Novel Strategies for the Control of Wireworm in Potato Crops

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Abstract

The biological control of wireworm (*Agriotes* spp.) in potato crops has not yet caught up to conventional controls at a time in which deregistration of pesticides is increasing, leaving a paucity of options for growers. Some measure of control has been achieved with use of entomopathogenic fungi (EPF), and to a lesser extent, entomopathogenic nematodes (EPN), with varying success. Factors affecting efficacy range from instability of pathogens within a soil matrix to difficulty in accurately targeting a subterranean pest in a heterogeneous population. The overall aim of this project was to identify areas for improvement for biological control strategies for wireworm, through identification of bioactive compounds and exploring synergies between these and existing entomopathogens. Using historical research to identify botanical extracts for bioactivity against invertebrate pests, tea tree (*Melaleuca alternifolia*) and rosemary (*Salvia rosemarinus*) oils were found to elicit repellent properties in wireworm, positively associated with mortality. Cedarwood (*Cedrus atlantica*) oil produced the opposite effect, an attractant response with no adverse effects on larval health. The second aspect of the project posited a novel behavioural assay methodology, demonstrating wireworm plant preferences and complex behavioural responses to introduced botanical semiochemicals beyond that of a simple attraction or repulsion. Identified botanicals were then evaluated for compatibility with strains of *Metarhizium brunneum*, with each of the three exhibiting fungicidal and fungistatic effects on the EPF, but at lower concentrations improving mortality and rate of pathogenicity. Finally, wireworm behavioural responses to EPF inoculations were exploited with two fungal volatile organic compounds (VOC), 1-octen-3-ol and 3-Octanone, found to have direct bioactivity towards wireworm and marginal synergistic effects with a known biological control in the entomopathogenic nematode, *Heterorhabiditis bacteriophora.* The project has clearly demonstrated methods for improvement for existing biological controls and given strong evidence for use of botanicals as crop protectant compounds in an integration pest management system for potato crops.

Declarations & Statements

i.   This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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ii.   This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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iii.   I hereby give my consent for my work, if relevant and accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access approved by the University.**
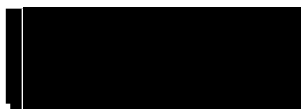
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iv.   The University's ethical procedures have been followed with ethics approval code: SU-Ethics-Student-291121/4808

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Abbreviations

Amu	Atomic Mass Unit
AI	Active Ingredient
AIC	Akaike's Information Criteria
ARI	Anthocyanin Reflectance Index
BBCH	Biologische Bundesanstalt, Bundessortenamt and CHemical industry (Plant Growth Stages)
CCCI	Chlorophyll Canopy Vegetation Index
CI	Chemotaxic Index
CLSI	Clinical Laboratory Standards Institute
CVI	Chlorophyll Vegetation Index
DMSO	Dimethyl Sulphoxide
DVI	Difference Vegetation Index
EI	Electron Ionization
EPF	Entomopathogenic Fungi
EPN	Entomopathogenic Nematodes
eV	Electron Volt
F_m	Maximum yield of fluorescence
F_v	Variable Fluorescence
GC/MS	Gas Chromatography Mass Spectrometry
GLM	Generalised Linear Model
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
ml	Millilitre

MPR	Mean Percentage Repellency
NDRE	Normalised Difference Red Edge
NDVI	Normalise Difference Vegetation Index
NIR	Near Infrared
PI	Percentage Inhibition
PPM	Parts per Million
PPO	Applied Plant Research of Wageningen UR (Praktijkonderzoek Plant & Omgeving van Wageningen UR)
PSII	Photosystem II
RE	Red Edge
RH	Relative Humidity
RLU	Relative Light Unit
SADIE	Spatial Analysis by Distance IndicEs
SDB	Sabouraud Dextrose Broth
SDA	Sabouraud Dextrose Agar
µl	Microlitre
V/V	Volume by Volume
W/V	Weight by Volume
W/W	Weight by Weight

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Chapter 1: Wireworm Control – Bridging the Gap between Conventional and Biological Controls in Integrated Pest Management Solutions

1.1 Introduction

Wireworm are the subterranean larval form of the click beetle (Coleoptera: Elateridae). Within the UK and Europe crops are primarily affected by few species of wireworm within the genus *Agriotes* and to a lesser extent, *Athous spp.* (Barsics *et al.*, 2013; Eckard *et al.*, 2014). A large range of crops are susceptible to damage from wireworm, their polyphagous edacity resulting in feeding on all below-ground plant organs and those at the surface, with potato tubers heavily affected (Johnson *et al.*, 2008; Sufyan *et al.*, 2011). This damage can result in stunted growth and cosmetic damage, potentially facilitating secondary infection of fungal and bacterial pathogens thus reducing yield and marketability (Kabaluk & Ericsson, 2007; Barsics *et al.*, 2013). Potatoes may be particularly susceptible to wireworm damage due to the availability and nutritional value of the tubers (Johnson *et al.,* 2008). It has been suggested that if over 10-15% of the harvested tubers are damaged then it is not economically viable to grade these out, thus making the crop ineligible for anything other than seed (Ansari *et al.*, 2009).

Potato crops are particularly susceptible to wireworm within the UK, not least due to their prominence in organic farming and therefore limitation there with chemical insecticide availability (Wraight *et al.*, 2008; Sufian, 2012). Aside from wireworm, other factors affecting potato production in the UK include other soil borne pests such as potato cyst nematode (PCN), which in 2009 was estimated to cost the industry £26 million annually (Kaczmarek *et al.*, 2019, Twining *et al.*, 2009). Disease is another factor with more than 20 pathogens affecting tuber marketability within the UK (BPC, 2001), principal of which is late blight, *Phytophora infestans* (Mont.) de Bary, causing similar levels of economic damages to PCN (Clarke, 2014). Cultural issues such as labour costs and availability, uncontrollable field rotation of rented land, water availability and pesticide deregistration are all hurdles facing the potato industry.
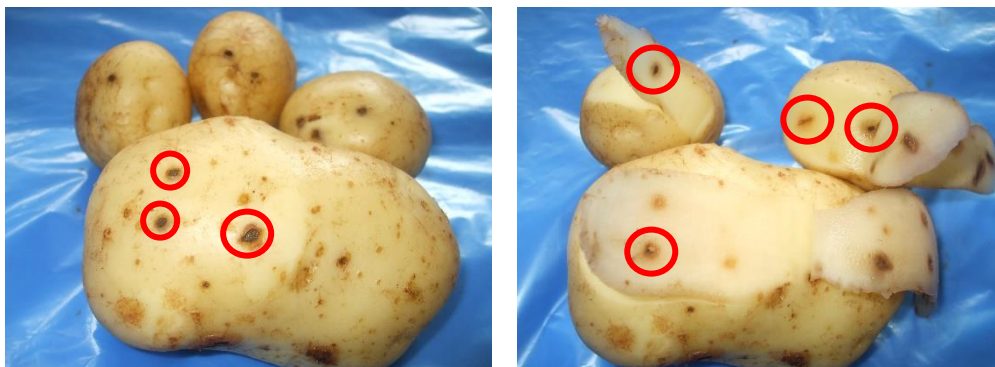
Fig. 1.1 Potato tubers undergoing inspection for marketability, exhibiting typical wireworm damage. Tubers are peeled to examine the extent of damage for grading.

From 2016 – 2021, potatoes accounted for just under 3% of total crop area within the UK, at about 106,000 ha, the value of which rests around £700 million (both as seed and for human consumption) (DEFRA, 2022). Although the total planted area of potatoes has decreased steadily in the UK over the last 30 years, yields have increased over that time, thanks to improvements in farming practice, technologies and agronomy (AHDB, 2018). Potatoes may be considered a higher value crop when compared to cereals per hectare, though there is a greater instability in market price fluctuations (AHDB, 2018). Within the food and drink retail market in the UK (as of 2018) potatoes account for just under 3% of consumer purchases, with 33% of that market attributable to fresh potatoes, and 32% to crisps (AHDB, 2018). Frozen and chilled potato products make up the rest of the retail market.

To identify wireworm damage to ensure suitability for market, the damage to the tuber can be distinguished from that caused by other pests, such as slugs, as a thin hole bored 1 – 2 cm into the flesh often resulting in pockmarks on the periderm (Parker, 2005; Syngenta, 2010). Examples of wireworm damage can be seen in Figure 1.1, with a commercial technique of peeling used to determine depth and extent of wireworm damage. The three species most problematic within UK potato crops are *Agriotes lineatus*, *Agriotes sputator* and *Agriotes obscurus (L.)* (Parker & Howard, 2001).

That these species have once again begun to establish themselves as economic pests has been attributed to shifts in agricultural practice over the last 15 years (Sufian, 2012). This shift includes the more rigorous demands of retailers for ever more high-quality products. Additionally problematic is growers' use of pasture to grow potatoes in a 'clean' environment free from common soil-borne pathogens, though a habitat associated with wireworm (Lole, 2010). Adult click beetles show a preference for oviposition within pasture and, to a lesser extent, cereal crops, and so pest populations are routinely replenished and may harbour a full range of larval instars (Furlan, 1996). There has been suggestion that the rise in wireworm populations may in part be attributed to a reduction the use of soil persistent insecticides. Directive 128/2009/EC directs growers to use non-chemical methods of control where possible. The organochlorine compounds aldrin and lindane provided control until their removal from the market; and with the widespread de-registration of effective organophosphates and carbamates, modern insecticides such as neonicotinoid seed treatments and pyrethroids appear to have limited effects on population control (Traugott *et al.*, 2015). In 2020, conventional insecticides accounted for approximately 5% of all pesticide usage on ware potatoes (grown for human consumption and thus most at risk of wireworm affecting marketability) and of these only 1% were used as having some intended effect for wireworm (Ridley

et al., 2020). This was predominantly the active ingredient fosthiazate (Nemathorin 10G – Syngenta UK), itself labelled as a nematicide for potato cyst nematode (PCN) with secondary activity for other pests, 'such as wireworm'. Insecticidal use in seed potatoes is limited to use against aphids to curb spread of viral infections.

1.1.1 Biology and Life Cycle

Wireworm are semivoltine with larvae persistent in the soil for up to five years (Eckard *et al.*, 2013; Vernon *et al.*, 2013; Ufyan *et al.*, 2014). This long-life cycle can be attributed to restriction of larval activity through abiotic factors, primarily moisture and temperature, with desiccation and cold-induced immobility hurdles to regular feeding. As such, Furlan (2004) suggests that larvae may spend as little as 24% of their time feeding, in spite of a polyphagous feeding strategy and food availability. They have thin, cylindrical bodies with three pairs of thoracic legs set behind a sclerotized head, characteristic of coleopterans. Their colour ranges from a light tan in early instars through to the darker copper colouration they are most associated with. The adults range from 1 – 3 cm in length and have an elongated, tapered abdomen, with the thorax often showing rear
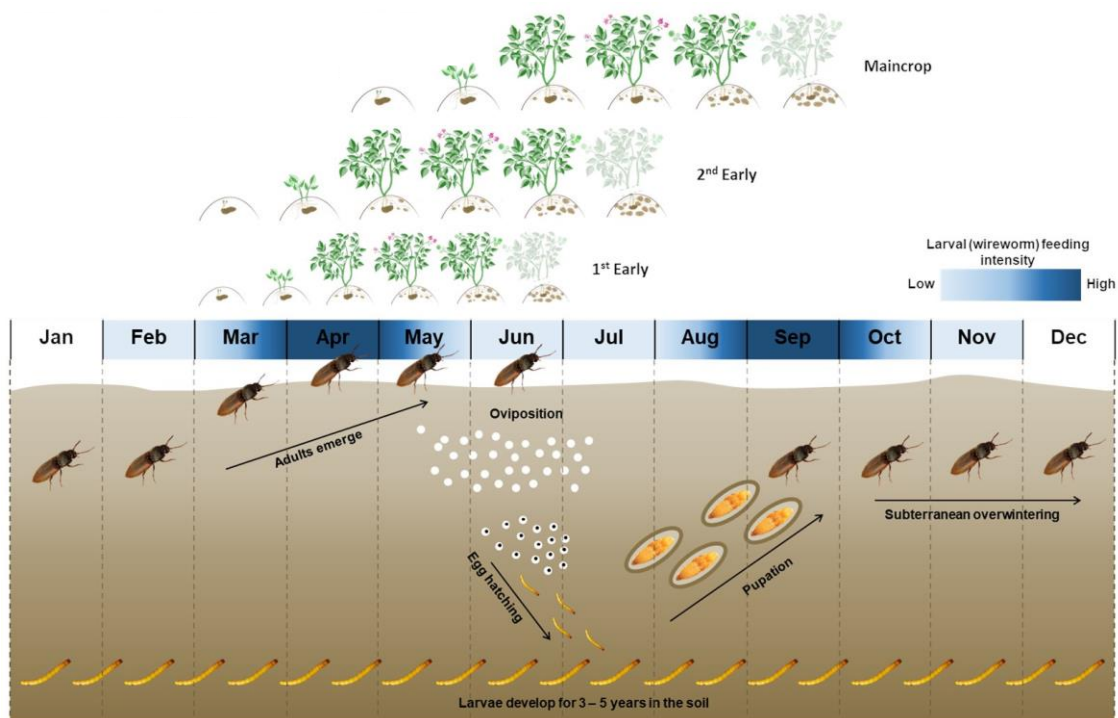
Fig. 1.2 The subterranean development of *Agriotes* spp*.* and adult emergence throughout the year in relation to the UK potato growth season. Indicates the semivoltine development of larvae within the soil profile, a major hurdle to control of the pest.

facing 'spiky' protrusions on the rear tips of the pronotum, and serrate antennae. The adults' common name is derived from the fashion in which they right themselves when turned over, which can produce an audible 'click.' Colouration is generally dark and dull in agriculturally significant species.

The life cycle and feeding trends *Agriotes* spp. can be seen in Figure 1.2, in relation to the UK potato growing season. It clearly outlines the subterranean development of *Agriotes* spp. and the year-round presence of the developing larvae. All three pest species of concern in the UK share similar enough life cycles to one another that control implementation should not have to vary in its timing for each species (Hicks & Blackshaw, 2008).

Having pupated and overwintered in cavities below-ground, adult click beetles emerge from April to August (Kozina *et al.*, 2015). Adults are not known to migrate large distances and will oviposit predominantly in grassland and heavily weeded areas, just below the surface. Hatched larvae will persist in the soil for multiple seasons, going through 2 – 4 instars each year (Kabaluk *et al.*; Furlan, 2004). Adults will live for several months, and in rare instances up to one year (Benefer *et al.*, 2012; Sufian, 2012).

Very little is known about the vertical and horizontal movement of wireworm larvae in soil. During the winter, *Agriotes spp.* larvae remain at depths of up to 60cm in periods of low temperatures, with activity decreasing rapidly once soil temperatures drop below 8°C (Parker & Howard, 2001; Blackshaw & Vernon, 2008; Landl *et al.*, 2011). Positive correlations between bait trap catches and rising soil temperatures in Spring have been observed in the UK rising ten-fold from early March to April, with soil temperatures rising from 2 to 8°C (Parker, 2005). The active periods appear to be a response to a mixture of soil temperature and moisture; and as such may be affected by the soil type (Furlan, 2007; Benefer *et al.*, 2012). Recent work in attempting to use wireworm soil preferences as a population prediction tool (Jung *et al.*, 2014), concluded that an optimum soil temperature range of 11 – 13°C and soil moisture content of approximately 31% may be considered favourable conditions for the pest, this would seem to be supported by previous behavioural work with the larvae, reaching similar conclusions (LaFrance, 1968).

Aside from the risks of larval desiccation, novel work using 3D X-ray tomography noted that soils with a moisture content below 10% would lack the structural integrity to maintain wireworm burrows (van Herk & Vernon, 2008). The importance of this is due in part to wireworm preferences to use existing burrows within the soil profile to facilitate movement (Lees 1943; van Herk & Vernon, 2007). Vertical migration to forage in these

active periods is generally reported to be in early to mid-Spring (March to May) and late Summer to Autumn (September to October). This migration may be affected by local factors such as the age and species of larvae present, plants available as a food source, and variable abiotic factors due to seasonal weather fluctuations. Furlan (1998) concludes that movement is increased in later instars and an ability to migrate vertically more quickly will combat desiccation and feed more prolifically. Furthermore, larvae will feed intermittently (breaks from 1 – 10 days) at 8 – 11 °C soil temperature and continuously at temperatures from 20 – 24 °C (Furlan, 1996 & 1998; Parker & Howard 2001). It is suggested that as long as soil moisture is adequate, temperature is the main determining factor of larval feeding habits, with a sustained moisture content of at least 31%, as mentioned above, considered favourable (LaFrance 1968; Furlan 1998; Parker & Howard 2001).

Increasingly, studies have focused on the horizontal movement of wireworm in response to conspecifics and food sources. It has been suggested that migration habits of wireworm can be affected not only by the density of food available but also by the presence of conspecifics in those areas, potentially due to cannibalism. Using adult *Agriotes spp.* emergence in conjunction with climatic and soil analysis, Kozina *et al.* (2015) further suggest that abundance may be influenced by soil pH, consistency, amount of humus, rainfall levels and air temperatures. Indeed, early works on wireworm migration suggest that soil moisture may influence migration with larvae often observed moving out of dry environments to areas of high moisture (Thorpe *et al.*, 1946; Crombie & Darrah, 1947). As such, correspondence with growers' knowledge and geographical field and area history may be vital to forming any accurate system of prediction which might influence application of a control method. Wireworm response to pH may be species specific, with the agriculturally important *Agriotes* spp. observed to prefer acidic soils (Milosavljevic *et al.*, 2016), with some research also suggesting that soils high in organic matter (OM) may lead to increased risk of damage from the genus, although OM itself may not be considered a significant food source (Furlan *et al.*, 2017).

1.1.2 Monitoring

Monitoring for wireworm presence within prospective field sites is key to implementing control measures, with the heaviest infestations inhibiting planting altogether. The primary manual sampling technique involves the collection of soil samples dug approximately 10 x 10 x 15 cm along a "W' shaped transect covering a minimum area of four hectare, with larvae counted cumulatively. Approximately 20 samples should be

considered a sufficient estimate of infestation (Simmons *et al.*, 1998). This method is very labour intensive though it is noted to be the only way to gauge wireworm population size over a given area. Barsics *et al.* (2013) note that growers may be reluctant to invest time into a labour-intensive trapping program and so there is potential for improvement of trapping systems with identified semiochemicals or trap design. This is addressed within both Chapter 2 and 3 with relation to wireworm responses to essential oils and the inception of a wireworm capture methodology. The limit of detection given for this technique is 62,500 wireworms per hectare, though it is likely that lower population densities than this would still cause economic damage (Parker, 2005; Barsics *et al.*, 2013), with recommendations for treatment often based on either simple presence of absence. Sampling should occur in early Spring, with consistent soil temperatures of eight degrees or more to ensure wireworm activity (Parker, 1996), though post-harvest sampling throughout the autumn is increasingly employed to identify problem fields for future planting, when wireworm may still be active but food availability is low (Landl *et al.*, 2011; Chabert & Blot, 1992).

Another useful method may be the use of bait traps for surveying over a longer period. A variety of food sources for traps have been describe such as rolled oats and both wheat and rice flours. All work on the principle of wireworm attraction to a gradient of carbon dioxide (CO_2). Often used are mesh begs filled with grain and then soaked, before placement into a plastic container with drilled holes large enough for the larvae (Parker & Howard, 2001). Containers should be buried to a depth of at least 20cm and left for a period of 10 – 14 days. When burying the trap, over-compaction of the soil should be avoided, and the site preferably covered - both for protection and relocation. The trap may then be examined for wireworm both inside the container and in the immediate vicinity, with the presence of a single larvae a suggested threshold.

The use of pheromone traps is currently used for the detection of adults, with sex pheromones described for the three *Agriotes* spp. mentioned previously (Furlan *et al.*, 2003; Tóth & Furlan, 2005). This method is principally used for the identification of species within an area. It has be a suggested that there is a difference in the speeds of movement between the three species (Fastest = *A. lineatus* $\rightarrow$ *A.obscurus* $\rightarrow$ *A. sputator* = slowest), thus potentially misrepresenting in the traps the community of species in the field (Vernon & Van Herk, 2013). As such, timing, placement, and quantity of traps should be considered. Adults within the UK will emerge and mate exclusively in the Spring, and pheromone trapping should occur as soil temperatures begin to rise in March through to early June (Parker, 2005; Barsics *et al.,* 2013).

Although work has been done to establish a connection between both population size and larval location with adults caught in traps, this has yielded no positive results (Blackshaw & Vernon, 2008). Interestingly, due to the regular capture of both sexes within such traps one study has suggested that the described compounds should potentially be classed as 'aggregation' rather than 'sex' pheromones (Tóth, 2013).

1.2. Management of Wireworm

1.2.1 Integrated Pest Management

The principle of integrated pest management (IPM) is not simply to reduce conventional pesticide inputs but a holistic approach to tackle pests, weeds and diseases within a crop using all methods at the grower's disposal (Stenberg, 2017). Stenberg (2017) uses the reference of a modified 'IPM' pyramid to expand the elements of cultural, conventional and biological controls within IPM to establish a fluid central tier that focuses on an ecologically based IPM approach. Whilst still acknowledging that the pyramid decreases towards the top from methods of prevention to intervention, correlating with the increasing toxicity of the input, the tier of biological control has many elements that feed into it. These include biorational synthetic volatiles; intrinsic heritable plant resistance; plant vaccination and inter- and intra-specific botanical diversity. What this thesis aims to highlight are the gaps in this ecological knowledge concerning wireworm with crops, resulting in a knowledge gap and subsequent lack of controls for wireworm as a pest species (Parker & Howard, 2001; Barsics *et al.*, 2013; LaForgia & Verheggen, 2019).

With reference to wireworm, it has been suggested that in crops such as maize and sugar beet less than 5% of planted area will need to be treated with conventional insecticides (Furlan, 2005). As a result, a secondary effect of wireworm presence may be pollution through applied conventional pesticides without confirming that an economic threshold of the pest population has been met. Furlan concludes that this is due to a lack of a fundamental knowledge base necessary to apply an effective IPM solution for wireworm. This must focus on a species-specific ecology, effective monitoring to predict levels of infestation and density and a comprehensive review of the economic threshold for different crops. The latter of which is not widely or accurately recorded with reference to specific pests, let alone wireworm.

1.2.2 Cultural Controls

The most effective control of wireworm is to plant crops in a plot free of the pest. As mentioned previously, the history of a plot can have an impact on the wireworm presence if it was occupied by a favourable habitat such as grass pasture (Benefer *et al.*, 2012; Wallinger *et al.*, 2014). Indeed, even surrounding fields of suitable habitat for wireworm may act as 'reservoir' populations for a plot perceived as being free from infestation (Hicks & Blackshaw, 2008). It is imperative to know the history of crops within a field and avoid rotations of preferred wireworm habitats such as pasture, ensuring also that fields are well weeded, as these conditions encourage click beetle oviposition (Schepl & Paffrath, 2005). Although weeds are mentioned as key oviposition sites in more than one review of wireworm management (Barsics et al., 2013; Poggi et al., 2021), research on specific plant species or communities are lacking, with instead emphasis placed on females requiring cover to reduce dessication of laid eggs (Furlan, 1996; Parker & Howard, 2001; Toepfer *et al.,* 2007). Initial monitoring of a plot is key to assessing the extent of infestation through described sampling methods for both larvae and adults. A particular problem facing UK growers is the use of rented land in which to plant potatoes. Such sites are regularly used as grazed pasture for years previous and this provides the ideal habitats for wireworm populations to flourish (Parker, 2005; Ritter & Richter, 2013). As previously stated, wireworm are particularly polyphagous pests of

1.2.3 Conventional Controls

All current chemical control methods must be implemented prior to planting as a protective treatment; there are no curative control measures once the crop is established (Vernon *et al.*, 2005). The UK relevant applications recommended through the Red Tractor crop module and AHDB Potatoes wireworm factsheet were, until 2020, ethoprophos (Mocap 15g) and fosthiazate (Nemathorin 10g); both organophosphorous insecticides (Potato Council, 2011; Red Tractor Assurance, 2016), each applied in granular form. Both treatments have activity against wireworm, but only state a 'reduction' in wireworm damage. Mocap 15g and Nemathorin 10g have a recommended application rate of 6kg/ha (max. dose) and 1.5kg/ha of active ingredient (AI) respectively, with the former applied pre-planting and the latter at time of planting (as per product labels). The active ingredient in Nemathorin, fosthiazate, is an organophosphate chemical eliciting a neurotoxic effect through inhibition of the enzyme acetylcholinesterase, resulting in an overload and shutdown of neurotransmitters (Fukuto, 1990). As of 2020, the deregistration of ethoprophos removed Mocap as a

control option for UK growers. The 119-day harvest interval of Nemathorin limits its viability as a like-for-like alternative. Both of these rely on the principal of interrupting wireworm as they begin actively foraging for food in Spring (Vernon *et al.*, 2016).

It is suggested that treatments for other crops used in rotation may have a suppressive effect on wireworm populations, such as neonicotinoid and pyrethroid insecticides. In the Pacific Northwest, Esser et al. (2015) found up to 31% yield increase In Spring wheat through using neonicitinoid (Thiamethoxam) seed treatments, though found that the control only effective for *Limonius californicus*. In experiments with *L. infuscatus* there was no yield increase despite a reduction in pest numbers (Esser *et al.*, 2015). Though within the UK, options are limited due to pesticide deregistrations over the last couple of decades, most recently losing access to granular, organophosphate-based product Mocap 15g, withdrawn by European regulators in 2019. Previous research has incorporated the use of wheat seed treated with the insecticides fipronil and thiamethoxam, intercropped between rows of potatoes, working simply on the principal of the kairomonic effects of CO_2 on wireworm (Vernon *et al.*, 2016). It is currently accepted that wireworm will orient towards food sources through CO_2 detection within the soil profile through klinotaxis, (orientation through regular lateral movement of the head) (Horton & Landolt, 2002; Brandl *et al.*, 2017; Cooper *et al.*, 2019). The findings presented in Vernon *et al.* (2016) suggest a reduction in potato damage caused by wireworm compared to a conventional insecticide, Thimet 15g (AI is phorate, reduction observed 81.2% compared to 83.4%), and wireworm population densities (89 – 100% compared to 59.2%). Although still concerned with chemical treatment, the reduced rates of insecticide used to achieve results in the attract and kill (A&K) method (3.5g of AI/ha compared to 3250g AI/ha in the granular treatment) demonstrates the use of the system in increasing treatment efficiency by maximizing contact with the target pest (Vernon *et al.*, 2016).

Recently there have been an increased number of pesticides deregistered in Canada (a major geographical area of infestation for wireworm) due to links with poisoning at higher trophic levels. This coupled with the longer soil persistency of some pyrethroids and phenylpyrazoles, although applied at reduced rates, point to the negative environmental impacts these products may be having (Vernon *et al.*, 2005; van Herk *et al.*, 2013; Traugott *et al.*, 2015). As a result, there are increasing efforts into better understanding of wireworm biology and chemical ecology so as to improve upon biological control and increase the efficacy of novel biopesticides, with the aim of reducing chemical input.

To find novel solutions for the unreliability of insecticide applications, Vernon *et al.* (2016) carried out investigations into the viability of trap cropping with wheat in potatoes , though treated the seed with one or both of fipronil (phenylpyrazole) and thiamethoxam (neonicotinoid). The results showed a comparable reduction in wireworm damaged tubers to an available granular application (Thimet 15g) and using a much lower concentration of AI also. This was attributed to the trap crop providing precise and direct contact to the target pest. Although chemical insecticide focused, such results demonstrate the benefits of incorporating an attractant, in this case a more desirable phagostimulant than the main crop, in a system of delivery for a biological control agent. These results highlight that further work is needed to assess the effects of the system on the quality and yield of tubers due to resource competition from the trap crop.

1.2.4 Biological Control

Biological control within IPM can be split into three categories; conservation, augmentative & classical. Classical biological control involves the management of an invasive pest population with a natural predator or parasitoid species which is not already local, to establish a permanent population (Michaud, 2002; Hoy 2012). Augmentative control concerns the large-scale release of a predator or parasitoid species with the aim of bolstering local populations and achieving a bolstered level of control. Perhaps the broadest aspect of biological IPM, conservation biological control aims to counteract habit fragmentation and loss resulting from intensive agricultural practices through increasing plant diversity and landscape complexity to sustain populations of natural enemies (Begg *et al.*, 2017).

The commercial aspect of biological control of wireworm in potatoes is primarily concerned with the application of biopesticides created with entomopathogenic fungi (EPF)(la Forgia & Verheggen, 2019). Of these EPF, the most successful species occur within the genus *Metarhizium*. *Metarhizium* is a Hypocrealean genus within the family Clavicipitaceae, that exists ubiquitously throughout the soil profile. Commonly referred to as the 'green muscardine' fungus for its sporulated appearance it is a common source of arthropod biological controls and a generalist entomopathogen with varieties exhibiting some species specificity, reviewed here in the case of wireworm (Fox, 1960). Conidia germinate upon contact with a suitable host and penetrate through the insect's cuticle, thereafter the host's haemolymph may distribute hyphal bodies throughout the body (Fox, 1960; Zacharuk & Tinline, 1968). At this point toxins produced by the fungi will kill the host and germinating bodies will form outside of the cadaver and multiply

(Zacharuk, 1973), to maximise potential for contact dispersal through other soil invertebrates (Kabaluk & Vernon, 2007). Often the fast germination and growth of *Metarhizium* are cited as pathogenetic related traits (Sinha *et al.,* 2016)), though comparatively its slow time-to-kill may be a hindrance, as larvae may continue to feed during infection and the biological control will fail to protect the crop.

There are several species and strains of EPF screened against *Agriotes spp.* for potential for use as biopesticides (la Forgia & Verheggen, 2019; Poggi *et al.*, 2021). These are primarily within the genus *Metarhizium (var. anisopliae* & *brunneum)*, with *Beauveria bassiana* and *Paecilmyces fumosoroseus* also noted as possible control species (Shapiro-Ilan *et al.*, 2004; Kabaluk *et al.*, 2007; Sharma *et al.*, 2020). Two commercial products were granted temporary emergency approval in Germany within the last five years, Attracap® (BIOCARE GmbH, Germany – registered 19/02/20 – 17/06/20) and Velifer® (BASF, registered 15/02/17 – 14/06/21). Attracap® is a granular product consisting of yeast and suspended conidia of a strain of *M. brunneum* within a biodegradable matrix. The respiration of the yeast allows for a steady gradient of CO_2 to act as an attractant for the wireworm and bring them into contact with the EPF conidia released by the slowly degrading granule. Velifer® is an oil dispersion formulation designed for direct conidial contact with the target pest, making it more widely accepted for use against foliar pest such as thrips or hemipterans.

Unlike Velifer ®, Attracap® has been commercialised specifically for use against wireworm in potato crops. Whilst largely experimental until recently, the biological plant protection company Biocare, in collaboration with EU research project INBIOSOIL, launched the encapsulated biocontrol product within the last 5 years. The product is recommended for application at planting and relies on the emission of CO_2 to attract and inoculate larvae with the EPF *M. brunneum*, suppressing populations before damage to the potatoes can occur. That such a product is beginning to obtain approval demonstrates the system's worth within a pest management market that is continually shifting away from an over-reliance on chemical controls. Furthermore, it builds a foundation for improvement on an attract-and-kill system that has shown promising initial results. As mentioned previously, incorporation of elements such as specific host plant volatiles may increase the efficacy of an attract and kill system; at this stage, Attracap® is recommended for 'light to average' infestations of *Agriotes* spp. with a 60% efficacy level (Hermann *et al.*, 2017). The strain of *M. brunneum* included within the product was found to control each of the agriculturally significant *Agriotes* spp., though *A. obscurus* showed the greatest sensitivity. Although, no specifics on the thresholds for 'light to average' infestations are provided by Biocare.

With biological control with EPF, strains of *Metarhizium brunneum* have been the primary focus of research due to their specific pathogenicity toward wireworm. Regardless of lethality in field collected strains, the factor of stability remains a pertinent one as any strains of EPF cultured on a commercial basis must retain virulence under a process of mass cultivation through subsequent infections (Eckard *et al.*, 2014). High percentages of mortality have also been found in studies undertaken on both adult and larval stages of *Agriotes* spp. though in these cases the time-to-kill was highlighted as a drawback in the process. Eckard *et al.* (2014) also compared the virulence of three strains of *M. brunneum*; V1002 (a UK identified strain), ART2825 from Switzerland and BIPESCO 5/F52 – a commercially registered strain. Of these, the Swiss strain was identified as highly virulent and additionally stable with a relatively low time to achieve a median lethality. As the study suggests, this may be another suitable candidate for use in a commercial biopesticide, though of course would require further screening against all relevant pest species.

Another possible approach within potato cropping systems system is the inclusion of a reduced concentration of insecticide within applications of EPF (Wraight *et al.*, 2008). Disadvantages of pest control using EPF often center on the time taken to kill the pest, which can be three to four weeks (Ansari *et al.*, 2009; Eckard *et al.*, 2014). Whilst inoculations of EPF can achieve high mortality rates in wireworm populations, the length of time until death may result in feeding damage that could be avoided, and the stability of the entomopathogen within the soil is precarious. Therefore, incorporation of conventional insecticides to the delivery system at a much-reduced concentration may introduce a factor of 'stress-and-kill,' in which the wireworm would be weakened to an extent that an EPF would achieve mortality in the target much faster. The integration of the two methods could also potentially reduce the selection pressure of the more commonly used insecticide. One example of this relationship has shown that a 'naturalyte' insecticide (a product derived from naturally occurring soil microbes) with the AI spinosad, applied with the EPF *M. anisopliae* can reduce the time to kill of the EPF and increase efficacy (Ericsson *et al.*, 2007; Kabaluk, 2014)*.* Spinosad is comprised of spinosyns, bioactive compounds classified as macrolides, exhibiting neurotoxicity towards invertebrate pest via inhibition of nicotinal acetylcholine receptors (Mayes *et al.*; Williams *et al.*, 2003). Spinosad was tested for inhibitory effects on the development of *M. anisopliae* and the two were found to be compatible when used in combination as a control of wireworm, and other invertebrate pests. Applications of both spinosad and EPF combined achieved much higher mortality rates in *Agriotes* spp*.* than either treatment applied singularly (Ericsson *et al.*, 2007; Bourdon *et al.*, 2021), though the

mechanism behind this was not defined. Similar research carried out with spinosad and a strain of *M. anisopliae* also found increased mortality and significantly reduced time to kill when applied to house flies, *Musca domestica* L. (Sharififard *et al.*, 2011). Combinations of the EPF and Spinosad in the study found time-to-kill reduced by 50% when compared to EPF alone and up to 75% when compared to just spinosad, though again there is little suggestion as to the mechanism behind this relationship.

An area of research gaining ground in the race to make up for the rapid loss of conventional controls is the chemical ecology of wireworm and the manipulation of their behaviour to directly kill, or more specifically target them within the soil (Vernon *et al.*, 2016; la Forgia & Verheggen, 2019; Poggi *et al.*, 2021). Barsics *et al.* (2016) carried out research on which compounds may be most attractive to wireworms within the soil, emphasizing that whilst CO_2 may be an initial long-range kairomone, plant specific volatile compounds (VOC) will indicate how suitable a food source may be. Johnson & Nielsen (2012) also propose that CO_2 may act as a 'trigger' for subterranean root-feeding insects to intensify search efforts for more specific VOCs from suitable hosts. The study from Barsics *et al.* (2016) identified four aldehydes (hexanal, (E)-hex-2-enal, (E)-non- 2-enal, and (E,Z)-nona-2,6-dienal) using gas-chromatography – mass spectrometry (GC-MS) emitted from barley roots as VOCs. The conclusion of the work noted that whilst there was attraction and orientation towards a blend of the synthetic VOCs, it was not to the same extent as the live root samples; attributed to the lack of CO_2 emission. Nevertheless, this work points towards the possibility of synergy between a source of CO_2 emission and a more specific VOC for increased attraction and thus greater wireworm mortality and less tuber damage.

Although wireworm are recognized as polyphagous pests, little is known about host plant preferences and location (Staudacher *et al.*, 2011; Traugott *et al.*, 2015), with some suggesting little impact beyond general semiochemicals such as CO_2. Some limited work within potato crops has suggested that glycoalkoids may be a significant factor in identifying varietal susceptibility to wireworm (Johnson *et al.*, 2008), in addition to various volatiles discussed identified in both barley and maize (Barsics *et al.*, 2016; la Forgia & Verheggen, 2019). In some instances, the natural production of secondary metabolites and VOCs from various plants into the rhizosphere may initially serve as a chemically-mediated defence mechanicsm, though through possible habituation of a robust pest species (such as wireworm) may be serve as a phagostimulant or attractive cue (Johnson & Gregory, 2006; Johnson & Nielsen, 2012).

The source of potential VOCs to be used in an attract and kill system has so far only addressed in a handful of studies (Gfeller *et al.*, 2013; la Forgia *et al.*, 2020). More widely studied is the suitability of whole crops to be used as attractants in either the form of a planted trap crop (Adhikari & Reddy, 2017) or a buried bait trap (Parker & Howard, 2001; Vernon *et al.*, 2003). Wheat, *Triticum aestivum* L., has commonly been identified as a candidate for use as a trap crop for wireworm, and similarly used as a bait trap for sampling. Wheat used as an intercrop planted in advance of strawberries has been demonstrated to reduce mortality incurred through feeding damage of the economic crop by over 20% (Vernon *et al.*, 2003). Furthermore, the result was achieved in the absence of a biological or chemical control agent. Later research from this group found that wheat, oats, barley and rye all demonstrated suitability for use as bait for wireworms, and thus trap crops also (Vernon *et al.*, 2003), though concluded that suitability for use with specific crops should be studied further.

Although a growing area of study, the chemical ecology of wireworm responses with the soil and rhizosphere is lacking within the literature. Observations have been made at least on *A. obscurus* and *A. lineatus* of sensillae on the maxillary and labial palps used in the klinotaxic detection of VOCs (Crombie & Darrah, 1947; Barsics *et al.*, 2013). Early work towards the study of chemoreception in wireworm focused on their orientation towards extracted tuber and root juices, as well as specific sugars, amino acid compounds and botanicals (Thorpe *et al.*, 1946; Crombie & Darrah, 1947). The behavioural work carried out in these early studies focused on small pot laboratory studies evaluating wireworm responses to chemicals through general location in the substrate and through 'biting' behaviours observed on treated filter paper. There is a need to scale any promising compounds identified through this earlier material to more semi-field experiments to more thoroughly classify behavioural responses of wireworm to novel compounds, in particular essential oils, to take advantage of a rapidly expanding area of biopesticide research (Pavella & Benelli, 2016; Isman, 2020).

These earlier works were undertaken for the purpose of improving bait trapping techniques at the time, with control largely focused on now-banned chemical means. Nevertheless, it remains that this early research may contribute towards finding potential kairomones in the development of an integrated pest management system for wireworm control. Research has not currently caught up to provide adequate control to account for loss of conventional methods, and this early work may provide the answer to identifying or improving suitable biological controls.

