Combatting pesticide resistance in insects using botanical bio-synergists

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Over 600 species of pests have developed some level of resistance to pesticides, rendering many products ineffective and reducing the options for pest management. With increasing restrictions on future pesticide use and withdrawal of active ingredients, together with the decline in the rate of new insecticide discoveries, options need to be explored to extend the useful life of products currently available. Plant derived compounds (botanicals) have been shown to interfere with the activity of enzymes that detoxify insecticides and are involved in metabolic pesticide resistance in insects. The use of such compounds can increase the efficacy of insecticides, thereby reducing required application rates and/or counteracting metabolic resistance, allowing effective control to be restored without increasing application rates.

In vitro enzyme assays and efficacy testing were used to evaluate whether botanicals could enhance pesticide efficacy against insect pest species associated with brassicas. These included cabbage stem flea beetle (*Psylliodes chrysocephala*), peach-potato aphid (*Myzus persicae*), diamondback moth (*Plutella xylostella*) and cabbage root fly (*Delia radicum*). Botanicals were tested in combination with selected plant protection products and efficacy compared to single product applications, using standard laboratory efficacy testing methods. Data showed that the efficacy of pyrethroids against field-collected *P. chrysocephala,* which have developed resistance against this pesticide group, can be restored when used in combination with some botanicals, such as neem oil. Pyrethroid efficacy can also be enhanced against *M. persicae* and *P. xylostella* when combined with lemongrass oil and garlic oil. Similarly, efficacy of Spinosad against *D. radicum* can be increased when used in combination with botanicals such as parsley-seed oil and thymol. Enzyme activity assays (esterase, glutathione S-transferase and cytochrome P450 monooxygenase) have also indicated that several botanicals interfere with the function of certain enzyme groups that detoxify insecticides, some of which correlates with the mortality data from the bioassays.

Botanicals may counteract the metabolic resistance of some insect pests and thereby restore efficacy and/or allow the reduction of pesticide required for effective control, which could have implications for protecting oilseed rape and other high-risk crops.

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1.1 Introduction

It was around 10,000 years ago when humans first began collecting seeds and growing food plants. Since then we have successfully domesticated and selected thousands of variants from a number of species of plants and animals (Diamond, 2002), developed innovative technology such as gene editing (National Human Genome Research Institute, 2013) and even grown plants in space (NASA, 2021). Yet, as the practice of agriculture has progressed and evolved at a substantial rate, the pests and diseases that first challenged the beginnings of this industry so many years ago have kept pace. Even with the most advanced control measures in place, these threats continue to cause global crop losses of 20-40% (Centre for Agriculture and Bioscience International (CABI), 2019). Synthetic pesticides are heavily relied upon to minimise these losses, but the continued high input of these products is being challenged by the pressure of social and environmental responsibility, as well as the widespread development of heritable resistance in target pest populations (Hu, 2020; Insecticide Resistance Action Group, 2021; European Environment Agency, 2023; Pesticide Action Network – Europe, 2023).

One of the first pesticide ingredients to be used was pyrethrum, with variations of this compound continuing to be extensively used to control agricultural and human pests (McLaughlin, 1973). Pyrethrum is a naturally occurring axonic excitotoxin made up of a combination of six esters and originating in *Chrysanthemum cinerariaefolium* (Treviranus) flowers (Davies, 1985; Ray, 1991). The toxin interferes with the normal neurotransmission in insects and causes paralysis and death in susceptible individuals (Soderlund, 2012). The daisy-like flowers were initially ground into powder and used to protect stored grains from insect damage (Casida, 1980) but, as pressure intensified from insect vectors of disease and the costs of both yield losses and control measures increased, there was a drive towards identifying more effective and cheaper alternatives. The development of synthetic pesticides was revolutionised in the first half of the 20th century with the successful manufacture and deployment of a range of different chemical control products (e.g. organochlorines and carbamates), followed by the rapid development of various synthetic pyrethroids (Figure 1.). These pesticides were effective, cheap to make, highly toxic and only small amounts were required (Metcalf, 1980; Pretty and Bharucha, 2015). They could also be used for protection against pests that spread human disease as well as those that targeted crops (Ray, 1991).

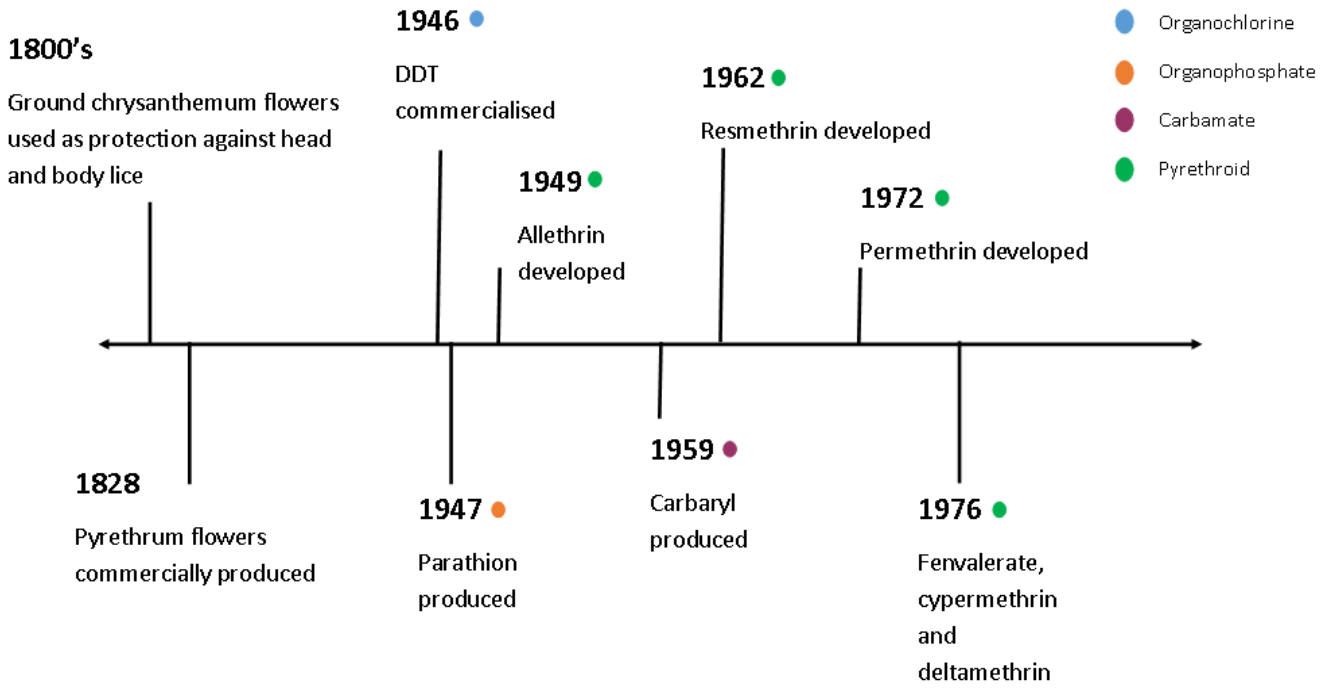

Figure 1.1. Timeline of pesticide product development (Casida, 1980; Metcalf, 1980; Bond *et al.,* 2016; International Agency for Research on Cancer, 2017; Ujihara, 2019)

Despite their apparently favourable attributes, investigations into these pioneering chemical controls revealed devastating environmental consequences. In response to initial reports and even before the application of the persistent organochlorine dichlorodiphenyltrichloroethane (DDT) became common in the control of insect crop pests, Strickland (1945) postulated that "should it even approach its claimed toxicity to plant-feeding insects in general, its *widespread* employment over large connected areas might constitute an entomological disaster of the first magnitude". As the capabilities of pesticides for controlling everything from body lice on the Western Front of World War 1 to the gypsy moth in residential trees became apparent, so too did its more harmful effects on non-target species such as bees, fish and other organisms throughout the world's food webs (Wigglesworth 1945; Carson, 1962; Fry, 1995). As a result, the European Union banned DDT in 1983 (Commission Directive 83/131/EEC, 1983) and the regulation of plant protection products (PPP) and pesticides to protect human and environmental health began gathering momentum.

In 2009, the Sustainable Use Directive (SUD, Commission directive (EC) 2009/128/EC) was introduced by the European Parliament with an aim to reduce the impacts and risks associated with pesticide use on both human and environmental health. Council regulation (EC) 1107/2009 presents the guidance and restrictions placed on the development and use of PPPs in member countries, from approval of active substances to risk assessments. It also works in combination with others such as Council regulation (EC) 396/2005 to dictate maximum acceptable residue levels in food. The SUD also requires member countries to devise their own National Action Plans in relation to these aims to encourage an Integrated Pest Management (IPM) approach, employ non-chemical alternatives where available and use chemical control with care and only where absolutely necessary. The first two progress reports, however, suggest mixed success towards these goals among member states with all of them banning aerial pesticide derogation, some producing measurable targets and others appearing reluctant to quantify theirs (European Commission, 2017; 2020). Amid growing recognition of the changing climate, environmental degradation and mounting research confirming catastrophic impacts of pesticide use (European Environment Agency, 2020; Wagner, 2020), the European Green Deal was devised by the European Commission in December 2019 to reduce the existential threat produced by unsustainable practices. At the centre was the Farm to Fork Strategy (F2F) with two key PPP targets focussed on 1) reducing chemical pesticide usage and risk by 50% and 2) reducing the use of 'more hazardous' pesticides by 50% – both by 2030 (European Commission, 2019). Despite a proposal to make these targets legally binding in a regulation to replace the SUD being ultimately rejected by member states due to concerns over lack of comparable alternatives and disagreement over achievability of targets (European Parliament, 2023), the focus remains on overall reduction of negative PPP effects and sustainable food and feed production.

The UK government, for example, offer financial support under the Sustainable Farming Incentive (SFI) and Countryside Stewardship grants for producers who meet certain criteria to protect and improve the environment on their land. Activities such as establishing and maintaining hedgerows or woodland, planting legume fallow, flower-rich margins or field blocks and not using insecticides on arable crops earn monetary reward for demonstrating a commitment to sustainability, in line with EU directives (UK Government, 2024a; 2024b).

1.2 Integrated Pest Management

As a cornerstone of the SUD, the integrated pest management approach is mandatory for all professionals in the food and feed production industry. It essentially comprises four tiers:

a) Prevention

The first step is to attempt to protect the crop from initial infestation where possible or, failing that, prevent the growth of insect pest populations from reaching a level that would result in crop failure or significant loss of yield. This may be achieved through practices such as crop rotation as it could reduce damage if soil-borne or overwintering pests emerge to find unsuitable hosts. This can be further supported by covering the crops with mesh that both restricts exit, so they cannot travel to find suitable crops, and entry to pests that would attack. Under-sowing or planting companion crops is a common tactic that may attract natural predators and distract pests from the marketable crop. Adapting sowing dates in response to predicted pest emergence each year may help to protect vulnerable seedlings from attack until they are more established and resilient to infestation. Resistant cultivars of commonly attacked crops are also being developed to support production without the need for chemical control. Finally, planting herbal and flower-rich margins or in-field blocks support natural predators and other beneficial organisms that may help to reduce the impact of pest infestation.

b) Monitoring

Pest monitoring systems vary from fairly straightforward but labour-intensive deployment of sticky, pit-fall, light and pheromone traps throughout the fields to more automated remote sensing using Global Positioning Systems (GPS) and Geographical Information Systems software. Both are useful to track infestation levels, as well as general crop health, and allow growers to react rapidly if insect threshold levels are reached.

c) Threshold setting

The threshold level of an insect pest is reached when the number per area, per plant or per part of a plant is deemed such that if control measures are not used, significant economic damage will be sustained (Ramsden *et al.*, 2017). They should be based on robust scientific evidence and reviewed to keep updated in line with evolving agricultural practices and cultivars. However, of the 34 main arable crop pests in the UK, eight of them have no identified threshold, most do not have evidence that has been peer reviewed or

recorded support at all and many of them were established over 20 years ago (Ellis, Berry and Walters, 2009; Ramsden *et al.*, 2017).

d) Control

If thresholds have been reached, plant protection measures should be implemented. In the first instance, non-chemical products such as biological control should be considered first but if these are impractical for the circumstances (e.g. outdoor cultivation) then targeted chemical pesticide applications should be used only at the necessary levels (Department of Agriculture, Environment and Rural Affairs (DAERA), 2017; European Commission, no date).

IPM uses all suitable techniques and methods to maintain pest populations at levels below those causing economic damage with chemical control, preferably specific with the least impact on non-target species, the final solution when other options have been exhausted (DAERA, 2017). Despite the guidance, a lack of confidence in thresholds based on outdated or unknown research, cheap and possibly effective pyrethroids, variation amongst crop tolerance, time-consuming monitoring systems and fear of potential economic loss can lead growers to carry out 'insurance sprays' in an attempt to ensure maximum yield (Pannell, 1991; Pedersen *et al*., 2012; Dewar, 2016; Ramsden *et al.*, 2017). Unnecessary application of insecticides may undermine profits by adding further costs, cause environmental degradation and increase the risk of developing pest resistance (Hillocks, 2012; Bass *et al.,* 2014).

1.3 Insecticide resistance

Although current regulations and policy trends to reduce chemical pesticide use are welcome to safeguard against biodiversity loss and environmental harm, they have also largely restricted UK and European farmers to using insecticides containing a limited number of active ingredients (Garthwaite *et al.,* 2016). Synthetic pyrethroids now remain one of the few classes of pesticides that can be consistently applied to control crop pests in the UK, but even these are dwindling in their efficacy (Slater *et al.,* 2011; Bass *et al.,* 2014; Højland *et al.,* 2015). The Insecticide Resistance Action Committee (IRAC) have defined resistance as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (IRAC, 2025). In 2015, around 600 insect species were identified as

exhibiting resistance to one or more pesticides and likely more have followed but this data has not been collectively reviewed since (Sparks and Nauen, 2015). There is also evidence to suggest that substantial loss of agricultural yield and profits due to insect damage is affecting supply of certain crops with farmers now opting out of growing those that are heavily targeted by resistant insects. For example, oilseed rape and the cabbage stem flea beetle (*Psylliodes chrysocephala*) (Scott and Bilsborrow, 2018; AHDB, 2024a).

The development of resistance in insects is dependent on a range of environmental and genetic factors such as fecundity rates of the species, generation time, ability to disperse and any fitness costs associated with the resistance mechanisms. The combination of these with selected biochemical pressures including dosage, frequency and consistency of use as well as the persistence of the pesticides in the environment are responsible for the insects' ability to rapidly develop resistance to synthetic chemical control (Tabashnik, 1990; Panini *et al.,* 2016). Interestingly, for example, female house flies exhibited 23% faster metabolism of chrysanthemic esters than males. This suggests that the level of resistance is enhanced depending on likelihood of exposure, with females needing to land on/around the affected plants more often to lay eggs (Bridges *et al.,* 1957).

Although there are variances between authors regarding the number and classification of insect resistance cases (Bass *et al.,* 2014; Panini *et al.,* 2016; Dang *et al.,* 2017) there is an overall acceptance that the mechanisms are predominantly physiological. The following four mechanisms have been identified as the most significant (IRAC, 2024a):

i) Cuticle penetration resistance – as many pesticides are neurotoxic, the pesticide must first enter the body of the insect in order to have an effect. The cuticle presents a barrier to desiccation, physical attack and harmful chemical absorption. Insects exhibiting this type of resistance have a slower rate of toxin absorption than susceptible insects. This not only reduces the amount of toxin absorbed but may also provide more time for other mechanisms (below) to provide further defence. The diamondback moth (*Plutella* xylostella) (Linné) (Lepidoptera: Plutellidae), for example, is a significant pest of brassicas and is known to have cuticular resistance to the pyrethroid fenvalerate (Noppun *et al.*, 1989).

ii) Target site mutation – pesticide compounds are often intended to bind to certain target sites within an insect (such as the sodium channels, acetylcholinesterase enzymes or GABA-gated chloride channel receptors) and thereby disrupt normal

neurotransmission, eventually leading to death in susceptible insects. A minor difference in the genetic code defining the development of these areas can lead to varying levels of insecticide resistance. This is the primary cause of knockdown resistance (kdr and super-kdr) in insects such as the house fly (*Musca domestica*) (Linné) (Diptera: Muscidae) (Busvine, 1951; Farnham *et al.,* 1987).

iii) Behavioural resistance – some insects have been observed to modify their behaviour in order to reduce their exposure to pesticides. Malaria mosquitoes (*Anopheles* sp.) (Diptera: Culicidae), for example, have been observed to rest on untreated external walls rather than internal walls impregnated with DDT (Gerold, 1977; Sundararaman, 1958). It has also recently been suggested that 'avoidance behaviour' may be a resistance mechanism in the peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). The resistant strains appeared to show higher rates of movement and an ability to locate and remain on control leaves more frequently than any other strain, suggesting they may do the same in the field and perhaps avoid treated areas (Fray *et al.,* 2014).

iv) Metabolic resistance – the most common form of insecticide resistance, whereby detoxification enzymes are consistently present in greater numbers (gene amplification) or demonstrate higher catalytic activity in resistant insects. These enzymes work to break down or otherwise alter the molecular structure of the insecticidal compound to ensure it is less toxic than the original form. There are several subsidiaries under discussion that also include sequestration of insecticidal compounds and increased excretion (Devonshire and Moores, 1982; Panini *et al.*, 2016).

1.4 Enzyme resistance

Herbivorous insects have been engaged in an arms race with plants since their origins, with one striving to not be eaten while the other gamely endeavours to eat. Thus, many plants contain a range of naturally occurring allelochemicals, such as alkaloids, phenols and terpenes, that have evolved as potentially toxic/repellent/feeding deterrent protection against insect pests. As a result, various insects have established select groups of detoxification enzymes to overcome these compounds, specifically esterases, mixed function oxidases (MFOs) and glutathione S-transferases. It is these metabolic enzymes that also confer resistance to the

different classes of insecticides, many of which have been developed from, or are synthetically similar to, botanical products (Isman, 2006).

Detoxification of an insecticidal product occurs in two phases: 1) hydrolysis or oxidation of insecticidal compounds and 2) excretion (Li et al., 2007; Hollingworth and Dong, 2008; Berenbaum and Johnson, 2015). It is also often accompanied by sequestration, whereby the insecticide is stored within the body and therefore prevented from interacting with the selected target sites (Devonshire and Moores, 1982), although it is unknown whether this process incurs fitness costs.

The consistent exposure to limited and related insecticidal compounds has also driven the efficacy of these mechanisms towards delivering an even more challenging outcome for pesticide efficacy: cross-resistance. This is where the enzymes groups and other defensive strategies involved in resistance to one insecticide may also be able to confer comparable protection to other insecticides of similar structure and function. This can consequently result in insects showing resistance traits to insecticides that they have never been exposed to, rendering new formulations and minor variations to known insecticidal compounds less and less valuable or useful (IRAC, 2024b). For example, in 2003 wild-caught Colorado potato beetle, *Leptinotarsa decemlineata,* demonstrated 309-fold resistance to imidacloprid (a neonicotinoid) that was used in the field and an, albeit lower, resistance to nine other neonicotinoid compounds that had never been used there (Mota-Sanchez *et al*., 2006). Many insect species also employ more than one of these resistance mechanisms, known as multiple resistance, thereby presenting significant challenges to their control (Buhler, no date). Multiple resistance means that the pest population is likely to survive in the crop despite deployment of pesticides with different modes of action through the season, which is an often-recommended tactic to try and avoid development of cross-resistance (IRAC, 2024b). The diamondback moth, for example, has developed resistance to 101 insecticidal active ingredients to date (Arthropod Pesticide Resistance Database, no date).

1.5 Alternatives

In more recent years, the focus for plant protection and pest management has returned to the original beginnings of pesticide use – botanical products. Indeed, the last 30 years has seen a significant increase in the number of publications related to botanicals and pest

management (Isman, 2020). Of these botanical extracts, pyrethrum remains the most commonly used across the world, although the majority of it now is not used for agricultural purposes (Isman, 2020). Neem oil (A*zadirachta indica* Juss*.,* Meliaceae) is a broad-spectrum insecticidal extract from which several commercial products have been developed (Campos *et al.,* 2016). Many other plant-derived products, such as essential oils, terpenoids, sterols, alkaloids, flavanones and polyketides have been identified as having pesticidal activity, low toxicity to humans, are readily biodegradable, can even have some target specificity and are considered more compatible with IPM strategies (Dang *et al.,* 2012). Crucial to their potential for use as pest management tools in this respect, some have even been identified as having a synergistic effect in combination with synthetic and naturally derived pesticides (Metcalf, 1967; Joffe *et al.,* 2012, Tong and Bloomquist, 2013; Tak and Isman, 2017; Zihao *et al.*, 2017).

1.6 Synergy

Synergistic action is determined when compound one (the synergist) has a negligible or non-toxic effect on the insect, but when combined at the same dosage with compound two (the insecticide), it causes a more significant effect than when compound two is applied alone (Metcalf, 1967). Early demonstration of this effect by Lindquist *et al*. (1947) highlighted that applying piperonyl cyclonene, sesamin, or N-isobytlundecycleneamide to house flies anywhere between 4 and 24 hours before exposure to pyrethrins resulted in enhanced knockdown effect. Further results from Wilson (1949) and Winteringham *et al.* (1955) respectively, showed that piperonyl cyclonene blocked 100% of metabolism of pyrethrins when compared to 50-54% metabolism with no synergist and that the effect was the same regardless of whether the application site for synergist and insecticide was the same or not. Lindquist *et al.* (1947) also noted that temporal application of the compounds was significant as no synergistic effect was observed when pyrethrins were applied prior to the synergists. This suggests that the synergists were interfering with the cuticular penetration, activity at target site and/or detoxification and metabolism processes, allowing the successive application of insecticide to exert an increased effect. Subsequent studies have indicated that more than one of these factors can be affected by selected synergists (Tak and Isman, 2017; Zihao *et al.*, 2017), although the complex underlying mechanisms are yet to be fully explained (Caesar and Cech, 2019).

There is, however, some evidence to suggest that synergy appears to be heavily influenced by the presence of a methylenedioxyphenol (MDP) ring in the structure (although not necessary) and variations of the attached functional groups and side chains (Haller *et al.* 1942; Beroza and Barthel, 1957; Moore and Hewlett, 1958; Cassida, 1970). Piperonyl butoxide (PBO) contains this structure and exhibits a synergistic effect with a wide range of insecticide classes including pyrethrins, pyrethroids, carbamates, organophosphates and organochlorines (Wachs, 1947; Joffe *et al.,* 2012; Tong and Bloomquist, 2013; Gupta, 2014; Arena *et al.,* 2018). It is reported that the MDP ring binds to the haem group of the P450 enzyme and subsequently blocks their ability to begin detoxifying the insecticide (Wilkinson, Murry and Marcus, 1984 in Moores *et al.,* 2008). As such, PBO is in widespread use across the world, particularly the US, and is a common component of many veterinary medications to treat fleas, ticks and mites in a range of animals (Cross *et al.,* 2017). However, it is not approved for use in agricultural production or with plant protection products in the UK (Osimitz, 2010; Lewis *et al.,* 2016) and due to its synthetic nature, it is also not classed as an organic product and can have a significant negative impact on amphibians in their tadpole stage (Cross *et al.,* 2017) – facts that conflict with the current socio-political drivers to use more natural approaches to pest management to deliver improved environmental protection. As such, interest has grown in the investigation of botanical compounds that may exhibit similar synergistic effects.

Recent research has demonstrated that a wide range of individual botanical compounds and essential oils have a synergistic effect with pyrethrins and pyrethroids within selected insects (Scott *et al.*, 2003; Joffe *et al.,* 2012; Tak and Isman, 2017; Marchand *et al.*, 2018). The specific mechanisms involved in synergy are being heavily investigated as it has also been determined that the MDP ring is not the sole reason denoting the success of a compound as a synergist. There are also several essential oils and their components that do not possess this type of chemical structure and yet demonstrate themselves to be potent synergists and enzyme inhibitors. For example, as well as possessing strong insecticidal and repellent characteristics (Koul, Isman and Ketkar, 1989), neem oil is reported to have significant synergistic properties when combined with various pesticidal products (Khot, 2009; War *et al.,* 2014). Dillapiole, apiole and myristicin, compounds commonly found in parsley-seed oil, dill oil and nutmeg oil, share structural similarities to the MDP ring and have proven to be strong synergists when combined with pyrethrum (Joffe, 2011).

There are a number of studies that continually challenge the hypotheses drawn from these studies, however, due to the varied successes and failures of insecticide/botanical combinations. For example, Tong and Bloomquist (2013) demonstrated that some essential oils had synergistic effects on larvae of *Aedes aegypti* but not adults; others demonstrate synergy as combined products or applied as binary formulations, but not when separated into their constituent compounds (Tamiru *et al.*, 2016) and Faraone *et al.,* (2015) demonstrated that lavender oil, thyme oil and their constituents synergised imidacloprid in *Myzus persicae* but, interestingly, showed antagonistic effects when they were combined with spirotetramat.

In the context of insecticide resistance, the use of a botanical synergist in combination with existing pyrethroid-based (and other) pesticides may offer an alternative to PBO that is more likely to be accepted by UK regulators and environmental groups alike. It would also provide a more economically efficient pest management option by reducing the amount of expensive, synthetic active ingredient required to demonstrate the desired mortality rate. Furthermore, due to the possible specificity of certain botanicals, a plant-based synergist also has the potential to reduce non-target species impact, simultaneously promoting natural pest predators, and partially or completely restoring effective control of insect pest populations. Finally, due to the biodegradable nature of botanical products, there would be minimal environmental persistence and impact long-term from plant-based synergist use (Isman, 2000; Koul, Walia and Dhaliwal, 2008). As such, despite the challenges of managing synergistic and antagonistic properties, specificity and varying degrees of success with insecticide combinations, botanicals may hold the key to a more economically valuable and environmentally friendly agricultural industry.

1.7 Target insects

1.7.1 Psylliodes chrysocephala (Linnaeus) (Coleoptera: Chrysomelidae), cabbage stem flea beetle

Figure 1.2. Cabbage stem flea beetle adult (Slater, 2020) and larva (San Martin, 2016)

The cabbage stem flea beetle (CSFB) is a significant brassica crop pest with established populations throughout Europe, North Africa, Asia, Canada and North America (Bonnemaison, 1965; Gruev and Döberl, 1997; CABI, 2021a). In the UK it has one generation per year and was previously limited to parts of East Anglia but has since spread to cover England, Wales and even parts of Scotland. The adults are around 3-5mm long, iridescent black and have enlarged hind legs and an adapted exoskeleton that allows them to spring powerfully away when disturbed (CABI, 2021a; Figure 1.2). They are also capable of short flights when temperatures reach more than 16°C, meaning they can disperse over two to three miles and migrate from spring to winter rape crops (Hoarau *et al.* 2022). Adults cause shot-holing to mature leaves and further damage to stems and pods of oilseed rape and other brassicaceous flora (Williams, 2010). A single female can lay up to 1000 eggs (Kaufmann, 1941 in Hoarau, 2022) in the soil at the base of plants and larvae will burrow through petioles into the stems to grow through three instars before dropping to the soil to pupate (Figure 1.3) Larvae that emerge in time with sowing the winter rape can cause entire crop failure if they feed on the growing point of seedlings (AHDB, 2024b). Established oilseed rape plants can tolerate high levels of larval infestation but their activities can cause distorted mature plants, reduced vigour, delayed flowering, increased susceptibility to frost damage and even total plant destruction should they reach sufficient numbers (Williams and Carden, 1961; Graham and Alford 1981, Nilsson, 1990; Winfield 1992; Nilsson, 2002 in Hoarau *et al.,* 2022). Furthermore, presence of this pest is also associated with fungal, bacterial and viral infections (CABI, 2021a).

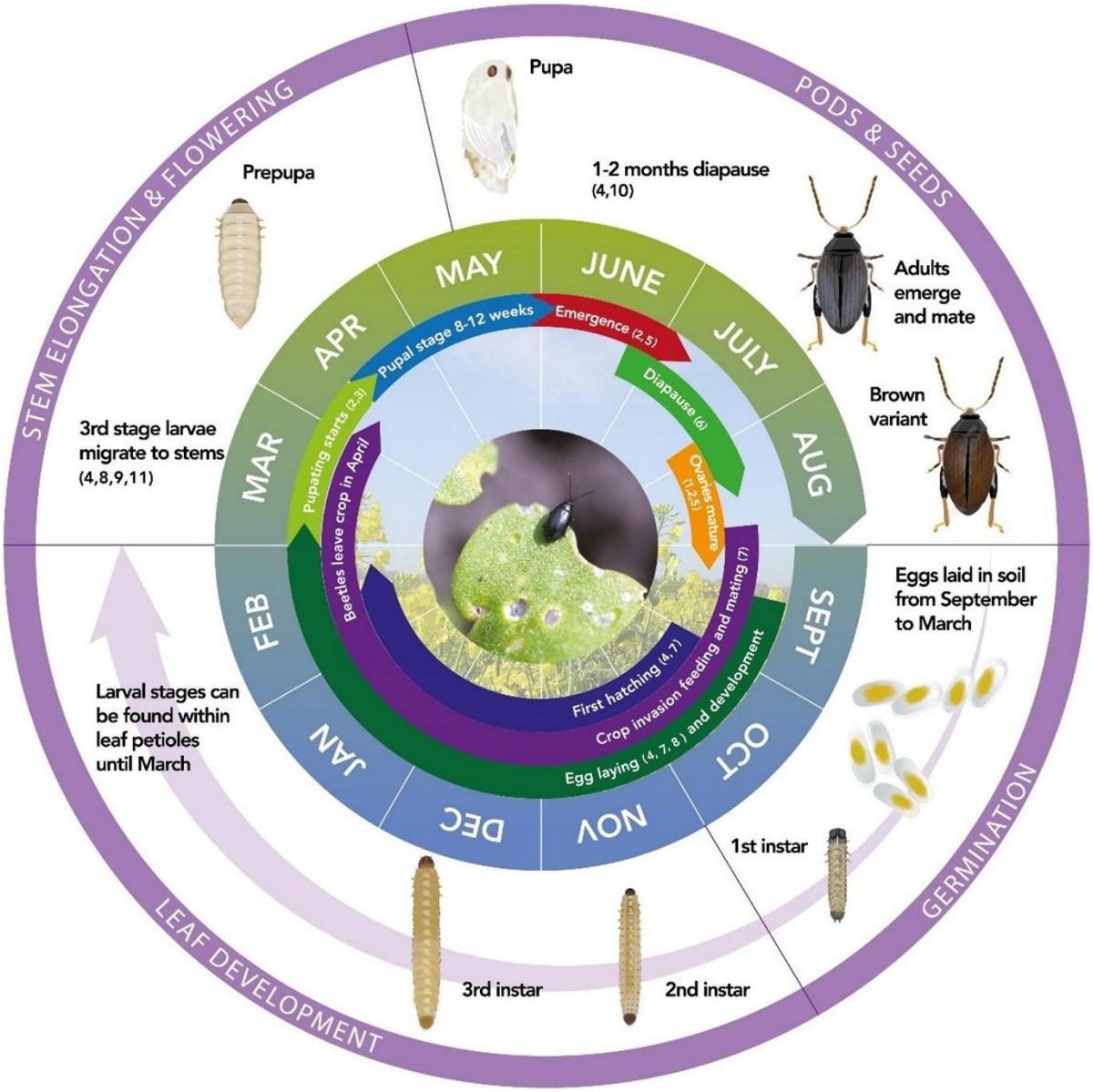

Figure 1.3. Cabbage stem flea beetle life cycle, relative to oilseed rape development (Source: Penny Greeves in Hoarau *et al.,* 2022, fig.1). Figures in brackets represent the following references in Hoarau *et al.,* 2022: 1: Börner and Blunck, 1920; 2: Kaufmann, 1941; 3: Godan, 1951; 4: Ebbe-Nyman, 1952; 5: Williams and Carden, 1961; 6: Bonnemaison, 1965; 7: Alford, 1979; 8: Alford *et al.,* 2003; 9: Nilsson, 1990; 10: Cox, 1998; 11: White, 2015.

The cabbage stem flea beetle has been determined as the most important pest of oilseed rape in Europe (Zheng *et al.*, 2020). Around 76% of the UK crop was affected by just the adults in 2014 (Nicholls, 2016) and it was also directly responsible for an approximate loss of 5.4% in the year 2016/17, which rose to 15% in some counties (John Innes Centre, 2024). Monitoring and reporting systems reveal that between ~5-15% of crop is lost each year to this species,

which equates to around £70m (Thursfield, 2019). An integrated pest management approach is recommended to try and mitigate their impact, historically with a focus on neonicotinoid seed treatments that helped to protect the crops during their most vulnerable seedling stage, followed by foliar sprays of pyrethroids (Williams, 2010; Hoarau *et al.*, 2022; AHDB, 2024c). However, on 1st December 2013 the use of neonicotinoid seed coats was banned by the European Commission due to the detrimental effects on bees and other non-target species (Council regulation (EU) 485/2013). Even prior to this, the first reports of pyrethroid-resistant CSFB were confirmed in Germany in 2008 (Heimbach and Müller, 2013) but the continued and sustained use of pyrethroids increased the selection pressure further until 100% resistant CSFB were recorded in the UK in 2020 (Willis *et al.,* 2020). Furthermore, Scott and Bilsborrow (2019) found that the area dedicated to oilseed rape production on more than 200 farms in England decreased in the seasons immediately following the ban, citing CSFB as a major influencing factor. However, despite a 40.6% reduction in oilseed rape area between 2012 and 2021, the total crop yield only reduced from 3.6 tons/ha to 3.2 tons/ha (Hoarau *et al.,* 2022) – this could suggest that applied research into alternative control measures and calculated IPM strategy (AHDB, 2024c) may be leading to some success.

Cabbage stem flea beetle was selected for this study due to its resistance status and high agricultural and economic impact worldwide. They were also the only species in this study to be collected from the field and known to be repeatedly exposed to pyrethroids.

1.7.2 Delia radicum (Linnaeus) (Diptera: Anthomyiidae), cabbage root fly

Figure 1.4. Cabbage root fly adult (AfroBrazilian, 2017) and larvae (Rasbak, 2004).

The cabbage root fly is present throughout Europe, Asia, North America, Canada and reports from parts of Africa are under review (CABI, 2021b). They have two to three overlapping generations per year in the UK, depending on temperature, and their larvae can cause significant damage to the roots of cruciferous vegetables (Capinera, 2020; AHDB, 2024d). Adults are grey-brown, vary in size between 5-7mm and feed on nectar, thus causing no damage to plants themselves (Capinera, 2020; Figure 1.4). The larvae, however, will feed on the root systems and sometimes the stems of various Brassicaceae species (Figure 1.4), often unnoticed, until the aerial part of the plant begins to wilt, delay maturity or show stunted growth (Smith, 1989; Santolamazza-Carbone *et al.,* 2017). Young or newly transplanted plants are particularly vulnerable to complete failure (Ferry *et al.,* 2009). Damage may not even be apparent until harvest and this can be a particular problem when the marketable part of the plant is the root e.g. turnip, swede, radish (Smith, 1989; AHDB, 2024d).

Current control methods involve seed treatments and module drenches before transplanting and/or at seedling emergence to ensure the soil is treated at the most vulnerable stage of plant growth. Once plants are established, foliar sprays have limited effect due to the females laying eggs in the soil surrounding the base of plants and larvae residing below the surface (AHDB, 2016; Capinera, 2020). Due to a lack of available chemical or commercial biological control, recommendations include prophylactic treatment using non-chemical control such as fine mesh netting, horticultural fleece and/or brassica collars to prevent females laying on crops, crop rotation to non-brassicas in successive years to prevent overwintered adults emerging under mesh, trap cropping and encouraging natural enemies (AHDB, 2016; Lamy *et al.,* 2020; RHS, 2024). However, these methods may not be practical for vast hectares of crop

coverage or consistently effective enough for a marketable quantity and quality of crop yield, particularly if more than one of these tactics is required to ensure sufficient production (Collier, *et al.,* 2020).

Regular use of insecticides remains an integral part of effective IPM strategy against cabbage root fly in Europe but the number of both registered and approved products is decreasing due to their detrimental environmental and human health impacts (Brühl and Zaller, 2019; Srivastava and Kesavachandran, 2019). Additionally, with brassica crop production worldwide being worth more than $20 billion, the UK market for the brassica vegetables and oilseed rape regularly reaching £1 billion per year (John Innes Centre, 2024) and that alternative options have limited efficacy, the threat that cabbage root fly may pose in the not-too-distant future could have significant economic impact. Despite this species having no current known pesticide resistance, the high potential for it to develop and threaten cruciferous vegetable production made this an important pest to investigate alternative control measures with. A reduction in the amount of applied active ingredient in the presence of a botanical synergist would also reduce the selection pressure for cabbage root fly, and other pest species, to develop pesticide resistance in future generations, thereby maintaining the efficacy of chemical control when required.

1.7.3 Myzus persicae (Sulzer) (Hemiptera: Aphididae), peach-potato aphid

Figure 1.5. Peach-potato aphids (San Martin, 2021).

The peach-potato aphid is a globally distributed and economically significant crop pest with a strikingly rapid generation time and ability, not just to withstand, but thrive in highly variable environmental conditions (Capinera, 2014; CABI, 2021c; Alyokhin, Wenninger and Jensen, 2022). Aphids are hemipteran and this species can feed on both the phloem and xylem fluids of hundreds of different plants in over 40 plant families (Capinera, 2014). They exhibit a wide range of colours and are 1-2mm long (Figure 1.5). *M. persicae* is parthenogenetic throughout most, if not all, of the year meaning it can exhibit exponential population growth, has both unwinged (apterous) and winged (alate) forms so it can travel between hosts with ease and shows remarkable plasticity in temperate climates by producing sexual adults in autumn so that fertilised and genetically varied eggs can be laid and overwintered. Alate individual generation is stimulated by increased population numbers and poor host health so they can effectively self-manage their own infestation to maintain optimal production conditions (Dedryver, Le Ralec and Fabre, 2020).

Excessive population density is normally not enough in itself to cause crop failure, although it can do if the seedlings are young enough. It can, however, cause leaf-curling, chlorosis and stunting at high infestation levels, as well as sooty mould associated with honeydew production. Due to its life cycle, ubiquitous distribution and polyphagous nature Myzus persicae is a formidable vector of over 100 plant viruses such as Turnip yellows virus (TuYV), Potato leafroll virus (PLRV) and Beet mosaic virus (BtMV). TuYV infected crops can reduce oilseed rape yield by up to 30% and affect the oil content of the harvest (AHDB, 2024e). PLRV is the most destructive virus affecting potato crops, significantly impacting growth and production and causing tuber necrosis, among other symptoms (Agrios, 2005). Yield losses of

up to 50% have also been seen in cases of beet yellows, alongside an increased level of impurities seen in the sugar extracted from the surviving plants (CABI, 2021c).

Attempts to reduce aphid numbers are seen as the only way to try and reduce infection rate. However, control of virus transmission is incredibly difficult as it may only take a small number of aphids and a short amount of time to infect entire fields. Added to this, *M. persicae* has been recorded as showing exceptionally high resistance to almost all classes of insecticide to be produced, including neonicotinoids (Bass et al., 2014; Mottet *et al*., 2024). Their rapid generation time and ability to move between sexual and asexual forms allows the species to both evolve quickly in response to intense selection pressures (such as consistent exposure to chemical pesticides) and create vast numbers of resistant individuals. The only group of insecticides that allows growers to still retain some control over this species are neonicotinoids, but several were withdrawn from outdoor use in the UK in 2013 (Council regulation (EU) No 485/2013). Emergency approval has, however, been granted for the use of thiamethoxam on sugar beet in England each year since 2021 due to the predicted impact of beet yellows spread by, among others, *M. persicae*. The resulting outcry by environmental groups and concern for the widespread effects the use of this pesticide could have (development of further resistance, impact on pollinators, environmental contamination etc.) makes the investigation of potential alternative control of this species of the utmost importance and why this species was selected for this project.

1.7.4 Plutella xylostella (Linnaeus) (Lepidoptera: Plutellidae), diamondback moth

Figure 1.6. Diamondback moth adult (International Institute of Tropical Agriculture) and caterpillar (Fk, 2006).

The diamondback moth is a universally distributed and highly invasive lepidopteran which has a presence wherever cruciferous vegetation exists. The adult's migratory nature and ability to travel long distances in the wind make it particularly adept at dispersing across the world. The adult moths are grey-brown and around 6mm in length with a distinctive diamond pattern on the folded wings, hence the name. Only the larvae of this species cause damage to plants as the adults will feed on nectar of flowering plants. Larvae have four instars and are voracious in appetite, feeding on all parts of the plant including leaves, flowers and seed pods, before pupating in a silken cocoon (Figure 1.6). Development time depends largely on temperature and adults begin to mate and lay eggs on the day of emergence so they can have up to 20 overlapping generations in a single year (CABI, 2022).

Larvae can affect plants at every growth stage, mining the leaves in the first instar before emerging and continuing to cause shot-holes and often a total loss of foliage. Seedlings can be destroyed outright, with affected growth points causing distortion and stunting in adult plants if they survive. Damage caused to flowers and seeds also affects the reproductive ability of the plants and significantly reduces crop yields (Canola Council of Canada, 2024). Additionally, larval feeding can leave the plant vulnerable to fungal infection, causing powdery mildew and blight. The current control recommended for this species is to take an IPM approach. Like with other pests, there are alternative field management considerations such as inter- and trap cropping, sprinkler irrigation and use of covers but these have had limited success and are often not practical or cost-effective on a commercial scale (CABI, 2022). The IPM method invariably includes use of chemical control but this species has now consistently

shown resistance to pyrethroid pesticides and some are now developing it to the newer classes, such as diamides (Troczka *et al.,* 2012). Diamondback moth is susceptible to a range of natural enemies (CABI, 2022) but use of pyrethroids in an area where resistance is present may now only serve to clear the arthropod predators from the crops, leaving the population free to erupt and cause further loss. It was also the first crop pest to develop resistance to the common Cry-toxin-containing *Bacillus thuringiensis* bio-pesticide, however the specific mechanisms for it are currently debated (Crickmore, 2016).

