap of *A. gossypii* DEGs after extract 68N.M treatment.

Venn diagram of differentially expressed genes, <-3 Log_2FC >3, in *A. gosspyii* from (**A**) each treatment outcome compared to control (**B**) dead specimens compared to surviving or dead from 0.25% w/v. (**C**) Heatmap of the 57 shared differentially expressed genes identified between each treatment and control. The bar on the right side of the heatmap represents relative expression levels of DEGs. Venn diagrams where generated by Venny 2.1, heatmap was generated by TBtools (v 0.6735).

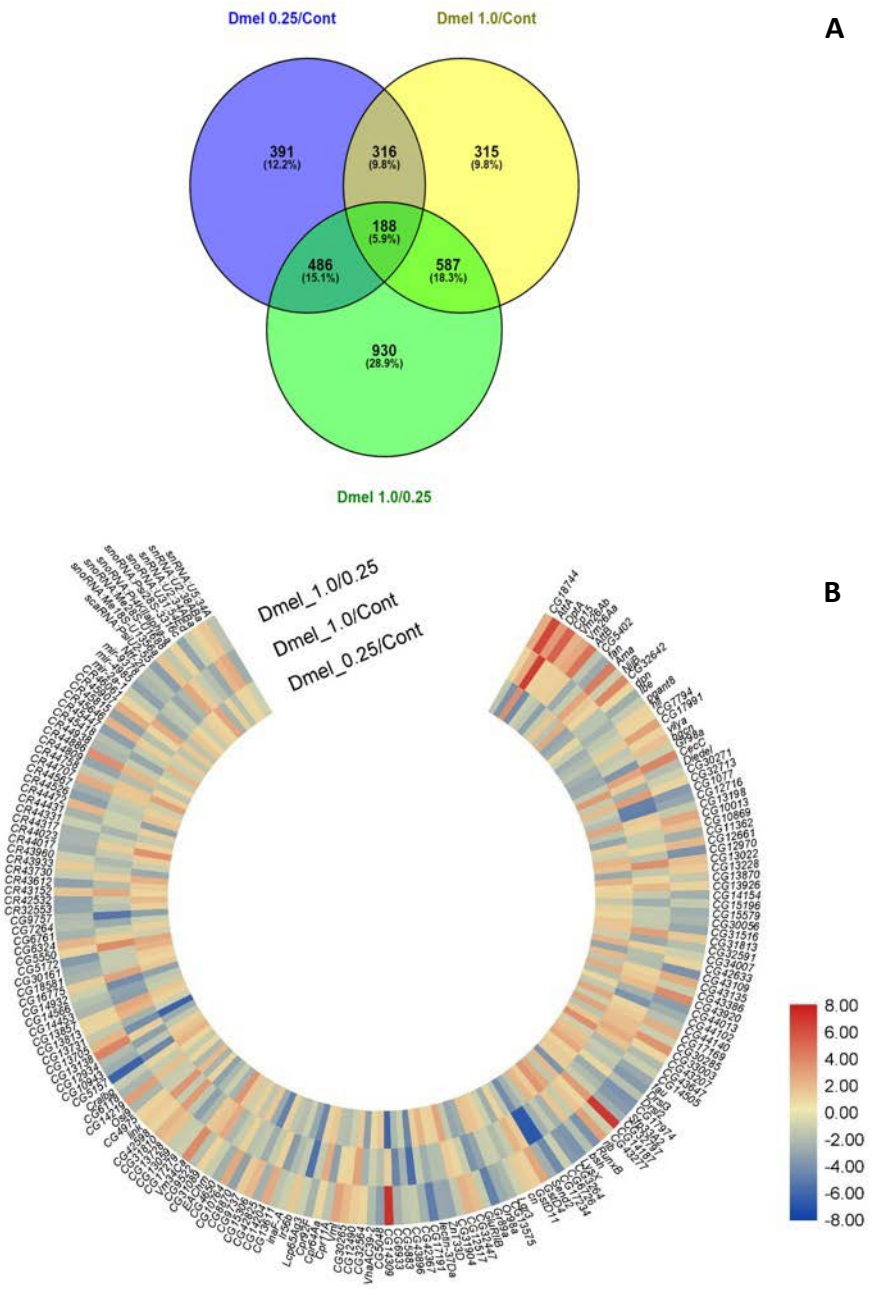


Figure 6-10 Venn diagram and heatmap of *D. melanogaster* DEGs after 68N.M treatment.

(**A**) Venn diagram of differentially expressed genes, <-1 Log_2FC >1 from each treatment outcome 0.25% w/v and 1.0% w/v compared to control, and 1.0% w/v compared to 0.25% w/v. All *D. melanogaster* survived each treatment. (**B**) Heatmap of the 188-shared, differentially expressed genes identified all treatment groups. The bar on the right side of the heatmap represents relative expression levels of DEGs. Venn diagrams where generated by Venny 2.1, heatmap was generated by TBtools (v 0.6735).

Table 6-2 Summary of 39 high-confidence DEGs out of the 57 overlapping DEG common to all treatments of *A. gossypii* shown in Figure 6-9A & C.

Gene locus	Ag_0.25 Dead/Cont	Ag_0.25 Alive/Cont	Ag_1.0 Dead/Cont	E.value	similarity	*Gene name*
g.Unigene028136	-3.68	-6.10	-5.89	1.10E-50	50.49	*2,3-bisphosphoglycerate-independent phosphoglycerate mutase-like [Plutella xylostella]*
g.Unigene013487	-4.22	-3.41	-5.88	1.29E-24	42.16	*2-oxoglutarate-dependent dioxygenase At3g111800 [Folsomia candida]*
g.Unigene020591	-4.42	-4.95	-5.74	2.88E-55	77.33	*40S ribosomal protein S19a [Dinoponera quadriceps]*
g.Unigene023028	-3.03	-4.98	-4.76	3.52E-28	88.09	*40S ribosomal protein S26-like [Parasteatoda tepidariorum]*
g.Unigene024261	-3.40	-3.98	-4.72	3.87E-84	77	*40S ribosomal protein S3a [Nephila clavipes]*
g.Unigene021125	-3.25	-6.20	-5.21	2.29E-62	80.33	*40S ribosomal protein SA-like [Limulus polyphemus]*
g.Unigene021315	-3.10	-3.92	-5.23	4.29E-98	72.8	*60S acidic ribosomal protein P0 [Apis cerana]*
g.Unigene026424	-4.57	-4.52	-3.52	5.04E-27	83.13	*60S ribosomal protein L15-like [Limulus polyphemus]*
g.Unigene019950	-3.53	-3.25	-5.07	2.21E-65	86.51	*60S ribosomal protein L19 [Halyomorpha halys]*
g.Unigene021141	-3.98	-5.52	-4.52	2.05E-26	65.05	*60S ribosomal protein L24-like [Rhagoletis zephyria]*
g.Unigene018302	-3.35	-4.96	-6.26	1.75E-85	77.99	*60S ribosomal protein L7 [Pseudomyrmex gracilis]*
g.Unigene021346	-3.87	-6.82	-6.60	4.40E-150	75.95	*adenosine kinase 2 [Dinoponera quadriceps]*
g.Unigene014084	-3.13	-5.97	-6.23	2.20E-174	95.25	*alpha-tubulin-1 [Coptotermes formosanus]*
g.Unigene012733	-3.07	-5.07	-6.88	1.61E-40	54.68	*aquaporin AQPAe.a-like isoform X1 [Hyalella azteca]*
g.Unigene016962	-3.81	-5.76	-4.03	0	91.37	*ATP synthase subunit beta, mitochondrial [Agrilus planipennis]*
g.Unigene014954	-5.11	-6.28	-5.51	3.00E-143	56.58	*Fructokinase-1-like [Monomorium pharaonis]*
g.Unigene021742	-3.86	-5.39	-4.40	1.05E-31	64.7	*cytochrome b5 [Bactrocera latifrons]*
g.Unigene014842	-3.10	-7.10	-3.79	1.80E-48	44.01	*cytochrome c peroxidase, mitochondrial-like [Folsomia candida]*
g.Unigene022900	-3.84	-5.79	-4.80	7.04E-49	80.24	*cytochrome c-like isoform X1 [Galendromus occidentalis]*
g.Unigene024974	-3.51	-4.46	-4.24	7.81E-13	57.18	*Endoplasmic reticulum protein ERp29, putative [Pediculus humanus corporis]*
g.Unigene016283	-3.93	-4.24	-5.33	0	79.72	*Enolase [Nilaparvata lugens]*
g.Unigene021842	-3.57	-4.52	-4.52	1.20E-102	81.72	*Enolase-like [Tetranychus urticae]*
g.Unigene019025	-3.52	-5.79	-5.58	5.91E-17	50.88	*Fructokinase-1-like [Monomorium pharaonis]*

g.Unigene028377	-3.84	-4.79	-3.80	3.39E-25	60.44	*GH14669 [Drosophila grimshawi]*
g.Unigene013264	-3.08	-6.30	-6.23	2.24E-20	49.29	*glutathione S-transferase 1-like [Spodoptera litura]*
g.Unigene013966	-3.19	-4.64	-5.63	0	90.66	*heat shock 70 kDa protein [Haemaphysalis flava]*
g.Unigene020992	-3.21	-4.74	-3.75	1.63E-23	73.28	*heat shock 70 kDa protein cognate 4 [Solenopsis invicta]*
g.Unigene021183	-3.85	-6.12	-5.13	1.93E-66	81.82	*heat shock protein 70 kDa, partial [Necora puber]*
g.Unigene033669	-4.94	-4.89	-4.67	3.70E-15	68.17	*hypothetical protein D910_05527 [Dendroctonus ponderosae]*
g.Unigene012731	-3.25	-4.50	-5.49	4.00E-148	72.47	*hypothetical protein QR98_0103270 [Sarcoptes scabiei]*
g.Unigene017265	4.04	3.34	4.32	1.95E-78	84.81	*neurogenic locus notch homolog protein 1-like, partial [Diuraphis noxia]*
g.Unigene018538	-4.95	-3.38	-3.28	5.72E-89	55.94	*nicotianamine synthase-like [Bemisia tabaci]*
g.Unigene022901	3.93	3.78	3.07	1.53E-38	84.21	*protein lozenge-like isoform X1 [Diuraphis noxia]*
g.Unigene033859	-3.92	-3.86	-3.65	2.02E-39	87.21	*titin, partial [Acyrthosiphon pisum]*
g.Unigene018788	-4.31	-4.25	-4.04	2.63E-22	84.39	*ubiquitin/ribosomal S30 fusion protein [Penaeus monodon]*
g.Unigene015960	4.13	4.61	4.09	2.44E-22	72.35	*uncharacterized protein LOC107165419 isoform X1 [Diuraphis noxia]*
g.Unigene017274	-3.60	-5.36	-5.37	6.99E-13	47.34	*uncharacterized protein LOC108680782 [Hyalella azteca]*
g.Unigene013979	3.52	4.17	3.11	4.91E-16	63.14	*uncharacterized protein LOC111032991 isoform X1 [Myzus persicae]*
g.Unigene028197	-3.98	-3.93	-3.94	2.79E-11	57.37	*uncharacterized protein LOC111681881 [Lucilia cuprina]*

Note: Genes without gene names highly differentially expressed at cut-off thresholds of <-3 Log_2FC >3, have been removed, leaving only high-confidence DEGs.