More recent study has begun to build on the findings in this older research, for example Horton *et al.* (2012) identified the phagostimulant properties of several sugars in assays with wireworm, combining the positive effects with botanicals to enhance the effects. Additionally, there are a wide variety of secondary plant metabolites or VOCs identified that produce a whole suite of responses in insect pests and beneficials (van der Meijden, 1996; Bruce & Pickett, 2011). Whilst this can range from antifeedant to insecticidal and a host of other interspecies and inter-trophic effects, the semiochemical properties of these metabolites, as attractants or repellents, may provide a solution to improving the efficacy of existing biocontrols such as entomopathogenic fungi or nematodes (Cherry & Nuessly, 2010). A renewed interest in essential oils as naturally derived insecticides or semiochemicals may provide an underexplored avenue for direct control or greater targeting of subterranean pests, like wireworm, with other entomopathogens. In Table 1 may be seen a selection of essential oils or plant extracts, their potential mode of action and insect groups that have had some bioactivity identified in response. Primarily these are oils which have been further explored within this thesis for their bioactivity towards wireworm, with neem and garlic included as comparisons already well established within the literature. Predominantly, studies focus on both stored product and foliar pests (Rosette et al.; Umpiérrez et al., 2017; Chaudhari et al., 2021; Zimmermann et al., 2021), with subterranean invertebrates, including wireworm, a comparatively ignored study area for the use of essential oils ((Thorpe *et al.*, 1946; Waliwitiya, 2005; Brandl *et al.*, 2016)

Table 1.1 Examples of essential oils and their potential mode of action. Includes those screened for study within Chapter 2, 3 & 4 of this thesis and includes Neem and Garlic as two regularly studied plant extracts within IPM. Indicates focus of study on whether they have incorporated insecticidal effects and / or behavioural responses

Plant		Mode of Action	Insecticidal Effects	Behaviuoral Response	Potential Insect Targets	References
Common	Binomial					
Rosemary	*Rosmarinus officinalis*	Affect GABAergic receptors; acetylcholinesterase enzyme inhibition	✓	✓	Hemiptera, Coleoptera, Lepidoptera	Tak et al., 2015; Mossa AH, 2016; Amiri & Bagheri, 2021;
Tea Tree	*Melaleuca* spp.	Neurotoxic, limited effects of acetylcholinesterase (AChE) and Glutathione S-transferase (GST) inhibition.	✓	✓	Diptera, Ixodida, Acari	Jang *et al.*, 2016;
Citrus spp.	*Citrus*	Potentially affecting GABAergic receptors, Octopaminergic systems or disrupting endocrine and respiratory systems	✓	✓	Coleoptera, Hemiptera, Lepidoptera	Kanikor *et al.*, 2021
Cedarwood	*Cedrus atlantica*	Non-toxic mode of action or unexplored	✓	✓	Isoptera, Lepidoptera, Siphonaptera, Coleoptera	Baker *et al.*, 2018
Neem	*Azadirachta indica*	Neurotoxicity through binding to actylcholine receptors	✓	✓	Lepidoptera, Diptera, Coleoptera, Hemiptera	Grdiša & Gršić, 2013; Boate & Abalis, 2020
Garlic	*Allium sativum*	Lectin interaction with midgut receptor proteins	✓	✓	Hemiptera, Lepidoptera, Coleoptera	Upadhyay & Singh, 2012; Plata-Rueda *et al.*, 2017

1.3 Overview of Thesis

With a loss of effective conventional controls and limitations on arable land available for growing potato in the UK, there is a pressing need for a sustainable IPM solution to wireworm control, with scope to incorporate novel biological controls. Promising initial research in entomopathogens, bacterial derived insecticides, biofumigation & plant derived extracts and metabolites provide an exciting future for crop protection against the pest. However, there is no single commercially viable solution and it is clear that a multi-pronged approach incorporating aspects of each of these must be considered in place of the 'silver bullet' approach of sole applications of conventional insecticides. Little is known about how these biological solutions interact with one another or whether they

might be compatible at all but through linking historical research to a growing research base of wireworm chemical ecology this thesis aims to suggest a route forward.

This thesis takes a multi-faceted approach to improving the biological control of wireworm in potato crops. The work firstly builds on historical data on wireworm behaviours to identify and screen a range of botanical extracts for their bioactivity towards wireworm in **Chapter 2** (Lees, 1943; Thorpe *et al.*, 1946; Crombie & Darrah, 1947). Of these, five essential oils – lemon, citronella, tea tree, rosemary and cedarwood were taken forward for detailed study. These were evaluated with both mortality and behavioural assays in both laboratory and field studies with projections for future applications.

In **Chapter 3**, a novel methodology was proposed for the evaluation of subterranean invertebrate behaviours in a semi-natural arena. This was tested using *Agriotes sp.* wireworm as a model species, examining the plant preferences of the larvae and categorising responses to semiochemicals identified in Chapter 1. Identified botanicals were screened against strains of *M. brunneum* found to be most pathogenic towards wireworm in **Chapter 4**. After determining required concentrations in fungitoxicity assays, botanicals were tested in synergy with EPF for use in an integrated control approach, both stress-and-kill and lure-and-kill. Fieldwork was carried out with a commercial EPF biopesticide to highlight areas of improvement for the control method. Finally, **Chapter 5** uses identified VOCs of known entomopathogens and behavioural antagonists of wireworm to propose further control solutions. Both 1-octen-3-ol and 3-Octanone, volatile constituents of *M. brunneum*, were assessed for bioacitivty against both wireworm and a commercial strain of EPN. Potential for synergy between the two was evaluated, with suggestions for inclusion in a push-pull or direct fumigant application for wireworm control.

1.3.1 Aims

The overall aim for this thesis was to determine novel strategies for the control of wireworm within potato crops by identifying and evaluating bioactive compounds for both their suitability for direct control and compatibility with existing entomopathogenic biocontrols.

- **Chapter 2: Identify botanical extracts that exhibit bioactivity towards wireworm and quantify the behavioural and insecticidal effects.**

- **Chapter 3: Demonstrate the use of a novel methodology to evaluate and quantify subterranean invertebrate behaviours in a semi-natural arena**
- **Chapter 4: Determine the fungitoxicity of identified bioactive botanicals toward *M. brunneum* and evaluate synergistic potential with one another in an integrated control approach for wireworm.**
- **Chapter 5: Evaluate bioactive effects of VOCs obtained from *M. brunneum* on both wireworm and entomopathogenic nematodes to determine suitability for an integrated control approach for wireworm**

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Chapter 2: Bioactivity of Essential Oils Against Wireworm and Their Potential as Bioprotectants in Potato Crops

Abstract

The loss of conventional controls for wireworm (*Agriotes* spp.) and the larvae's ability to render potato crops unmarketable has given rise to a need for alternative, environmentally friendly control options. Prior to the implementation of broad-spectrum chemical insecticides in the mid-20th century a range of sugars, acids and botanicals were screened against wireworm to examine their behavioural effects. Botanicals extracts have gained traction as alternative biological control options within integrated pest management systems for both their insecticidal and semiochemical properties, often with the benefit of fewer environmentally negative side effects. However, their effects on subterranean invertebrate pests, like wireworm, is understudied. Here we demonstrate the bioactivity of tea tree, rosemary and cedarwood oils against *Agriotes* larvae as both fumigants and semiochemicals with the soil matrix. It was found that both tea tree and rosemary exhibit repellent effects on wireworm, positively correlating with mortality for each, with a suppression of feeding response in laboratory assays. The converse was found for cedarwood, with strong attractant effects observed, and no insecticidal effects against the larvae, and evidence of phagostimulation. These properties were reduced within the field, though comparable effects were observed in areas of lower infestation. It is suggested that there is strong potential for development of a novel control solution for wireworm using these botanicals, with formulations taking advantage of the specific bioactive constituents or through development of push-pull systems benefitting from their behavioural effects.

2.1 Introduction

In the earlier part of the 20th century a range of biological compounds were screened against wireworm for their potential chemotactic and anti-feedant properties (Falconer, 1944; Thorpe *et al.*, 1946; Crombie & Darrah, 1947). These included various acids, sugars, and essential oils (Thorpe et al. 1946). The content of these studies focused on behavioural bioassays in simple substrate filled choice arenas, in which feeding behaviours were quantified by counted bit marks on treated filter paper and wireworm location after a set duration. The limitation of these experimentations on accurately defining the observed behavioural response beyond a binary attraction or repellence is addressed and this thesis aims to, in part, address these methodological hurdles in studying subterranean invertebrates (Chapter 3). Interest in this research was largely put aside following the introduction and widespread use of chemical insecticides (Parker & Howard, 2001).

There has been a renewed interest in botanicals and other natural products for the control of wireworm and other soil dwelling pests (Horton *et al.*, 2012; Brandl *et al.*, 2016; Atanasova & Leather, 2018). Key drivers include the withdrawal of many conventional chemical pesticides due to the risks they pose to humans and the environment, and Directive 128/2009/EC which obliges EU members to use non-chemical methods of control. The use of botanicals can be low-risk and cost-effective, as well as environmentally safer than conventional alternatives (Regnault-Roger *et al.*, 2011; Pavela & Benelli, 2016).

One of the most challenging pests to control is the wireworm, the subterranean larval stage of the click beetle (Barsics *et al.*, 2013; la Forgia & Verheggen, 2019). A constant hurdle in combating soil dwelling pests with a heterogenous distribution within a crop is accurate targeting of infestations. Behaviour modifying chemicals or semiochemicals can play a crucial role. Attractant compounds could be used to monitor pests or lure them to a control agent (Vidal, 2013; Brandl *et al.*, 2016). These may be further used within a push-pull strategy in which repellent compounds could protect plants and drive them towards a trap crop where the pest would be more concentrated and easier to control. Some botanicals have been shown to have insecticidal activity (Karamaouna *et al.*, 2013; Benelli *et al.*, 2017). A revived interest in botanicals, especially essential oils, has identified a wide range of plant compounds that show promise for use in pest management programmes (Isman, 2020). Most studies focus on the management of foliar and stored grain pests (Rosette *et al.*; Umpiérrez *et al.*, 2017; Chaudhari *et al.*, 2021; Zimmermann *et al.*, 2021). There are comparatively few studies on the use of

essential oils for control of soil dwelling pests such as wireworm. This study focuses on evaluating selected essential plant oils, including promising candidate compounds identified in the 1940's, for use in wireworm pest management (Thorpe *et al.*, 1946; Waliwitiya, 2005; Brandl *et al.*, 2016).

2.1.1 Aims

- **Identify botanical extracts that exhibit bioactivity towards wireworm and narrow down the selection based on the strength of the response**
- **Quantify the insecticidal and behavioural effects of identified botanicals towards wireworm**
- **Evaluate botanicals in laboratory, semi-field, and field experiments, to conclude on their suitability for inclusion in IPM strategies for wireworm control and areas for synergy to improve quantified effects**

2.2 Materials & Methods

2.2.1 Maintenance of Insects

Agriotes lineatus larvae were initially provided by Applied Plant Research of Wageningen UR (Praktijkonderzoek Plant & Omgeving van Wageningen UR, PPO). Additional wireworm were collected from agricultural soils in Pembrokeshire, Wales. On-farm collections were carried out during field preparation in May and April during the destoning process with 50 – 300 individuals collected per day. Up to 1000 individuals were maintained in culture at any one time. Establishing a larval colony may be difficult as raising hatched specimens from trapped adults will take at least two years to attain an experimentally appropriate instar, and the in-field collections rely heavily on labour intensive methods and experience in spotting exposed larvae.

Larvae were maintained in 1L pots filled with a blend (1:1 v/v) of loam and sand and kept in controlled environment chambers at 15°C ± 1°C (60% RH ± 5%, 16:8 L:D) photoperiod and fed with fresh potato slices (approximately 1 x 1 x 2cm, inserted centrally and flush with the soil surface) every three days. Five larvae were contained in each pot. Larvae were starved 2 weeks before each experiment to encourage host seeking behaviour. Late instar larvae were used in studies, with the age being determined on length and weight (Furlan, 2004).

2.2.2 Essential Oils

Eleven essential oils (Table 2.1) were selected from literature that suggested a potential chemotactic or feeding stimulant effect in either a wireworm species, or similar subterranean crop pest (Thorpe *et al.* 1946; Waliwitiya 2005; Brandl *et al.* 2016). The oils, prepared by steam distillation, included: tea tree (*Melaleuca alternifolia*, Cheel), rosemary (*Salvia rosmarinus*, Spenn), citronella (*Cymbopogon winterianus*, Jowitt ex Bor), lemon (*Citrus limon*, L.), cedarwood (Atlas, *Cedrus atlantica*, Endl.), caraway (*Carum carvi*, L.), carrot seed (*Daucus carota* subsp. *sativus*, Hoffm.), geranium (*Pelargonium graveolens*, L'Hér), and coriander seed (*Coriandrum sativum,* L.). All the oils were obtained from BiOrigins (Madar Corporations Ltd) except grapefruit and garlic which were purchased from Sigma Aldrich, the oils were kept in sealed 1.1 L aluminium bottles at room temperature in the dark to prevent photooxidation. The oils were assayed as outlined in section 2.2.4 and the five oils eliciting the strongest response (rosemary, lemon, citronella, cedarwood, tea tree) were selected for more in-depth evaluation. The relative abundance (>0.5% by peak area) of constituents of these oils was determined by GC/MS at the start and end (1 year later) of the study with no significant differences recorded. Full GC/MS analyses may be seen in Chapter 3. Oils were applied neat, without carrier, unless otherwise specified.

Table 2.1 Initial screening of essential oils in a terrarium soil assay. Behavioural observations carried out at regular intervals and responses were averaged from 5 replicates over a 48-hour period.

Botanical	CAS No.	Observed behavioural response	Inclusion for further study (Y/N)
Rosemary	8000-25-7	Negative; repellent effect	Y
Lemon	8008-56-8	Negative; repellent effect	Y
Grapefruit	8016-20-4	No obvious response	N
Citronella	8000-29-1	Negative; repellent effect	Y
Caraway	8000-42-8	No obvious response	N
Carrot Seed	8015-88-1	No obvious response	N
Cedarwood	8000-27-9	Positive; clear orientation towards source, possible attractant	Y
Tea tree	85085-48-9	Negative; repellent effect	Y
Geranium	8000-46-2	No obvious response	N
Garlic	8000-78-0	Some apparent anti-feedant effects, minimal response	N
Coriander seed	84775-50-8	No obvious response	N

2.2.3 Gravimetric Analysis of Essential Oils

To determine the volatility of oils as an indication of duration bioactivity, gravimetric analysis was carried out under controlled conditions (24°C ± 2°C, 60 % RH ± 10%). Filter tips (6 mm diameter, Sharrow Ltd) were inoculated with either 100 or 500 µl of oil and weighed initially (Sartorius Microbalance, sensitivity: 1 ng), with subsequent measurements taken at 3, 6, 12, 24, 48 hours and then every 7 days thereafter until 21 days. Filter tips were left unsealed in 20 ml glass vials to avoid contamination or absorption into any porous material. Two volumes were used to gauge the effects of loading capacity on the rate of gravimetric loss.

2.2.4 Fumigation Studies using Petri Dishes

A Petri dish fumigation assay was used to determine the fumigation properties of the oils as outlined by Khoja *et al.* (2019). Briefly, a filter disc (6 mm diameter, Whatman® Intl. Ltd.) was adhered to a glass coverslip using the liquid tension of applied essential oils, which had been fixed to the lid of a 9 cm diameter Petri dish, in turn using the water tension of a drop of distilled water. This ensured no contact between the oil and the plastic of the arena. The discs were treated with 5, 10, 20 or 50 µl of oil. The base of the Petri dish was lined with filter paper (Whatman® Intl. Ltd.) and covered with 5 g of soil mixture (4:1 Multipurpose compost with John Innes: Vermiculite). The compost for the soil mixture was selected for two primary reasons – the consistent availability of the product and the texture and density allowing natural tunnelling behaviours whilst retaining moisture consistently to avoid desiccation. Exact data on the organic matter (OM) content of the soil was not found, or analysed, but as the John Innes component was expected to raise the percentage of OM, any clear behavioural responses were considered strong. A preference for humus rich soils has been observed in wireworm, and specifically *Agriotes* spp., though the effect this may have on foraging behaviours and feeding habits is understudied (Gui, 1935; Cherry & Stansley, 2008; van Herk *et al.*, 2021).

Soil was desiccated in a drying chamber for 48h prior to experimentation to minimise condensation within the dish and ensure consistency of conditions, the arena was then covered with 1 ml of water using a pipette to avoid larval desiccation. This was found to reduce condensation for the duration of the study period almost completely and ensure larval survival in the controls.

A single larva was introduced to the centre of the Petri dish and the arena was sealed with Parafilm®. After a 30-minute acclimation period, wireworm behaviour and mortality were noted at 3, 6-, 12-, 24- and 48-hour intervals. Larvae were determined to be either 'alive' (active movement, regular activity in palps and / or antennae, or static position with dorsal surface showing at rest), 'moribund' (inactive, possible slight twitching of palp and / or antennae, body at rest on unnatural lateral surface), or 'dead' (inactive, no movement in palps and / or antennae, body in apparent rigor mortis on lateral surface). Behavioural categories for the resultant ethogram were adapted from van Herk *et al.* (2015). Petri dishes were not opened to assess larval behaviour with any ambiguous replicates observed under dissection microscope for a maximum of one minute to not further influence larval reactions. This was sufficient in all cases and used only to differentiate between moribund and dead individuals. The experiment contained 15 replicates for each dose.

2.2.5 Concentration Dependent Behavioural Response – Petri dish fumigation experiment

Behavioural observations were conducted in the Petri dish arenas as in 2.2.4, in fumigation dose studies with identical conditions. Larvae were exposed to 20 µl of oil at 50, 10 and 1 % concentrations, diluted with DMSO (v/v). Observations were made at 3, 6-, 12-, 24- & 48-hour time points and behaviours were recorded in an adapted ethogram detailing proportion of time exhibiting behaviours along with any evidence of mortality. Larval observations were carried out without opening Petri dishes, as in 2.2.4, with each larva observed for five minutes at each time point. Behavioural categories for the resultant ethogram were adapted from van Herk *et al.* (2015) as in 2.2.4. The experiment contained 15 replicates for each concentration.

2.2.6 Soil Fumigation

To assess the buffering capacity of soil on the efficacy of the oils in killing wireworm, assays were conducted in soil filled tubes. Universal tubes (30 ml, polystyrene) were filled with 5 ml of soil mixture (as in section 2.4) and a filter tip (50 mm x 7.13 mm filter tip (Sharrow, Wilson & Co. Ltd)) treated with 6, 12 or 30 µl of oil (Tea Tree, Rosemary or Cedarwood) was placed on top. This was then covered with an additional 20 ml of soil and left for one hour uncovered to permeate through the soil. One larva was then introduced, and the tube covered with 5 ml of substrate and sealed, then left in complete

darkness in controlled conditions (24°C ± 2°C, 70 % RH ± 10%). Larvae were checked every 24 hours for position within the tube and for their condition (alive, moribund – twitching thoracic legs & mouthparts, or dead – immobile legs & mouthparts with characteristic rigor mortis). Immobile individuals (i.e., those presumed dead) were removed to fresh soil to see if they recovered by burying themselves in the soil. Larvae were monitored for active feeding seven days post removal from experimental arena. Fifteen replicates were used for each treatment and an untreated control.

2.2.7 Soil Fumigation – Degradation effects

Degradation of the oils was determined by repeating the above soil fumigation assay at the highest bioactive concentration but introducing the larvae 1, 2 and 3 weeks after initiating the experiment. Tubes were sealed with Parafilm® until insertion of the larvae, and then sealed with the cap and checked at 24-hour intervals for mortality and position within tube until a final 96-hour check. Immobile individuals were removed as outlined earlier to see if they recovered. There were 15 replicates per oil and for each time point.

2.2.8 Terraria Studies with and without Plants

Terrarium experiments in the absence of plants were conducted in a greenhouse (24 °C ± 2 °C, 63.5 % RH ± 15%) to directly observe wireworm responses to the oils. Terraria were filled with a blend of a multipurpose compost containing John Innes and vermiculite (4:1, v/v, 550 g ± 4 g). Each terrarium consisted of two glass plates (37 x 27 cm) with a 0.6 cm space between the clear panels (Figure 2.1). Spacer bars were placed at the edges of plates with manual wingnut fasteners for simple deconstruction and sterilisation. During experimentation terraria were placed vertically in complete darkness in controlled environment chambers (24°C ± 2°C, 70 % RH ± 10%).

Terraria were divided into arbitrary coordinates in which x ranged from 0 – 10, and y from 0 – 8. A control filter tip (20 µl water) was inserted at point x = 2.5, y = 4.5, and a treatment filter (20 µl oil) at x = 8.5, y = 4.5. A single wireworm was introduced at the centre line, approximately 5 cm deep and covered with substrate. After a 30-minute acclimation period, larval location was recorded every 30 mins over a 6hr period and then again at 24 hours. There were ten replicates per treatment. For each treatment, a mean percentage repellency (MPR) score was calculated as follows:

$$MPR\ (-100\ to\ +100) = \frac{(T_c - T_t)}{(T_c + T_t)} \times 100$$

Where T_c and T_t are equal to the designated control and treatment halves of the terrarium, respectively. The MPR score ranged between -100 and +100 with the most repellent products having a score of +100 and the attractant having a score of -100. A zero-value denoted that the oil was neither a repellent nor an attractant.

Larval behavioural responses to oils in the presence of plants was investigated using methods adapted from Brandl *et al.* (2016). Each terrarium had a 10 x 8 cell grid overlay with arbitrary coordinates applied for spatial analysis. These were filled with substrate (as above, 550 g, ± 10g), at 20% (v/v) water capacity.
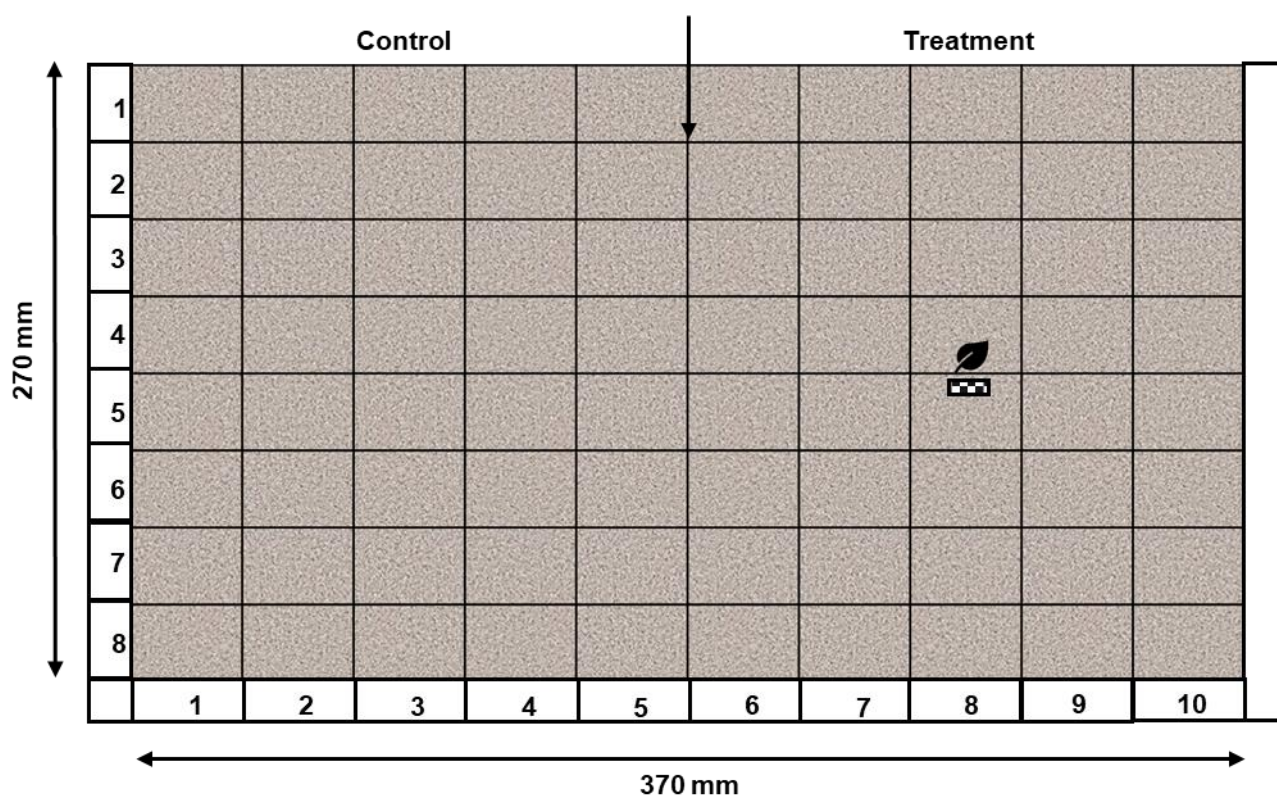
Fig. 2.1 Diagram of terraria set-up indicating germinated seed and treated filter tip at point x = 8, y = 4.5. The superficial grid is overlaid on the screen surface to aid in spacial analysis, with each cell 37 x 34 mm. The point of insertion for wireworm is indicated by the central arrow at x = 5.5, y = 1.5.

Germinated maize seeds (BBCH 08) were placed at point x = 8, y = 4.5, adjacent to a treated filter tip inoculated with 200 µl of essential oil. One-hour post-inoculation a single wireworm (late instar larvae, at least 1.5 cm length) was introduced to point x = 5.5, y = 6.5 and entry point covered. Terrariums were covered and left in complete dark at 24°C ± 2°C, 70 % RH ± 10%. Wireworm position was recorded at 2, 4 and 6 hours for initial behavioural response and to ensure larval survival. Subsequent checks at 12, 24, 48

and 72-hour were collated for analysis of behavioural response to introduced stimuli. Controls included untreated maize seed to ensure a feeding response and no seed, to ensure a random foraging response in the absence of stimuli. Larvae used in experimentation were not used for subsequent studies.

2.2.9 Germination – Fumigation of Seeds Exposed to Oils

Since the oils may be deployed during planting or sowing, germination assays were conducted in 9cm diameter Petri dishes at constant conditions (24 $^{\circ}$C ± 2 $^{\circ}$C, 70 % RH ± 10%), within an incubator in the absence of light. Either three pre-soaked maize (*Zea mays* L.) seeds or cut potato eyes (*Solanum tuberosum* L., var. 'Maris piper', tuber plug approximately 0.5 cm^3) were placed on two 90 mm Whatman filter papers (soaked with 2 ml water) in dishes. Filter discs were inoculated with 5, 10 or 20 µl of essential oil, adhered to a glass cover slip, held on the inner surface of the lid with water tension. Petri dishes were then sealed and checked daily for germination. The effect of the oils on successful germination, speed of germination, and for maize the length of successfully emerged radicles were measured over an 8-day period. Successful germination was determined by the emergence of radicles from maize seeds or potato eyes, with a binary success or failure recorded over eight days. Three seeds were used per replicate and four replicates were carried out.

2.2.10 Field Experiments

Field trials were conducted to determine the field efficacy of the repellent (tea tree, rosemary, lemon, citronella) and attractant (cedarwood) oils. The oils were dispensed using biodegradable vials with a 3mm diameter aperture. Each vial contained 1 ml of pure oil and was inserted into the ridge of a potato crop.

Order of treatments applied in a row (Figure 2.2) was randomised by allocating each treatment and control an integer from 1 to 6 and using the runif function in R (Version 1.0.153 – © 2009-2017 RStudio, Inc). From this start point each treatment was shifted along for each of the 10 rows to ensure an even distribution for each treatment within the limited field plot, to counteract a heterogenous pest population. Each plot was approximately one metre square, containing 10 plants. There was a minimum distance of one metre and an untreated row of plants between plots to minimise cross-interference of treatments. Ethoprophos (MOCAP 15g) was used to treat the entire field at bed preparation. This organophosphate based granular insecticide (15%
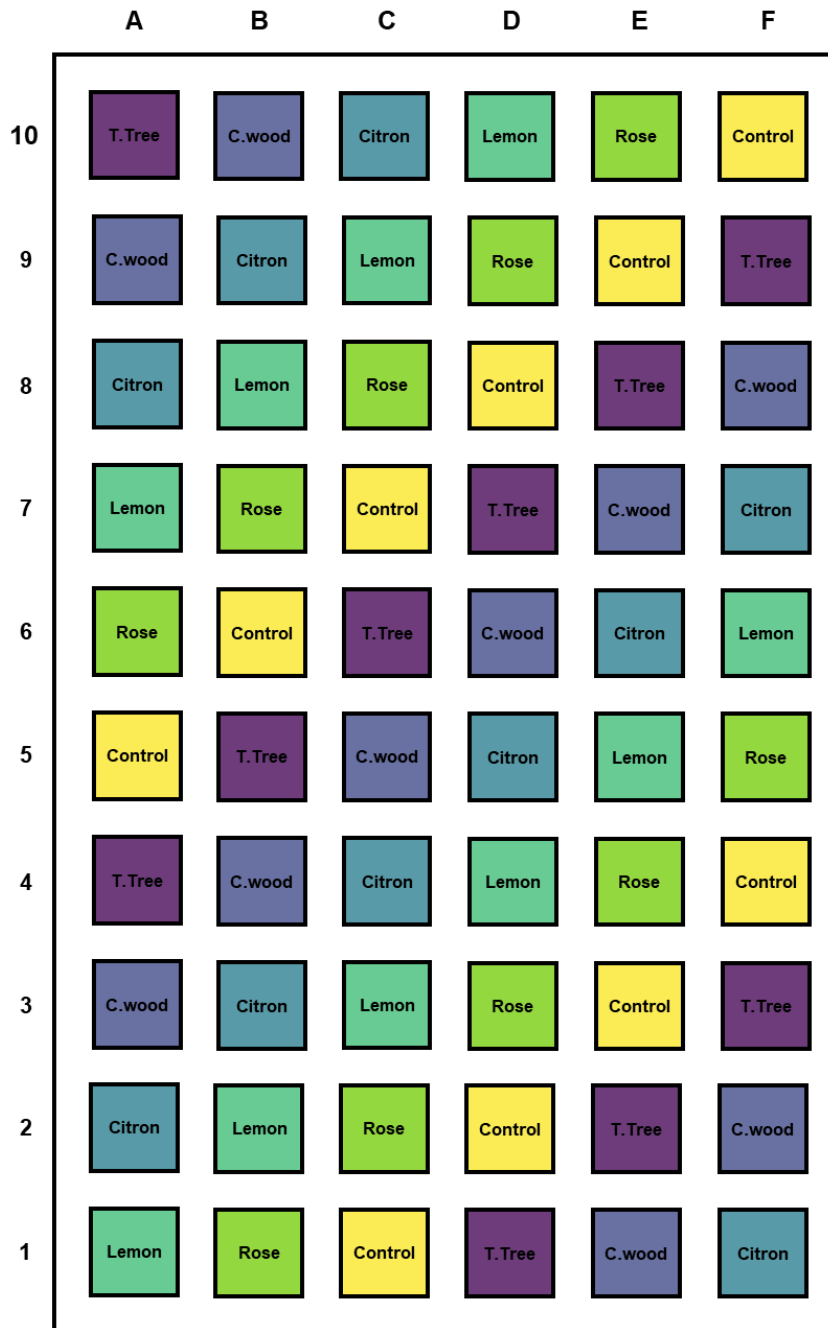
Fig 2.2 Trial plot lay out. Individual plots measured 1 m^2 with at least 1 m of untreated crop between. Row one was randomised and treatments shifted across one co-ordinate up to row 10, to account for a heterogenous population within the field as much as possible. MOCAP treated plot replicates were obtained from 1 m^2 plots placed randomly within the main crop due to necessitating tractor mounted application. Where 'T.Tree' = Tea Tree, 'C.wood' = Cedarwood, 'Rose' = Rosemary, 'Lemon' = Lemon & 'Citron' = Citronella. 'Control' were untreated plots.

ethoprophos, w/w) is now de-registered as a control within the UK but was licensed for use against potato cyst nematode (PCN) and for a reduction, rather than control, of wireworm numbers. As a result of application timing and method, it was included as a reference to applied treatments but not as a true control. MOCAP treated tubers from the main crop were similarly harvested in 1m^2 plots, located at random intervals (at least

two metres apart) outside the main trial area, at a maximum distance of three metres away from the border of the trial plot. As such, only harvested tubers were compared to MOCAP plots, with spatial analysis confined to the main trial plot boundaries.

The vials were placed at intervals of 10 cm to ensure an even distribution over the plot (seed tubers planted 25 cm apart) and manually injected with an adapted tool (metal rod, 50 cm in length, with terminal welded 'socket' for vial placement, marked at 5 cm intervals) to a depth of 15 cm, adjacent to the tubers where necessary. Application of treatment was carried out immediately post-topping of the main crop's haulm (8-weeks post planting for the Angle site and 7-weeks post planting for Marteltwy) to establish treatments for the late season feeding period of wireworm. This also ensured the bioactivity of oils for a six-week period until harvest. Gravimetric studies for biovials were used to determine release rates and hence the volume and quantity of vials necessary for each plot.
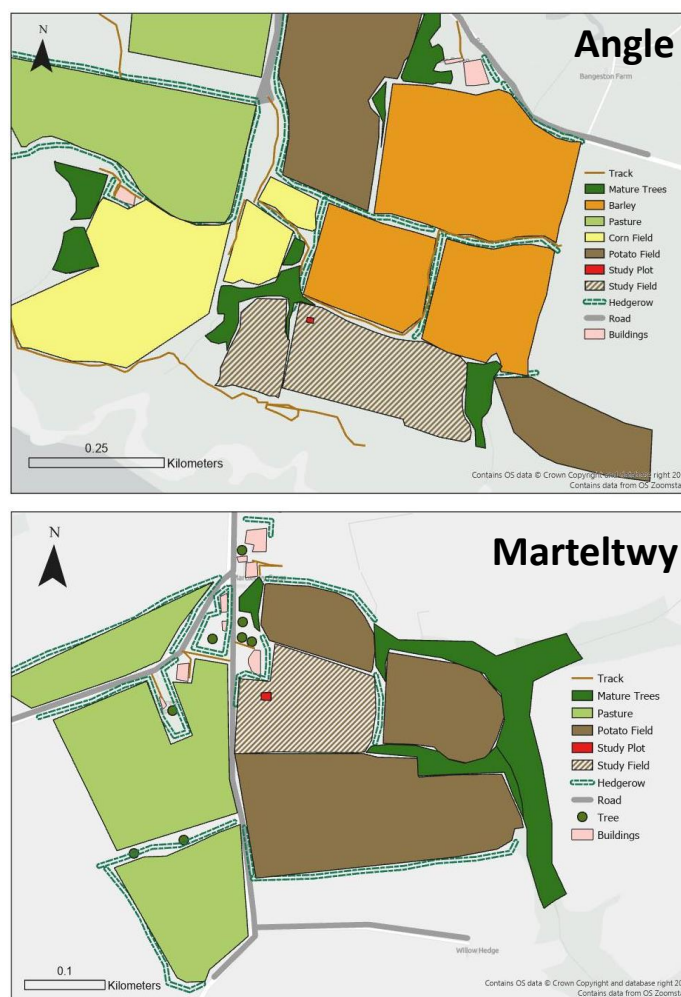
Fig. 2.3 GIS maps of field sites indicating surrounding field usage at time of trial and position of trial plots within potato crop.

Two farm sites (Angle, Marteltwy) 40 miles apart, in Pembrokeshire, Wales, previously used as pasture were used for field trials (Figure 2.3). Both sites were sampled for wireworm prior to selection. Sampling consisted of walking five transects (100m in length, at least 20 m apart) following de-stoning machinery, with an average of one larva being recovered every five meters from overturned soil in the Angle site, and every ten meters in the Marteltwy site. The Angle site was planted with early varieties (experimental plot was 100% 'Orla') and situated on headland with a soil-scape of freely draining, slightly acid, loamy soil. The Marteltwy site was similar but was planted with maincrop varieties (experimental plots was 100% 'Maris Piper'). Each site was managed by the same grower and only differed in the timings of varieties, planting and harvest. Figure 2.2 shows the sites and surrounding land usage which provided favourable wireworm habitats.

Tubers were harvested manually and separated into treatment and plot replicate number for assessment. Only final damage was assessed due to lack of regular access to the field site across the growing season and no regular sampling. Wireworm numbers at harvest were compared though with no initial baseline for comparison, considered a significant weakness of the study. Each replicate was visually assessed for presence and quantity of disease and pest damage – bacterial, fungal, invertebrate (wireworm & slug) and mechanical (machinery) damage – in accordance with commercial quality control. Thresholds for crop acceptance were incorporated as a measure of crop protectant quality, as supplied by the commercial grading team at Puffin Produce Ltd. This was primarily concerned with wireworm and slug damage, in which only damage that remained after more than two peels with a handheld vegetable peeler was counted. Tubers were then weighed and counted as an additional indication of crop quality or phytotoxic effects of the treatment application.

2.2.11 Statistical Analyses

Spatial Analysis by Distance IndicEs (SADIE) was used to investigate spatial patterns as outlined by Winder *et al.* (2019). SADIEShell Version 2.0 software (Conrad, K. F©, 2008) was used to calculate the centre of gravity of counts and aggregation indices for spatial analysis. N_AShell Version 1.0 (Conrad, K. F©, 2008) was used for the comparison of spatial associations between treatments and controls. RStudio Version 1.2.5019 (RStudio Inc©, 2019) was used for ANOVA and production of filled contour heatmaps, with the R package 'epiphy' (version 0.3.4, 2018) used for 'red-blue' plots for visualisation of clustering indices and 'emmeans' (version 1.6.0, 2021) used for post-

hoc analysis and pairwise interactions. The 'dose.p' function from the 'MASS' package (version 7.3-51.6, 2021) in R was used for calculation of LD50, 90 & 95 values. All statistical analyses were carried out with R (version 4.1.2, 2021).

For terrarium assays, through comparison of the counts with randomised arrangements of crowding or irregularity, SADIE was used to identify patches or gaps within the data, providing indices to identify degrees of aggregation, randomness, or regularity (Perry, 1995; Winder *et al.*, 2019).

An index of aggregation (I_a) was used to indicate the extent of spatial association (Perry, 1995, 1998; Winder *et al.*, 2019). Where $I_a > 1$ may be considered an aggregated count, $I_a = 1$ a random dispersion and where $I_a < 1$ individuals are at regularity. Probability thresholds (P_a) for I_a range from < 0.025 for aggregation, $0.025 - 0.975$ for random dispersion and >0.975 for regularity.

SADIE may also indicate the extent to which counts primarily occupy the edge or the centre of a set sampling area through calculation of a centre of gravity (Perry, 1998). This is defined as delta (δ) – the distance between the centroid of the sampling unit (P) and the centroid of the counts within the data (C) (Perry *et al.*, 1999). To assess the degree to which identified treatments attracted or repelled larvae within the arena, a separate metric was created (here δ_1) to measure the distance between C and point of treatment inoculation (T).

Patterns of clustering in the count data were identified by calculating indices for patches (v_i) or gaps (v_j) within arbitrary contours ranging from > 1.5 (v_i) to < -1.5 (v_j), each attributed a significance value P (P_{vi} & P_{vj} for patch and gap respectively). Treatments (for field trials larval damage and presence) were compared between one another and controls to identify similarities or differences in clustering patterns. This index of local spatial association (Perry *et al.*, 1999; Brandl *et al.*, 2016) suggests positive association at $X > 0$ or spatial disassociation at $X < 0$. This metric may be tested for significance (after Dutilleul adjustment for spatial autocorrelation (Dutilleul *et al.*, 1993)) with $P_x < 0.025$ indicating significant association and $P_x > 0.975$ significant disassociation.

2.3 Results

2.3.1 Gravimetric Analysis of Essential Oils

A statistically significant difference was found in gravimetric release rates (Figure 2.4) by both treatment (100 µl: $F_{(4,36)}$ = 159.1, p < 0.0001; 500 µl; $F_{(4,140)}$ = 1189.9, p < 0.0001) and time (100 µl: $F_{(1,36)}$ = 82.81, p < 0.0001; 500 µl; $F_{(1,4)}$ = 407.4, p < 0.0001), with a slower rate of release seen in the larger volume inoculated filters ($F_{(1,4)}$ = 790.61, p < 0.0001).