The average global production of cruciferous vegetables is nearly 90 million tonnes per year, with just three types (cabbage, cauliflower and broccoli) accounting for US$32.3 billion and oilseed brassicas valued at US$35 billion in 2020 alone (Food and Agriculture Organization of the United Nations, 2023). The diamondback moth is directly responsible for an estimated US$4-5 billion loss of this production every year (Furlong, Wright and Dosdall, 2013), and this combined with a dwindling availability/efficacy of chemical or any other control, rising concerns over resistance status and potential disruption of global food supply makes for an exceptionally important pest for this study to investigate an effective control method for.

1.8 Summary and the project

While there is a general societal and policy-driven transition away from the widespread use of synthetic chemical pesticides, a complete and immediate halt on applying them would create a global, life-threatening risk to food security. Cultural methods and biopesticides alone can have variable effects on crop pest management and subsequent yield results, as well as often being impractical on large-scale outdoor commercial production. As such, they are not yet at the stage where they can fully replace the use of chemical control but remain an important part of the IPM strategy. However, given the ever-increasing number of active ingredients/modes of action that insects are becoming significantly or 100% resistant to and the dwindling number approved for use, the onus is now on finding more sustainable and less detrimental alternatives. Botanical synergists present a valuable opportunity for the development of novel crop protection products that may exhibit lower selection pressure for resistance and a less impactful outcome on human and environmental health.

With the above in mind this project had three main aims:

1) Investigate if selected botanical extracts have *in vitro* inhibitory effects on the main groups of detoxification enzymes involved in metabolic resistance in the target insect species.
2) Determine whether the same botanical extracts have potential as synergists when combined with selected plant protection products *in vivo* in a range of crop pests: cabbage stem flea beetle *Psylliodes chrysocephala*, cabbage root fly *Delia radicum*, peach-potato aphid *Myzus persicae* and diamondback moth *Plutella xylostella*.
3) Investigate a possible correlation/cause and effect between any apparent synergism *in vivo* and enzyme inhibition *in vitro*.

Chapter 2. General Materials and Methods

2.1 Insects

2.1.1 Psylliodes chrysocephala

Adult cabbage stem flea beetles were collected using a mechanical pooter (Watkins & Doncaster) from fresh rapeseed (*Brassica napus* L.) in storage sheds within 1 day post-harvest in July 2021 and 2022, at a commercial farm in North Yorkshire, UK. The crops were known to have been treated with a Lambda-cyhalothrin based pesticide product in both years. They were kept in BugDorms (Watkins and Doncaster, UK) at 20°C, 60% relative humidity and a 16h:8h light:dark cycle. Fresh leaves from 2-3 week old untreated oilseed rape plants were added twice weekly and dried leaves removed. Adult beetles were prepared for enzyme activity assays within 2 weeks of collection. Bioassays were completed within 2 months of collection using mixed-age adults.

2.1.2 Delia radicum

Cabbage root fly were reared according to an adapted method from Finch & Coaker (1969) with no exposure to pesticides over the generations. They were maintained at 20°C, 60% relative humidity and a 16h:8h light:dark cycle.

Adult flies were kept in BugDorms and provided with water in a deli pot with capillary matting to act as a wick. Dry diets were presented in 30ml plastic pots and consisted of dry yeast powder and dry granulated sugar. Water and diets were replaced every two weeks. Flies were also supplied with a Petri dish containing dry sand and a small piece of swede to attract oviposition.

Each week, two 8-inch diameter plant pots were lined with muslin cloth and approximately an inch of dry sand poured in. A whole swede, sand to fill half the pot and water were added, followed by the fly eggs sprinkled around the swede. More sand was filled to the top of the pot, water poured on to dampen and pots were placed in the above conditions for 35 days.

After 35 days, the remains of the swede were removed and the sand flushed through a sieve (aperture size 1.7mm) with cold water, leaving the pupae behind. The pupae were then collected into deli pots on top of a layer of vermiculite and put into a clean BugDorm. Fresh

cages were prepared every week to hold the pot of new pupae and the previous two weeks' pupae were also added. Flies used for enzyme activity assays and bioassays were between 0- and 8-days post-emergence.

2.1.3 Myzus persicae

Green peach aphids were kept in plastic and fine mesh insect culture cages (York Plastics (Eng) Ltd.) with the open side set flat onto water trays. They were kept at 20°C, 60% relative humidity and a 16h:8h light:dark cycle. Two 1L pots containing approximately 3-week old Chinese cabbage (*Brassica rapa* L. var. *yuki*) grown in compost were placed into the water tray and infested with aphids. One fresh cabbage plant was added to the cage twice per week and the oldest plant removed. All plants were grown from seed under glasshouse conditions at Fera Science Ltd. (Sand Hutton, York). Adult aphids were collected from the culture using a soft-bristled paintbrush and prepared for the enzyme activity assays. Aphid nymphs used for bioassays were between 0- and 24-hours old.

2.1.4 Plutella xylostella

The diamondback moth culture was started from caterpillars obtained from a stock culture kept at the University of Warwick, UK. They were kept in BugDorm insect rearing tents (Megaview Science Co. Ltd., Taiwan) on Chinese cabbage (*Brassica rapa* L. var. *yuki*) at 20°C, 60% relative humidity and a 16h:8h light:dark cycle. Twice weekly, 2-3 cabbage plants (approximately 3 weeks old and grown as in section 2.1.3) were added to each tent, any severely damaged plants were removed and all plants were watered. Cotton wool soaked with 10% sugar solution (w/v water) was provided and replaced weekly in the adult moth tents only. Caterpillars used for enzyme activity assays and bioassays were 3rd instar.

2.2 Insecticides

Insecticides were selected based on current approval status for the UK and recommendations for control of nominated insect pests (Liaison, 2024). Analytical grade cypermethrin and lambda-cyhalothrin were purchased from Sigma (UK). Field rate solutions and serial dilutions were prepared in acetone. Field rates for analytical products were determined by calculating recommended application doses for the corresponding formulated products (Cythrin and Clayton Sparta respectively) containing the active ingredients by area or by volume depending on bioassay method.

Tracer (Dow Agrosciences – now under Corteva Agriscience, Cambridge, UK) containing the active ingredient Spinosad (480g/L) was diluted in water to field rate concentration (1ml/L) and serial dilutions made thereafter. Tween 20 (Sigma-Aldrich) is a nonionic detergent that was added to Tracer solutions and the water control at 0.1% to allow the droplet to spread onto the insect.

PREV-AM (Oro Agri) is a formulation manufactured as an insecticide, fungicide and acaricide, with the active ingredient orange oil (60g/L). Recommended field application rate is 0.4-0.8% diluted in water. The maximum of 0.8% was used in the bioassays.

FLiPPER (Crop Science/Bayer) is a bioinsecticide and acaricide containing 479.8g/L fatty acids C7-C20. Recommended field application rate is 1.6% diluted in water.

2.3 Synergists

All potential synergists tested in bioassays and enzyme assays are referred to as 'synergist' throughout this thesis, even if they have not then shown synergistic activity. As PREV-AM's active ingredient is listed as orange oil, this product was tested as a potential synergist. FLiPPER was also tested as a potential synergist.

Table 2.1. Details of potential synergists used in bioassays and enzyme activity assays.

Synergist (common name)	Source	Description
Piperonyl butoxide	Sigma-Aldrich	Technical grade, 90%
Neem oil	Ourons	100%, 1500ppm azadirachtin
Garlic oil	Nikura	100%, *Allium sativum*
Thymol	Sigma-Aldrich	≥98.5%
Eugenol	Alfa Aesar/Thermo Scientific	99%
Geraniol	Sigma-Aldrich	98%
Sesame oil	Naissance	100%, *Sesamum indicum*
Bergamot oil	Nikura	100%, *Citrus bergamia*
Rapeseed oil	Biona Organic	100%, *Brassica napus*
Black pepper oil	Nikura	100%, *Piper nigrum*
Orange oil	Sigma-Aldrich	*Citrus sinensis*
Rosemary oil	Nikura	100%, *Rosemarinus officinalis*
Tea tree oil	Sigma-Aldrich	*Melaleuca alternifolia*
Borage seed oil	Sigma-Aldrich	*Borago officinalis*
D-limonene	MP Biomedicals	96.9%
Lemongrass oil	Nikura	100%, *Cymbopogon citratus*
Parsley seed oil	Nikura	100%, *Petroselinum sativum*
PREV-AM	Oro Agri	60g/L Orange oil
FLiPPER	Crop Science/Bayer	479.8g/L fatty acids C7-C20

2.4 Enzyme activity assays

All essential oils were diluted in acetone to 10%, 1% and 0.1% for use in the enzyme assays. PBO was diluted in acetone to 1%, 0.1% and 0.01% stock concentration to measure inhibition tenfold either side of, and including, the recommended application dose. PREV-AM and FLiPPER were diluted in water and also tested to measure inhibition tenfold either side of, and including, the recommended application doses (0.8% and 1.6% respectively). The final well concentrations are shown in the figure legends.

2.4.1 Esterase activity

Esterase activity was determined by measuring the rate at which the enzymes hydrolysed para-nitrophenyl acetate to produce para-nitrophenol, using a kinetic method adapted from Joffe *et al.* (2012). Insects were homogenised in 0.02M sodium phosphate buffer, pH 7.0 at the following quantities: *D. radicum*: ~20 flies/ml; *P. chrysocephala*: ~25/ml; *M. persicae:* ~50/ml; *P. xylostella*: ~20/ml. The homogenate was centrifuged at 10,000 x g for 15 minutes, the supernatant decanted to fresh Eppendorf tubes to remove some of the debris and centrifuged for another 15 minutes at 10,000 x g. Supernatant was then transferred to fresh Eppendorf tubes, flash frozen in liquid nitrogen and kept at -80°C until use.

Preliminary tests were run using varying amounts of insect homogenate to ascertain a level of activity to show significant inhibition by the synergists. To a 96-well plate, 47µl of insect homogenate and buffer were added to each test well, followed by 3µl of synergist in acetone or water. They were left for 30 minutes to incubate at room temperature. After incubation, 200µl of 1mM para-nitrophenyl acetate (initially diluted in ethanol and then added to the homogenisation buffer) was added to each well and a kinetic assay run on a Labsystems Multiskan MS plate reader at 405nm for 10 minutes with 10 second intervals between readings. The software Genesis was used to monitor and calculate vmax values. Each synergist and control test were run in triplicate and either acetone or water used as the corresponding control. To determine synergist interference, buffer blanks were run for each synergist by adding the equivalent amount of buffer in place of enzyme homogenate.

2.4.2 Glutathione S-transferase activity

Glutathione S-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate, with methods adapted from Zhu *et al*. (2007) and a Sigma assay kit. Insects were homogenised in 0.1M sodium phosphate buffer, pH 8.0 at the following quantities: *D. radicum*: ~25 flies/ml; *P. chrysocephala*: ~25/ml; *M. persicae*: 50/ml; *P. xylostella*: ~20/ml. The homogenate was centrifuged at 10,000 x g for 15 minutes, the supernatant decanted to fresh Eppendorf tubes to remove some of the debris and centrifuged for another 15 minutes at 10,000 x g. Supernatant was then transferred to fresh Eppendorf tubes, flash frozen in liquid nitrogen and kept at -80°C until use.

Preliminary tests were run using varying amounts of insect homogenate to ascertain a level of activity to show significant inhibition by the synergists. Due to extensive activity, cabbage root fly and cabbage stem flea beetle homogenates were used at 1:5 and 1:8 dilutions respectively. To a 96-well plate, 47µl of insect homogenate and buffer were added to each test well, followed by 3µl of synergist in acetone or water. They were left for 30 minutes to incubate at room temperature. A master mix containing 200mM L-Glutathione reduced and 100mM CDNB in buffer was made immediately before the reaction was started. After incubation, 150µl master mix was added to each well and a kinetic assay run on a BioTek Epock 2 microplate reader at 340nm for 10 minutes with 30 second intervals between readings. The software Gen5 (version 3.10) was used to monitor absorbance levels. The GST specific activity was calculated using the following steps:

1. Plot the absorbance over time.
2. Calculate the change in absorbance in the linear range:

   ΔA_{340}/min = change in absorbance per minute

$$\Delta A_{340}/\text{min} = \frac{A_{340}(\text{final read}) - A_{340}(\text{initial read})}{\text{reaction time (mins)}}$$

3. Subtract the change in absorbance of the blank from that of the sample.
4. Calculate GST specific activity:

$$\left[\frac{(\Delta A_{340}/\text{min}) \times V}{\varepsilon_{mM} \times V_{enz}}\right] \times \text{dil} = \mu\text{mol}/\ \mu\text{l}\ /\text{min}$$

where:

V = reaction volume (µl)

dil = dilution factor of the original sample

ε_{mM} ($mM^{-1}cm^{-1}$) = the extinction coefficient for CDNB conjugate at 340nm; 5.3 mM^{-1}

V_{enz} = volume of enzyme sample (µl)

2.4.3 P450 Monooxygenase activity

Insect P450 monooxygenase activity was measured by using an adapted method from Ullrich and Weber (1972) and de Sousa *et al.* (1995). Insects were homogenised in 0.1M sodium phosphate buffer, pH 7.6 at the following quantities: *D. radicum*: ~20 flies/ml; *P. chrysocephala*: ~33/ml; *P. xylostella*: ~15/ml. The homogenate was centrifuged at 10,000 x g for 5 minutes and the supernatant decanted to fresh Eppendorf tubes to remove some of the debris. The supernatant was then used immediately as the enzyme source.

To a 96-well plate, 90µl of insect homogenate and buffer were added to each test well followed by 3 µl of synergist in acetone or water. They were left to incubate for 30 minutes at room temperature then 2µl of 20mM 7-ethoxycoumarin in acetone and 10µl of 10mM nicotinamide adenine dinucleotide phosphate (NADPH) in water were added. The plate was incubated again for 30 minutes at 30°C while shaking (400rpm). With these substrates, the P450 enzymes should produce umbelliferone. As NADPH fluoresces within the same wavelength as umbelliferone and to avoid distorting the measurement of enzyme activity, the remaining NADPH was oxidised to the non-fluorescent NADP+ by adding 10µl of 100mM oxidised glutathione in water and 1.3 units of glutathione reductase to each well. The plate was incubated for another 10 minutes at room temperature before 125µl of acetonitrile and TRIZMA base buffer (0.05M, pH10, 50% vol:vol) were added to stop the enzyme activity. Fluorescence was then measured with a Tecan Infinite 200 Pro plate reader (Tecan Group Ltd.) at 460nm and exciting at 380nm. The specific enzyme activity was determined using an umbelliferone standard curve. To determine synergist interference, buffer blanks were run for each synergist by adding the equivalent amount of buffer in place of enzyme homogenate. Each synergist and control test were run in triplicate and either acetone or water used as the corresponding control.

2.4.4 Protein analysis

Protein content of each insect homogenate sample was analysed using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as the standard. Reagent and BSA sourced from Sigma (UK).

2.5 Bioassays

Ten individuals of each insect were used per replicate, with three or six replicates per treatment.

2.5.1 Dose-response

Dose-response bioassays for each pesticide were completed using modified IRAC susceptibility test methods described below (sections 2.5.2, 2.5.3, 2.5.4). Serial dilutions of the pesticides were made either in water (formulated product) or acetone (analytical grade active ingredient). Knockdown and mortality were assessed over a 48-72 hour period. The LC_{10} was selected for each insect and used in the synergist bioassays. Synergists were also tested, as above, for inherent insecticidal properties using 1% concentration or recommended field rate dose.

*2.5.*2 Psylliodes chrysocephala *bioassays*

Psylliodes chrysocephala bioassays were completed using modified IRAC susceptibility tests (No. 011, IRAC, 2009) for pollen beetles. Pesticide in acetone (1ml total volume) was pipetted into 30ml glass vials which were then rotated for an hour on a tube roller (model SRT6, Stuart Scientific) to evenly coat the inner surface and evaporate the acetone. Synergist solution (1µl) was applied to the dorsal surface of anaesthetised beetles and they were left for 30 minutes. After this time, the beetles were transferred to the vials together with a 22mm diameter disc cut from the leaf of an oilseed rape plant and a ventilated lid. Treatment groups where acetone was applied in place of the synergist and pesticide were included as controls. They were kept as per the culture conditions described above (2.1.2). Beetles were recorded as dead if they did not move within 30 seconds of the vial being disturbed. They were recorded as knocked down if they were on their backs upon initial inspection and could not right

themselves/show coordinated movement upon disturbance. Knockdown and mortality were monitored daily over a 72-hour period.

2.5.3 Delia radicum bioassays

Bioassays for *Delia radicum* were done using a modified IRAC susceptibility test (No. 029, IRAC, 2013) for stink bugs. Anaesthetised flies had 1µl of synergist solution applied to their dorsal surface using a micropipette and were left for 30 minutes before 1µl of pesticide was applied in the same way. Treatment groups where acetone or water and Tween 20 were applied in place of the synergist and pesticide were included as corresponding controls. They were kept in pots with a 20% sucrose solution as a food and water source and conditions as described for the cultures above (Section 2.1.2). Flies were recorded as dead if they did not move within 30 seconds of disturbance. They were considered knocked down if they were not standing upright and/or unable to make coordinated movement. Mortality and knockdown were monitored daily over a 48-hour period.

2.5.4 Myzus persicae bioassays

Myzus persicae bioassays were completed using modified IRAC susceptibility tests (No. 027 and No. 029, IRAC, 2012; 2013) for pollen beetles and stink bugs. Pesticide in acetone (1ml total volume) was pipetted into 30ml glass vials which were then rotated for an hour on a tube roller (model SRT6, Stuart Scientific) to evenly coat the inner surface and evaporate the acetone. Synergist solution (1µl) was applied to the dorsal surface of nymphs and they were left for 30 minutes. They were then introduced to the vial for a 2-hour period, before being transferred to a Petri dish containing a 22mm diameter disc of Chinese cabbage mounted in 1% agar. Treatment groups where acetone was applied in place of the synergist and pesticide were included as controls. Aphids were recorded as dead if they did not move after disturbance with a soft paintbrush. Aphids that were unable to stand and/or show coordinated movement after disturbance were recorded as knocked down. Mortality and knockdown were recorded after a 48-hour period.

2.5.5 Plutella xylostella *bioassays*

Plutella xylostella bioassays were completed using a modified IRAC susceptibility test (No. 018, IRAC, 2010) for this species. Third instar larvae had 1µl of synergist solution applied to their dorsal surface using a micropipette and were left for 30 minutes. Leaf discs (22mm diameter) cut from a Chinese cabbage were dipped and agitated for 10 seconds in pesticide before being left to air dry on paper towel, abaxial surface up. Five leaf discs were placed on top of filter paper in each pot and the larvae added. Treatment groups where acetone or water were applied in place of the synergist and pesticide were included as corresponding controls. Larvae were kept at conditions described above (Section 2.1.2) and recorded as dead if they did not move after disturbance with soft tweezers. Mortality was recorded after a 48-hour period.

2.6 Data analysis

Enzyme data sets were analysed in R Studio (R Core Team, 2020) using a parametric linear mixed effects model. Residuals were assessed for normal distribution using a Shapiro-Wilk test and variance assessed using a Levene's test. One data set (*P. chrysocephala* P450 assay, highest concentration) was transformed using log+1 scale in order to fit these assumptions. Homogeneity of variance was true in all cases. Slight abnormal distribution of the data set was accepted as the model is robust enough to present an accurate result. The p-values from the linear mixed effects model were adjusted using the Bonferroni-Holm correction. Post-hoc comparisons were conducted to assess differences between treatments using the Tukey Honest Significant Difference (TukeyHSD) test. The model outputs were subsequently converted to show activity as percentage of the control to allow a more direct comparison to be made between similar published studies.

Dose-response assay data were analysed using probit analysis to calculate LC_{10} values (Finney, 1971) with SPSS (IBM SPSS Statistics 29).

Abbott's formula was used to correct for control mortality in the bioassay data, where this occurred (Abbott, 1925).

Bioassay data were analysed by calculating the co-toxicity factor of each synergist using the formula:

$$\left[\frac{(\text{observed \% mortality}) - (\text{expected \% mortality})}{(\text{expected \% mortality})}\right] \times 100$$

Where expected % mortality = % mortality with pesticide alone + % mortality with synergist alone. A co-toxicity factor of >20 means there is synergistic activity, <-20 suggests antagonistic activity and a factor that falls between these two means the result is simply the additive effect of the two components (Mansour *et al.* 1966).

As the data did not meet the assumptions of a parametric test, a Spearman's correlation was used to ascertain if there was a relationship between the mortality data and the enzyme activity data, using R studio (R Core Team, 2020).

Chapter 3. The evaluation of selected botanicals and products as esterase, glutathione s-transferase and cytochrome P450 monooxygenase inhibitors

3.1 Summary

The enzyme groups involved in the detoxification of insecticides – esterases, glutathione s-transferases and cytochrome P450 monooxygenases – are an important focus for potential synergists. The inhibition of any of these enzymes within the insect could remove a significant layer of defence and allow more of the pesticide to reach its intended target before it is metabolised, thereby enhancing its efficacy and potentially restoring control of metabolically resistant insect pests. Several of the botanical extracts and products tested in this study demonstrated significant inhibition of these enzymes, although some also appeared to increase their activity. Effects on enzyme activity also varied depending on the enzyme group being tested, species of insect and dose of the botanical.

3.2 Introduction

Select groups of enzymes within the bodies of insects have developed to overcome plant defensive compounds to ensure they can continue to feed safely on their hosts. It is these groups that have developed to combat the effects of chemical insecticides and thus provide the insect with a level of protection, known as metabolic resistance.

Esterases are a large group that cover various roles in the body of an insect. Acetylcholinesterase (AChE), for example, is an enzyme involved in the successful transmission of nerve impulses across synapses and interference, with its function being the target of some insecticides, including organophosphates and carbamates (Siegfried and Scott, 1990; Gunning, Moores and Devonshire, 1998). Mutations in the genes encoding AChE have resulted in resistance to these compounds as the enzymes are overproduced and/or demonstrate enhanced activity whereby pesticides are rapidly detoxified before they can have a lethal effect on the insect. Carboxylesterase, on the other hand, has an important role in detoxification of xenobiotics (those foreign to the body) within the insect, e.g. plant defensive compounds, but have more recently adapted to confer the same protection from synthetic insecticides. Gene amplification and subsequent overproduction of the specific carboxylesterase E4 in the green peach aphid (*Myzus persicae*) has been identified as being

integral to their resistance to pyrethroids, carbamates and organophosphates (Devonshire and Moores, 1982). Carboxylesterases catalyse the hydrolysis of ester bonds in the pesticide actives, altering their ability to bond with target sites within the body, and produces polar metabolites of acid and alcohol groups that can then be more easily excreted from the body (Panini *et al.*, 2016). Esterases can also sequester insecticidal compounds so they cannot bind with their intended target sites (Wang *et al.,* 2018). One or more of these adaptations are responsible for the pesticide resistance shown in several crop pest species, including the diamondback moth (*P. xylostella*), Colorado potato beetle (*Leptinotarsa decemlineata*) and cotton aphid (*Aphis gossypii*).

Cytochrome P450 monooxygenases (P450s) are a varied group of phase 1 enzymes with roles in a diverse range of processes throughout the insect body, including metabolising and synthesising endogenous substances such as fatty acids, hormones and pheromones. Phase 1 enzymes carry out oxidation, reduction and hydrolysis and usually render harmful compounds less toxic and facilitate excretion of them (Kshatriya and Gershenzon, 2024). As such, they are also a principal group involved in detoxifying xenobiotics such as insecticidal compounds, originating from plant or anthropogenic sources (Feyereisen, 2005). Despite issues such as difficulties in obtaining high enough yields of P450s and their relative instability, it has been demonstrated that resistant insects show increased levels of these enzymes, either due to upregulation or gene amplification (Feyereisen, 2005; Amichot *et al*., 2004; Wondji *et al*., 2009).

Like P450s, glutathione s-transferases (GSTs) are multifunctional within the insect body and are also involved in detoxification of xenobiotics. As phase 2 enzymes, they catalyse the conjugation of electrophilic substances – such as phase 1 products, allelochemicals and insecticidal compounds – with reduced glutathione and thereby reduce their toxicity (Konanz and Nauen, 2004; Després *et al.,* 2007; Kshatriya and Gershenzon, 2024). The resulting molecules are more easily soluble in water and excreted from the body (Habig *et al.,* 1974). The role of GSTs in this type of resistance has been identified in *P. xylostella*, conferring protection against parathion and methyl parathion (Kao and Sun, 1991).

Successful inhibition of any of these enzyme groups could result in the insects showing a higher susceptibility to the pesticides, thereby restoring their efficacy in resistant insects and enhancing control. As such, assessing the effects of various botanical compounds and formulated products on the activity of these three enzyme groups *in vitro* would highlight any

that have strong inhibitory characteristics and may, therefore, be promising candidates for synergists to use in combination with pesticides in bioassays.

3.3 Esterase activity results

In all cases, the plate was a significant influencing random factor on the response of the model ($p<0.05$, see Appendix A). Due to the number of synergists tested, the esterase activity assay for each insect species required seven microtitre plates. To determine synergist interference, buffer blanks (where the equivalent amount of buffer was added in place of enzyme homogenate) were included for each synergist on each plate. The relevant uninhibited controls for the synergists being tested were also included on every plate. In place of the botanical oils and PBO, this meant the equivalent amount acetone was added. In place of PREV-AM and FLiPPER, the equivalent amount of water was added.

The Tukey honest significant difference test was then run between the synergists and the relevant control on the plate they were tested on to ensure that the plate variation and any difference between controls were considered in the results. The model estimates were subsequently converted to show activity as percentage of the uninhibited control. The upper and lower 95% confidence limits are shown by the error bars as they are a more complete representation of the uncertainty of the model estimates than the standard error in this analysis.

3.3.1 Psylliodes chrysocephala

All synergists tested showed significant inhibition of esterase activity at the highest and intermediate concentrations when compared with the control ($p<0.05$). The most effective inhibitors at these concentrations were black pepper oil, garlic oil, lemongrass oil, parsley-seed oil, rosemary oil and thymol, reducing activity by around 50% or more in each case (Figure 3.01 A and B). Similarly, these synergists also significantly reduced activity at the lowest concentration, although the reduction in activity was comparatively less severe (Figure 3.01 C). Sesame oil, rapeseed oil and borage oil showed the least potency as esterase inhibitors across all concentrations tested in this species.

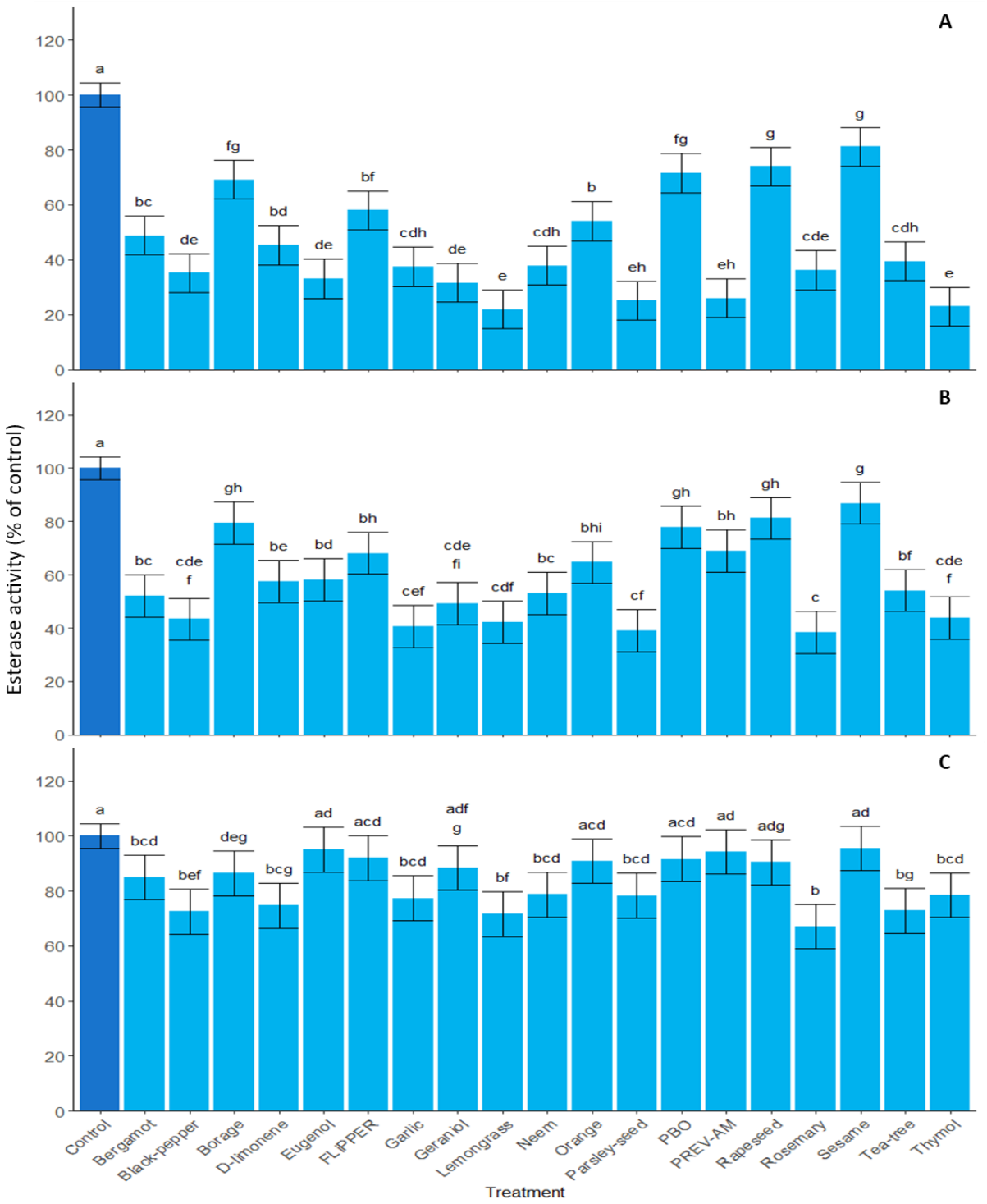

Figure 3.01. Effect of treatment on *Psylliodes chrysocephala* esterase activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.3.2 Delia radicum

Esterase activity of *D. radicum* was significantly affected by eight of the synergists tested at the highest concentration, with eugenol, neem oil, parsley-seed oil, PBO, PREV-AM and thymol being the most potent (Figure 3.02 A). This was also reflected when tested at the intermediate concentration although the reduction in activity was less evident (Figure 3.02 B). At the lowest concentration, only five of the synergists – eugenol, orange oil, PBO, PREV-AM and thymol demonstrated significant inhibition of cabbage root fly esterase (Figure 3.02 C). Interestingly, orange oil appeared to inhibit the esterase at the lowest concentration but not at the higher concentrations. Also, in contrast to all other treatments, geraniol increased esterase activity to higher than the uninhibited control at both the highest and intermediate concentrations.

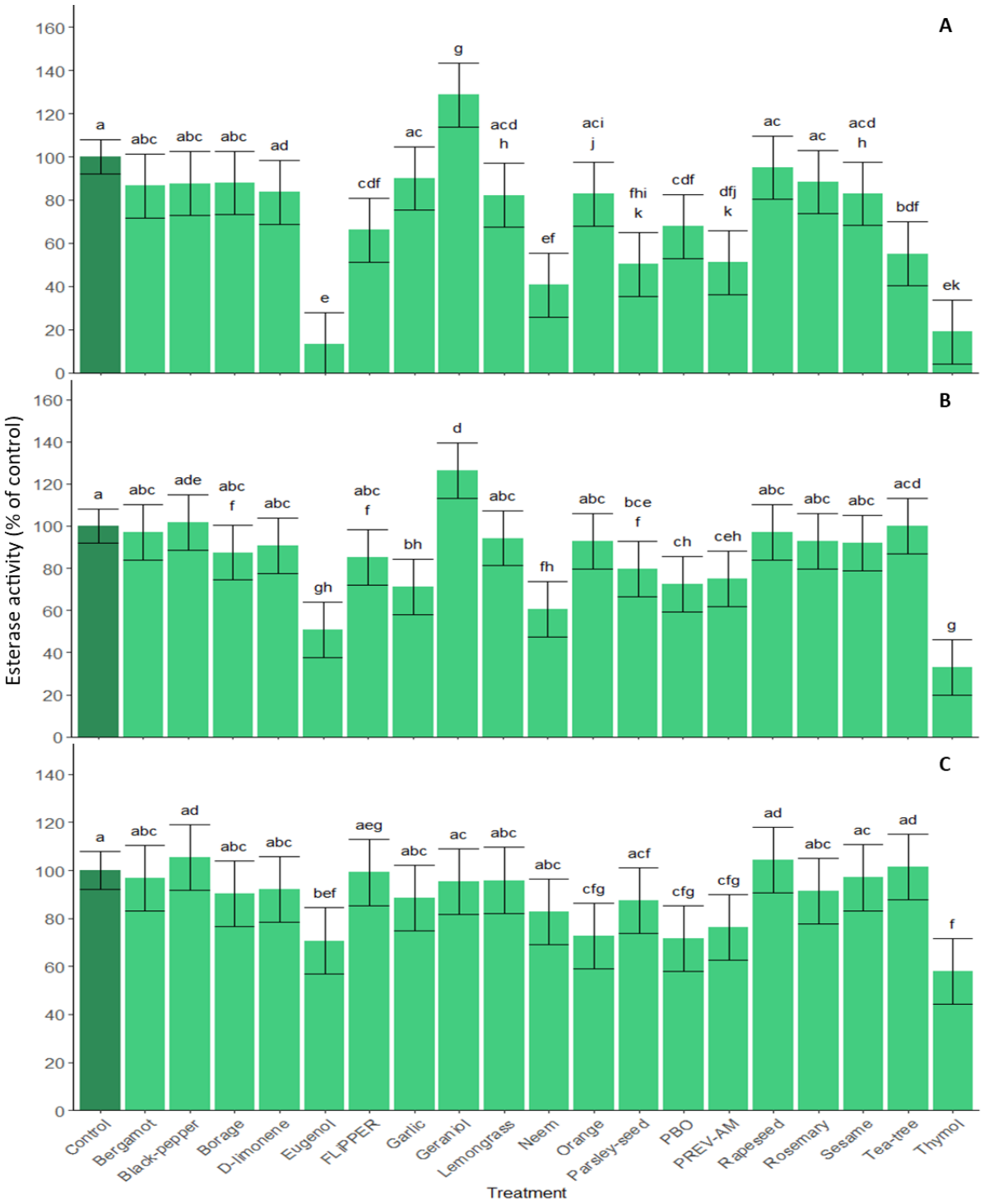

Figure 3.02. Effect of treatment on *Delia radicum* esterase activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.3.3 Myzus persicae

Few of the synergist treatments had any significant effect on esterase activity in the *M. persicae* homogenate, particularly at the lower concentrations. Indeed, at highest concentration, only eugenol, lemongrass oil, parsley-seed oil, PREV-AM, rosemary oil, tea-tree oil and thymol had any significant effect but still retained more than 50% activity compared to the uninhibited control (Figure 3.03 A). Although these synergists tended to also demonstrate significant inhibition of activity at the intermediate concentration, the level was greatly reduced (Figure 3.03 B). There was no significant inhibition of *M. persicae* esterase by any of the synergists at the lowest concentration. There was also no difference in effect on activity between any of the treatments at this dose (Figure 3.03 C).

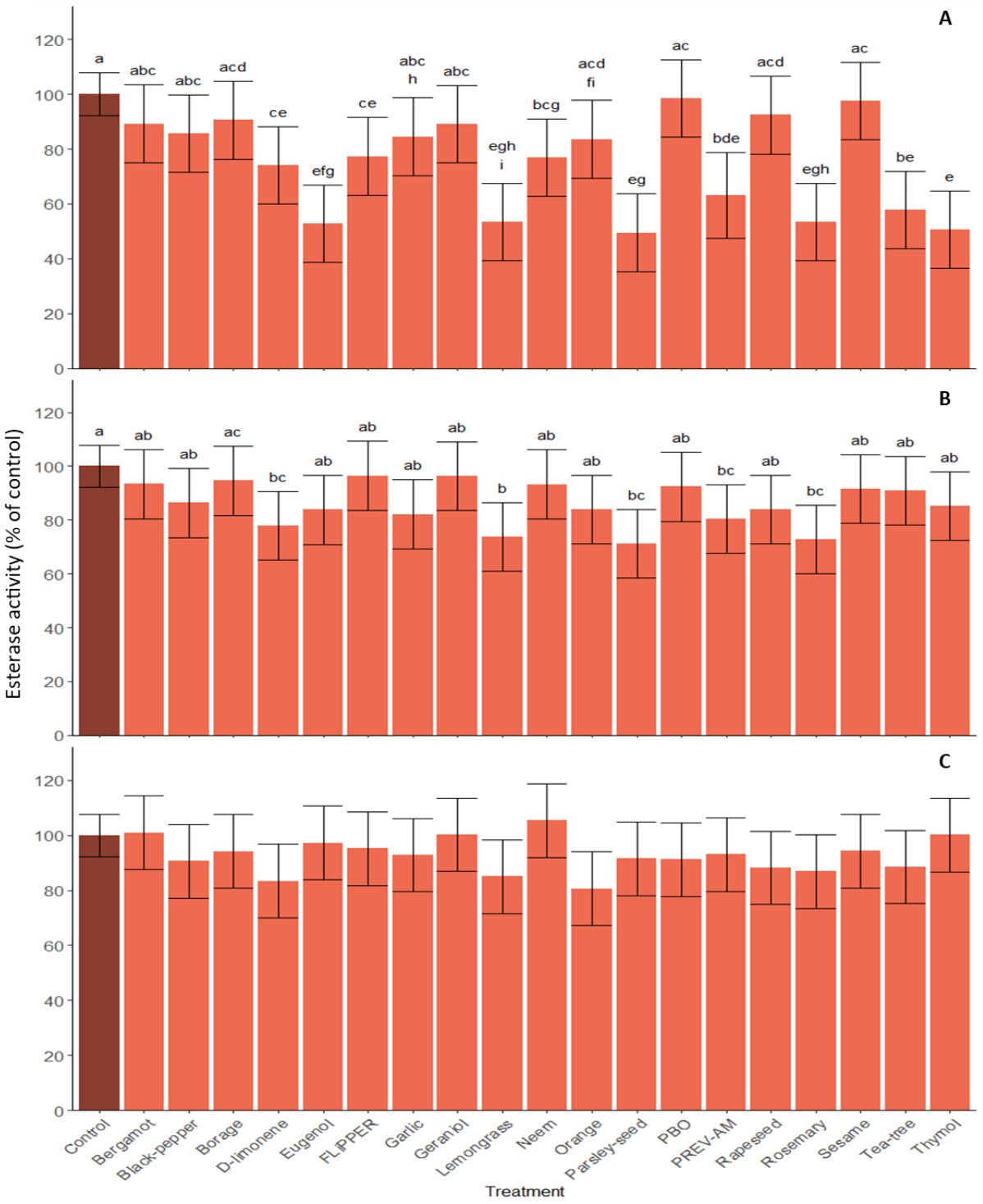

Figure 3.03. Effect of treatment on *Myzus persicae* esterase activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.3.4 Plutella xylostella

Several synergists were highly effective at inhibiting *P. xylostella* esterase at the highest concentration, with eugenol, FLiPPER, neem oil and thymol proving the most potent. However, others such as orange oil, rosemary oil and geraniol appeared to have no significant effect on activity compared with the control, even at this high dose (Figure 3.04 A). A ten-fold reduction in the concentration of synergists appeared to critically impact their inhibitory effect as only four (eugenol, FLiPPER, neem oil and thymol) still significantly reduced activity levels (Figure 3.04 B). Finally, neem oil appeared to be the most effective even at the lowest concentration. FLiPPER and thymol still significantly impacted enzyme activity at this dose but the reductions they caused were far less substantial (Figure 3.04 C).