Table 6-3 Summary of 51 high-confidence DEGs out of 188 overlapping DEGs common to all treatments of *Drosophila melanogaster* shown in Figure 6-10A & B.

Gene locus	Gene name	Dmel_0.25/Cont	Dmel_1.0/Cont	Dmel_1.0Alive/0.25Alive	Gene description
FBgn0000071	*Ama*	-1.55	2.83	4.39	Amalgam
FBgn0012042	*AttA*	-3.88	2.56	6.44	Attacin-A
FBgn0041581	*AttB*	-3.33	1.41	4.75	Attacin-B
FBgn0004581	*bgcn*	-2.87	-1.09	1.78	benign gonial cell neoplasm
FBgn0000529	*bsh*	3.46	1.67	-1.79	brain-specific homeobox
FBgn0000279	*CecC*	-1.26	2.82	4.08	Cecropin C
FBgn0035636	*Cralbp*	2.33	1.14	-1.19	Cellular retinaldehyde binding protein
FBgn0000355	*Cp15*	-4.26	1.15	5.41	Chorion protein 15
FBgn0000337	*cn*	-2.62	-1.26	1.36	cinnabar
FBgn0030394	*Cpr11A*	-1.75	-3.37	-1.62	Cuticular protein 11A
FBgn0035510	*Cpr64Aa*	1.56	2.62	1.05	Cuticular protein 64Aa
FBgn0038819	*Cpr92F*	1.13	-1.27	-2.4	Cuticular protein 92F
FBgn0010109	*dpn*	-2.6	-1.59	1	deadpan
FBgn0004240	*DptA*	3.66	6.87	3.21	Diptericin A
FBgn0052279	*Drsl2*	2.75	1.05	-1.7	Drosomycin-like 2
FBgn0052283	*Drsl3*	2.02	-1.54	-3.56	Drosomycin-like 3
FBgn0036470	*EAChm*	-1	-2.14	-1.14	Enhancer of Acetyltransferase Chameau
FBgn0028379	*fan*	1.6	-1.17	-2.77	farinelli
FBgn0020429	*GluRIIB*	-2.6	-3.85	-1.25	Glutamate receptor IIB
FBgn0038029	*GstD11*	1.09	2.29	1.19	Glutathione S transferase D11
FBgn0010040	*GstD4*	-1.39	1.4	2.79	Glutathione S transferase D4
FBgn0038440	*Gr89a*	1.75	-3.04	-4.79	Gustatory receptor 89a
FBgn0039520	*Gr98a*	-2.67	-3.71	-1.04	Gustatory receptor 98a
FBgn0028519	*hll*	-2.62	-1.49	1.13	heimdall

FBgn0034456	*Ir56b*	-2.84	-1.3	1.54	Ionotropic receptor 56b
FBgn0011278	*lbe*	1.01	-1.61	-2.62	ladybird early
FBgn0086611	*Lcp65Ag3*	-4.51	-5.56	-1.04	Larval cuticle protein
FBgn0039354	*Lgr3*	-1	-2.01	-1.01	Leucine-rich repeat-containing G protein-coupled receptor 3
FBgn0004431	*LysX*	1.41	-2.43	-3.84	Lysozyme X
FBgn0262377	*mir-2a-1*	-1.55	-2.82	-1.27	mir-2a-1 stem loop
FBgn0263546	*mir-4985*	1.6	-1.61	-3.21	mir-4985 stem loop
FBgn0283551	*mir-9378*	1.02	-1.17	-2.18	mir-9378 stem loop
FBgn0036822	*NijB*	2.62	1.54	-1.08	Ninjurin B
FBgn0032680	*Ntf-2r*	-1.12	-2.89	-1.77	Nuclear transport factor-2-related
FBgn0039551	*Or98a*	1.59	-1.19	-2.78	Odorant receptor 98a
FBgn0037416	*Osi9*	1.03	-1.17	-2.19	Osiris 9
FBgn0036529	*pgant8*	-1.13	-2.39	-1.25	polypeptide GalNAc transferase 8
FBgn0003254	*rib*	-1.42	1.59	3	ribbon
FBgn0259162	*RunxB*	2.68	-1.2	-3.89	Runt related B
FBgn0259963	*Sfp33A2*	1.48	-2.02	-3.49	Seminal fluid protein 33A2
FBgn0263485	*scaRNA:PsiU2-55*	1.24	-2.12	-3.37	small Cajal body-specific RNA : PsiU2-55
FBgn0004191	*snRNA:U2:34ABa*	1.01	2.14	1.14	small nuclear RNA U2 at 34AB a
FBgn0003922	*snRNA:U2:38ABa*	1.02	2.61	1.59	small nuclear RNA U2 at 38AB a
FBgn0003935	*snRNA:U5:34A*	-1	-2.61	-1.61	small nuclear RNA U5 at 34A
FBgn0264253	*Send2*	-4.25	-6.72	-2.47	Spermathecal endopeptidase 2
FBgn0039058	*VhaAC39-2*	1	-1.02	-2.02	Vacuolar H[+] ATPase AC39 subunit 2
FBgn0003979	*Vm26Aa*	-1.49	1.24	2.73	Vitelline membrane 26Aa
FBgn0003980	*Vm26Ab*	-1.07	1.21	2.29	Vitelline membrane 26Ab
FBgn0003983	*Vm34Ca*	-2.39	1.5	3.89	Vitelline membrane 34Ca
FBgn0085362	*Vml*	-1.92	1	2.92	Vitelline membrane-like
FBgn0051860	*ZnT33D*	1.24	-1.82	-3.06	Zinc transporter 33D

6.4. Discussion

6.4.1. Gene expression analysis indicates the potential of 82N targeting cellular signaling and ion channels of neurons in *Drosophila melanogaster*

Nitric oxide (NO) is a secondary messenger molecule and free radical acting as a neurotransmitter, hormone, neuronal and synapse development signalling agent (Ball et al. 1998; Truman et al. 1996), and ion and fluid regulator. NO is synthesised by many cells and involved in multiple processes (Zayas et al. 2000). In invertebrates, the family of enzymes that form nitric oxide are called nitric oxide synthases (NOS) and seem to be controlled by a single gene in *D. melanogaster, Dmel\NOS* (Davies 2000; Rivero 2006). Inducible nitric oxide synthases (iNOS) are well known for their involvement in immunity and inflammation responses, increasing *Drosophila* survival rates against bacterial and other pathogenic infections (Rivero 2006). Studies of long-term adaptation and short-term stress response to beta-cypermethrin in resistant cockroaches has shown an up-regulation of NOS production in beta-cypermethrin-resistant strains compared to susceptible strains but a down-regulation NOS as a short-term response in those same beta-cypermethrin-resistant strains after direct exposure (Yang et al. 2019). Our results indicate the *NOS* gene related to nitric oxide production may play a role in response to the exogenous challenge of extract 82N (Figure 6-2).

No action potential upregulation in *D. melanogaster* after exposure to 82N 2.0% w/w (Figure 6-2) may indicate this extract is a nerve toxin, since *nap* affects nerve-membrane excitability. Initially this was found to cause temperature-sensitive paralysis but later mutant studies showed this gene affects sodium-channel activation and inactivation, causing nerve-membrane abnormalities regardless of temperature (Ganetzky et al. 1986). *Open rectifier K_+ channel 1* (*ORK1*) gene codes for a potassium channel which functions to rectify K^+ conductance along myelinated nerves, located in the central and peripheral nervous systems Although this gene is not heavily investigated in terms of it being an insecticidal mode of action, it may be the likely target for extract 82N found to date. My MIFE ion flux studies on

D.mel-S2 cells support the inference that K^+ loss is a likely MOA, with detrimental effects resulting in immediate efflux that cannot be recovered in a time-sensitive manner and remains significantly different from pre-treatment conditions during the stabilisation phase (Figure 5-11). Three genes *Irk1, Irk2 and Irk3* encoding inwardly rectifying potassium channels in the *Drosophila* genome resulted in a statistical significance by 82N treatment between either control and one or two treatment groups of adult flies. *Irk2* is thought to play a role in fly osmoregulation due to its high expression in malpighian tubules and the hindgut, but is also expressed in the head, eyes and brain, indicating that this may also regulate neuronal signalling (Luan et al. 2012). *Irk2* which has been previously reported to show high expression in *Drosophila* adults in digestive and excretive tissues, corresponding to its role in immune responses (Luan et al. 2012). This study showed that *Irk2* was significantly upregulated in sick and surviving treated flies compared to control survivors, giving clues to the extract's possible target sites. Interestingly, the significant upregulation of *IRK3* recorded in sick flies had reverted to normal expression in survivors, leading one to propose if this gene may be a candidate for future resistance to this extract. This may also indicate that extract 82N's MOA is in some way involved in GABAergic processes since some GABA receptors are known to couple with IRK channels, and starvation experiments that knockdown Irk3 in *D. melanogaster* elicited the same starvation-stress response as the $GABA_BR2$ knockdown mutants (Enell et al. 2010). Furthermore, IRK3 is reported to be susceptible to reactive oxygen species, unlike other potassium channels (Duprat et al. 1995).

The lack of significant regulation of *GSTE1* and *GLUCL* by 82N suggests that their related oxidative stress detoxification pathway and synaptic transmission mediation roles, respectively, can be ruled out as possible MOAs. *KCMF1* has not been investigated for its role in insecticide survival; however, I chose to include it because some investigations suggest that *KCMF1* is a key component in multiple pathways. KCMF1 binds to RAD6, allowing a bridge to UBR4 which is involved in bulk lysosome-mediated degradation and autophagy. Hong et al. (2015) suggests that this is important, as a disruption of the RAD6-KCMF1-UBR4 complex may lead to a toxic build-up of proteins to negatively affect neuronal function in humans. Ashton-Beaucage et al. (2016) report that *KCMF1* is also a key component modulating mitogen-activate protein kinase (MAPK) levels and may act similarly to the Ras/MAPK negative

regulating protein purity of essence (*POE*) gene. Reports of the importance of the MAPK-signalling pathway in terms of insecticide resistance and xenobiotic responses is increasing (Canton et al. 2015; Guo et al. 2015; Wei et al. 2019; Yang et al. 2020). The highly upregulated *nap* reflected the Na^+ flux seen in the electrophysiology data. A neuropathology study in 2006 suggests that impaired neuronal excitability (seen in *nap* mutants) as well as increased excitability can trigger neurodegeneration (Fergestad et al. 2006). *Drosophila melanogaster nap* larval mutant experiments show increased sensitivity to the potent neurotoxin tetrodotoxin (TTX), which binds to voltage-gated Na^+ channels, blocking Na^+ and inhibiting action potentials (Ganetzky et al. 1986).

6.4.2. Whole organism transcriptome provides insight into understanding the molecular target of novel extract 68N.M

In the transcriptome profiling, the wider scatter plots of *A. gossypii* (Figures 6-4 and 6-5) in comparison to *D. melanogaster* (Figures 6-6) may indicate that the physiology of *D. melanogaster* has provided protection from the effects of 68N.M, and that the active compound/s may not have been able to reach their target site/s. It is also possible that *D. melanogaster* has a superior signalling transduction, receptor, detoxification or other genetic mechanism by which to resist its toxic effects (Tables 6-2 and 6-3).