Only water showed complete evaporation before the end of the study duration (72 hours for 100 µl, 336 hours for 500 µl), and all treatments differed significantly from this control (p < 0.0001). Cedarwood showed a significant increase from initial weight across both volumes (**100 µl**: est = 18.7, t = 5.49, p = 0.0003; **500 µl**: est = 74.822, t = 14.25, p < 0.0001). Conversely, rosemary (**100 µl**: est = -76.1., t = -22.294, p < 0.0001**; 500 µl**: est =-222.041, t = -42.29, p < 0.0001) and tea tree (**100 µl**: est = -46.5, t = -13.628, p < 0.0001; **500 µl**: est = -86.472., t = -16.469, p < 0.0001) both showed a significant loss in volume from initial weights.

For both 100 and 500 µl, water lost volume at the fastest rate, with compete evaporation in each. This was followed by rosemary (**100 ul**: -87.54% total volume; **500 ul**: -49.84%) and then tea tree (**100 ul**: -42.57% total volume; **500 ul**: -19.14). As above, only cedarwood oil showed a gravimetric increase (**100 ul**: +12.29% total volume; **500 ul**: +10.69%).
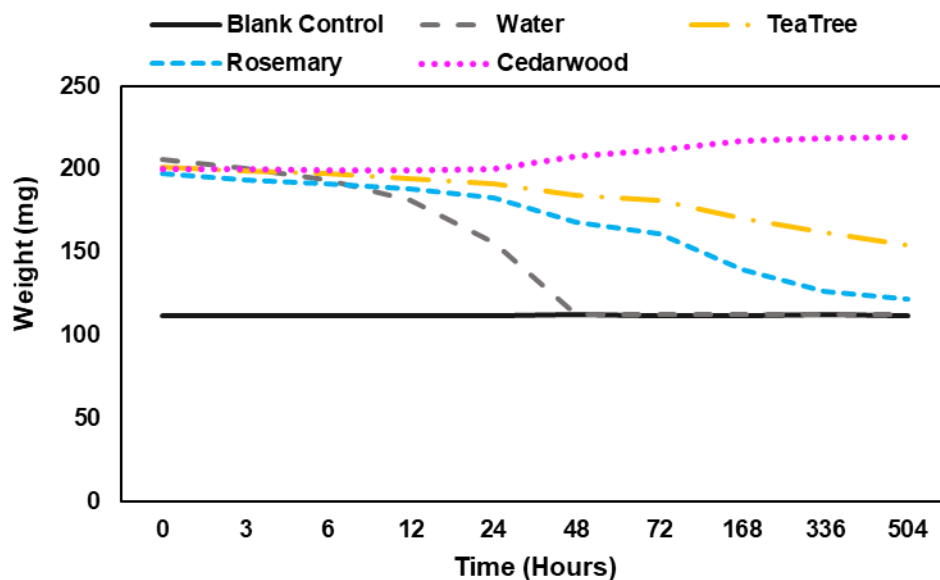
Fig. 2.4 Gravimetric release rates of compounds from filter tips at 100 ul total essential oil. Results at 100 ul correlated with those at 500 ul with no significant difference. Baseline of untreated filter tip may be seen as comparison and guide for release rate

2.3.2 Fumigation Studies Using Petri Dishes
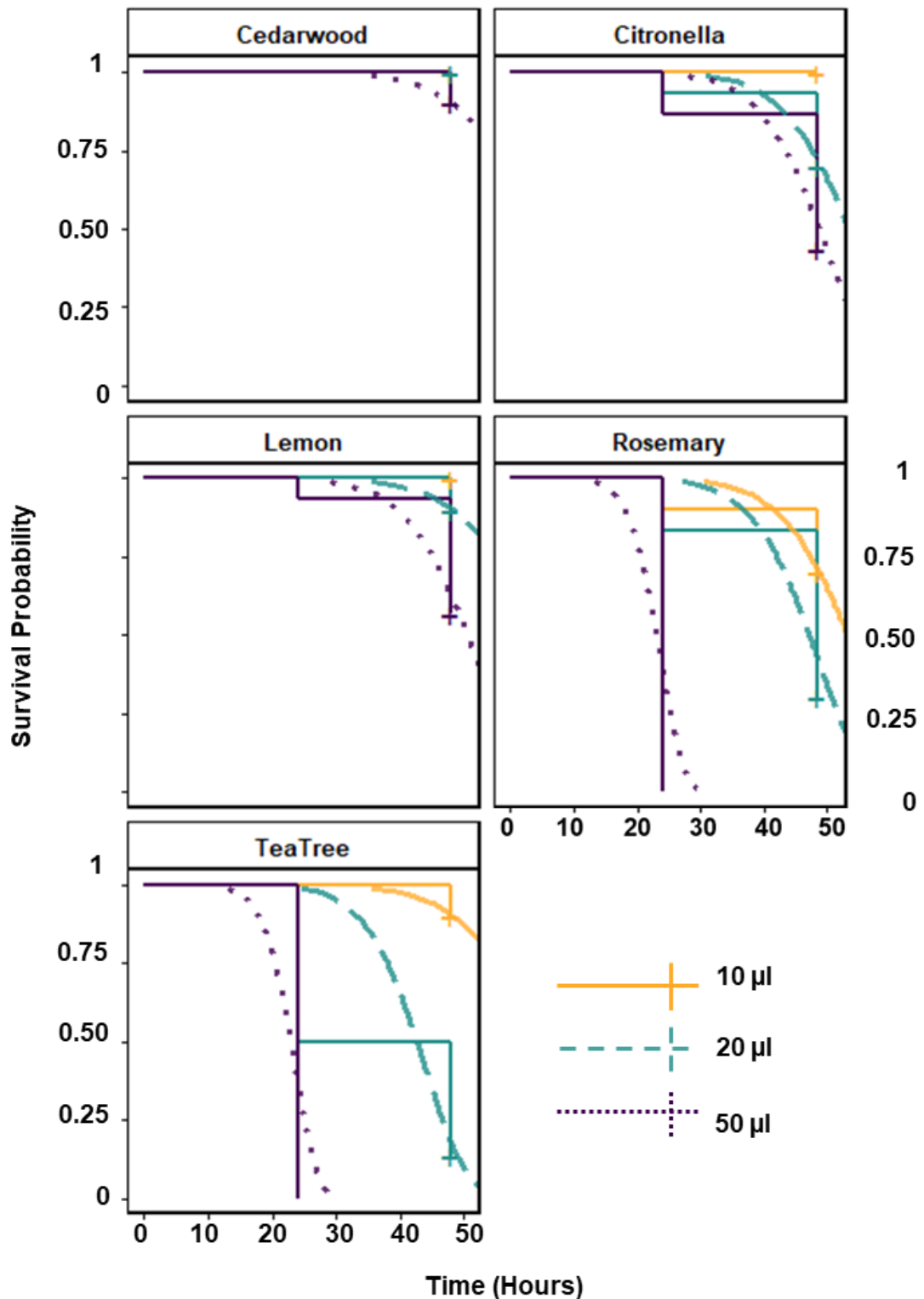
Fig. 2.5. Petri-dish fumigation assay to evaluate dose-dependent response of wireworm mortality exposed to botanical extracts. Kaplan-Meier plots of survival probability for wireworm with fitted survival curves for each dose are as shown in the legend.

Model selection was based on Akaike's Information Criteria (AIC) for both Petri dish and soil fumigation assays. Within Petri dish fumigation assays (Figure 2.5), both treatment and dose were found to have significant effects on both larval survival and between one another (χ^2 = 506.99, df = 9, p < 0.0001). Both rosemary and tea tree oils were found to be the most insecticidal (Table 2.2) with complete mortality at the highest dose (50 µl). Lemon and citronella oils killed no more than 50% of wireworm across the study period at the highest dose, with no clear difference between the two treatments. Wireworm exposed to cedarwood oil showed survival comparable to untreated controls with marginal (< 10%) mortality at the highest dose. Except for cedarwood and untreated controls, there was a clear dose dependent response on larval survival across all treatments, with higher doses greatly reducing time-till-death.

Table 2.2 Lethal dose (LD) 50, 90 & 95 values for wireworm exposed to five essential oils in a Petri dish fumigation assay over 48h. Neat doses were used at 5, 10, 20 & 50 µl. Only two larvae were found dead at the highest dose for Cedarwood treated replicates.

Treatment	LD	Dose	SE
Cedarwood	50	n/a	n/a
	90	n/a	n/a
	95	n/a	n/a
Lemon	50	52.12	4.1
	90	75.4	7.99
	95	83.32	9.54
Citronella	50	43.93	3.86
	90	70.58	7.33
	95	79.64	8.73
Rosemary	50	15.84	1.17
	90	24.34	2.15
	95	27.23	2.59
Tea Tree	50	15.56	0.95
	90	20.62	1.32
	95	22.35	1.54

2.3.3 Concentration Dependent Behavioural Response – Petri dish fumigation experiment

There was no mortality across all treatments at any concentration. Behaviours were observed and recorded in an adapted ethogram. Final ethograms were converted to proportions of time spent in each behaviour for each treatment. The data for the highest concentration (50%) was presented as a proportion of time spent for each time point. Details of both behaviours observed and results can be seen in Table 2.3 (a&b). For cedarwood at the highest concentration, most larvae were observed at rest, with > 60% of individuals observed at all time points except 24 hours. During this period, equal numbers of larvae were observed with active forward movement as at rest (40%), this was likely a foraging response. As no food source was included in the experimental arena, larvae at rest or with regular active forward movement were considered to be the least stressed.

Larvae exposed to tea tree oil at 50% concentration initially spent most of the time (up till 12 hours) in a moribund or resting state. From 12 hours onwards, there was evidence of recovery with increased movement across > 70% of larvae up until a majority return to rest or moribund state at a final 48-hour check.

Rosemary elicited the strongest response in larva exposed at 50%. From three hours up until 12, all larvae were seen to be in a state of extreme agitation, exhibiting behaviours of excessive writhing. This involved a rapid and irregular contraction and release of the body along its entire length, with the larva unable to move from that location. This had slowed to a slower and regular writhing behaviour by 24 hours and at 48 hours, all larvae were observed as moribund.

Table 2.3 (a & b). Proportion of observed behaviours within oil concentration dependent Petri dish fumigation assay, with associated codes for analysis. Description of observed behaviours may be seen in 4a. Proportion of wireworm time across behaviours may be seen in 4b, with values given for highest concentration (50%) as no mortality was recorded across all used (1, 10 & 50%).

Behaviour		Code	Description
Inactive	Rest (Edge)	Re	Larva at rest, no adverse effects, at edge of arena
	Rest (Central)	Rc	Larva at rest, no adverse effects, central in arena, under substrate
	Moribund	M	Larva inactive, on lateral side
	Slight twitching	Slt	Inconsistent but regular movement of legs and sensory organs (palps & antennae) with no locomotion
	Excessive twitching	Ext	Consistent and regular movement of legs and sensory organs (palps & antennae) with no locomotion
Locomotion	Regular movement (Edge of arena)	RegE	Consistent forward locomotion through thoracic leg movement around edge of arena
	Regular movement (Central in arena)	RegC	Consistent forward locomotion through thoracic leg movement within centre of arena
Agitation	Slight writhing	Slw	Larva stationary, with inconsistent but regular contraction and extension of body along length
	Excessive writhing	Exw	Larva stationary, with rapid and irregular contraction and extension of body along length
Dead	Dead	D	Larva exhibiting no signs of life, with no recovery

Treatment	Time (Hours)	Behaviour (Proportion of wireworm observed)								
		Re	M	Tw	RegE	RegC	Slm	Slw	Exw	D
Cedarwood	3	1	0	0	0	0	0	0	0	0
	6	0.73	0	0	0.27	0	0	0	0	0
	12	0.67	0.33	0	0	0	0	0	0	0
	24	0.40	0.20	0	0.40	0	0	0	0	0
	48	0.67	0	0	0.20	0.13	0	0	0	0
Rosemary	3	0	0	0	0	0	0	0	1	0
	6	0	0	0	0	0	0	0	1	0
	12	0	0.2	0.2	0	0	0	0	0.6	0
	24	0	0.2	0	0	0	0	0.8	0	0
	48	0	1	0	0	0	0	0	0	0
Tea Tree	3	0.40	0.6	0	0	0	0	0	0	0
	6	0.20	0.47	0	0.13	0.2	0	0	0	0
	12	0	0.2	0	0.2	0.6	0	0	0	0
	24	0.13	0.13	0	0.13	0.6	0	0	0	0
	48	0.47	0.20	0	0.13	0	0	0.2	0	0
Control	3	0.4	0	0	0.5	0.1	0	0	0	0
	6	0.7	0	0	0.1	0.2	0	0	0	0
	12	0.73	0	0	0.27	0	0	0	0	0
	24	0.67	0	0	0.13	0.2	0	0	0	0
	48	0.7	0	0	0.1	0.2	0	0	0	0

For both cedarwood, tea tree and control treatments, all larvae were recovered within one hour of removal from the experimental arena. For rosemary, all larvae were recovered by 24 hours.

2.3.4 Soil Fumigation

Both treatment and dose were found to have significant effects on larval mortality ($x^2 = 28.62$, df = 3, p < 0.0001) but no interaction between the two was appropriate for fitted models in the comparison of Akaike Information Critiea (AIC). The model with the lowest AIC value accepted as the 'best' fitting model here. Larval mortality was reduced within a soil substrate (Figure 2.6), as was time-to-kill, for both tea tree and rosemary exposed wireworm.

There were negligible differences between doses at 50 µl and 100 µl for either oil, but both killed significantly more larvae than the lowest dose (20 µl). Survival was lowest for rosemary treated replicates (75% at 100 µl), though only slightly increased for tea tree
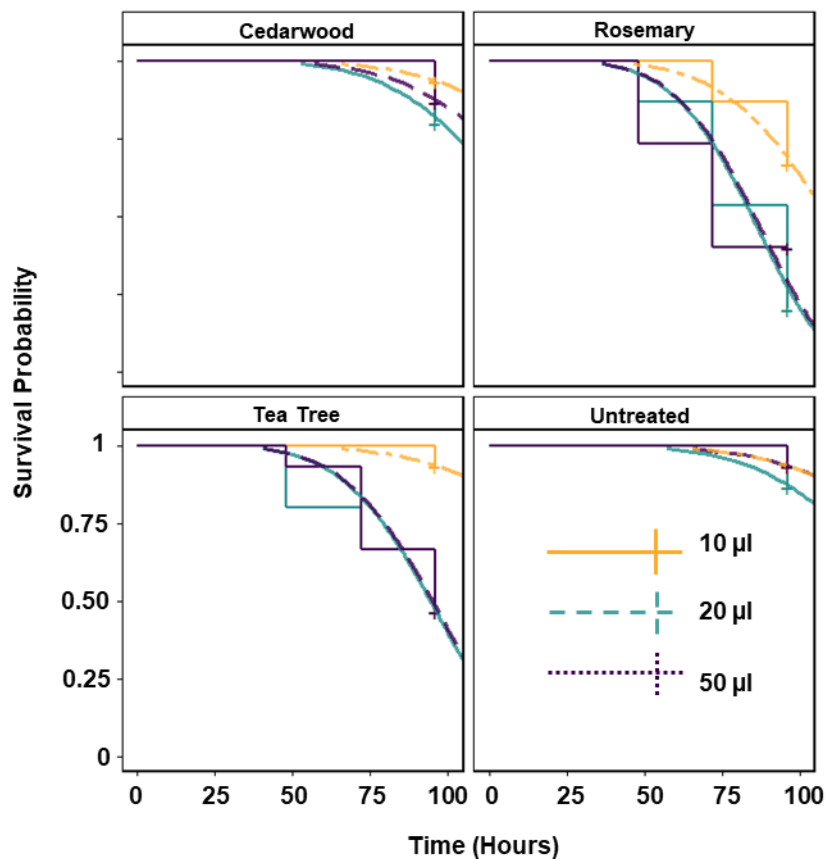
Fig. 2.6. Soil fumigation assay to evaluate dose dependent response of wireworm mortality when exposed to three doses of botanical extract. Kaplan-Meier plots of survival probability for wireworm with fitted survival curves for each dose are as shown in the legend.

(55% at 100 μl), with cedarwood showing comparable survival to untreated controls with > 85% across all doses.

2.3.5 Soil Fumigation – Degradation Effects

In Figure 2.7 can be seen the mortality effects of oils at the highest dose (100 μl) in a soil assay at four different time points. Larvae exposed to cedarwood aged at each of
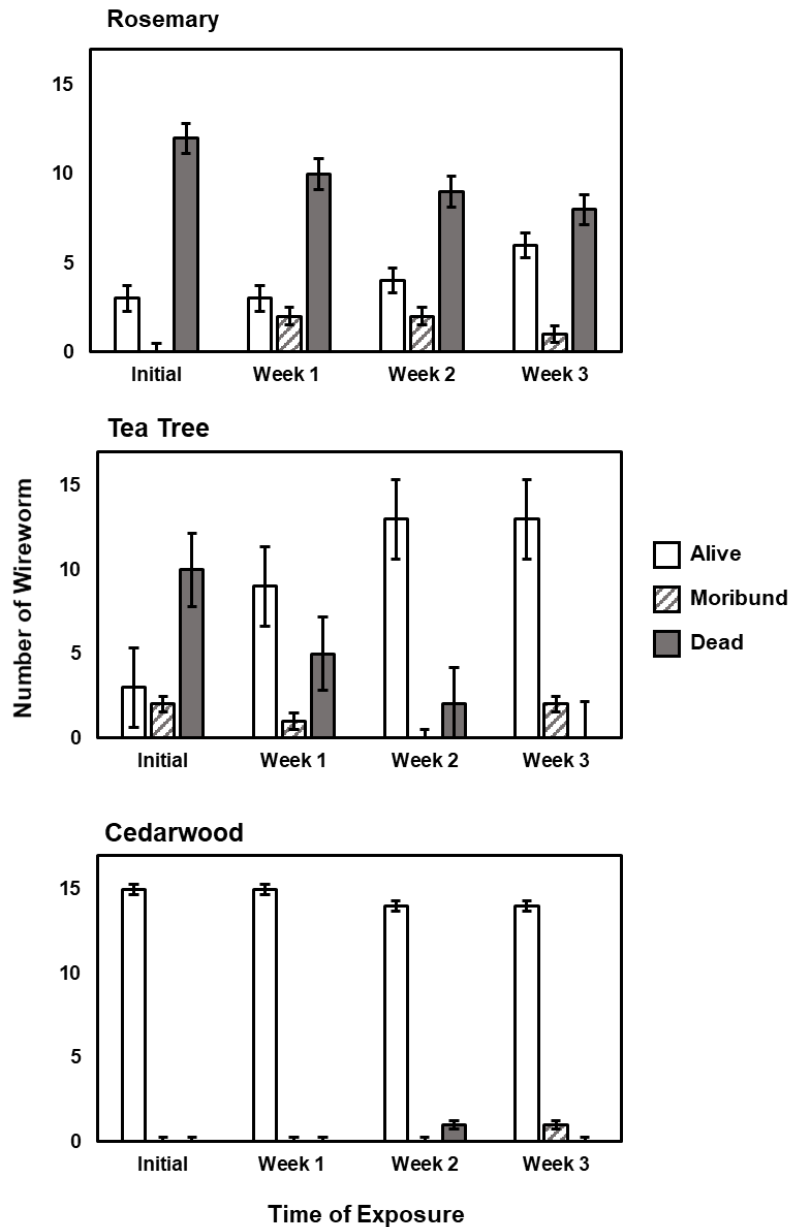
Fig. 2.7. Mortality data for wireworm exposed to oils in a soil fumigation assay. Larvae exposed to oils aged at seven-day intervals over a 21-day period. Oils were inoculated into experimental arena at an initial time point and larvae introduced on indicated day. Mortality / moribundity was then checked after four days for each time point.

the four timepoints showed adverse effects or mortality. For those exposed to tea tree, mortality dropped significantly (approximately 50% in those exposed after one week and after two weeks), after three weeks mortality did not differ significantly from cedarwood or the untreated control. Mortality exposed to rosemary aged over the 4-week period did not drop below 50%, significant when compared to both tea tree and cedarwood at any time point after the initial exposure. There was a clear persistence in the bioactivity of rosemary throughout the study period.

2.3.6 Terraria Studies with and without Plants

For terraria experiments with both a known attractant (germinated seed) and an introduced treatment (essential oil) only rosemary, tea tree and cedarwood were used due to restrictions in larval numbers and the lack of fumigation effects observed in citronella and lemon compared to the other two repellent oils. As before, cedarwood was included to evaluate attractant properties.

For the terraria study with no seed, MPR scores can be seen in Figure 2.8. The greatest repellent effect can be seen in citronella (54.5%). Alhough this did not differ significantly from the other oils eliciting a repellent effect (citronella – rosemary (43.6%) & lemon (40%): p > 0.75). Additionally, citronella was not significantly different, albeit marginally, from tea tree (citronella – tea tree (23.6%): est = 30.91, t = 2.77, p = 0.07).

The least repellent, or most attractant, effect can be seen in cedarwood at -49.1%. This differed significantly from all repellent treatments (cedarwood – tea tree, rosemary, lemon & citronella: p = < 0.001).

The untreated control showed a slightly negative MPR at -10.9% but this result being close to zero was as expected, with larval movement covering the entirety of the arena in a foraging response in the presence of no food source. The control differed significantly from all treatments, greatly when compared to rosemary, lemon and citronella (p < 0.001), and to a lesser extent when compared to cedarwood (est = 38.18, t = 3.419, p = 0.013) and tea tree (est = -34.55, t = -3.093, p = 0.034).

In all terraria treated with oils, larval movement was regular and unhindered but generally constricted to the untreated half (for repellent oils) or the treatment (in the case of cedarwood). In cedarwood treatments, there was some visual evidence that larvae had attempted to feed on the treated filter tip, with the behaviour not persisting.
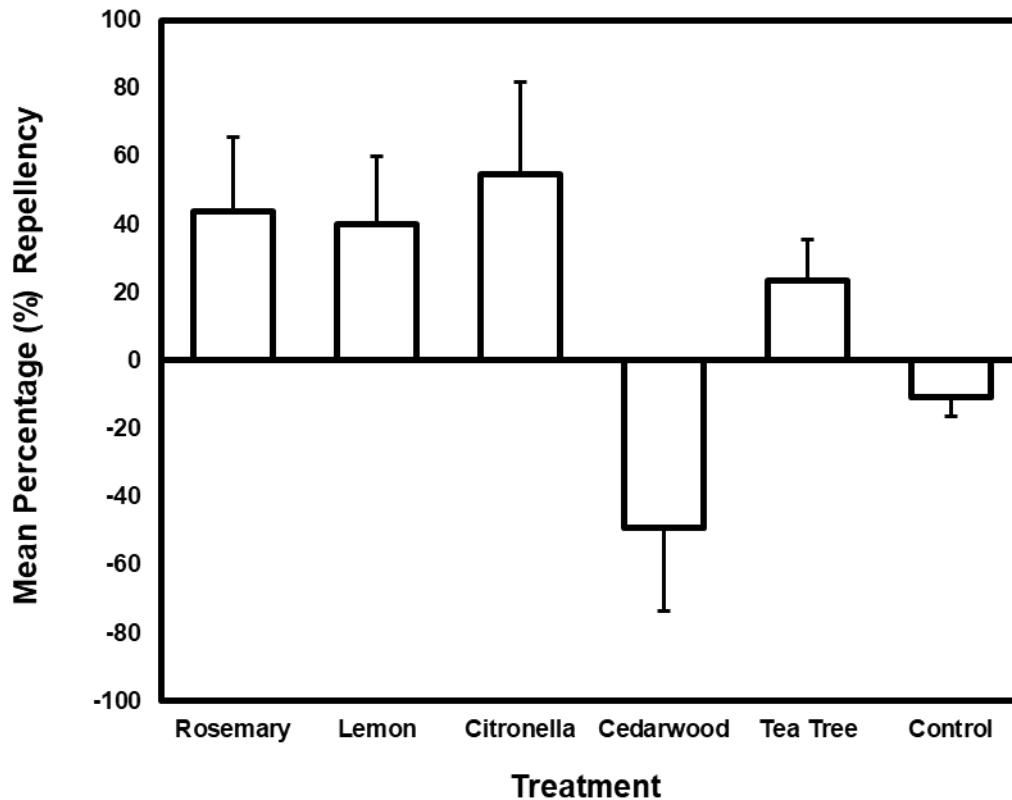
Fig. 2.8. Mean percentage repellency scores for wireworm exposed to five oils tested in a no-choice terrarium soil assay. A positive value indicates repellency, a negative value indicates attraction. Binary data was used by designating each half of the terrarium as 'control' or 'treatment' sections. Error bars indicate 95% CI.

As described in section 2, the δ^1 (distance between centroid of counts and point of treatment application) was compared between treatments and controls, and then aggregation and cluster indices were calculated for each treatment, and their associations analysed. For those containing germinated seeds, comparison of terraria up to four hours showed that treatment had no significant effect on δ^1, with all pairwise interactions showing any significant comparisons between treatments ($p > 0.05$). Centroid of counts for each treatment at this time point remained within the middle third of terraria with no clear directional movement toward or away from the germinated seed (Figure 6).

As can be seen in Figure 2.9, after 72 hours, treatment was shown to have a significant effect upon δ^1 ($F(4,55) = 29.55$, $p < 0.0001$) with tea tree and rosemary showing repellency compared to the positive control with germinated seed (Tt: Est = 3.823, $p < 0.0001$; Ro: Est = 3.789, $p < 0.0001$) and cedarwood treated terraria (Tt: Est = 3.823, $p < 0.0001$; Ro: Est = 3.789, $p < 0.0001$). There was no significant difference in the strength of repellent effect between tea tree and rosemary (Est = 0.034, $p = 1$). Cedarwood showed an attraction response comparable to the positive control with

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germinated seed (Est = 0.981, p = 0.268) with larvae observed feeding on the germinated seed across replicates for both these treatments. In control replicates in which no seed was present there was uniform movement across the whole terraria,
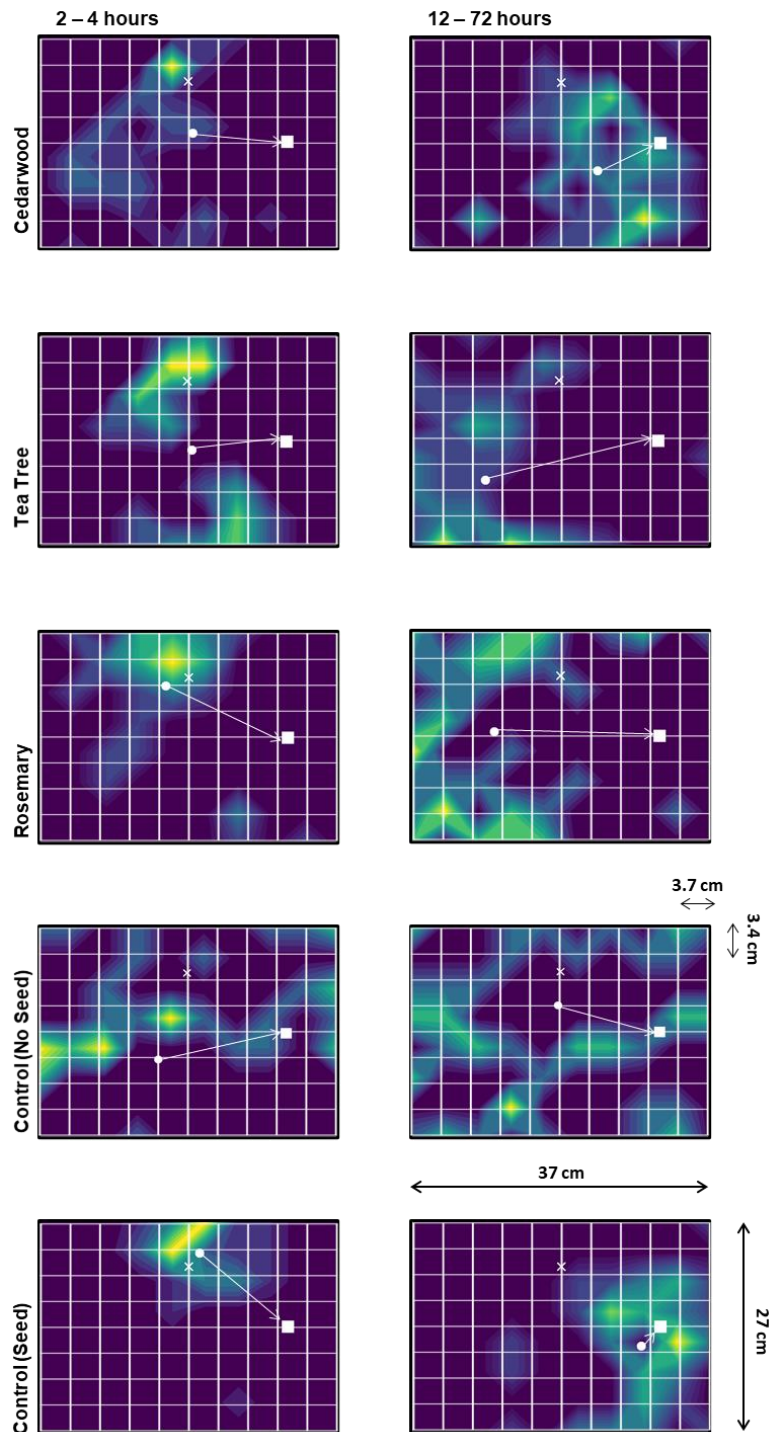
Fig. 2.9. Filled contour heatmaps of average observed counts of 12 wireworm in terraria containing a food source (germinated maize seed) and inoculations of treatment. Controls included seed without treatment and no seed at all. Where □ = Inserted maize seed and filter tip containing 200 µl of treatment, ○ = centroid of counts for wireworm location (C), x = insertion point of larvae, → = δ_1, distance between C and treatment.

suggesting a random foraging response in the absence of olfactory cues. There was no significant difference in δ^1 values for initial counts (up to four hours) compared to those up to 72 hours (t = 1.365, df = 22, p = 0.186).

Clustering patterns across all treatments can be seen in Figure 2.7, with results summarised in Table 2.4. Significant disassociation ($\chi < 0$) was observed in clusters observed in positive control (with germinated seed) terraria compared to both rosemary ($\chi = -0.529$, $P_\chi > 0.999$ ***) and tea tree ($\chi = -0.4887$, $P_\chi > 0.999$ ***) treated terraria. Cedarwood appeared to act as an attractant, with clusters centred towards the treatment and seed, showed strong disassociation of clusters compared to tea tree ($\chi = -0.0178$, $P_\chi > 0.8808$), and significant disassociation compared to rosemary ($\chi = -0.4173$, $P_\chi > 0.999$ ***), both eliciting a repellent response with clusters centred away from treatments.

Both rosemary and tea tree showed significant association ($\chi < 0$) between clusters ($\chi = 0.5083$, $P_\chi > 0.001$), suggesting no clear difference in strength of repellent response between treatments. The same was true between cedarwood treated terraria and the positive control ($\chi = 0.4971$, $P_\chi > 0.001$). These associations between spatial patterns supported comparisons between treatments for calculated δ^1.

Aggregation and cluster indices were calculated for all treatments up to 72-hours. All treatments showed an aggregated distribution of wireworm counts ($I_a > 1$) up to 72-hours (Figure 2.7). Rosemary ($I_a = 2.21$, $P_a < 0.001$) and tea tree ($I_a = 2.41$, $P_a < 0.001$), and the positive control ($I_a = 2.26$, $P_a < 0.001$) exhibited the strongest aggregation of wireworm observations.

Both rosemary and tea tree treated terraria showed aggregations in the untreated half of the terraria, with tea tree counts forming a significant singular cluster in the lower far left edges of the arena ($J_a = 1.33$, $Q_a < 0.001$). Rosemary, meanwhile, whilst counts were firmly concentrated in the control half, showed no clear singular or multiple clusters ($J_a = 1.04$, $Q_a < 0.26$), with increased movement of larvae throughout the study. This agitation in wireworm movement focused away from the rosemary treated terraria quadrants could possibly be linked to stressed behaviours seen in section 2.3.3.

Cedarwood treated terraria ($J_a = 1.44$, $Q_a < 0.001$) and positive controls ($J_a = 1.60$, $Q_a < 0.001$ ***) also showed a pattern of a singular cluster of counts, centred near to the seed and treatment quadrants. This was supported with behavioural observations of larval feeding throughout the study duration. Minimal larval tracks for these treatments indicate a focused foraging response compared to controls with no seed ($J_a = 0.96$, $Q_a < 0.59$), showing a random dispersal across the entire terraria with no evidence of clustering.

Patch and gap cluster indices support results from indices of aggregation (Figure 2.10). Rosemary ($V_j = -2.21$, $P_{vj} = 0.001$) and tea tree ($V_j = -2.42$, $P_{vj} = 0.001$) both had significant areas of spatial disassociation around the treatment and seed, to re-enforce the repellent effect supported with significantly strong clustering in areas far removed from the treatment quadrant (**Tt**: $V_i = 2.53$, $P_{vi} < 0.001$; **Ro**: $V_i = 2.23$, $P_{vi} < 0.001$). Both cedarwood and positive control terraria showed the converse trend, with strong clustering near to the treatment quadrants (**Cw**: $V_i = 1.64$, $P_{vi} < 0.003$; **Pc**: $V_i = 1.92$, $P_{vi} < 0.001$) and significant spatial disassociation (**Cw**: $V_i = -1.63$, $P_{vi} < 0.002$; **Pc**: $V_i = -2.24$, $P_{vi} < 0.001$) beyond this small radius of attraction.
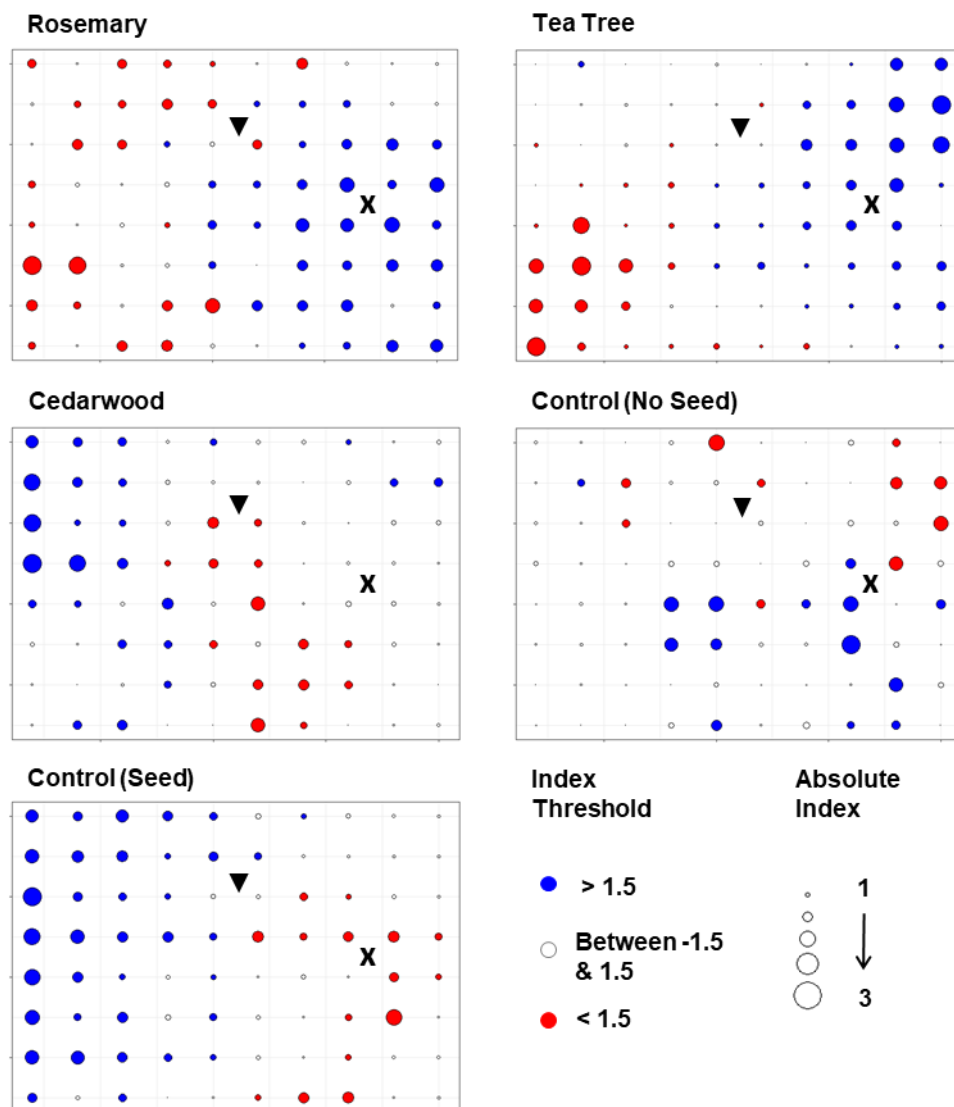
Fig. 2.10. Red – Blue plots illustrating clusters of association and disassociation of wireworm counts in response to treatment (x) in a soil-filled terrarium assay. Counts were collated over a 72 hour period. Blue points show gaps, or negative association, within the counts and Red shows patches or positive association, the size of point indicates the strength of the response. Where ▼ = larval insertion point.

Table 2.4. Significant associations of wireworm clusters (χ) between treatments within soil-filled terrariums. Where $\chi > 0$ is equal to positive association of calculated clusters and $\chi < 0$ the converse. The associated statistical test (P_χ) shows the significance of the interaction, with $P_\chi < 0.025$ indicative of a significant association, and $P_\chi > 0.975$ indicative of significant disassociation.

Treatment Comparison	χ	P_χ
Control (Seed) – Rosemary	-0.5920	> 0.999 ***
Control (Seed) – Tea Tree	-0.4887	> 0.999 ***
Control (Seed) – Cedarwood	0.4971	< 0.001 ***
Cedarwood – Rosemary	-0.4173	0.999 ***
Cedarwood – Tea Tree	-0.0178	0.8808
Rosemary – Tea Tree	0.5083	< 0.001 ***
Control (Seed) – Control (No Seed)	-0.3090	0.9950 ***

2.3.7 Germination – Fumigation of Seeds Exposed to Oils

Cumulative numbers of germinated seeds exposed to three doses of essential oils can be seen in Table 2.5. All potato seeds (cut eyes from chitted seed potato) germinated across all treatments after ten days except for rosemary (37% germination) and tea tree (67%) at the highest dose (20 µl). For maize, all seeds germinated across all treatments after ten days except for rosemary at 20 µl (78% germination) and tea tree at 10 µl (78%) and 20 µl (67%).

Radicle length of germinated maize seeds after eight days was dependent on both treatment and dose of essential oil. For each of rosemary, tea tree and cedarwood, average radicle length decreased from exposure to the lowest dose (5 µl) to 10 µl, with the lowest average lengths measured at the highest dose of 20 µl. The only significant decrease however was cedarwood from the lowest to highest dose (Est = 48.33, t = 4.714, p > 0.0001).

Table 2.5. Cumulative number of germinated seeds given for both potato (cut eyes from seed potato) and maize seeds in a Petri dish fumigation assay exposed to different doses of essential oils. Where '–' indicates all seeds have germinated from that time point on, to visualise comparisons between treatments and doses.