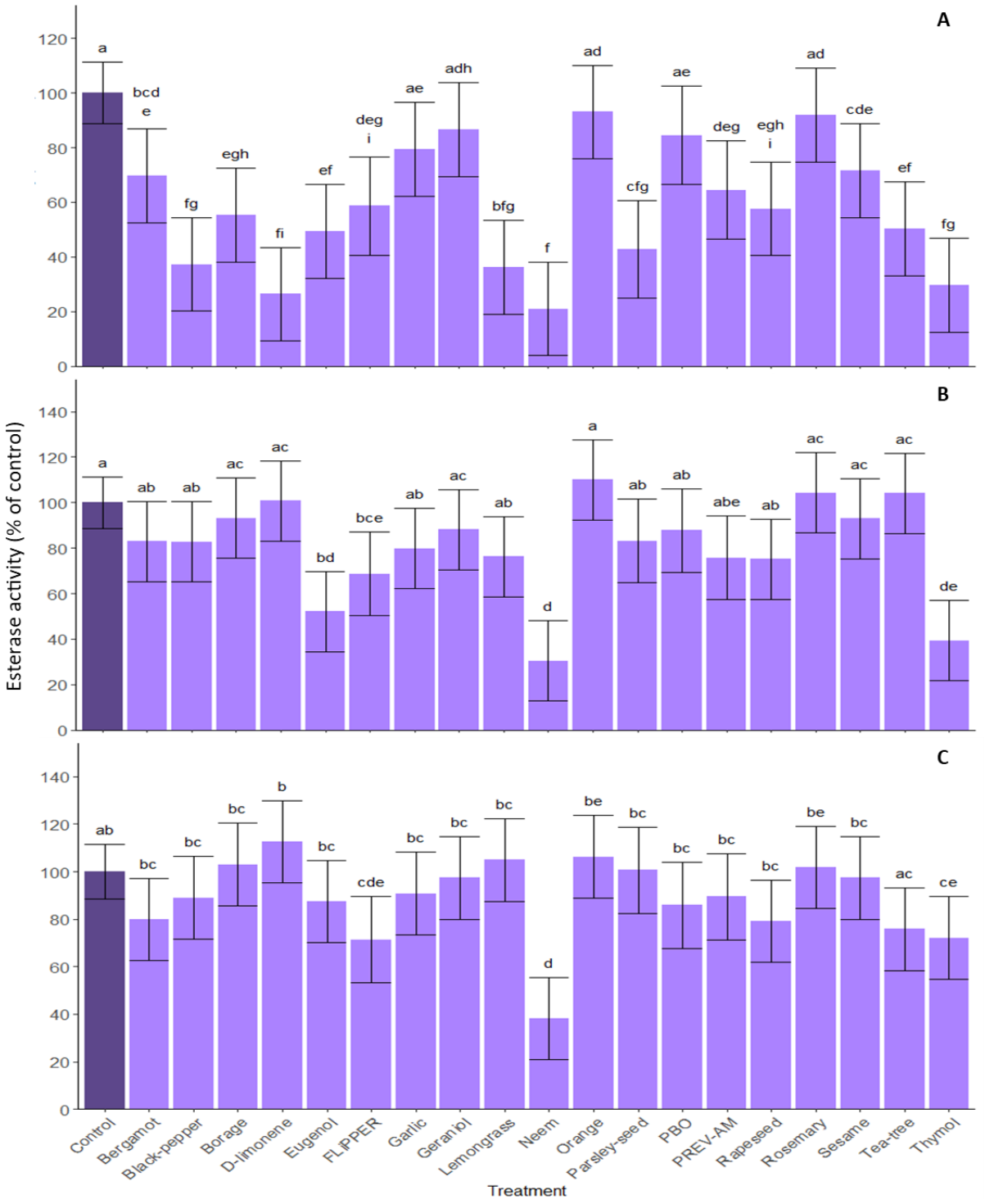

Figure 3.04. Effect of treatment on *Plutella xylostella* esterase activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.4 Glutathione S-transferase (GST) activity results

As the glutathione S-transferase catalyses the conjugation of the reduced glutathione to the CDNB to produce 1-(S-glutathionyl)-2,4-dinitrobenze (GS-DNB) which absorbs at 340nm, the change in absorbance can be attributed to the enzyme activity so glutathione S-transferase specific activity was calculated as per the formula in section 2.4.2.

In all cases, the plate was a significant influencing random factor on the response of the model ($p<0.05$). The Tukey honest significant difference test was then run between the synergists and the relevant control on the plate they were tested on to ensure that the plate variation was considered in the analysis. The model estimates were subsequently converted to show activity as percentage of the control. The upper and lower 95% confidence limits are shown by the error bars as they are a more complete representation of the uncertainty of the model estimates than the standard error in this analysis.

3.4.1 Psylliodes chrysocephala

GST specific activity in *P. chrysocephala* was significantly affected by all synergists at the highest concentration, with eugenol and geraniol appearing to halt activity completely. FLiPPER, garlic oil, lemongrass oil, neem oil, parsley-seed oil and thymol were also potent inhibitors (Figure 3.05 A). Apart from parsley-seed oil, these synergists showed similar effects when they were tested at a ten-fold lower concentration (Figure 3.05 B). However, there was a distinct increase in overall GST activity in the treatment groups when the synergists were applied at the lowest concentration. Garlic oil was the only synergist that had a particularly strong inhibitory effect when compared to the control at this dose (Figure 3.05 C). Although still demonstrating some inhibition at the highest concentration, orange oil, PBO and rosemary oil were less effective compared to the other synergists and had no significant effect at all on GST activity at the other concentrations.

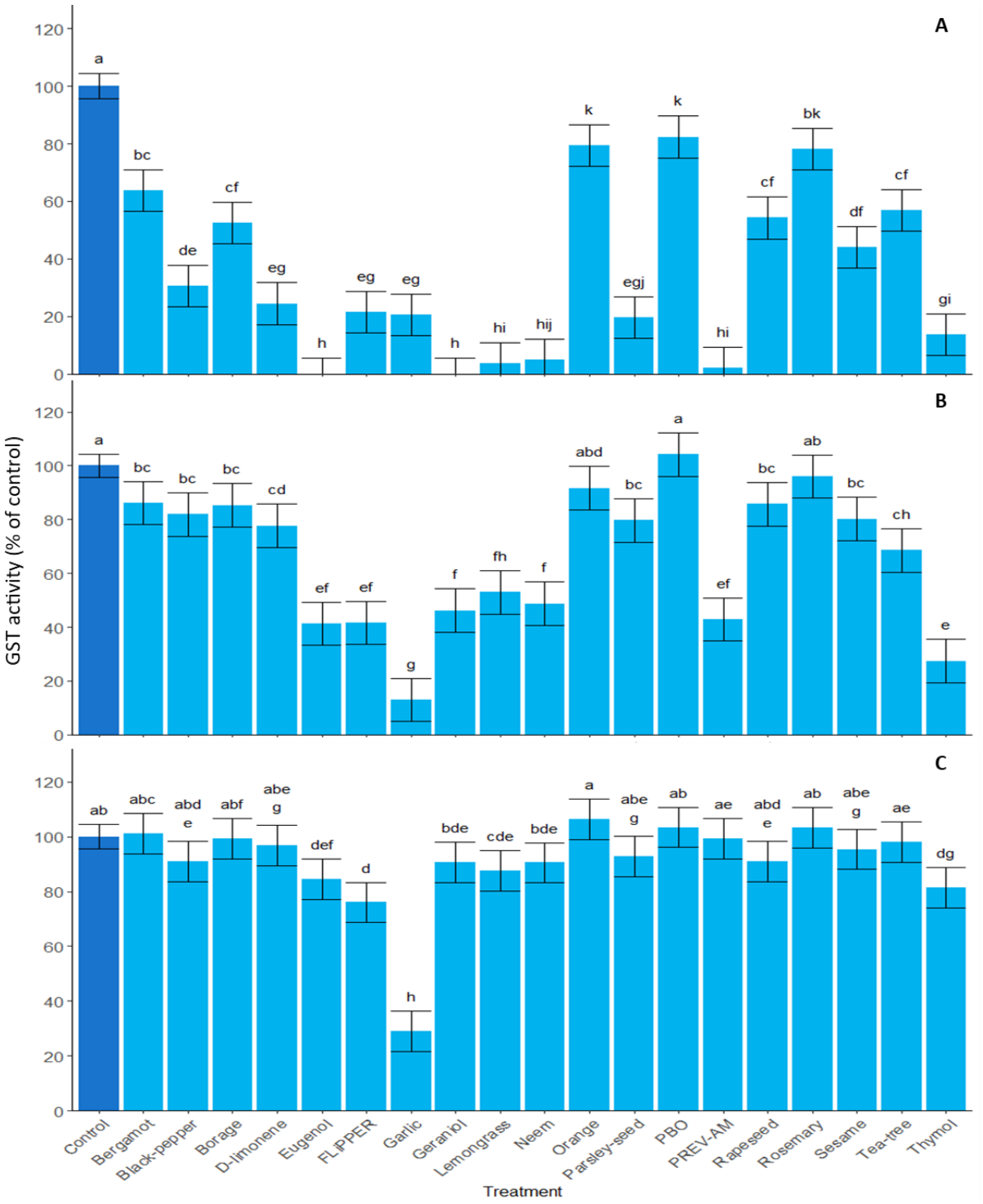

Figure 3.05. Effect of treatment on *Psylliodes chrysocephala* GST activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.4.2 Delia radicum

Eugenol and neem oil halted *D. radicum* GST activity at the highest concentration, with garlic oil and geraniol reducing activity by up to 100% when compared with the uninhibited control. Black pepper and thymol were also particularly effective at this dose (Figure 3.06 A). Eugenol, garlic oil, neem oil and thymol were similarly effective at the intermediate concentration. Additionally tea-tree oil appeared to lower activity at 0.06% more strongly than at 0.6% and black pepper became markedly less effective with the ten-fold reduction in concentration (Figure 3.06 B). There was very little inhibition of *D. radicum* GST shown when the synergists were tested at the lowest concentration. Despite some statistically significant reductions in activity by eugenol, FLiPPER, neem oil, rapeseed oil and thymol, these were comparatively small when compared with garlic oil at this dose (Figure 3.06 C). Bergamot oil, borage oil, orange oil and rosemary oil did not appear to have a particularly potent effect, even at the highest concentration.

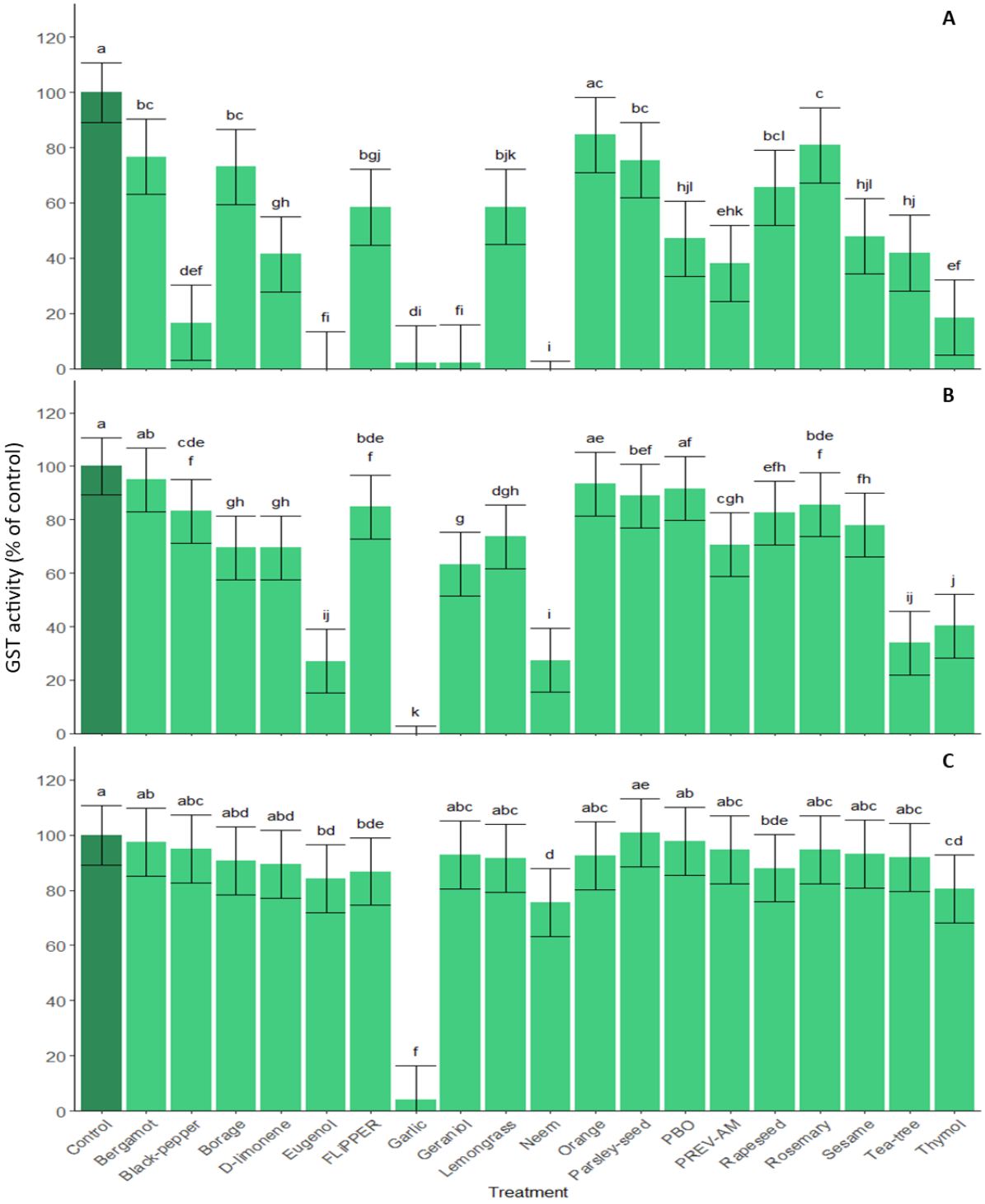

Figure 3.06. Effect of treatment on *Delia radicum* GST activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.4.3 Myzus persicae

At the highest concentration tested, borage oil, FLiPPER and PREV-AM all reduced *M. persicae* GST activity to less than 4.5% of the uninhibited control, with lemongrass oil halting activity completely. Garlic oil, parsley-seed oil and thymol were also very effective as enzyme inhibitors at this concentration. Geraniol, PBO and tea-tree oil showed no significant effect at this dose (Figure 3.07 A). A ten-fold decrease in synergist concentration allowed far more enzyme activity, with only FLiPPER, garlic oil, lemongrass oil, neem oil, PREV-AM and thymol having any distinct effect compared to the control (Figure 3.07 B). When tested at the lowest concentration, apart from PREV-AM, these synergists remained significantly effective as enzyme inhibitors, albeit with a lower comparative reduction in activity. Garlic oil and neem oil were the most potent at this dose. Interestingly, eugenol appeared to significantly increase activity (Figure 3.07 C).

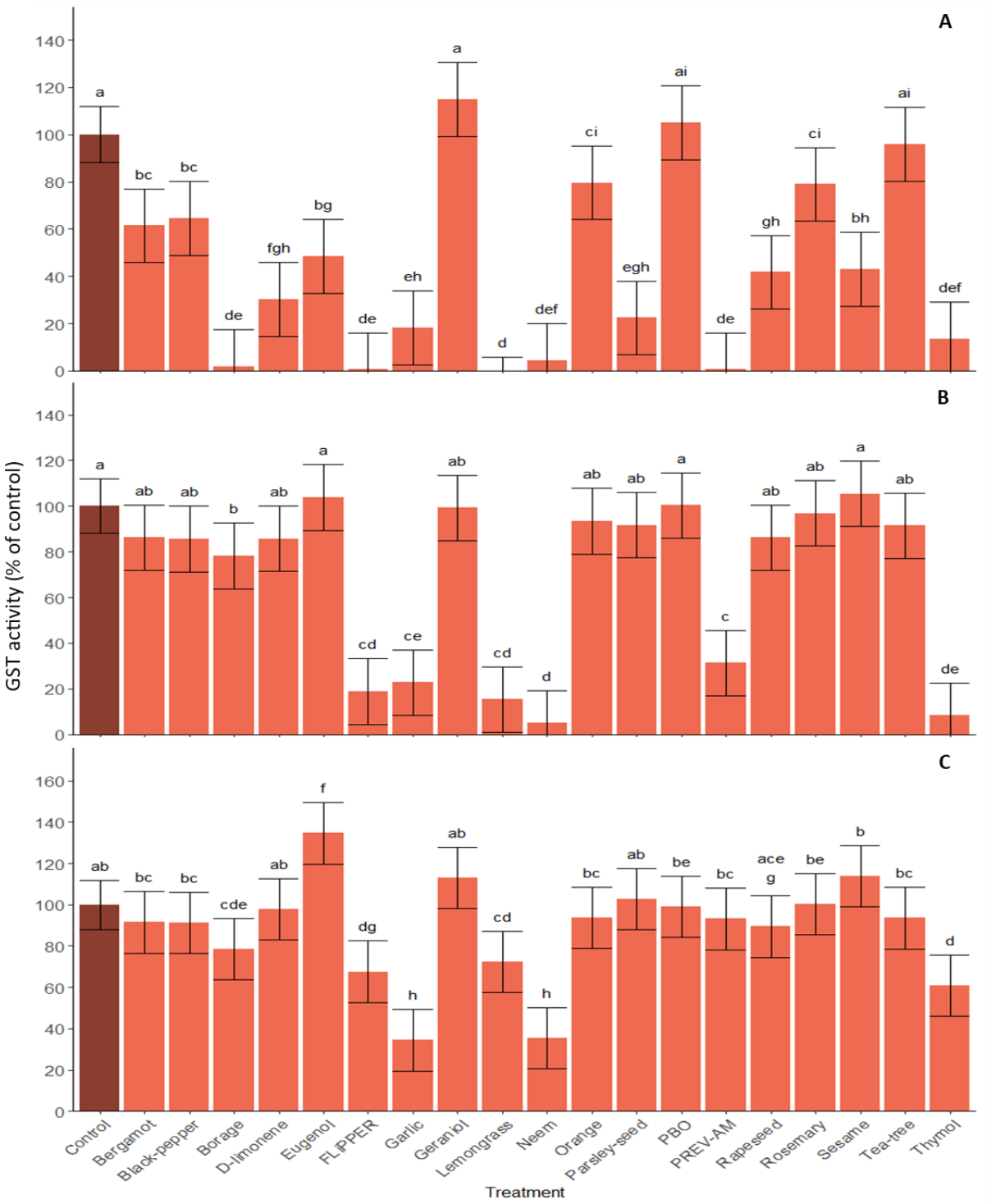

Figure 3.07. Effect of treatment on *Myzus persicae* GST activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.4.4 Plutella xylostella

At the highest concentration, all synergists tested significantly reduced *P. xylostella* GST activity and was completely halted by parsley seed oil. Additionally, eugenol, garlic oil, lemongrass oil, neem oil and PREV-AM demonstrated particularly potent effects at this dose (Figure 3.08 A). Despite an increase in activity over all treatment groups when synergists were tested at the intermediate concentration, those that were particularly effective at the higher dose remained so. Garlic oil was the most effective enzyme inhibitor at this dose, reducing activity by more than 92% of the uninhibited control (Figure 3.08 B). At the lowest concentration only garlic oil, neem oil, parsley-seed oil and thymol significantly inhibited enzyme activity but the reduction was not as strong as with the higher doses (Figure 3.08 C).

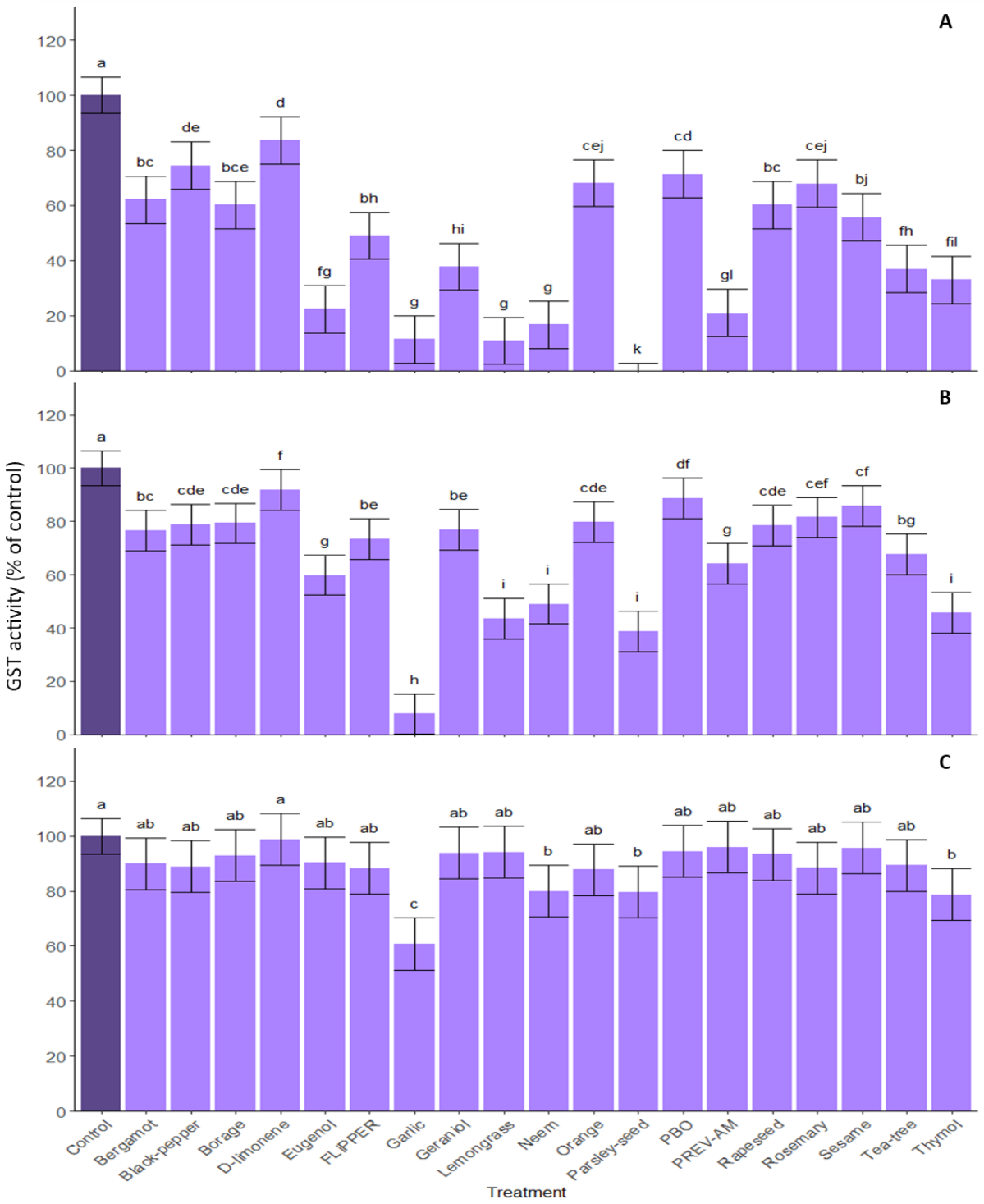

Figure 3.08. Effect of treatment on *Plutella xylostella* GST activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.5 P450 monooxygenase activity results

When investigating P450 activity, it was apparent that the presence of neem oil, particularly at the highest concentrations, interfered with the fluorescence readings of the fluorometer. The readings of the neem-containing wells were heightened to the upper limits of the machine and thereby reduced the readings of all other wells to below base-line or negligible levels. Thus, neem oil was tested at all concentrations with all possible controls on a separate plate. The highest concentration of neem oil in a well with just buffer also produced a fluorescence level that obscured any possible calculation of enzyme activity in any well where it was combined with insect homogenate, so it was only tested for P450 inhibition at 0.06% and 0.006% concentrations.

P450 monooxygenase activity and inhibition was not tested in *M. persicae*. Wu *et al.* (2022) concluded that "determination of P450 activity in aphids has variable success and there is no reliable method yet". Preparation of aphid homogenate to use as the enzyme source, in the same way as other insects, releases compounds that inhibit P450 activity and can therefore compound the results, particularly when the inhibitory effects of other specific compounds are being assessed. Wu *et al.* (2022) successfully recorded P450 activity in aphids by homogenising a single individual directly in the well. However, in order to accurately evidence a significant reduction in activity and conclude that it is due to the presence of the synergists, activity of the uninhibited control should be as high as possible to ensure the difference to the treatment group is clear and unambiguous when variation and standard error are taken into account. Having reviewed the numbers required to reach this high level of activity in the esterase and GST assays, a number of aphids may need to be homogenised within the wells in the P450 assay to raise the activity to that level and this would invariably also release more inhibition compounds. At this point, the decision was made not to run this assay with *M. persicae*.

Due to the significant inhibitory effects that the highest concentrations of the synergists had on *P. chrysocephala* P450 activity, the data was log+1 transformed in order to meet the assumptions of the model and be accurately analysed. The plate also had no significant difference in this case ($P>0.05$), but it did not change the outcome of the model results.

In all other species, the data confirmed to the required assumptions and the plate was a significant influencing random factor on the response of the model ($p<0.05$). The Tukey honest significant difference test was run between the synergists and the relevant control on the

plate they were tested on to ensure that the plate variation was considered in the analysis. The model outputs were subsequently converted to show activity as percentage of the control. The upper and lower 95% confidence limits are shown by the error bars as they are a more complete representation of the uncertainty of the model estimates than the standard error in this analysis.

3.5.1 Psylliodes chrysocephala

P450 monooxygenase activity was significantly inhibited by all synergists tested at the highest concentration, reducing activity to at least 50% of the uninhibited control by all treatments. Indeed, bergamot oil, eugenol, FLiPPER, garlic oil, geraniol, parsley-seed oil, PREV-AM, tea-tree oil and thymol all halted enzyme activity at this dose (Figure 3.09 A). There was a similar response when the synergists were tested at the intermediate concentration with only borage oil not significantly affecting the enzymes. FLiPPER, parsley-seed oil and PREV-AM completely ceased enzyme activity at this dose (Figure 3.09 B). When tested at the lowest concentration many of the synergists still significantly inhibited enzyme activity but their potency was greatly reduced compared with the higher doses. Borage oil, D-limonene, eugenol, neem oil and orange oil did not significantly affect enzyme activity at this dose. In contrast, FLiPPER and PREV-AM maintained their ability to terminate enzyme activity completely even at the lowest concentration (Figure 3.09 C). PBO – the synthetic P450 inhibitor – demonstrated the least inhibition compared to any of the other synergists when applied at the highest dose and was neither particularly effective nor particularly ineffective compared to the other treatments when tested at the other two concentrations.

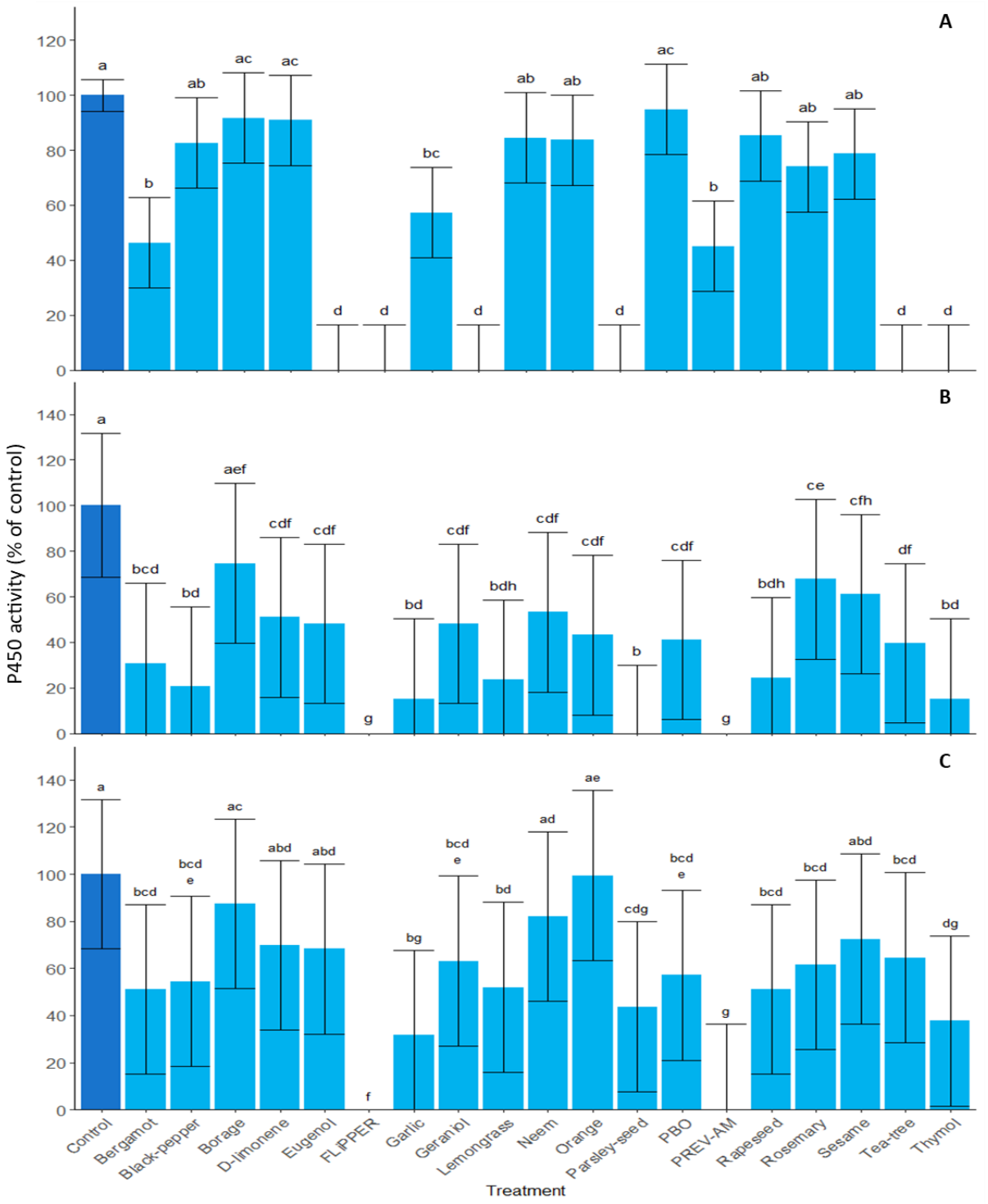

Figure 3.09. Effect of treatment on *Psylliodes chrysocephala* P450 activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.5.2 Delia radicum

At the highest concentration, all synergists demonstrated significant inhibition of *D. radicum* P450 enzymes. Eugenol, thymol, garlic oil, geraniol and rosemary reduced activity to less than 2% of the uninhibited control and thymol ceased activity completely. Garlic oil, geraniol and rosemary oil were also particularly effective (Figure 3.10 A). At the intermediate concentration, only bergamot showed no significant inhibition of enzyme activity. Eugenol, garlic oil, geraniol and thymol continued to strongly reduce activity at this dose but neem oil was the most effective in that it halted activity completely (Figure 3.10 B). The majority of the synergists tested did not significantly reduce enzyme activity at the lowest dose, compared with the control. Of those that did, garlic oil, geraniol and thymol were the most effective and all reduced activity to below 50% of the control while the remaining were less so and reduced activity to a minimum of 59% of the uninhibited control (Figure 3.10 C).

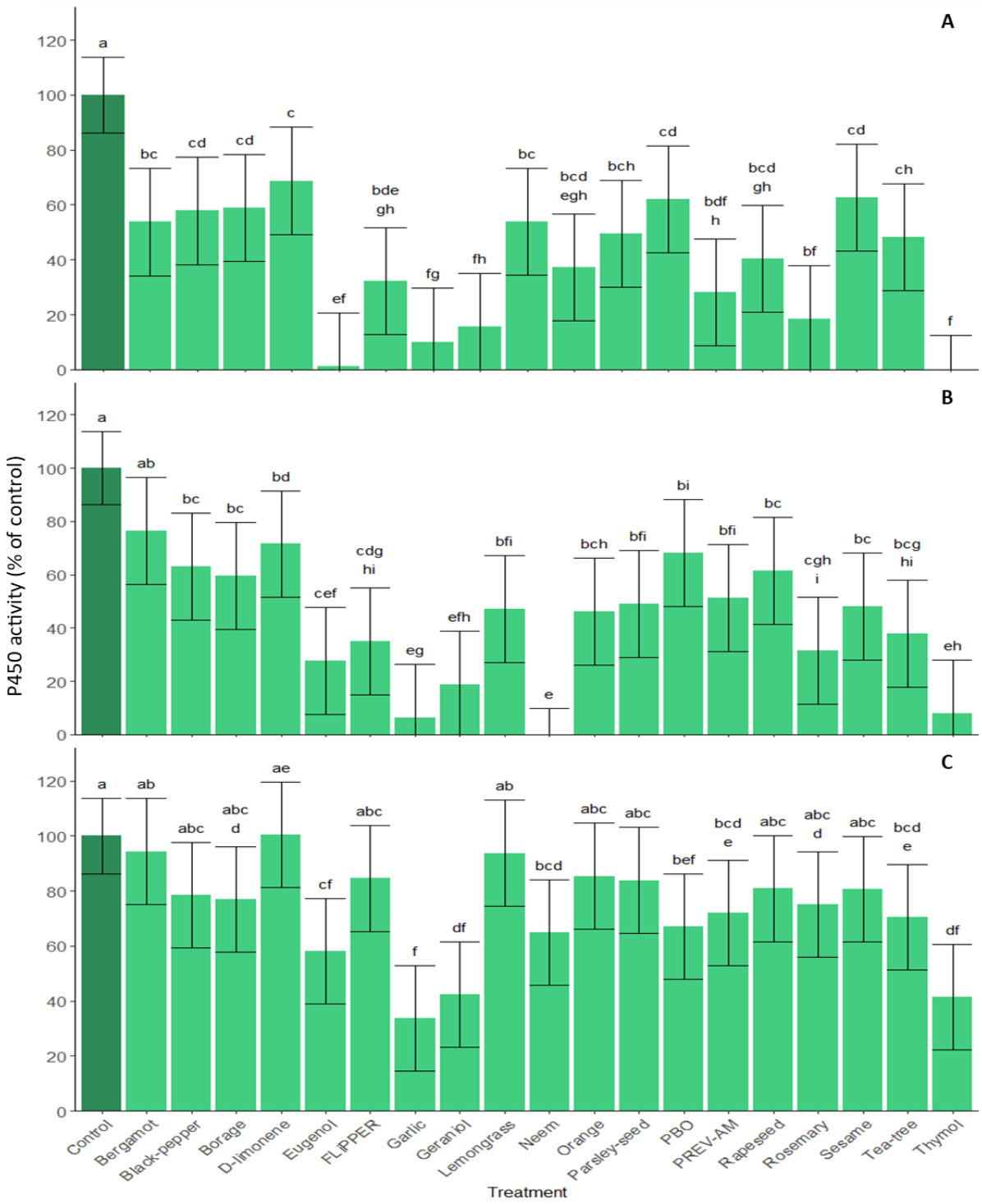

Figure 3.10. Effect of treatment on *Delia radicum* P450 activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.5.3 Plutella xylostella

The inhibitory effects of the synergists varied considerably between the concentrations tested on *P. xylostella* P450 activity. At the highest concentration, none of the synergists tested ceased *P. xylostella* P450 enzyme activity completely. The most effective inhibitor at this dose was eugenol, reducing activity to 21.5% of the uninhibited control. D-limonene, lemongrass oil, orange oil, rosemary oil, tea-tree oil and thymol all had no significant effect at all on the activity of the enzymes. Interestingly, 0.6% garlic oil appeared to significantly increase P450 activity in this species (Figure 3.11 A). When applied at 0.06%, eugenol was again the most effective enzyme inhibitor. Black-pepper oil, geraniol, neem oil, PBO, PREV-AM and sesame oil all reduced enzyme activity by a similar amount compared with the control. Bergamot oil, lemongrass oil, orange oil, rapeseed oil and rosemary oil had no significant effect on enzyme activity at this dose, even appearing to increase activity slightly compared to the control (Figure 3.11 B). Finally, at the lowest concentration, none of the synergists significantly inhibited enzyme activity. However, borage oil and D-limonene significantly increased P450 activity by 72% and 60%, respectively, above that of the control (Figure 3.11 C).

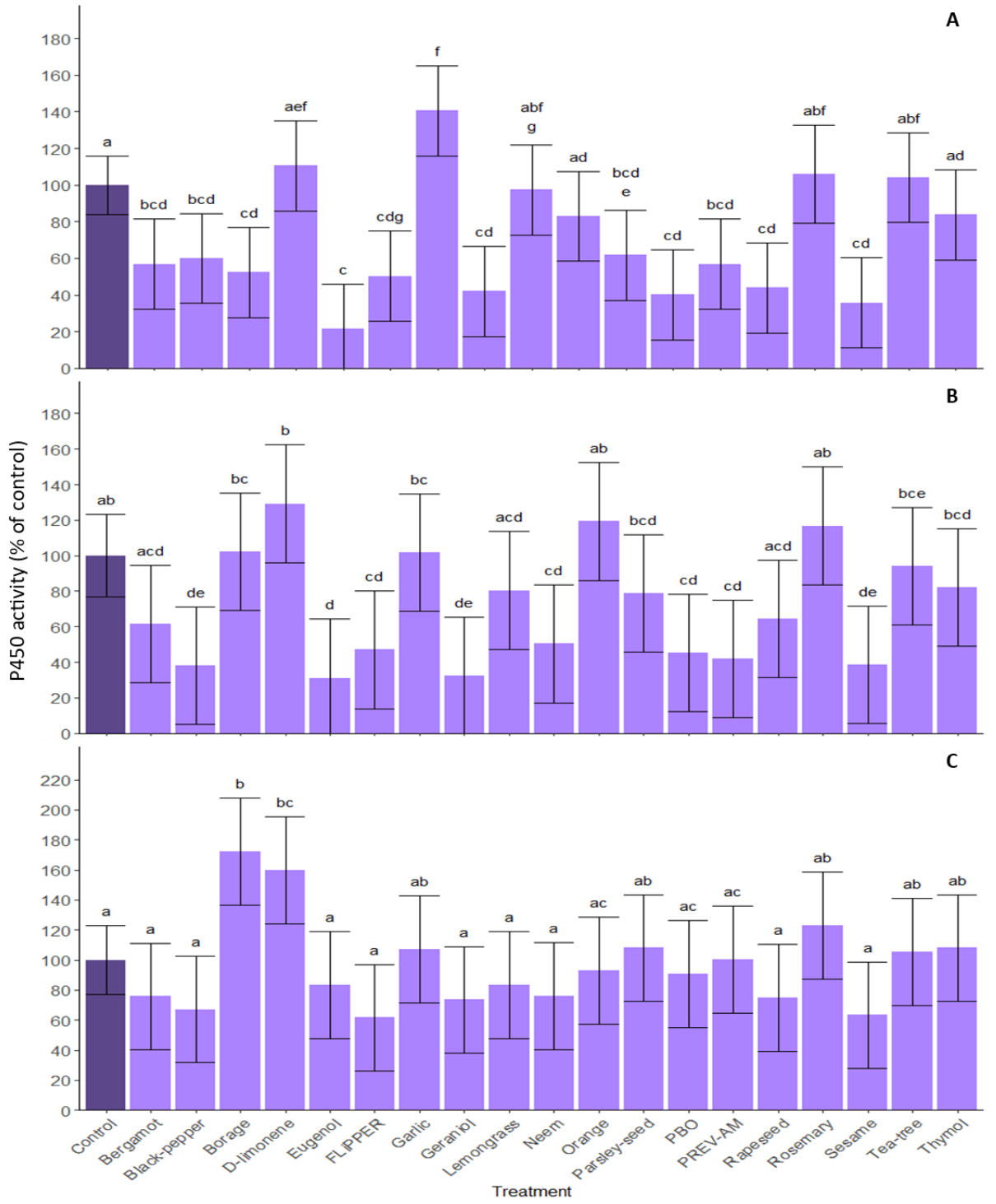

Figure 3.11. Effect of treatment on *Plutella xylostella* P450 activity shown as percentage of the uninhibited control (model estimates, n=3-24). A: botanical oils tested at 0.6%, FLiPPER at 0.96%, PBO at 0.06% and PREV-AM at 0.48%. B: botanical oils tested at 0.06%, FLiPPER at 0.096%, PBO at 0.006% and PREV-AM at 0.048%. C: botanical oils tested at 0.006%, FLiPPER at 0.0096%, PBO at 0.0006% and PREV-AM at 0.0048%. Error bars represent upper and lower limits at 95% CI. Bars showing the same letter do not differ significantly from one another (TukeyHSD, $p<0.05$).

3.6 Protein Analysis

The protein content of each sample of homogenate used for all enzyme assays was assessed using the Bradford method and the levels used as an indication of enzyme presence. The lowest protein content was in *M. persicae*, followed by *P. xylostella* with *P. chrysocephala* and *D. radicum* having similar protein levels per µl (Table 3.1). Interestingly, the highest GST and P450 activity levels per µg of protein was found in the insect with the highest susceptibility to the effects of the selected insecticide out of all the species tested: *P. xylostella*. Esterase activity was highest in *M. persicae* but this species also exhibited the lowest GST activity out of all insects tested. Per µg of protein, *P. chrysocephala* showed the lowest P450 activity and *D. radicum* the lowest esterase activity (Table 3.2).

Table 3.1. Protein content of insect homogenate samples for each species and enzyme group (µg/µl).

Insect species / Enzyme group	*P. chrysocephala*	*D. radicum*	*M. persicae*	*P. xylostella*
Esterase	0.405	0.453	0.036	0.103
GST	0.454	0.539	0.043	0.103
P450	0.427	0.525		0.123

Table 3.2. Average activity per µg protein for each species and enzyme group.

Insect species / Enzyme group	*P. chrysocephala*	*D. radicum*	*M. persicae*	*P. xylostella*
Esterase (ΔA/min)	0.0395	0.0088	0.1389	0.0874
GST (µmol/min)	33.915	16.960	3.163	41.215
P450 (RFU)	774.640	1072.191		1756.730

3.7 Discussion

Despite the general functions of the enzymes in the esterase, GST and P450 groups being largely similar between insect species, as they are generally identified as having important roles in detoxification (Fan *et al.,* 2023), there were striking differences in the effects that the same synergists had on the different target pests used in this study. For example, some synergists that proved particularly effective on *P. chrysocephala* esterase, such as lemongrass oil, had comparatively little effect on the same enzymes in *D. radicum*. In this study, field-collected *P. chrysocephala* esterase also showed significant inhibition of activity when combined with black pepper oil, garlic oil, lemongrass oil, parsley-seed oil, rosemary oil and thymol at any concentration. In contrast, *D. radicum* esterase was inhibited more by eugenol, PBO and PREV-AM and *P. xylostella* esterase by FLiPPER and neem oil. *M. persicae* was most similar to *P. chrysocephala* in terms of which botanicals had more of an impact but was demonstrably more resistant to any inhibition at all. Similar variation was observed in the GST and P450 activity assays, although it appeared that a greater number of synergists had significant effects compared to the esterase assays. These synergists also appeared to have higher overall efficacy in that many of them halted GST and P450 enzyme activity altogether.