Gene ontology and the KEGG pathway enrichment analysis can offer insight into compound-target interactions and assist in classifying pathway for MOA investigation (Chen et al. 2015). In my study, the KEGG pathway was useful in placing DEG from the *A. gossypii* species into categories that may otherwise prove challenging given the multiple genomes used. In any case, this allowed me to summarise the potential MOAs of extract 68N. For instance, two of the categories identified cytochrome P450 pathways; this is not surprising as cytochrome P450s (CYPs) have been frequently reported in both insecticidal detoxification and resistance (Coelho et al. 2015). Although P450 genes are highly conserved and form one of the largest superfamilies, the P450 enzymes are also located in multiple insect tissues (Feyereisen 1999). Early studies of insect feeding on plants such as tobacco, lead to the hypotheses that midgut P450 enzymes where responsible for detoxification following ingestion of plant toxins (Feyereisen 1999; Krieger et al. 1971). In mammals the cytochrome P450 reductase seems to

be a close homolog for NOS (Bredt et al. 1994), additionally both cytochrome P450 and NOS can generate ROS species (Lambeth et al. 2014). It is not surprising then, that this pathway would be identified as involved in extract 68N.M's MOA. However, additional molecular information will be revealed when a complete *A. gossypii* genome is available in the near future.

6.4.3. Transcriptomic insights in response to extract 68N.M in *A. gossypii*

There was an overall trend of downregulation of the common DEGs in response to 68N.M in *A. gossypii*. There are some key genes identified in this study. For the only three upregulated DEGs, g.Unigene017265 was mapped to the predicted neurogenic locus notch homolog protein 1-like using the Russian wheat aphid genome, *Diuraphis noxia* (Hemiptera: Aphididae). Notch is a highly conserved family of type-1 transmembrane proteins that form a core component of the Notch signalling pathway in animals. Notch proteins is a receptor for membrane-bound ligands to regulate cell-fate and represses differentiation of neurons and muscle cells (Mirandola et al. 2009), indicating that 68N.M may target this receptor to affect the function of neurons and muscles in *A. gossypii* for insecticidal action. Another novel upregulated gene, g.Unigene022901, was mapped to the predicted protein lozenge-like isoform 1 in *D. noxia* (Table 6-2). The *lozenge* gene was demonstrated to be potentially important in pre-patterning photoreceptor precursors in fruit fly developing eyes (Daga et al. 1996), regulating neuronal activity (Goulding et al. 2000) and causing programmed cell death (Wildonger et al. 2005), which may also be linked to insecticidal activity.

In response to 68N.M, the most abundant DEGs in *A. gossypii* were those encoding 40S and 60S ribosomal proteins (Table 6-2). It has been demonstrated that the 40S and 60S ribosomal proteins have an extra-ribosomal role as an endonuclease involved in the repair of animal DNA damage. Although 40S and 60S ribosomal proteins were not well-documented in aphids, they are reported to have a key role in RNA-binding and translational repression in *D. melanogasterl* (Gebauer et al. 2003). Downregulation of these genes is likely to result in the malfunction of RNA-binding and protein translation, which could be lethal to any living organisms. Moreover, some of the glutathione S-transferases are associated with feeding adaptation to host plants (Zou et al. 2016) and in response to detoxification and oxidative

stress (Xu et al. 2016) in *Spodoptera* spp. Therefore, the downregulation of *glutathione S-transferase 1-like* in *A. gossypii* by 68N.M may reduce the capacity of chemical detoxification and increase oxidative stress in cotton aphids. Last, but not least, the ApAQP1 transcript in pea aphid (*Acyrthosiphon pisum*) was localised to the stomach and distal intestine, and RNAi-mediated knockdown of its expression resulted in elevated osmotic pressure of the haemolymph, suggesting that ApAQP1 contributes to the molecular basis of water cycling in the aphid gut (Shakesby et al. 2009). In this study, a -5.07 downregulation of aquaporin *AQPAe.a-like isoform X1* by 1.0% w/v 68N.M in *A. gossypii* may indicate the loss of function in water flux to sustain the normal physiology and osmoregulation of the cotton aphid.

6.4.4. Transcriptomic insights in response to extract 68N.M in *D. melanogaster*

In response to 68N.M, the gene expression pattern in *D. melanogaster* was substantially different from that in *A. gossypii*. There were a number of receptor genes (usual targets of chemicals) induced by the novel botanical extract 68N.M in *D. melanogaster* including *Gr89a* and *Gr98a* encoding gustatory receptors, *Glutamate receptor IIB* (*GluRIIB*), Ionotropic receptor 56b (Ir56b) Leucine-rich repeat-containing G protein-coupled receptor 3 (Lgr3) and *Odorant receptor 98a* (*Or98a*) (Table 6.3; Figure 6-10). The gustatory receptors are a divergent group of transmembrane chemoreceptors that are expressed in gustatory sensilla receptor neurons of *D. melanogaster*, which detect non-volatile compounds via contact chemosensation (Montell 2009). Moreover, it was shown that Gr28b is required for thermotaxis, Gr43a serves as a nutrient sensor, and Gr21a and Gr63a act in the detection of CO_2 (Montell 2013; Ni et al. 2013). GluRIIB was reported to have glutamate-gated calcium ion channel activity and ionotropic glutamate receptor activity involved in regulation of postsynaptic membrane of *D. melanogaster* (Qin et al. 2005). Ir56b receptor is a membrane-bound ligand-gated ion channel, which have been identified as chemosensory receptor able to detection internal and external chemical stimuli in *D. melanogaster* (Benton et al. 2009). Lgr3 is one of the neuropeptide and protein hormone receptors belonging to the large superfamily of G-protein-coupled receptors (GPCRs), which modulate many important processes such as development, reproduction, homeostasis and behavior (Hauser et al. 2006). Odorant receptor 98a (Or98a) belongs to the multi-transmembrane chemoreceptor

superfamily that mediates response to volatile chemicals in insects (Hallem et al. 2006). Moreover, *brain-specific homeobox* (*bsh*) encodes a putative transcription factor required for the specification of neural type identities in the adult brain (Hasegawa et al. 2013). Therefore, these DEGs encoding the key receptors and transcription factors may be the novel targets for functional analysis in flies and aphids using gene overexpression and RNAi technology in the future.

From the 188 overlapping DEGs of *D. melanogaster* after all treatment by 68N, I investigated the most commonly occurring protein families and found six down-regulated genes belonged to the Trypsin family (PF00089), five down-regulated belonging to the insect cuticle protein family (PF00379), three highly down-regulated belonging to the carbohydrate-binding module family (PF01607), and two up-regulated genes belonging to the attacin N-terminal region family (PF03768) (Figure 6-10). The genes belonging to the trypsin family are enzymes involved in protein metabolism, with all genes belonging to the S1A serine proteases. The down-regulation of this family of genes suggests extract 68N may have a protease inhibitor MOA. Proteinase inhibitors bind to digestive enzymes, and reduce protein digestion, causing a range of effects from anti-feeding, growth retardation and reduced reproduction (Babu et al. 2010; Pilon et al. 2018). Pilon et al. (2018) report the reversible competitive trypsine and serine protease inhibitor, benzamidine, as having insecticidal characteristics causing reduced midgut proteolytic activity, increased larval mortality, and reduced feeding and oviposition. When we consider insect immunity mechanisms, the cuticle plays a dual role of both a physical and chemical barrier to microbe attack and pesticide application (James et al. 2012). From my transcriptomic data, most cuticle genes (CG42367, Cpr11A, Cpr92F, Lcp65Ag3) were down-regulated in every treatment group and range from -1.04 to -5.56 (Lcp65Ag3), whilst one gene (Cpr64Aa) was up-regulated in every group from 1.05 to 2.62. Both are inferred from a sequence model in flybase.org as being involved in chitin-based cuticle development.

Two very highly upregulated attacin genes, *AttA* and *AttB*, (were identified in the transcriptomic analysis of *D. melanogaster* after extract 68N treatment. Attacins have been identified as antimicrobial proteins (AMPs) involved in multiple defence signalling pathways and are strongly expressed in injured flies (Dushay et al. 2000; James et al. 2012). In a study

of the synergistic effects between the neonicotinoid insecticide imidacloprid and the entomopathogenic fungus *B. bassiana,* Farooq et al. (2018) reported attacin gene expression greatly increased after insecticide application (79-fold increase), but to a far lesser degree after entomopathogen application (3-fold), suggesting that attacin plays a role in insecticidal detoxification beyond its known antimicrobial defence. James & Xu (James et al. 2012) suggest that attacins play a role in the complex cellular immune response, which involves melanin production leading to formation of reactive oxygen species, which are in turn regulated through the phenoloxidase cascade, resulting in an interdependent defensive system. Attacins are involved in detoxification mechanisms utilized by insects to prevent damage from environmental toxins (James et al. 2012), providing a potentially useful target for biopesticide discovery and insect immunity.

In summary, the two commonly used methods, qPCR and RNA-seq, for gene expression studies have provided useful insights for the potential insecticidal MOA of the novel extracts of 82N in *D. melanogaster* and 68N.M in both *D. melanogaster* and *A. gossypii.* However, detailed functional analysis of these key DEGs should be conducted to further elucidate the insecticidal activity of these extracts and their different efficacy in *D. melanogaster* and *A. gossypii.*

Chapter 7: General Discussion

7.1. General discussion of the thesis

The extraction, refining, formulation and employment of plant secondary compounds for pest management has a long history of use in agriculture (Bassman 2004; Isman et al. 2007; Siegwart et al. 2015; Zhu et al. 1999). The search for new biopesticides with either novel MOAs, sub-lethal effects that can be incorporated with existing chemistry to extend their longevity and slow down the adaption of resistance, or which are highly selective is a high priority task for modern agriculture (Amarasekare et al. 2016). Furthermore, many non-selective insecticides have detrimental effects on pollinators and beneficial natural enemies (Cloyd et al. 2011). The Australian cotton industry quickly adapts technologies and directs substantial resources into supporting research and development. There are over 100 pests that affect Australian cotton, resulting in the high prioritisation of novel 'softer' chemical discovery, verification and commercialisation to support existing biotechnologies and management strategies such as integrated pest and resistance management. Therefore, screening of botanical extracts that go further into elucidating the possible MOA and addressing future resistance and detoxification potential, as seen in this study, are essential in supporting the longevity and sustainability of this industry.

Throughout my study, I have placed a heavy emphasis on nervous system changes elicited from the botanical extracts; this is because the nervous system is central to insect survival and historically has been a target for insecticides. It is integral to multiple functions and inter-related behaviours, such as the detection of environmental cues through sensations, the coordination of motor responses, homeostasis through secretory activity, and the processing of information (Scharf et al. 2008). Thus, nerve-poisoning type MOA account for the vast majority of insecticides. Researchers over many decades have focussed on elucidating how nerve-poisons bind with target sites, mimic and interrupt messenger molecules and trigger genetic regulation and mutations. For instance, the *Drosophila* gene *para* encodes for functional voltage-dependant sodium channels (Ganetzky 1986; O'Dowd et al. 1989), which have similarly conserved homologous domains in rat brain sodium channels (Loughney et al.