Seed	Treatment	Dose (µl)	Cumulative Germination - Days Post-Inoculation				Total	
			4	6	8	10	Num.	%
Potato	Rosemary	5	27	-	-	-	27	100%
		10	27	-	-	-	27	100%
		20	4	6	10	10	10	37%
	Tea Tree	5	27	-	-	-	27	100%
		10	6	27	-	-	27	100%
		20	0	6	18	18	18	66%
	Cedarwood	5	27	-	-	-	27	100%
		10	27	-	-	-	27	100%
		20	27	-	-	-	27	100%
	Untreated		27	-	-	-	27	100%
Maize	Rosemary	5	21	27	-	-	27	100%
		10	27	-	-	-	27	100%
		20	18	21	21	21	21	77%
	Tea Tree	5	8	27	-	-	27	100%
		10	15	21	21	21	21	77%
		20	15	18	18	18	18	66%
	Cedarwood	5	15	24	27	-	27	100%
		10	27	-	-	-	27	100%
		20	18	24	27	-	27	100%
	Untreated		27	-	-	-	27	100%

The radicle length of rosemary treated seeds differed significantly from untreated seeds at 20 µl (Est = -55.11, t = -5.374, p < 0.0001) and at 10 µl doses (Est = -36.33, t = -3.543, p < 0.0001) though not at 5 µl (Est = -27.11, t = -2.644, p = 0.271). Tea tree followed this same pattern, differing significantly from controls at 20 µl (Est = -63.11, t = -6.155,

p < 0.0001)) and 10 µl (Est = -55.78, t = -5.439, p < 0.0001), though not at the lowest dose (Est = -29.57, t = -2.88, p = 0.271). Cedarwood treated seeds however, whilst longest on average at the lowest dose, showed no significant difference in radicle length compared to untreated controls at 5 µl (Est = 8.78, t = 0.856, p < 0.999), or 10 µl (Est = -11.67, t = -1.138, p < 0.992), until decreasing in length significantly at 20 µl (Est = -39.56, t = -3.856, p < 0.011).

Between treatments, at the lowest dose, cedarwood treated seeds exhibited significantly greater length than both rosemary (Est = 35.89, t = 3.5, p =0.0039) and tea tree (Est = 38.33, t = 3.738, p = 0.0018), with latter two showing no difference between one another (Est = 2.44, t = 0.238, p = 0.9952). At 10 µl, cedarwood treated seeds had only marginally longer radicles compared to rosemary (Est = 24.67, t = 2.406, p = 0.0828), though significantly longer when compared to tea tree (Est = 44.11, t = 4.302, p = 0.0002). Again, though average lengths decreased less in seeds exposed to rosemary, there was no significant difference in length observed between rosemary and tea tree (Est = 19.44, t = 1.896, p = 0.2365) at this dose. Finally, at the highest dose – 20 µl - there was no significant difference in radicle length between cedarwood and either rosemary (Est = 15.56, t = 1.517, p = 0.4314) or tea tree (Est = 23.56, t = 2.297, p = 0.1059) or between the latter two (Est = 8, t = 0.780, p = 0.8633), with all treatments inhibiting radicle growth.

2.3.8 Field Experiments

For the gravimetric release from biovials used in trials, all oils showed evidence of decrease in weight over the four weeks, except for cedarwood which showed a marginal increase. Up until week 2 there was no significant difference in loss between treatments. Into week 3, the control (water) had decreased greatly compared to all treatments (p < 0.001). Rosemary (est = - 1281.4, t = 3.5, p = 0.01) and lemon (est = -1677, t = 4.6, p > 0.001) dropped in weight at a faster rate than cedarwood at this point also (est = -1281.4, t = 3.5, p = 0.01), though did not significantly differ from other essential oils. By week 4, water again continued to evaporate at a consistent rate, more than five times the rate of the nearest oil, lemon (est = -4442, t = 12.07, p > 0.0001). Rosemary (est = -1629.2, t = 4.43, p = 0.004) and lemon (est = -2139, t = 5.82, p > 0.0001) again showed a faster rate of loss than cedarwood, which marginally gained weight, though only lemon differed significantly from its starting weight (est = 1394.2, t = 3.791, p = 0.044).

There were more tubers harvested per plot for the Marteltwy site (t = 5.924, df = 137.91, p < 0.0001) than at Angle, though this is likely due to differences in varieties planted as no differences were recorded in average weights per plot (t = -0.168, df = 137.96, p = 0.8665). For both sites there was no significant pairwise interactions recorded in tuber number or tuber weight (per plot) across all treatments. There were many more wireworm collected across harvested plots in the Angle site (216) than at Marteltwy (69), with a positive linear relationship between length and weight (Figure 2.11), indicating a uniform spread of larval stages (Angle: R^2 = 0.9019; Marteltwy: R^2 = 0.8826). For both sites, there was no significant difference in wireworm numbers between each treatment.
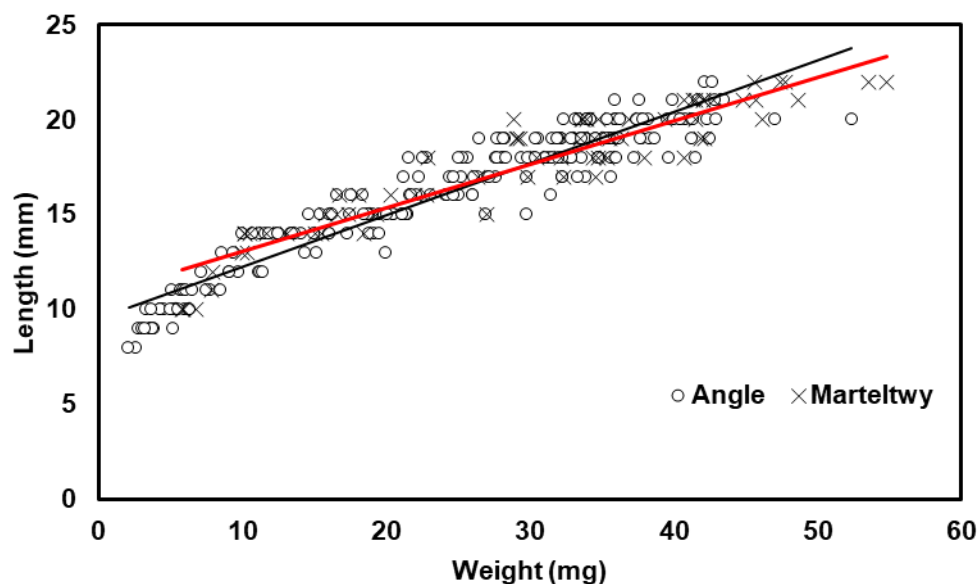
Fig. 2.11. Relationship between length and weight for larvae collected at harvest for both sites, independent of treatments. Shows no significant differences between the population age range for both sites, though there was a clear difference in the amount of wireworm collected in Angle (216) to Marteltwy (69) across the trail plots.

For wireworm damage, the Angle site showed a much higher proportion of tubers affected, reflective of the high numbers of wireworm found. However, for both sites, there was mostly no significant difference recorded in the proportion of tubers showing wireworm damage across all treatments (Figure 2.12). The exception was a marginal increase in wireworm damage in cedarwood treated plots for the Marteltwy site when compared to tea tree (est = 0.15, z = 3.049, p = 0.0372), with all other treatments showing no pairwise interactions. This relationship was not repeated in the Angle site (p > 0.999). Pest and disease damage of tubers was converted to a proportion of total tubers harvested for each plot. Wireworm and slug were the only invertebrate pest damages recorded across both sites. Common scab (*Streptomyces*) was the only

bacterial disease identified in both sites, with soft rot (*Pectobacterium*) identified in Marteltwy. Two fungal diseases occurred in both sites, these were black scurf (*Rhizoctonia solani*), and silver scurf (*Helminthosporium solani*), with the Martelwy site alone showing tubers infected with black dot (C*olletotrichum coccodes*). All diseases were identified visually post-harvest and confirmed by an agroecologist – Tony Little, Sustainable Farming Consultancy, Llwynbedw, Ceredigion.
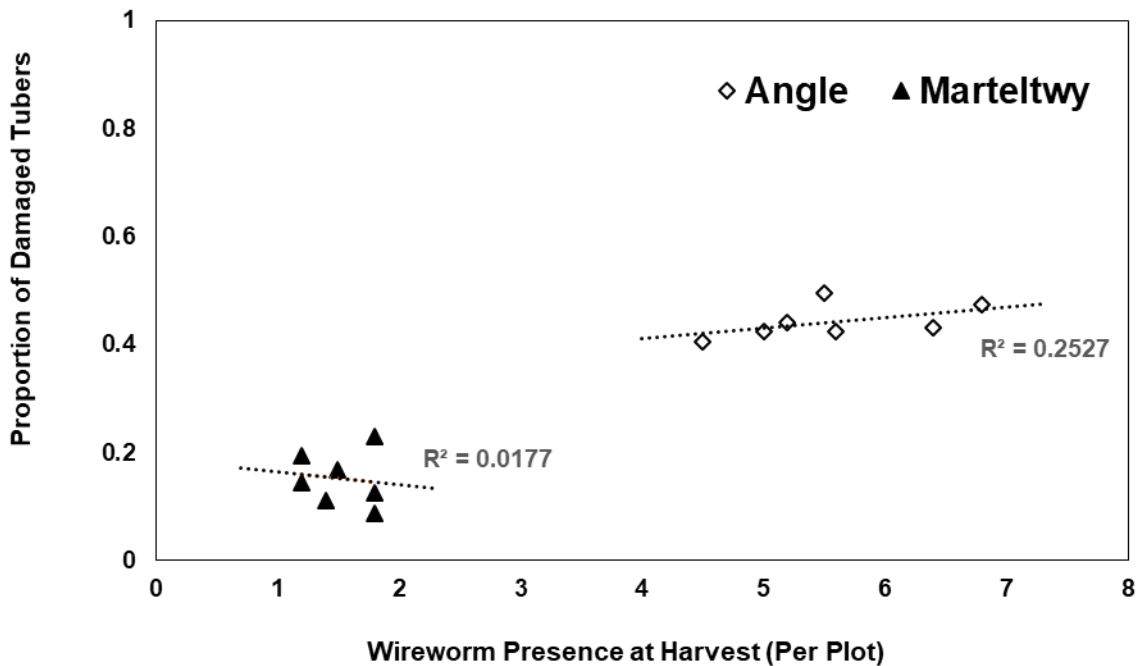
Fig. 2.12. Proportion of damaged tubers as a function of wireworm presence at harvest across all treatments for the two sites.

There was significantly more common scab recorded in the Marteltwy site than the Angle trial site (t = 13.898, df = 50.035, p-value < 0.0001). This was attributed to a spike in temperature and decrease in rainfall across the period of tuber initiation, conditions concomitant with proliferation of the pathogen. Between treatments there was some variation also. Tea tree treated plots were found to have the least amount of infection with scab, differing from all treatments and controls (p < 0.0001) except lemon, though the similarities were marginal (est = -0.135, z = -2.728, p = 0.0912). Cedarwood treated plots revealed the highest incidences of scab, more so than tea tree (est = 0.386, z = 7.818, p < 0.0001), lemon (est = 0.251, z = 5.089, p < 0.0001) & mocap (est = 0.157, z = 3.180, p = 0.0248), with just a marginal similarity to untreated plots (est = 0.137, z = 2.784, p = 0.0788). Citronella treated plots also had more scab infected tubers than

lemon plots (est = 0.178, z = 3.605, p = 0.0058). There was no significant difference between treatments for the Angle site.

Heatmaps of larval presence and wireworm damage across both sites can be seen in Figure 2.13, with geographical orientation in relation to surrounding sites seen in Figure
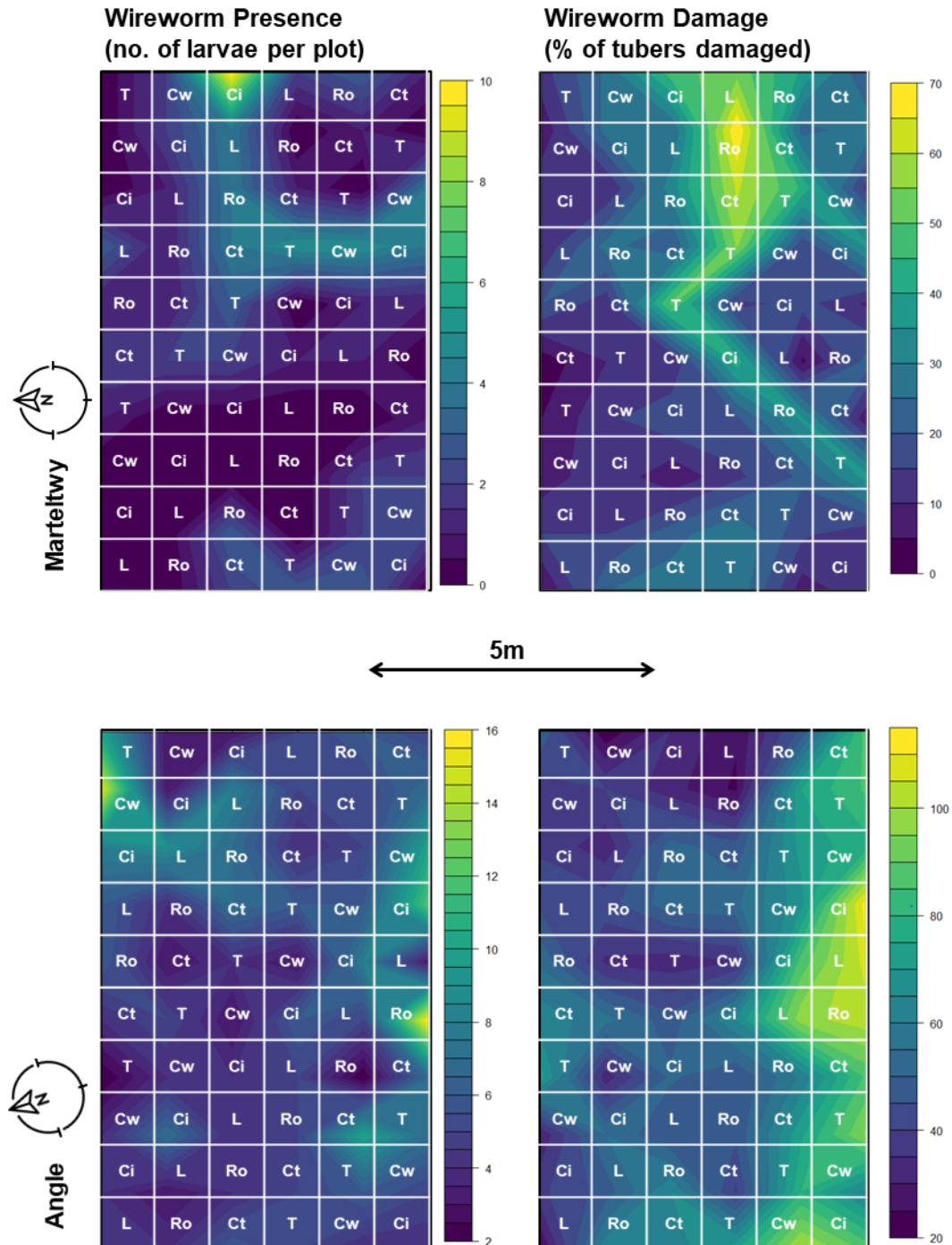
Fig. 2.13. Wireworm presence (larvae per plot) and wireworm damage (total tubers damaged) across trial plots at harvest for both field sites. Indicates higher numbers of both larvae collected and tubers damaged for the Angle site and areas of high presence and damage for both. Orientation relates to GIS maps, fig. 7. T = Tea Tree, Cw = Cedarwood, Ci = Citronella, L = Lemon, Ro = Rosemary, Ct = Control

1. Comparison of cluster association (χ) between wireworm damage and wireworm presence (Figure 2.14) across trial plots indicated a significantly negative association between the two variables for the Angle site (χ = 0.2577, p_χ = 0.0223 *), though conversely, a significantly positive association between the two for the trail plot at Marteltwy (χ = -0.2493, p_χ = 0.9567 *).
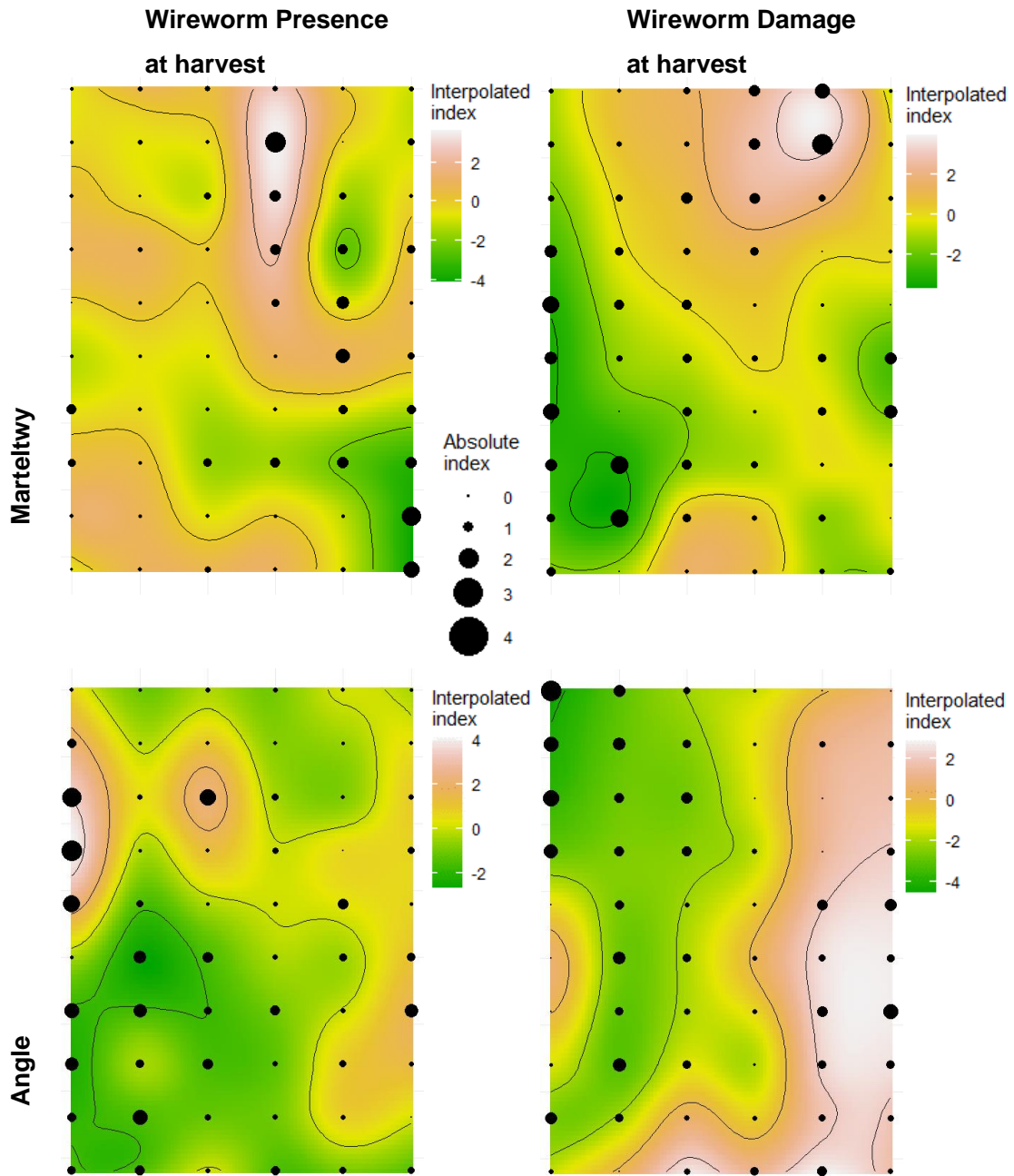
Fig. 2.14. Interpolated contour plots indicating wireworm clusters for both presence and tuber damage at harvest, across both field sites. Where positive index values, or v_i (> 1.5), indicate patches of counts and negative, or v_j (< -1.5), indicate gaps in distribution of counts. Values between 1.5 to -1.5 indicate no significant clustering. Marteltwy shows positive association between wireworm damage and wireworm presence. Angle shows negative association between wireworm damage and wireworm presence.

However, for the Angle site, there were no statistically significant differences between mean cluster indices for each treatment, for either wireworm damage of tubers ($F_{(5,54)}$ = 0.5804, p = 0.7147), or wireworm presence in harvested plots ($F_{(5,54)}$ = 0.2857, p = 0.9189). The same was found for the Marteltwy site with neither clusters of wireworm damage ($F_{(5,54)}$ = 1.082, p = 0.3807) or wireworm presence ($F_{(5,54)}$ = 0.6303, p = 0.6774), significantly determined by treatment. Subsequently, the relationships between wireworm damage and presence for each site must have been influenced by a suite of factors, including abiotic, soil and chemicals conditions, beyond the behavioural response to botanical treatments.

Future trials should look to incorporate a more traditional randomised block design, focusing on fewer treatments with greater distance between to act as a buffer. Additionally, regular sampling over the study period should have been carried out to determine damage over the season and larval movements within and between plots. Heterogenous wireworm populations across both sites in an overly condensed experimental plot design masked any treatment effects that may have been present. Natural infestations have shown themselves to be consistently patchy within any given site and though an inoculation of the pest might seem an effective method to combat this heterogeneity, obtaining wireworm in great enough numbers is a significant hurdle. Site selection should instead rely heavily on grower knowledge and multi-year sampling of sites incorporating data on field history and agronomic practices. Even then, any field trial should be accompanied by small-pot trials in similar conditions to corroborate and further detail effects.

2.4 Discussion

Plant essential oils are low-risk biopesticides with repellent, insecticidal, and growth-reducing effects on a variety of insects and have been used effectively to control a range of phytophagous insects and as insect repellents (Isman, 2020). These oils are often complex mixtures of terpenoids and other compounds including aldehydes, ketones, esters and alcohols. The oils differ in their spectrum of activity but often the overall bioactivity is a result of synergy among constituents. The current study shows that wireworm respond to some oils more than others with rosemary, lemon, citronella, and tea tree being repellent and cedarwood being an attractant. Wireworm did not appear to respond to the other oils (geranium, garlic, coriander, carraway, carrot, grapefruit) in spite of them being well known insecticides or repellents of a wide range of pest species,

including wireworm (Machial *et al.*, 2010; Sharaby, 2015; Eckard *et al.*, 2017; Mohamed *et al.*, 2017; Rosa *et al.*, 2020). Of the repellent oils, rosemary and tea tree show much promise, killing through fumigation activity as revealed in Petri dish and soil assays. The potency of these compounds, reflected in percentage mortality and speed of kill, was dose dependent which is virtually true for all essential oils. Higher doses were required to kill wireworm in soils, presumably due to the buffering capacity of the soil. The movement of VOCs through the soil is reliant on diffusion through soil air spaces (Insam & Seewald, 2010), as such this may be affected by abiotic factors, most notably temperature and soil water content. This does suggest that the fumigation results observed within soils were conspicuous in their efficacy, with high mortality for each of tea tree and rosemary, though a clear degradation in the potency of tea tree was observed over time. No data was obtained on the dose required to achieve a fumigation effect in a semi-natural arena or field scale, though it follows that this would be unfeasibly high for any real-world application and studies focused predominantly on the behavioural effects.

Wireworm mortality was similar for cedarwood oil and controls, suggesting it had no negative effect on the wireworm. Terraria studies suggested it had mild attractant properties. Cedarwood oil is highly repellent for some insects such as potato psyllid (*Bactericera cockerelli)* adults but an attractant for others such as the flour beetle (*Tribolium confusum)* (Diaz-Montano & Trumble, 2013; Martynov *et al.*, 2019). The behavioural response of psyllids was carried out in y-tube olfactometers and over a range of doses, from 1 to 2000 µl, with repellency observed across all (Diaz-Montano & Trumble, 2013). The attractant effect observed on flour beetles was much less pronounced, though only a small dose (6 µl) was tested, and the results suggests a degree of species specificity in response to essential oils (Martynov *et al.,* 2019).

Exactly how these oils killed the wireworm is unclear. Determining the mode of action of the essential oils, or the specific bioactive components, may contribute to formulating a viable product for effective application of the compound once a behavioural effect is determined. Jankowska *et al.* (2018) suggest a number of neurologically important enzymes that may be inhibited by components of essential oils, and molecular targets that could be used to determine their roles over the course of the insects' exposure to them. Modes of action of essential oils seem to vary but may include neurotoxicity, regulation of insect growth, deterioration of the waxy layers of the insect cuticle, impeding digestive enzymes, and inhibition of glutathione-S-transferase (Shaaya & Rafaeli, 2007; Norris, 2018; Gaire *et al.*, 2019).

Writhing was only noted for wireworm exposed to rosemary and tea tree suggesting some action on the insect's nervous system. Since the response was delayed in tea tree, it suggests the active compounds are less abundant or released slower than those of rosemary. Since the greatest movement was observed in wireworm exposed to cedarwood, tea tree and controls suggests that the tea tree actives had to build up to reach a specific threshold to prove damaging to the insect. The fact that moribund wireworm recovered following exposure to the oils suggest these insects have excellent detoxification systems.

Since the active components of essential oils differ in their volatility and mode of action, this could influence the speed and potency of the oil. Relationship between the disparate essential oil components is poorly understood, however, studies by Tak & Isman (2015, 2017) suggest the actives act synergistically with some components enhancing the action of others. For example, camphor and 1,8-cineole (eucalyptol), two actives in rosemary oil, worked in combination in penetrating the cuticle of the cabbage looper, *Trichoplusia ni* (Tak & Isman, 2015, 2017). In evolutionary terms, plants produce a plethora of actives and exploit synergy between actives which would potentially prevent herbivory and concomitantly prevent habituation to the deterrents. Exactly why cedarwood would be attractive is unclear, but two salient features of its GC-MS volatile profile (analysed further within Chapter 3) were that it differed radically from that of the repellent compounds and lacked any of the actives found in the repellent or toxic essential oils. Clearly, the potential exists to exploit the cedarwood oil in lures for improved wireworm monitoring or lure and kill control programmes.

Spatial analysis of wireworm in terraria highlights repellent effects of both rosemary and tea tree. A strong clustering in tea tree indicates a positive geotaxis with wireworm remaining as far away from the source as possible. Greater movement across the rosemary treated terraria corroborate the behavioural findings in the oil eliciting an agitated response, suggestive of an effect on the nervous system (Norris, 2018; Gaire *et al.*, 2019), resulting in seemingly random movements away from the oil source. Cedarwood, however, elicited a consistent attraction towards the treatment area, similar to untreated positive controls, with larvae displaying regular movement and feeding in the presence of the oil. Although this is not conclusive evidence of an additive attraction response, further study might exploit this behaviour within a push-pull strategy, or lure-and-kill with identified entomopathogens. The need to classify the exact behavioural response to the oils relies on more developed methodologies, evaluated within Chapter 3, and can indicate suitable for use with known entomopathogens of wireworm, such as *Metarhizium* spp., evaluated in Chapter 5.

Field evaluation of the oils resulted in inconclusive results. There was a clear relationship between wireworm presence and proportion of damaged tubers in the more infested Angle site, with no clear relationship in the Marteltwy site, but differences between treatments in both sites were not conclusive. It is possible that the heavy infestation and heterogenous population in the Angle site masked these differences. The results at the less populated Marteltwy site mirrored those of the laboratory studies, though not significantly. It is possible, that increasing the application rate of the oils could have given greater protection or if the oils had been used as part of a push pull strategy, driving the pest into a trap crop which would allow for targeted control with conventional pesticides. The trap crop could also intercept wireworm migrating from adjacent field which were high conducive breeding sites.

Spatial analysis indicates edge effects around the plot even though there was no clear relationship between larval presence or damage and treatment. There was a positive association between wireworm damage and presence in the least infested site, Marteltwy, with a negative association observed between the two for the more heavily populated Angle site.

There is clearly scope for developing essential oils for use in wireworm management programmes. Future research should focus on identifying the actives in the oils which are most repellent and either using these alone or as a blend in push-pull IPM programmes with cedarwood and other promising attractants being used to lure the pest into as a trap crop.

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Chapter 3: A Simple Soil Bioassay for the Evaluation of Behavioural Response of Subterranean Invertebrates

Abstract

The study of subterranean invertebrate movement is made problematic by the inability to directly observe the organisms themselves within a substrate. Solutions such as narrow terrariums can expose the organism to unwanted abiotic stimuli such as light, whereas other proposed olfactometry equipment can restrict movement and thus limit findings on taxis within a more natural arena. As such, subterranean invertebrate taxis in response to external stimuli can be unpredictable when scaling up to field setting from laboratory studies. With this work, a simple assay is designed in which invertebrate taxis can be assessed (here using wireworm, *Agriotes* spp.) in a substrate filled, semi-natural arena allowing omnidirectional movement of the organism in response to introduced stimuli, such as volatile organic compounds or plants. Using this novel capture tube (CT), we have demonstrated wireworm plant preferences and complex behavioural responses to introduced botanical semiochemicals beyond that of a simple attraction or repulsion. The presence or absence of organisms within the CT, and proximity in relation to it, can give a range of information such as feeding preferences, speed of movement, and chemotactic response. This methodology has the potential to provide a bridge between laboratory and field evaluated natural products to evaluate subterranean invertebrate responses in a more natural arena.

3.1 Introduction

Soil dwelling, root feeding insect pests are responsible for major crop losses in the agriculture, horticulture, and forestry sectors (Johnson *et al.*, 2016). Notable examples include western corn rootworm, white grubs, cutworms, leatherjackets and wireworm which cause billions of dollars losses each year (la Forgia & Verheggen, 2019; Lojewski & Wenninger, 2019; Poggi *et al.*, 2021) The success of these pests is partly due to the withdrawal of many chemical pesticides which posed a risk to human health and the environment, development of resistance in pest populations, and difficulty in detecting them to allow for timely targeted control (Parker, 2005; Barsics *et al.*, 2013). Numerous studies have been conducted to elucidate the impact of biotic (e.g. crop, soil biota) and abiotic (e.g. soil texture, temperature, moisture) factors on pest behaviour and distribution in the soil profile (Benefer *et al.*, 2012; Furlan *et al.*, 2016; Sandhi *et al.*, 2021). Particular interest has been given to the behaviour-modifying compounds (semiochemicals) produced by host and non-host plants as well as soil microbiota (Gfeller *et al.*, 2013; la Forgia *et al.*, 2020; Hummadi *et al.*, 2021).The reason for this is that the attractant and repellent compounds could be used in pest monitoring and control programmes.

Different approaches have been used to study the below ground behaviour of root feeding pests including use of olfactometers and terraria as well as non-invasive tools (Johnson *et al.*, 2007; Mankin *et al.*, 2008; Eckard *et al.*, 2017). Ideally, the assays should closely resemble the natural environment. Each assay design has its advantages and disadvantages (Johnson *et al.*, 2018). For example, dual or multi-arm olfactometers facilitate screening of semiochemicals to identify attractant and repellent compounds (Gfeller *et al.*, 2013; Barsics *et al.*, 2014; la Forgia *et al.*, 2020), or the use of x-ray to visualise burrowing behaviour or galleries (Booth *et al.*, 2020). However, such equipment is often costly and complex to create without adequate skills or materials. A popular choice for evaluating more observable, multi-directional pest behaviours is the use of a terrarium where a thin layer of substrate is sandwiched between two clear sheets of glass or Perspex through which insect movement may be observed over a given layout (van Herk, 2008; Brandl *et al.*, 2016). The advantage of this method lies primarily in visual observations allowing more complex spatial analysis to be carried out on larval responses to introduced stimuli (Brandl *et al.*, 2016; Eckard *et al.*, 2017; Winder *et al.*, 2019). However, to allow visual confirmation of pest presence the dimensions of experimental set-ups must be restrictive, limiting the three dimensions of a more natural soil setting.

Here is described a simple assay design to further study behaviours of soil dwelling pests in a more natural arena, using wireworm as a model. Wireworm, subterranean larval stages of the click beetle, can live several years before pupating and emerging as adults (Parker, 2005; Traugott *et al.*, 2015). Essential oils identified within Chapter 2 were initially screened to identify a more binary response of bioactivity, namely attraction or repellence. When a greater spectrum of behaviours was found in sealed fumigation assays at reduced concentrations, it was clear that a more complex behavioural classification was required beyond that evaluated within simple sealed arenas or terraria.

The intention of the design is to be able to germinate and grow seedlings within a 'capture tube' (CT) that may be easily introduced and removed from a given arena without damage to the plant and with minimal disruption to the substrate. The removal of the tubes themselves allows for regular observations of wireworm presence, or larval entry / exit from the tubes. It is suggested that production of volatile organic compounds from damaged plants may have a chemotaxic effect on wireworm (Gfeller *et al.*, 2013; Pinto-Zevallos *et al.*, 2016; la Forgia *et al.*, 2020), thus the limited disruption of the root complex allows for repeated observations within a given experiment duration whilst maintaining more controlled conditions. The CT also provide a containment unit for the introduction of semiochemicals for evaluation of behavioural response through presence or absence of larvae in the tubes over a given timeframe. This study demonstrates the benefits of the capture tube methodology when assessing wireworm behavioural responses to host plants and selected essential oils with known attractant and repellent properties.

3.1.1 Aims

- **Demonstrate the use of a novel methodology to evaluate and quantify subterranean invertebrate behaviours**
- **Use the capture tube methodology to assess behavioural responses to both plants and semiochemicals**
- **Use GC/MS to quantify constituent degradation of semiochemicals and their movement through a soil substrate to augment results from wireworm behavioural experiments**

3.2 Materials & Methods

3.2.1 Essential Oils

Essential oils of Tea Tree (*Melaleuca alternifolia*, Cheel, CAS No: 85085-48-9), Rosemary (*Salvia rosmarinus*, Spenn, CAS No: 8000-25-7), Citronella (*Cymbopogon winterianus*, Jowitt ex Bor, Cas No: 8000-29-1), Lemon (*Citrus limon*, L., Cas No: 8008-56-8), and Cedarwood (Atlas, *Cedrus atlantica*, Endl., Cas No: 8000-27-9) were used in described assays. The commercial oils, obtained from BiOrigins (Madar Corporations Ltd), were stored sealed in 1.1 L aluminium bottles at 21°C, in the dark to avoid photooxidation. Oils were applied neat, without carrier, unless otherwise specified

3.2.2 Insects

Wireworm (*Agriotes spp*) collected from agricultural land in Pembrokeshire, Wales, were maintained in one litre pots filled with a soil: vermiculite substrate (4:1, v/v) in a controlled temperature room (15°C ± 1°C, 60% RH ± 5%, 16:8 L:D photoperiod). Larvae were fed with fresh potato slices every three days, with seven days starvation prior to experimentation. Late instar larvae were used in experimentation, with age determined from morphometric measurements as in Furlan (2021). Specimens were identified to species level using the key from Klausnitzer (1994), with > 85% species determined to be *Agriotes obscurus* (L.), with the remainder *A. lineatus* (L). .
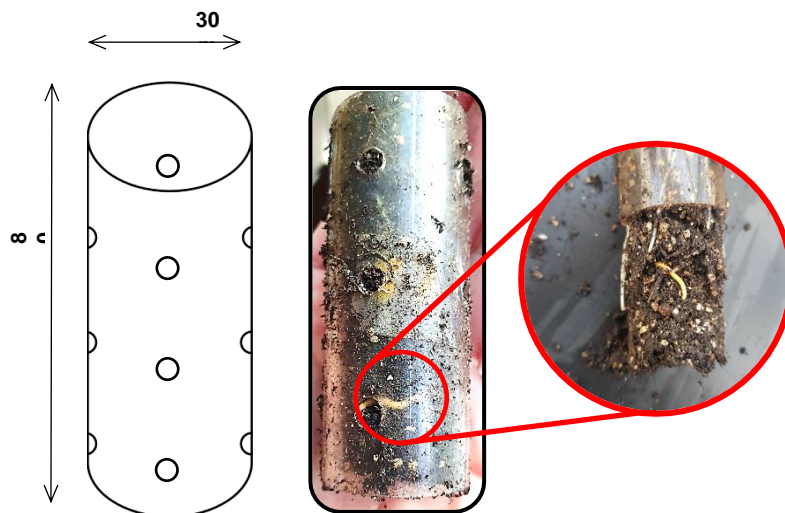
Fig. 3.1. Capture tube design and example of captured larvae feeding on maize roots. Tubes allow for efficient capture of larvae and for addition of biological (various plant species) or chemical incentive to stay or evaluate behavioural responses.

3.2.3 Capture Tubes and Experimental Arenas

Capture tubes consisted of acrylic cylinders (80mm x 32mm OD x 28 ID) with three 5 mm diameter holes spaced at 15, 40 and 65 mm from the bottom, in each quarter of the tube face, as shown in Figure 3.1. Preliminary studies had shown these dimensions facilitate seedling growth and wireworm capture.

Single tube capture assays (Figure 3.2) were carried out in black pots (Diameter 13cm x Depth 13 cm) filled with compost and vermiculite (4:1, v/v, Multipurpose w/ John Innes: Vermiculite) with a one-litre volume (750g (±25g)) at approximately 20% moisture content (v/v). Moisture content was determined in 25L batches using a moisture meter (ML3 ThetaKit, ©Delta-T Devices Ltd), and then aliquoted out for each replicate as necessary.