The sheer variation in success and failure of the same synergists and their components on enzymes sourced from different insects in this study and throughout the literature (see later) demonstrates that the level of inhibition of detoxification enzymes appears to be a product of factors such as, but not limited to, chemical structure of the synergist(s), structure of the enzyme, application dose, whether the synergist is composed of one or more different compounds, their relative proportions, if/how they interact and finally the interaction of all of these aspects (Jankowska *et al.,* 2017).

Across all insects in this study, a handful of synergists were identified as being potent influencers on enzyme activity, where this was also shared across the enzyme groups. Three were individual components of essential oils: eugenol, thymol and geraniol; the other three were essential oils in their entirety: garlic oil, neem oil and lemongrass oil. Eugenol is a monoterpenoid compound found in high levels in cloves and comparatively lower amounts in other spice and herb plants such as cinnamon, nutmeg and basil (Van Roon, Parsons and Govers, 2005). These spices are typically imported as pre-processed products and none of the plants themselves are native to the UK, unlike the target insect species of the current study. Thymol is a monoterpenoid phenol primarily found in plants of the mint (Lamiaceae) family. It

is most commonly found in the thyme plant (*Thymus vulgaris*) but is also present in lower quantities in oregano (*Oreganum vulgare*), basil (*Ocimum* basilisum) and marjoram (*Origanum majorana*) (Burt, 2004). Geraniol is a monoterpene alcohol commonly found in a range of citrus essential oils as well as those of flowering plants such as geranium and rose (Bakkali *et al.,* 2008, Koul *et al.,* 2008).

All of the individual components showing a particular affinity for enzyme inhibition in this study are monoterpenoids, which are a large group of plant secondary metabolites principally classified by the number of carbon atoms they possess (Boncan *et al.,* 2020). A variety of terpenoids have been isolated and tested on insect AChE and the majority of those reviewed were found to inhibit the enzyme (Lee *et al.,* 2001; Picollo *et al.,* 2008; Anderson and Coats, 2012; Park, 2014; Seo, 2014; Yeom, 2015; Park *et al.,* 2016; Reegan *et al.,* 2016). The most effective were monoterpenoid structures, with the exception of nootkatone, which is a sesquiterpene (Anderson and Coats, 2012), suggesting that this structural assembly comprises an effective selection of compounds for consideration as synergists, particularly if the esterase group is responsible for the case of insecticide resistance under investigation. However, it should be noted that although AChE activity is important for insect survival and is, in fact, the target for carbamate and organophosphate pesticides (IRAC, 2024c), it is not involved in the detoxification of insecticides. It appears that many of the studies (such as those above) investigating esterase inhibition by plant extracts and essential oils focus solely on ACHE, rather than those more heavily involved in detoxification. Further studies on the potential of botanicals to inhibit enzymes and result in metabolic synergy with a pesticide would need to examine the activity of detoxification enzymes such as carboxylesterase, which has been identified in *Myzus persicae* as being the primary source of insecticide resistance in at least one strain of this species (Devonshire and Moores, 1982). For example, thymol is well-reported in the literature for reducing AChE activity in a range of insects (Shahriari *et al.,* 2018; Kumrungsee *et al.,* 2022; Lazarević *et al.,* 2022) and this inhibition may well be replicated amongst the wider esterase group as it significantly inhibited those of all insects tested in the present study, but this would need to be confirmed for it to be considered further in combatting metabolic insecticide resistance. Eugenol was comparatively less effective on esterase in the current study, particularly for *P. chrysocephala*, contrasting with work elsewhere in which eugenol had a relatively high binding affinity to AChE compared to a selection of other monoterpenes (Lee *et al.,*2001). However, this research was conducted with

Sitophilus oryzae at a different relative dosage which could explain this apparent discrepancy – particularly as the current work supports variability in esterase inhibition between target species and synergist dose rates.

However, there are some studies that do investigate these enzymes more closely and have identified that botanicals may well have a similar effect on detoxifying enzymes as AChE. For example, eugenol inhibited the activity of all three groups of detoxification enzymes in *Aedes aegypti*, suggesting it has a wide-ranging-spectrum mode of action and affinity for a variety of enzyme structures, but again the dosage and species differentiation may well account for differences in inhibition levels between this and the current study (Adhikari, Khanikor and Sarma, 2022). Evidence also indicates that monoterpenoids such as thymol, carvacrol and limonene can be effective inhibitors of insect GST and cytochrome P450 enzymes, further explaining the results seen across all three enzyme assays in the present work (Koul and Walia, 2009; Anderson and Coates, 2012; Pavela, 2015).

As promising as monoterpenoids first appear as potent enzyme inhibitors, there is also evidence to the contrary. Thymol, one of the most effective inhibitors in the current study, proved to have no significant effect on either esterases or P450s in the cabbage looper, *Trichoplusia ni* (Tak, Jovel and Isman, 2017). It did, however, have an inhibitory effect on GSTs. Comparatively, Shahriari *et al.* (2018) found that thymol significantly elevated GST activity in Mediterranean flour moth (*Ephestia* kehniella) caterpillars, rather than reducing it. Geraniol had this effect on *D. radicum* esterase in the current study but, in contrast, decreased activity in *P. chrysocephala* and then had no significant effect at all in *M. persicae* or *P. xylostella* when applied at the same concentration. Geraniol also induced activity in P450 enzymes in the Japanese beetle, *Popillia japonica* (Adesanya, Held and Liu, 2017) but significantly reduced activity in the same enzyme of all species tested in the current study.

Showing similar contrasts, garlic oil was particularly effective at inhibiting some GST and P450s in the present work (*P. chrysocephala* and *D. radicum*) but increased activity of *P. xylostella* P450 enzymes. Garlic oil is extracted from the garlic plant (*Allium sativum*) and contains a number of sulfur-based compounds as well as its well-known active – allicin (Batiha *et al.,* 2020) but is low in monoterpenoid compounds. This suggests further that, as well as the structural format of the synergist, both mode of action of the compounds and the species of insect appear to play significant parts in what effect, if any, a particular synergist will have on a specific enzyme.

Despite a vast number of research papers investigating the insecticidal and synergistic nature of essential oils and their constituents (Chapters 4 and 6), the majority do not delve into the explicit mode of action and enzyme kinetics behind the results. The available research suggests that some synergists work as competitive inhibitors – where they compete with the substrate for access to the active site of the enzyme, reducing the ability of the enzyme to bind to the intended target. Others are uncompetitive inhibitors, where they bind to allosteric sites on the enzyme and/or the enzyme-substrate to reduce overall activity (Jankowska *et al.*, 2017). Both positions mean that the active site is not fully open to bond with the target substrate/site or pesticidal components (depending on the enzyme's function), meaning the insect is then more vulnerable to insecticidal effects. Furthermore, López *et al.,* (2015) found that size of the synergist molecule may be positively correlated with inhibitory effect and that two monoterpenoid molecules could bind simultaneously to the enzyme, although this was again in AChE and may or may not be replicated with enzymes involved in detoxification. Eugenol, thymol and geraniol are all relatively large molecules, compared to other monoterpenes so this may assist their efficacy as enzyme inhibitors (Bakkali *et al.,* 2008). It would appear then that the mode of action of the synergist and whether its structure will allow it to bind to either or both of these sites will, therefore, heavily dictate its efficacy as an inhibitor. Correspondingly, in this study, differences between enzyme inhibition between species using the same synergists, even at the same concentration, appear to suggest that the structures of the enzymes may differ between species and have a defining influence on inhibition as well.

The differing diets of herbivorous insects, and therefore their exposure to different plant toxins, as well as contact with various classes of insecticide have affected the composition and organisation of the gene families encoding their detoxification enzymes (Rane *et al.,* 2019; Volonté *et al.,* 2022). Genome-sequencing and comparative genomic analysis has revealed that in *P. xylostella* the genes encoding detoxification metabolism, including P450, GST and carboxylesterase enzymes, have undergone significant amplification (You *et al.,* 2013). Volonté *et al.* (2022) also found that this expansion was evident across certain hemipteran species where the taxonomic group with the broadest diet of those investigated had the greatest selection of detoxification enzymes. This may help to explain why the enzymes found in *M. persicae* homogenate appear to be the most resilient out of the species tested in this study. The peach-potato aphid is a highly polyphagous species with hosts in over 40 different

plant families (CABI, 2021c) and may have undergone evolution and gene amplification while exposed to a wide range of plant hosts and their compounds. The varying evolutionary and environmental pressures leading to adaptations to different ecological niches and the differences in genetic sequencing will have therefore resulted in structural differences to the enzymes between species. As previously alluded to, the species differences seen in this study and throughout the literature suggest this is an important component in whether a synergist is an effective enzyme inhibitor. A lack of exposure may be part of the reason as to why some of the synergists tested appear to affect the targeted enzymes more than the others – e.g. if they have not been present during the evolution or adaptive processes of the insect and, as such, remain effective in their role as plant defensive compounds against herbivores. However, this may exert a relatively small influence on the overall results as the effective oils noted in the current study are also not produced by the Brassicaceae family thus indicating that higher impact factors are involved. Although the enzyme structure has not been examined in this study, its influence on a synergists' ability to inhibit enzyme activity is understood and further work to visualise the enzyme structures in various insects will help to clarify the kinetics and mode of action required for a synergist to be effective in different species.

The timescale within which the enzyme is treated with a synergist may also affect the impact it has. Nasr *et al.* (2015) noted that *P. xylostella* GST was significantly inhibited by oregano essential oil but enzyme activity appeared to increase after 24 hours of exposure, suggesting that the bonds between synergist and enzyme were reversible and synergism efficacy reduces over time. The exposure time for the current study was 30 minutes – following a method in Joffe *et al.* (2011) – but there appears to be no approved timing for this type of assay and there is huge variation in the literature. Incubation times can range from 10 minutes (Seo *et al.*, 2014) to 6 hours (Tak, Jovel and Isman, 2017). Repeating the assays in this study with a temporal variable, intermittently adding substrate so levels do not reach exhaustion and periodically assessing enzyme activity over time may help to elucidate whether the inhibition seen is reversible, how long it takes for the enzymes in each species to recover, if they do, and whether a higher concentration or repeated exposure may be required to achieve control. For maximum impact, an irreversible bond would be desirable. Grundy and Still (1985) found that pulegone-1,2-epoxide extracted from the electric eel (*Lippia stoechadifolia*) bonded irreversibly to AChE and shows promise but there appears to be no specific reports of

irreversible enzyme inhibition by essential oil components or plant extracts against detoxification enzymes in insect species. Additionally, methanethiosulfonate-containing compounds have been found to irreversibly inhibit AChE in a range of insects including aphids, mosquitoes and cockroaches (Pang *et al.,* 2009; Polsinelli *et al.,* 2010; Dou *et al.,* 2013). It may be possible to review the chemical structures of these compounds, screen essential oils for similar assemblies that may function the same way and carry out assays to test them in order to attain a natural product source of effective synergists or standalone insecticides.

From this, it is apparent that the dose of synergist applied is a strongly influencing factor on the extent to which they inhibit enzyme activity. Due to their effect on cellular and molecular function, essential oils and their components are inherently toxic at high doses but there appears to be variation in the level required to bring about a toxic or inhibitory effect, which is also ostensibly different between enzyme groups and insect species. In all insect species tested in the present study, there was a general increase in enzyme activity as the concentration of synergist was reduced. However, this is important when considering the role these compounds are being investigated to play in an IPM approach. For a synergist to be classed as such it is required to have sub-lethal effects at the applied dose, as does its companion substance, but applied together they bring about an effect that exceeds the sum of their additive effect on target pest mortality (Metcalf, 1967). An optimum concentration of a synergist would severely impact the activity of the insect enzymes in order to allow the pesticidal components to exert their full effects, but not so much that the synergist itself is a standalone insecticide. As with the factors already discussed, and to complicate matters further, the required dose appears to be different for each synergist and each species. The highest stock concentrations of synergists had a very different effect on the groups of enzymes extracted from *P. chrysocephala*. Although not directly compared, the data suggests that none of the synergists completely halted esterase activity in this species but some, namely eugenol and geraniol, did effectively terminate both GST and P450 activity. A similar trend was found in *D. radicum, M. persicae* and *P. xylostella* although the effects did not appear to be quite as strong. This lends weight to the theory that chemical structure and the enzyme/synergist mode of interaction may be largely responsible for this variation.

Comparatively, the concentration at which the synergists failed to have a significant effect on enzyme activity differed hugely between synergists and, again although not directly compared, appeared to also differ between species. At least one synergist tested at the lowest

dose in all assays caused a significant reduction in enzyme activity in all target insects – apart from *M. persicae* esterase and *P. xylostella* P450. However, the relative proportions of these enzyme groups in the homogenate may have had an effect. If there were more numerous esterase enzymes than GST or P450 in the homogenate of the insect, for example, then it may take a higher dose of the synergist to inhibit the esterase group to the same level as the other groups. This information is not forthcoming in the literature so may be an interesting avenue to clarify in this field. However, the variation seen in the results suggests that, for each synergist and insect, there is an optimum dose required to bring about the desired effect. A complete lack of enzyme activity, such as that delivered by lemongrass oil to *M persicae* GST activity and neem oil to *D. radicum* P450 activity, could suggest that the enzymes have denatured completely and the level of synergist has become toxic, rather than just inhibitory (Tiwari *et al.,* 2009). Denaturation of proteins and malfunction of significant enzymes would likely be fatal to the insect so this concentration would need to be reduced to find inhibition and possible synergism.

In contrast, orange oil appeared to inhibit *D radicum* esterase activity at 0.1% stock concentration but not at the higher concentrations. It could be that the higher doses of certain oils and components elicited a stress response, activating other enzymes and heat-shock proteins to protect the enzymes (Farahani and Bandani, 2023). In fact, increased enzyme activity in response to exposure to essential oils and their components is not an uncommon observation in this area of research. For example, *T. castaneum* GST activity levels were found to be elevated after the insects were exposed to various essential oils (Shojaei *et al*, 2017; Ramachandran, Baskar and Jayakumar, 2022). Given that these enzymes have a role in detoxification, they may respond to stress conditions by increasing activity in an attempt to protect the insect from damage (Farahani and Bandani, 2023). This may also explain why geraniol appeared to significantly increase *D. radicum* esterase activity compared to the uninhibited control at both 10% and 1% stock concentrations. This effect was also apparent with garlic oil in that it was an effective inhibitor, particularly for GST and P450, across the insects in this study but significantly increased activity of *P. xylostella* P450 enzymes at the highest dose.

Alternatively in these cases, the botanical compounds may have acted as positive allosteric modulators i.e. they bound to the enzyme in a location other than the active site and change the conformation such that the enzyme has greater binding affinity with the substrate and/or

show improved efficacy (van Westen, Gaulton and Overington, 2014). This would then have resulted in the enzyme showing activity higher than that of the uninhibited control when exposed to those botanicals. As illustrated by the number of aforementioned studies, an increase in insect detoxification enzyme activity upon exposure to certain botanical compounds is not a new observation but the mode of action involved in this activity induction appears relatively understudied as there are few papers exploring it.

So, as well as selecting a synergist for its promising chemical structure (where a monoterpenoid is apparently useful, but not absolutely required), an ability to bind to an enzyme's active site or an allosteric site conducive to inhibition rather than induction (preferably irreversibly, or at least for long enough for an insecticide to carry out its role), the synergist must be at a concentration high enough to elicit an inhibitory response and impact the number and type of enzymes present and low enough not to denature the proteins – but not so low as to stimulate a stress response that heightens the enzyme activity rather than reducing it – all while bearing in mind the species that is being targeted.

Evidence also suggests that the components of the essential oils and their relative proportions within the treatment affect how insect enzymes respond. Jankowska *et al.* (2017) states that "the majority of essential oils exhibit greater activity than their single components" but Jukic *et al.* (2007) found that thyme essential oil exerted less activity on AChE than its individual components. Mills *et al.* (2006) found that tea tree essential oil acted as an uncompetitive inhibitor of AChE, but its components were competitive inhibitors. Further investigation is required on the exact mode of inhibition exerted by botanical compounds on enzymes responsible for pesticide detoxification in insects. However, these studies suggest that the individual components may work collectively or antagonistically, depending on their interaction and relative amounts in the mixture applied. D-limonene makes up around 90.5% percent of orange oil (Kvittingen, Sjurness and Schmid, 2021). However, despite both exhibiting some inhibitory effects on all enzyme groups in the current study, neither were remarkable in their efficacy, at least when compared to thymol and neem oil for example. Their insecticidal effect may then be due to factors other than enzymatic influence.

Lemongrass oil proved to be particularly effective at inhibiting detoxification enzymes in this study – most notably in *M. persicae* and *P. xylostella* GST enzyme assays and the *P. chrysocephala* P450 and GST enzyme assays. Lemongrass oil is extracted from *Cymbopogon* sp. and several of its primary components include those already known to have insecticidal,

repellent and inhibitory effects: citral, geraniol, limonene and citronellol (Mukarram *et al.*, 2022). Crucially, these are monoterpenoids which, as previously discussed, trend towards inhibiting insect detoxification enzymes. Interestingly, *M. persicae* GSTs were strongly inhibited by lemongrass oil and to a lesser, albeit still significant, extent by D-limonene but geraniol alone showed no significant reduction in activity in the same assay. The modes of action of the individual components of lemongrass oil may be interacting synergistically or antagonistically within the whole essential oil, leaving the overall effect in this case as inhibitive. Indeed, Savalev *et al.* (2003) investigated similar phenomena using lavender oil and found both synergism and antagonism between individual compounds depending on their combination. Lemongrass oil's major component, comprising around 68%, is citral (an isomer mix of neral and geranial), with geraniol around 6% and limonene less than 1% (Mukarram *et al,* 2022). This would suggest then that citral may be having the major influence on the enzyme, with the excitatory effect of geraniol being muted by being present as a small proportion. This would, however, need to be more thoroughly investigated by testing each individual component.

Similarly, neem oil was particularly effective in this study as it significantly inhibited the activity of all enzymes across all species. This oil is extracted from the neem tree (*Azadirachta indica*) and is already well-known to demonstrate activity in the study of insecticides, repellents and antifeedants (Koul, Isman and Ketkar, 1989). In the present work, it dramatically reduced GST activity in both *P. chrysocephala* and *M. persicae* as well as *D. radicum* P450 enzymes. The primary active ingredient in neem oil is azadirachtin but it is also made up of several terpenoids, flavonoids and fatty acids (Campos *et al.,* 2016). Senthil-Nathan (2013) highlighted that although a lot of work has focussed on azadirachtin, the other compounds have also shown significant activity. Azadirachtin is a limonoid compound (Campos *et al.,* 2016), so does not fit the trend of the monoterpenoid influence, but as already discovered, chemical structure is only part of the complex mix of factors that dictate synergist potential.

In order to elucidate the particular effects of the whole essential oils and their individual components, a repeat of these enzyme assays would need to be undertaken with each essential oil and their components tested in their entirety and in their relative combinations. Miyazawa *et al.* (1998) conducted a similar study using essential oils and their main components from the *Mentha* family of plants and investigated their effects on AChE. They found that the inhibitory effects of the whole essential oil outweighed the summary effects of

the major components and that, perhaps more intriguingly, mixing an artificial 'essential oil' using the individual compounds produced the lowest inhibitory effect. However, the mix was made of the major oil components only, suggesting that trace molecules may also have a role to play. The AChE was also from a bovine source and not an insect so similar studies testing the effects of individual and artificially combined botanical components on the enzymes responsible for pesticide detoxification in insects would be valuable in assessing their potential as synergists. All of this indicates that the complex interactions of all of the individual components making up an essential oil, their various modes of action and their relative amounts in the synergist mixture dictate the overall effect on an enzyme's activity. Too much of one component may completely change the overall effect and cause an increase in activity, rather than the desired decrease. Consequently, a synergist may act as an inhibitor only in the presence of another: synergists within synergists.

All of these influencing factors builds a complex web of aspects that should be considered when evaluating whether or not a compound or essential oil is, in fact, going to function as an insect detoxification enzyme inhibitor and therefore have promise as a synergist in combination with a pesticidal component. To have a truly effective formulation involving a plant-based synergist, a combination of approaches will need to be used. Initially, the structures of the target enzymes within the problem insect should be examined to ensure the potential bonding sites are clarified to inform what would need to occur for a synergist to be a barrier to effective enzyme function. It may be that species or a higher taxonomic order of specificity can be achieved through designing a synergist for that role alone – producing a devastating effect on problem pests while avoiding impacts on pollinators, parasitoids, predators and decomposers that deliver valuable ecosystem services in the agricultural landscape. The structures of the individual components and their analogues within essential oils are already relatively well known (Essential Oil Chemical Reference database, 2024) so a literature review may first assist in highlighting which whole oils and their components are more well-suited to the target enzyme and species. The interaction between these components and how this may work *in* vivo and more expansively in the field is more difficult and is not as well researched. However, to begin with it may be useful to select whole essential oils that have shown efficacy in the target species *in vitro*, preferably with a particular enzyme group in mind, identify all individual compounds within and their relative proportions and experiment with various combinations in enzyme activity assays to explore which of the

individuals are effective inhibitors, which are synergists in themselves when combined and which produce antagonistic outcomes. These assays can then be refined further to optimise all of the individual doses and relative proportions required to bring about a high level of enzyme inhibition. Enzyme activity assays may also be conducted with pesticide ingredients and synergists in combination to examine further interaction and refine efficacy. Finally, bioassays using combinations of the successful formulations of synergists, components and pesticides can be used to examine the response in the insect itself, both in the lab and then the field.

Given that metabolic synergy is thought to be dependent on either an upregulation of and/or greater number of detoxification enzymes present within the resistant insects, it may be logical to assume that susceptible pests would contain fewer/less active enzymes than resistant species. However, this may not always be the case. In the current study, protein content in the homogenised insects used as the enzyme source for the activity assays was assessed using the Bradford method, which was then used as a proxy for enzyme level. These results suggest that the highest GST and P450 enzyme activity per µg of protein was present in *P. xylostella,* which was the species that showed the highest susceptibility to insecticide in the bioassay. A dose of just 0.074% of the recommended field rate of Sparta was required to achieve ~10% mortality, compared to 25% in both *P. chrysocephala* and *M. persicae*. The esterase activity of *P. xylostella* was also second highest per µg out of all species tested.

This may indicate that although enzyme activity is high in *P. xylostella*, particularly compared to the more resistant insects, those enzymes are not primarily engaged in detoxification. It is known that detoxification is just one of the roles of the esterase and P450 enzyme groups in insects and that they also carry out other critical physiological functions involved in behaviour, development and reproduction (Briegel and Freyvogel, 1971; Feyereisen, 1999; Browder *et al.,* 2001; Chertemps *et al.,* 2012). It appears then that perhaps the level of enzyme activity shown by *P. xylostella* may be connected to other processes (for example, developmental activities), otherwise the insect would likely show a greater level of insecticide resistance in the bioassays. However, insect GST enzymes in particular are known to be mainly associated with metabolism or sequestration of potentially harmful xenobiotics and protection from oxidative/chemical stress (Koirala, Moural and Zhu, 2022), so *P. xylostella* exhibiting nearly three times the activity per µg seen in the similarly susceptible *D. radicum* in this case is interesting. It would be useful to obtain a sample of *P. xylostella* that does show significant

resistance to lambda-cyhalothrin based pesticides and repeat the enzyme activity and protein assays of the current study to see if there is a considerable difference in activity level per µg of protein, particularly in the GST activity. If the resistant sample exhibits a higher level of GST activity per µg of protein, then this may be one source of the resistance but it still would not explain the discrepancy between susceptibility and enzyme activity observed in this study.

There is, however, evidence to suggest that diet of a caterpillar may play a role in activity of certain enzymes, including GST. Ji (2014) found that *Spodoptera exigua* exhibited greater GST, carboxylesterase and trypsin activity when fed on *Arabidopsis thaliana* compared to *Medicago truncatula* and *Solanum lycopersicum*, so perhaps the Chinese cabbage the caterpillars in the current study were fed on contained one or more allelochemical or plant metabolite that activated these enzymes. Rearing another sample on a different source plant and analysing their subsequent enzyme activity may indicate if this is the mechanism responsible. Additionally, there is an indication in the literature that the class of GST enzyme affects the type of pesticide that can be detoxified. Zhang *et al.* (2022) found that one insect-specific epsilon class of GST could metabolise malathion and DDT but, interestingly, that suppression of this particular enzyme only translated to increased susceptibility to malathion in the bioassays, not DDT. It is conceivable then that the majority of the GST enzymes present in the *P. xylostella* homogenate used in the current study may not have been of a specific structure to be able to successfully detoxify the lambda-cyhalothrin but they may be able to confer significant resistance to another class of insecticide. The background and conditions in which the caterpillars were collected and maintained is unknown so they may have been exposed to a pesticide containing an active ingredient other than lambda-cyhalothrin and still carry the resistance in their GST activity. Further tests with insecticides of different classes and/or modes of action may help to clarify this.

Perhaps unsurprisingly, the second highest GST activity per µg of protein was observed in the homogenate of *P. chrysocephala* and the insects exhibited particularly strong resistance to cypermethrin in the bioassays. Moreover, the esterase and P450 activity per µg of protein for this species was lower than both *M. persicae* and *P. xylostella,* which suggests that at least some of the resistance may be due to the GST activity, rather than the other two enzyme groups. That this resistance has a metabolic source is further supported by the corresponding bioassay and enzyme assay data indicating that certain botanical compounds, such as neem

oil, can vastly improve pesticide susceptibility and significantly inhibit enzyme activity respectively, which would not occur if a target site mutation was responsible.

Chapter 4. The investigation of selected botanicals and products as potential synergists when combined with pesticides against a range of crop pests

4.1 Summary

The bioassays showed that, like with inhibition in the enzyme activity assays, there was significant variation both between and within species as to whether or not a botanical proved successful as a synergist. *In vivo* testing of the botanicals that showed promise as potent enzyme inhibitors in Chapter 3 determined that several are indeed synergistic when paired with selected insecticides in the same insects. However, the co-toxicity analysis also showed that there were several botanicals that had additive effects or even antagonistic effects when they were applied prior to pesticide exposure, despite their efficacy as enzyme inhibitors. This suggests that there is more to synergy than the capability of a botanical to inhibit detoxification enzymes.

4.2 Introduction

Given the dwindling number of active ingredients currently approved for use as insecticides in the UK and European farming sector, and the rising number of crop pests exhibiting resistance to them, the pressure is increasing to restore control and safeguard the future of food production (Bass *et al.,* 2014; Sparks and Nauen, 2015; Garthwaite *et al.,* 2016). Indeed, the acreage assigned to oilseed rape production in the UK drastically reduced in the years since the withdrawal of authorisation for neonicotinoids, with many growers citing yield reduction and crop failure due to CSFB as a primary factor (Scott and Bilsborrow, 2019). Some UK populations of this species were recorded as showing 100% resistance to pyrethroids in 2020, indicating that there is a distinct lack of control using the remaining plant protection products available. There is a similar narrative regarding *M. persicae*, with the species showing resistance to almost every class of insecticide, including the neonicotinoids that have been approved for emergency use in the UK since 2021 in an attempt to control the devastating impact of beet yellows virus in sugar beet, of which *M. persicae* is a vector.

Further, an estimated US$4-5 billion loss of cruciferous crops each year is due to diamondback moth and the species' rising resistance to pyrethroids, biopesticides such as *Bacillus thuringiensis* and the more recent deployment of diamide pesticides (Troczka *et al.,* 2012;

Furlong, Wright and Dosdall, 2013; Crickmore, 2016). Although the cabbage root fly, *D. radicum,* has yet to reach these levels of economic impact and resistance, the reduction of available pesticide products and lack of effective alternatives may well result in the development of this species as another major threat to successful supply of cruciferous vegetables. However, with the benefits of plant-derived products, ranging from low toxicity to humans and being readily biodegradable to having the capacity to target specific insect pests and their compatibility with IPM strategies, the potential of using botanicals for pest control has been the focus of an increasing number of studies in recent years and may provide a promising route to reducing the impact of these species (Dang *et al.,* 2012; Isman, 2020).

Some studies have shown that many botanicals have synergistic properties, in that they are able to enhance the effects of several different classes of pesticides in a range of insects and could be used to combat insecticide resistance (Metcalf, 1967; Joffe *et al.,* 2012, Tong and Bloomquist, 2013; Tak and Isman, 2017; Zihao *et al.*, 2017). This synergism is thought to be due to the botanical compounds inhibiting the enzymes responsible for metabolising the insecticide before it can reach the intended target site(s) (Joffe *et al.*, 2012). Although there are a great number of studies exploring enzyme inibition by botanicals and their synergism with pesticides, there are comparatively few that combine the two approaches and investigate whether enzyme inhibition *in vitro* translates to synergism and improved pesticide efficacy *in vivo*.

Enzyme activity assays detailed in Chapter 3 highlighted several botanicals that significantly inhibited the detoxification enzymes and these, along with some that were not quite as potent, were selected for further investigation as potential synergists in the bioassays described here in Chapter 4. Insecticides were selected based on their approval status for the UK and recommendations for control of nominated insect pests (Liaison, 2024).

4.3 Dose-response

Dose-response effects of pesticides were generated to calculate lethal doses using probit analysis. These were used to determine LD_{10} values for use in subsequent combination assays with potential synergists. The LD_{10} was selected as it allowed for up to a 90% increase in mortality if a botanical exhibited synergistic activity in the combined treatment bioassay groups and would therefore be a clear and significant difference to the control.

4.3.1 Psylliodes chrysocephala

A dose-response assay was not used to determine LD values of cypermethrin with *P. chrysocephala* as initial tests showed that application of 100% of the recommended field dose provided 7.07% average pest mortality. As such, 100% field dose was selected as the discriminating dose to use in combination with potential synergists.

A full dose-response assay was conducted for technical grade lambda-cyhalothrin. Lethal doses were calculated by probit analysis (Figure 4.1). The LD_{10} for lambda-cyhalothrin was identified as 23.36% and rounded to 25% of the recommended field dose for further study (Table 4.1).

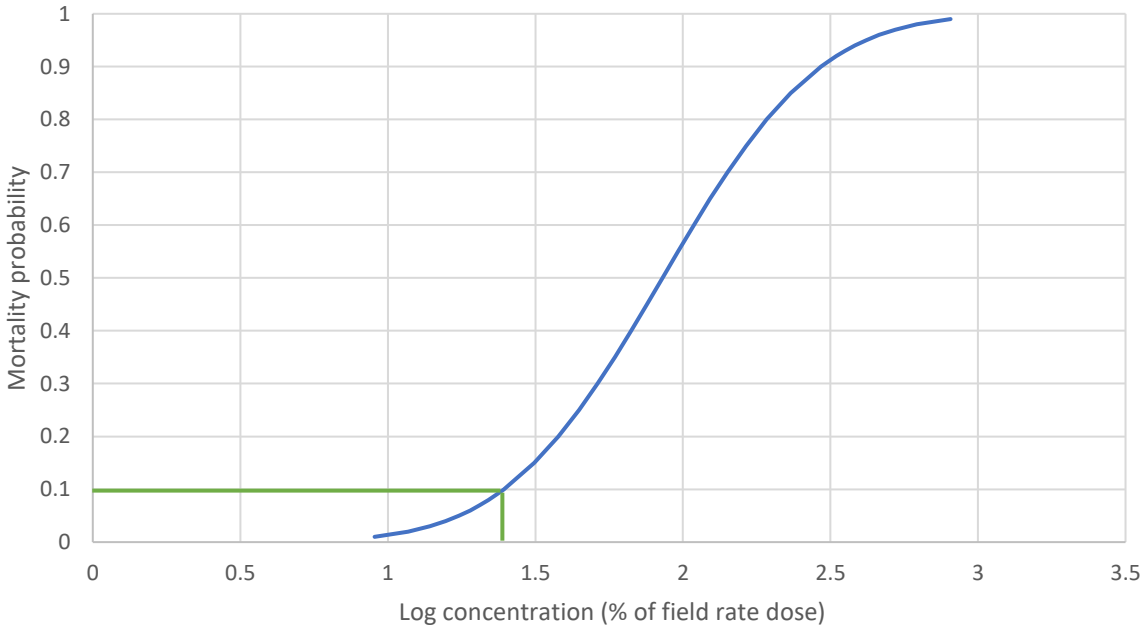

Figure 4.1. Probit analysis curve from *Psylliodes chrysocephala* and lambda-cyhalothrin dose-response assay. The LD_{10} log concentration is highlighted.

Table 4.1. Lethal dose values calculated by probit analysis from *Psylliodes chrysocephala* and lambda-cyhalothrin dose-response assay.

LD value	Log Concentration	% field dose λ-C
10	1.369	23.36
20	1.647	44.32
30	1.847	70.34
40	2.019	104.36
50	2.179	150.90
60	2.339	218.20
70	2.510	323.74
80	2.711	513.73
90	2.989	974.66
99	3.649	4460.13

n = 5, total number of beetles = 150, d.f. = 2, χ^2 critical value = 2.025.

4.3.2 Delia radicum

A dose response assay was completed for Tracer efficacy against *D. radicum*. Lethal dose values were calculated by probit analysis (Figure 4.2.). The LD_{10} was identified as 3.39% and rounded to 4% of the recommended field rate dose for further study (Table 4.2).

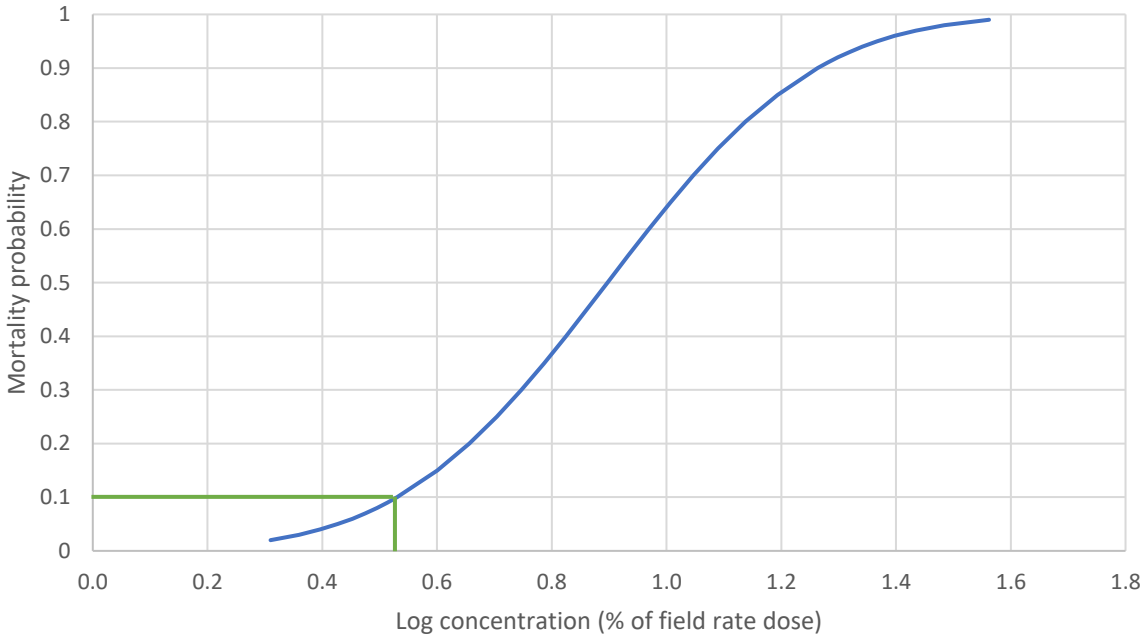

Figure 4.2. Probit analysis curve from *Delia radicum* and Tracer dose-response assay. The LD_{10} log concentration is highlighted.

Table 4.2. Lethal dose values calculated by probit analysis from *Delia radicum* and Tracer dose-response assay.

LD value	Log Concentration	% field dose Tracer
10	0.601	3.39
20	0.656	4.53
30	0.747	5.59
40	0.825	6.68
50	0.897	7.89
60	0.969	9.32
70	1.047	11.14
80	1.138	13.73
90	1.263	18.34
99	1.562	36.48

n = 8, total number of flies = 480, d.f. = 5, χ^2 critical value = 5.086.

4.3.3 Myzus persicae

A dose response assay was conducted for technical grade lambda-cyhalothrin and *M. persicae*. Lethal doses were calculated by probit analysis (Figure 4.3). The LD_{10} was selected as the discriminating dose found to be 24.73% rounded to 25% of the recommended field dose for further study (Table 4.3).

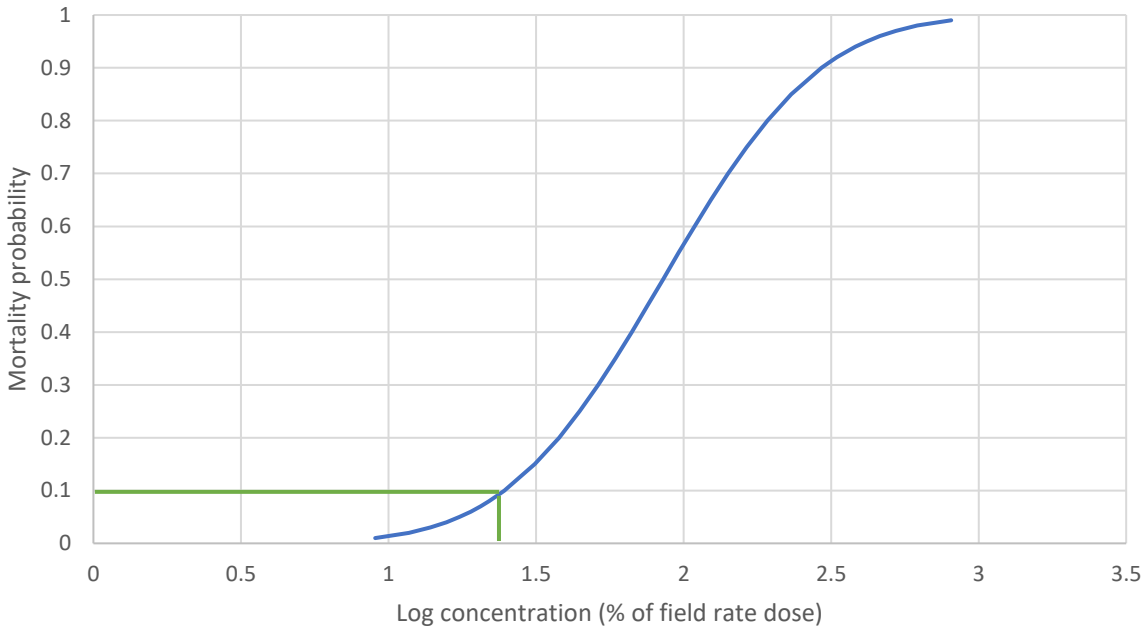

Figure 4.3. Probit analysis curve from *Myzus persicae* and lambda-cyhalothrin dose-response assay. The LD_{10} log concentration is highlighted.

Table 4.3. Lethal dose values calculated by probit analysis from *Myzus persicae* and lambda-cyhalothrin dose-response assay.

LD value	Log Concentration	% field dose λ-C
10	1.393	24.73
20	1.578	37.82
30	1.711	51.38
40	1.825	66.76
50	1.931	85.27
60	2.037	108.90
70	2.151	141.49
80	2.284	192.21
90	2.468	293.98
99	2.907	806.40

n = 5, total number of aphids = 300, d.f. = 2, χ^2 critical value = 4.021.

4.3.4 Plutella xylostella

A dose response assay was completed for Sparta and *P. xylostella*. Lethal dose values were calculated by probit analysis (Figure 4.4.). The LD_{10} was identified as 0.02% of the recommended field rate dose (Table 4.4). However, in practice this amount of pesticide caused lower than 10% mortality in the pesticide-only treatment group so the level was increased to LD_{20} of 0.074% of the field rate dose for further study.

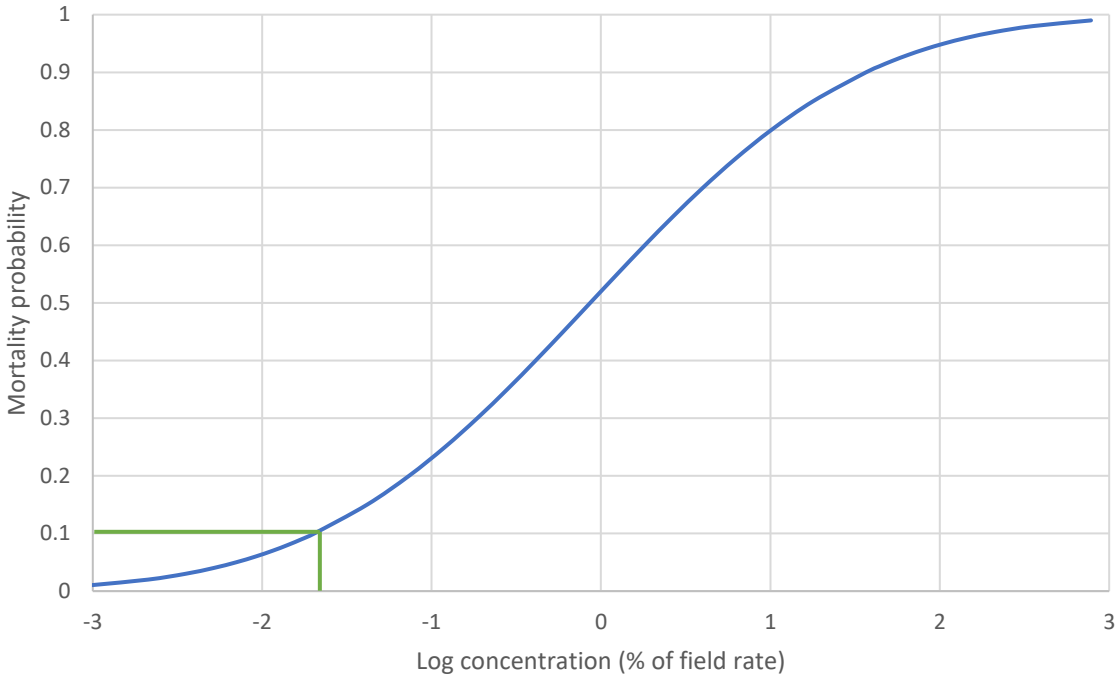

Figure 4.4. Probit analysis curve from *Plutella xylostella* and Sparta dose-response assay. The LD_{10} log concentration is highlighted.