1989), that have been shown to be involved in cation transport and VGSC activation (Lopez-Barneot et al. 1993). However, while studies on ion channels using model vertebrates can help us understand channel excitation and binding capabilities, the similarities may not be close enough to ensure insect-specific channel behaviours are captured. In my studies therefore, I used model insects and cell lines that can also offer the advantages of reference genomes, ease of culturing and a plethora of literature. I also employed key cotton pests (target species related to funding from CRDC) as well as commercially available cell line in the same family as the key pest, cotton bollworm, *H. armigera*. Furthermore, I have investigated beyond the nervous-system to encompass the broad potential MOAs that botanical insecticides may offer.

To recap my PhD studies and this thesis, chapter 1 provides a general overview of the use of pesticides in the Australian cotton industry and the benefit that biopesticides, such as *Bt* cotton, have provided, highlighting that new options are needed, and that novel botanical pesticides can be used to address this need. I have also provided a literature review that explores many of the MOAs currently identified in pesticides, and how some of these are being explored to understand resistance development in pests notwithstanding the complexity of the cross discipline knowledge needed to facilitate this understanding. Finally, I have outlined the approaches I selected with regard to screening novel insecticidal botanical extracts and elucidating their possible MOAs, with the aim of supporting the larger foundation project and addressing the goals of the Cotton Research and Development Corporation, on behalf of the Australian Cotton industry.

In chapter 2, as a general methods and materials chapter, I have provided a brief background to the selection and culturing of the target insects and plant extracts used in the foundation project and my studies as well as the bioassay techniques employed. I particularly addressed the cell lines used in my work and the general considerations implemented to ensuring the use of reliable protocols and data collection. I have also provided an overview of the results in the foundation project and how those led to the ranking and selection of the 20 extracts investigated in my study.

Chapter 3 has described novel methodologies and reported results that highlight the use of multiple, interdisciplinary techniques to assist in evaluating novel plant extract for medicinal, agricultural or entomological development. Although commercial drug-discovery uses more advanced, high throughput screening techniques than I have employed, several issues became apparent during my literature review:

1.) the open sharing of techniques and transparency of methodologies is limited, especially from a bio-prospecting point of view,

2.) limited numbers of similar cell lines, usually expressing particular MOA target sites are used for specific investigations, which leads to micro-specific inferences of results, making studies difficult to compare, and therefore, evaluation for industry applicability difficult,

3.) MOA elucidation is approached from a single-discipline framework, testing against highly-likely target sites based on a former knowledge of the chemical structure or pre-existing cross-resistance to long established commercial products.

Our manuscript offers a triangulation of methods, easily adopted in most laboratories to screen botanical extracts of unknown constituents for the quick and reliable assessment of insecticidal toxicity and insight into likely MOA pathways. This chapter primarily, along with parts of other chapters, addresses the first object to evaluate a range (including novel) of methodologies to investigate activity of botanical pesticides.

Chapter 4 has reported investigations of some of the top 20 extracts (Table 2-4) from the foundation project (Table 7-4, Appendix 3), using a variety of whole organism bioassays. In many cases, modern screening methodology has bypassed whole organism target pest bioassays in favour of high throughput, time efficient discovery assays which, at some point, must return to entomological studies to be verified. I have taken the opposite approach; verifying initially that extracts have insecticidal activity in vivo, and assessing a wide variety of modes of entry. These results allowed us to narrow my molecular investigations to just two extracts, 68N and 82N, as well as giving us multiple ranges of toxicity, alive (surviving), sick (detrimental) or dead (morbidity), effects to broaden our MOA understanding. Furthermore, it allowed me to rule out certain MOA in the test species at least, with regard to effects on

ovipositon, egg hatching and larval feeding, which can be common indicators of hormonal regulators or growth inhibitors.

Using the foundation project's whole organism bioassay on *H. armigera*, (Table 7-4, Appendix 3) in combination with the self-contamination bioassay of *D. melanogaster* and the Potter spray tower bioassays using *D. melanogaster* (chapter 4) and the cytotoxicity assays on two cell lines (chapter 5) goes some way to qualitatively meeting my second research objective: to challenge *Helicoverpa armigera* caterpillars and *Drosophila melanogaster* flies with selected botanical extracts and assess the correlation between the whole organism level and cellular level using *Spodoptera frugiperda* (Sf9) and Schneider's *Drosophila* 2 (D.mel-S2) cell lines.

Chapter 5 used the methods developed in chapter 3 to investigate cytotoxicity on two "native" insect cell lines using the top 20 extracts. I have also reported on two additional extracts which have recently been identified as having insecticidal action, W11N and W44N. Although these are not novel, they were included for comparison as positive controls, supporting the argument that botanically-derived insecticides may have high potential to offer pest management solutions. Although the chemistry of extract W11N - *Hakea microcarpa* had not been studied, at least seven other *Hakea* species have been investigated and this genus has been found to produce long chain aromatic compounds such as the alkylresorcinol bilobol (Lytollis et al. 2015) and 2-methylgrevillol (Manju et al. 1977). *Hakea* species are also reported to produce toxic cyanogenic compounds (Hanley et al. 2009). This chemotaxonomic review of the literature suggested to us that bioassay guided fractionation of W44 was not a high priority, as this information was in the public domain.

From the foundation study, it was decided that extracts with high activity against *H. armigera* and other target arthropod species but with unexplained chemistry (i.e. the insecticidal activity was not easily attributable to obvious classes of compounds), such as extracts 68N, 72N and 82N, were also prioritised. During my cytotoxicity assays on D.mel-S2 cells however, some of these highly efficacious (non-polar) insecticides did not perform as well as I had expected. This may be explained by their fatty acid constituents, which possess a generic surfactant mode of action and work primarily by disrupting the insect cuticle or cell

membranes, leading to desiccation or cellular damage. However, such activity is relatively slow. Activity in the polar extracts was also deemed interesting since lipophilic compounds such as lipids possess insecticidal activity by virtue of their ability to block the spiracles of insects - thus causing mechanical suffocation. However, non-polar fractions were also found to be directly toxic to cells.

Table 7-1 Summary of extracts efficacious against arthropods and their cytotoxicity to D.mel-S2 cells

Extract	*T. urticae* 24 HAT	*A. gossypii* 24 HAT	*H. armigera* 24 HAT	D.mel-S2 cells 4 HAT
10N	100.00***	100.00***	85.19***	14.4
25N	88.96***	60.00**	11.11	31.2
28N	100.00***	47.06	70.00**	47.5
33N	79.53**	100.00***	100.00***	5.7
55N	96.63***	81.22***	0.00	23.3
62N	83.72***	100.00***	0.00	26.8
68N	98.86***	100.00***	57.81**	34.5
68P	38.57	100.00***	0.00	25.2
72N	28.57	100.00***	100.00***	11.2
72P	13.33	16.67	100.00***	21.9
82N	100.00***	100.00***	100.00***	25.8
82P	49.87	73.68**	0.00	22.2
100N	86.67***	52.50**	14.29	17.9

Note: Very high efficacy was determined as ≥80% mortality and denoted by ***, high efficacy was determined as ≥50% but <80% mortality and denoted by **, whole organisms treated at 1.0% w/v, D.mel-S2 cells treated at 0.01% w/v.

In chapter 5, I reported the effects that a selection of extracts had on ROS production and highlighted that this as an area that while being investigated from a molecular view point, can also provide rapid and complementary screening information. During the ROS tests, it became evident that the same extracts at the same concentration, using the same methods (e.g., 68N at 0.01% w/v), resulted in very different cellular responses from two different insect cell lines. This study met my third objective; namely, to challenge cell lines with potentially insecticidal extracts and measure the elicited stress responses by measuring ROS production of the two cell lines using confocal fluorescence microscopy.

Last, my electrophysiological studies into ion flux movement against the top three extracts provided an excellent insight into MOA potential and multi modal target elucidation. This, in my view, is a significant insight which is very difficult to obtain from all other single-target-focused discovery screening. Although I did not see any ion flux changes in Ca^{2+} or Mg^{2+} in D.mel-S2 cells, there is a greater scope available for researchers than the K^{+}, Na^{+} and Cl^{-} enquiries that I have included. That being said, my results point to all three of the highest priority extracts having significant effects on ion flux, in particular extract 68N, which was

superior to the widely-used botanical insecticide pyrethrum in its ability cause massive efflux in Na^+ flux. Surprisingly, this was not its only MOA, as Cl^- influx was immediate and unrecoverable. These two quantitative measurements make it easy to understand how *A. gossypii* can be rendered immobile directly after direct applications, with haemorrhaging and death within 1 h. This study fulfils my fourth objective: to measure ion transport across membranes of the two cell lines using electrophysiology techniques to discover the target sites and MOAs of novel insecticidal extracts and compounds.

Chapter 6 used two molecular techniques, RT-qPCR and RNA sequencing transcriptomics from two whole organisms, a key cotton pest, *Aphis gossypii* and a model organism with a fully sequenced genome, *Drosophila melanogaster*, to investigate several lines of enquiry. First, extract 82N using *D. melanogaster* flies identified several genes linked to the ion flux results, *ORK1*, *Irk1*, *Irk2*, *Irk3, KCMF1*, which point to K^+ regulation and its roles in neuronal signalling, osmoregulation, immune response and oxidative stress as a possible MOA. Likewise, *nap* and its links to Na^+ regulation support the Na^+ ion flux recorded in MIFE adding to the proposition that botanical insecticides may offer multiple target sites or complex MOA that are advantageous in slowing the resistance potential in target insects. This study also enabled the ruling out of some genes that are often reported as involved in either MOA or insecticide resistance.

Second, the transcriptomic data comparing *A. gossypii* to *D. melanogaster* highlighted a large number of highly differentially regulated genes in *A. gossypii* which have added to our knowledge of the pathways and target sites influenced by 68N. Some gene families targeted by 68N supported my other lines of investigation such as the glutathione S-transferases which encode for proteins involved in oxidative stress, while other groups such as the ribosomal proteins offer insight into new avenues of investigation. Still other genes such as aquaporins may offer hints as to why small, soft bodied species such as *A. gossypii* and *T. urticae* were affected by extracts that had minimal toxicity to *D. melanogaster* or *H. armigera*. The differences reported in down-regulation of DEGs in *A. gossypii* in comparison to the up-regulations in flies may also broadly explain evolutionary differences that could enable *D. melanogaster* to mitigate the toxic effects of extract 68N. However, this would require a

separate investigate which is outside of the scope of this project. Both of the molecular studies of 82N against *D. melanogaster* using RT-qPCR, and of extract 68N against *D. melanogaster* and *A. gossypii* using transcriptomics, highlighted the differentially expressed genes involved in the mortality or sub-lethal effects of these two premium extracts, thereby satisfying my last objective: to measure changes in differential gene expression of *D. melanogaster* and *A. gossypii* after exposure to novel botanical extracts.