Multi-choice capture assays (Figure 3.3) to determine bioactivity of botanicals were carried out in five litre plastic tubs (Width - 15 cm x Height - 14 cm x Length - 34 cm – Figure 3) filled with a compost and vermiculite mixture as above, with a volume of 4.5
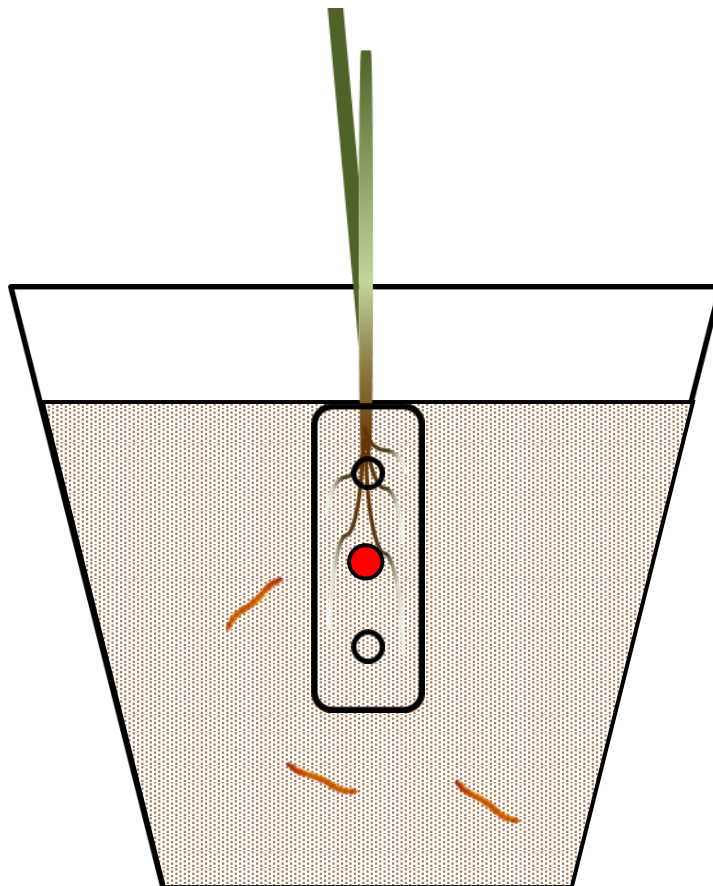
Fig. 3.2. Diagram of experimental set-up for capture tube no-choice assay with germinated seedling. The red point indicates insertion of inoculated filter tip.

litres (3900 g ± 100 g) at 20% moisture content. Arenas were split into superficial thirds – 'treatment A', a central control, and 'treatment B'. Details of specific experimental set ups are as listed below.
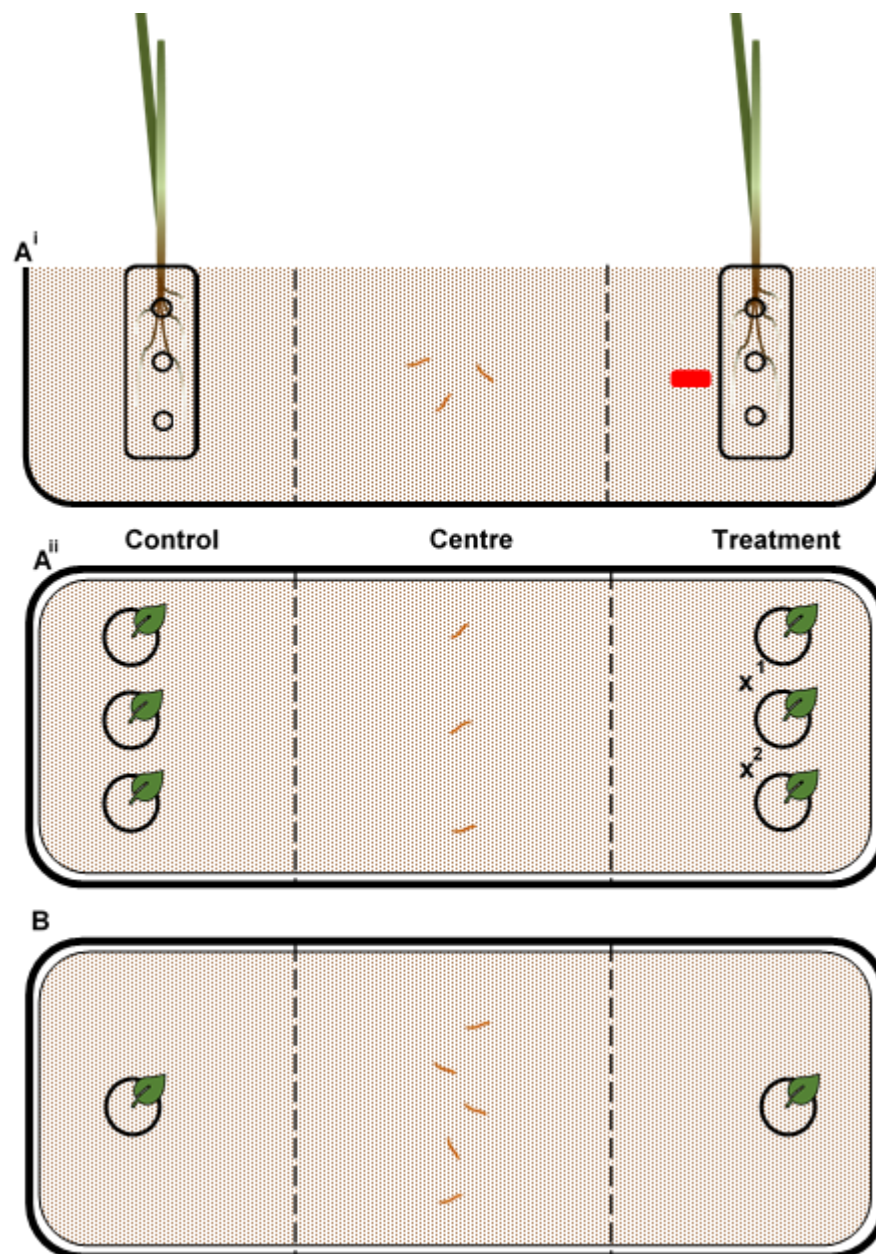
Fig. 3.3. Diagram of experimental set-up for capture tube choice assay to examine behavioural effects of essential oils (A^i & A^{ii}). The introduction of the three larvae is indicated in the central section. Where x (1 & 2) indicates inoculated filter tips. In plant preference experiments (B), five larvae were used per replicate.

3.2.4 Plants

Capture tubes contained seedlings of various crop species – wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), and potato (*Solanum tuberosum* L., var. Maris Piper, cut eyes with 0.5 cm^3 tuber) all seeds were supplied through Kings Seeds Direct (E.W King & Co Ltd, Essex, UK) apart from seed potatoes, supplied by Puffin Produce Ltd (Havorfordwest, UK). Seeds were germinated directly in capture tubes in the same substrate as in 3.2.3, with successfully germinated seedlings used in experimentation. In the case of potato, eyes were cut from chitted tubers and these were grown within tubes. Filled capture tubes were maintained in glasshouse conditions from planting through to use in experiments (22°C ± 4°C, 60% RH ± 10%). Seedlings were compared in a no choice feeding assay to determine wireworm preferences and to demonstrate the robustness of the capture tube as a monitoring device.

3.2.5 Plant Preference: No-choice Capture Assay

Seven day old seedlings were selected for experiments (growth stage BBCH 12 – 14, as in Lancashire *et al* (1991)), grown from seeds within capture tubes filled with the same substrate as in 3.2.3. Selected tubes were placed in the centre of the experimental arena and left for one hour to allow for the settling of the substrate. Five larvae were then introduced at even points > 2 cm from the tubes' outer edge. The tube was checked for larval presence after 48 hours. Ten replicates were used for each crop type and controls. An empty tube was used as a negative control, with main crop species (maize & potato) used as positive comparisons.

3.2.6 Plant Preference Dual-choice Capture Assay

Dual-choice capture assays to determine plant preferences were used to further distinguish crop preferences. Single capture tubes containing potential trap crop candidates (wheat, barley, rye or oat, at growth stage *BBCH 12 - 14*) were placed at the opposite end of the arena from tubes containing either potato or maize – identified main crops at risk of wireworm damage. Potato and maize were also compared to one another. Five larvae were introduced to the central third of the arena, evenly spaced apart (at least 2 cm). After 72 hours, the arenas were emptied and each section and

tube were assessed for wireworm presence. Ten replicates were used for each comparison.

3.2.7 Botanicals Bioactivity: No-choice Capture Assay

Capture tubes containing maize seedlings (in the same substrate, seven days post-germination, selected for consistency in growth stage, (BBCH 12 - 14) were treated with a 50 mm x 7.13 mm filter tip (Sharrow, Wilson & Co. Ltd) loaded with 200 µl (200 ppm) of either rosemary, tea tree or cedarwood oil.

Tubes were placed centrally and left for one hour in experimental conditions for the settling of the substrate and dispersion of volatile organic compounds (VOCs). Three larvae were then introduced equidistant from the tube rim and pot margin and more than 2.5 cm from one another, and left for a 48-hour period. Tubes were then checked, and the arena examined for individuals either inside the tube, adjacent to it or absent. The experiment had ten replicates per treatment.

3.2.8 Botanicals Bioactivity: *Mean Relative Growth Rate*

Larval growth rate within the same no-choice treated capture tube assay set up as in 3.2.7 was used to further specify larval feeding behaviours when exposed to the oils in a soil environment. Here citronella and lemon were included alongside rosemary, tea tree & cedarwood after initial screening processes in Chapter 2, section 2.2.2. Larval availability precluded these oils from further study, with those oils causing a stronger behavioural response selected for more detailed experiments. Larvae were removed from culture, weighed (Time point 1; T1) to within 0.001mg (Sartorius CP2 P Microbalance) and placed within individual pots with 30 ml of soil mix (as in 2.5) with no food source. After seven days larvae were weighed again (T2) and placed into a prepared arena under the same conditions as single tube pot assays, with a single larva in separate pots. Finally, larvae were removed from these pots after seven days and weighed for a final time (T3). The mean relative growth rate (MRGR) was calculated for each treatment as follows:

$$MRGR = \frac{\ln T3 - \ln T2}{\text{Time between measurements (Days)}}$$

The capture rate of larvae in tubes was compared to MRGR to specify the bioactivity of each oil. Ten replicates were used for each treatment.

3.2.9 Botanicals Bioactivity: Dual-choice Capture Assay

For larger arena choice experiments, three tubes were placed adjacent in opposite thirds of the arena buried so the top was flush with the soil level. Tubes contained maize seedlings, in the same substrate, seven days post-germination and were selected for both above and below ground consistency in growth stage. Four wireworm were introduced to the central third. After 72 hours, arenas were emptied and separated into the treated, untreated and central thirds with the number of wireworm counted in each. Each tube was assessed for entry holes and then emptied to count wireworm numbers and evaluate feeding damage. The experiment contained twelve replicates.

3.2.10 GC/MS: Constituent Composition of Botanicals

Percentage change in chemical composition of the essential oils was examined over a three-week period, to correlate with gravimetric studies and mortality assays and identify the bioactive constituents. Biodegradable vials were filled with 1 ml of undiluted oil and left in controlled conditions (24°C, 60% RH ± 5%). An initial sample of 100 µl was taken from this and then every seven days up until 3 weeks. Samples were taken in triplicate and then stored in a freezer (-20°C, ± 1°C) until GC/MS analysis.

For GC/MS analysis of essential oils an Agilent 7890A GC was used with a 5974C MS detector and DB-W column. Oils were diluted 1:100 (v/v) in hexane and a liquid injection of 1 µl was used in splitless mode with a flow of helium at 1 ml min^{-1}, with a 9683B series autosampler. For the GC program, the oven was held at 50°C for 5 mins, then ramped to 160°C at a rate of 5°C min^{-1}, before finally ramping to 260°C at the same rate of 5°C min^{-1}. The injector was at 250°C and the detector held at 280°C. The MS detector was set with a three-minute solvent delay and a scan from 40 to 400 EI+ up to the end of the method. Significant (>0.05% relative abundance by peak area) peak mass spectra were identified through comparison to the literature and NIST Mass Spectral Library database.

3.2.11 GC/MS: Diffusion through Soil

To assess the buffer capacity of a soil substrate and to determine the movement of the essential oil VOCs through the medium, GC/MS analysis of CT were taken from a soil-filled arena inoculated with the studied botanicals. One litre glass beakers were washed initially with water and subsequently with acetone before baking in an oven (VWR Ventline) at 300°C. These were then held at 110°C until used in experiments. Baked beakers were then filled with 150ml of compacted substrate (soil mix as in 2.2.3, at 20% soil moisture content, determined as stated above and re-tester once within beaker) and a filled biovial (2 ml vial, with 1.5 ml oil) with a 3 mm aperture placed 2 cm in from the edge. A filled CT was placed in the centre of the beaker and the remaining substrate were added and compacted and topped up to the rim. The substrate was moistened to field capacity prior to addition to the beaker.

After 72 hours, the CT was removed from the beaker. Three sections of the CT were analysed: the top, the middle and the bottom thirds. Soil cores were frozen prior to separation of sections to ensure even thirds for analysis. This was repeated at seven days post-inoculation. From these sections 200 mg of substrate was sealed in a 20 ml headspace vial for analysis.

The samples were analysed with a Perkin Elmer TurboMatrix 110 headspace autosampler, injecting into a Perkin Elmer Clarus 580 GC with an Elite 5 MS column (30 m, 0.25 mm ID, 0.25 µm df), connected to a Clarus SQ 8S mass spectrometer. For the headspace method, vials were baked at 80°C for 10 minutes, pressurised for two minutes then an injection of 0.07 minutes withdrawn at 0.2 minutes. Needle temperature was held at 90°C and the transfer line at 110°C, with injection and column pressures at 15 PSI.

For the GC method, the oven was held at 40°C for one minute, ramped to 150°C at a rate of 5°C min-1, held for two minutes and then ramped to 250°C at a rate of 20°C min -1 with a final hold of one minute. The MS detector was set with a three-minute solvent delay and a scan from 40 to 400 EI+ up to the end of the method. Significant (>0.05% relative abundance by peak area) peak mass spectra were identified through comparison to the literature and NIST Mass Spectral Library database. Additionally, an n-alkane reference standard mixture (C10 – C40) was run under the same conditions as the samples and resulting retention indices (RIs) standard curve used to compare RIs of sample compounds and match against the literature.

3.2.12 Statistical Analysis

All data from capture tube experiments were analysed with fitted generalised linear models (GLM), selected for best fit with comparison of Akaike's Information Criteria (AIC) for models with the addition / removal of variables ('AICcmodavg' – ver. 2.3-1, 2020). *Any post-hoc analysis to highlight pairwise relationships was carried out with comparisons of estimated marginal means* ('emmeans' – ver.1.7.0, 2021). *Plant preference experiments were compared between treatments with Student's T-test comparisons of means.* All statistical analyses were performed using RStudio Build 351, utilising R version 4.1.2 (2021). All GC/MS data was processed as stated in section 3.2.7.

3.3 Results

3.3.1 Plant preference: No-choice Capture Assay

Capture rates of wireworm in tubes containing various crop plant species can be seen in Figure 3.4. Crop species was found to have some effect on tube capture rates though only rye (60% capture) was seen to be significantly more attractive to wireworm when compared to potato (30% capture, est = 1.5, z = 3.947, p = 0.0015), barley (30% capture, est = 1.5, z = 3.947, p = 0.0015) and oat (36% capture, est = 1.2, z = 3.157, p = 0.0266), with no clear pairwise interactions found between any other crop species. Wheat and maize planted tubes showed 48% and 50% larval capture respectively. Single choice
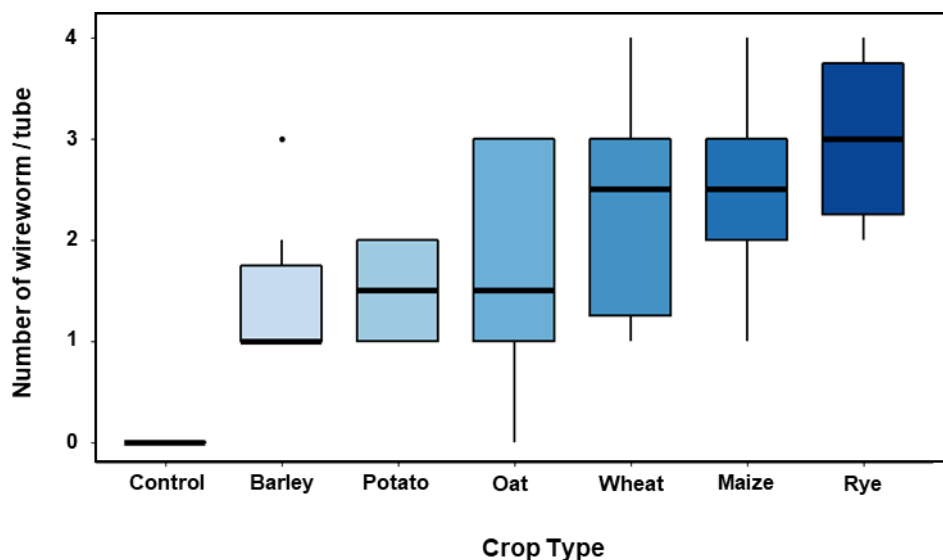
Fig. 3.4. Number of wireworm found inside tube in single tube pot experiment for differing crop species. Five larvae were introduced per replicate and the tube checked for larval presence after 48h. Crop types were 7-day old seedlings germinated within tubes.

tube capture assays proved to be inconclusive when limited to a single variable, though useful as proof of concept for the efficacy of the capture tube itself.

3.3.2 Plant Preference: Dual-choice Capture Assay

Results of dual choice tube capture assays for plant preference can be seen in Table 3.1. When given a choice between potato and various trap crop species, both wheat (t = 2.304, p = 0.034) and maize (t = -2.484, p = 0.023) were preferred food sources for wireworm. No clear differences were observed between potato and oat (t = 0, p = 1) or rye (t = -0.943, 0.358). Barely was the only potential trap crop to which potato was a significantly preferred food source (t = 3.143, p = 0.005).

Table. 3.1. Comparison of wireworm capture in dual-choice crop type preference capture tube experiment. Wireworm capture is indicated for main crop, trap crop and null indicate larvae located in central 'neutral zone'. T-tests were carried out on capture in each with significance presented.

Capture Tube		Larval Capture			T	P
Main	**Trap**	**Main**	**Trap**	**Null**		
Potato →	Oat	13	13	4	0	1
	Wheat	8	19	3	-2.304	0.034*
	Barley	20	5	5	3.143	0.005**
	Rye	11	16	3	-0.943	0.358
Potato →	Maize	7	19	4	-2.484	0.023 *
Maize →	Oat	17	6	7	2.952	0.388
	Wheat	16	10	4	1.260	0.224
	Barley	16	9	5	1.512	0.148
	Rye	18	7	5	3.051	0.007**

With maize a main crop at high risk of wireworm damage, it was included as both a 'trap' crop comparison to potato and as a 'main' crop comparison in further choice studies. A preference was shown for maize across comparison to oat (t = 2.952, p = 0.388), wheat (t = 1.260, p = 224), barley (t = 1.512, p = 0.148) and rye (t = 3.051, p = 0.007), though only the latter was found to highlight a significant difference.

For each crop type, wireworm were found within tubes, with evidence of feeding, unless specified 'null'. On average, 14% of larvae for each comparison were considered a 'null' result, defined as found within a neutral central section, with no clear plant preference.

3.3.3 Botanicals Bioactivity: No-choice Capture Assay

Capture rates for single tube essential oil experiments can be seen in Figure 3.5. Treatments had a significant effect on both wireworm capture and wireworm exclusion from tubes, with larvae found adjacent to tubes having no obvious differences.
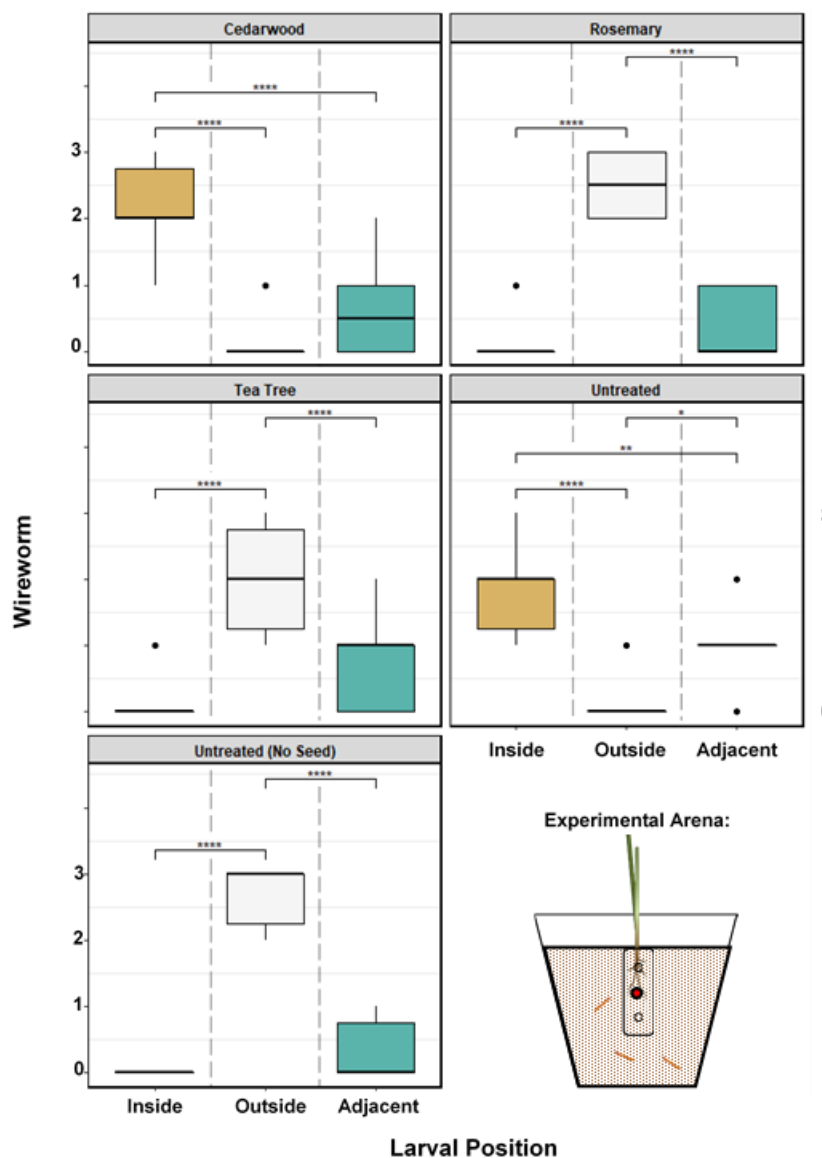
Fig. 3.5. Mean wireworm capture for single tube, no-choice pot assay to examine bioactivity of botanicals in the presence of maize. Larval location given as inside, adjacent to or outside capture tube. Significant pairwise comparisons of estimated marginal means are indicated for each treatment.

*Both rosemary and tea tree treated tubes showed minimal capture rates (3 and 6% respectively), evidence of significant repellency compared to both cedarwood (**Ro**: est = -2, z = -7.842, p < 0.0001; **Tt**: est = -1.9, z = -7.450, p < 0.0001) and untreated positive controls (**Ro**: est = -1.7, z = -6.666, p < 0.0001; **Tt**: est = -1.6, z = -6.274, p < 0.0001). Cedarwood treated tubes showed the highest rate of capture (70%), and this was significantly more attractive than untreated positive controls (60% capture: est = 0.3, z = 1.176, p = 0.765). Evidence of feeding was found in both cedarwood and untreated positive controls, with no feeding damage in the larvae captured in either tea tree or rosemary treated tubes.*

3.3.4 Botanicals Bioactivity: Mean Relative Growth Rate

Treatment was found to have a significant effect on the mean relative growth rate (MRGR) of larvae exposed to essential oils in a treated capture assay (F(6,53) = 5.763, p = 0.0001). Positive growth differences were found in untreated controls (0.011), cedarwood (0.013) and lemon oil (0.008) treated replicates, with negative values obtained for larvae exposed to citronella (-0.003), tea tree (-0.004) and rosemary (0.005) oils. Capture rates as compared to MRGR can be seen in Figure 3.6 (A & B).

*No significant differences were found between treatments with positive MRGR (**Cw – Con**: p = 0.997; **Cw – Lem**: p = 0.854, **Con – Lem**: p = 0.978). Both cedarwood treated seeds and untreated controls showed evidence of feeding and high tube capture at the end of the feeding period, with a marginally higher capture rate in cedarwood treatments, suggesting possibly a slight phagostimulant effect or simply an absence of adverse effects elicited by the presence of the oil.*

*For lemon treated tubes, whilst MRGR was positive, capture rate was low (30%). Although some feeding was observed, damage was minimal, indicating a possible antifeedant effect of the treatment. Neither capture rates, nor difference in MRGR, were found to be significantly different between lemon and all treatments with negative MRGR (**Lem – Ci**: est = 0.011, t = 2.443, p = 0.1602; **Lem – Tt**: est = 0.012, t = 2.578, p = 0.1204; **Lem – Ro**: est = 0.013, t = 2.841, p = 0.0661), suggesting the positive MRGR could also be due to anomalous results that could be focused with increased replication.*

*No significant difference was found between treatments with negative MRGR either, (**Ci – Tt**, p = 1; **Tt – Ro**, p = 0.9998, **Ci – Ro**, p = 0.9986), with all treatments showing a positive relationship between MRGR and capture rate (**Ci**: 40%; **Tt**: 0%; **Ro**: 0%). There*

was present, but minimal, damage to seeds in citronella treated replicates. This was, as seen with lemon, evidence of a possible antifeedant effect of the oil.
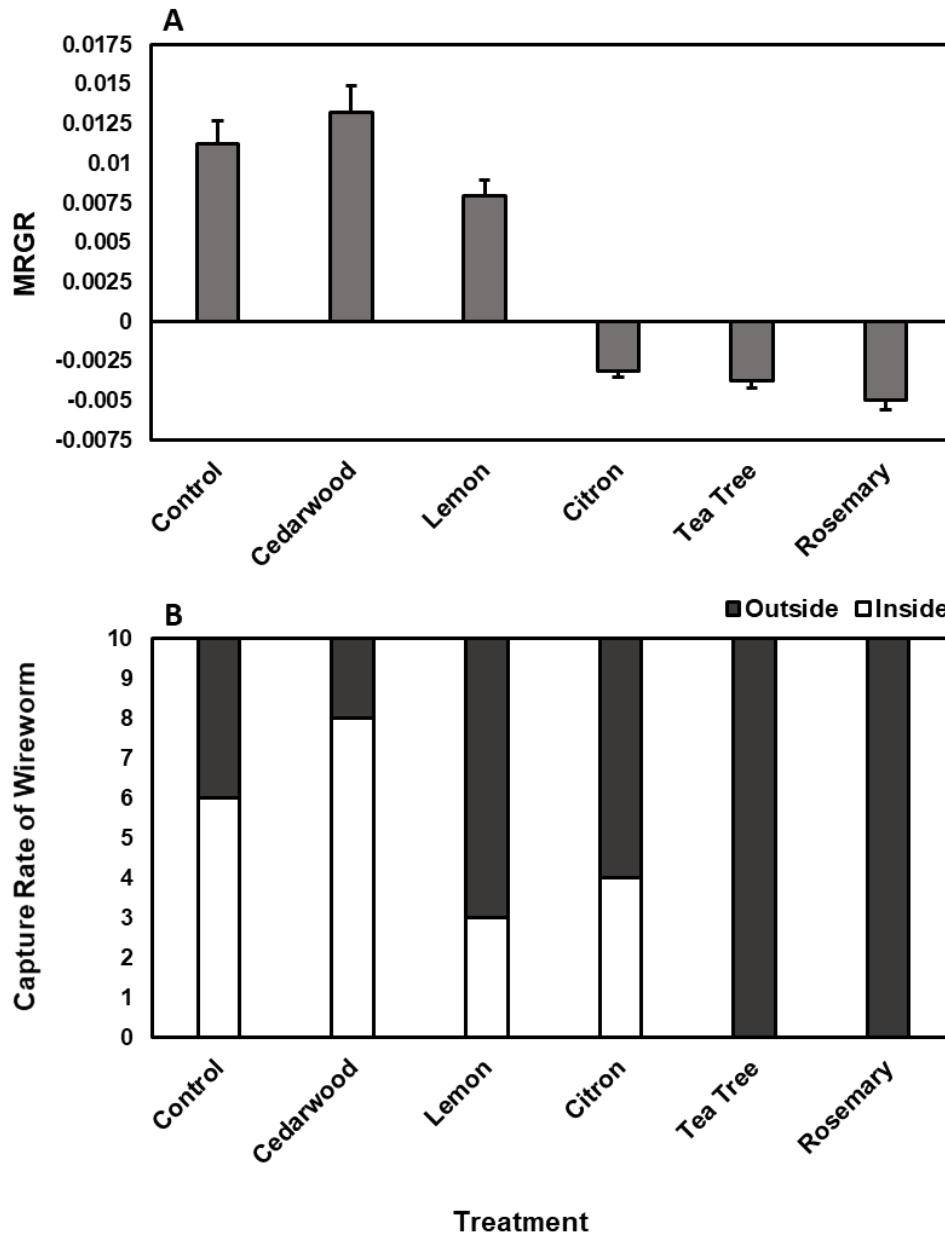
Fig. 3.6. Mean relative growth rate of wireworm (A) exposed to botanicals in a no-choice capture tube assay containing maize seedlings, over a 7-day period. With positive results indicating net weight gain and negative results the converse. Capture rate (B) indicates total wireworm found inside or outside capture tube at collection point for final weighing.

3.3.5 Botanicals Bioactivity: Dual-choice Capture Assay

Wireworm in cedarwood and untreated controls (one sided, plants in treatment section) showed clear preference for treatment sections (Cw: est = -3, t = -4.376, p < 0.001; UC: est = -2.625, t = -3.829, p = 0.005). Only tea tree treated replicates revealed wireworm with a significant preference for the control planted tubes (est = 2.250, t = 3.282, p =

*0.0347). Neither rosemary nor untreated controls (planted both sides) showed any significant difference in wireworm found in either section (Ro: est = -0.750, t = -1.094, p = 0.9854; **UC**: est = 1, t = 1.459, p = 0.9083).*
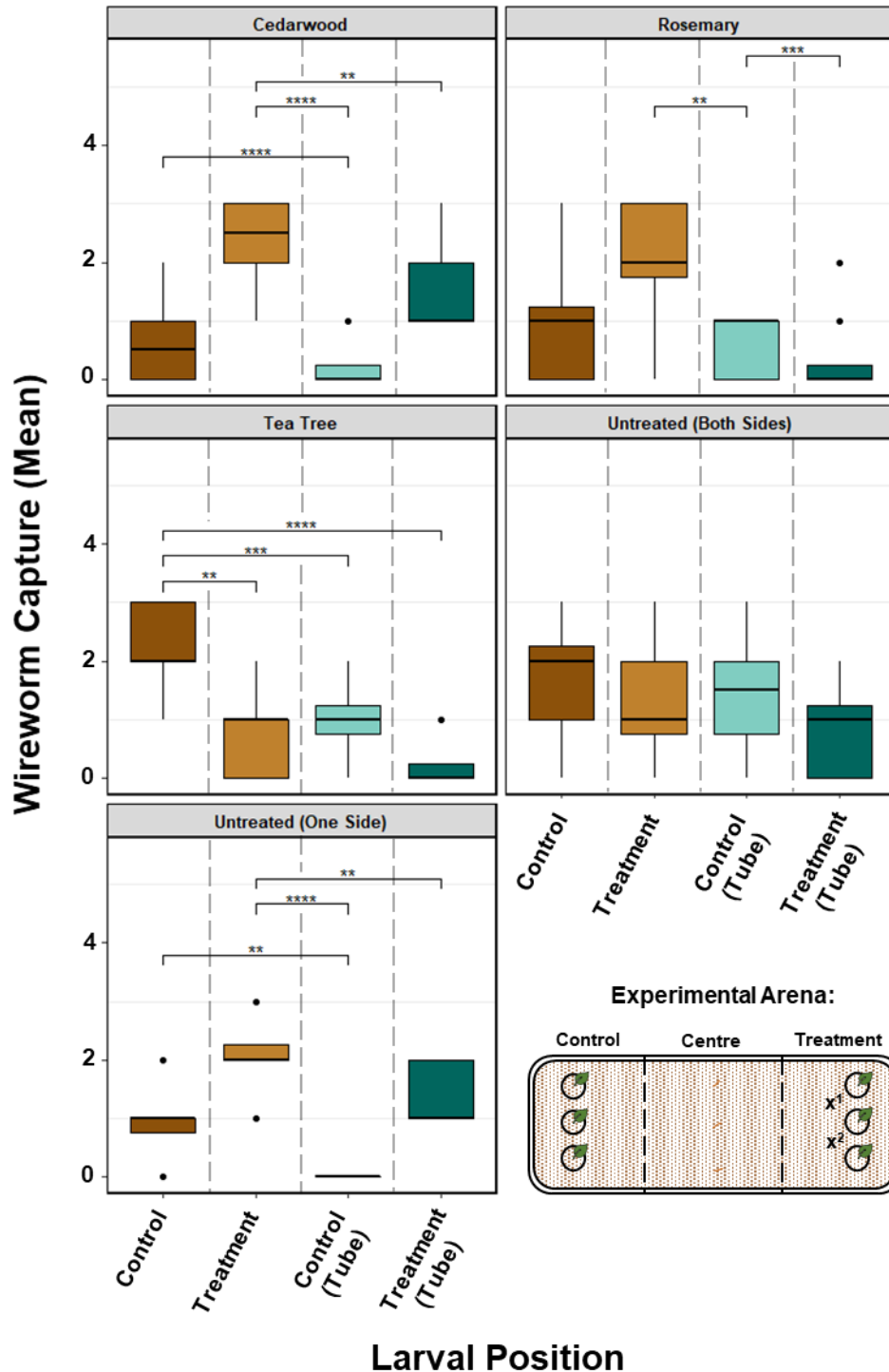
Fig. 3.7. Mean captures of wireworm in CT and arena section to evaluate bioactivity of botanicals in a dual-choice experiment in the presence of maize seedlings. For one-sided untreated controls, the 'treatment' section contained tubes with maize, the control section contained soil only. Statistical significance presented as pairwise comparisons of estimated marginal means

For capture rates in choice arenas, comparisons are visualised in Figure 3.7 between larval presence in each section of the arena, and presence within tubes.

*Cedarwood (79% of larvae) showed a significantly greater level of wireworm presence in treatment sections than tea tree (25%: t = 4.478, d.f. = 13.964, p = 0.007), with more larvae capture within tubes in treatment sections also (**Cw** – 50%; **Ro** – 8%; t = 3.987, d.f. = 11.603, p = 0.013). However, there was no significant difference between wireworm capture in the treatment sections of cedarwood (79% of larvae) and rosemary (67%; t = 0.814, d.f. = 12.493, p = 0.507), though cedarwood had greater larval capture in tubes, with obvious evidence of feeding (**Cw** – 50%; **Ro** – 12%; t = 3, d.f. = 13.996, p = 0.04).*

There was no significant difference in wireworm capture across untreated controls with planted tubes in each section (p > 0.999). Wireworm found within tubes showed evidence of feeding on plants in each. As with cedarwood and one-side untreated controls, there was evidence of feeding in all tubes.

3.3.6 *GC/MS: Constituent Composition of Botanicals*

Peak overlays of the main constituents from each of the three oils over the three-week period can be seen in Figure 3.8.

For tea tree oil, eleven compounds were identified above the 0.05% relative peak area threshold with the majority of these terpenoid class compounds. The percentage change of constituents over the three-week period can be seen in Table 3.2. 4-Terpineol and terpinolene were the dominant compounds comprising > 40% of the total composition each. The breakdown of Terpinolene saw a consistent loss each week with an overall percentage change of -27.03%. The predominant compounds at the end of the three weeks were 4-Terpineol and o-Cymene, with 51.01% and 19.53% of relative peak area respectively.

Sixteen constituents in total were identified in rosemary oil (Table 3.3) above the arbitrary threshold, similarly, made up of mainly terpenes. Eucalyptol (27.74%), limonene (26.64%) and camphor (+)- (24.97%) constituted the largest relative peak area. Over the three-week period, no individual constituent displayed more than a 2% percentage change, with all main compounds remaining consistent.

Cedarwood oil showed eighteen compounds above the arbitrary threshold (Table 3.4), again terpene was the dominant chemical class. α-Longipinene (48.67%), α-Himachalene (23.15%) and Longifolene-(V4) (15.84%) were the main constituents. As with rosemary oil, over the three-week period the greatest percentage change was minimal, with α-Longipinene the greatest difference at -1.36%.
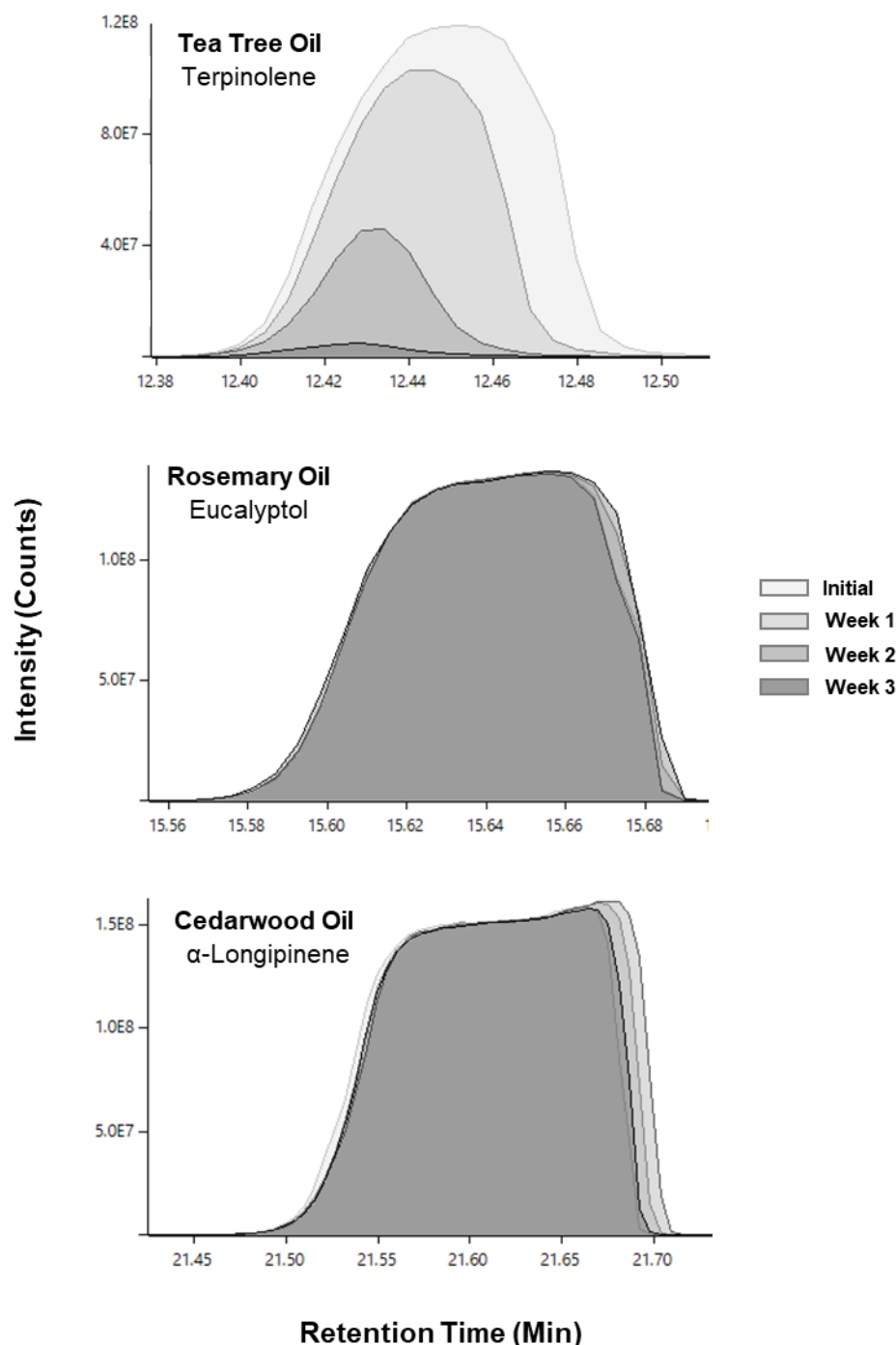
Fig. 3.8. The peak overlay of main botanical constituents within the GC/MS spectra of each oil over a three-week period. Indicates negligible change of a main constituent, consistent with all constituents > 0.05% within the sample, except for tea tree indicating a significant loss of terpinolene.

Table 3.2. GC/MS analysis of tea tree oil constituents and their breakdown over a three-week period. Samples taken from an initial inoculation into 2 ml biodegradable vial, with aliquots removed every seven days and diluted in hexane (1:100) for analysis. Percentage change is presented as a difference from the previous week's abundance, with the overall change presented as a final difference between week 3 and the initial sample. Constituents < 0.05% abundance within the mass spectra were omitted as an arbitrary threshold.