Table 4.4. Lethal dose values calculated by probit analysis from *Plutella xylostella* and Sparta dose-response assay.

LD value	Log Concentration	% field dose Sparta
10	-1.692	0.020
20	-1.133	0.074
30	-0.73	0.186
40	-0.386	0.412
50	-0.064	0.864
60	0.258	1.813
70	0.603	4.006
80	1.006	10.136
90	1.565	36.719
99	2.893	780.843

n = 6, total number of caterpillars = 630, d.f. = 2, χ^2 critical value = 2.807.

4.4 Results

To investigate potential enhancement of pesticide efficacy, all essential oils were initially tested at 1% concentration for all insect species. In *P. chrysocephala* assays, however, this was reduced to a 0.5% and 0.1% dose in the garlic oil and thymol tests respectively because the 1% synergist-only groups resulted in 20-50% mortality, which was considered too high to show any synergistic activity in the combined treatment groups. PREV-AM and FLiPPER were tested at their recommended field rate doses. PBO was tested at 0.1% following methods from Snoeck *et al.* (2017).

4.4.1 Psylliodes chrysocephala

When tested in combination with field-rate doses of cypermethrin, the synthetic P450 inhibitor PBO, neem oil, orange oil and thymol all showed synergistic activity (Figure 4.5). Although the co-toxicity factor was greatest for PBO (Table 4.5), the combined effects with cypermethrin only increased efficacy to 56%, whereas the combination with neem oil increased mortality to 87% (Figure 4.5). Black pepper oil, garlic oil and FLiPPER showed no synergistic effect. FLiPPER did cause a mortality rate of 44% when combined with cypermethrin but no synergism was observed as the mortality rate was the same in the FLiPPER only treatment group (Figure 4.5). Rapeseed oil and PREV-AM appeared to show some antagonistic activity when combined with cypermethrin (Table 5.5).

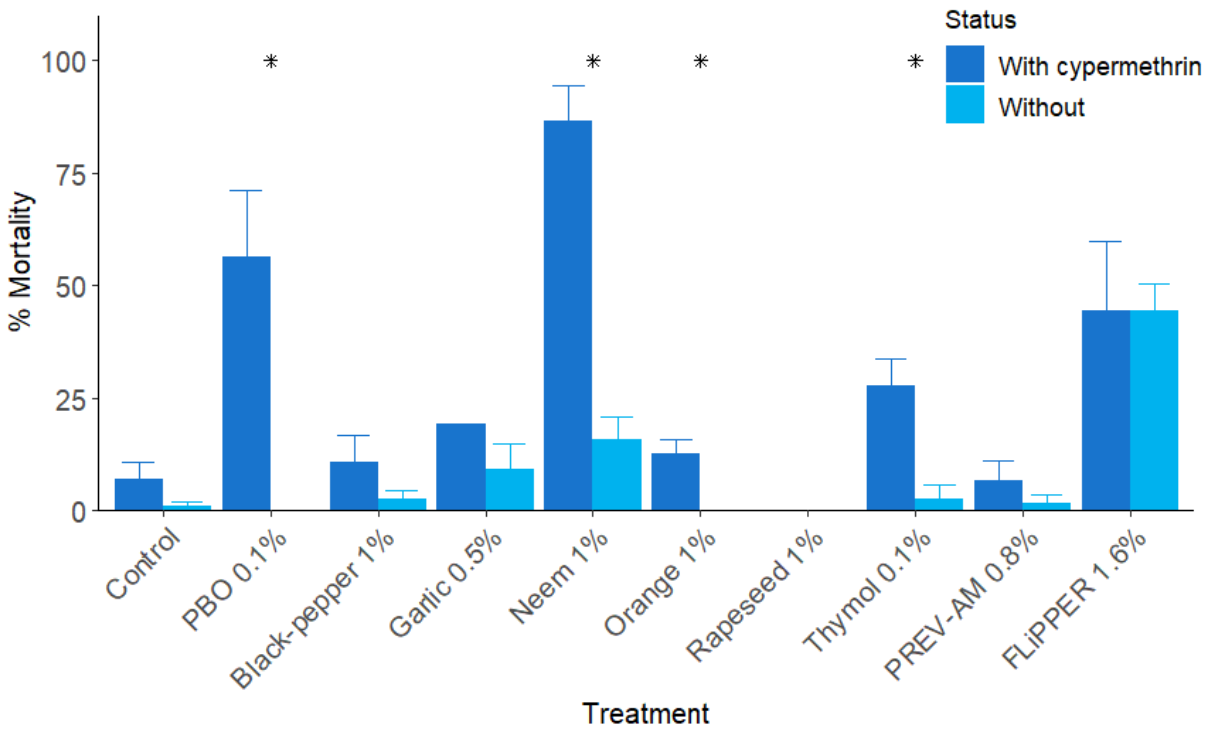

Figure 4.5. Percentage mortality of adult *Psylliodes chrysocephala* when treated with synergists separately and when combined with the recommended field rate dose of cypermethrin. Means ±SE, n=3-15. Asterisks highlight treatments that demonstrated synergistic activity.

Table 4.5. Co-toxicity factors* and synergy status for synergists tested in combination with cypermethrin on *Psylliodes chrysocephala*.

Synergist	Co-toxicity factor	Status
PBO	695.24	Synergistic
Black pepper oil	14.29	Additive
Garlic oil	18.75	Additive
Neem oil	277.94	Synergistic
Orange oil	76.19	Synergistic
Rapeseed oil	-100.00	Antagonistic
Thymol	192.86	Synergistic
PREV-AM	-23.70	Antagonistic
FLiPPER	-13.73	Additive

*As noted in section 2.6, a co-toxicity factor of >20 means there is synergistic activity, < -20 suggests antagonistic activity and a factor that falls between these two means the result is simply the additive effect of the two components (Mansour *et al.*, 1966).

Neem oil appeared to show a strongly synergistic effect which resulted in high mortality when combined with cypermethrin. This was further investigated by running the same test with a lower concentration of pesticide to see if efficacy could be maintained. The concentration of cypermethrin was thus reduced to 10% of the recommended field rate dose and the combination of this with 1% neem oil produced a 76% (±5.58) mortality rate, as compared with 32% (±5.58) when the additive effects of both components were considered separately (Figure 4.6). The co-toxicity factor of 1% neem oil with 10% field dose cypermethrin was 141.29 showing significant synergistic activity.

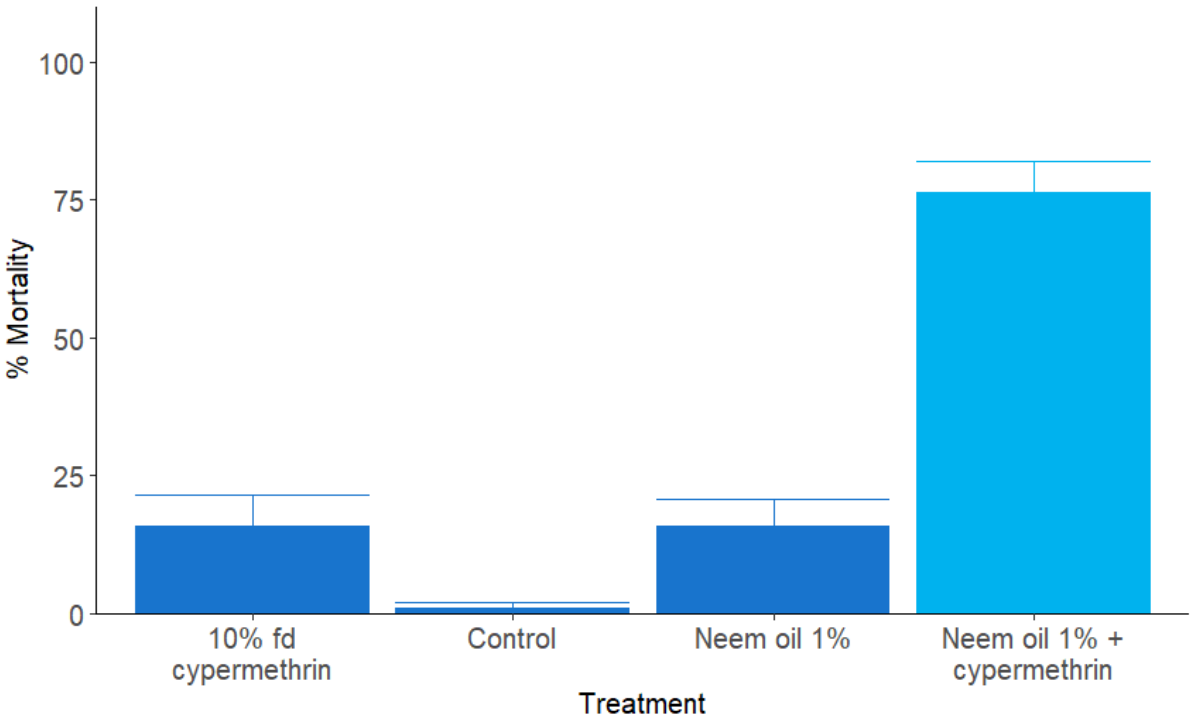

Figure 4.6. Percentage mortality of adult *Psylliodes chrysocephala* treated with 10% of the field rate dose of cypermethrin, water control and 1% neem oil separately (dark blue bars) and when combined (light blue bar). Means ±SE, n=6.

4.4.2 Delia radicum

Out of the 11 botanicals tested in bioassays with *D. radicum,* only garlic oil and FLiPPER demonstrated an additive effect when combined with Tracer and none appeared to be antagonistic. Black pepper oil, eugenol, neem oil, orange oil, parsley-seed oil, rapeseed oil, thymol, PREV-AM and PBO all demonstrated synergistic activity (Table 4.7). The highest co-toxicity factors were with PBO and PREV-AM as there was 0% mortality in their respective synergist-only treatment groups. The highest mortality rate (80%) was achieved by combining thymol and Tracer but this showed a lower co-toxicity factor due to the inherent toxicity of the botanical causing 22% mortality when applied separately (Figure 4.7).

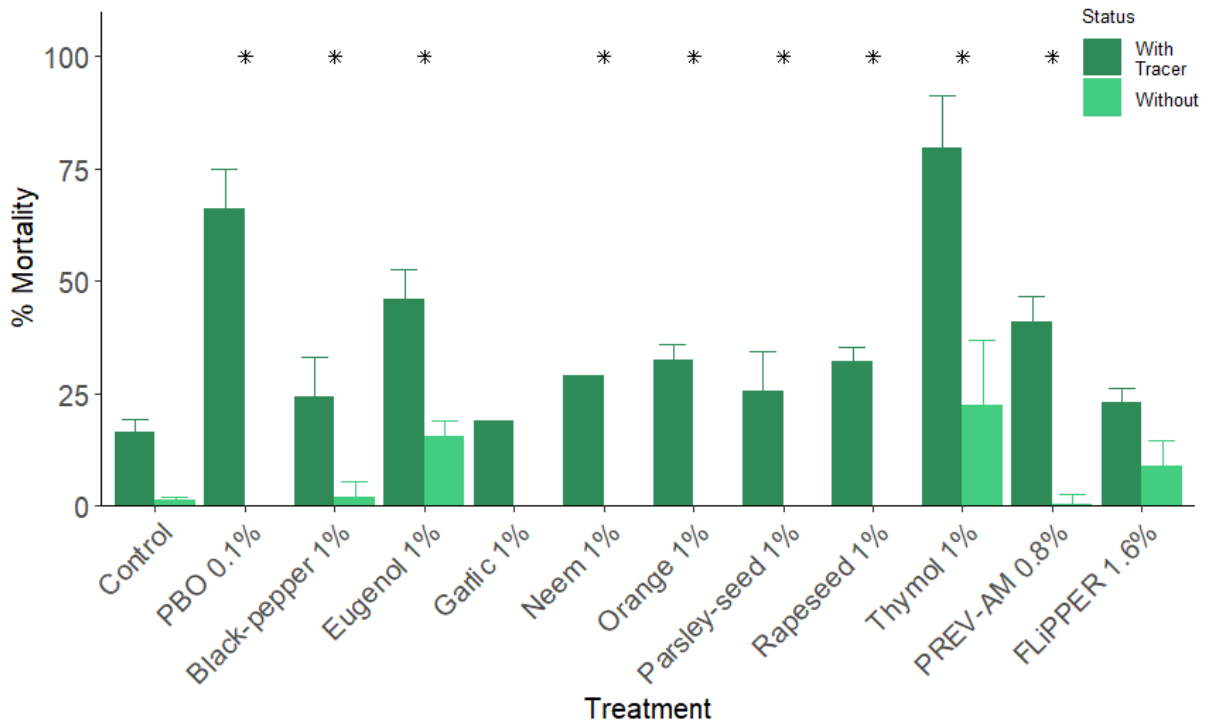

Figure 4.7. Percentage mortality of *Delia radicum* when treated with synergists separately and when combined with the recommended field rate dose of Tracer. Means ±SE, n=3-30. Asterisks highlight treatments that demonstrated synergistic activity.

Table 4.7. Co-toxicity factors and synergy status for synergists tested in combination with Tracer on *Delia radicum*.

Synergist	Co-toxicity factor	Status
PBO	300.85	Synergistic
Black-pepper oil	30.50	Synergistic
Eugenol	43.43	Synergistic
Garlic oil	14.33	Additive
Neem oil	75.72	Synergistic
Orange oil	96.19	Synergistic
Parsley-seed oil	55.26	Synergistic
Rapeseed oil	94.88	Synergistic
Thymol	105.60	Synergistic
PREV-AM	143.07	Synergistic
FLiPPER	-8.89	Additive

4.4.3 Myzus persicae

Garlic oil, lemongrass oil and parsley seed oil all showed synergistic activity when combined with 25% of the recommended field rate dose of lambda-cyhalothrin (Table 4.8). They also significantly increased mortality rate to over 70% in the bioassay compared with 25% mortality in the pesticide-only control (Figure 4.8). Neem oil and orange oil only appeared to show an additive effect but thymol and Prev-am demonstrated antagonistic activity when combined with the pesticide (Table 4.8).

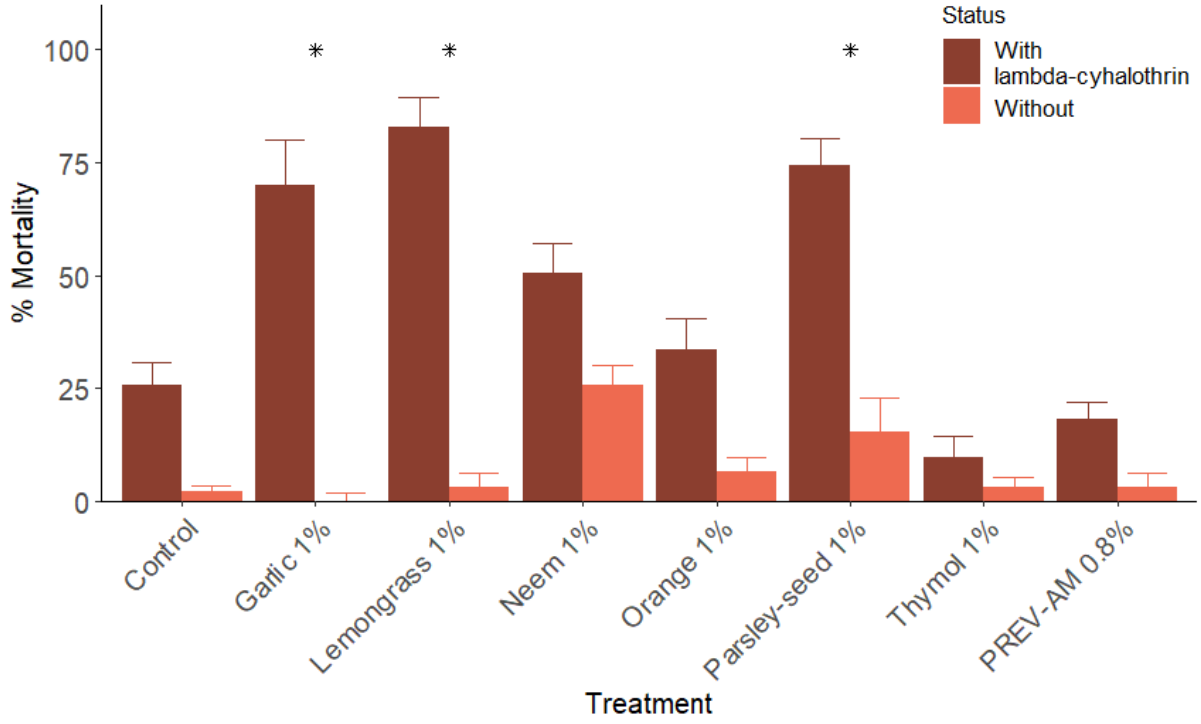

Figure 4.8. Percentage mortality of *Myzus persicae* when treated with synergists separately and when combined with 25% of the recommended field rate dose of lambda-cyhalothrin. Means ±SE, n=6-30. Asterisks highlight treatments that demonstrated synergistic activity.

Table 4.8. Co-toxicity factors and synergy status for synergists tested in combination with lambda-cyhalothrin on *Myzus persicae*.

Synergist	Co-toxicity factor	Status
Garlic oil	172.64	Synergistic
Lemongrass	189.37	Synergistic
Neem oil	-1.77	Additive
Orange oil	4.22	Additive
Parsley-seed oil	81.77	Synergistic
Thymol	-66.05	Antagonistic
PREV-AM	-36.08	Antagonistic

4.4.3 Plutella xylostella

Garlic oil, lemongrass oil, neem oil and thymol all showed synergistic activity when combined with 0.074% of the recommended field rate dose of Sparta. Only PREV-AM showed an additive effect. Conversely, eugenol, parsley seed oil and FLiPPER appeared to show antagonistic activity when combined with this pesticide (Table 4.9). Despite showing synergistic activity, none of the botanicals or products tested increased mortality rate above 50% in the combination bioassay (Figure 4.9).

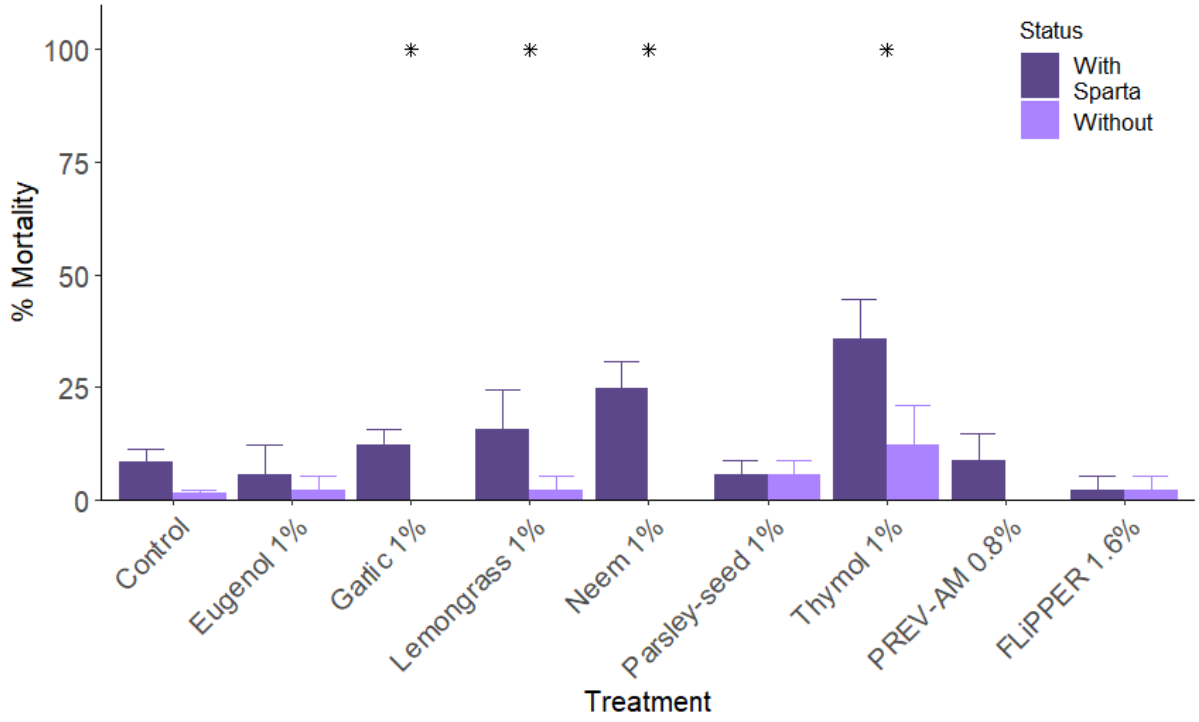

Figure 4.9. Percentage mortality of *Plutella xylostella* when treated with synergists separately and when combined with 0.074% of the recommended field rate dose of Sparta. Means ±SE, n=3-15. Asterisks highlight treatments that demonstrated synergistic activity.

Table 4.9. Co-toxicity factors and synergy status for synergists tested in combination with Sparta on *Plutella xylostella*.

Synergist	Co-toxicity factor	Status
Eugenol	-47.83	Antagonistic
Garlic oil	45.95	Synergistic
Lemongrass	50.00	Synergistic
Neem oil	197.97	Synergistic
Parsley-seed oil	-60.66	Antagonistic
Thymol	74.73	Synergistic
PREV-AM	5.41	Additive
FLiPPER	-80.43	Antagonistic

4.5 Discussion

The results of the initial dose-response assays to determine the LD_{10} for each species highlighted the substantial impact of insecticide resistance and extent of the problem that growers are confronted by. This was most clearly demonstrated *P. chrysocephala* showing high levels of resistance to cypermethrin in the current study, which resulted in only 7% pest mortality at the full field rate dose. This level of resistance in the test population of *P. chrysocephala* used in this study could likely be explained as beetles were collected immediately prior to testing from fields known to have been treated for many years with pyrethroid pesticides, and more recently sprayed with lambda-cyhalothrin based formulations. Lambda-cyhalothrin, again applied at 100% of the recommended field rate dose, demonstrated higher efficacy than cypermethrin in the current study but still only achieved 35% pest mortality after 72 hours (Figure 4.1). Despite the mode of action of both insecticides targeting the sodium channels (IRAC, 2024c), the different relative concentrations of the active ingredients in the selected formulations used as reference may have resulted in the difference in mortality seen between them. In either case, this level of resistance, in addition to the commercial crop damage caused by *P. chrysocephala* each year demonstrates why it continues to present a significant challenge to oilseed rape growers in the UK and across Europe.

By contrast, the dose-response curves for *D. radicum* and *P. xylostella* indicated that the LD_{10} of the pesticide formulations for both species were below 5% of the suggested field doses for these products (Figures 4.2 and 4.4 in turn), being 4% Tracer for *D. radicum* and just 0.074% of Sparta for *P. xylostella*. Both pest cultures were developed from field-caught individuals, though both had been lab-reared for an extended length of time with no deliberate selection for insecticide resistance. The resistance status of the culture-founding individuals at the point of collection is unknown. Although, as discussed in Chapter 1, *D. radicum* control is difficult to achieve with any current method and *P. xylostella* are known to have developed significant pesticide resistance in field populations. It is also well-established that pesticide resistance can incur fitness costs in insects when compared to susceptible individuals (Kliot and Ghanim, 2012). It is, therefore, entirely possible that whilst the individuals used to establish both cultures possessed significant resistance, this had declined over generations in culture as it was no longer selected for, resulting in their apparent susceptibility to pesticides in the current study.

The population of *M. persicae* had also been maintained in a lab culture for a substantial period without resistance selection pressure but the LD_{10} for treatment with lambda-cyhalothrin (25% active ingredient) was similar to the selected dose of Sparta for the field-caught *P. chrysocephala*. Amongst consideration of species differences and the unknown mode of resistance exhibited by *P. chrysocephala,* this perhaps suggests that the target-site mutation conferring resistance in this *M. persicae* population may have little impact on other fitness factors and has, therefore, been maintained without selection pressure.

As with the enzyme activity assays, the combination bioassay results of this study showed significant variation in the relative success and failure of the potential synergists to act as such, both between and within species. PBO consistently had the highest co-toxicity factor out of all compounds tested but this is likely due to the fact that PBO has little effect on the mortality rate when applied in isolation (Figures 4.5 and 4.7). Conversely, botanicals such as neem oil and thymol did show inherent toxicity and caused mortality when applied alone and this affected the output of the calculations accordingly. Like with the enzyme activity assays, these two botanicals also proved effective across the insect species tested in the bioassays. They both demonstrated synergistic activity and significantly increased mortality rates (compared to the control) when combined with pesticides in *P. chrysocephala, D. radicum* and *P. xylostella* but, interestingly, did not have the same effect on *M. persicae*. This suggests that, as with the enzyme activity assays, although some botanicals may appear to be more broad-spectrum in their effects, there may be several factors influencing the overall outcome (discussed further below).

As previously discussed in chapter 3, insecticide synergy using essential oils and their components may be, at least in part, due to the botanical extracts sufficiently inhibiting the insect's defensive detoxification enzymes and subsequently allowing the pesticide to cause fatal effects. This may account for some of the significantly increased mortality rates seen in several of the combined treatment groups in the present work but correlations suggest that this is not the sole reason for it (Chapter 5). Other contributory factors may include a) increased cuticular penetration, b) behavioural changes, c) additional attack on different target sites, d) increased physiological stress and e) non-detoxification enzyme inhibition caused by the botanical products. These factors are not mutually exclusive either and may interact within an insect species, also differing in impact between insect species.

The cuticle of an insect is composed of a lipophilic external layer and a hydrophilic internal layer (Tak and Isman, 2015). It is also known that some resistant insects possess thickened and/or modified integuments that afford them a certain level of protection against insecticides (Ahmad, Denholm and Bromilow, 2006; Lin, Zeng and Lu, 2012; Balabanidou, Grigoraki and Vontas, 2018). Despite this, there appear to be no studies that examine how to enhance the penetrative properties of a pesticide through the insect cuticle using essential oils. There are, however, parallels drawn between the insect integument and human skin due to their similar functions, and investigations do exist focussed on enhancing penetration of both lipophilic and hydrophilic (human) medications. Combining lipophilic and hydrophilic drugs with non-polar and polar terpenes respectively resulted in increased penetration of skin, with 1,8-cineole (commonly found in eucalyptus and tea-tree oils) proving particularly effective. It is thought that this enhancement may be due to 1,8-cineole increasing lipid fluidity in the skin barrier (Gao and Singh, 1997; Narishetty and Panchagnula, 2005; Heard, Kung and Thomas, 2006). A subsequent study by Tak and Isman (2015) suggested that this compound could increase the affinity of camphor when applied to a waxy substance (such as an insect integument) by reducing surface tension, potentially allowing further penetration and greater spread. Although this was initially tested on beeswax, a further study by the same authors in 2017 confirmed 1,8-cineole to be capable of improving penetration of camphor (both major components of rosemary oil (*Rosmarinus officinalis*)) in the cabbage looper (*Trichoplusia ni*).

This suggests that both polar and non-polar terpenoid compounds, such as those found in essential oils, may indeed affect the properties of the insect cuticle in such a way that they may be able to enhance the penetration of pesticides – by influencing solubility and/or surface tension. It could be that, in the current study, some of the increased mortality seen in some of the treatment groups of the bioassay was due to the oils affecting the integrity of the insect integument and its secretions, thereby allowing the pesticide greater access to the insect body. Although they were applied with acetone, itself a solvent and therefore potentially capable of affecting physical and chemical properties at the cuticle, the relevant controls showed that acetone alone had a relatively minor, if any, effect when applied prior to pesticide exposure. Considering the penetration enhancement of camphor afforded by 1,8-cineole, this could be an open area for significant further research regarding improved delivery of insecticidal components to the target sites of pest insects.

As well as some essential oils and their components being well-known for their insecticidal effects, many also have been shown to possess both insect repellent and antifeedant properties (Lee, 2018, Espinoza *et al.,* 2021). Eugenol, neem oil and garlic oil, all of which were included in the current study's bioassays, are some such examples that have proven successful in repelling a variety of insects (Abiy *et al.,* 2015; Plata-Rueda *et al.,* 2017; Lv *et al.,* 2022). Both thymol and eugenol have also demonstrated antifeedant properties when exposed to insects and mites (Isman, 2000; Valcárcel *et al.,* 2021). Assuming that such behavioural effects were replicated in the current study, then this could have impacted the survival of the individuals in treatment groups, providing yet another benefit to the use of botanical extracts in pest control. As each treatment group was provided with a source of food and water for the duration of the study, tainting of these resources could be one way in which this could have occurred, with treated insects potentially leaving traces of botanicals on food and water reserves. If the selected synergist was an effective repellent and/or antifeedant then the insects in the treated groups may have spent less time around the food tainted with the essential oil, fed less often and ingested a lower amount overall thereby potentially reducing survival (Huang, Hee and Ho, 1998; Jeon and Tak, 2024). This would affect the synergist only groups too but the overall impact in both the singular and combined treatment groups would still positively affect the outcome from a pest management viewpoint – the insect would be more likely to remove themselves and feed less from a tainted crop than an untreated one. This process may even be exploited so that insects spread the products further in a field setting (George *et al.,* 2010).

It is this behavioural influence, rather than just the physiological/insecticidal capability of botanicals, that some studies are beginning to investigate with a view to exploiting it for pest control in the field. Essential oils and their components have been shown to disrupt other common but essential insect behaviours, such as grooming. Members of the Diptera, Coleoptera, Hemiptera and Lepidoptera orders of insects are all known to engage in grooming behaviours in order to maintain their sensory organs and overall health by removing particulate debris and parasites, spreading cuticular excretions and optimising flight or adhesive structures (Thelen and Farish, 1977; Phillis *et al.,* 1993; Ozaki *et al.,* 2005; Seid and Brown, 2009; Hosoda and Gorb, 2011; Pritchard, 2016). Some studies have shown that insects can be stimulated to engage in grooming behaviour when they are subjected to physical, chemical or mechanical irritation (Zhukovskaya, Yanagawa and Forschler, 2013). In the current

bioassays, all insects were treated with the botanical synergists by direct application to their bodies, which could have stimulated more frequent and/or longer grooming periods in an attempt to remove the offending solutions (Reingold and Camhi, 1978; Xing *et al.*, 2023). Further, exposure to pyrethroid pesticides, even with a time delay, has been shown to stimulate more frequent grooming behaviours in insects (Wiles and Jepson, 1994).

It is unknown whether the grooming behaviour of these insects would effectively remove the botanical synergist and/or pesticides before being absorbed, thereby reducing their effects, or if it would serve to spread the compounds more widely over the insect body to increase them, so additional investigation is required in this area. If it were to increase the exposure of the insect to both synergist and pesticide, this could contribute to the higher mortality rate seen in some of the combination treatment groups. Additionally, some insects use their mouthparts and/or their antennae (run through their mouthparts) to dislodge particles whilst grooming (Zhukovskaya, Yanagawa and Forschler, 2013), which may facilitate ingestion of both the pesticide and botanical synergist and provide another route of delivery. Given that many essential oils and their components are also known to be disruptive and/or toxic if ingested, the combined treatment insect groups may have suffered from attack at multiple points of exposure (Barbosa *et al.,* 2021).

Grooming has also been suggested as a displacement behaviour involved in the insect stress response. Octopamine is a molecule that mimics the structural and physiological characteristics of vertebrate noradrenaline and plays an integral role in the regulation of the insect stress response (Jankowska *et al.,* 2017). When an insect is stressed, octopamine is released into the haemolymph which results in a higher state of arousal (Davenport and Evans, 1984). Certain components of essential oils, such as eugenol and geraniol, can act in a similar way to octopamine and stimulate a higher level of activity, aggression and heart rate, resulting in increased energy usage (Jankowska *et al.*, 2017, Enan, 2001, Xing *et al.,* 2023). However, this reaction is dependent on concentration of the compounds and higher doses have been shown to dampen the nervous response, potentially caused by damage to the neuronal membranes (Enan, 2001). Exposure to both the synergists and pesticides in the bioassays could have caused an increased physiological stress response in the insects, resulting in displacement behaviours such as grooming and an overall higher energy use and fitness cost (Jacquet *et al.,* 2012). This could have further enhanced the detrimental effects of the pesticides and increased mortality rate in the combined treatment groups. Additionally, if

sufficient levels of the synergists were absorbed, damage to the nervous system of the insect may also have negatively affected their survival in the trial.

As previously explained in chapter 3, exposure of these botanical compounds to various enzymes within the insect body may have significantly impacted their ability to carry out essential processes. Botanical compounds such as essential oils and their components are known to inhibit the activity of enzymes involved in a range of functions vital for survival within the body of an insect, not just detoxification. Acetylcholinesterase (AChE) is an enzyme of the esterase group that is largely concentrated at cholinergic synapses throughout the insect nervous system and terminates the transmission of the nervous impulse once it has passed. It does this by hydrolytically cleaving the acetylcholine that acts as the synaptic transmitter, thus ending the signal. If this enzyme is disrupted, as with organophosphate pesticides, it is unable to complete its role and the acetylcholine continues to stimulate the synaptic receptors. This means that the impulse is repeatedly delivered, resulting in sustained muscle contraction or spasm, difficulty respiring and neuronal fatigue (Siegfried and Scharf, 2001). There is an extensive body of evidence suggesting that many botanical compounds inhibit AChE and this alone has a detrimental effect on the survival of the insect (Table 1. in Jankowska *et al.,* 2017). Indeed, preliminary tests with the synergists used in this study found that the concentrations of garlic oil and thymol both needed to be reduced for the bioassays due to their inherent toxicity. These findings suggest that, even applied in isolation, the botanical compounds could function as an organic alternative to the synthetic chemistry presently available and provide an option for reducing their use.

In addition, if the botanical synergists are combined with synthesised conventional pesticides, as with the bioassays in the current study, they may further incapacitate the insect pest by targeting different functional mechanisms to those of the pesticides and causing a fatal response, perhaps even in resistant individuals. The pesticides cypermethrin and lambda-cyhalothrin, for example, are pyrethroids that disrupt the sodium-gated ion channels responsible for the successful transmission of an action potential along the neurons of the insect nervous system. The chemicals in this class of insecticides typically bind to the ion channels which prevents them from closing after effective transmission of an action potential and results in continual firing of the impulse leading to spasm, paralysis and eventual death (IRAC, 2024c). In the bioassays using *D. radicum*, the pesticide Tracer contains the active ingredient Spinosad, the primary mode of action of which involves binding to the nicotinic

acetylcholine receptors of the insect neural network, and results in the same end-point: spasms, paralysis and death (IRAC, 2024c). The increased mortality rate seen in the combined treatment groups for several of the tested botanical synergists may be due, at least in part, to the cumulative effect of the pesticides and botanicals acting on different target sites.

While all of the above mechanisms could have potentially resulted from co-application of the botanical and pesticide treatments in the current study, their combined effects may not be interpreted as exerting true synergy – i.e. the outcome of the combination being higher than the additive effects of the singular. Nevertheless, even an additive effect alone, with mechanisms acting simultaneously on the body of the insect, will certainly have had a cumulative effect on pest mortality, thereby increasing the overall efficacy of the combined treatment groups. This is a promising result regardless of whether the botanicals were determined to be synergistic, as the improvement in pest control afforded may translate to an increased yield and reduced crop damage in the field. Stacking these benefits with the synergistic effects of inhibiting the insect detoxification enzymes may allow enhanced control of metabolically resistant pests and a reduced application rate of conventional pesticides. This is particularly apparent in Figure 5.6, where a 90% reduction of applied pesticide combined with neem oil in *P. chrysocephala* can be seen to maintain a comparable mortality rate to the recommended field rate dose. The bioassays also suggest that neem oil may not be the only plant-based compound that could be used in this way. Further investigation using other essential oils and components, such as thymol, in combination with reduced pesticide concentrations, may reveal a range of potential synergists that have this effect. These products can therefore be used, not only to restore control in resistant insects and more effectively control susceptible species, but also to reduce the amount of synthetic chemicals required to bring about that control. The results of these bioassays also suggest that, similar to the enzyme assays, different botanicals will have a higher or lower impact depending on the insect species they are applied to.

As well as PBO, the botanicals showing significant synergistic activity in *P. chrysocephala* were neem oil, orange oil and thymol. PBO had the highest co-toxicity factor but produced a lower mortality rate than neem oil when combined with cypermethrin. Thymol and orange oil also had synergistic co-toxicity factors and, although they significantly increased mortality rates compared to the control, they were not as effective as PBO or neem oil (Figure 5.5, Table 5.5). Interestingly, despite orange oil showing apparent synergy in this species, PREV-AM – whose

active ingredient is listed as orange oil – appeared to be marginally antagonistic. However, PREV-AM contains only 6% orange oil and a 0.8% solution of PREV-AM was used, so the beetle was actually exposed to 0.048% orange oil in that treatment group. This greatly reduced proportion of botanical combined with some variation in the effect of cypermethrin within the beetle population, may account for the slight antagonism seen in the calculations.

Rapeseed oil, however, showed a much lower co-toxicity factor and appeared to be considerably antagonistic for *P. chrysocephala* (Table 5.5). Perhaps predictably for an insect that regularly feeds on oilseed rape, treatment with just the oil extracted from this plant resulted in 0% mortality (Figure 5.5). To ensure safe and continued feeding on the chosen plant host, evolution would have adapted this species to mitigate any possible detrimental effect of the oilseed rape plant's defensive compounds or metabolites. This would explain an additive effect of the treatments, but an antagonistic response suggests that the beetle may have adapted further to then use the essential oils and/or components in a defensive manner, which is a tactic known to be used by the striped flea beetle (*Phyllotreta striolata*) when feeding on cruciferous vegetation (Beran *et al.,* 2014). Although this system is slightly different, there may be a similar chemistry allowing the beetle to protect itself by making the pesticide less effective in the presence of rapeseed oil but this would need further investigation.

The majority of the botanicals and products tested in bioassays against *D. radicum* demonstrated synergism with Tracer (Table 5.7) and significantly increased mortality rates compared with the control group (Figure 5.7). The higher number of botanicals that demonstrated synergistic activity in this species compared to the others is likely to be due to the different mode of action of Spinosad to pyrethroid pesticides and its compatibility with that of the botanicals. Spinosad works by overstimulating the insect acetylcholine receptors, resulting in spasm, neuronal fatigue and death (IRAC, 2024c). If the botanicals do inhibit the enzymes responsible for the breakdown of acetylcholine, also resulting in overexcitation of the neurons, the overall impact is likely to be magnified. This, combined with the other identified botanical effects, may be why several showed such success in synergising Tracer. Thymol and PBO were the most effective at increasing mortality rate compared to the control, followed by eugenol and PREV-AM (Figure 5.7). Despite garlic oil and FLiPPER slightly increasing mortality compared to the control group, analysis showed that this was an additive effect, rather than synergism and not significant (Table 5.7). As previously discussed, the

precise reasons for these differences may lie within the varying chemical composition of each botanical extract or product and the resulting molecular interactions. While interesting, disentangling these interactions was beyond the scope of this study and would require intense further examination.

In a now familiar pattern to these studies, a selection of botanicals different to those effective in the other species caused a significant increase in mortality of *M. persicae*, when combined with lambda-cyhalothrin. In this species, garlic oil, lemongrass oil and parsley-seed oil were particularly effective in raising mortality to above 70% in each case, with little effect in the oil-only treatment groups (Figure 5.8). Similar to the results of the enzyme assays, this variation in which oils affected which species may well be closely linked to evolutionary history and specific chemical interaction. In contrast, thymol and PREV-AM were both antagonistic in effect on the aphid (Table 5.8). In work elsewhere, synergism with a neonicotinoid has been observed in this species using thymol, although it was observed that whole thyme essential oil had a comparatively greater effect, again suggesting an underlying interaction between various components that may synergise and/or antagonise one another (Faraone, Hillier and Cutler, 2015). It should also be highlighted that the strain of *M. persicae* used in the current bioassay has an identified target-site resistance. As botanical extracts have been established to be active on various targets within an insect (Jankowska *et al.,* 2017), some compounds may show higher or lower efficacy in this strain of *Myzus* compared to others depending on precisely how the adapted target-site is structured and whether it is compatible.