Moreover, the qPCR and RNA-seq analysis in Chapter 6, have extended the observations at whole organism bioassay and cell physiology level to molecular level. Although differentially expression of genes encoding major K^+, Ca^{2+}, Cl^- and Na^+ channels in insects was observed in the qPCR experiments in *D. melanogaster*, the initially proposed targets of ion channels for the novel extract were not found in the transcriptome profiling of *D. melanogaster* or *A. gossypii*. This could be due to the low abundance nature of the ion channels in insect neurons. In the future, tissue and cell specific RNA-seq (Zhao et al. 2019) should be conducted to potentially identify these ion channels, which have been proven to be the targets of pesticides (Costa et al. 2008; Narahashi 1992). Interestingly, the novel extract 68N.M showed distinct effects on the transcriptome of *D. melanogaster* and *A. gossypii*, where cotton aphids had a major downregulation of key DEGs and fruit flies showed a mixture of upregulation and downregulation of the key DEGs. Major 68N.M-induced DEGs *A. gossypii* was found to be in the drug metabolism, RNA-binding and translational repression, chemical detoxification and oxidative stress, indicating that the cotton aphids were not able to cope with even the low concentration of 68N.M. However, the novel 68N.M-induced DEGs in *D. melanogaster* were transmembrane chemoreceptors, glutamate-gated calcium ion channel, ligand-gated ion channel, and transcription factor, which have diverse functions in the detection of chemical stimulus for fruit fly. These DEGs may equip *D. melanogaster* with superior capacity to tolerate 68N.M, which were indicated by the very high survival rate even at high concentrations of 68N.M. All these key DEGs can be considered for more detailed functional analysis in both *D. melanogaster* and *A. gossypii* to elucidate the gene function for a better and faster discovery of biopesticides for Australian and global agricultural industry in the future.

7.2. Summary of key findings

- Insect cell line cytotoxicity responses to the active compounds in the novel plant extracts are comparable to whole organism mortality from bioassays, provided there is no significant whole organism barriers or metabolic detoxification.

This study has shown that cell lines, which do not possess external barriers or complex detoxification systems, once exposed to novel plant extracts are not comparable to their whole organism counterparts. Therefore, I reject this hypothesis and while cell lines are useful and will allow target specific investigations, they cannot replace whole organism bioassays and target pest-specific studies.

- In model insect cell lines, the over-production of ROS indicates a high level of cellular stress-response to individual extracts, while an under-production in ROS indicates extracts which are less toxic to insect cells or have antioxidant properties.

This study generated some very interesting and extract-specific trends in ROS production. However, it also illuminated the influence that different cell lines may have on ROS production or on oxidative stress mitigation. Furthermore, the known antioxidant caffeic acid did not scavenge free-radicals at the concentration used with sufficient statistical data to satisfy the hypothesis. Further studies are needed to investigate and retest this theory. Therefore, I must reject the hypothesis.

- In model insect cell lines, the increase of ion flux indicates a high level of cellular toxicity resulting from novel extracts with nerve-poisoning-type modes of action.

From the results of my study, ion-flux measurements have been supported by molecular differential gene expression using two of the extracts tested, 68N and 82N, by two different methods, RT-qPCR and transcriptomic. Therefore, I accept this hypothesis. This can be further verified, if require, by employing known channel blockers which disrupt or

inactivate nerve impulses, however careful selection of the appropriate cell type(s) is advised.

- The differentially expressed genes (DEGs) of dead insects of one pest species and one model species following exposure to novel plant extracts indicate key genes controlling the insecticidal modes of action.

During my molecular studies, I found highly differentially expressed genes which I believe play some role in the mode/s of action for the extracts used. However, it is difficult to be definite due to the tolerance of *D. melanogaster* flies to 68N and the lack of an assembled genome for *A. gosspyii*. Under those circumstances, I must currently respect the scientific rigor and reject the hypothesis. However, when a fully mapped assembly for *A. gossypii* becomes available, I will revisit this hypothesis.

- The DEGs of surviving insects exposed to novel plant extracts elucidate key genes in the detoxification pathways.

As with the previous hypothesis, very interesting results led me to believe that this hypothesis is true, but it is unable to be fully explored at this time. In any case, there was certainly a large number of identifiable DEGs which gave insight into detoxification for both species. In addition, there were a number of DEGs identified in my investigations for which functions are currently not designated.

7.3. Limitations and future research

I believe this body of work is beneficial to the scientific community, and useful to the Australian cotton industry in that it is unique by employing a wide range of methods in novel ways to elucidate novel botanical insecticide MOA. However, I have also identified some considerations and limitations in interpreting the outcomes of my work.

Based on my studies of a number of selected plant extracts, I recommend that the three highly prioritised extracts I have investigated, *Podolepis jaceoides*, *Schoenia filifolia* subsp. *subulifolia* and *Lechenaultia biloba* 'Big Blue' be subjected to further investigations, especially focusing on the MOAs of 68N. This extract was highly efficacious in all studies, even when

extraction protocols varied. It was found to elicit a moribund state within 1 h in *A. gossypii* and substantially influenced multiple and opposing ion fluxes.

In this study, I obtained the *D. melanogaster* cells that were commercially available in Australia, but these may not necessarily be the most highly aligned with neural characteristics, since they originate from late embryo tissue material. Since that time I have discovered cell lines, presumably available, listed in the USA from the Harvard Medical School and the *Drosophila* Genomics Resource Centre that were cultured in the early 1990s from the central nervous system of 3rd instar larvae (*Drosophila* Genomics Resource Center 2020; Harvard Medical School 2020; Ui et al. 1994). Whilst selection of a different cell line origin of *D. melanogaster* is not anticipated to change the results of my study, since my cells had the appropriate cellular processes required for the investigations, the magnitude of the changes, especially for cell flux investigations, may be larger. Furthermore, I recommend the use of cell lines of other species if available, as the use of both D.mel-S2 and Sf9 was of substantial benefit to this study.

Based on the broad range of cross-disciplinary skills and knowledge that I have brought to this study, it should now be possible for future investigations to focus on multiple MOA pathways; for example, K^+, Na^+ or Cl^- ion flux and relegated gene expression, using the addition of ion channel blockers to limit and verify types of currents, channels or co-transporters, and a narrower selection of the genes associated with them.

Throughout this study, I have taken care to use a range of known insecticides as positive controls. In some cases, this have been beneficial and provided useful confidence in the data; in other situations, it has added complexity which may not have been entirely beneficial. Some of these insecticides were chosen because they were well known single compounds, such as synthetic pyrethroids. In other cases, consistent with my tested extracts, they were multi-constituent plant extracts, such as commercially available pyrethrum, or wild collected W11N and W44N, previously studied at Western Sydney University. I included these latter two extracts as secondary positive controls alongside pyrethrum because although their chemistry is known and their insecticidal properties have been reported, they have not yet progressed to market or registration, or because their MOA has not yet been (e.g. tasmanone

and flavasone). I decided that if I was going to employ novel techniques not previously utilised for insecticidal MOA discovery on polar and non-polar fractions of crude plant extracts, I should employ at least some complex, botanically-derived insecticides as my positive controls. During my study, it was not my intention to investigate these other extracts in any detail, as it was beyond the scope of my work; I merely used them as benchmarks. Nevertheless, given their activity I recorded in my work and that the β-triketones tasmanone and flavesone have been published as major components of *Campomanesia viatoris* (Matos et al. 2015), *Baeckea frutescens* (Tam et al. 2004) as well as various *Eucalyptus* species (Bignell et al. 1997), I recommend that their MOA also be investigated. Last, I believe that once the full genome of *A. gossypii* has be published, my transcriptome data will once again benefit from being revisited for a thorough and in-depth evaluation of the many highly DEG that could not be identified in this current study.

I look forward to this work being further progressed, and the possible development of IPM compatible insecticides being a useful and practical outcome from my studies.

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Appendix 1

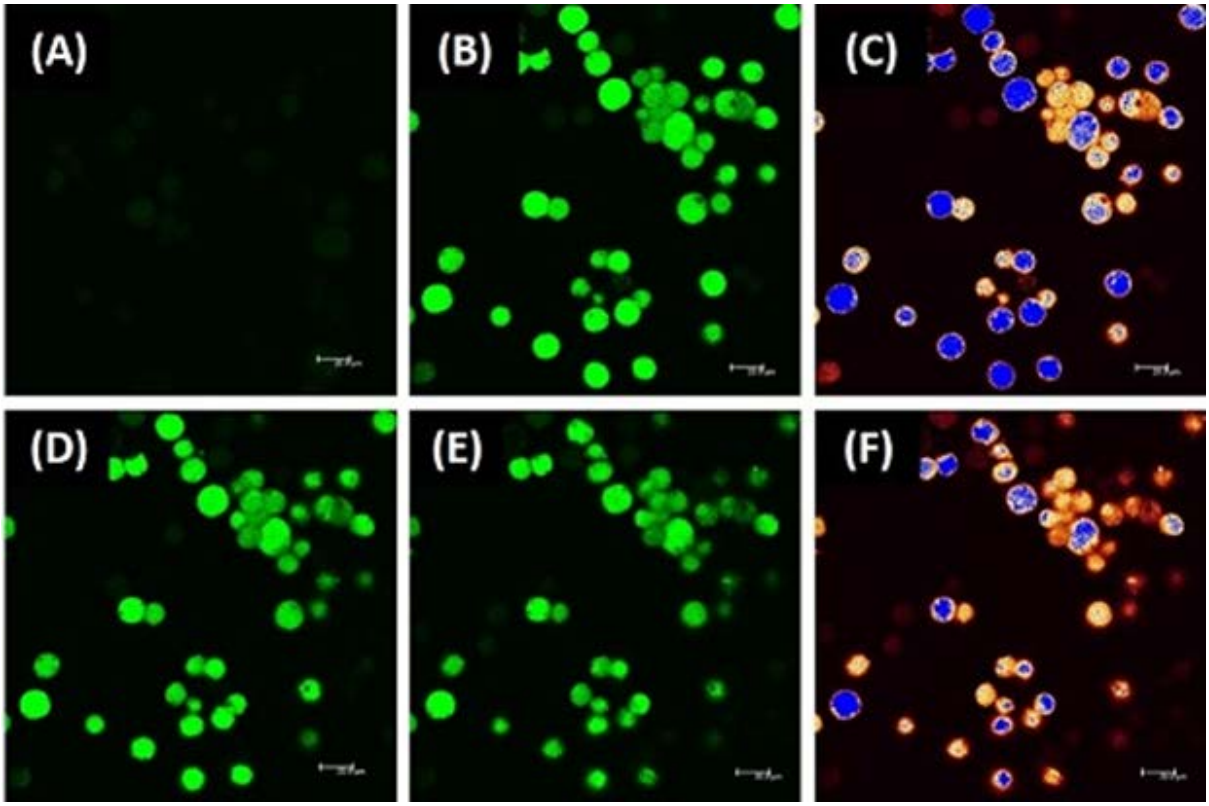

Figure 7-1 CSLM lamp effects resulting in extreme ROS production

Images of cells with natural ROS fluorescence following light-avoidance methodologies referred to in this paper and the detrimental effects of light stress induced by a short, 10 s exposure, to the fluorescence lamp commonly used in CSLM. (**A**) Cells without exposure to fluorescence lamp. (**B**) ROS fluorescence immediately after lamp exposure and (**C**) the over-exposure image at the same time point**.** (**D**) Image taken 5 min after the single exposure. (**E**) Image taken 15 min after exposure shows persistent ROS production and (**F**) over-exposure**,** verifying that cells exposed to lamp are unusable for exogenous toxin ROS detection. White horizontal scale bars in the lower right-hand corner indicate a 20 µm distance.

Appendix 2

Table 7-2 Pyrethroid types, molecular formulas, origins and ratios of constituent compounds used in this study.