Tea Tree Oil	Percentage composition (Initial)	Percentage Change			Final Percentage Composition	Overall Change
		Week 1	Week 2	Week 3		
4-Terpineol	42.09643%	1.237%	4.458%	3.216%	51.008%	8.911%
Terpinolene	40.221%	-6.454%	-11.755%	-8.823%	13.189%	-27.032%
Artemiseole	8.56724%	-0.297%	0.849%	1.129%	10.248%	1.681%
α-Pinene	2.48038%	-1.142%	-0.468%	-0.417%	0.454%	-2.026%
γ-Gurjunene	2.240%	-0.099%	0.300%	0.541%	2.983%	0.743%
o-Cymene	1.887%	7.077%	6.444%	4.118%	19.52645%	17.639%
Limonene	1.071%	-0.322%	-0.133%	-0.115%	0.50093%	-0.570%
Eucalyptol (1,8-cineole)	0.547%	-0.048%	0.059%	0.020%	0.577%	0.031%
Valencene	0.385%	-0.003%	0.022%	0.018%	0.421%	0.036%
Isopinocarveol	0.06516%	0.002%	-0.004%	0.008%	0.071%	0.006%
Globulol	0.000%	0.000%	0.006%	0.008%	0.062%	0.062%

Figures highlighted as green text indicate positive change from, with red highlighted figures the converse

Table 3.3 GC/MS analysis of rosemary oil constituents and their breakdown over a three-week period. Samples taken from an initial inoculation into 2 ml biodegradable vial, with aliquots removed every seven days and diluted in hexane (1:100) for analysis. Percentage change is presented as a change from the previous week's abundance, with the overall change presented as a final difference between week 3 and the initial sample. Constituents < 0.05% abundance within the mass spectra

Rosemary Oil	Percentage composition Initial	Percentage Change			Final Percentage Composition	Overall Change
		Week 1	Week 2	Week 3		
Eucalyptol (1,8-cineole)	27.739%	-0.218%	0.157%	-0.188%	27.490%	-0.249%
Limonene	26.639%	-0.348%	-0.755%	-0.875%	24.660%	-1.978%
Camphor, (+)-	24.974%	0.092%	0.838%	0.673%	26.57720%	1.603%
o-Cymene	11.223%	1.171%	0.603%	0.197%	13.19478%	1.972%
α-Pinene	3.281%	-0.454%	-0.391%	-1.821%	1.460%	-1.821%
p-menth-1-en-8-ol	1.716%	0.136%	-0.079%	0.248%	2.021%	0.305%
L-borneol	1.373%	0.199%	-0.043%	0.354%	1.884%	0.511%
Isoborneol	0.583%	0.054%	-0.009%	0.088%	0.716%	0.133%
Caryophyllene	0.539%	0.017%	-0.064%	-0.114%	0.378%	-0.161%
Camphene	0.510%	-0.146%	0.295%	-0.272%	0.238%	-0.272%
Linalool	0.308%	0.027%	-0.005%	0.042%	0.372%	0.064%
γ-Terpinene	0.279%	-0.210%	0.230%	-0.230%	0.000%	-0.279%
Acetic acid	0.251%	0.032%	-0.001%	0.049%	0.332%	0.080%
Terpinolene	0.130%	-0.072%	-0.020%	-0.008%	0.030%	-0.100%
γ-Terpineol	0.123%	0.009%	-0.006%	0.001%	0.128%	0.004%
α-Phellandrene	0.058%	-0.036%	-0.013%	-0.006%	0.004%	-0.055%

Table 3.4. GC/MS analysis of cedarwood (Atlas) oil constituents and their breakdown over a three-week period. Samples taken from an initial inoculation into 2 ml biodegradable vial, with aliquots removed every seven days and diluted in hexane (1:100) for analysis. Percentage change is presented as a change from the previous week's abundance, with the overall change presented as a final difference between week 3 and the initial sample. Constituents < 0.05% abundance within the mass spectra were omitted as an arbitrary threshold.

Cedarwood Oil	Percentage composition (Initial)	Percentage Change			Final Percentage Composition	Overall Change
		Week 1	Week 2	Week 3		
α-Longipinene	48.669%	-0.999%	-0.872%	0.508%	47.306%	-1.363%
α-Himachalene	25.153%	0.158%	0.164%	0.053%	24.644%	-0.509%
Longifolene-(V4)	15.842%	0.545%	0.503%	-0.359%	16.532%	0.689%
Tumerone	1.933%	-0.363%	-0.268%	-0.427%	1.569%	-0.364%
Bisabolol	1.668%	0.009%	0.327%	-0.110%	0.875%	-0.794%
Germacrone	0.905%	0.125%	0.119%	-0.069%	2.225%	1.320%
α-Caryophyllene	0.719%	0.130%	0.112%	0.004%	1.080%	0.361%
Caryophyllene oxide	0.578%	0.097%	0.217%	0.376%	0.965%	0.388%
ñ-4-Acetyl-1-methylcyclohexene	0.546%	0.021%	0.043%	-0.040%	1.267%	0.721%
Himalchalene-1	0.457%	-0.001%	-0.014%	-0.094%	0.570%	0.113%
o-Cymene	0.240%	0.023%	0.030%	0.002%	0.348%	0.108%
Isolongifolene	0.228%	-0.179%	-0.049%	0.036%	0.295%	0.067%
Valencene	0.188%	0.017%	0.022%	0.005%	0.363%	0.175%
Vestitenone	0.184%	0.184%	-0.605%	-0.088%	0.232%	0.048%
Perillol	0.150%	0.023%	0.029%	0.008%	0.210%	0.060%
Bicyclo[3.1.1]hept-3-ene, 4,6,6-trimethyl-2-vinyloxy-	0.103%	-0.008%	-0.005%	-0.009%	0.080%	-0.023%
Viridiflorol	0.096%	-0.363%	0.008%	0.003%	0.116%	0.020%
(Z,Z)-α-Farnesene	0.086%	-0.021%	0.009%	-0.020%	-0.020%	-0.106%

3.3.7 GC/MS: Diffusion through Soil

Headspace analysis of botanical volatile diffusion through soil yielded chromatograms for both rosemary and tea tree, but soil cores from cedarwood treated arenas showed no peaks identifiable as constituents of the oil at either 3 or 7-day time points. Peaks observed at three days were compounds consistent with column bleed effects. Column bleed peaks were observed at 3-day time points for both rosemary and tea tree treated soil cores additionally, albeit alongside volatile constituents of the oils.

For both rosemary and tea tree, ion counts were low at both 3-day (~5.00e7) and 7-day time points (6.00e6 – 2.00e7). There was also no evidence of constituent peaks from oils at the top sections of oil core for both 3 and 7-day time points.

Significant peaks for oil constituents of rosemary can be seen in overlay plots in Figure 3.9. The bottom third of soil cores contained the most evidence of compounds, with eucalyptol, D-Limonene, and o-Cymene present at 3-days, with o-Cymene not present in soil cores at 7 days. Ion counts of both eucalyptol and D-Limonene did not decrease significantly over the 7 days, concomitant with findings in section 3.3.2.

For tea tree oil, similar constituents were observed (Figure 11, with peaks for eucalyptol, D-Limonene and o-Cymene identified at both 3 and 7-day time points, in addition to terpinolene and γ-Terpinene. Middle and top sections of soil cores showed no evidence of oil constituents, except for minimal presence of o-Cymene in the middle core at 3-days post-inoculation. There was a decrease in ion intensity from 3 to 7-days for each constituent identified, concomitant with earlier findings.
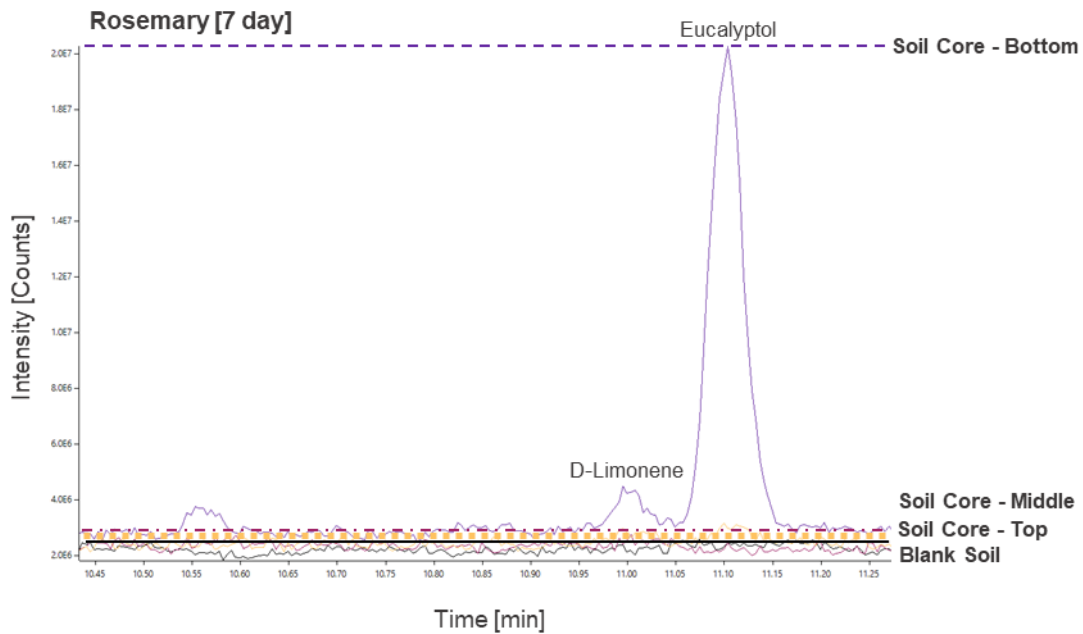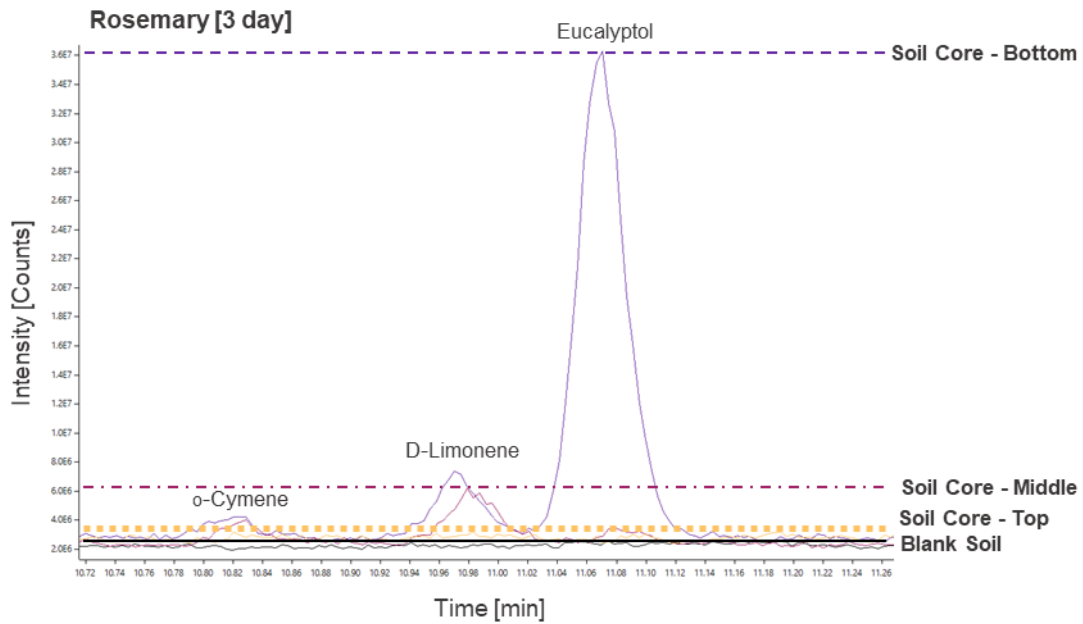
Fig. 3.9 (a & b). Peak overlays for main observed constituents of rosemary for each depth of the soil core, at both 3 day and 7-day time points. Marked line overlays were included to indicate highest point of compound for corresponding soil core section as a visualisation of constituent decrease. The blank soil baseline is included for comparison.
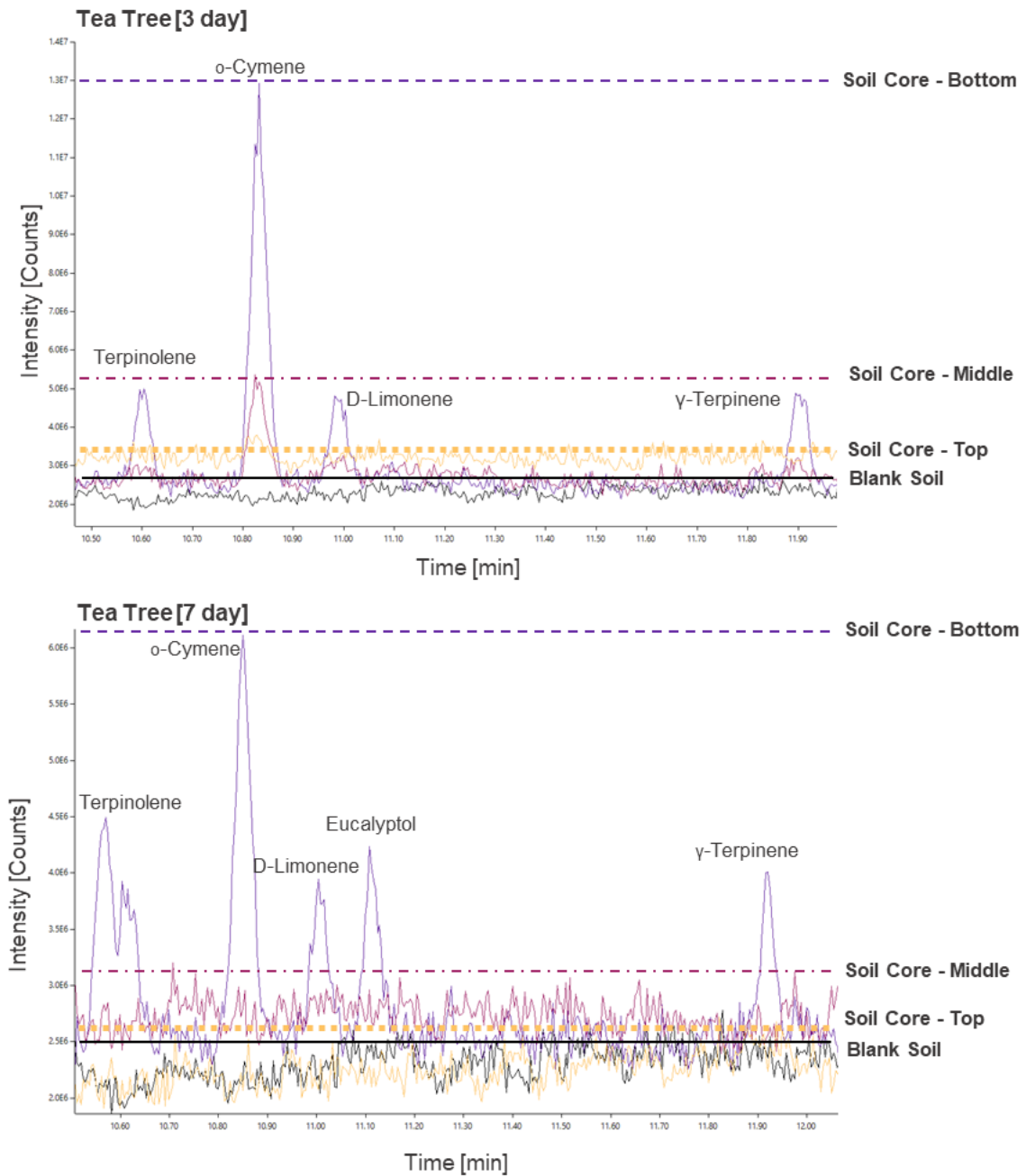
Fig. 3.10 (a & b). Peak overlays for main observed constituents of tea tree for each section of the capture tube, at both 3 day and 7-day time points. Marked line overlays were included to indicate highest point of compound for corresponding soil core section as a visualisation of constituent decrease. The blank soil baseline is included for comparison.

3.4 Discussion

The ability to accurately determine behavioural responses of subterranean invertebrates to introduced stimuli is an ongoing problem in integrated pest management (van Herk, 2008; Campos-Herrera *et al.*, 2013). There is a clear need to develop efficient and cost-

effective strategies to bridge a divide between laboratory studies and efficacy in the field (Barnett & Johnson, 2013; la Forgia *et al.*, 2020). In this study it was found that novel CTs can be used to quantify simple wireworm responses to stimuli through presence or absence within tubes. Further specificity in behavioural response may be considered with observations of both entry holes to the tube and feeding damage to included food source, plant materials or otherwise. As described here, analysis of the larval condition itself, such as MRGR, may be used in conjunction with no-choice capture experiments to ensure specificity in behavioural classification. Time dependent responses of larvae can be incorporated to experimental design with removal and replacement of the CT ensuring root systems of seedlings remain intact, avoiding damage to the plant releasing secondary volatiles (Figure 3.11A). The presence of clear entry holes in tubes not containing wireworm (Figure 3.11C), show that larval behaviours can be qualified further than simple attraction or repellence by correlating this information with seedling damage.

It was found that, in instances of plant comparisons, choice experiments revealed a clearer picture of wireworm food preferences. No-choice pot experiments with food source as the only variable highlighted the polyphagous edacity of wireworm (Adhikari & Reddy, 2017), with no significant differences between plant species observed. Although, when presented with a choice, wireworm showed a clear preference for maize over any of the identified cereal 'trap crops', and over potato additionally. Recent studies have posited that volatile organic compounds (VOCs) of maize were highly attractive to wireworm (la Forgia *et al.*, 2020). These findings and other less conclusive (mixed larval responses to VOCs of Barley – Barsics *et al.*, 2013) may be more robustly evaluated with use of the developed methodology presented here, and provide more conclusive behavioural classifications. Any introduced attractant, crop or compound, must acknowledge that it may act as a buffer strip for sustaining pest populations. As a result, the most viable strategy for wireworm control would be the incorporation of findings into a lure-and-kill programme to ensure eradication or suppression of the pest.

Pot assay plant preference results minimises the importance of plant choice in CT assays to evaluate semiochemicals, requiring simply a known food source. However, analysis of root volatile profiles should be considered in further research to understand the balance of compounds within the soil when targeting specific crop species. There is a possibility that introduced botanicals could activate, or mimic, plant defences (Erb *et al.*, 2009; Dewhirst *et al.*, 2012). This may indicate multiple modes of action of the essential oils, further impressing the need to identify the mechanisms by which the oils affect both insect and plants, explored further in Chapter 2, section 2.3.3.

The inclusion of semiochemicals to assess responses of larvae despite a clear food source indicated a range of behaviours exceeding a simple binary reaction. Tea tree and rosemary showed a complete repulsion of the larvae from the CT, with a negative MRGR suggesting a complete suppression of any feeding and a deleterious effect on larval condition. Citronella oil elicited the same response, with some capture of larvae

Fig. 3.11 (A – C). Details of capture tube in-situ and removed from experimental arenas (A) Detail of whole tube removal from experimental arena, with inclusion of captured larvae. Images taken from dual-choice capture tube experiments. **(B)** Active entry of wireworm into capture tube at highest point, adjacent to seed and above inoculation point. **(C)** Evidence of an entry hole **(Cⁱ)** present on an empty tube. This would suggest wireworm foraging movement affected by antifeedant effect of lemon oil on arrival at food source, especially with limited evidence of feeding and small positive weight gain.

indicating a weaker effect. Disparity between limited larval capture and positive MRGR in lemon oil suggests an antifeedant effect, corroborated by a lack of feeding damage compared to both untreated controls and cedarwood oil. Cedarwood showed marginally more capture and MRGR in larvae compared to untreated control suggesting a mild phagostimulant effect.

Choice assays highlighted the effects of the botanicals in a larger, semi-natural arena. The effects of both cedarwood and tea tree on larvae in no-choice pot assays were replicated. Wireworm in cedarwood treated replicates showed a clear attraction towards treated plants and presence within CT again suggested a phagostimulant effect. Tea tree treated arenas however repelled larvae almost entirely into control sections, with limited feeding observed. Interestingly, rosemary treated arenas showed a larval

presence in treated sections comparable to cedarwood. However, little or no wireworm were found within CT. This would suggest a possible neuro-inhibitory effect of the botanical (Tak *et al.*, 2016; Chaudhari *et al.*, 2021), causing random non-specific movement and disruption of the foraging response. This supports this studies hypothesis that the use of the CT in a larger semi-natural arena can indicate further behaviours masked by simple binary experiments.

Analysis of constituent breakdown of essential oils used in capture tube studies showed a clear loss of key constituents in tea tree oil compared to both cedarwood and rosemary. Previous research has indicated, in cases of antimicrobial activity, that complete oils provide a greater bioactive effect than individual constituents (Tabassum & Vidyasagar, 2013; Teixeira *et al.*, 2013). Though the loss of significant compounds (> 40% relative abundance) from the established constituent matrix of the oils may have an impact on both the oils' ability to have a lasting bioactive effect in the soil on any invertebrate behaviours or mortality, and on the ability of the oils VOCs to permeate through a soil matrix. Headspace analysis of soil cores would support this hypothesis, with only the significant compounds detected at the base of the CT with limited diffusion up through the soil profile. Indeed, this supports knowledge that CO_2 is the most significant long-range phagostimulant for wireworm (Johnson & Gregory, 2006; Schumann *et al.*, 2014). Although, behavioural findings here lend weight to growing evidence that short range cues have a significant impact on larval responses (Horton *et al.*, 2012; Johnson & Nielsen, 2012) and can have significant repellent or protectant effects even in cases of initial wireworm presence (Cherry & Nuessly, 2010; Brandl *et al.*, 2016; Eckard *et al.*, 2017). Incorporation of essential oils and CO_2 in a common system would be a complex challenge, existing formulations rely on the incorporation of yeast to consistently produce a release of CO_2 and essential oils are likely to have an adverse effect on their growth (addressed further in Chapter 4). This may have a significant impact on any biological control methodologies relying on manipulating invertebrate behaviours. As such, the formulation of any novel product should be heavily informed by both the timing and area of application.

There is potential for use of the CT device for monitoring in the field, with work need to adapt the design to protect against disruption from weather and larger fauna. Subterranean pitfall traps exist with a design emphasising collection of specimens in the field (Jowett *et al.*, 2021), as well as rough monitoring devices requiring burying and sealing (Parker & Howard, 2001). The examples shown here in evaluating behavioural responses in two separate arenas suggest that the novel CT are best utilised as a bridge between laboratory and field work.

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Chapter 4: Entomopathogenic Fungi and Essential Oils: Compatibility in an IPM strategy for Wireworm Control

Abstract

The biological control of wireworm (*Agriotes* spp.) is currently most advanced in the use of entomopathogenic fungi (EPF), primarily from the Hypocrealean genus *Metarhizium*. The use of EPF against wireworm in a field setting however is made problematic by an inability to directly target a heterogenous, subterranean pest population, and abiotic factors affecting the EPFs stability and efficacy. Research has turned its focus to improving the efficacy of EPF by manipulating wireworm behaviours, or reducing their pathogenic defences, through inclusion of bioactive compounds in partnership with the fungi. Here we show that previously identified botanicals, tea tree, rosemary and cedarwood do exhibit fungicidal and fungistatic effects on strains of *Metarhizium brunneum*, but at lower concentrations can improve mortality and rate of pathogenicity. Furthermore, through field evaluation of an existing EPF biocontrol, we highlight the need to improve pest targeting with short-range cues in the soil, in addition to CO_2. The variability in behavioural effects of the three botanicals may allow for implementation of a push-pull system of control, or simply improve an existing template for an attract-and-kill system.

4.1 Introduction

The control of wireworm (agriculturally significant elaterid larvae) is currently problematic for growers with the deregistration of accepted conventional controls (Ritter & Richter, 2013; Poggi *et al.*, 2021) and a lack of commercially ready biological alternatives (la Forgia & Verheggen, 2019). The biological control of wireworm currently shows most promise with the application of biopesticides created with entomopathogenic fungi (EPF), with several products available for other invertebrate pests. The primary groups identified for pathogenicity towards wireworm occur within Hypocreales; *Metarhizium brunneum* (Petch), *Beauveria bassiana* (Balsamo) and *Isaria fumosorosea* (Wize) (Faria & Wraight, 2007; Kabaluk, 2014; Sharma *et al.*, 2020).

Wireworm exhibit particular robust defences against the physical penetration of EPF hyphae and the action of enzymes (Eckard *et al.*, 2013; Butt *et al.*, 2016; Aw & Hue, 2017). Previous research has demonstrated the efficacy of EPF Metarhizium in controlling wireworm under laboratory conditions (Ansari *et al.*, 2009; Eckard *et al.*, 2014), but application in the field is restricted by the ability to accurately target the larvae and the time taken to kill and spread infection (Tharp *et al.*, 2007; van Herk *et al.*, 2013; Traugott *et al.*, 2015). Additionally, the impact of abiotic factors (e.g. moisture, temperature, pH) can affect both the stability and efficacy of the EPF once applied to the soil (Sharma *et al.*, 2020). Although research into soil type on the efficacy of EPF is understudied, it is known that percolation of conidia is slow through the substrate (Ensafi *et al.*, 2018), emphasing the need for greater targeting of the pest. Examples of abiotic factors such as tempterature and moisture may be more readily available for EPF, with Mishra *et al.* (2015) finding optimum conditions for *Beauveria bassiana* L. of 80 – 100% soil moisture content and temperature of approximately 30 °C when applied as loose conidia. As such, any field-scale application of EPF must be appropriately formulated to not only retain some sort of shelf life prior to application, but also a shield against fluctuating abiotic factors. The registered biological control Attracap® (BIOCARE GmbH, Dassel-Markoldendorf) makes use of yeast-produced CO_2 as a known long-range attractant for wireworm to bring them into direct contact with biodegradable granules loaded with a strain of *Metarhizium brunneum* (Hermann *et al.*, 2017). These capsules ensure a measure of protection for the biocontrol agents against abiotic stresses. Furthermore, the factor of stability remains a pertinent one as any strains of EPF cultured on a commercial basis must retain virulence under a process of mass cultivation through subsequent infections (Eckard *et al.*, 2017).

This manipulation of wireworm behaviours to bring them into contact with a known entomopathogen is increasingly being explored to overcome the hurdles of targeting pests within a heterogenous population (Kabaluk *et al.*, 2015; Adhikari & Reddy, 2017; Brandl *et al.*, 2017). Other work has attempted to augment the lure-and-kill model with botanical extracts that may increase chemotactic responses or even work synergistically with EPF to reduce the LD_{50} or decrease time-to-kill (Eckard *et al.*, 2017). Previous work carried out within the research group (Hummadi *et al.*, 2021) highlights the role of volatile organic compounds produced by *M. brunneum* and the role those may have in either direct bioactivity on invertebrates (both insect and nematode) or behavioural manipulation. Building on the initial mortality screening here, the effect of these on wireworm is explored further in Chapter 5.

This study focuses on the use of botanicals to work synergistically with EPF for wireworm control to specifically target the pest and improve efficacy, in terms of both lethality and timeframe. Building on behavioural and sub-lethal effects of bioactive botanicals identified in Chapter 2, this study also builds on existing work by Butt *et al.*, (2013) in exploring the use of caspase activity as an indicator of stress in insects. The role of caspases within insects is particularly understudied but it is hypothesised here that it may be used in conjunction with bioassays to indicate stress in wireworm exposed to compounds eliciting clear distress (Chapter 2, section 2.3.3).

4.1.1 Aims

- **To determine the most virulent strains of *Metarhizium* against *Agriotes* spp. and identify any disagreements within existing literature**
- **To determine the contact and volatile fungitoxicity of identified bioactive botanicals**
- **To assess whether EPF may be used in synergy with botanical bioprotectants to suggest a more effective approach to biocontrol within potato crops**

4.2 Methods

4.2.1 Insects

Wireworm were collected from the field and supplied by the Applied Plant Research station, Wageningen Field Crops (PPO, Lelystad, Netherlands). Larvae were identified to genus and a subsection identified to species to be representative of the collected population (Klausnitzer, 1994). Individuals were separated into aliquots of 10 and

checked for signs of infection prior to sequestration into culture. Specimens used in all assays were late instar larvae (25 – 45mg, 15 – 25mm as in Furlan, (2004)).

Larvae were then maintained in 1L pots filled with a blend (1:1 v/v) of loam and sand and kept in controlled environment chambers at 15°C ± 1°C (60% RH ± 5%, 16:8 L:D) photoperiod and fed with fresh potato slices (approximately 1 x 1 x 2cm, inserted centrally and flush with the soil surface) every three days. Five larvae were contained in each pot. Larvae were starved 2 weeks before each experiment to encourage host seeking behaviour.

4.2.2 Entomopathogenic Fungi

Several strains of *Metarhizium* spp. were used across all assays, with strains selected from those previously identified as having some entomopathogenic effect towards wireworm (Ansari *et al.,* 2009). These were primarily strains of *Metarhizium brunneum* with some strains not sequenced beyond genus. Strains of EPF were taken from existing frozen stock cultures (-80°C) originally obtained from USDA ARSEF culture collection and passaged through *Tenebrio molitor* larvae with an immersion inoculation, with 10 second submersion in a solution of 10^8 colony forming units (CFU) in Tween 80. Streaks were taken from sporulated cadavers on placed onto Saboraud dextrose agar (SDA) plates with antibiotics (chloramphenicol), before final inoculations onto pure SDA plates (90 mm diameter Petri dish, 20 ml SDA). Final plates were stored in sterile fridge conditions (4°C, ±1°C) until ready for use.

4.2.3 Chemicals

Essential oils were obtained from BiOrigins (Madar Corporations Ltd), selected for CAS numbers matching those from Sigma Aldrich (Merck Life Science Uk Ltd). Extracts from plant materials were obtained through cold-pressing raw materials or steam distillation. The oils included: tea tree (*Melaleuca alternifolia*, Cheel), rosemary (*Salvia rosmarinus*, Spenn), cedarwood (Atlas, *Cedrus atlantica*, Endl.) Oils were applied neat, without carrier, unless otherwise specified. Full GC/MS analyses may be seen in Chapter 3.

4.2.4 Comparison of EPF Strains

Seven strains of Metarhizium were selected for experiments after narrowing down the most virulent strains tested against wireworm in previous research. Spores from EPF cultured as in 4.2.2 were obtained through mass production on broken rice grains and separation from the substrate with a custom 'mycoharvester'. Spores were then stored in sterile conditions at 4°C. Prior to use in experimentation spores from each strain were tested for germination in a 24-hour assay on SDA plates.

Spores of each strain were homogenised within a soil substrate (mixed compost with John Innes & sand, 3:1, 20% moisture, v/v) to a concentration of 2.5 - 5 x 10^8 colony forming units (CFUs). The mix was aliquoted into 50 ml lidded pots (30 g ± 1 g), and five larvae were introduced into each arena. A carrot slice was placed on top, with this changed every three days to avoid introducing secondary infections. Larval mortality was checked every three days with dead individuals removed to clean arenas (Petri dish, 45 mm diameter, single filter paper base) to check for fungal infection and subsequent sporulation. The final check occurred at 27 days post-inoculation. There were five larvae per replicate, and there were six replicates for each strain.

4.2.5 Fungitoxicity

An adaptation of the 2-fold serial dilution technique from the CLSI document M38 was used to determine the minimum inhibitory concentration (MIC) of essential oils against EPF used in mortality assays. Microplate wells were pipetted with 50 µl of Sabouraud dextrose broth (SDB, 10 g/L mycological peptone, 40 g/L glucose, in distilled water), and 100 µl of essential oil diluted 50% in DMSO was added to the first row. From well one onwards, 50 µl was pipetted forward in 2-fold serial dilutions ranging from initial 20% to 0.020%.

An EPF suspension in Sabouraud dextrose broth was prepared from sporulated colonies on SDA plates, the number of colony forming units (CFU) quantified with a haemocytometer and diluted to a density of 1 – 5 x 104 CFU/ml. This was then added to the dilutions for a total volume of 200 µl per well. Plates were stored in an incubator maintaining conditions of 27°C, 60% RH. The MIC was determined by the lowest concentration of oil that completely inhibited any fungal growth. A further metric MIC <50 was determined by wells that showed less than 50% of growth compared to the untreated control.

Any fungicidal effect was determined through pipetting 10 µl of broth from completely inhibited wells onto SDA agar plates and assessing growth over a 72-hour period. The

lowest concentration that did not result in mycelial growth was determined the minimum fungicidal concentration (MFC). *Metarhizium brunneum* strains V275, ARSEF 4556 and ARSEF 3297 were used for screening and all treatments were repeated three times.

To differentiate between contact and volatile fungitoxicity, essential oils were screened in a fumigation assay. Filter discs (5 mm diameter) were loaded with different concentrations of oils (10 µl total volume, cedarwood, rosemary & tea tree) and adhered to a glass covered slip attached to the centre of a Petri dish lid fixed with water tension (1 µl distilled water). Concentrations of oils began at 10% (w/w, diluted with DMSO) and decreased in two-fold serial dilutions to 0.078%. A single agar plug (7 mm) was taken from SDA plates of *Metarhizium brunneum* (strains 4556, 3297 & V275) 10-days post inoculation and placed conidial-surface-down in the centre of a fresh SDA plate (20 ml) and the treated lid attached.

Petri dishes were sealed with Parafilm M™ and left for 72 hours to incubate at 27 °C, 60% RH. The zone of inhibition was measured and compared between treatments, with inhibition of growth calculated as in Kordlali et al (2009), with measurements of growth adapted for area in place of diameter:

$$Percentage\ Inhbition\ (PI) = \frac{C - T}{C} \times 100$$

Where C is equal to average of growth area of controls and T is equal to average of growth area of treated plates. Four replicates were used for each concentration of each treatment and controls.

Concentrations used were those ranging from the MIC and MFC identified in the broth dilution assay, with an untreated control and neat oil as a positive comparison.

4.2.6 Caspase Activity

The caspase activity was assessed in wireworm exposed to each of 1-octen-3-ol and 3-octanone. Caspase activity methods were adapted from those presented within Butt *et al.* (2013), in which the method was used to evaluate stress responses in mosquito larvae (*Aedes aegypti* L.) to *Metarhizium* spp. exposure. Although specific caspases are understudied in relation to insects, it was hypothesised that utilising these methods for wireworm exposed to oils may indicate a basis for stress observed in behavioural assays in Chapter 2, section 2.3.4.

Larvae were exposed to doses of oils shown to elicit mortality after 24 hours (20 µl) in the same Petri dish arena outlined in Chapter 2, section 2.2.4, but removed from the fumigation arena after three and six hours, timepoints at which stress behaviours were observed but mortality did not occur. Controls containing untreated larvae were removed at those time points to evaluate stress based on handling as a comparison.

Larvae (three wireworm per treatment) were then frozen with liquid nitrogen upon removal from an initial experimental arena and then homogenized mechanically. Total homogenates were resuspended in 420 µL 0.5% Triton lysis buffer (Tris 20mM, NaCl 100mM, EDTA 500mM, 0.5% Triton X-100) and agitated gently before incubating on ice for 10 min. Homogenates were then centrifuged at 14, 000 g for 10 min and 38 µL aliquots of supernatant were added to 3 replicate wells in a white walled 96-well microtiter plate (Costar, Corning). Luminometric assays for caspase-2, caspase-3, caspase-7 and caspase-8 activity were performed in accordance with the manufacturer's guidelines using the Caspase Glo 2, Caspase Glo 3-7 and Caspase Glo 8 assay kits (Promega) by adding equal volume (38 µL) of Caspase Glo reagent to the sample.

4.2.7 Stress-and-Kill

Larvae were exposed to doses of oils whilst contained within EPF-mixed substrate to examine any effect on the time-to-kill or efficacy of the entomopathogen, suggesting their potential for a stress-and-kill approach to control. The experimental arena consisted of a 6-well cell culture plate, with each well filled with 3 ml of spore-mixed substrate (multipurpose compost with John Innes & *Metarhizium strain* ARSEF4556 at 2.5 – 5 x 10^7 CFUs) and a filter tip adhered to the base of each cell inoculated with a concentration of essential oil. A single larva was introduced to each well.

Cedarwood, rosemary and tea tree oils were used in experiments at ten-fold serially diluted concentrations of 10, 1 & 0.1%, with DMSO as a solvent. EPF-only and 10% oil replicates were used as controls. Experimental arenas were kept in constant conditions (25°C, 65% RH) in the absence of light and checked for mortality every three days under red light. Six individuals were used per 6-well plate and each concentration of each oil was repeated in triplicate. Dead individuals were removed to a fresh arena as in section 2.4.

4.2.8 Statistical Analyses

For mortality data in comparing fungal strains and oil / EPF exposure assays, Kaplan-Meier plots were used for visualisations with comparisons between variables made with survival regression models, plotted as model curves (CRAN packages: 'survival' - ver. 3.2-13, 2021; 'survminer' – ver. 0.4.9, 2021). Continuous measurements made for both antifungal assays compared treatments with analysis of variance, with any pairwise interactions compared with Tukey's post-hoc analysis ('multcomp' – ver. 1.4-17, 2021). Analysis of growth area for fungitoxicity experiments (dilution and volatile effects) were carried out with ImageJ (1.53 K, NIH, USA). All statistical analyses were performed using RStudio Build 351, utilising R version 4.1.2 (2021).

4.3. Results

4.3.1 Comparison of EPF strains

Survival probability of wireworm exposed to strains of *M. brunneum* can be seen in Figure 4.1. Strain was found to have a significant effect on larval mortality ($\chi^2 = 31.76$, df = 5, $p < 0.0001$), with all strains eliciting some mortality, whilst wireworm exposed to untreated controls showed complete survival over the four-week period. Strain ART2825
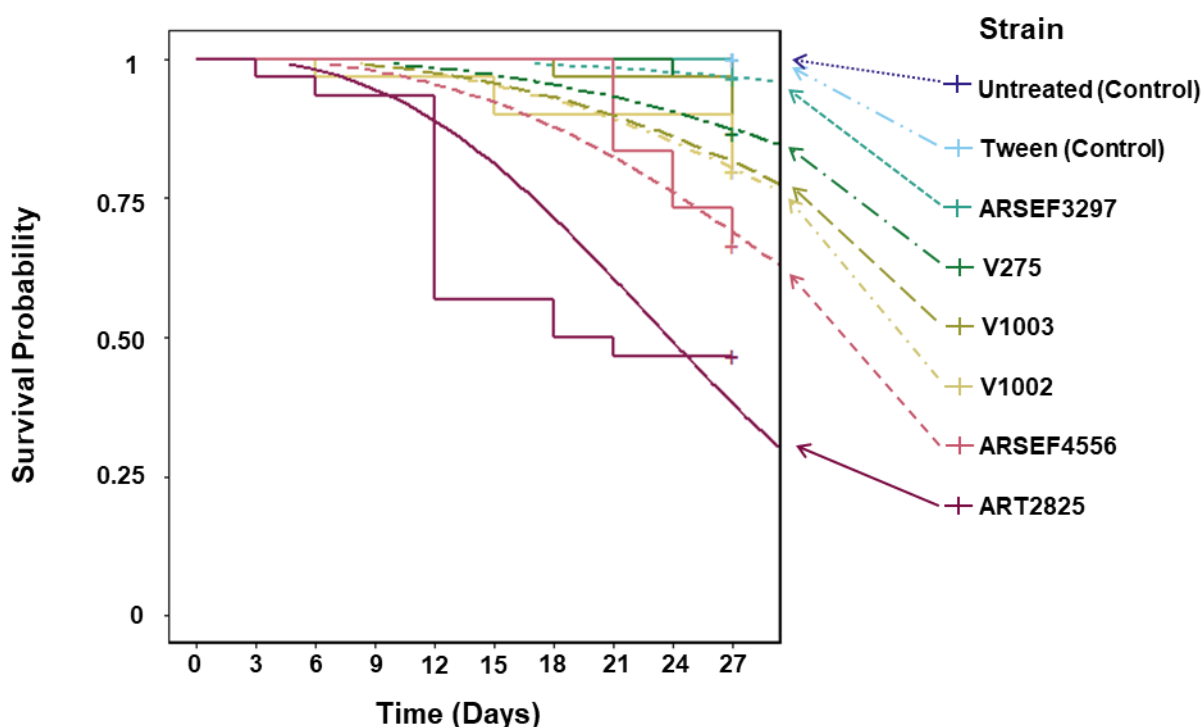
Fig. 4.1. Survival of wireworm exposed to strains of *M. brunneum*. A spore suspension was mixed in substrate at concentrations of 2.5 – 5 x 10^8. Untreated larvae and Tween at 0.03% were used as controls. Five larvae were used per replicate, with three replicates per treatment, repeated twice. Censoring occurred across all treatments.

was the most pathogenic towards wireworm with just over 50% mortality over four weeks. Strain ARSEF4556 was found to be the next effective strain with 27% larval mortality. All other treatments had a larval survival rate of over 80%.

4.3.2 Fungitoxicity

Highlighted in Figure 4.3 are a section of broth microdilution replicates for ARSEF4556 exposed to each oil, after 72-hours. The difference in MIC and MIC (<50%) can be seen in the strength of sporulated growth, indicated with annotations.