Neem oil and thymol were particularly synergistic when applied to *P. xylostella* in combination with Sparta. To a lesser extent, garlic oil and lemongrass oil were also effective synergists. However, even the highest co-toxicity factor of neem oil (197.97) did not seem to translate directly into exceptionally high efficacy in the bioassay, as it only achieved 25% mortality. Thymol, although having a lower co-toxicity factor than neem oil due to higher inherent mortality, was the most effective in combination but still only raised mortality to 35% (Figure 5.9). This apparent resilience of *P. xylostella* to synergistic action of essential oils and pesticide could be due to the various mechanisms already discussed but there is an extra layer of complexity when the larval stages of this species moult. Although there are insecticides targeted towards disrupting the process of moulting (growth regulators, IRAC 2024c), there is no mention in the literature of what happens when an insect exposed to a contact pyrethroid immediately moults or has already begun moulting when exposed. It was noted during the

bioassay with this species that surviving individuals continued through the developmental process and shed skins were apparent in their test arenas. It could be that traces of the pesticide and applied botanicals were shed during the process of moulting so, in some cases, very little of either may actually have been absorbed into the body of the insect to have any effect. Zhang *et al.* (2014) also concluded that moulting fluid secreted during the process of ecdysis contains proteins related to immunity, anti-microbial action and detoxification, further reporting that it also effectively protected silkworm larvae (*Bombyx mori*) from infection by *Beauveria bassiana*. In addition, it is possible that this fluid could act as a buffer that does not allow the oils or pesticide to pass effectively between the old and new cuticles to reach their target sites. The larvae selected for this test were all third instar but their exact stage in development was not calculated by day as it would have been difficult to achieve the requisite number of individuals required for testing with exacting timing. It may have been that newly shed third instars were more vulnerable to penetration by both botanical and insecticide and those that were nearing ecdysis towards fourth instar were more protected. Similar queries may relate to *M. persicae* as skins were also visible during these bioassays, but they were selected for use between 0 and 24 hours old so they may not have been quite as close to ecdysis during the trial. Significant further examination of this process, timing and its impact on pesticide efficacy would be required to investigate any impacts of moulting more fully.

It is clear from these bioassays that some essential oils and their components do indeed synergise the effects of the selected conventional insecticide chemistries when tested in the identified species. It is also apparent that there is significant variation in which botanical extracts and some products demonstrate synergy depending on the species used. Likewise, there is evidence that some will even elicit an antagonistic response when combined with a particular pesticide – and that this is further dependent on species. These effects and the extent of their impact on survival appear to be related to a range of factors including the ability of both botanical extract and pesticide to penetrate the insect cuticle, cause physiological and behavioural stress, impact on the same or different targets, inhibit enzymes involved in detoxification and modulate other enzymes related to normal biological functions within the body of the insect. These factors are also not mutually exclusive and likely to not only occur simultaneously, but to interact with one another. Although the initial idea of this study was to explore the botanical synergy through detoxification enzyme inhibition and bioassay, the correlation of which will be discussed next, it has become apparent that there are many other

factors to consider when reviewing the broader context of this work. As such, the following chapter will first explore these findings within the scope of this study and then through a wider, applied perspective, to consider how all of this fits within an integrated pest management and optimised sustainable farming framework.

Chapter 5. The correlation between enzyme inhibition and synergism with pesticides

5.1. Summary

Work undertaken in Chapter 5 demonstrates that there was no significant correlation between the bioassay data (Chapter 4) and the enzyme activity data (Chapter 3), overall. There were, however, certain botanicals that appeared to strongly inhibit enzyme activity and result in high mortality when combined with pesticides in the bioassays in some species. In direct contrast, there were also examples where treatments showed strong inhibitory properties but this did not result in synergy, and vice versa. In many cases the overall relationship was negative, suggesting that there was a general trend that mortality increased as activity decreased. This all suggests that although synergism may be influenced by the ability of a botanical to inhibit insect detoxification enzymes, it is not solely due to this effect.

5.2. Introduction

The theory of metabolic synergy is based on the idea that certain compounds, in this case botanicals, interfere with the activity of insect detoxification enzymes, thus allowing the pesticide to exert its full and fatal effects without interference from these defensive measures (Metcalf, 1967). However, the modes of action contributing to enhanced mortality when synergism between selected compounds and/or a pesticide is observed in insect bioassays could range from improved cuticular penetration, heightened attack on the same target site or different target sites being affected, physiological and behavioural stress and inhibition or modulation of enzymes involved in detoxification as well as other survival processes (Tak and Isman, 2015; Barbosa *et al.,* 2021; Lee *et al.,* 2001; Adhikari, Khanikor and Sarma, 2022; Xing *et al.*, 2023; Jeon and Tak, 2024).

Various studies have focussed on the inhibitory capabilities of botanicals on the detoxification enzymes involved in metabolic pesticide resistance (Koul and Walia, 2009; Anderson and Coates, 2012; Pavela, 2015) and yet more studies have tested the potential of botanical extracts to be used as pesticide synergists, with varied success achieved by some products and insect combinations (Scott *et al.*, 2003; Khot, 2009; Joffe *et al.,* 2012; Tak and Isman, 2017; Marchand *et al.*, 2018). Despite there being speculation as to the mode of action involved in cases of successful synergy, and parallels drawn between known enzymatic sources of

resistance in insects and treatments appearing to overcome that resistance, the literature is scarce in providing direct correlation analyses between enzyme inhibition by botanicals and mortality in bioassays.

To determine whether or not the synergistic effects observed in the bioassays of the current study were connected to the enzyme inhibition seen in the enzyme assays, a correlation analysis was run between the two datasets for each enzyme group and insect. This would then help to clarify whether botanicals identified as promising synergists were acting on the enzymes only, and could thus be applied to target metabolically resistance species, or if they were more broad-spectrum in their effects and could also help to control insects with different types of resistance, such as target-site mutations.

5.3. Results

5.3.1 Psylliodes chrysocephala

There was no statistically significant correlation between the mortality data collected in the bioassays and the enzyme activity data in *P. chrysocephala* (Esterase: p>0.05; GST: p>0.05; P450: p>0.05). However, in each case the correlations were negative suggesting that, overall, as the enzyme activity reduced, the mortality increased (Figures 5.1-5.3).

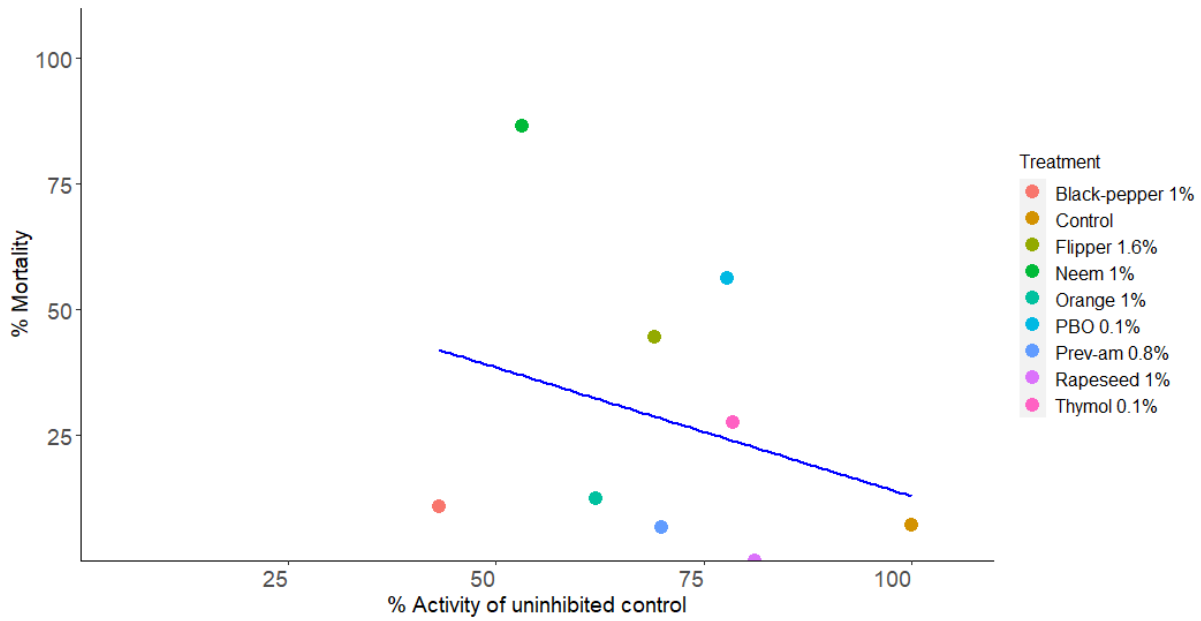

Figure 5.1. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the esterase activity in *Psylliodes chrysocephala*, n=9.

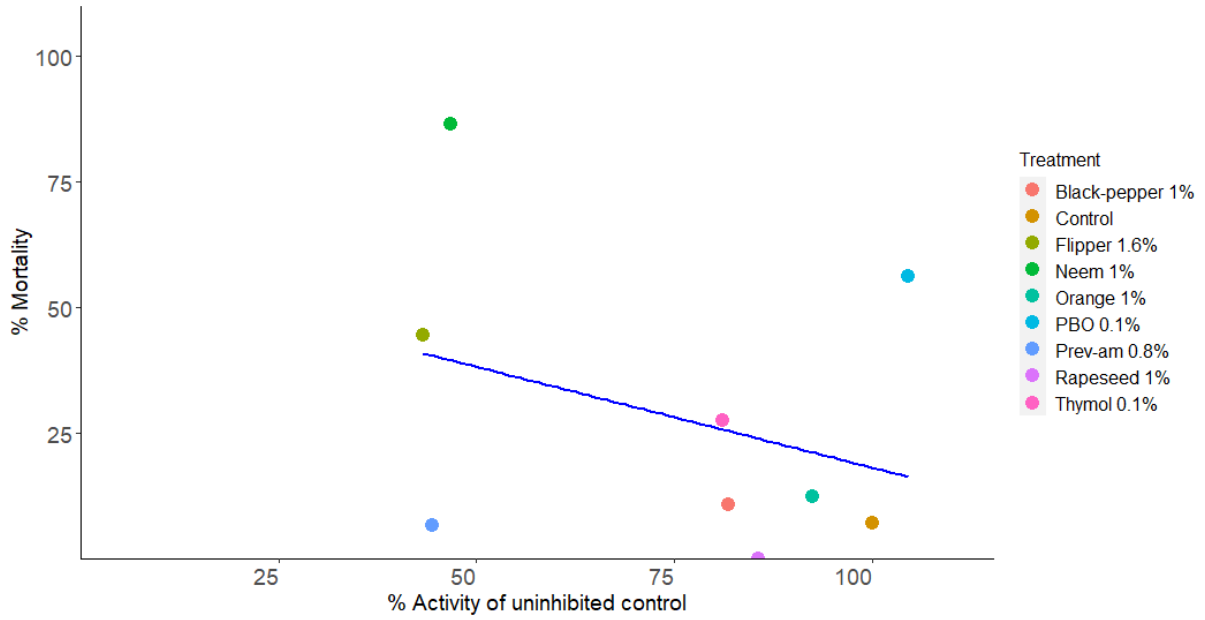

Figure 5.2. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the GST activity in *Psylliodes chrysocephala,* n=9.

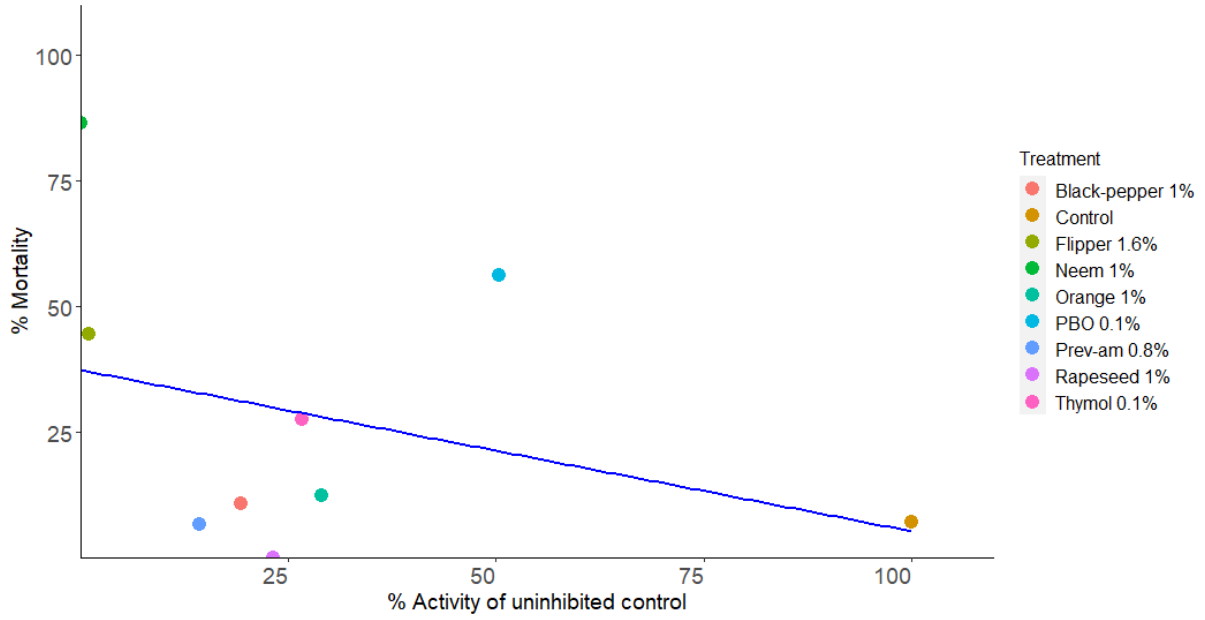

Figure 5.3. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the P450 activity in *Psylliodes chrysocephala*, n=9.

5.3.2 Delia radicum

The correlation between the *D. radicum* mortality data from the bioassays and the esterase data appear to show a negative relationship (Figure 5.4) but this proved to be marginally not significant (p=0.06). There was also no statistically significant correlation between the mortality data collected in the bioassays and the GST (Figure 5.5) or P450 (Figure 5.6) activity data in *D. radicum* (GST: p>0.05; P450: p>0.05). However, in each case the correlations were negative suggesting that as the enzyme activity reduced, the mortality increased (Figures 5.4-5.6).

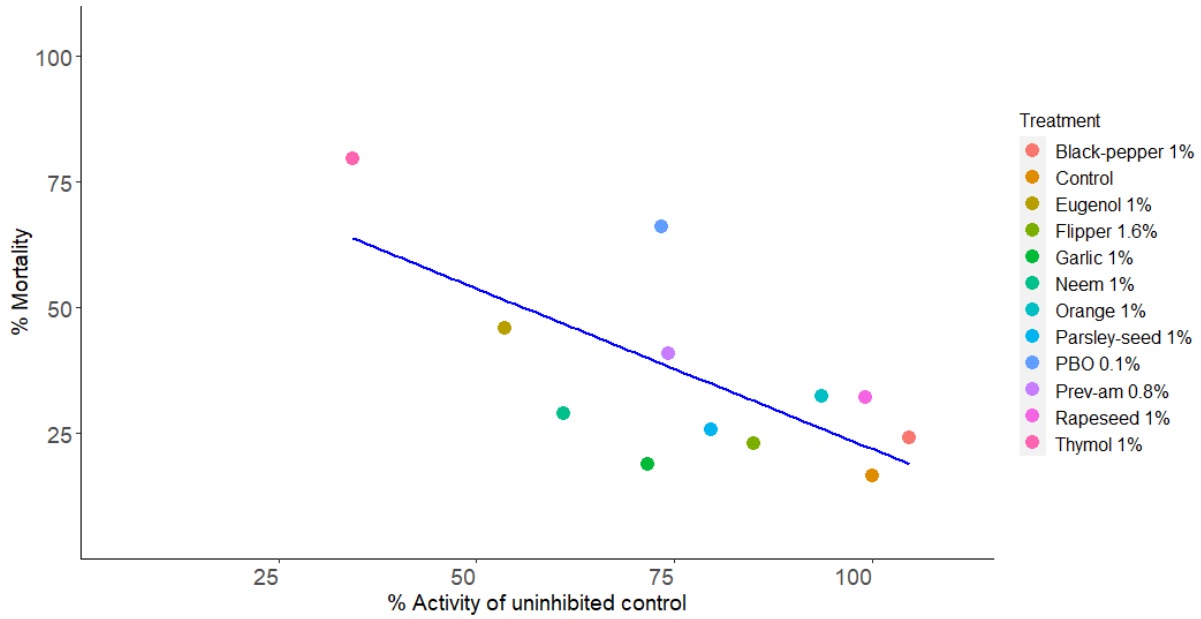

Figure 5.4. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the esterase activity in *Delia radicum*, n=12.

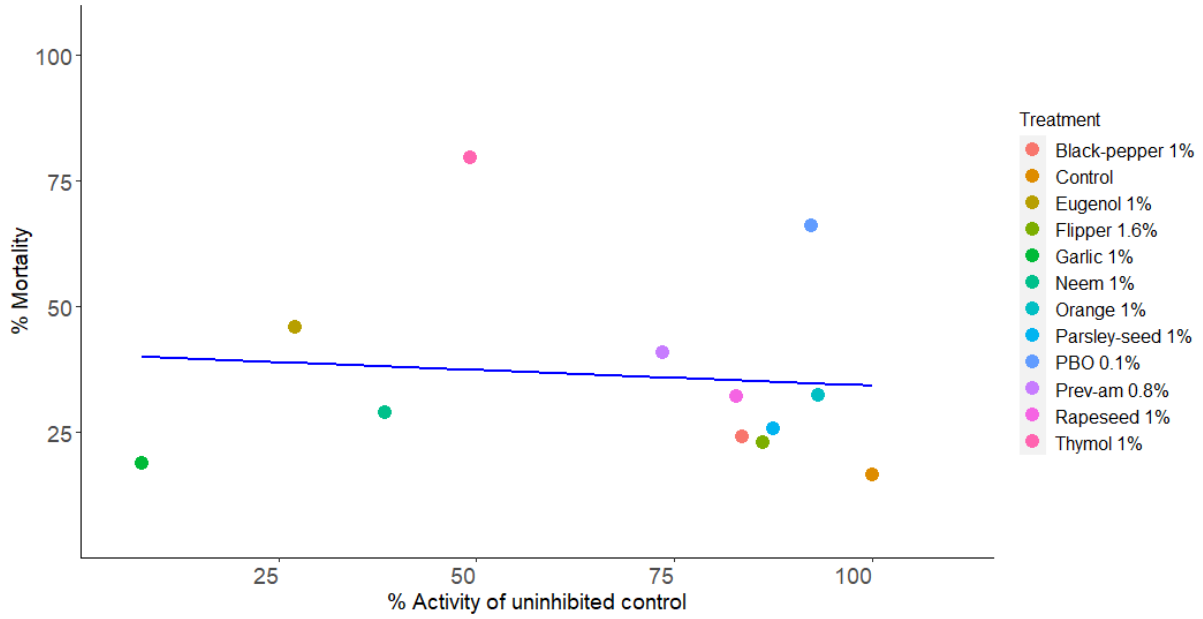

Figure 5.5. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the GST activity in *Delia radicum*, n=12

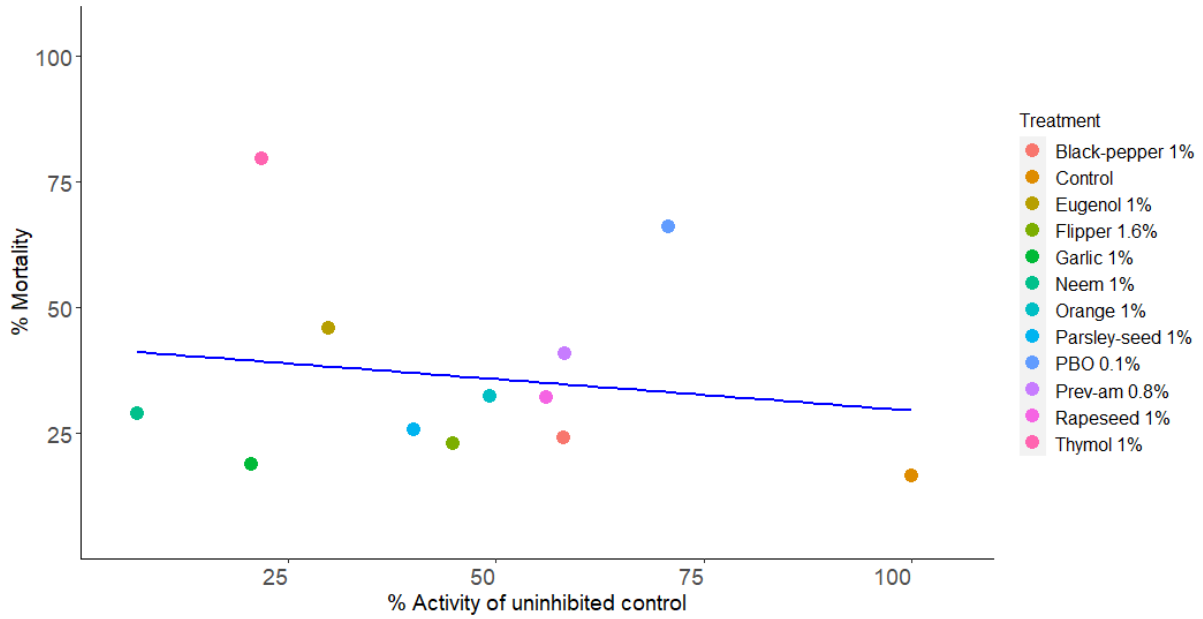

Figure 5.6. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the P450 activity in *Delia radicum*, n=12.

5.3.3 Myzus persicae

There was no statistically significant correlation between the mortality data collected in the bioassays and the enzyme activity data in *M. persicae* (Esterase: p>0.05; GST: p>0.05). However, the correlation did suggest that as esterase activity decreased, the mortality increased (Figure 5.7). There appeared to be no such relationship between the mortality and GST activity data (Figure 5.8).

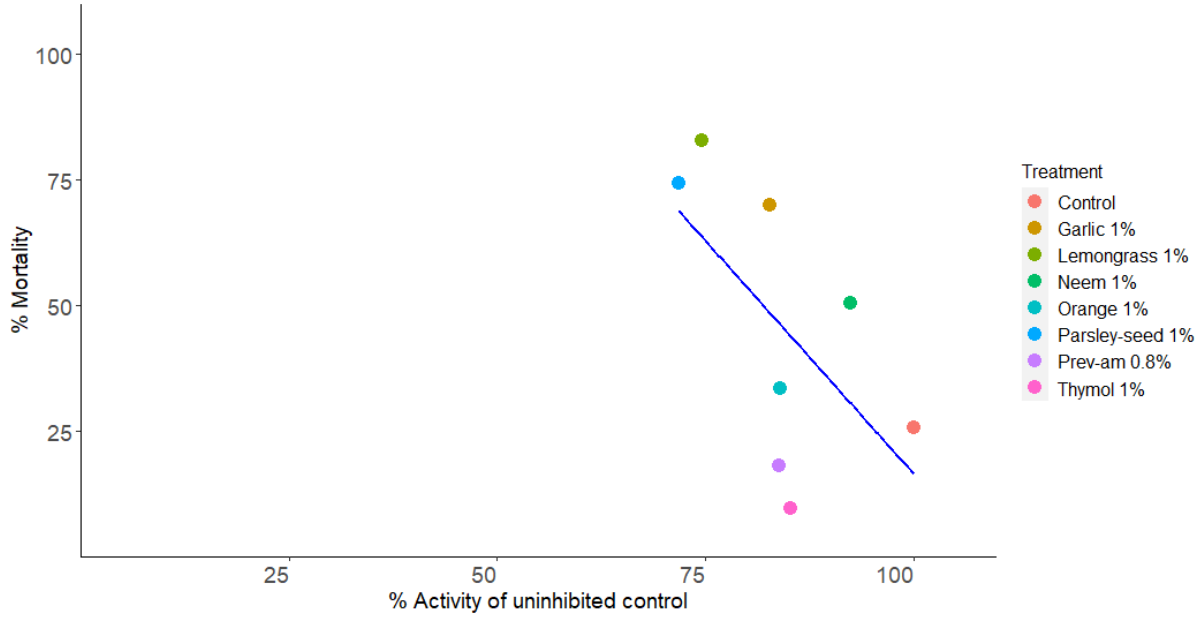

Figure 5.7. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the esterase activity in *Myzus persicae*, n=8.

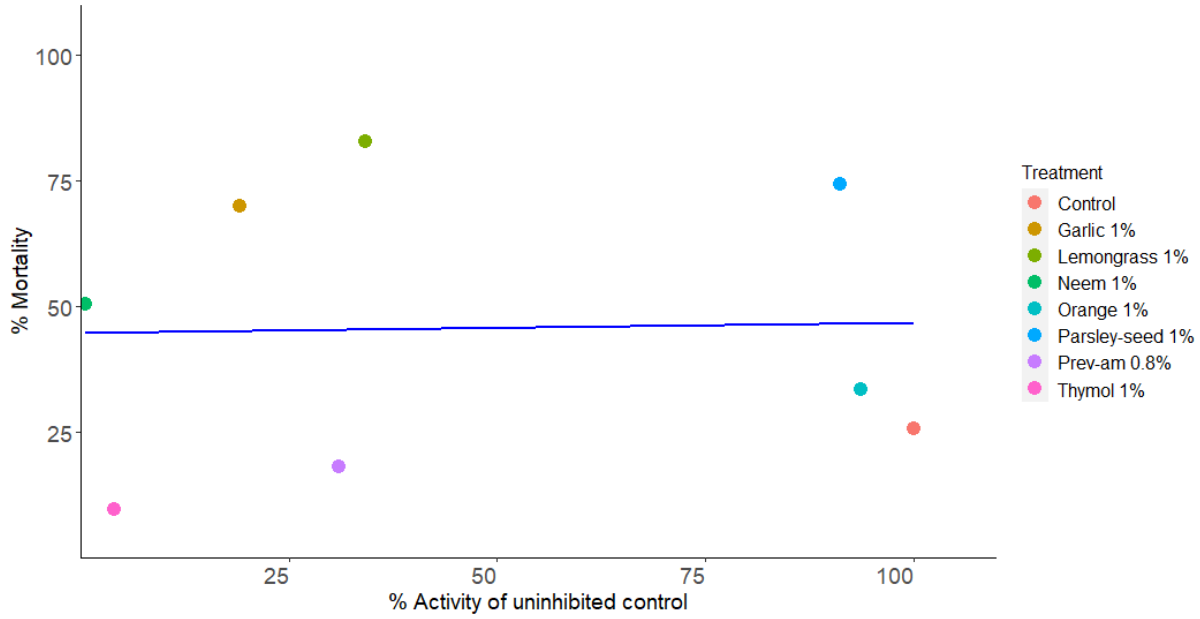

Figure 5.8. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the GST activity in *Myzus persicae*, n=8.

5.3.4 Plutella xylostella

There was no statistically significant correlation between the mortality data collected in the bioassays and the enzyme activity data in *P. xylostella* (Esterase: p>0.05; GST: p>0.05; P450: p>0.05). However, the relationship between the mortality data and the esterase and GST activity data appeared to be negative, suggesting that as enzyme activity reduced, mortality increased (Figures 5.9-5.10). There was no such relationship indicated in the P450 enzyme activity/mortality correlation (Figure 5.11).

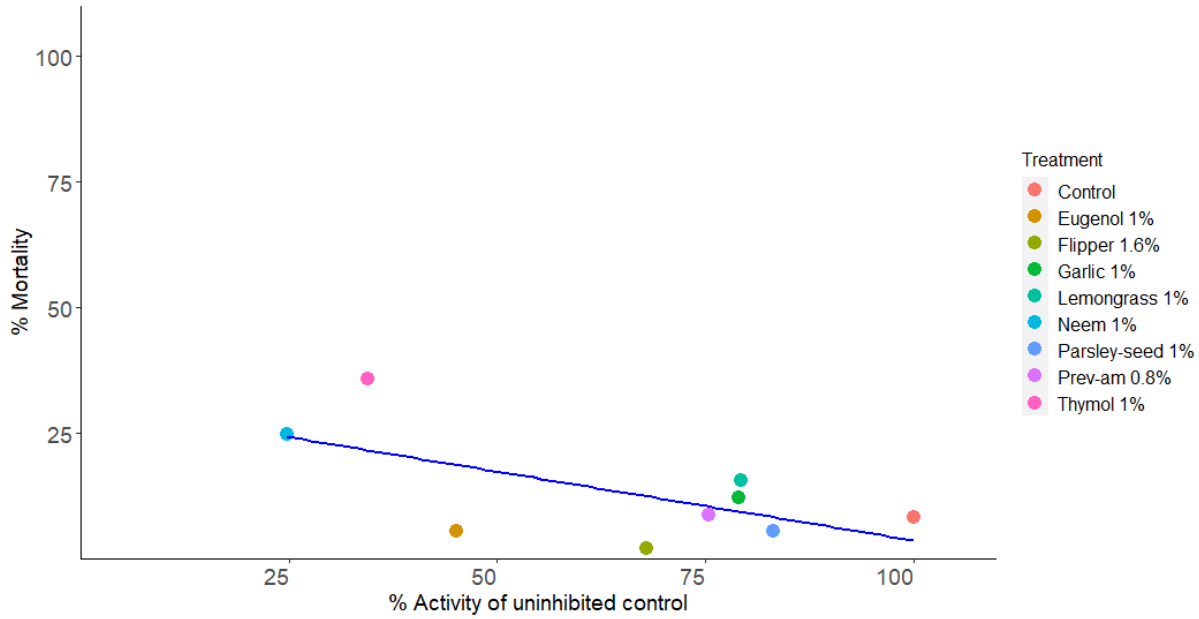

Figure 5.9. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the esterase activity in *Plutella xylostella*, n=9.

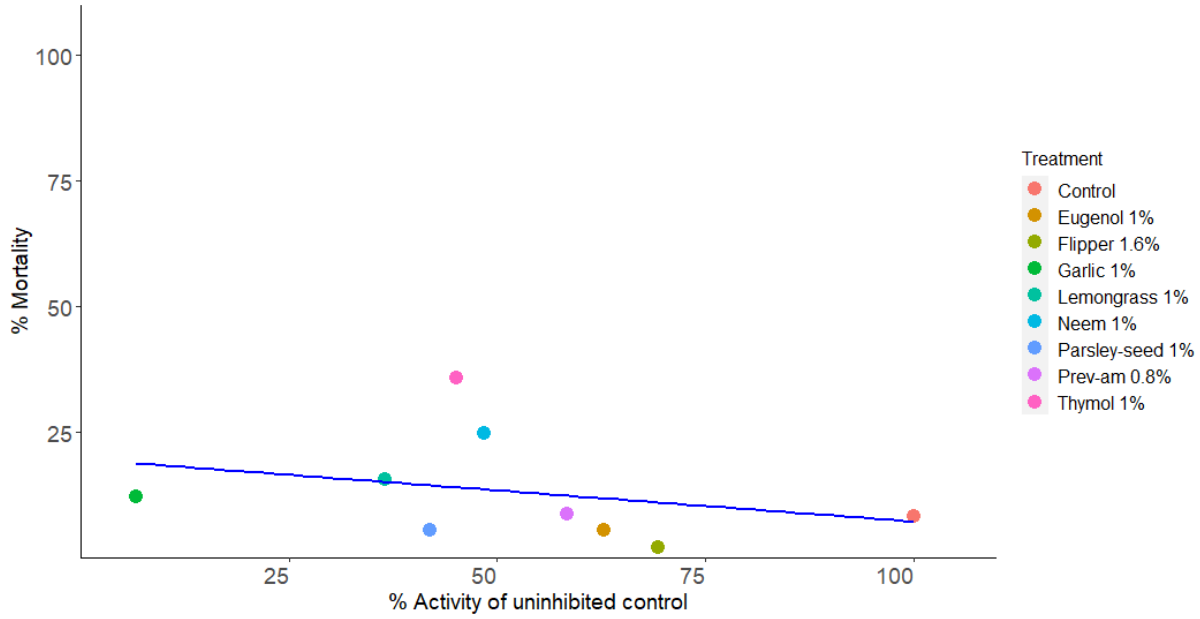

Figure 5.10. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the GST activity in *Plutella xylostella*, n=9.

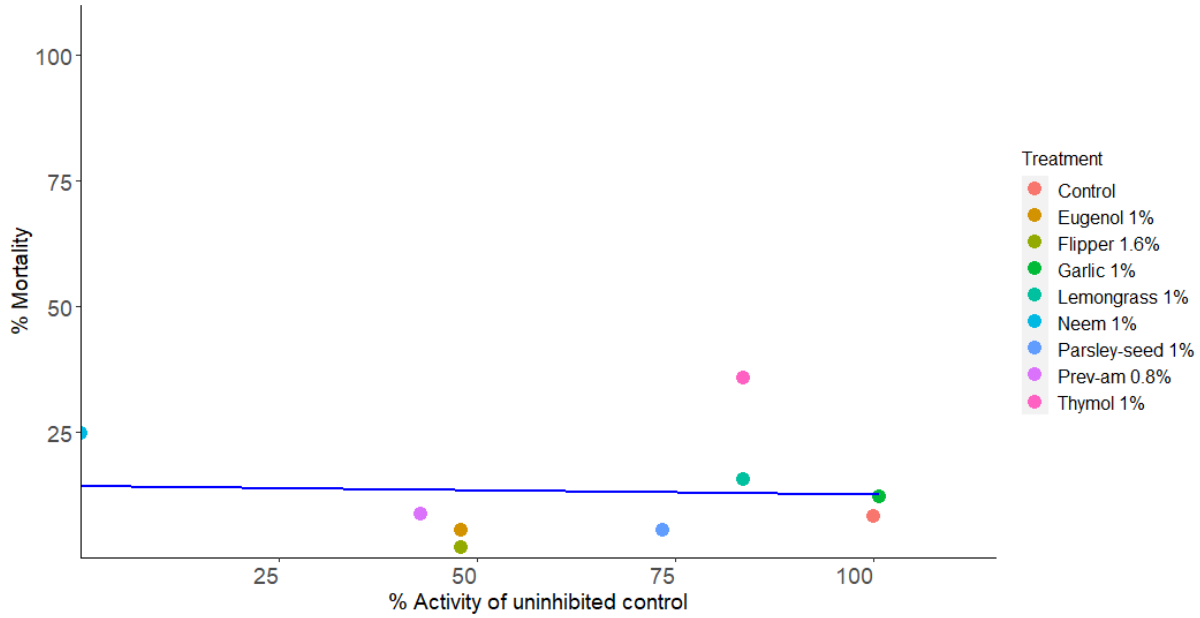

Figure 5.11. Correlation between percentage mortality of the combined pesticide/synergist group in the bioassay and the P450 activity in *Plutella xylostella*, n=9.

5.4 Discussion

The results of the correlations in this study indicate that, as discussed in previous chapters, the mitigation of metabolic resistance in insects in order to restore control of crop pests is indeed a significantly complex problem involving numerous interacting factors, ranging from botanical chemical structure to life history. It is also apparent that it is not one that is easily resolved by simply selecting a botanical product that showed promise in reducing detoxification enzyme activity *in vitro* and combining it with a previously ineffective pesticide, *in vivo*. It is known that metabolic pesticide resistance in insects is due to an upregulation and/or increased presence of the detoxification enzymes responsible for metabolising the insecticidal ingredients before they have chance to incur fatal consequences. The theory behind enhancing the effects of a pesticide using botanical extracts is that this detoxification process may be subdued by the botanical compounds inhibiting the activity of those enzymes, thereby allowing the pesticide sufficient access to their required targets to carry out their role. The hypothesis then, was that as the activity of any enzyme group assays decreased, the mortality of the insects within the combined treatment groups in the bioassays would increase, and that this relationship would be statistically significant.

In an initial challenge to this hypothesis, none of the enzyme activity data had any statistically significant correlation with the mortality data for any of the insect species tested. The correlation between *D. radicum* esterase and bioassay mortality was only marginally non-significant ($p=0.06$), however. It should be noted that the minimum suggested sample size for a Spearman's correlation can be 5 pairs of data, ideally at least 8-15, and although each correlation in this study consisted of at least 8 pairs of data, the strength and reliability of such an analysis are improved with a higher sample size (Field Studies Council, no date). It may be that testing a greater range of botanicals in the bioassays and increasing the number of replicates in both assays could improve the accuracy of the analysis and help clarify borderline relationships such as that of *D. radicum* esterase and the corresponding insect mortality. The current work sought to explore effects across a range of insect species, a wide variety of botanicals and to include all enzyme groups of interest so elements such as number of replicates were necessarily limited to ensure completion within the allotted time.

More promisingly, and despite a lack of statistical significance, the majority of the correlation analyses did appear to indicate that the relationships between enzyme activity and mortality rate were indeed negative, as predicted. The slopes of the correlations suggest that as activity

in all enzyme groups extracted from both *P. chrysocephala* and *D. radicum* decreased, the mortality rate observed in the corresponding botanical treatment groups generally increased. The same trend was observed in *M. persicae* and *P. xylostella*, apart from with the GST and P450 enzyme groups respectively (Appendix B). These exceptions may point towards the influence of factors such as the chemical structure of botanical compounds, potential antagonism between compounds and their incompatibility with binding to enzymes in different species, as discussed in chapter 3. It also offers evidence to support the idea that botanicals could be selected for species-specific impact when targeting crop pests and potentially provide some relief for beneficial insects.

Further to this, there were certain botanical products tested in the current work that were notable as potential metabolic synergists for individual species. For example, neem oil was very effective as an inhibitor across all detoxification enzyme groups in *P. chrysocephala* while also causing more than 50% mortality in the bioassay combinations. Similarly, thymol significantly inhibited *D. radicum* esterase and achieved more than 80% mortality when combined with Tracer in the bioassay. Although *M. persicae* esterase appeared particularly resilient to inhibition by any botanical, lemongrass oil did have significant impact on its activity and proved synergistic by achieving more than 83% mortality in combination with Sparta in the bioassays. These cases appear to signify that, in those treatments at least, the theory of botanical metabolic inhibition resulting in improved pesticide efficacy is supported.

In contrast, there were some data pairs within these treatments that appeared to have little to no connection at all i.e., whereby the botanical may significantly inhibit one or more enzyme groups in one insect species but this is not translated to significant mortality in the corresponding bioassay, and vice versa. This was notably evident in tests using *M. persicae* as while thymol had an almost completely inhibitory effect on the GST enzyme in this pest species, the bioassay results demonstrated that it had very little effect on the mortality rate of the insect when applied with the pesticide. Given that the strain of *M. persicae* tested in this study has a known target-site resistance, this suggests that botanical synergists may be of little help when attempting to restore control in species whose primary resistance mechanism is not metabolic.

However, there were also cases where this occurred in *P. xylostella,* which demonstrated very little, if any, resistance at all when treated with Sparta. Although several of the botanical products significantly inhibited the detoxification enzymes in this species, the botanical that

achieved the highest mortality when combined with Sparta in the bioassays was with thymol at 35%, a relatively small increase from the control when compared with the other insect data. It may be that, in these cases, the botanicals and/or the pesticidal ingredients are not penetrating the insect cuticle in sufficient quantities to deliver the inhibitory and toxic components to the target sites within. Due to the range of botanicals tested and their varying species of source plants, both native and not, it is perhaps unlikely that this species has developed cuticular resilience to all of them through their evolutionary history. It is more likely that moulting and the secretion of defensive and detoxification fluids during that process may have protected the caterpillars from penetration by the treatments (Zhang *et al.,* 2014). The absence of this during the bioassays involving *P. chrysocephala* and *D. radicum* may account for some of the difference in outcomes.

The presence of an apparent relationship between the enzyme data and bioassay data when an insect is treated with one botanical and its absence when treated with another will have affected the slope of the curve when combined into one correlation analysis. Consequently, these opposing relationships may be cancelling out the effects of one another, resulting in a lack of significance and flattening of the relationships that do exist. For example, thymol reduced activity of GST in *D. radicum* to 49% of the uninhibited control and produced 80% mortality when combined with Tracer, which suggests it may be a potent metabolic synergist. However, while garlic oil reduced GST activity to 7% of the uninhibited control, it only had an additive effect in the bioassay and produced 19% mortality. This meant that the data were more scattered in their distribution, rather than grouped along a negative slope, and reduced the possibility of a significant correlation between them. It also suggests that factors discussed in chapters 3 and 6, such as chemical structure, evolutionary life-history and botanical mode of action, could indeed influence whether or not synergy is achieved in a given scenario as significant enzyme inhibition clearly does not routinely translate into improved mortality *in vivo*.

Based on the results of the current study, it is difficult to confidently clarify the relationship between higher mortality and enzyme inhibition, given that the enzyme activity assays and combination bioassays were necessarily conducted on different groups of insects. The enzyme assay required destruction of the insects during the extraction process and therefore it was not possible to gather data for both assays from the same individuals. So, although it has been robustly ascertained that any significant statistical differences between the control and

treatment groups in enzyme activity and mortality of both assays are likely due to the treatment effect of the botanical products, the correlation analyses showed that the outcomes of these effects were often not directly associated. They also suggested that any relationship that may actually be present could be diluted by grouping the botanicals into single data points. However, the methods used could potentially be adapted to provide parallel studies using insects from the same batch of eggs, stage of life and conditions to allow each botanical to be examined separately, rather than grouped by insect. Although a statistician would need to be consulted on the particulars, each replicate in the enzyme assay and the bioassay could be randomly paired and the correlation analysis then performed on the dataset from each botanical treatment to identify a possible relationship. This would remove the confounding effects that the differing outcomes of each product had on the overall analysis and clarify significant connections between metabolic inhibition and apparent synergism in the bioassays. From this, the botanicals with both attributes could be selected for larger scale glasshouse and/or field trials to develop more effective formulations or combination treatments for the selected pest species.

Chapter 6. General Discussion and Conclusions

There is a growing demand, both by consumers and legislative bodies, to reduce the application of and reliance on chemical pesticides for the control of insect crop pests, due to rising concerns over their health and environmental impacts. However, to sustain the supply of food to an increasing human population, access to these products remains critical within an effective IPM approach as alternatives, such as biopesticides and cultural practices, have yet to consistently match their capabilities and practicality. To add further pressure, the available options for chemical control have been greatly reduced in the UK and EU by product withdrawals and tightening regulation on approved ingredients. This has, in turn, forced selection in targeted insect pests towards the development of complex and interactional resistance mechanisms to combat the remaining formulations, resulting in significantly or completely resistant populations that cause devastating damage and yield loss year on year. Botanical extracts and their components have long been known to inhibit the activity of the detoxification enzymes that grant some species their insecticide resistant status so they may provide a valuable resource for use as synergists when combined with reduced levels of existing pesticide chemistry.