Pyrethroid Type	Compound	Ratio in Fluka Pestanal® Pyrethrum	Molecular Formula	Origin	Sigma-Aldrich Catalogue #
Type I	Cinerin I	2.3%	$C_{20}H_{28}O_3$	Natural compound	33739
Type I	Jasmolin I	1.6 %	$C_{21}H_{30}O_3$	Natural compound	33739
Type I	Pyrethrin I	26.4 %	$C_{21}H_{28}O_3$	Natural compound	33739
Type II	Cinerin II	1.9 %	$C_{21}H_{28}O_5$	Natural compound	33739
Type II	Jasmolin II	1.3 %	$C_{22}H_{30}O_5$	Natural compound	33739
Type II	Pyrethrin II	15.7 %	$C_{22}H_{28}O_5$	Natural compound	33739
Type II	Alpha-Cypermethrin	99.8 %	$C_{22}H_{19}Cl_2NO_3$	Synthetic compound	45806
Type II	Esfenvalerate	98.9 %	$C_{25}H_{22}ClNO_3$	Synthetic compound	46277

Table 7-3 Pyrethrum dose response 96-well plate template.

Date: **Experiment/File Name:** D.mel OD600 Pyrethrum Dose Response Template

	1	2 No Control Cells Only	3 -ve Control 1% DMSO	4 Conc. 1 0.01% Pyrethrum	5 Conc. 2 0.005% Pyrethrum	6 Conc. 3 0.0025% Pyrethrum	7 No Control Cells Only	8 -ve Control 1% DMSO	9 Conc. 1 0.01% Pyrethrum	10 Conc. 2 0.005% Pyrethrum	11 Conc. 3 0.0025% Pyrethrum	12
A	Empty Wells →											Empty Wells ←
B		50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	
C		50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	
D		50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	
E		50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	
F		50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	
G		50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	50µl Cells 50µl Media	50µl Cells 50µl 2% DMSO	50µl Cells 50µl 0.02% Pyr	50µl Cells 50µl 0.01% Pyr	50µl Cells 50µl 0.005% Pyr	
H	Empty Wells →											Empty Wells ←

Appendix 3

Table 7-4 Corrected mortality for activity of plant extracts from the foundation project.

Sample Number	Sample	*Tetranychus urticae*		*Aphis gossypi*i		*Helicoverpa armigera*	
		24 HAT	48 HAT	24 HAT	48 HAT	24 HAT	48 HAT
1	1P	0.00	0.00	12.34	12.34	14.29	25.04
2	1N	0.00	7.69	0.00	0.00	0.00	0.00
3	2P	0.00	0.00	0.00	0.00	0.00	16.71
4	2N	40.00	50.00**	0.00	0.00	0.00	0.00
5	3P	5.00	21.05	0.00	0.00	0.00	0.00
6	3N	6.67	6.67	0.00	0.00	0.00	0.00
7	4P	0.00	5.00	0.00	0.00	4.76	33.33
8	4N	0.00	0.00	0.00	0.00	0.00	12.50
9	5P	4.96	4.96	0.00	0.59	16.71	16.71
10	5N	55.56	58.75**	0.00	0.00	0.00	0.00
11	6P	0.00	4.76	0.00	0.00	0.00	0.00
12	6N	0.00	11.11	0.00	0.00	0.00	14.29
13	7P	0.00	7.41	0.00	0.00	0.00	0.00
14	7N	0.00	0.00	0.00	0.00	0.00	0.00
15	8P	18.13	18.13	0.00	0.00	0.00	0.00
16	8N	23.61	53.22**	14.29	33.33	0.00	0.00
17	9P	6.17	17.90*	0.00	0.00	20.00	20.00*
18	9N	23.23	23.23*	0.00	14.29	0.00	25.00*
19	10P	4.04	4.04	0.00	0.00	0.00	0.00
20	10N	47.22	100.00***	87.50	100.00***	10.00	85.19***
21	11P	5.00	5.00	0.00	0.00	12.50	12.50
22	11N	0.00	8.84	0.00	0.00	10.00	20.00
23	12P	9.52	16.67	0.00	0.00	0.00	0.00
24	12N	1.48	8.52	0.00	0.00	12.50	33.33
25	13P	0.00	0.00	0.00	0.00	10.00	21.26
26	13N	23.48	37.83	0.00	0.00	10.00	10.00
27	14P	1.00	7.37	0.00	0.00	10.00	55.01**
28	14N	45.00	86.25***	0.00	0.00	11.11	12.51
29	15P	0.00	15.88	0.00	0.00	11.11	25.01
30	15N	6.92	45.00*	0.00	20.00	0.00	32.51*
31	16P	7.37	9.41	0.00	0.00	10.00	43.76
32	16N	0.00	10.63	0.00	12.50	0.00	43.76
33	17P	5.00	22.22	0.00	16.67	30.00	9.91
34	17N	13.04	13.04	0.00	0.00	30.00	54.95**
35	18P	50.00	66.67**	0.00	0.00	10.00	32.43
36	18N	11.11	11.11	7.69	7.69	10.00	10.00

37	19P	2.22	2.22	0.00	0.00	38.27	38.27
38	19N	3.41	3.41	0.00	0.00	0.00	0.00
39	20P	0.75	0.75	0.00	0.00	10.00	11.11
40	20N	5.71	5.71	0.00	0.00	44.44	88.88***
41	21P	5.56	16.19	0.00	14.29	1.23	1.23
42	21N	6.27	6.27	0.00	0.00	25.93	25.93
43	22P	1.96	12.50	0.00	0.00	0.00	50.00**
44	22N	36.51	66.25**	0.00	0.00	20.00	33.33
45	23P	0.00	0.00	0.00	0.00	0.00	25.00
46	23N	7.41	7.41	0.00	0.00	10.00	12.50
47	24P	0.00	0.00	0.00	0.00	0.00	0.00
48	24N	1.96	7.35	0.00	0.00	11.11	33.33
49	25P	0.00	0.00	0.00	0.00	0.00	25.00
50	25N (PT)	82.50	88.96***	50.00	60.00**	11.11	11.11
51	26P	7.62	11.51	0.00	0.00	0.00	11.11
52	26N	8.89	11.90	20.00	20.00	0.00	40.00
53	27P	0.00	0.00	0.00	0.00	0.00	10.00
54	27N	2.89	3.51	0.00	0.00	11.11	30.00
55	28P	25.23	55.05**	0.00	0.00	40.00	40.00
56	28N	91.57	100.00***	47.06	47.06	0.00	70.00**
57	29P	0.00	0.00	0.00	0.00	0.00	10.00
58	29N	0.00	5.75	0.00	0.00	0.00	0.00
59	30P	10.88	10.88	0.00	0.00	11.11	20.00
60	30N	6.86	24.60	0.00	0.00	11.11	30.00
61	31P	20.69	20.69	11.27	24.00	14.29	14.29
62	31N	12.19	12.19	0.00	0.00	0.00	0.00
63	32P	10.92	27.20	0.00	5.88	25.00	28.57
64	32N	79.21	92.59***	0.00	13.79	0.00	0.00
65	33P	0.00	0.00	15.12	21.74	50.00	57.14**
66	33N	79.53	79.53**	78.30	100.00***	87.50	100.00***
67	34P	0.00	0.39	0.00	0.00	25.00	25.00
68	34N	14.41	2.14	0.00	0.00	0.00	0.00
69	35P	0.00	0.00	0.00	0.00	0.00	0.00
70	35N	0.00	0.00	0.00	0.00	0.00	0.00
71	36P	0.00	2.60	0.00	0.00	0.00	12.50
72	36N	33.69	95.35***	22.73	62.50**	0.00	0.00
73	37P	0.00	30.60	0.00	0.00	0.00	0.00
74	37N	1.82	4.33	25.00	0.00	0.00	0.00
75	38P	0.00	0.00	0.00	0.00	0.00	0.00
76	38N	0.00	0.00	0.00	0.00	0.00	0.00
77	39P	4.99	4.99	0.00	0.00	0.00	0.00
78	39N	100.00	100.00***	25.06	25.06	25.00	25.00
79	40P	0.00	0.00	0.00	0.00	25.00	25.00
80	40N	1.82	1.82	0.00	0.00	0.00	0.00

81	41P	0.18	6.94	0.00	4.00	0.00	14.29
82	41N	2.51	5.43	0.00	0.00	14.29	14.29
85	42P	15.43	25.63	0.00	10.49	0.00	12.50
86	42N	0.00	0.00	0.00	0.00	0.00	42.86
87	Termilone	33.23	51.39**	4.89	15.93	0.00	0.00
88	QCIDE	100.00	100.00***	65.38	100.00***	37.50	50.00**
89	43P	50.00	60.95	0.00	0.00	14.29	14.29
90	43N	15.63	33.09	4.00	16.00	0.00	0.00
91	44P	6.25	13.49	0.00	4.17	0.00	0.00
92	44N	12.12	20.34	0.00	0.00	12.50	12.50
93	45P	6.52	12.27	33.33	50.00**	0.00	0.00
94	45N	10.26	81.70***	0.00	4.17	14.29	14.29
95	46P	0.00	0.00	8.11	8.11	66.67	66.67**
96	46N	0.00	0.00	23.53	77.61**	0.00	0.00
97	47P	2.78	2.78	25.81	44.12	42.86	42.86
98	47N	1.85	1.96	22.58	57.70**	0.00	23.81
99	48P	27.66	41.38	14.81	63.64**	25.00	71.43**
100	48N	2.15	23.33	3.85	89.85***	0.00	0.00
101	49P	16.67	36.59	15.38	39.13	0.00	23.81
102	49N	27.40	38.89	8.33	56.07	0.00	0.00
103	50P	23.68	27.08	27.27	33.33	14.29	14.29
104	50N	44.44	44.44	26.67	70.39**	0.00	14.29
105	51P	26.67	74.07**	0.00	0.00	0.00	0.00
106	51N	23.08	72.97**	0.00	0.00	0.00	0.00
107	52P	30.77	40.91	38.12	50.00**	0.00	0.00
108	52N	55.17	66.67**	42.11	42.11	0.00	0.00
109	53P	0.00	0.00	0.00	0.00	0.00	0.00
110	53N	0.00	0.00	0.00	0.00	0.00	0.00
111	54P	0.00	0.00	0.00	0.00	0.00	0.00
112	54N	0.00	0.00	0.00	3.33	0.00	0.00
113	55P	33.33	34.78	0.00	0.00	0.00	0.00
114	55N	91.18	96.63***	80.65	81.22***	0.00	0.00
115	56P	1.33	1.33	0.00	0.00	0.00	0.00
116	56N	0.00	0.00	3.33	3.33	0.00	0.00
117	57P	18.65	34.15	20.69	20.69	0.00	0.00
118	57N	28.64	42.11	25.00	25.00	0.00	0.00
119	58P	16.50	20.97	0.00	0.00	0.00	0.00
120	58N	0.00	12.20	4.76	4.76	0.00	0.00
121	59P	0.00	0.00	0.00	0.43	0.00	0.00
122	59N	0.00	0.00	0.00	0.00	0.00	11.11
123	60P	37.93	78.07**	0.00	52.09**	0.00	0.00
124	60N	12.90	30.01	25.64	32.89	0.00	0.00
125	61P	32.35	89.66***	32.35	56.50**	0.00	0.00
126	61N	10.26	22.45	0.00	5.41	0.00	0.00