Of the three *M. brunneum* strains tested, ARSEF3297 showed the least resistance to the oils with MICs of 0.26% for rosemary, 0.21% for tea tree and 4.12% for cedarwood (Table 4.1). Results for MIC (<50%) were considerably lower than MICs with results of 0.02% for rosemary, 0.02% for tea tree and 0.16% for cedarwood.
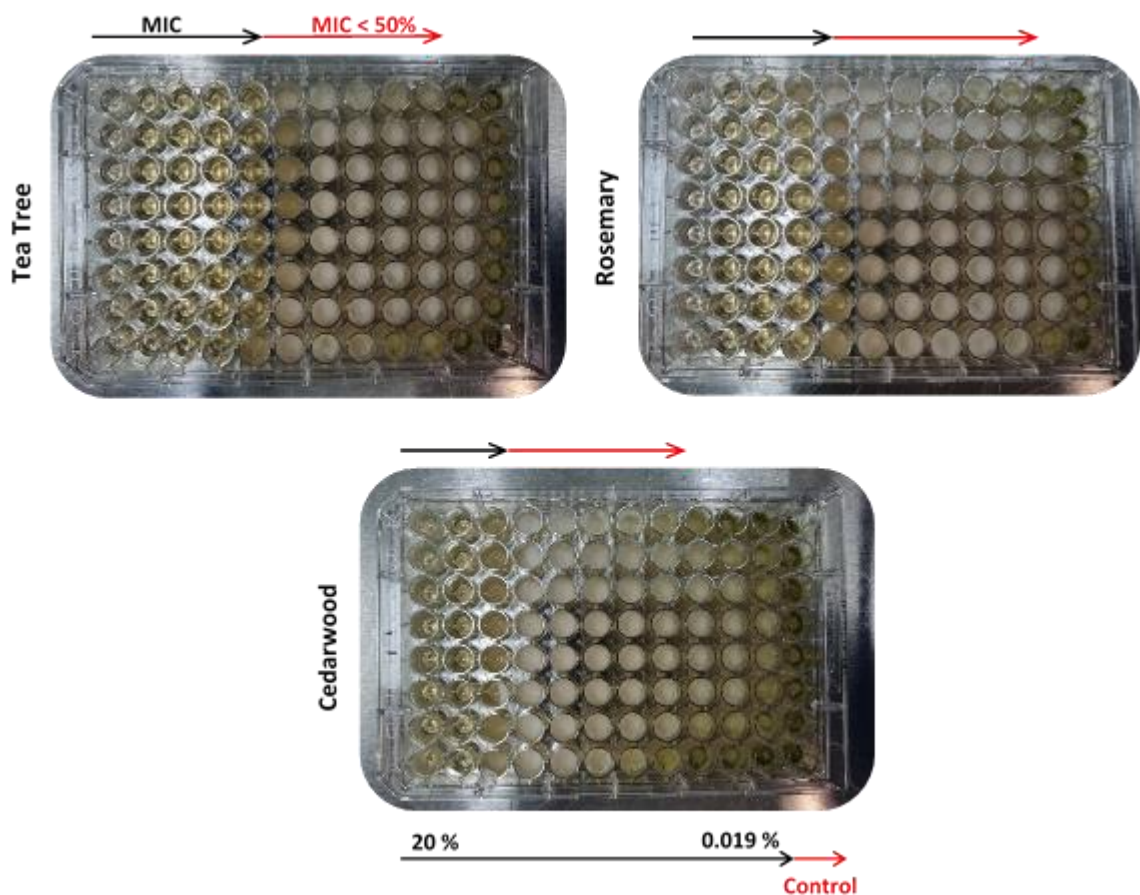
Fig. 4.3. Broth dilution fungitoxicity assay to evaluate response of *Metarhizium brunneum* strain ARSEF4556 to botanical extracts. Initial wells contained 100µl of 20% dilution of oil in DMSO and SDA broth, with two-fold serial dilutions to 0.019% in well 11. The final well contained an untreated control of SDA broth. All wells inoculated with 100µl of $1 - 5 \times 10^{5}$ spore suspension. **MIC** = Minimum inhibitory concentration. **MIC < 50%** = less than 50% growth compared to control. Each after 72hrs. The broth dilution was carried out three times for each strain.

Strains ARSEF4556 and V275 showed greater resistance to the essential oils with comparable results to one another for each. Again, cedarwood prompted the least resistance with MICs of 10% for both strains, and low MIC (<50%) values observed (4556 = 0.21%, V275 = 0.16%). Rosemary treated strains gave MIC values up to five times lower than cedarwood (4556 = 2.92%, V275 = 1.67%) with MIC (<50%) values approximately three times lower than those observed for cedarwood (4556 = 0.07%, V275 = 0.05%). Tea tree treated strains were comparable to rosemary, with MIC values the same for both (1.67%) and MIC (<50%) values only marginally lower (4556 = 0.04%, V275 = 0.07%).

Table 4.1. Minimum inhibitory (MIC), MIC < 50% and Minimum Fungicidal Concentration (MFC) of three strains exposed to essential oil treatments in a microbroth dilution assay.

Treatment		Fungitoxicity Values (µl)		
		ARSEF 3297	ARSEF 4556	V275
Rosemary	MIC	0.26	2.92	1.67
	MIC <50%	0.02	0.07	0.05
	MFC	1.67	5	4.17
Tea Tree	MIC	0.21	1.67	1.67
	MIC <50%	0.02	0.04	0.07
	MFC	1.25	6.67	6.67
Cedarwood	MIC	4.12	10	10
	MIC <50%	0.16	0.21	0.16
	MFC	13.3	16.67	20

After inoculation of SDA plates with broth solutions from inhibited wells, there was evidence of fungal growth beyond the MIC for each strain exposed to each strain. Cedarwood continued to require higher concentrations of oil to have any fungitoxic effect, with 3297 showing no fungal growth at 13.3%, 4556 at 16.67% and V275 at the highest concentration tested at 20%. Rosemary treated strains gave much lower MFC values, with 3297 again the least resistant at 1.67%, and then V275 at 4.17%, with 4556 with complete fungitoxicity at 5% concentration. Tea tree gave similar values to rosemary with MFC values of 3297 (1.25%), 4556 and V275 elicited the same MFC as one another for both tea tree and rosemary (6.67%).

Inoculated SDA plates with the highest (10%) and lowest (0.078%) concentrations of oils in volatile inhibition assays can be seen in Figure 4.4 for each oil tested compared to an untreated control of strain ARSEF4556.

Table 4.2 indicates the percentage inhibition (PI) of each strain at the highest and lowest concentration of essential oil and the difference between the two values. At 10%, cedarwood treated plates gave the lowest PI. With 38.66% for ARSEF3297, 69.9% for 4556 and the lowest for V275 at 5.96%. At the same concentration, tea tree and rosemary treated plates showed comparable inhibition for both 3297 (**Tt** = 50.01%, **Ro** = 48.52%) and 4556 (**Tt** = 78.83%, **Ro** = 79.97%), though tea tree appeared more pathogenic towards V275 (32% PI) than rosemary (17.76%).

Table 4.2. Percentage Inhibition (PI) of *M. brunneum* strains exposed to volatiles of essential oils in decreasing two-fold serial dilutions. Given here are the highest dose (10%) and the lowest (0.078%) and the difference between them. The higher the percentage inhibition, the greater the inhibitory effects of the volatiles in comparison to an untreated control. A negative difference between the two concentration end points indicates a concentration dependent response in the fungal inhibition of the volatiles.

Treatment	Concentration	Percentage Inhibition (PI) of Metarhizium Strain		
		ARSEF 3297	ARSEF 4556	V275
Rosemary	10%	48.52%	79.97%	17.76%
	0.078%	35.05%	81.47%	14.13%
	Diff.	-13.48%	1.50%	-3.63%
Tea Tree	10%	50.01%	78.83%	32.11%
	0.078%	18.81%	78.74%	12.44%
	Diff.	-31.19%	-0.09%	-19.67%
Cedarwood	10%	38.66%	69.90%	5.96%
	0.078%	20.97%	19.89%	17.20%
	Diff.	-17.68%	-50.00%	11.23%

At the lowest concentration (0.078% oil) PI values were lower for each treatment of each strain except for 4556 exposed to rosemary (81.47%, difference of 1.5) and V275 exposed to cedarwood (17.2%, difference of 11.23%). This result for rosemary treated plates of 4556 was low and only marginally diverged from difference in PI for tea tree replicates (-0.09% difference), and as such was not considered anomalous.

The result for cedarwood treated plates of V275 was a larger difference between concentrations and elicited the highest PI value for that strain (17.2%, difference of
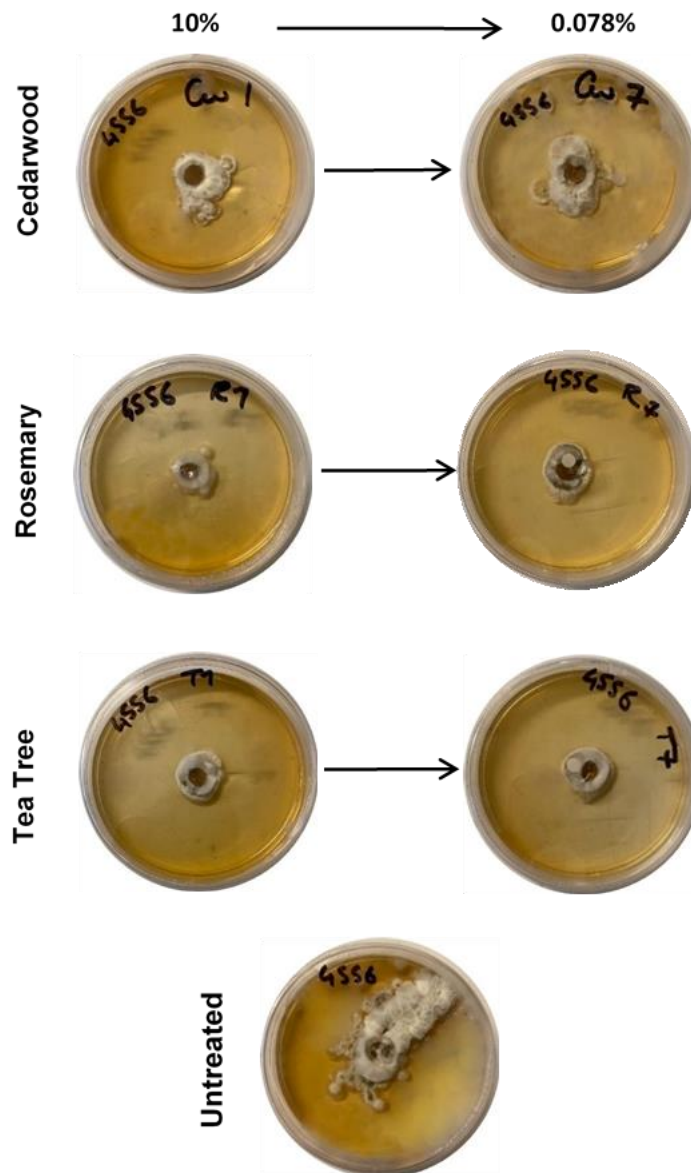
Fig. 4.4. Volatile fungal inhibition assay with concentrations of essential oil decreasing in two-fold serial dilutions. Shown here are plates of Metarhizium strain ARSEF4556 exposed to oils (Cedarwood, Rosemary & Tea tree) at a starting concentration of 10% (diluted in DMSO) and the final concentration (0.078%) after 96 hours, with an untreated control below. Oils were inoculated into filter discs and adhered to glass coverslips above agar plugs of sporulated EPF.

11.23%), compared to both tea tree (12.44%) and rosemary (14.13%). Whilst a potential anomaly for that strain, PI of strains exposed to cedarwood oil volatiles at that concentration was similar (3297 = 20.95%, 4556 = 19.89%). The considerably lower PI value for the higher dose (5.96%) could suggest a growth stimulant effect, but this would be in direct contrast with PI values seen for both 3297 (38%, -17.68% difference) and 4556 (69.9%, -50% difference) when exposed to cedarwood oil VOCs.

4.3.3 Caspase Activity

For all caspase fluorescence assays, only tea tree and rosemary were included (with untreated controls) due to both availability of materials and the fact cedarwood had elicited no adverse effects or mortality across all doses and concentrations. Comparisons of means and directional comparisons of 95% CIs can be seen in Figure 4.5.

Treatment type was found to have significant effects on the caspase activity of wireworm exposed to essential oils for caspase 3-7 ($F_{(2,218,)}$ = 9.357, p <0.0001), caspase 8 ($F_{(2,218)}$ = 16.863, p <0.0001) and caspase 9 ($F_{(2,218)}$ = 80.612, p <0.0001), with both tea tree and rosemary treated larvae showing increased caspase activity compared to untreated controls for each.

Timepoint showed a significant effect on the caspase activity for both caspase 3-7 ($F_{(1,218)}$ = 11.775, p = 0.0007) and caspase 8 ($F_{(1,218)}$ = 16.863, p < 0.0001), with the effects on caspase 9 only marginally outside the arbitrary significance cut off ($F_{(1,218)}$ = 3.530, p = 0.0616). For each treatment, there was a decrease in caspase activity at six hours exposure compared to three hours across all fluorometric assays. The exception to this was caspases 3-7 for rosemary treated larvae (est = -8305, t= -1.480, p = 0.6776) in which a slight increase was observed, though not found to be statistically significant.

At caspase 3-7 there was a significant decrease in activity for tea tree compared to the same treatment at 6 hours (est = 37820, t = 6.738, p < 0.0001). For caspase 8, activity decreased from three to six hours significantly for rosemary (est = 104753, t = 3.614, p = 0.0050), though marginally for tea tree treated larvae (est = 77175, t = 2.662, p = 0.0872). Activity of caspase 9 was not found to decrease significantly for either treatment from three to six hours fumigation.

At three hours post-inoculation, caspase activity was greater in tea tree than in rosemary for caspase 3-7 (est = 31726, t = 5.653, p <0.0001), though no significant difference was observed in activity between treatments for either caspase 8 (est = 8731, t = 0.301, p = 0.9997) or caspase 9 (est = 49523, t = 2.441, p = 0.1470). At six hours fumigation, caspase activity was not significantly different between treatments for caspase 3-7 (est = -14399, t = -2.565, p = 0.1104) or caspase 8 (est = 36309, t = 1.253, p = 0.8102), though was found to be significantly higher in wireworm exposed to tea tree for caspase 9 (est = 97413, t = 4.801, p < 0.0001).

At each time point, for each treatment, larval caspase activity was significantly greater than untreated controls (p < 0.0001).
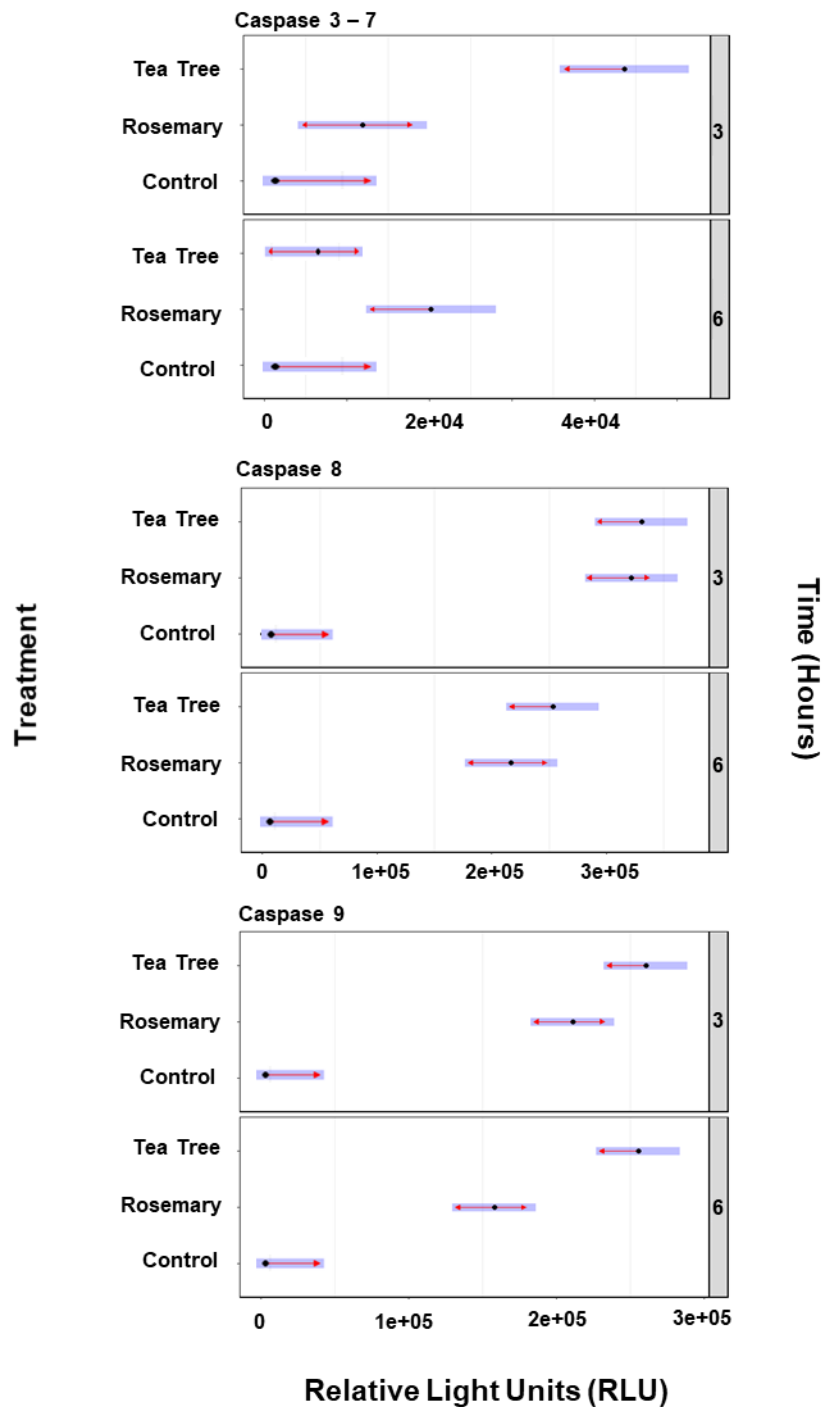
Fig. 4.5. Response plotted for estimated marginal means for RLUs obtained through luminometric assays for caspases 3-7, 8 & 9 for wireworm exposed to either tea tree or rosemary at a 20 μl dose. Three larvae were used per replicate and wireworm were exposed to botanicals for both 3- and 6-hour time points before mechanical homogenisation.

4.3.4 Stress-and-Kill

Survival probability of wireworm exposed to different concentrations of each botanical can be seen in Figure 4.6. Both treatment and concentration were found to have significant effects of wireworm mortality, with an interaction evident between the two (χ^2 = 34.04, df = 11, p = 0.0004). For each botanical, across all concentrations, mortality was increased compared to controls of EPF. No mortality was recorded in larvae exposed to the botanicals at the highest concentration (10%). Interestingly, similar levels of mortality were observed at 1% and 10% concentrations of both cedarwood oil and tea tree, though the time-to-kill for cedarwood exposed larvae was much quicker. Rosemary was not seen to reduce survival to the same extent but in each concentration, there was > 50% mortality. Larvae exposed to rosemary oil exhibited reduced fungal growth from cadavers compared to both cedarwood and tea tree.
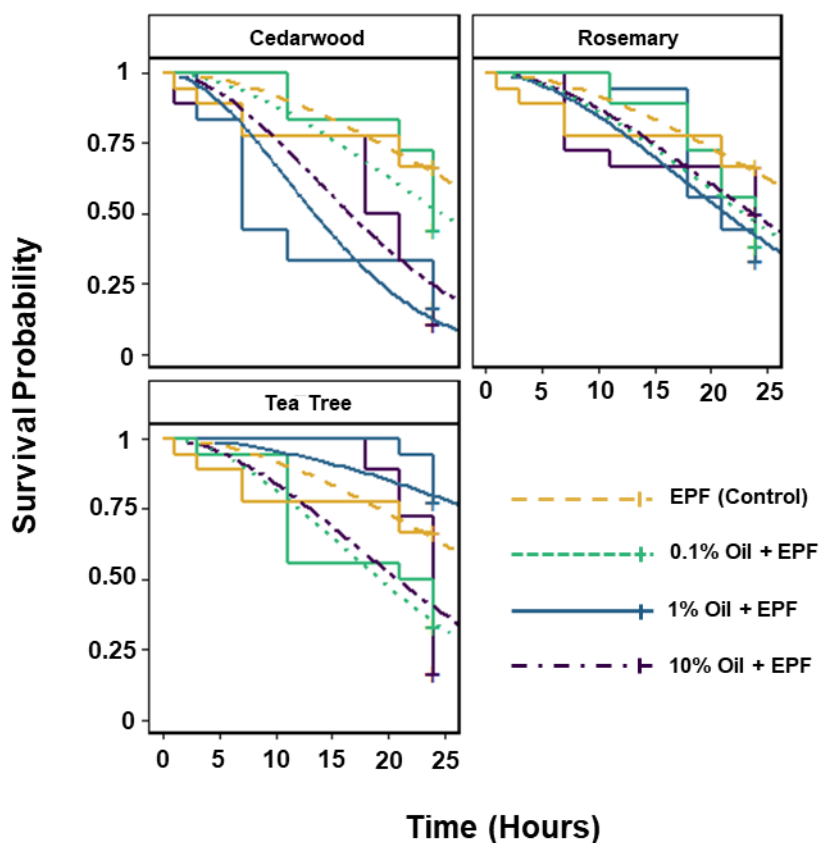
Fig. 4.6 Survival of wireworm exposed to a soil-mix of *M. brunneum* strain ARSEF4556 and decreasing concentrations of the three botanicals. Passaged and harvested spores were mixed in with substrate at concentrations of $2.5 – 5 \times 10^7$. Oils at 10% and EPF without oils were used as controls. Six replicates were used, with this repeated three times. Censoring occurred across all treatments.

4.4 Discussion

Entomopathogenic fungi, as naturally occurring microbes within a soil matrix, have great potential as environmentally friendly biopesticides against wireworm (Kabaluk *et al.*, 2015; Brandl *et al.*, 2017). The EPF *M. brunneum* has shown great promise against a range of invertebrate pests, including wireworm (Wraight *et al.*, 2008; Butt *et al.*, 2013; Eckard *et al.*, 2014; Khoja *et al.*, 2019). Species specificity of strains can ensure that pest species can be accurately targeted without knock-on effects towards beneficials (Faria & Wraight, 2007; Sharma *et al.*, 2020). In addition, applications of *Metarhizium* may have further beneficial effects such as biostimulation and suppression of other harmful pathogens (Guigón-López *et al.*, 2021; Praprotnik *et al.*, 2021).

Here it was found some strains clearly outperform others in their efficacy towards wireworm. The most virulent strain (ART2825) elicited just over 50% mortality in *A. lineatus*, with some potential in ARSEF4556 for inclusion in synergy experiments. Commercial strain V275 (Met52, registered for use against vine weevil) caused < 15% mortality. The strain ARSEF4556 has shown strong pathogenicity towards mosquitoes previously (Butt *et al.*, 2013), though its stability warranted inclusion in further studies.

This study built on work identifying bioactive effects of botanical extracts towards wireworm. It has been shown that botanicals can elicit both insecticidal and behaviour modifying effects towards the polyphagous pest (Brandl *et al.*, 2016; Eckard *et al.*, 2017; Chapter 2). Similarly studied are the fungicidal effects of some botanicals, such as tea tree and rosemary (Hammer *et al.*, 2003; Özcan & Chalchat, 2008). However, there is potential for a combined use in either a push-pull strategy to target larvae more specifically, or in a stress-and-kill strategy at lower concentrations of the botanicals (Pickett *et al.*, 2014; Butt *et al.*, 2016). In instances of botanicals exhibiting attractant effects, lure-and-kill strategies – already implemented for biological control of wireworm – may be augmented and further improved (Brandl *et al.*, 2017; Hermann *et al.*, 2017).

Here fungicidal effects of tea tree and rosemary were demonstrated towards three strains of the EPF *M. brunneum*, in a microbroth dilution assay. For each botanical, there was inhibition of EPF in a clear fungistatic effect across lower doses. Fungistatic properties against *Metarhizium* have been observed in both insect defences and with secondary plant metabolites (Butt *et al.*, 1995; Tripathi *et al.*, 2008; da Silva *et al.*, 2013). For the most pathogenic strain towards wireworm, the minimum fungicidal concentration was > 5% for both tea tree and rosemary and >15% for cedarwood.

This fungistatic effect was replicated in assays monitoring fungal growth exposed to the volatiles of botanicals. Although, in this instance, the commercial strain V275, was inhibited far less with only 18% and 32% percentage inhibition (PI) compared to untreated controls for both rosemary and tea tree respectively and only 6% for cedarwood at the highest concentration. For the most pathogenic strain, 4556, PI was approximately 80% for tea tree and rosemary, and 70% for cedarwood, when compared to untreated controls. Even with high PI however, there was still mycelial growth evident, and no fungicidal effects observed at the highest dose.

Evidence of stress in caspases 8 & 9 in larvae exposed to both tea tree and rosemary. This corroborates behaviours observed in chapters one and two, and the short-range diffusion of botanicals within the soil suggest larvae may remain in an area of stress long enough to facilitate infection with EPF. Obstacles remain in the application and formulation of the two biocontrols, with a granular product a potential solution (Bruck & Donahue, 2007; Sharma *et al.*, 2020). Mortality of wireworm exposed to EPF strain ARSEF4556, and low concentrations of botanicals showed synergy between the two with increased mortality for each of cedarwood, rosemary and tea tree compared to controls. Infected cadavers with sporulated ARSEF4556 (Figure 4.7) indicate clear fungal growth despite exposure to each oil (at 10% concentration).
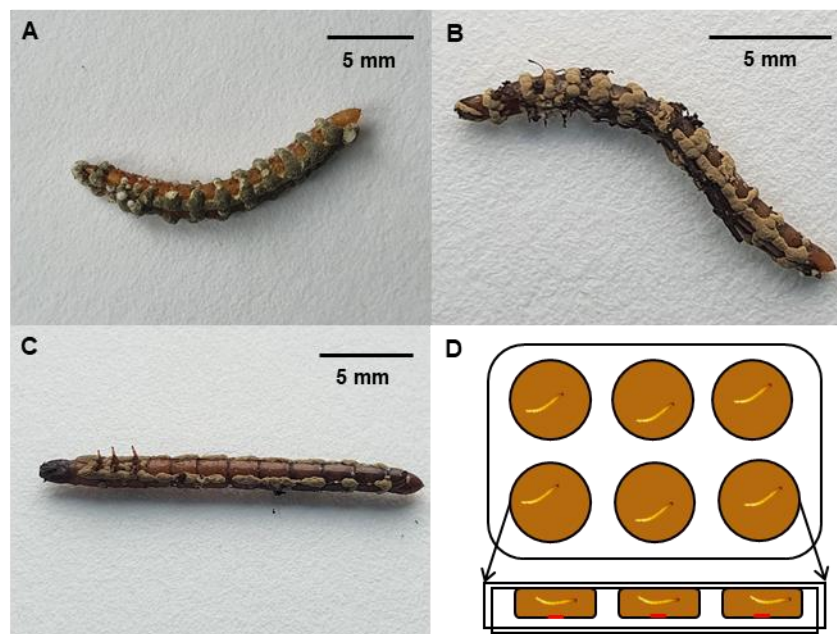
Fig. 4.7 (A – C). Wireworm cadavers infected with *M. brunneum* ARSEF4556 in spore / soil mix mortality assay in the presence of botanicals. In **(A)** can be seen an infected larva exposed to cedarwood oil, **(B)** exposed to tea tree and **(C)** exposed to rosemary. As in **(D)**, assays were conducted in 6-well culture plates filled with substrate with $2.5 - 5 \times 10^{7}$ concentration of spores, with filter tip inoculated with 10 µl of diluted oil (v/v in DMSO) placed at the base (red disc). This was completed in triplicate for each concentration of each botanical.

The stress caused by tea tree and rosemary in wireworm, seen in both behavioural assays (Chapter 2) and in caspase results here suggest a viable stress-and-kill approach. This strategy has precedence in synergy between a biocontrol agent and insecticide for wireworm control (Eckard *et al.*, 2017; Isman, 2020; Bourdon *et al.*, 2021). The results for cedarwood appear anomalous in that no adverse effects were observed in larvae exposed to the botanical. Although the increased mortality may be explained by possible phagostimulation effects of the oil opening entry routes into the haemocoel for the EPF via the mouthparts. The findings here point towards suitability for a push-pull strategy to enable more specific targeting of the pest with EPF.

Research conducted on field applications of *Metarhizium brunneum* (Vernon *et al.*, 2003; Kabaluk, 2014) raises the possibility of introduced biological control agents, such as EPF, having knock-on effects of either failing to control the initial target organism, or encouraging other invertebrate pests, such as nematodes (Mwaura *et al.*, 2017; Hummadi *et al.*, 2021). Further work would be required to provide more evidence for this hypothesis, but early results lend support for the inclusion of a protectant element for the crop itself as well as the attract-and-kill approach, for a two-pronged IPM solution. Any attraction effect would have to ensure that it was not simply sustaining a pest population in an area in which it may increase damage without control. There currently exists a need to bridge a gap between the deregistration of conventional insecticides and field-ready biological alternatives. A reduced rate of conventional applications may allow for synergy with biological controls (Vernon *et al.*, 2008; da Silva *et al.*, 2013) in order to provide a short-term solution until more complete formulations are identified.

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Chapter 5: Volatile Organic Compounds of Metarhizium brunneum – Bioactivity against Wireworm and Synergy with Entomopathogenic Nematodes

Abstract

The identification of volatile organic compounds (VOCs) from naturally occurring bioactive compounds or microbes is a growing area of study within integrated pest management. The biological control of wireworm – subterranean coleopteran larval pests (*Agriotes* spp.) – has progressed most significantly with applications of entomopathogenic fungi (EPF), commonly *Metarhizium* spp, or entomopathogenic nematodes, (EPN). Within this research, the larvae have exhibited repulsion behaviours when exposed to high concentrations of the EPF within a soil matrix. Additionally, evidence of synergy exists between EPF and EPN in the control of other invertebrate pests. The constituent composition of *Metarhizium* spp. could yield specific VOCs which can more accurately elicit behavioural or deleterious effects on wireworm and improve the efficacy of EPN applications. Here we have demonstrated the fumigant properties of 1-octen-3-ol and 3-Octanone, VOCs of *M. brunneum,* towards both wireworm and a commercial strain of *Heterorhabditis bacteriophora*, and a potential for exploiting positive relationships between the two in the control of the former. Results indicate some direct synergy between low concentrations of 3-Ocatnone and the EPN. However, repulsion effects of the VOC on wireworm in behavioural studies, indicate the suitability of a push-pull system to more accurately target the larvae with the pathogen.

5.1 Introduction

Wireworm, the soil-dwelling, polyphagous larvae of click-beetles, are notoriously resistant to infection with entomopathogens (Kabaluk, 2014; Sandhi *et al.*, 2020) and are long-lived within the soil, providing a major hurdle to biological control solutions (Eidt & Thurston, 1995; Rahatkhah *et al.*, 2015). They are a major invertebrate pest within a range of crop species and as conventional pesticides are rapidly being de-registered with no existing alternatives for adequate control, there is a pressing need for development of novel management solutions (Ritter & Richter, 2013; la Forgia & Verheggen, 2019). Increasingly, research has focused on the manipulation of wireworm behaviours within a soil matrix to target the pest more accurately with entomopathogens or lower their natural defences to ensure a higher rate of mortality.

Secondary metabolites of plants, fungi and other micro-organisms are identified as sources for bioactive compounds in insect pest management solutions (Johnson & Nielsen, 2012; Bojke *et al.*, 2018). These may be applied as direct fumigants, as semiochemicals or as an integrated pest management solution alongside known biological controls such as entomopathogenic fungi (EPF) or entomopathogenic nematodes (EPN) (Barsics *et al.*, 2016; Brandl *et al.*, 2017; Eckard *et al.*, 2017). Baverstock *et al.* (2010) review behavioural responses of insects towards EPF, highlighting examples of avoidance behaviour across several insect orders, including Coleoptera. This includes more detailed description of avoidance behaviour of infected conspecifics identified in the Isoptera (Chouvenc *et al.*, 2008) with examples of more species-specific aggregation and defensive behaviours towards infected individuals of *Reticulitermes flavipes* (Kollar) – the Eastern subterranean termite - with *Metarhizium anisopliae* (Myles, 2002). Additionally, there is evidence of wireworm moving away from areas of high concentration of soil-conidia of *Metarhizium robertsii* (Sorokin) (Kabaluk & Ericsson, 2007a), though behaviour towards infected conspecifics is relatively unknown. Evidence of conventional insecticides being used to reduce locomotion in root weevil larvae (*Diaprepes abbreviates* L.) showed an increase in infection with EPF due to a decrease in avoidance behaviours (Quintela & McCoy, 1998; Roditakis *et al.*, 2000). This lays a foundation for manipulation of wireworm behaviour to overcome the hurdles of application of biocontrols such as EPF or EPN to a heterogenous subterranean pest population.

Entomopathogenic nematodes have already shown promise as control agents against wireworm. Although, both commercial and natural nematode strains generally have

reduced efficacy against wireworm species (Morton & Garcia-del-Pino, 2017; La Forgia *et al.*, 2021) compared to conventional controls. Some synergy has been observed been both EPN and entomopathogenic fungi in controlling some invertebrate pests (Shapiro-Ilan *et al.*, 2004; Ansari *et al.*, 2010). Recent work has focused on taking advantage of the volatile organic compounds (VOCs) of *Metarhizium sp.* to amplify the effects of the bioactive constituents (Hummadi *et al.*, 2021). Several of these constituents have been identified to have pesticidal and semiochemical effects on EPN, plant parasitic nematodes (PPN), molluscs, insects and other fungal pathogens (Groen & Whiteman, 2014; Khoja *et al.*, 2019, 2021; Guigón-López *et al.*, 2021; Hummadi *et al.*, 2021).

In this study two VOCs of *Metarhizium brunneum*, 1-octen-3-ol and 3-Octanone, identified to have bioactive effects on three species of EPN (Hummadi *et al.*, 2021) were screened against wireworm for insecticidal and behavioural effects. They were further tested against a commercially available strain of *Heterorhabditis bacteriophora* (Poinar) to assess suitability for an integrated control solution for wireworm.

5.1.1 Aims

- **Identify lethal dose of identified VOCs through fumigation assays**
- **Quantify the bioactive and behavioural effect of VOCs on EPN**
- **Determine the viability of VOCs in a combined approach to wireworm control with EPN**

5.2 Methodology

5.2.1 Insect and Nematode Cultures

Entomopathogenic nematodes were obtained from commercially available sources as follows: *Steinernema carpocapsae* (Nemasys C®) & *S. feltiae* (Nemasys M®) – BASF Ltd, *H. bacteriophora* (Larvanem®) – Koppert Ltd. De-hydrated infective juveniles (IJs) were stored at 4°C until re-hydration with tap water immediately prior to experimental use. Only populations with < 10 % initial mortality were considered viable for experiments.

Wireworm used in experiments were field collected specimens from pre-prepared potato fields in Pembrokeshire, Wales, identified to species with a translated key, with an 8:2 split, *Agriotes obscurus : A. lineatus*. (Klausnitzer, 1994). Wireworm used in stress-and-

kill experiments in section 5.2.10 were *A. lineatus* provided by Applied Plant Research of Wageningen UR (Praktijkonderzoek Plant & Omgeving van Wageningen UR, PPO). Larvae were kept in conditions as described in Chapter 4, section 4.2.1.

5.2.3 Chemicals

Both 1-octen-3-ol (CAS No: 3391-86-4**)** and 3-Octanone (Cas No: 106-68-3) were obtained from Simga-Alrich (Merck Life Science UK Ltd). Each compound was at a purity of 98% (analytical standard) and stored in brown glass to avoid photooxidation, kept in fridge conditions at 4°C.

5.2.4 Phytotoxicity – Volatile Effects on Tomato Seedlings

The phytotoxic effects of VOCs (1-octen-3-ol & 3-Octanone) was assessed in a glasshouse growth assay with tomato (*Solanum lycopersicum* L.) seedlings. Uniform germinated seedlings were selected for assays seven days post emergence of cotyledons. Plastic pots (8 cm x 8 cm x 8 cm) were filled with mixed compost (multipurpose compost with John Innes and vermiculite – 4:1, v/v) up to a depth of 2 cm where an inoculated filter tip (6 mm D, Sharrow Ltd) was placed before the addition of another 2 cm of substrate. Finally, a seedling was placed and a further 3 cm of substrate packed in.

A range of doses (100, 200, 300 & 400 µl) were tested for each compound with untreated controls for comparison. Fourteen days post-inoculation, photosynthetic capacity was measured with an advanced continuous excitation chlorophyll fluorometer (Handy PEA+, Hansatech Instruments Ltd), taking an Fv / Fm reading (the maximum quantum efficiency of photosystem II (PSII)) with values ~ 0.8 Fv / Fm assumed consistent with unstressed leaves (Björkman & Demmig, 1987). Eight replicates in total were used for each dose of each treatment with untreated controls.

5.2.5 Fumigation – Still air, Dry soil and Wet soil Tube Bioassay

Dose-dependent fumigation effects of VOCs were quantified in a sealed bioassay. A single larva was introduced to a universal tube (30 ml), in which a filter tip (50 mm x 7.13 mm filter tip (Sharrow, Wilson & Co. Ltd)) was fixed to the underside of the lid with a fine mesh. The filter was inoculated with 5, 10 15 or 20 µl of either 1-octen-3-ol or 3-octanone

and the arena sealed. A cross section of filter tip pipetted with 20 µl of water was included in the arena to avoid desiccation of the wireworm.

Larvae were assessed for mortality at 3, 6-, 12-, 24- & 48-hour time points, with mortality considered as rigid larvae with no response in thoracic legs or sensory organs to mechanical stimulation. Fifteen replicates were carried out, with an untreated control. Larval recovery was recorded as active subterranean movement once removed to a fresh soil arena.

The buffer capacity of soil against insecticidal effects of the VOCs was tested with a modification of the no-soil fumigation assay. Universal tubes (30 ml) were filled with 5 ml of substrate (3:1, v/v, Multipurpose w/ John Innes: Vermiculite) and a filter tip inoculated with 5, 10, 15, 20, 30 or 40 µl of either 1-octen-3ol or 3-octanone was placed inside. This was then covered with the remaining 25 ml of substrate and allowed to diffuse through the soil for one hour before introduction of a single wireworm. The tube was then sealed, and wireworm position and mortality recorded at regular time points. Tubes were kept in controlled conditions (21°C, 60% RH, 0:24 LD). Fifteen replicates were used for each treatment, with an untreated control. Soil fumigation was assessed for both a 'dry' substrate and a 'wet' substrate.

For the dry soil fumigation, the substrate mix was dried on a tray in a controlled temperature room under plant grow lights (27°C, 60% RH) for 24 hours before setting up the experimental arena. For the wet soil fumigation assay the soil was prepared in the same way and then re-moistened to field capacity (Rai *et al.*, 2017) before establishment of the experimental arena. Larval mortality and recovery process was as in 2.3.

5.2.6 Capture Tube – Behavioural Response in Soil Arena

Capture tube methodologies for both no-choice and choice experiments were as outlined in Chapter 2. Differences in applications for the evaluation of responses to VOCs are outlined here.

Single tube capture assays were carried out in black pots (Diameter 15cm x Depth 13.5cm) filled with compost and vermiculite (3:1, v/v, Multipurpose w/ John Innes: Vermiculite) with a one-litre volume (750g (±25g)) at 10% moisture content (v/v).

Capture tubes containing maize seedlings (in the same substrate, seven days post-germination, selected for both above and below ground consistency in growth stage)

were inoculated with a 50 mm x 7.13 mm filter tip (Sharrow, Wilson & Co. Ltd) loaded with 200 µl of either 1-octen-3-ol or 3-Octanone.