In this study, the enzyme activity assays conducted confirmed that certain botanicals are indeed effective metabolic inhibitors but their efficacy was affected to various degrees by several different factors. For example, the detoxification enzymes were categorised into esterases, GSTs and cytochrome P450 monooxygenases and the extent to which selected botanicals inhibited their activity appeared to differ depending on the group. For example, 1% rosemary oil significantly inhibited both esterases and P450s extracted from *P. chrysocephala* but had no substantial effect on GST from the same species. These assays also demonstrated that dose may well a significant influence on how effective these botanicals are at inhibiting those enzymes, with this also differing between products. It was commonly observed that activity would reduce as concentrations of the botanical increased, but there were also exceptions, such as where activity of *D. radicum* esterase was significantly reduced by 0.006% orange oil but not by 0.06% or 0.6%. There was also significant evidence to suggest that different botanicals exerted varying levels of enzyme inhibition depending on the species of insect – an encouraging result when species-specific effects targeting selected crop pests and maintaining beneficial insect populations would be a highly prized outcome of chemical

control. Not only that, but these factors also appeared to interact. Geraniol, for example, appeared to significantly increase activity of *D. radicum* esterase rather than inhibit it, but only when applied at the highest and intermediate concentrations and this was not observed in the other enzyme groups or species. A similar observation was made when *M. persicae* GST was treated with eugenol, but only at the lowest dose in that enzyme group and species.

The bioassays generated equally variable results in that some botanicals appeared to synergise the pesticide they were combined with, and to differing extents depending on the botanical and the species of insect. For example, neem oil significantly raised mortality of *P. chrysocephala* to 86% when combined with cypermethrin, compared to the separate treatments. Perhaps more importantly, this effect was maintained and achieved almost the same mortality rate even when the combined pesticide dose was reduced by 90%. The same oil, however, did not achieve synergism at all in *M. persicae* as it appeared to have stronger toxic effect when applied separately in this species. Other products tested in the bioassays, rather than being synergistic or simply having an additive effect when combined with the insecticide, they appeared to demonstrate antagonism where the mortality rate was lower with treatment than the controls. It is clear from these results that similar factors to those causing variation in the enzyme assay data, such as chemical structure and mode of action, may be influencing the ability of certain botanicals to perform as effective synergists.

This appeared to be confirmed, at least in part, by the results of the correlation analyses indicating that there was no significant relationship between enzyme activity and mortality in any of the combinations tested. In some cases, such as with GST from *D. radicum, M. persicae* and *P. xylostella,* the flat graph lines show that there was no association between the two variables at all. This would suggest that factors other than enzyme inhibition are the source of any apparent synergism observed in the corresponding bioassays. Other correlations, however, such as with esterase extracted from *P. chrysocephala, D. radicum* and *M. persicae*, the slopes generated appear to indicate that a negative relationship may exist in those circumstances. It is likely then that metabolic inhibition may be responsible for part of, but not all, synergism subsequently observed in the bioassays, and that the variation observed between botanicals and species may again be related to a complex network of other interacting effects.

In terms of the advancement of research into botanicals as synergists for application in the field, the results of the correlation suggests that although initial screening of botanical

products using enzyme inhibition assays may help to flag potential synergists and direct further investigation, this strategy should be used in combination with other tests such as combination bioassays. As has been shown in the current study, a botanical may be a potent enzyme inhibitor but this may not translate to mortality in the bioassays, and vice versa. Just using one method of screening for promising synergists would omit potentially useful compounds for control of pests in the future. Additionally, if a selected botanical synergist is primarily effective because it does inhibit the detoxification enzymes responsible for the metabolism of the insecticide with little effect elsewhere in the insect, it may have limited efficacy when applied to insects with alternative modes of resistance. Target site insensitivity, for example may not be mitigated or affected at all by the synergist so alternatives would have to be explored depending on the target insect. However, as demonstrated in the bioassay, some botanicals may have a synergistic effect without showing strong inhibitory characteristics in the enzyme assays so these may still hold promise.

Given the combination of results obtained from this study, it seems likely that the use of botanicals may indeed present promising opportunities for novel crop protection. However, the variation in outcome caused by factors such as dose, insect species, the individual botanical components and their interactions mean that transferring this to a larger-scale field-application is a multifaceted challenge, made more difficult by the additional environmental influences and practicalities of doing so. To start, a formulation containing one or more botanicals would ideally be designed to have maximum effect on the targeted species and less so on others – with the selection of botanical components, their proportions within the solution and effective delivery of both synergist and pesticidal ingredients steered towards this goal.

An investigation into how much of a botanical or compound actually reaches the internal structures of an insect after cuticular exposure may help to direct the external dose required to achieve metabolic synergy when combined with a pesticide. Initially, a factor of ten difference between concentrations of botanicals used in the current study's enzyme activity assays was used to identify an approximate dose of each botanical required to achieve significant inhibition in each species and allows for further refinement. Data from the current study suggests that the dose at which enzyme inhibition reached statistically significant levels for each botanical assessed for each species and enzyme group lies between the final concentrations tested. These assays could be repeated using reduced concentration intervals

to more accurately identify the optimal dose to ensure the desired inhibitory effect. This information could then be used to determine a target concentration for each botanical, relative to insect species and enzyme group for use in further tests. Previous studies have shown that both enzyme inhibition and internal product detection can be attained after externally treating a live insect with a botanical (Liao, *et al.,* 2016; Rizvi *et al.,* 2018). These methods could be applied with the insects used in the current study to identify the extent of cuticular penetration by each compound, enzyme inhibition levels from cuticular exposure and match these to the exact external dose of each required to achieve the optimum levels identified in previous enzyme inhibition tests. The individual components of the most promising botanicals could also be tested in this way, with various combinations and proportions of each, in order to identify the optimal cocktail for the selected species. Further additions to the formulation could ensure that more than one enzyme group is affected and perhaps more than one site of action targeted to maximise efficacy in pests exhibiting multiple resistance processes and slow the speed with which they may develop future resistance mechanisms.

Few studies have been conducted into how quickly a pest may develop resistance to natural plant products but we know from studying life-history and evolution of herbivorous insects that they can overcome a plant's defences to continue feeding on them (Rane et al., 2019; Volonté et al., 2022). It stands to reason then that repeated and consistent exposure to botanicals will cause insects to develop resistance to them in much the same way that they have to pesticides. Indeed, Feng and Isman (1995) demonstrated that exposing aphids to purified azadirachtin resulted in a 9-fold increase in resistance after 40 generations. In contrast, the same study demonstrated that after exposure to neem oil as a raw collection of its various components, aphids showed no resistance to its effects after the same number of generations. This suggests that the 'cocktail' approach to the formulation, mixing a selection of effective components from either the same or different botanical extracts would help to ensure that the possibility of resistance development by the pests to the botanical is kept to a minimum. A similar approach is currently recommended for the use of pesticides in that, where possible, there should be alternation or rotation of compounds with different modes of action (IRAC, 2024c).

Refinement of an effective synergist/pesticide formulation combining all of these approaches for a specific insect pest would involve a complex set of studies and a significant effort, but

such work would likely yield significant returns. It would, for example, refine the doses of each component required for each species, reducing waste and needless overexposure to chemicals, as well as provide a multi-faceted approach that would confound the ability of even the most adaptive and plastic species to develop resistance. Despite this, adaptation and development of resistance to natural products is not outside the realms of possibility. Afterall, as previously discussed, many herbivorous insects have already developed strategies to mitigate their host plant defence systems, including secondary metabolites. A successful strategy in reducing opportunity for resistance development in insects is already deployed in crop protection with growers being advised to rotate the various modes of action (where possible) of their selected pesticides. A similar approach should be adopted if botanical compounds are combined and applied either as synergists or standalone insecticides. Furthermore, review of the formulations for registration would explore the effects on beneficial species and the environment, with revisions and recommendations to reduce both. However, the approval and registration for novel active ingredients and/or products can take up to 10-12 years (Sparks, 2013) and a speedier option may be available for more rapid control where pest pressure and risk of crop loss is highest, in oilseed rape crops for example.

Several biopesticides identifying at least one of the botanicals tested in the current study as the active ingredient are available on either or both of the EU and UK markets – but with restrictions for use only under controlled glasshouse conditions and/or specified crops (Liaison, 2024). Additional investigations pairing the use of these products with conventional chemical insecticides in bioassays would be required to confirm, but the current study suggests that these biopesticides would act as synergists in the same way as the raw botanicals, thereby enhancing the insecticidal effects, restoring control of previously resistant pest species and lowering the required dose of pesticidal ingredient in the application. Furthermore, due to these formulations being designed as standalone biopesticides, they may contain adjuvant substances that heighten the insecticidal efficacy and synergism more so than even the raw botanicals, therefore potentially lowering the required dosage of both applications significantly compared to when they are used separately. In order to legitimately and safely use these biopesticides in a combined application to combat resistant and high-impact crop pests, such as *P. chrysocephala* in oilseed rape, they would need to have a label extension approval granted. If these product manufacturers have previously collected data on aspects such as ecotoxicology and environmental fate, this approval may be conferred within

six months to a year, possibly longer depending on whether new studies are required – but still presents a far more rapid and practical route than novel product development (Health and Safety Executive, no date).

A drawback to this 'ready-made' approach is that maximising the synergistic effects of the biopesticide in a field setting would require a time delay between product applications. Studies have shown that the effects of metabolic synergy are optimised when there is a temporal lag between synergist and insecticide application, thereby allowing time for the synergist to be absorbed and inhibit the enzymes before the toxic ingredient is introduced (Moores, Bingham and Gunning, 2005; Bingham *et al.,* 2007*;* 2008, Young, Gunning and Moores, 2006). Indeed, Young, Gunning and Moores (2005) showed that PBO was able to reach maximum esterase inhibition in *H. armigera* (cotton bollworm) three to four hours after topical exposure and that this corresponded to maximum mortality rates in subsequent bioassays. Herron *et al.* (2014) also demonstrated that a four hour delay between exposure to PBO and Spinosad in Western flower thrips (*Frankliniella occidentalis*) was sufficient to reduce resistance from 577 to 72-fold. Although, Joffe (2011) found that there was no significant difference in mortality between pre-treatment times ranging from 30 minutes to 8 hours when *H. armigera* larvae were exposed to PBO and pyrethrum. This variation in temporal synergism may be dependent on the dose of the synergist, as this differed between studies, and may be related to other factors such as how quickly and/or easily the synergist is absorbed through the insect cuticle (Joffe, 2011).

Given this, investigations into the optimal pre-treatment times for each product would need to be carried out in order to maximise their synergistic capabilities for field applications in combination with a conventional pesticide. An alternative and longer-term option to consider for development of a new product would be to ensure that the botanical synergist is freely available in the formulation for immediate exposure and the pesticidal ingredient microencapsulated for a slow-release delivery. It would also be possible to include additional encapsulated botanicals in such a formulation, which would serve to protect them from UV degradation and provide a longer-lasting exposure system (Moores *et al.,* 2005; Makame *et al.,* 2023).

Regardless of whether a new or existing product is made available, its application would need to be included as part of an integrated pest management plan, combining pest management approaches such as monitoring, use of chemistry and promotion of beneficial insects to deliver

overall satisfactory control of target pests. As has been noted in this study, the efficacy of a combination of approaches can produce a significantly enhanced result when compared to the summary effects of its individual components, with IPM itself now embedded within agricultural policy in many countries (European Commission, no date; UK Government, 2024a; 2024b). One of the concerns over use of chemical pest control is the detrimental effects that have been observed on beneficial species which may compromise IPM (European Commission, no date). In the event that a botanical synergist could be applied prior to a conventional pesticide as part of a management strategy in a crop, the required dosage of the insecticidal ingredient to achieve sufficient pest control is likely to be drastically reduced. This may then reduce the detrimental impact that the chemical control has on beneficial insects and the wider environment – particularly if the botanical was selected with consideration of further investigations into the apparent differentiation seen between insect orders.

The present study also demonstrated that a 90% reduction in the field recommended dose of pesticide applied to *P. chrysocephala* pre-treated with neem oil was sufficient to achieve more than 75% mortality. This was in a laboratory setting with the necessary limitations but if these results could be replicated and confirmed using a botanical biopesticide in place of the raw essential oil, this could theoretically lead to a rapid and significant dilution of the applied rate of insecticides currently used in oilseed rape crops. Following validation, approval of product usage and national/international adoption of this strategy, this approach could be applied to a range of crops and resistant pest species to combat the growing pandemic of insecticide resistance and subsequent crop loss. Within the European Union if the application of chemical pesticide was reduced to even 50% of current rates, member states could immediately reach the target of the European Green Deal. Not only this, if fewer applications of chemical pesticide were required each season, the cost of widespread crop protection could also drop considerably, it would address both environmental and health concerns of pesticide use and perhaps work favourably with natural predators and plant defences.

Correct formulation and use of botanical products has the potential to even enhance the crops natural defences in a field setting. It is known, for example, that the release of plant metabolites caused by damage from herbivorous insects can attract both predatory species and parasitoids (Turlings and Wäckers, 2004), encourage attendance by pollinators and deter further feeding by herbivorous insects (Sosenski and Parra-Tabla, 2019). Use of these metabolites has subsequently been explored for utility in IPM systems where Farid *et al.*

(2019) showed that the spider (*Pardosa pseudoannulata*) was attracted by a combination of black pepper (*Piper nigrum*) and may chang (*Litsea cubeba*) oils. However, they also observed that there was a reduction in predatory behaviour by the spiders with increasing concentrations of plant extracts – again highlighting the importance of dose in application and need for conscientious testing before use of botanicals. Evidence also suggests that, even where the exposure level is not fatal to an individual, the depressive effects of the selected combination and dose may reduce the frequency of insect feeding, mating and egg-laying of crop pests (Jankowska *et al.,* 2017). This may not only benefit the crop in the season the formulation is applied but could even reduce pest pressure in the following season by reducing the number emerging in the next generation. Plant extracts have also been found to deter oviposition and even have ovicidal activity (Jeyasankar *et al.,* 2013), thus providing both short- and long-term crop protection. The inclusion of selected botanical extracts in a given synergist-pesticide formulation could thus further enhance crop health, beyond that of direct insecticidal effects.

This study has shown that there is significant promise in the use of botanicals as synergists against insect pests in both susceptible and metabolically resistant species. Nevertheless, there is a vast body of evidence in the literature suggesting that whilst a variety of botanicals have potential as both insecticides and synergists, they show varied efficacy depending on species (Isman, 2000; 2006; 2020). There is also a concern with botanical extracts that their composition is affected by a number of factors such as the geographical distribution of source plants, processing methods, climatic and seasonal conditions, harvesting period and genetics of the plants involved (Lammerink, Wallace and Porter, 1989; Baser *et al.,* 1997; Sefidkon, Abbasi and Khaniki, 2005; Kara and Baydar, 2011; Cannon *et al.,* 2013). Given that, as discussed in Chapters 3 and 4, the relative proportions of botanical constituents in a treatment may have a significant effect on enzyme inhibition, cuticle penetration and synergistic success when combined with a pesticide, their source and consistency should be an important consideration.

Added to this is the fact that many botanical compounds are rapidly biodegradable – both an asset and a problem when a biopesticide is considered. The biodegradable nature of botanicals is a benefit as it means the residue and longevity of the substances in the environment, and the possible negative impacts of that, are greatly reduced. However, it also means that the product may have to be applied repeatedly and under dry conditions to ensure

it continues to control pests through the season and not be washed off or degraded too quickly. Despite this, the inclusion of botanicals as active ingredients in biopesticides such as orange oil in PREV-AM (RovensaNext, no date) and garlic oil in Nemguard SC (Ecospray, no date) suggest that these issues can be managed effectively to provide a consistent product.

Finally, while some botanicals may be produced as side-products of an existing industry (e.g. orange oil) others, such as neem oil, would require dedicated production. The availability of the source plant of a botanical would also limit whether or not it would be a commercially viable option. Some plants naturally produce more essential oil per unit of biomass so this would also affect supply. There are also various methods of extraction (e.g. cold-pressing, hydrodistillation, steam distillation etc.) and some are more suited to certain plants than others to achieve sufficient yield. However, they do also have various benefits and drawbacks so some essential oils may be significantly more cost-effective and have better environmental credentials than others, which would also affect their viability as a synergist (Chakravarty, Parmar and Mandavgane, 2021).

One of the other difficulties in developing effective products lies in teasing out exactly what combination of botanical compounds will have the most effective impact for the selected pest, how to enhance their delivery to ensure sufficient exposure and how all of this may translate to an agricultural-scale field application. In an ideal setting, the selected insect pest and crop would have a bespoke treatment programme, including a specifically designed botanical/pesticide formulation that would have as little residual effect on the environment and beneficial species as possible. Despite the success of this study and excitement that these results may elicit from the industry, care must be taken to highlight that the application of this knowledge in the field would be a significant challenge and any adoption of botanical treatments should only be as part of an optimised integrated pest management strategy. Chemical pest control remains the final option for use when all other preventative tactics have been explored and the pest pressure is still high enough to cause significant or total yield loss. Given the limited chemical pesticide options available now in the UK and the rising levels of resistance seen in high-impact pest species, such as the cabbage stem flea beetle, botanical synergists may nevertheless provide an effective and environmentally friendlier option for supporting enhanced use of synthetic chemistry for pest control in future years, ensuring that remaining active ingredients are retained in the IPM toolbox for as long as possible.

Appendix A

A.1 Esterase activity: Linear mixed effects model results

A.1.1 Psylliodes chrysocephala

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: CSFB10lm

REML criterion at convergence: -243.6
Scaled residuals:

Min	1Q	Median	3Q	Max
-3.10084	-0.37602	-0.03211	0.44565	2.60357

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0013399	0.03661	0.63408	0.013833	7.00000	45.8370	6.15E-10
Residual		0.0005729	0.02394					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Psylliodes chrysocephala* esterase assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.32451	0.019002	59.10751	-17.0775	1.10E-23
Black-pepper	-0.41118	0.019002	59.10751	-21.6384	1.16E-28
Borage	-0.19517	0.019002	59.10751	-10.2711	3.66E-14
D-limonene	-0.34684	0.019002	59.10751	-18.2526	4.57E-25
Eugenol	-0.42402	0.019002	59.10751	-22.3141	2.46E-29
FLiPPER 0.96%	-0.26590	0.019002	59.10751	-13.9930	1.02E-19
Garlic	-0.39655	0.019002	59.10751	-20.8688	6.54E-28
Geraniol	-0.43335	0.019002	59.10751	-22.8053	8.32E-30
Lemongrass	-0.49517	0.019002	59.10751	-26.0587	8.03E-33
Neem	-0.39389	0.019002	59.10751	-20.7284	8.45E-28
Orange	-0.29128	0.019002	59.10751	-15.3288	1.76E-21
Parsley-seed	-0.47444	0.019002	59.10751	-24.9674	7.32E-32
PBO 0.06%	-0.18014	0.019002	59.10751	-9.47971	5.39E-13
PREV-AM 0.48%	-0.46890	0.019002	59.10751	-24.6760	1.30E-31
Rapeseed	-0.16518	0.019002	59.10751	-8.69247	7.35E-12
Rosemary	-0.40428	0.019002	59.10751	-21.2755	2.60E-28
Sesame	-0.11968	0.019002	59.10751	-6.2984	4.09E-08
Tea-tree	-0.38361	0.019002	59.10751	-20.1879	3.00E-27
Thymol	-0.48822	0.019002	59.10751	-25.6928	1.64E-32

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: CSFB1lm

REML criterion at convergence: -224.2

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.58869 -0.63988 0.08144 0.63988 2.22007

Random effects:
 Groups   Name   Variance Std.Dev. Estimate Std. Error df   t value   Pr(>|t|)
 Plate   (Intercept) 0.0012573 0.03546 0.63408   0.013833   7.00000 45.83699 6.15E-10
 Residual   0.0008209 0.02865
Number of obs: 81, groups: Plate, 8

Table 2. Linear mixed model results for *Psylliodes chrysocephala* esterase assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.30440	0.022460	60.46707	-13.5526	6.65E-19
Black-pepper	-0.35906	0.022460	60.46707	-15.9865	3.28E-22
Borage	-0.13030	0.022460	60.46707	-5.80144	7.72E-07
D-limonene	-0.26897	0.022460	60.46707	-11.9753	1.20E-16
Eugenol	-0.26539	0.022460	60.46707	-11.8160	1.89E-16
FLiPPER 0.096%	-0.20207	0.022460	60.46707	-8.99655	5.61E-12
Garlic	-0.37617	0.022460	60.46707	-16.7481	3.71E-23
Geraniol	-0.32206	0.022460	60.46707	-14.3389	5.28E-20
Lemongrass	-0.36664	0.022460	60.46707	-16.3236	1.25E-22
Neem	-0.29750	0.022460	60.46707	-13.2456	1.73E-18
Orange	-0.22406	0.022460	60.46707	-9.97595	1.51E-13
Parsley-seed	-0.38675	0.022460	60.46707	-17.2191	9.78E-24
PBO 0.006%	-0.14086	0.022460	60.46707	-6.27148	1.68E-07
PREV-AM 0.048%	-0.19640	0.022460	60.46707	-8.74426	1.25E-11
Rapeseed	-0.11906	0.022460	60.46707	-5.30101	3.42E-06
Rosemary	-0.39006	0.022460	60.46707	-17.3667	6.71E-24
Sesame	-0.08306	0.022460	60.46707	-3.69799	0.000471
Tea-tree	-0.29073	0.022460	60.46707	-12.9441	4.42E-18
Thymol	-0.35650	0.022460	60.46707	-15.8725	4.35E-22

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: CSFB0.1lm

REML criterion at convergence: -219.5

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.47813 -0.67951 0.04765 0.59039 2.13866

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0012324	0.03511	0.63408	0.013833	7.00000	45.83699	6.15E-10
Residual		0.0008954	0.02992					

Number of obs: 81, groups: Plate, 8

Table 3. Linear mixed model results for *Psylliodes chrysocephala* esterase assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.09546	0.023367	60.7236	-4.08532	0.00131
Black-pepper	-0.17413	0.023367	60.7236	-7.45186	6.74E-09
Borage	-0.08621	0.023367	60.7236	-3.68931	0.004343
D-limonene	-0.16021	0.023367	60.7236	-6.85613	6.26E-08
Eugenol	-0.03134	0.023367	60.7236	-1.34107	0.376036
FLiPPER 0.0096%	-0.05098	0.023367	60.7236	-2.18181	0.132016
Garlic	-0.14315	0.023367	60.7236	-6.12629	1.02E-06
Geraniol	-0.07367	0.023367	60.7236	-3.15272	0.020108
Lemongrass	-0.18021	0.023367	60.7236	-7.71203	2.55E-09
Neem	-0.13515	0.023367	60.7236	-5.78393	2.99E-06
Orange	-0.05807	0.023367	60.7236	-2.48503	0.094353
Parsley-seed	-0.13757	0.023367	60.7236	-5.88747	2.38E-06
PBO 0.0006%	-0.05321	0.023367	60.7236	-2.27717	0.131558
PREV-AM 0.0048 %	-0.03632	0.023367	60.7236	-1.55415	0.376036
Rapeseed	-0.06046	0.023367	60.7236	-2.5875	0.08454
Rosemary	-0.20840	0.023367	60.7236	-8.91853	2.32E-11
Sesame	-0.02867	0.023367	60.7236	-1.22695	0.376036
Tea-tree	-0.17240	0.023367	60.7236	-7.37791	8.50E-09
Thymol	-0.13615	0.023367	60.7236	-5.82673	2.77E-06

1.1.2 Delia radicum

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: CRF10lm

REML criterion at convergence: -312.6

Scaled residuals:
    Min   1Q Median   3Q   Max
-1.76642 -0.39615 -0.09548 0.35806 3.11423

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0002627	0.01621	0.162125	0.0064	7.00000	25.332	3.81E-08
Residual		0.0001950	0.01396					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed model results for *Delia radicum* esterase assay with synergists at 0.6% unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.02171	0.010896	60.76731	-1.99263	0.304795
Black-pepper	-0.02004	0.010896	60.76731	-1.83966	0.353518
Borage	-0.0196	0.010896	60.76731	-1.79912	0.353518
D-limonene	-0.0266	0.010896	60.76731	-2.44157	0.122854
Eugenol	-0.14071	0.010896	60.76731	-12.9145	8.51E-18
FLiPPER 0.96%	-0.05481	0.010896	60.76731	-5.03014	6.02E-05
Garlic	-0.01623	0.010896	60.76731	-1.48969	0.353518
Geraniol	0.04662	0.010896	60.76731	4.278725	0.000745
Lemongrass	-0.02894	0.010896	60.76731	-2.65572	0.100922
Neem	-0.09623	0.010896	60.76731	-8.83198	2.90E-11
Orange	-0.02787	0.010896	60.76731	-2.55744	0.11751
Parsley-seed	-0.08056	0.010896	60.76731	-7.39337	7.96E-09
PBO 0.06%	-0.05218	0.010896	60.76731	-4.7889	0.000134
PREV-AM 0.48 %	-0.07914	0.010896	60.76731	-7.26342	1.25E-08
Rapeseed	-0.00804	0.010896	60.76731	-0.73832	0.463163
Rosemary	-0.01887	0.010896	60.76731	-1.73143	0.353518
Sesame	-0.02771	0.010896	60.76731	-2.54349	0.11751
Tea-tree	-0.07287	0.010896	60.76731	-6.68748	1.13E-07
Thymol	-0.13123	0.010896	60.76731	-12.0442	1.73E-16

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: CRF1lm

REML criterion at convergence: -333.4

Scaled residuals:
   Min   1Q Median   3Q   Max
-1.6754 -0.3915 -0.0579 0.3761 3.7051

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0002834	0.01684	0.162125	0.0064	7.00000	25.332	3.81E-08
Residual		0.0001328	0.01152					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed model results for *Delia radicum* esterase assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.00456	0.009126	59.42134	-0.49917	1
Black-pepper	0.002444	0.009126	59.42134	0.267854	1
Borage	-0.02033	0.009126	59.42134	-2.2271	0.297414
D-limonene	-0.01533	0.009126	59.42134	-1.67923	0.885227
Eugenol	-0.07955	0.009126	59.42134	-8.71657	5.78E-11
FLiPPER 0.096 %	-0.02412	0.009126	59.42134	-2.64341	0.115274
Garlic	-0.04648	0.009126	59.42134	-5.09265	6.13E-05
Geraniol	0.042784	0.009126	59.42134	4.688038	0.000232
Lemongrass	-0.00933	0.009126	59.42134	-1.02178	1
Neem	-0.06414	0.009126	59.42134	-7.02847	3.98E-08
Orange	-0.01151	0.009126	59.42134	-1.26135	1
Parsley-seed	-0.03282	0.009126	59.42134	-3.59608	0.007897
PBO 0.006%	-0.04464	0.009126	59.42134	-4.89133	0.00012
PREV-AM 0.04 8%	-0.04046	0.009126	59.42134	-4.43312	0.000529
Rapeseed	-0.00489	0.009126	59.42134	-0.53569	1
Rosemary	-0.01151	0.009126	59.42134	-1.26135	1
Sesame	-0.01322	0.009126	59.42134	-1.44813	1
Tea-tree	0.000155	0.009126	59.42134	0.017023	1
Thymol	-0.10848	0.009126	59.42134	-11.8863	4.57E-16

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: CRF0.1lm

REML criterion at convergence: -325.2

Scaled residuals:
   Min   1Q Median   3Q   Max
-1.8247 -0.3757 -0.0537 0.4293 3.4590

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0002762	0.01662	0.162125	0.0064	7.00000	25.332	3.81E-08
Residual		0.0001543	0.01242					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed model results for *Delia radicum* esterase assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.00519	0.009788	60.00629	-0.52984	1
Black-pepper	0.008814	0.009788	60.00629	0.900466	1
Borage	-0.01573	0.009788	60.00629	-1.60695	1
D-limonene	-0.01273	0.009788	60.00629	-1.30046	1
Eugenol	-0.04759	0.009788	60.00629	-4.86235	0.000157
FLiPPER 0.0096 %	-0.00136	0.009788	60.00629	-0.13901	1
Garlic	-0.01873	0.009788	60.00629	-1.91304	0.726222
Geraniol	-0.00759	0.009788	60.00629	-0.77577	1
Lemongrass	-0.00673	0.009788	60.00629	-0.68747	1
Neem	-0.02806	0.009788	60.00629	-2.86657	0.080012
Orange	-0.0442	0.009788	60.00629	-4.51545	0.000482
Parsley-seed	-0.02051	0.009788	60.00629	-2.09539	0.524742
PBO 0.0006%	-0.04603	0.009788	60.00629	-4.70277	0.000263
PREV-AM 0.004 8%	-0.03836	0.009788	60.00629	-3.91909	0.003458
Rapeseed	0.007147	0.009788	60.00629	0.730192	1
Rosemary	-0.01386	0.009788	60.00629	-1.41646	1
Sesame	-0.00493	0.009788	60.00629	-0.50333	1
Tea-tree	0.002469	0.009788	60.00629	0.252229	1
Thymol	-0.06806	0.009788	60.00629	-6.95315	5.71E-08

1.1.3 Myzus persicae

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: MP10lm

REML criterion at convergence: -309.4

Scaled residuals:
    Min   1Q Median   3Q   Max
-1.75942 -0.49359 -0.05752 0.54843 2.24533

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0002711	0.01646	0.167500	0.0065	7.00000	25.947	3.23E-08
Residual		0.0001870	0.01368					

Number of obs: 80, groups: Plate, 8

Table 1. Linear mixed model results for *Myzus persicae* esterase assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.01806	0.010699	59.56055	-1.68774	0.544676
Black-pepper	-0.02406	0.010699	59.56055	-2.24852	0.19779
Borage	-0.01579	0.010699	59.56055	-1.47553	0.581344
D-limonene	-0.04345	0.010699	59.56055	-4.06134	0.001735
Eugenol	-0.07906	0.010699	59.56055	-7.38923	9.57E-09
FLiPPER 0.96%	-0.03804	0.010699	59.56055	-3.55502	0.007477
Garlic	-0.02596	0.010699	59.56055	-2.42639	0.14636
Geraniol	-0.01839	0.010699	59.56055	-1.71916	0.544676
Lemongrass	-0.07812	0.010699	59.56055	-7.30138	1.27E-08
Neem	-0.03863	0.010699	59.56055	-3.61025	0.006913
Orange	-0.02757	0.010699	59.56055	-2.57707	0.112138
Parsley-seed	-0.0846	0.010699	59.56055	-7.90673	1.41E-09
PBO 0.06%	-0.00259	0.010699	59.56055	-0.24227	1
PREV-AM 0.48 %	-0.06154	0.012068	58.75966	-5.09898	4.97E-05
Rapeseed	-0.01272	0.010699	59.56055	-1.18928	0.717152
Rosemary	-0.07791	0.010699	59.56055	-7.28136	1.29E-08
Sesame	-0.00406	0.010699	59.56055	-0.37952	1
Tea-tree	-0.07057	0.010699	59.56055	-6.59597	1.76E-07
Thymol	-0.08263	0.010699	59.56055	-7.72261	2.74E-09

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: MP1lm

REML criterion at convergence: -332.5

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.12122 -0.48838 -0.05746 0.43092 2.57917

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0002885	0.01699	0.167500	0.0065	7.00000	25.947	3.23E-08
Residual		0.0001346	0.01160					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed model results for *Myzus persicae* esterase assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.01121	0.009191	59.40773	-1.2202	1
Black-pepper	-0.02288	0.009191	59.40773	-2.48961	0.140457
Borage	-0.00907	0.009191	59.40773	-0.98729	1
D-limonene	-0.03707	0.009191	59.40773	-4.03388	0.002542
Eugenol	-0.02716	0.009191	59.40773	-2.95553	0.058113
FLiPPER 0.096%	-0.00592	0.009191	59.40773	-0.64413	1
Garlic	-0.02995	0.009191	59.40773	-3.2583	0.025982
Geraniol	-0.00616	0.009191	59.40773	-0.6706	1
Lemongrass	-0.04407	0.009191	59.40773	-4.79552	0.000192
Neem	-0.01128	0.009191	59.40773	-1.22724	1
Orange	-0.02693	0.009191	59.40773	-2.93045	0.058113
Parsley-seed	-0.04832	0.009191	59.40773	-5.25795	3.96E-05
PBO 0.006%	-0.01276	0.009191	59.40773	-1.38833	1
PREV-AM 0.048 %	-0.03292	0.009191	59.40773	-3.58191	0.010323
Rapeseed	-0.02688	0.009191	59.40773	-2.92484	0.058113
Rosemary	-0.0456	0.009191	59.40773	-4.96151	0.000111
Sesame	-0.01416	0.009191	59.40773	-1.54105	0.900307
Tea-tree	-0.01527	0.009191	59.40773	-1.66104	0.815791
Thymol	-0.02495	0.009191	59.40773	-2.71427	0.086807

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: MP0.1lm

REML criterion at convergence: -324.6

Scaled residuals:
   Min   1Q Median   3Q   Max
-1.9286 -0.5073 0.0267 0.4743 2.4172

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0002814	0.01678	0.167500	0.0065	7.00000	25.947	3.23E-08
Residual		0.0001559	0.01248					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed model results for *Myzus persicae* esterase assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	0.001507	0.00984	59.97881	0.153128	1
Black-pepper	-0.01583	0.00984	59.97881	-1.60839	1
Borage	-0.00982	0.00984	59.97881	-0.99824	1
D-limonene	-0.02782	0.00984	59.97881	-2.82751	0.114605
Eugenol	-0.00466	0.00984	59.97881	-0.47379	1
FLiPPER 0.0096%	-0.00803	0.00984	59.97881	-0.8161	1
Garlic	-0.01208	0.00984	59.97881	-1.22746	1
Geraniol	0.000338	0.00984	59.97881	0.034338	1
Lemongrass	-0.02516	0.00984	59.97881	-2.55651	0.223085
Neem	0.008922	0.00984	59.97881	0.906693	1
Orange	-0.03259	0.00984	59.97881	-3.31187	0.029892
Parsley-seed	-0.01416	0.00984	59.97881	-1.43949	1
PBO 0.0006%	-0.01483	0.00984	59.97881	-1.50679	1
PREV-AM 0.0048 %	-0.0117	0.00984	59.97881	-1.18873	1
Rapeseed	-0.01983	0.00984	59.97881	-2.0149	0.726066
Rosemary	-0.02192	0.00984	59.97881	-2.22786	0.474436
Sesame	-0.00966	0.00984	59.97881	-0.98192	1
Tea-tree	-0.01926	0.00984	59.97881	-1.95686	0.770337
Thymol	0.000255	0.00984	59.97881	0.025932	1

1.1.4 Plutella xylostella

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: Px10lm

REML criterion at convergence: -236.3

Scaled residuals:
   Min   1Q Median   3Q   Max
-2.9114 -0.2516 0.0074 0.1672 4.5042

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0015433	0.03929	0.26077	0.01476	7.12716	17.666	3.80E-07
Residual		0.0005072	0.02252					

Number of obs: 78, groups: Plate, 8

Table 1. Linear mixed model results for *Plutella xylostella* esterase assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.07923	0.017987	55.14266	-4.40494	0.000344
Black-pepper	-0.16357	0.017987	55.14266	-9.09346	2.20E-11
Borage	-0.11654	0.017987	55.14266	-6.47932	2.92E-07
D-limonene	-0.19188	0.017987	55.14266	-10.6675	9.13E-14
Eugenol	-0.13212	0.017987	55.14266	-7.34545	1.31E-08
FLiPPER 0.96%	-0.10776	0.019778	57.05591	-5.44824	1.01E-05
Garlic	-0.05395	0.017987	55.14266	-2.99924	0.02028
Geraniol	-0.03512	0.017987	55.14266	-1.95273	0.178415
Lemongrass	-0.16621	0.017987	55.14266	-9.24054	1.36E-11
Neem	-0.20595	0.017987	55.14266	-11.4497	6.42E-15
Orange	-0.0181	0.017987	55.14266	-1.00625	0.491645
Parsley-seed	-0.1492	0.019778	57.05591	-7.54364	5.50E-09
PBO 0.06%	-0.04062	0.019778	57.05591	-2.05364	0.178415
PREV-AM 0.48%	-0.09276	0.019778	57.05591	-4.68984	0.00014
Rapeseed	-0.11057	0.017987	55.14266	-6.14692	9.23E-07
Rosemary	-0.0211	0.017987	55.14266	-1.17304	0.491645
Sesame	-0.07412	0.017987	55.14266	-4.12094	0.000767
Tea-tree	-0.12943	0.017987	55.14266	-7.19584	2.13E-08
Thymol	-0.18328	0.017987	55.14266	-10.1895	4.68E-13

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
  Data: Px1lm

REML criterion at convergence: -231.2

Scaled residuals:
   Min   1Q Median   3Q   Max
-2.7757 -0.2114 -0.0211 0.1409 4.2844

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0015261	0.03907	0.260756	0.014775	7.139475	17.648	3.76E-07
Residual		0.0005595	0.02365					

Number of obs: 78, groups: Plate, 8

Table 1. Linear mixed model results for *Plutella xylostella* esterase assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.04466	0.018849	55.50132	-2.36916	0.2247
Black-pepper	-0.04499	0.018849	55.50132	-2.38685	0.2247
Borage	-0.01774	0.018849	55.50132	-0.94117	1
D-limonene	0.001927	0.018849	55.50132	0.102213	1
Eugenol	-0.12488	0.018849	55.50132	-6.6253	2.53E-07
FLiPPER 0.096%	-0.08158	0.020692	57.38204	-3.94244	0.003548
Garlic	-0.05251	0.018849	55.50132	-2.78605	0.08745
Geraniol	-0.03088	0.018849	55.50132	-1.63831	0.856124
Lemongrass	-0.06174	0.018849	55.50132	-3.2755	0.025489
Neem	-0.18118	0.018849	55.50132	-9.61222	3.91E-12
Orange	0.026215	0.018849	55.50132	1.390797	1
Parsley-seed	-0.0439	0.020692	57.38204	-2.12164	0.343784
PBO 0.006%	-0.03199	0.020692	57.38204	-1.54606	0.893118
PREV-AM 0.048%	-0.06324	0.020692	57.38204	-3.05643	0.044164
Rapeseed	-0.06499	0.018849	55.50132	-3.44791	0.016274
Rosemary	0.011215	0.018849	55.50132	0.595	1
Sesame	-0.01821	0.018849	55.50132	-0.9663	1
Tea-tree	0.010548	0.018849	55.50132	0.559631	1
Thymol	-0.15818	0.018849	55.50132	-8.392	3.38E-10

Lowest stock concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Max.rate ~ Treatment + (1 | Plate)
Data: Px0.1lm

REML criterion at convergence: -233.9

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.8468	-0.2567	0.0000	0.1844	4.3996

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0015355	0.03918	0.260766	0.014768	7.132811	17.658	3.78E-07
Residual		0.0005311	0.02305					

Number of obs: 78, groups: Plate, 8

Table 1. Linear mixed model results for *Plutella xylostella* esterase assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.05227	0.018387	55.30889	-2.84291	0.087519
Black-pepper	-0.02861	0.018387	55.30889	-1.5558	1
Borage	0.007823	0.018387	55.30889	0.425471	1
D-limonene	0.032823	0.018387	55.30889	1.78509	0.972508
Eugenol	-0.03265	0.018387	55.30889	-1.77572	0.972508
FLiPPER 0.0096%	-0.07483	0.020203	57.21332	-3.70373	0.008163
Garlic	-0.02405	0.018387	55.30889	-1.3082	1
Geraniol	-0.00698	0.018387	55.30889	-0.37984	1
Lemongrass	0.012823	0.018387	55.30889	0.697395	1
Neem	-0.16139	0.018387	55.30889	-8.77705	8.73E-11
Orange	0.016406	0.018387	55.30889	0.892236	1
Parsley-seed	0.00145	0.020203	57.21332	0.071777	1
PBO 0.0006%	-0.03666	0.020203	57.21332	-1.81473	0.972508
PREV-AM 0.0048 %	-0.02749	0.020203	57.21332	-1.36088	1
Rapeseed	-0.05427	0.018387	55.30889	-2.95168	0.069442
Rosemary	0.004739	0.018387	55.30889	0.257747	1
Sesame	-0.00698	0.018387	55.30889	-0.37984	1
Tea-tree	-0.06326	0.018387	55.30889	-3.44042	0.017794
Thymol	-0.07272	0.018387	55.30889	-3.95493	0.003958

A.2 GST activity: Linear mixed effects model results

A.2.1 Psylliodes chrysocephala

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: CBG10lm

REML criterion at convergence: 148.8

Scaled residuals:
   Min   1Q Median   3Q   Max
-2.5658 -0.3503 0.0000 0.4064 2.8150