127	62P	24.90	52.94**	6.67	5.26	0.00	11.11
128	62N	76.50	83.72***	93.18	100.00***	0.00	0.00
129	63P	0.00	10.64	0.00	2.94	0.00	0.00
130	63N	23.53	85.71***	10.20	22.41	0.00	0.00
131	64P	2.17	8.70	0.00	4.88	0.00	0.00
132	64N	56.25	78.95**	2.63	40.00	0.00	0.00
133	65P	0.00	0.00	2.78	2.78	0.00	0.00
134	65N	50.00	86.11***	7.89	7.89	0.00	0.00
135	66P	3.03	17.86	3.03	3.03	0.00	0.00
136	66N	72.73	77.14**	48.48	73.08**	0.00	0.00
137	67P	2.56	12.98	3.57	9.68	0.00	0.00
138	67N	14.41	41.03	0.00	3.85	0.00	0.00
139	68P	24.20	38.57	92.86	100.00***	0.00	0.00
140	68N	94.86	98.86***	100.00	100.00***	57.81	57.81**
141	69P	9.30	9.30	0.00	0.00	0.00	0.00
142	69N	77.78	80.07***	88.89	92.59***	11.11	11.11
143	70P	6.25	6.25	0.00	0.00	0.00	0.00
144	70N	2.08	2.08	0.00	0.00	0.00	0.00
145	71P	0.00	10.34	0.00	0.00	0.00	0.00
146	71N	26.67	29.17	4.76	4.76	0.00	0.00
147	72P	NR	13.33	NR	16.67	NR	100.00***
148	72N	NR	28.57	NR	100.00***	NR	100.00***
149	73P	NR	41.67	NR	0.00	NR	50.00**
150	73N	NR	88.89***	NR	0.00	NR	0.00
151	74P	9.09	11.76	8.33	28.57	0.00	0.00
152	74N	29.41	29.41	70.00	70.00**	0.00	0.00
153	75P	11.76	15.38	7.69	10.00	0.00	0.00
154	75N	7.14	25.00	43.75	60.00**	0.00	0.00
155	76P	20.00	37.50	14.29	20.00	0.00	0.00
156	76N	29.41	35.71	22.73	22.22	0.00	0.00
157	77P	21.74	30.43	13.25	13.31	0.00	0.00
158	77N	14.29	28.57	27.43	27.43	0.00	33.33
159	78P	9.52	20.00	0.00	0.00	0.00	0.00
160	78N	19.05	19.05	29.69	31.58	0.00	0.00
161	79P	5.00	11.11	22.66	27.98	0.00	0.00
162	79N	41.18	56.25**	78.60	78.60**	77.42	79.40**
163	80P	4.76	4.76	0.00	0.00	0.00	25.00
164	80N	0.00	11.11	0.00	0.00	0.00	33.33
165	81P	15.38	22.22	0.00	0.00	0.00	0.00
166	81N	9.52	14.29	7.73	5.82	0.00	66.67**
167	82P	42.11	49.87**	66.85	73.68**	0.00	0.00
168	82N	94.74	100.00***	90.43	100.00***	100.00	100.00***
169	83P	5.82	11.36	9.52	13.31	0.00	50.00**
170	83N	62.50	66.67**	90.00	94.15***	0.00	0.00

171	84P	26.32	29.82	0.00	0.00	0.00	0.00
172	84N	0.00	0.00	0.00	13.04	0.00	0.00
173	85P	0.00	0.28	10.53	0.00	0.00	0.00
174	85N	10.53	53.33**	5.00	49.66	0.00	0.00
175	86P	44.86	47.37	0.00	0.00	25.00	50.00**
176	86N	27.78	27.78	9.52	9.77	0.00	0.00
177	87P	5.00	5.00	0.00	0.00	0.00	50.00**
178	87N	100.00	100.00***	5.88	6.43	0.00	0.00
179	88P	0.00	0.00	0.00	0.00	0.00	0.00
180	88N	41.18	42.11	24.00	24.00	0.00	0.00
181	89P	0.00	0.00	1.14	1.14	0.00	0.00
182	89N	0.00	0.00	25.47	25.47	0.00	0.00
183	91P	8.84	8.56	43.81	43.81	0.00	0.00
184	91N	25.00	25.00	30.43	30.43	0.00	0.00
185	92P	16.18	19.58	0.00	9.26	0.00	0.00
186	92N	83.33	83.33***	30.43	33.60	0.00	0.00
187	93P	15.56	15.56	9.57	9.57	25.00	25.00
188	93N	100.00	100.00***	53.62	69.57**	0.00	0.00
189	94P	5.00	11.76	10.20	10.20	0.00	0.00
190	94N	82.35	82.35	0.00	0.00	0.00	0.00
191	95P	11.76	11.76	5.31	0.00	0.00	0.00
192	95N	37.50	40.00	57.89	57.89**	0.00	0.00
193	96P	11.11	6.25	0.00	0.00	0.00	0.00
194	96N	88.89	88.89***	11.11	11.11	0.00	0.00
195	97P	0.00	0.00	0.00	0.00	0.00	0.00
196	97N	0.00	0.00	21.43	38.46	0.00	0.00
197	98P	0.00	0.00	15.53	15.53	0.00	0.00
198	98N	0.00	0.00	7.14	10.56	0.00	0.00
199	99P	11.76	0.00	9.52	23.81	0.00	0.00
200	99N	0.00	0.00	34.69	34.69	0.00	0.00
201	100P	14.29	14.29	5.88	5.88	0.00	14.29
202	100N	66.67	86.67***	52.50	52.50**	14.29	14.29
203	101P	6.67	6.67	30.77	30.77	0.00	0.00
204	101N	10.00	10.00	15.79	34.03	0.00	12.50
205	102P	0.00	35.29	0.00	12.04	0.00	0.00
206	102N	0.00	0.00	0.00	15.56	11.11	44.44
207	W1P	17.65	39.13	4.17	7.69	0.00	0.00
208	W1N	11.11	17.39	8.70	11.11	0.00	0.00
209	W2P	60.00	77.78**	0.00	21.43	0.00	0.00
210	W2N	80.95	100.00***	100.00	100.00***	66.67	66.67**
211	W3P	0.00	15.79	0.00	0.00	0.00	0.00
212	W3N	0.00	29.41	0.00	0.00	0.00	0.00
213	W4P	95.24	95.24***	32.00	81.25***	0.00	0.00
214	W4N	61.54	61.54**	100.00	100.00***	0.00	0.00

215	W5P	56.52	56.52**	9.09	44.44	0.00	0.00
216	W5N	5.26	5.26	30.00	100.00***	50.00	50.00**
217	W6P	88.89	90.00***	25.00	85.00***	0.00	0.00
218	W6N	47.06	50.00**	42.31	42.31	0.00	0.00
219	W7P	0.00	0.00	15.38	58.82**	0.00	0.00
220	W7N	9.09	9.09	5.88	26.32	0.00	0.00
221	W8P	0.00	0.00	0.00	0.00	0.00	0.00
222	W8N	21.05	21.05	0.00	0.00	22.40	33.33
223	W9P	0.00	0.00	0.00	0.00	0.00	0.00
224	W9N	28.57	28.57	33.33	33.33	22.00	33.33
225	W10P	4.76	4.76	3.23	3.23	0.00	0.00
226	W10N	10.71	10.71	0.00	0.00	0.00	0.00
227	W11P	100.00	100.00***	0.00	0.00	0.00	50.00**
228	W11N	100.00	100.00***	53.57	53.57**	-	100.00***
229	W12P	93.30	93.30***	87.50	100.00***	0.00	0.00
230	W12N	37.97	37.97	16.67	16.67	0.00	0.00
231	W13P	79.04	79.04**	15.38	15.38	0.00	0.00
232	W13N	85.42	85.42***	72.73	72.73**	0.00	25.00
233	W14P	100.00	100.00***	8.33	8.33	0.00	0.00
234	W14N	100.00	100.00***	86.96	86.96***	0.00	0.00
235	W15P	64.71	64.71**	6.67	6.67	0.00	0.00
236	W15N	14.03	14.03	0.00	0.00	0.00	0.00
237	W16P	12.50	12.50	0.00	5.41	0.00	0.00
238	W16N	4.35	4.35	7.14	7.14	0.00	0.00
239	W17P	50.00	65.33**	0.00	3.57	0.00	0.00
240	W17N	7.14	10.86	0.00	0.00	0.00	0.00
241	W18P	27.78	36.44	14.29	14.29	0.00	0.00
242	W18N	12.50	48.00	8.33	15.00	0.00	0.00
243	W19P	23.53	32.71	3.13	3.13	0.00	0.00
244	W19N	5.88	5.88	3.57	3.57	0.00	0.00
245	W20P	0.00	0.00	6.25	6.25	0.00	0.00
246	W20N	7.69	7.69	3.45	3.45	0.00	0.00
247	W21P	23.73	23.73	5.00	0.00	0.00	0.00
248	W21N	10.53	12.42	0.00	0.00	0.00	0.00
249	W22P	14.35	29.41	0.00	0.00	0.00	0.00
250	W22N	16.67	22.00	0.00	3.13	0.00	0.00
251	W23P	80.00	83.58***	0.00	0.00	0.00	0.00
252	W23N	0.00	5.45	0.00	0.00	0.00	0.00
253	W24P	100.00	100.00***	11.11	11.11	0.00	33.33
254	W24N	86.76	82.86***	5.00	5.00	0.00	0.00
255	W25P	31.93	53.68**	7.69	7.69	0.00	0.00
256	W25N	7.35	7.35	0.00	0.00	0.00	0.00
257	W26P	9.24	10.20	0.00	0.00	0.00	0.00
258	W26N	84.87	84.87***	0.00	0.00	0.00	0.00