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.7942	0.8912	15.3952	0.3379	7.0000	45.556	6.42E-10
Residual		0.3581	0.5984					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Psylliodes chrysocephala* GST assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-5.58734	0.47446	59.29048	-11.7762	1.47E-16
Black-pepper	-10.6859	0.47446	59.29048	-22.5222	8.54E-30
Borage	-7.30575	0.47446	59.29048	-15.398	1.54E-21
D-limonene	-11.6244	0.47446	59.29048	-24.5003	1.04E-31
Eugenol	-15.6367	0.47446	59.29048	-32.9568	1.51E-38
FLiPPER 0.96%	-12.0832	0.47446	59.29048	-25.4673	1.40E-32
Garlic	-12.2145	0.47446	59.29048	-25.7439	8.48E-33
Geraniol	-15.6367	0.47446	59.29048	-32.9568	1.51E-38
Lemongrass	-14.8431	0.47446	59.29048	-31.2843	2.37E-37
Neem	-14.6379	0.47446	59.29048	-30.8518	4.84E-37
Orange	-3.17981	0.47446	59.29048	-6.70195	1.69E-08
Parsley-seed	-12.3531	0.47446	59.29048	-26.0361	4.97E-33
PBO 0.06%	-2.71727	0.47446	59.29048	-5.72708	3.60E-07
PREV-AM 0.48 %	-15.0685	0.47446	59.29048	-31.7594	1.08E-37
Rapeseed	-7.04646	0.47446	59.29048	-14.8516	7.28E-21
Rosemary	-3.38106	0.47446	59.29048	-7.12614	4.85E-09
Sesame	-8.60102	0.47446	59.29048	-18.128	5.86E-25
Tea-tree	-6.6515	0.47446	59.29048	-14.0191	8.79E-20
Thymol	-13.2906	0.47446	59.29048	-28.0121	9.64E-35

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: CBG1lm

REML criterion at convergence: 168.2

Scaled residuals:
    Min   1Q Median   3Q   Max
-1.99641 -0.55007 0.02341 0.44474 2.49777

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.7425	0.8617	15.3952	0.3379	7.0000	45.556	6.42E-10
Residual		0.5134	0.7165					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Psylliodes chrysocephala* GST assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-2.1371	0.560545	60.61229	-3.81254	0.001298
Black-pepper	-2.79119	0.560545	60.61229	-4.97942	3.92E-05
Borage	-2.26808	0.560545	60.61229	-4.04621	0.000898
D-limonene	-3.4337	0.560545	60.61229	-6.12564	7.33E-07
Eugenol	-9.03812	0.560545	60.61229	-16.1238	2.29E-22
FLiPPER 0.096%	-8.98578	0.560545	60.61229	-16.0304	2.87E-22
Garlic	-13.3959	0.560545	60.61229	-23.8979	2.87E-31
Geraniol	-8.2834	0.560545	60.61229	-14.7774	1.30E-20
Lemongrass	-7.24083	0.560545	60.61229	-12.9175	5.57E-18
Neem	-7.89482	0.560545	60.61229	-14.0842	1.16E-19
Orange	-1.28731	0.560545	60.61229	-2.29652	0.075351
Parsley-seed	-3.13276	0.560545	60.61229	-5.58876	5.18E-06
PBO 0.006%	0.639135	0.560545	60.61229	1.140201	0.517376
PREV-AM 0.048 %	-8.80129	0.560545	60.61229	-15.7013	7.44E-22
Rapeseed	-2.20419	0.560545	60.61229	-3.93222	0.001095
Rosemary	-0.61645	0.560545	60.61229	-1.09972	0.517376
Sesame	-3.05069	0.560545	60.61229	-5.44237	8.01E-06
Tea-tree	-4.85125	0.560545	60.61229	-8.65451	3.84E-11
Thymol	-11.182	0.560545	60.61229	-19.9485	4.57E-27

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: CBG0.1lm

REML criterion at convergence: 152

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.46530 -0.43510 0.06798 0.43510 2.75592

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.7868	0.8870	15.3952	0.3379	7.0000	45.556	6.42E-10
Residual		0.3804	0.6167					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Psylliodes chrysocephala* GST assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	0.164609	0.488038	59.52828	0.337287	1
Black-pepper	-1.37837	0.488038	59.52828	-2.82431	0.070806
Borage	-0.11349	0.488038	59.52828	-0.23254	1
D-limonene	-0.49923	0.488038	59.52828	-1.02294	1
Eugenol	-2.38493	0.488038	59.52828	-4.88678	0.000129
FLiPPER 0.0096%	-3.69222	0.488038	59.52828	-7.56544	5.10E-09
Garlic	-10.9385	0.488038	59.52828	-22.4132	1.98E-29
Geraniol	-1.43734	0.488038	59.52828	-2.94515	0.059799
Lemongrass	-1.89965	0.488038	59.52828	-3.89243	0.0038
Neem	-1.4626	0.488038	59.52828	-2.99691	0.055625
Orange	0.986308	0.488038	59.52828	2.020967	0.43009
Parsley-seed	-1.10954	0.488038	59.52828	-2.27348	0.266191
PBO 0.0006%	0.525914	0.488038	59.52828	1.07761	1
PREV-AM 0.0048 %	-0.11989	0.488038	59.52828	-0.24566	1
Rapeseed	-1.39514	0.488038	59.52828	-2.85867	0.070264
Rosemary	0.508321	0.488038	59.52828	1.04156	1
Sesame	-0.70778	0.488038	59.52828	-1.45026	1
Tea-tree	-0.28833	0.488038	59.52828	-0.59078	1
Thymol	-2.86302	0.488038	59.52828	-5.8664	3.57E-06

A.2.2 Delia radicum

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: CFG10lm

REML criterion at convergence: 135.9

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.45619 -0.55964 -0.04145 0.44564 2.54256

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	1.8525	1.3611	9.1431	0.4922	7.0000	18.578	3.25E-07
Residual		0.2557	0.5057					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Delia radicum* GST assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-2.13789	0.408923	56.01044	-5.22811	7.89E-06
Black-pepper	-7.63056	0.408923	56.01044	-18.6601	1.57E-24
Borage	-2.46997	0.408923	56.01044	-6.04019	6.53E-07
D-limonene	-5.35257	0.408923	56.01044	-13.0894	1.33E-17
Eugenol	-9.1506	0.408923	56.01044	-22.3773	2.35E-28
FLiPPER 0.96%	-3.79865	0.408923	56.01044	-9.28941	4.92E-12
Garlic	-8.95034	0.408923	56.01044	-21.8876	6.76E-28
Geraniol	-8.94794	0.408923	56.01044	-21.8817	6.76E-28
Lemongrass	-3.79597	0.408923	56.01044	-9.28285	4.92E-12
Neem	-10.1261	0.408923	56.01044	-24.7628	1.42E-30
Orange	-1.40451	0.408923	56.01044	-3.43467	0.001124
Parsley-seed	-2.24346	0.408923	56.01044	-5.48627	4.10E-06
PBO 0.06%	-4.83933	0.408923	56.01044	-11.8343	7.21E-16
PREV-AM 0.48 %	-5.65924	0.408923	56.01044	-13.8394	1.32E-18
Rapeseed	-3.14942	0.408923	56.01044	-7.70176	1.44E-09
Rosemary	-1.74519	0.408923	56.01044	-4.26776	0.000154
Sesame	-4.76381	0.408923	56.01044	-11.6496	1.22E-15
Tea-tree	-5.31437	0.408923	56.01044	-12.996	1.66E-17
Thymol	-7.45138	0.408923	56.01044	-18.222	4.60E-24

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: CFG1lm

REML criterion at convergence: 83

Scaled residuals:
   Min   1Q Median   3Q   Max
-1.7669 -0.4771 -0.0422 0.4227 2.1644

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	1.90573	1.38	9.1431	0.4922	7.0000	18.578	3.25E-07
Residual		0.09607	0.31					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Delia radicum* GST assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.46758	0.252164	54.77251	-1.85429	0.069085
Black-pepper	-1.53677	0.252164	54.77251	-6.09432	8.04E-07
Borage	-2.78821	0.252164	54.77251	-11.0571	1.86E-14
D-limonene	-2.78821	0.252164	54.77251	-11.0571	1.86E-14
Eugenol	-6.67178	0.252164	54.77251	-26.4581	1.26E-31
FLiPPER 0.096%	-1.38647	0.252164	54.77251	-5.49828	6.21E-06
Garlic	-9.97713	0.252164	54.77251	-39.5661	1.07E-40
Geraniol	-3.35417	0.252164	54.77251	-13.3015	1.20E-17
Lemongrass	-2.40561	0.252164	54.77251	-9.53988	3.06E-12
Neem	-6.63856	0.252164	54.77251	-26.3264	1.54E-31
Orange	-0.60008	0.252164	54.77251	-2.37973	0.04167
Parsley-seed	-1.0156	0.252164	54.77251	-4.02754	0.0007
PBO 0.006%	-0.77239	0.252164	54.77251	-3.06306	0.010182
PREV-AM 0.048 %	-2.6915	0.252164	54.77251	-10.6736	5.95E-14
Rapeseed	-1.59966	0.252164	54.77251	-6.34373	3.62E-07
Rosemary	-1.31811	0.252164	54.77251	-5.22721	1.38E-05
Sesame	-2.01769	0.252164	54.77251	-8.00151	8.02E-10
Tea-tree	-6.05082	0.252164	54.77251	-23.9956	1.56E-29
Thymol	-5.4698	0.252164	54.77251	-21.6915	2.19E-27

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: CFG0.1lm

REML criterion at convergence: 99.2

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.34210 -0.46551 -0.02613 0.56734 2.06570

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	1.8945	1.3764	9.14308	0.49216	7.0000	18.578	3.25E-07
Residual		0.1298	0.3603					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Delia radicum* GST assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.23029	0.292729	55.03927	-0.7867	1
Black-pepper	-0.4609	0.292729	55.03927	-1.57449	0.587807
Borage	-0.85847	0.292729	55.03927	-2.93264	0.058677
D-limonene	-0.96329	0.292729	55.03927	-3.29073	0.022718
Eugenol	-1.45685	0.292729	55.03927	-4.97678	0.000107
FLiPPER 0.0096%	-1.20714	0.292729	55.03927	-4.12374	0.001903
Garlic	-8.7723	0.292729	55.03927	-29.9673	1.73E-34
Geraniol	-0.6602	0.292729	55.03927	-2.25534	0.224871
Lemongrass	-0.75889	0.292729	55.03927	-2.59246	0.13399
Neem	-2.23142	0.292729	55.03927	-7.62281	6.45E-09
Orange	-0.69717	0.292729	55.03927	-2.38164	0.186485
Parsley-seed	0.062358	0.292729	55.03927	0.213022	1
PBO 0.0006%	-0.19968	0.292729	55.03927	-0.68213	1
PREV-AM 0.0048 %	-0.48911	0.292729	55.03927	-1.67086	0.587807
Rapeseed	-1.09507	0.292729	55.03927	-3.7409	0.006146
Rosemary	-0.49277	0.292729	55.03927	-1.68337	0.587807
Sesame	-0.62351	0.292729	55.03927	-2.13001	0.263599
Tea-tree	-0.73386	0.292729	55.03927	-2.50697	0.15159
Thymol	-1.79117	0.292729	55.03927	-6.11885	1.75E-06

A.2.3 Myzus persicae

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: MPG10lm

REML criterion at convergence: -355.5

Scaled residuals:
   Min   1Q Median   3Q   Max
-2.6488 -0.3046 0.0000 0.5067 1.8008

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0004926	0.02220	0.135676	0.008064	7.0000	16.824	6.42E-07
Residual		0.0000830	0.00911					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Myzus persicae* GST assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.05224	0.007351	56.40813	-7.10596	1.56E-08
Black-pepper	-0.04817	0.007351	56.40813	-6.552	1.11E-07
Borage	-0.13315	0.007351	56.40813	-18.1117	5.67E-24
D-limonene	-0.09475	0.007351	56.40813	-12.8887	2.07E-17
Eugenol	-0.06997	0.007351	56.40813	-9.51839	1.97E-12
FLiPPER 0.96%	-0.13501	0.007351	56.40813	-18.3646	3.26E-24
Garlic	-0.11091	0.007351	56.40813	-15.0866	2.43E-20
Geraniol	0.020141	0.007351	56.40813	2.739757	0.02465
Lemongrass	-0.14885	0.007351	56.40813	-20.2484	2.85E-26
Neem	-0.12972	0.007351	56.40813	-17.6453	1.86E-23
Orange	-0.02764	0.007351	56.40813	-3.75955	0.001625
Parsley-seed	-0.10528	0.007351	56.40813	-14.3213	2.32E-19
PBO 0.06%	0.006938	0.007351	56.40813	0.943805	0.698589
PREV-AM 0.48 %	-0.13501	0.007351	56.40813	-18.3646	3.26E-24
Rapeseed	-0.079	0.007351	56.40813	-10.7462	2.88E-14
Rosemary	-0.02857	0.007351	56.40813	-3.88617	0.001351
Sesame	-0.07742	0.007351	56.40813	-10.5313	5.58E-14
Tea-tree	-0.00565	0.007351	56.40813	-0.76822	0.698589
Thymol	-0.11738	0.007351	56.40813	-15.9677	1.93E-21

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: MPG1lm

REML criterion at convergence: -379.5

Scaled residuals:
   Min   1Q Median   3Q   Max
-3.2054 -0.3827 -0.0121 0.4263 2.3438

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	5.025E-04	0.022417	0.1356764	0.0080645	7.0000	16.824	6.42E-07
Residual		5.325E-05	0.007298					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Myzus persicae* GST assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.01865	0.005914	55.57299	-3.15318	0.026041
Black-pepper	-0.01958	0.005914	55.57299	-3.31058	0.019674
Borage	-0.02944	0.005914	55.57299	-4.97825	8.54E-05
D-limonene	-0.01932	0.005914	55.57299	-3.26655	0.020552
Eugenol	0.005037	0.005914	55.57299	0.851741	1
FLiPPER 0.096%	-0.10998	0.005914	55.57299	-18.5967	2.52E-24
Garlic	-0.10482	0.005914	55.57299	-17.7239	2.34E-23
Geraniol	-0.00105	0.005914	55.57299	-0.1779	1
Lemongrass	-0.11484	0.005914	55.57299	-19.4195	3.31E-25
Neem	-0.12892	0.005914	55.57299	-21.7999	1.25E-27
Orange	-0.00886	0.005914	55.57299	-1.49798	0.838791
Parsley-seed	-0.01126	0.005914	55.57299	-1.90365	0.434975
PBO 0.006%	0.00042	0.005914	55.57299	0.071099	1
PREV-AM 0.048 %	-0.09322	0.005914	55.57299	-15.7636	5.09E-21
Rapeseed	-0.01865	0.005914	55.57299	-3.15318	0.026041
Rosemary	-0.0042	0.005914	55.57299	-0.71099	1
Sesame	0.007442	0.005914	55.57299	1.258353	1
Tea-tree	-0.01165	0.005914	55.57299	-1.97017	0.430476
Thymol	-0.12442	0.005914	55.57299	-21.0391	6.91E-27

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: MPG0.1lm

REML criterion at convergence: -369.2

Scaled residuals:
   Min   1Q Median   3Q   Max
-3.2485 -0.4061 0.0870 0.4061 1.7112

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	4.988E-04	0.022335	0.135676	0.008065	7.0000	16.824	6.42E-07
Residual		6.437E-05	0.008023					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Myzus persicae* GST assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.01146	0.006492	55.88943	-1.76601	0.667339
Black-pepper	-0.01181	0.006492	55.88943	-1.81979	0.667339
Borage	-0.02903	0.006492	55.88943	-4.47247	0.000499
D-limonene	-0.00285	0.006492	55.88943	-0.43963	1
Eugenol	0.047074	0.006492	55.88943	7.25164	2.15E-08
FLiPPER 0.0096%	-0.04389	0.006492	55.88943	-6.76175	1.30E-07
Garlic	-0.08892	0.006492	55.88943	-13.6979	3.14E-18
Geraniol	0.017753	0.006492	55.88943	2.734858	0.091804
Lemongrass	-0.03741	0.006492	55.88943	-5.76298	5.18E-06
Neem	-0.08752	0.006492	55.88943	-13.4828	5.89E-18
Orange	-0.00844	0.006492	55.88943	-1.30017	1
Parsley-seed	0.003893	0.006492	55.88943	0.59973	1
PBO 0.0006%	-0.00135	0.006492	55.88943	-0.20849	1
PREV-AM 0.0048 %	-0.00922	0.006492	55.88943	-1.42047	1
Rapeseed	-0.01426	0.006492	55.88943	-2.19618	0.322448
Rosemary	0.000519	0.006492	55.88943	0.079954	1
Sesame	0.018801	0.006492	55.88943	2.896172	0.064589
Tea-tree	-0.00867	0.006492	55.88943	-1.33602	1
Thymol	-0.05308	0.006492	55.88943	-8.17735	6.81E-10

A.2.4 Plutella xylostella

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: PXG10lm

REML criterion at convergence: -8.9

Scaled residuals:
   Min   1Q Median   3Q   Max
-2.7836 -0.4353 0.0000 0.6310 2.1079

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.14363	0.3790	4.2453	0.1377	7.0000	30.823	9.77E-09
Residual		0.02438	0.1561					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Plutella xylostella* GST assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-1.60838	0.125993	56.4245	-12.7657	1.68E-17
Black-pepper	-1.08428	0.125993	56.4245	-8.60584	1.48E-11
Borage	-1.69062	0.125993	56.4245	-13.4184	2.67E-18
D-limonene	-0.69271	0.125993	56.4245	-5.49804	9.63E-07
Eugenol	-3.29549	0.125993	56.4245	-26.1562	4.39E-32
FLiPPER 0.96%	-2.16542	0.125993	56.4245	-17.1868	4.33E-23
Garlic	-3.763	0.125993	56.4245	-29.8668	4.92E-35
Geraniol	-2.6414	0.125993	56.4245	-20.9647	2.88E-27
Lemongrass	-3.78496	0.125993	56.4245	-30.041	3.83E-35
Neem	-3.53519	0.125993	56.4245	-28.0586	1.26E-33
Orange	-1.35348	0.125993	56.4245	-10.7425	1.16E-14
Parsley-seed	-4.48537	0.125993	56.4245	-35.6001	4.48E-39
PBO 0.06%	-1.21616	0.125993	56.4245	-9.65257	4.50E-13
PREV-AM 0.48 %	-3.3541	0.125993	56.4245	-26.6213	1.87E-32
Rapeseed	-1.69015	0.125993	56.4245	-13.4146	2.67E-18
Rosemary	-1.36186	0.125993	56.4245	-10.8091	1.15E-14
Sesame	-1.8804	0.125993	56.4245	-14.9246	2.72E-20
Tea-tree	-2.67633	0.125993	56.4245	-21.2419	1.63E-27
Thymol	-2.84337	0.125993	56.4245	-22.5677	8.24E-29

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
  Data: PXG1lm

REML criterion at convergence: -45.3

Scaled residuals:
   Min   1Q Median   3Q   Max
-1.8447 -0.4701 -0.0188 0.4440 2.2563

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.14762	0.3842	4.24528	0.13773	7.0000	30.823	9.77E-09
Residual		0.01243	0.1115					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Plutella xylostella* GST assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.99372	0.090493	55.26604	-10.9811	1.65E-14
Black-pepper	-0.89938	0.090493	55.26604	-9.93859	4.62E-13
Borage	-0.87911	0.090493	55.26604	-9.71462	8.89E-13
D-limonene	-0.34661	0.090493	55.26604	-3.83027	0.000329
Eugenol	-1.70311	0.090493	55.26604	-18.8203	1.48E-24
FLiPPER 0.096%	-1.13021	0.090493	55.26604	-12.4894	1.10E-16
Garlic	-3.91752	0.090493	55.26604	-43.2906	4.76E-43
Geraniol	-0.97565	0.090493	55.26604	-10.7814	2.97E-14
Lemongrass	-2.39903	0.090493	55.26604	-26.5105	7.07E-32
Neem	-2.1628	0.090493	55.26604	-23.9001	1.22E-29
Orange	-0.85793	0.090493	55.26604	-9.48056	1.74E-12
Parsley-seed	-2.60314	0.090493	55.26604	-28.766	1.11E-33
PBO 0.006%	-0.47711	0.090493	55.26604	-5.27237	4.61E-06
PREV-AM 0.048 %	-1.52224	0.090493	55.26604	-16.8216	2.75E-22
Rapeseed	-0.90776	0.090493	55.26604	-10.0313	3.78E-13
Rosemary	-0.78455	0.090493	55.26604	-8.66972	2.76E-11
Sesame	-0.60668	0.090493	55.26604	-6.70411	3.38E-08
Tea-tree	-1.36946	0.090493	55.26604	-15.1332	3.15E-20
Thymol	-2.30536	0.090493	55.26604	-25.4754	5.10E-31

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: GST.activity ~ Treatment + (1 | Plate)
Data: PXG0.1lm

REML criterion at convergence: 14.5

Scaled residuals:

Min	1Q	Median	3Q	Max
-3.8175	-0.1628	-0.0108	0.2920	2.3684

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.13923	0.3731	4.24528	0.13773	7.0000	30.823	9.77E-09
Residual		0.03758	0.1939					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Plutella xylostella* GST assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-0.42955	0.155396	57.61215	-2.76422	0.0765
Black-pepper	-0.46938	0.155396	57.61215	-3.02054	0.045096
Borage	-0.3001	0.155396	57.61215	-1.93121	0.467052
D-limonene	-0.05063	0.155396	57.61215	-0.3258	0.745758
Eugenol	-0.4152	0.155396	57.61215	-2.67188	0.088121
FLiPPER 0.0096%	-0.50003	0.155396	57.61215	-3.21778	0.029709
Garlic	-1.66663	0.155396	57.61215	-10.725	4.50E-14
Geraniol	-0.26426	0.155396	57.61215	-1.70054	0.566538
Lemongrass	-0.24979	0.155396	57.61215	-1.60743	0.567134
Neem	-0.84902	0.155396	57.61215	-5.46356	1.67E-05
Orange	-0.517	0.155396	57.61215	-3.32698	0.022996
Parsley-seed	-0.86585	0.155396	57.61215	-5.57191	1.18E-05
PBO 0.0006%	-0.23855	0.155396	57.61215	-1.53509	0.567134
PREV-AM 0.0048 %	-0.16879	0.155396	57.61215	-1.08621	0.735211
Rapeseed	-0.2849	0.155396	57.61215	-1.83335	0.503433
Rosemary	-0.49184	0.155396	57.61215	-3.16509	0.032201
Sesame	-0.1825	0.155396	57.61215	-1.17439	0.735211
Tea-tree	-0.45201	0.155396	57.61215	-2.90876	0.056618
Thymol	-0.90562	0.155396	57.61215	-5.82781	4.80E-06

A.3 P450 activity: Linear mixed effects model results

A.3.1 Psylliodes chrysocephala

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Log ~ Treatment + (1 | Plate)
Data: CBP10lm

REML criterion at convergence: 228.3

Scaled residuals:

Min	1Q	Median	3Q	Max
-4.0344	-0.0893	0.0000	0.1291	2.5426

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	0.0	0.000	9.7135	0.2814	59.0000	34.521	<2E-16
Residual		1.9	1.378					

Number of obs: 78, groups: Plate, 8

Table 1. Linear mixed effects model results for *Psylliodes chrysocephala* P450 assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-5.21546	0.844141	59.0000	-6.17842	7.18E-07
Black-pepper	-1.69225	0.844141	59.0000	-2.0047	0.347133
Borage	-0.80775	0.844141	59.0000	-0.95689	0.897121
D-limonene	-0.88442	0.844141	59.0000	-1.04772	0.897121
Eugenol	-9.71353	0.844141	59.0000	-11.507	1.86E-15
FLiPPER 0.96%	-9.71353	0.844141	59.0000	-11.507	1.86E-15
Garlic	-4.15226	0.844141	59.0000	-4.91892	7.31E-05
Geraniol	-9.71353	0.844141	59.0000	-11.507	1.86E-15
Lemongrass	-1.50145	0.844141	59.0000	-1.77867	0.402227
Neem	-1.58176	0.844141	59.0000	-1.87381	0.395472
Orange	-9.71353	0.844141	59.0000	-11.507	1.86E-15
Parsley-seed	-0.50028	0.844141	59.0000	-0.59265	0.897121
PBO 0.06%	-5.32949	0.844141	59.0000	-6.3135	4.66E-07
PREV-AM 0.48 %	-1.42888	0.844141	59.0000	-1.69271	0.402227
Rapeseed	-2.52581	0.844141	59.0000	-2.99217	0.036355
Rosemary	-2.07062	0.844141	59.0000	-2.45293	0.137159
Sesame	-9.71353	0.844141	59.0000	-11.507	1.86E-15
Tea-tree	-9.71353	0.844141	59.0000	-11.507	1.86E-15
Thymol	-5.21546	0.844141	59.0000	-6.17842	7.18E-07

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Fluorescence ~ Treatment + (1 | Plate)
  Data: CBP1lm

REML criterion at convergence: 1186.9

Scaled residuals:
   Min   1Q Median   3Q   Max
-2.3293 -0.4167 0.0000 0.3326 2.4153

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	62526487	7907	16773.63	2654.44	8.0000	6.319	0.000228
Residual		2664416	1632					

Number of obs: 84, groups: Plate, 9

Table 1. Linear mixed effects model results for *Psylliodes chrysocephala* P450 assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-11602.5	1328.616	56.6924	-8.73275	4.86E-11
Black-pepper	-13283.8	1328.616	56.6924	-9.99823	5.63E-13
Borage	-4247.23	1328.616	56.6924	-3.19673	0.002274
D-limonene	-8222.57	1328.616	56.6924	-6.18882	3.59E-07
Eugenol	-8685.55	1328.616	56.6924	-6.53729	1.34E-07
FLiPPER 0.096%	-34004.3	1328.616	56.6924	-25.5938	1.49E-31
Garlic	-14208.4	1328.616	56.6924	-10.6942	5.20E-14
Geraniol	-8685.55	1328.616	56.6924	-6.53729	1.34E-07
Lemongrass	-12800.2	1328.616	56.6924	-9.63426	1.99E-12
Neem	-7852.71	1328.616	56.6924	-5.91045	8.19E-07
Orange	-9527.09	1328.616	56.6924	-7.17069	1.36E-08
Parsley-seed	-17604.8	1328.616	56.6924	-13.2505	8.93E-18
PBO 0.006%	-9870.36	1328.616	56.6924	-7.42905	5.68E-09
PREV-AM 0.048 %	-29393	1328.616	56.6924	-22.123	2.60E-28
Rapeseed	-12656.7	1328.616	56.6924	-9.52622	2.74E-12
Rosemary	-5419.76	1328.616	56.6924	-4.07925	0.000286
Sesame	-6536.55	1328.616	56.6924	-4.91982	2.34E-05
Tea-tree	-10124.4	1328.616	56.6924	-7.62028	3.04E-09
Thymol	-14208.4	1328.616	56.6924	-10.6942	5.20E-14

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Fluorescence ~ Treatment + (1 | Plate)
  Data: CBP0.1lm

REML criterion at convergence: 1203.9

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.80669 -0.45081 -0.04761 0.39674 2.36010

Random effects:
 Groups   Name   Variance Std.Dev.
 Plate   (Intercept) 62209960 7887
 Residual   3613996 1901
Number of obs: 84, groups: Plate, 9

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	62209960	7887	16773	2654.44	8.0000	6.319	0.000228
Residual		3613996	1901					

Number of obs: 84, groups: Plate, 9

Table 1. Linear mixed effects model results for *Psylliodes chrysocephala* P450 assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-8190.07	1545.635	56.93659	-5.29883	2.70E-05
Black-pepper	-7632.07	1545.635	56.93659	-4.93782	7.99E-05
Borage	-2114.55	1545.635	56.93659	-1.36808	0.353327
D-limonene	-5060.21	1545.635	56.93659	-3.27387	0.009035
Eugenol	-5335.16	1545.635	56.93659	-3.45176	0.00634
FLiPPER 0.0096%	-23493	1545.635	56.93659	-15.1996	2.03E-20
Garlic	-11451.1	1545.635	56.93659	-7.40866	1.13E-08
Geraniol	-6189.16	1545.635	56.93659	-4.00428	0.001458
Lemongrass	-8057.88	1545.635	56.93659	-5.21331	3.22E-05
Neem	-3003.54	1545.635	56.93659	-1.94324	0.17081
Orange	-93.8941	1545.635	56.93659	-0.06075	0.951773
Parsley-seed	-9446.23	1545.635	56.93659	-6.11155	1.42E-06
PBO 0.0006%	-7191.45	1545.635	56.93659	-4.65275	0.0002
PREV-AM 0.0048 %	-16747	1545.635	56.93659	-10.835	3.35E-14
Rapeseed	-8197.73	1545.635	56.93659	-5.30379	2.70E-05
Rosemary	-6442.89	1545.635	56.93659	-4.16844	0.00095
Sesame	-4621.5	1545.635	56.93659	-2.99003	0.016459
Tea-tree	-5946.23	1545.635	56.93659	-3.84711	0.002131
Thymol	-10446.8	1545.635	56.93659	-6.75887	1.29E-07

A.3.2 Delia radicum

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Fluorescence ~ Treatment + (1 | Plate)
  Data: CRP10lm

REML criterion at convergence: 1075.8

Scaled residuals:
   Min   1Q Median   3Q   Max
-2.3883 -0.2884 0.0434 0.3685 3.0947

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	9948208	3154	16885.29	1159.29	7.0000	14.563	1.72E-06
Residual		2419180	1555					

Number of obs: 78, groups: Plate, 8

Table 1. Linear mixed effects model results for *Delia radicum* P450 assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-7814.89	1248.952	55.20978	-6.25716	4.26E-07
Black-pepper	-7121.56	1248.952	55.20978	-5.70203	2.40E-06
Borage	-6937.38	1248.952	55.20978	-5.55456	3.30E-06
D-limonene	-5279.05	1248.952	55.20978	-4.22678	8.97E-05
Eugenol	-16670.7	1248.952	55.20978	-13.3478	1.08E-17
FLiPPER 0.96%	-11454.4	1248.952	55.20978	-9.17125	1.31E-11
Garlic	-15176.5	1248.952	55.20978	-12.1514	4.98E-16
Geraniol	-14242.4	1248.952	55.20978	-11.4035	5.83E-15
Lemongrass	-7772.38	1248.952	55.20978	-6.22312	4.26E-07
Neem	-10609.6	1248.952	55.20978	-8.49483	1.47E-10
Orange	-8534.12	1248.952	55.20978	-6.83302	5.57E-08
Parsley-seed	-6402.59	1248.952	55.20978	-5.12637	1.17E-05
PBO 0.06%	-12136.8	1248.952	55.20978	-9.71757	1.93E-12
PREV-AM 0.48 %	-10054.9	1248.952	55.20978	-8.05066	7.03E-10
Rapeseed	-13757.3	1248.952	55.20978	-11.0151	2.08E-14
Rosemary	-6325.41	1248.952	55.20978	-5.06457	1.17E-05
Sesame	-8740.3	1248.952	55.20978	-6.99811	3.36E-08
Tea-tree	-18087.2	1248.952	55.20978	-14.4819	3.39E-19
Thymol	-7814.89	1248.952	55.20978	-6.25716	4.26E-07

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Fluorescence ~ Treatment + (1 | Plate)
  Data: CRP1lm

REML criterion at convergence: 1118.6

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.70857 -0.31747 0.06909 0.52113 1.65584

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	9841872	3137	16885.3	1159.5	7.0000	14.563	1.72E-06
Residual		2738189	1655					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Delia radicum* P450 assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-3973.91	1325.775	57.70251	-2.99742	0.004011
Black-pepper	-6218.57	1325.775	57.70251	-4.69052	6.86E-05
Borage	-6852.05	1325.775	57.70251	-5.16834	1.85E-05
D-limonene	-4809.05	1325.775	57.70251	-3.62735	0.001216
Eugenol	-12200	1325.775	57.70251	-9.20216	9.69E-12
FLiPPER 0.096%	-10967.7	1325.775	57.70251	-8.27268	2.89E-10
Garlic	-15810.7	1325.775	57.70251	-11.9257	5.97E-16
Geraniol	-13700.7	1325.775	57.70251	-10.3341	1.54E-13
Lemongrass	-8916.39	1325.775	57.70251	-6.72541	8.65E-08
Neem	-18616.1	1325.775	57.70251	-14.0417	5.49E-19
Orange	-9097.27	1325.775	57.70251	-6.86185	5.63E-08
Parsley-seed	-8608.48	1325.775	57.70251	-6.49316	1.69E-07
PBO 0.006%	-5393.84	1325.775	57.70251	-4.06844	0.000436
PREV-AM 0.048 %	-8233.72	1325.775	57.70251	-6.21049	4.36E-07
Rapeseed	-6512.24	1325.775	57.70251	-4.91202	3.89E-05
Rosemary	-11576.3	1325.775	57.70251	-8.7317	5.38E-11
Sesame	-8775.66	1325.775	57.70251	-6.61927	1.17E-07
Tea-tree	-10511.6	1325.775	57.70251	-7.92865	1.00E-09
Thymol	-15571.4	1325.775	57.70251	-11.7451	1.06E-15

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Fluorescence ~ Treatment + (1 | Plate)
  Data: CRP0.1lm

REML criterion at convergence: 1108.8

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.46121 -0.43139 -0.04565 0.48520 2.28554

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	9993168	3161	16885.3	1159.5	7.0000	14.563	1.72E-06
Residual		2284297	1511					

Number of obs: 81, groups: Plate, 8

Table 1. Linear mixed effects model results for *Delia radicum* P450 assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-945.506	1214.781	57.14447	-0.77833	1
Black-pepper	-3629.17	1214.781	57.14447	-2.98751	0.03725
Borage	-3883.35	1214.781	57.14447	-3.19675	0.022656
D-limonene	59.98326	1214.781	57.14447	0.049378	1
Eugenol	-7086.5	1214.781	57.14447	-5.83356	4.27E-06
FLiPPER 0.0096%	-2605.6	1214.781	57.14447	-2.14492	0.181118
Garlic	-11207	1214.781	57.14447	-9.22555	1.23E-11
Geraniol	-9714.84	1214.781	57.14447	-7.99719	1.16E-09
Lemongrass	-1049.35	1214.781	57.14447	-0.86382	1
Neem	-5936.03	1214.781	57.14447	-4.8865	0.00013
Orange	-2463.52	1214.781	57.14447	-2.02795	0.188945
Parsley-seed	-2717.51	1214.781	57.14447	-2.23704	0.175206
PBO 0.0006%	-5572.98	1214.781	57.14447	-4.58764	0.00035
PREV-AM 0.0048 %	-4709.94	1214.781	57.14447	-3.87719	0.003303
Rapeseed	-3228.84	1214.781	57.14447	-2.65796	0.076446
Rosemary	-4213.85	1214.781	57.14447	-3.46882	0.011009
Sesame	-3257.84	1214.781	57.14447	-2.68183	0.076446
Tea-tree	-4982.52	1214.781	57.14447	-4.10158	0.00171
Thymol	-9871.03	1214.781	57.14447	-8.12576	7.52E-10

A.3.3 Plutella xylostella

Highest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Fluorescence ~ Treatment + (1 | Plate)
  Data: PP10lm

REML criterion at convergence: 1039.3

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.06811 -0.47729 -0.01115 0.47366 2.04490

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	5380661	2320	10804.08	865.18	7.0000	12.488	4.86E-06
Residual		1823009	1350					

Number of obs: 77, groups: Plate, 8

Table 1. Linear mixed effects model results for *Plutella xylostella* P450 assay with synergists at 0.6% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-4658.35	1077.675	55.13708	-4.32259	0.000708
Black-pepper	-4329.35	1077.675	55.13708	-4.01731	0.001439
Borage	-5152.67	1077.675	55.13708	-4.78128	0.00016
D-limonene	1133.331	1077.675	55.13708	1.051644	1
Eugenol	-8481.13	1077.675	55.13708	-7.86984	2.52E-09
FLiPPER 0.96%	-5382.02	1077.675	55.13708	-4.9941	8.18E-05
Garlic	4373.943	1077.675	55.13708	4.058685	0.001413
Geraniol	-6258.47	1077.675	55.13708	-5.80738	4.90E-06
Lemongrass	-292.003	1077.675	55.13708	-0.27096	1
Neem	-1839.56	1077.675	55.13708	-1.70697	0.560728
Orange	-4134.11	1077.675	55.13708	-3.83614	0.002265
Parsley-seed	-6463.44	1077.675	55.13708	-5.99757	2.58E-06
PBO 0.06%	-4662.02	1077.675	55.13708	-4.326	0.000708
PREV-AM 0.48 %	-6062.35	1077.675	55.13708	-5.6254	8.94E-06
Rapeseed	650.945	1210.462	54.39495	0.537766	1
Rosemary	-6947.13	1077.675	55.13708	-6.44641	5.11E-07
Sesame	446.445	1077.675	55.13708	0.414267	1
Tea-tree	-1749.39	1077.675	55.13708	-1.6233	0.560728
Thymol	-4658.35	1077.675	55.13708	-4.32259	0.000708

Intermediate concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Fluorescence ~ Treatment + (1 | Plate)
  Data: PP1lm

REML criterion at convergence: 1168.1

Scaled residuals:
    Min   1Q Median   3Q   Max
-1.73399 -0.44418 -0.05132 0.33483 2.98262

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	11101782	3332	9943.30	1150.28	8.0000	8.644	2.49E-05
Residual		2419788	1556					

Number of obs: 84, groups: Plate, 9

Table 1. Linear mixed effects model results for *Plutella xylostella* P450 assay with synergists at 0.06% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-3834.76	1250.851	59.21974	-3.06572	0.035958
Black-pepper	-6156.76	1250.851	59.21974	-4.92205	0.000122
Borage	213.7107	1250.851	59.21974	0.170852	1
D-limonene	2908.044	1250.851	59.21974	2.324852	0.211743
Eugenol	-6840.22	1250.851	59.21974	-5.46845	1.82E-05
FLiPPER 0.096%	-5264.65	1250.851	59.21974	-4.20886	0.001149
Garlic	189.7718	1250.851	59.21974	0.151714	1
Geraniol	-6736.22	1250.851	59.21974	-5.38531	2.35E-05
Lemongrass	-1942.96	1250.851	59.21974	-1.55331	0.879747
Neem	-4935.1	1250.851	59.21974	-3.94539	0.002565
Orange	1913.865	1250.851	59.21974	1.53005	0.879747
Parsley-seed	-2092.52	1250.851	59.21974	-1.67288	0.797014
PBO 0.006%	-5423.1	1250.851	59.21974	-4.33553	0.000802
PREV-AM 0.048 %	-5770.32	1250.851	59.21974	-4.61311	0.000326
Rapeseed	-3548.76	1250.851	59.21974	-2.83707	0.062242
Rosemary	1667.531	1250.851	59.21974	1.333117	0.879747
Sesame	-6091.22	1250.851	59.21974	-4.86966	0.000139
Tea-tree	-578.802	1250.851	59.21974	-0.46273	1
Thymol	-1787.56	1250.851	59.21974	-1.42908	0.879747

Lowest concentration

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: Fluorescence ~ Treatment + (1 | Plate)
  Data: PP0.1lm

REML criterion at convergence: 1183.7

Scaled residuals:
    Min   1Q Median   3Q   Max
-2.68182 -0.42491 -0.09981 0.39149 2.49711

Random effects:

Groups	Name	Variance	Std.Dev.	Estimate	Std. Error	df	t value	Pr(>|t|)
Plate	(Intercept)	10842329	3293	9943.30	1150.28	8.0000	8.644	2.49E-05
Residual		3198148	1788					

Number of obs: 84, groups: Plate, 9

Table 1. Linear mixed effects model results for *Plutella xylostella* P450 assay with synergists at 0.006% concentration unless otherwise stated.

Treatment	Estimate	Std. Error	df	t value	Adjusted_p
Bergamot	-2412.4	1430.828	60.15917	-1.68602	1
Black-pepper	-3271.73	1430.828	60.15917	-2.2866	0.386302
Borage	7196.274	1430.828	60.15917	5.029448	8.98E-05
D-limonene	5953.608	1430.828	60.15917	4.160953	0.001842
Eugenol	-1655.3	1430.828	60.15917	-1.15688	1
FLiPPER 0.0096%	-3821.24	1430.828	60.15917	-2.67065	0.165286
Garlic	714.8787	1430.828	60.15917	0.499626	1
Geraniol	-2631.97	1430.828	60.15917	-1.83947	0.990942
Lemongrass	-1673.06	1430.828	60.15917	-1.16929	1
Neem	-2382.47	1430.828	60.15917	-1.6651	1
Orange	-703.197	1430.828	60.15917	-0.49146	1
Parsley-seed	796.7428	1430.828	60.15917	0.55684	1
PBO 0.0006%	-914.622	1430.828	60.15917	-0.63923	1
PREV-AM 0.0048 %	31.75865	1430.828	60.15917	0.022196	1
Rapeseed	-2487.73	1430.828	60.15917	-1.73867	1
Rosemary	2289.803	1430.828	60.15917	1.600334	1
Sesame	-3645.63	1430.828	60.15917	-2.54792	0.214542
Tea-tree	538.8027	1430.828	60.15917	0.376567	1
Thymol	802.8787	1430.828	60.15917	0.561129	1

Appendix B

Correlation results:

Psylliodes chrysocephala

Esterase

S = 170, p-value = 0.2696

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

-0.4166667

GST

S = 132, p-value = 0.81

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

-0.1

P450

S = 146, p-value = 0.5809

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

-0.2166667

Delia radicum

Esterase

S = 446, p-value = 0.06275

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

-0.5594406

GST

S = 336, p-value = 0.5883

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

-0.1748252

P450

S = 300, p-value = 0.8863

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

-0.04895105

Myzus persicae

Esterase

S = 140, p-value = 0.08309

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

-0.6666667

GST

S = 74, p-value = 0.793

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

0.1190476

Plutella xylostella

Esterase

S = 163.18, p-value = 0.3415

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

-0.3598358

GST

S = 180.25, p-value = 0.1684

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

-0.5020964

P450

S = 100.92, p-value = 0.6828

alternative hypothesis: true rho is not equal to 0

sample estimates:

rho

0.1589972

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