259	W27P	3.74	3.74	0.00	8.70	0.00	0.00
260	W27N	66.17	66.17**	0.00	3.85	0.00	0.00
261	W28P	85.71	94.12***	5.26	10.00	0.00	0.00
262	W28N	22.86	43.53	0.00	3.13	0.00	0.00
263	W29P	9.09	9.09	0.00	0.00	0.00	0.00
264	W29N	0.00	0.00	0.00	0.00	0.00	0.00
265	W30P	0.00	0.00	0.00	0.00	0.00	0.00
266	W30N	0.00	0.00	0.00	0.00	12.50	12.50
267	W31P	5.56	5.56	0.00	0.00	0.00	0.00
268	W31N	0.00	0.00	0.00	0.00	22.22	33.33
279	W32P	9.09	9.09	0.00	0.00	0.00	0.00
270	W32N	0.00	0.00	0.00	0.00	0.00	0.00
271	W33P	0.00	0.00	0.00	0.00	0.00	0.00
272	W33N	0.00	0.00	0.00	0.00	0.00	0.00
273	W34P	0.00	5.56	0.00	0.00	0.00	0.00
274	W34N	0.00	0.00	0.00	0.00	0.00	0.00
275	W35P	0.00	41.18	0.00	0.00	0.00	0.00
276	W35N	75.00	75.00**	3.70	3.70	0.00	0.00
277	W36P	0.00	0.00	3.45	3.45	0.00	0.00
278	W36N	12.50	26.67	3.70	3.70	0.00	0.00
279	W37P	0.00	0.00	0.00	0.00	0.00	0.00
280	W37N	0.00	4.76	0.00	0.00	25.00	25.00
281	W38P	43.48	43.48	0.00	0.00	0.00	0.00
282	W38N	10.00	15.00	10.00	10.00	0.00	0.00
283	W39P	11.11	11.76	4.55	4.55	0.00	0.00
284	W39N	9.52	9.52	0.00	0.00	0.00	0.00
285	W40P	10.53	10.53	0.00	0.00	0.00	0.00
286	W40N	33.33	35.29	0.00	0.00	0.00	0.00
287	W41P	0.00	5.00	0.00	15.38	0.00	0.00
288	W41N	0.00	0.00	0.00	0.00	0.00	0.00
289	W42P	12.50	50.00**	0.00	0.00	0.00	0.00
290	W42N	12.50	50.00**	25.00	25.00	0.00	0.00
291	W43P	11.11	11.11	0.00	7.14	0.00	0.00
292	W43N	0.00	0.00	0.00	25.00	0.00	0.00
293	W44P	61.11	55.56**	7.14	7.14	0.00	0.00
294	W44N	80.00	100.00***	68.75	76.47**	0.00	0.00
295	W45P	0.00	0.00	0.00	0.00	0.00	0.00
296	W45N	0.00	0.00	0.00	1.48	0.00	0.00
297	W46P	0.00	0.00	0.00	0.00	0.00	0.00
298	W46N	0.00	0.00	4.55	4.55	16.67	16.67
299	W47P	27.78	52.94**	3.57	3.57	16.67	16.67
300	W47N	0.00	0.00	0.00	0.00	0.00	0.00
301	W48P	17.65	17.65	13.33	26.67	0.00	0.00
302	W48N	0.00	0.00	5.00	5.00	0.00	0.00

303	W49P	0.00	21.43	0.00	0.00	0.00	0.00
304	W49N	100.00	100.00***	0.00	0.00	0.00	0.00
305	W50P	0.00	0.00	0.00	0.00	0.00	0.00
306	W50N	0.00	0.00	0.00	0.00	0.00	0.00
307	W51P	0.00	0.00	0.00	0.00	0.00	0.00
308	W51N	0.00	0.00	0.00	0.00	0.00	0.00
309	W52P	77.78	78.95**	0.00	0.00	0.00	0.00
310	W52N	47.06	61.11**	0.00	0.00	0.00	0.00
311	W53P	25.00	31.58	0.00	0.00	0.00	0.00
312	W53N	4.76	4.76	0.00	0.00	0.00	0.00
313	W54P	0.00	0.00	0.00	8.11	0.00	0.00
314	W54N	0.00	0.00	0.00	0.00	0.00	0.00
315	W55P	1.23	1.23	6.25	6.25	0.00	0.00
316	W55N	0.00	0.00	1.92	5.77	0.00	0.00
317	W56P	0.00	0.00	0.00	0.00	0.00	0.00
318	W56N	0.00	0.00	0.00	0.00	0.00	0.00
319	W57P	4.00	20.00	0.00	0.00	0.00	0.00
320	W57N	0.00	4.31	0.00	0.00	0.00	0.00
321	W58P	76.00	87.37***	0.00	32.36	0.00	0.00
322	W58N	91.67	91.67***	26.09	37.03	0.00	0.00
323	W59P	36.84	36.84	0.00	0.00	0.00	16.67
324	W59N	0.00	0.00	4.76	4.76	0.00	0.00
325	W60P	0.00	45.83	5.71	8.24	0.00	0.00
326	W60N	8.70	52.63**	29.17	40.57	0.00	0.00
327	W61P	0.00	0.00	0.00	5.45	0.00	0.00
328	W61N	0.00	0.00	0.00	8.48	0.00	0.00
329	W62P	25.00	39.13	19.35	19.35	0.00	0.00
330	W62N	62.50	75.00**	19.35	33.82	16.67	16.67
331	W67P	82.61	82.61***	83.33	83.33***	0.00	0.00
332	W67N	95.45	95.45***	100.00	100.00***	66.67	66.67**
333	W68P	100.00	100.00***	22.58	26.11	0.00	0.00
334	W68N	0.00	0.00	5.00	5.00	12.50	16.67
335	W69P	0.00	18.36	6.98	6.98	0.00	0.00
336	W69N	0.00	0.00	31.82	79.48**	0.00	0.00
337	W70P	100.00	100.00***	80.70	100.00***	0.00	0.00
338	W70N	83.04	95.76***	89.47	98.01***	0.00	33.33
339	W71P	0.00	0.00	0.00	0.00	0.00	0.00
340	W71N	13.64	13.64	37.78	53.66**	16.67	16.67
341	W72P	30.77	30.77	59.18	60.42**	0.00	0.00
342	W72N	66.67	75.94**	79.59	96.00***	0.00	0.00
343	W73P	0.00	8.33	0.00	0.00	0.00	0.00
344	W73N	51.47	51.47**	50.00	84.00***	0.00	0.00
345	W74P	3.85	3.85	0.00	0.00	0.00	0.00
346	W74N	13.33	13.33	0.00	2.38	0.00	0.00

347	W84P	77.17	90.87***	68.97	68.97**	0.00	0.00
348	W84N	88.33	88.33***	93.48	97.78***	16.67	16.67
349	W95P	10.00	15.00	44.12	71.43**	0.00	16.67
350	W95N	90.45	90.45***	50.00	70.00**	14.29	14.29
351	W96P	33.18	35.00	26.67	51.52**	16.67	33.33
352	W96N	58.00	65.00**	54.39	71.43**	0.00	33.33
353	W98P	25.60	45.44	63.49	63.49**	0.00	0.00
354	W98N	70.24	79.17**	75.26	79.31**	16.67	16.67
355	W102P	15.87	18.48	26.50	52.98**	0.00	0.00
356	W102N	13.19	16.67	33.91	33.91	0.00	0.00
357	W105P	91.67	95.83***	64.02	64.02**	0.00	28.57
358	W105N	7.85	4.17	89.86	100.00***	66.67	100.00***

Note**:* Extracts efficacy was determined as: very high; mortality ≥80% (denoted), high; mortality ≥50%<80% (denoted**), moderate; mortality ≥35%<50, low; mortality ≥20%<35, negligible; mortality <20%, interesting non-fatal, semiochemical behaviour (denoted*). One extract was found to be phytotoxic (denoted PT). All extracts in greyed rows where subject to further assays in some respect.

Appendix 4

Table 7-5 Complete list of *D. melanogaster* primers designed for this study

Gene	Forward Primer	Reverse Primer	Action
*Dm.Act42A**	GCGTCGGTCAATTCAATCTT	AAGCTGCAACCTCTTCGTCA	Housekeeping gene
Dm.Atpα	GTGCCTGACCCTTACC	TTGAACACCCGACTGA	Na pump α subunit
Dm\Cad74A	CTTCAGGCAAGTTTACGC	GGTTGGTGGTCAGTGGTT	Resistance to Cry1Ac in *H. armigera* also associated with Ca^{2+} binding
Dm.ClCa	GGAGGAGAAGAAGAAACA	CTGGCGTAAACTGAGAAT	Chloride channel-a
Dm.ClCb	GATGTTTGCGGAGTAAGA	GATAGAGGTCCCTGAATGT	Chloride channel-b
Dm.ClCc	GACGGTGGACGATGTTGA	CCTCGATTAGACGCTTGG	Chloride channel-c
Dm.Clic	TGCTGGTAAAGAAGGACG	GCTGTGATACCCTGGAAC	Chloride intracellular channel, Cl^- channel activity & Ca^{2+} channel binding
Dm.GABA-B-R2	TTTCGTGGTTTCACAGAG	TTCGGGTCATAGGTCATC	Metabotropic GABA-B receptor subtype 2
Dm.GABA-B-R3	CCACCCACTCGGATAACT	CTCATTTCACGGCTTTGT	Metabotropic GABA-B receptor subtype 3
*Dm.GluClα**	GGAGCCTGGGTAGAACTG	GGGATGCGTATTGTGGAG	Glutamate-gated chloride channel subunits
*Dm.GstE1**	GCACCTGAGCGAGGAATA	CACCAAGTAGGCGGCAAT	Glutathione transferases enzymes
Dm.HisCl1	TTCCTCTTTGCCATTTCG	AGGTTTCATTCGCTTTCA	Histamine-gated chloride channel subunit 1
*Dm.IRK1.1 **	AATAAGTCGCCAGTCTCCAAC	TGAGTAACCACAATACGCCC	Inward rectifying K^+ channel 1
Dm.IRK1.2	AATAAGTCGCCAGTCTCCAAC	TGAGTAACCACAATACGCCC	Inward rectifying K^+ channel 1
*Dm.IRK2**	ACACGGAGACTTGGAAGA	GGAGAACAGGAAGCAGGA	Inwardly rectifying potassium channel 2
*Dm.IRK3**	TAAGGCGGTGGTAAGTAA	GTTTGTCCACGATGATGT	Inward rectifying K^+ channel 3
*Dm.KCNQ**	GGCACTTCCAGTCAACAC	TCCAGAAACTATCCACCC	KCNQ potassium channel
*Dm.Kcmf1**	GTTGCCAGATGTATGTGAGA	GCCGTATTTACTGAGGGTC	Potassium channel modulatory factor 1
Dm.Lcch3	AGGCAAGATCGGTAGGTC	GAGGGTGGCATTGTAGGT	Ligand-gated chloride channel homolog 3
*Dm.nap**	TGTCCCGTATTGGAAGTA	AAGGCACAAAGTCTGTCATC	No action potential
Dm.NaCP60E	CGCTACCACTACTTCACAG	GTCTTCCATCAGAATCCC	Na pump α subunit
Dm.Ncc69	AAGTTTACTCCTCGTCTCG	TTGGGAAGTCCACTGTTT	Sodium chloride cotransporter 69
*Dm.NKCC.1**	CGGCAGCCTAACTAAC	GGATGAGGAGGAGCAG	Sodium potassium chloride cotransporter

Dm.NKCC.2	CAAGCGTGAGTAAACAGC	ACCCTCCCTATGACAAGA	Sodium potassium chloride cotransporter
*Dm.Nos**	CAAATAATCTACTCGCTACG	CTCCTTACTTTCACCCTCT	Nitric oxide synthase
*Dm.Ork1**	ACAGGCTCTTTGGGTGAC	TGGTGCGAACTCTACTTACT	Open rectifier K^+ channel 1
Dm.para	AACACTAACTAATGCCTGAC	ATTCCTCCCACTTACAAC	Paralytic
Dm.Rdl.1	AAATGACTTCCTCCTCTG	GTGGCTGTTGTTTGTATG	Resistant to dieldrin
Dm.Rdl.2	GCCAAGTAGCAAGCGTTTATG	CTGGTGTTGCCTTGTTGTTATC	Resistant to dieldrin
Dm.tipE	CAAGAACGCCGAAGACAT	CAGGACACCCTCCAGACA	Temperature-induced paralytic E

Note: Genes that had two sets of primers designed from different sections of the gene code are denoted by and additional "*.#*" at the end of the gene name, for example *Dm.IRK1.1*, *Dm.IRK1.2*. The single * denotes the primers that were investigated using RT-qPCR against 82N 2.0% w/v.