Tubes were placed centrally and left for one hour in experimental conditions for the settling of the substrate and dispersion of volatile organic compounds (VOCs). Three larvae were then introduced and left for a 48-hour period. Tubes were then checked, and the arena examined for individuals either inside the tube adjacent to it or absent. The experiment had five replicates per treatment and was repeated twice.

Dual-choice capture assays were carried out in five litre tubs (W:15 x H:14 x L:34) filled with a compost and vermiculite mixture as in section 2., with a volume of four and a half litres (3,375g (± 100g)) at 10% moisture content (v/v). Three tubes were placed adjacent in opposite thirds of the arena buried so the top was flush with the soil layer with each containing maize seedlings, as above.Three wireworm were introduced to the central third.

After 72-hours, arenas were emptied and separated into the treated, untreated, and central thirds with the number of wireworm counted in each. Each tube was assessed for entry holes and then emptied to count wireworm numbers and evaluate feeding damage. The experiment contained three replicates for each dose and was completed four times.

5.2.7 Chemotaxis of EPN – Glass Slide Agar Assay

The chemotaxic response of EPN was evaluated using an adapted assay of that carried out by Laznik & Trdan (2013, 2016), seen in Figure 5.1. The surface of a standard glass slide (2.6 cm x 7.6 cm x 0.12 cm, Academy) was pipetted with 2.5 ml of water agar and allowed to set under sterile conditions.

The slide was superficially divided into three equal areas of 2.5 cm2. A 'treatment' section was pipetted with 1 µl of either 1-octen-3-ol or 3-Octanone, and an opposite 'control' section with 1 µl of distilled water. Within the central section was placed 30 µl (suspended in tap water, after rehydration from stock culture) of approximately 200 IJs, of either *Steinernema feltiae*, *S. carpocapsae* or *H.bacteriophora*.

Nematodes were counted in each section at both three hour and 24-hour time points, with mortality recorded. Concentrations of VOCs applied ranged from 100, 10 & 1%, diluted with DMSO. Water, DMSO and untreated slides were used as controls. The experiment contained five replicates and was repeated three times.
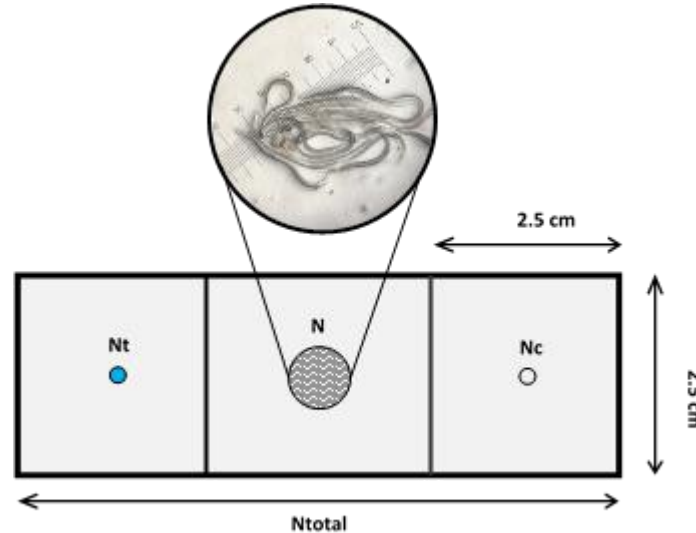
Fig. 5.1 Glass slide arena for EPN chemotaxis assay with exposure to VOCs of *M. brunneum*, 1-octen-3-ol & 3-Octanone.

The chemotaxic index (CI) for analysis was as used by Bargman & Horvitz (1991):

$$CI = \frac{Nt - Nc}{Ntotal}$$

Where Nt is equal to the number of nematodes in the treatment section, Nc to the number of nematodes in the control and Ntotal the total number of nematodes across the whole arena. The CI was interpreted as: 1 = complete attraction, 1 = complete repellency. Arbitrary degrees within CI defined the strength of response: ≥ 0.2 = considered as an attractant effect; 0.2 – 0.1 = minimal attraction; 0.1 – -0 .1 = no observable effects; -0.1 - -0.2 = minimal repellency; ≤ -0.2 = considered as a repellent effect. Nematodes located within the central third were considered a null result and only their mortality noted at that stage.

5.2.8 Mortality of EPN – Petri Dish Fumigation Assay

The dose dependent mortality effects of exposure to the VOCs were tested in a Petri dish fumigation assay (Figure 5.2). Agar plates (90 mm diameter) were filled with 20 ml of water agar medium. The optimal concentration of water agar (3.5 %, w/v) for nematode motility and survival was determined with prior comparisons of various

concentrations. In the centre of the dish, approximately 200 infective juveniles (IJs) of H. bacteriophora were applied, suspended in 100 µl of tap water. To the centre of the underside of the lid, a 22 mm² glass coverslip (Academy ltd) was adhered with water tension, and an inoculated filter disc (6mm) was similarly attached on to the coverslip. Filter discs were pipetted with either 5, 10 15 or 20 µl of 1-octen-3-ol or 3-octanone, or water for untreated controls. Finally, the Petri dishes were closed and sealed with Parafilm M©.

Nematode mortality was checked at 3-, 6- and 24-hour time points with counts taken through a binocular dissecting microscope. Assumptions of mortality were made morphologically with IJs characteristically straightened. The assay contained six replicates, with separate dishes used for each time point to allow temporal comparisons.
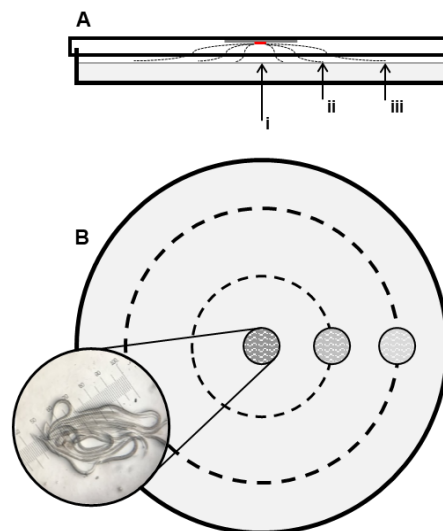
Fig. 5.2 Petri dish arena for EPN fumigation assay. A – cross-section of experimental set up, indicating inoculation point (i) of IJs, and points for counts (i, ii, iii) at each time point. Superficial 'zone of inhibition' of VOCs (introduced centrally – red disc) are demonstrated with dashed lines. B – plan view of Petri dish, inoculation point, and count chambers similarly shown in context of decreasing VOC zone of inhibition

5.2.9 Caspase activity of Wireworm exposed to VOCs in Fumigation Assay

The caspase activity was assessed in three wireworm per treatment. Larvae were frozen with liquid nitrogen upon removal from an initial experimental arena and then homogenized mechanically. Total homogenates were re-suspended in 420 µL 0.5% Triton lysis buffer (Tris 20mM, NaCl 100mM, EDTA 500mM, 0.5% Triton X-100), agitated gently before incubating on ice for 10 min. Homogenates were then centrifuged at 14,

000 g for 10 min and 38 µL aliquots of supernatant were added to 3 replicate wells in a white walled 96-well microtiter plate (Costar, Corning). Luminometric assays for caspase-3-7, caspase-8, and caspase-9 activity were performed in accordance with the manufacturer's guidelines using the Caspase Glo 3-7, Caspase Glo 8 and Caspase Glo 9 assay kits (Promega) by adding equal volume (38 µL) of Caspase Glo reagent to the sample.

Larvae were exposed to doses shown to elicit mortality after 24 hours but removed from the fumigation arena after at one and three hours to allow for fumigation effects without insecticidal effects. Wireworm were exposed to the VOCs at 10 µl.

5.2.10 Stress and Kill – VOC and EPN Mortality Assay

The relationship between *Metarhizium* VOCs and a commercially available EPN strain of *H. bacteriophora* was tested in a fumigation arena, adapted from that used in section 2.5 and as previously described in Hummadi *et al.* (2021).

Centrifuge tubes (50 ml, Falcon) were filled with autoclaved compost (multipurpose compost with John Innes, 20% moisture content, v/v) to eradicate any latent nematode population. Tubes were first filled with ~ 5 g of substrate and a filter tip (50 mm x 7.13 mm) placed on top, inoculated with 100 µl of either 1-octen-3-ol or 3-Octanone at 10, 1 or 0.1% concentrations (diluted with DMSO, v/v). A further ~ 15 g of substrate was added to the arena and the tubes left for an hour to allow for even dispersal of the VOC through the substrate, before a suspension of IJs (*H. bacteriophora*, 1000 IJs in 500 µl water) were applied on top. A single wireworm was introduced to the tube and the final 10 g of substrate added before the tube was sealed and placed under controlled conditions (24°C, 24 hr dark).

Larval mortality was checked every three days throughout a three-week period. Conditions for mortality were as described in 2.3. Larval recovery was recorded as in 2.3. All unrecovered wireworm cadavers were checked for nematode presence with a simplified White trap method (White, 1927).

5.2.11 Statistical Analysis

For mortality data from fumigation assays, data was visualised with Kaplan-Meier survival plots and data analysed with survival regression (CRAN packages: 'survival' - ver. 3.2-13, 2021; 'survminer' – ver. 0.4.9, 2021). Models were selected as appropriate

for data with comparisons of Akaike's Information Criteria ('AICcmodavg' – ver. 2.3-1, 2020). Capture tube study results compared larval presence between treatments and locations with Student's T-test. The 'dose.p' function from the 'MASS' package (version 7.3-51.6, 2021) was used for calculation of LD50, 90 & 95 values. Caspase and phytotoxicity measurements were analysed with ANOVA, with any pairwise interactions highlighted with comparisons of estimated marginal means ('emmeans' – ver.1.7.0, 2021). All statistical analyses were performed using RStudio Build 351, utilising R version 4.1.2 (2021).

5.3 Results

5.3.1 Phytotoxicity – Volatile Effects on Tomato Seedlings

Phytotoxicity of *S. lycopersicum* seedlings (Figure 5.3) was best explained by treatment, though the relationship was found to be insignificant ($F_{(1,78)} = 0.003$, $p = 0.959$), with dose not having any discernible effect on photosynthetic capabilities. Neither 3-Octanone nor 1-octen-3-ol incurred any measurable phytotoxic effect on seedlings compared to one another or untreated controls, with the same effects observed across all treatment doses. Across all doses, Fv/Fm values were marginally below suggest approximate optimal value range of 0.79 – 0.83 (Maxwell & Johnson, 2000), but
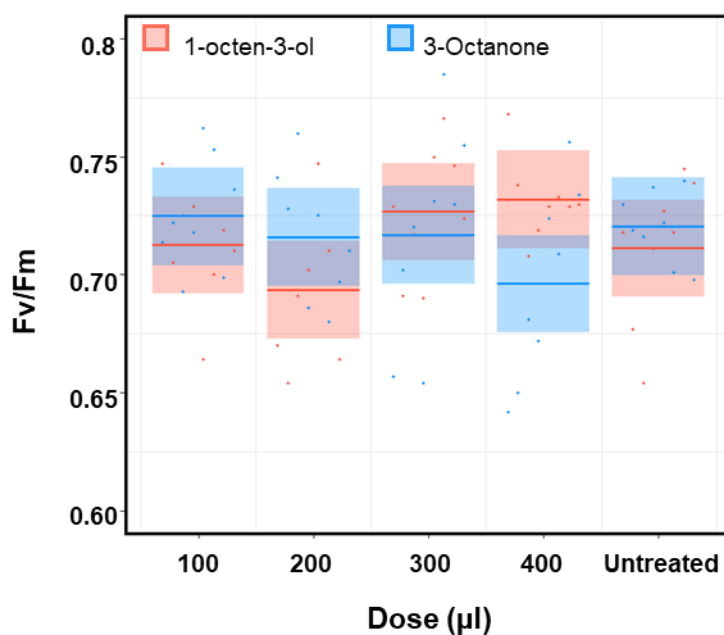
Fig. 5.3 Phytotoxicity index (Fv/Fm) for tomato seedlings inoculated with increasing doses of *Metarhizium* VOCs. Filter tips loaded with compound placed under germinated tomato seedlings with Fv/Fm readings taken 14-days post inoculation.

similarities in treatments and controls suggest any detrimental effects to plant health may be caused by abiotic factors rather than chemical inoculation.

5.3.2 Fumigation – Still air, Dry soil and Wet soil Tube Bioassay

Model selection criteria for survival regression of fumigation experiments was based on Akaike's Information criteria (AIC). In fumigation assays in the absence of soil, both treatment and dose were found to have a significant effect on larval survival ($\chi^2 = 60.37$, df = 2, $p < 0.0001$), with no clear interaction between the two (Figure 5.4). 1-octen-3-ol was found to be the most lethal, with all larvae dead after 6 hours at 10 and 20 µl and complete mortality at the lowest dose (5 µl) at 24 hours. For 3-Octanone, both the higher doses also caused mortality across all replicates but took the full time-period to do so. At the lowest dose, only 55% mortality occurred. With both treatments there was no recovery observed in individuals recorded as dead, with all doses for each differing significantly from untreated controls.
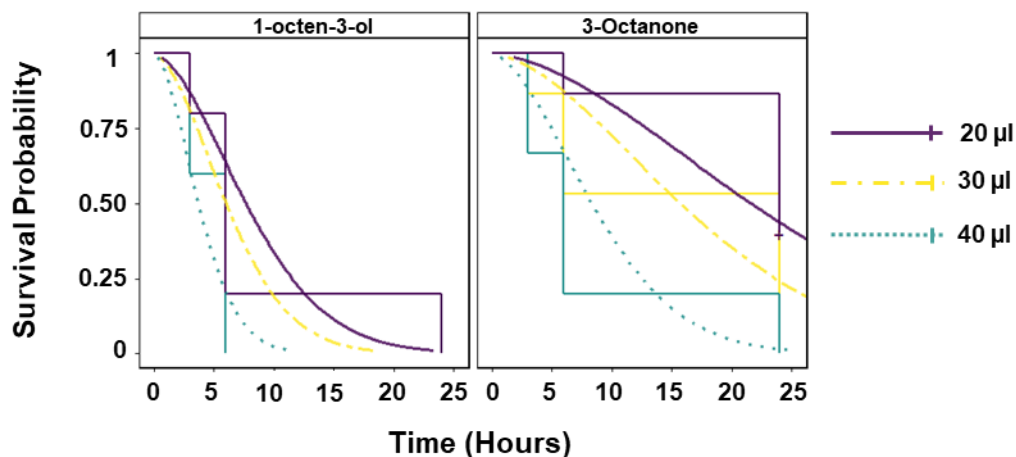
Fig. 5.4 Survival of wireworm in a fumigation assay with no substrate, exposed to *M. brunneum* VOCs 1-octen-3-ol & 3-Octanone. Fitted survival regression curves are plotted for each dose of each treatment. **In untreated controls survival > 95%.**

In dry soil fumigation assays, treatment and dose were found to have significant effects on wireworm survival ($\chi^2 = 117.76$, df = 3, $p < 0.0001$), with some interaction between one another (Figure 5.5). In a soil substrate, 3-octanone was more lethal towards wireworm than 1-octen-3-ol, converse to results found in soilless fumigation assays. There was a clear dose dependent response, with the highest doses, 40 & 30 µl, causing
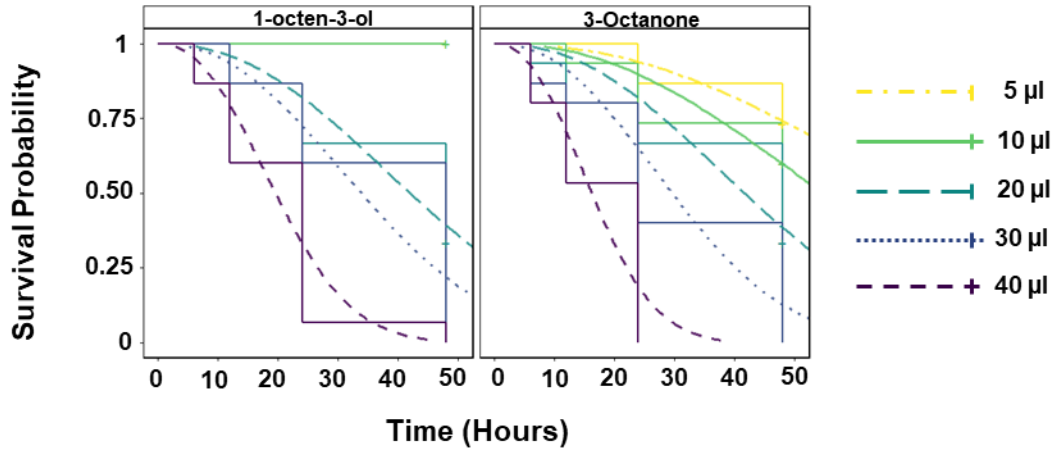
Fig. 5.5 Survival of wireworm in dry soil fumigation assay, exposed to *M. brunneum* VOCs 1-octen-3-ol & 3-Octanone. Fitted survival regression curves are plotted for each dose of each treatment. **In untreated controls survival > 95%.**

complete mortality after 24 & 48 hours respectively. Both 20 & 10 µl showed a 25% and a 50% survival rate respectively, and the lowest dose (5 µl) with a 75% survival rate after 48 hours. All doses of 3-octanone differed significantly from untreated controls.

For 1-octen-3-ol, there was complete mortality for all larvae at 30 & 40 µl after 48 hours, and just over 25% survival after the same time. For 5 & 10 µl there was complete survival after the study period. These doses of 1-octen-3-ol did not differ significantly from untreated controls.

Both treatment and dose had significant effects on wireworm survival in wet soil fumigation assays (χ^2 = 21.34, df = 2, p < 0.0001), though no interaction between the two was appropriate for fitted survival regression (Figure 5.6). Mortality across all doses
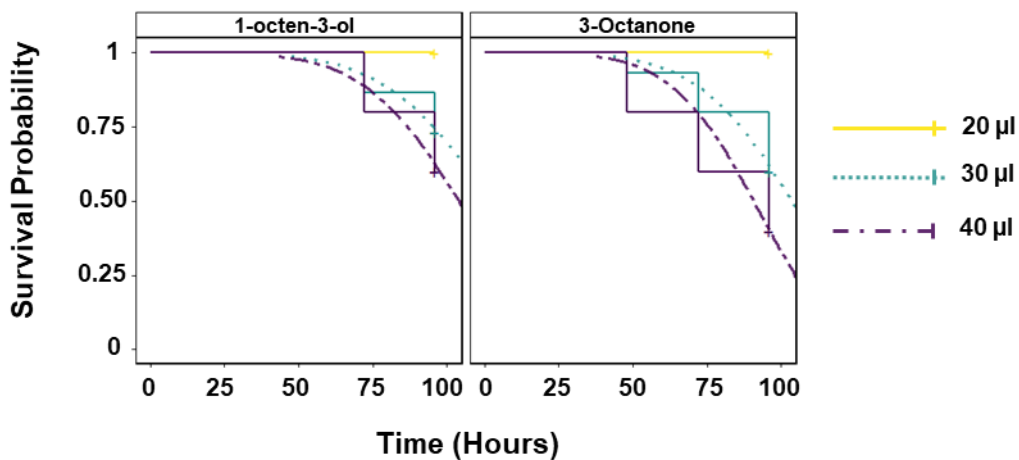
Fig. 5.6 Survival of wireworm in wet soil fumigation assay, exposed to VOCs 1-octen-3-ol & 3-Octanone. Fitted survival regression curves are plotted for each dose of each treatment.

was lower than respective doses in dry soil fumigation assays for each treatment. Only 3-octanone at 40 µl showed less than 50% survival of larvae. At 20 µl there was no mortality for either treatment, with no significant difference from untreated controls.

5.3.3 Capture Tube – Behavioural Response in Soil Arena

In Figure 5.7 can be seen the capture rates for wireworm in a single tube no-choice capture assay with seedlings in situ adjacent to VOCs. For each treatment, wireworm capture was limited with both 1-octen-3-ol (t = 7.6026, df = 13.235, p-value < 0.0001) and 3-Octanone (t = 6.6564, df = 15.68, p-value = < 0.0001), statistically different in capture rates compared to positive untreated controls (seedling only). There was no significant difference found in the capture rates between the two treatments (t = -0.6, df = 16.691, p-value = 0.5566). Untreated controls with no seed present showed no capture of larvae to re-enforce capture tube proof-of-concept seen in Chapter 3.

Capture rates for wireworm in a capture-tube choice assay can be seen in Figure 5.8. For both treatments, wireworm presence was found to differ when compared to control
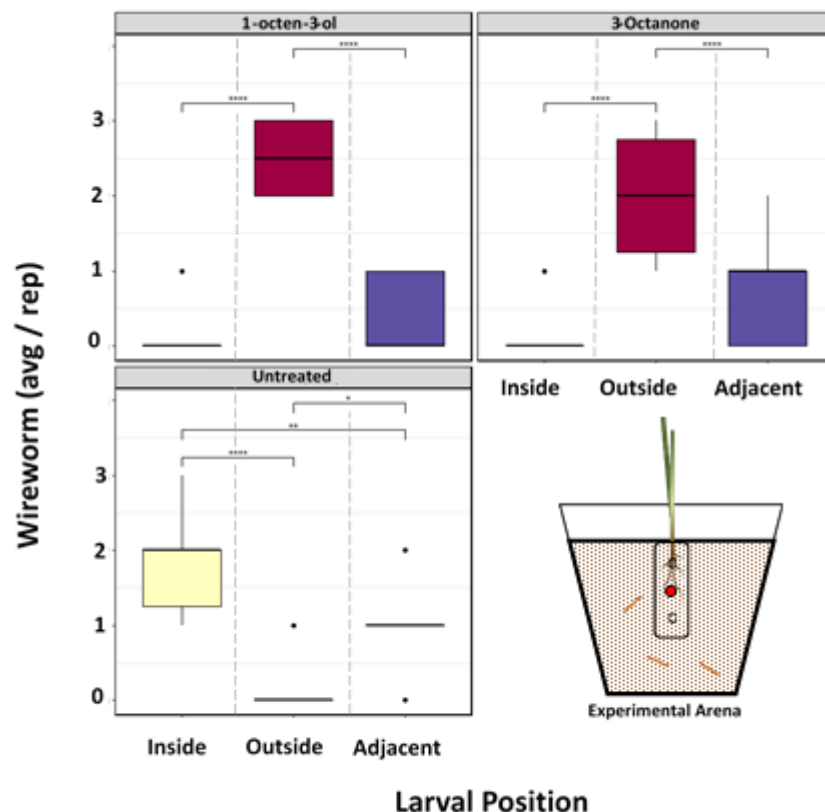
Fig. 5.7. Location of wireworm in no choice single-tube pot capture assay. Larval location given as inside, adjacent to, or outside capture tube, after 48-hours. Five replicates were used, with three larvae / pot and this was repeated twice. Significance is indicated as pairwise comparisons of estimated marginal means.

halves of the experimental arena, with significantly fewer larvae present in treatment sections (**1-ol:** t = -2.3406, df = 14, p-value = 0.03458; **3-Oct:** t = -2.9957, df = 14, p-value = 0.009633). There was no significant difference observed between wireworm presence in control sections between the treatments themselves (t = -0.67202, df = 13.126, p-value = 0.5132), suggesting no hierarchy of repellent effects between the two.

For capture within tubes there was minimal larval presence across both treatments in either the tubes in treatment or control sections (1-ol: t = -1.2104, df = 12.828, p-value = 0.2479; 3-Oct: t = -1.3229, df = 14, p-value = 0.2071), with no evidence of feeding
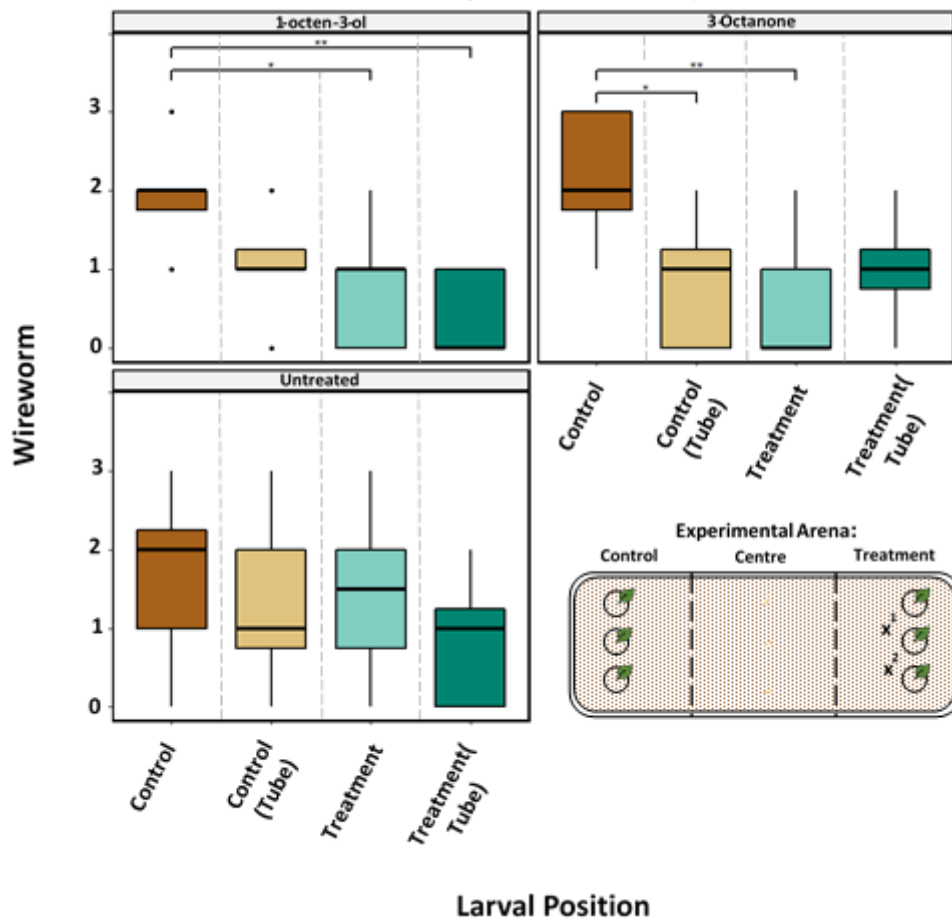
Fig. 5.8 Average captures of wireworm in dual-choice capture tube experiment. Wireworm location given as treatment or control with presence in capture tube for comparison. Five replicates were used, with three larvae / pot and this was repeated twice. Significance is indicated as pairwise comparisons of estimated marginal means.

behaviours. There was no significant difference found between larval capture in tubes within treated sections (t = 0.38592, df = 12.38, p-value = 0.7061). For untreated controls with seedlings in both sections, there was no statistical difference between wireworm presence in either section (t = -0.96609, df = 14, p-value = 0.3504) or tube capture (t = -1.0479, df = 13.266, p-value = 0.3134).

5.3.4 Chemotaxis of EPN – Glass Slide Agar Assay

The chemotaxis response of EPN species to VOCs (Figure 5.9) was found to be dependent on both species ($F_{(2,792)}$ = 4.592, p = 0.01) and concentration ($F_{(5,792)}$ = 3.059, p = 0.01), with significant interaction between the two in determining EPN preferences ($F_{(10,792)}$ = 4.253, p < 0.0001). Neither treatment nor time was found to have any significant effect on fitted models. Chemotaxis for across all treatments and concentrations for each species was low, with limited movement across the arena. The only replicates showing EPN chemotaxis outside the arbitrary thresholds of 'no observable effect' (-0.1 – 0.1 CI) were *S. feltiae* IJs exposed to 1-octen-3-ol, with mild repellent effects (-0.1 – -0.2) observed at the highest doses (100%) after 24 hours. This effect was reproduced, although a weaker response, for 3-octanone at 100% concentration and after 24 hours.
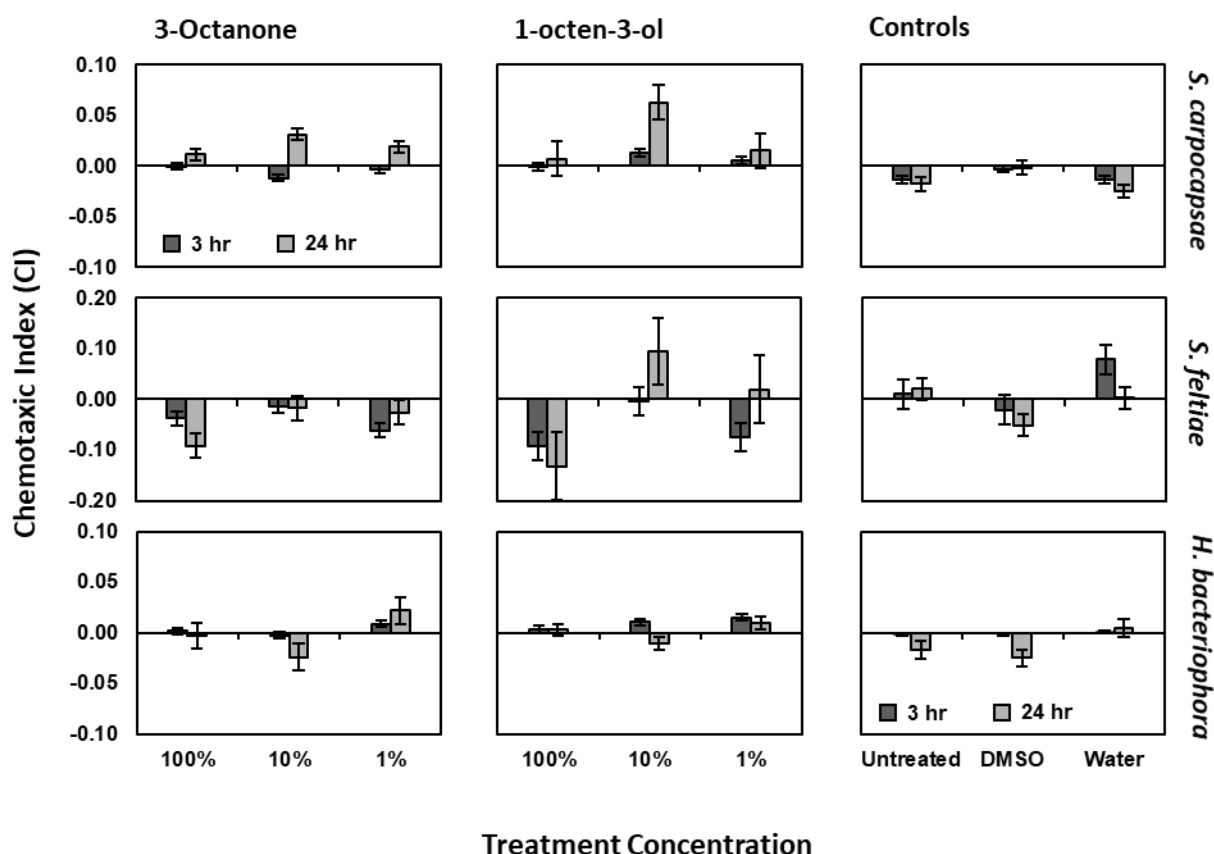
Fig. 5.9 Chemotaxic responses of three EPN species exposed to decreasing concentrations of VOCs of *M. brunneum*, 3-Octanone and 1-octen-3-ol, in a water agar choice arena. Controls included no treatment, DMSO (VOC solvent) and water. Chemotaxic index (CI) values given as follows: 1 = complete attraction, ≥ 0.2 = considered as an attractant effect; 0.2 – 0.1 = minimal attraction; 0.1 – -0.1 = no observable effects; -0.1 - -0.2 = minimal repellency; ≤ -0.2 = considered as a repellent effect, 1 = complete repellency.

5.3.5 Mortality of EPN – Petri Dish Fumigation Assay

The proportion of dead *H. bacteriophora* IJs (Figure 5.10) was determined to be affected significantly by compound, time, and dose, with interactions evident between compound and time (χ^2 = 7.744, df = 135, p < 0.0001).
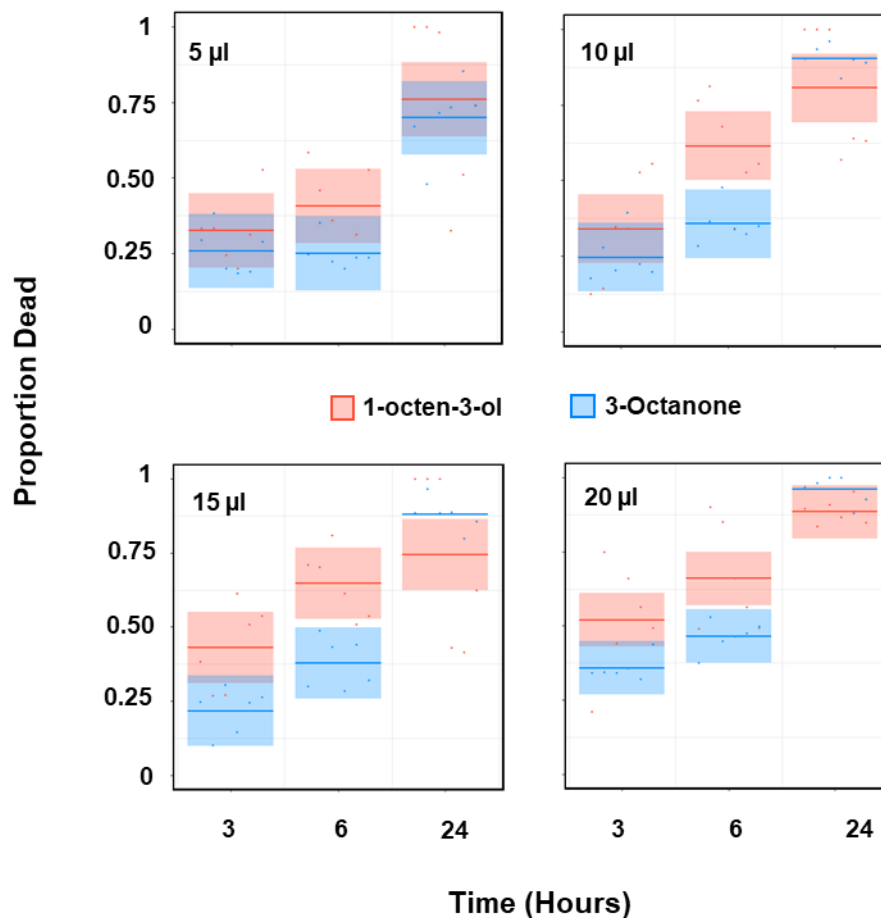
Fig. 5.10. Dose mortality plots for a commercial strain of EPN, *H. bacteriophora* (Larvanem, Koppert Ltd) known to be pathogenic towards wireworm. Proportion of dead nematodes are plotted with 95% CIs. Untreated controls showed <10% mortality across all time points.

There was a clear increase in mortality over time for each dose of both 3-Octanone and 1-octen-3-ol, with all doses showing > 75% mortality after 24 hours. Nematodes exposed to 3-Octanone showed complete mortality in reps at 10, 15 and 20 µl, and this was only

marginally significantly different from 1-octen-3-ol at 15 µl. At each dose, both compounds differed significantly from untreated control which showed < 10% mortality across all replicates. Lethal dose (LD) values at 50, 90 & 95% can be seen in Table 5.1, with 1-octen-3-ol causing 50% mortality at half the dose (0.9 µl) compared to 3-Octanone (1.89 µl) after 24 hours.

Table 5.1. Lethal dose (LD) 50, 90 & 95 values for EPN exposed to two VOCs of *M. brunneum.* Mortality data obtained from Petri dish fumigation bioassays with ~ 200 IJs and a filter disc inoculated with various doses of each VOC. Neat doses were used at 5, 10, 15 & 20 µl. Data for LD values taken at 24 hours.

Treatment	LD	Dose	SE
1-octen-3-ol	50	0.90	0.22
	90	4.11	0.27
	95	5.20	0.43
3-Octanone	50	1.89	0.05
	90	3.21	0.06
	95	3.66	0.08

5.3.6 Caspase activity of Wireworm exposed to VOCs in Fumigation Assay

Treatment was found to have significant effects on caspase activity for wireworm fumigated with VOCs (Figure 5.11) for caspase 3-7 ($F_{(2,217)}$ = 31.361, p > 0.0001), caspase 8 ($F_{(2,217)}$ = 52.31, p > 0.0001) and caspase 9 ($F_{(2,217)}$ = 26.27, p > 0.0001). At one hour of exposure to VOCs, wireworm in 1-octen-3-ol treated dishes showed significantly more caspase activity than untreated controls across each caspase. Larvae exposed to 3-Octanone at the same time point did not reveal the same statistical significance, with activity only marginally higher for each caspase. Significant differences
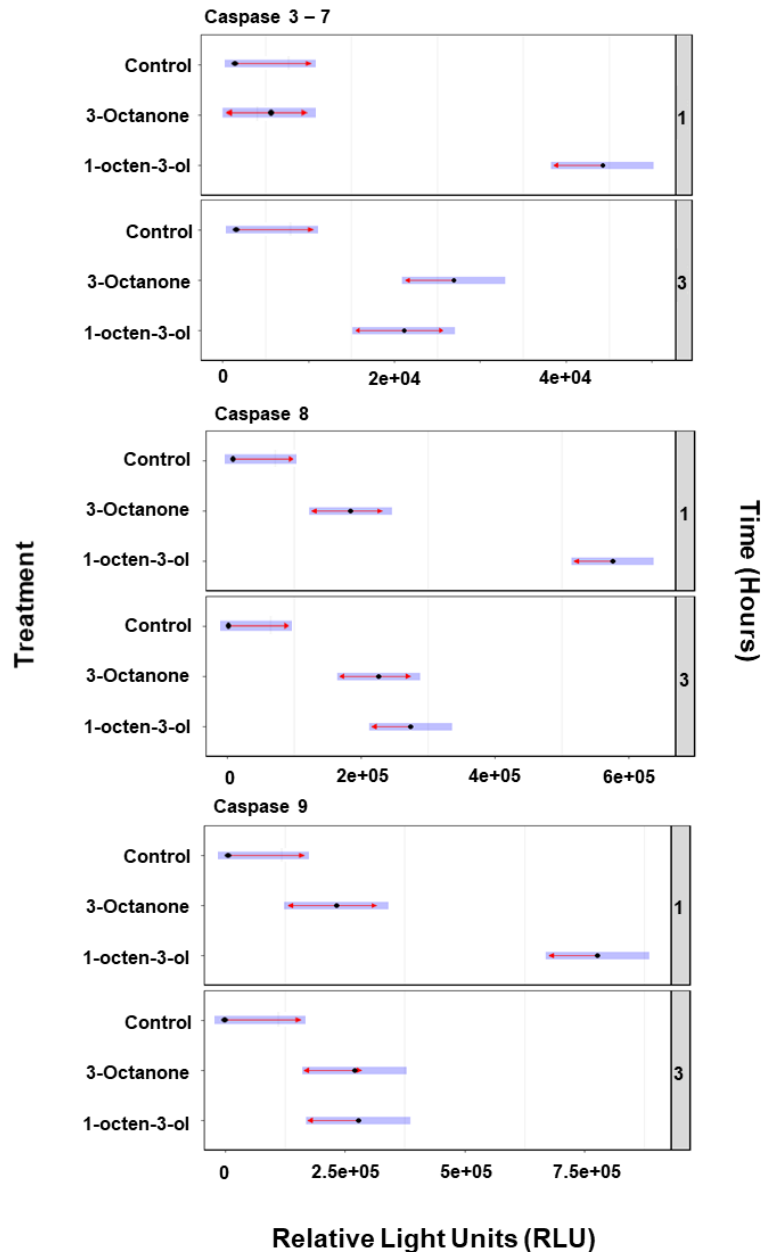
Fig. 5.11 Mean RLUs obtained through luminometric assays for caspases 3-7, 8 & 9 for wireworm exposed to VOCs, 1-octen-3-ol & 3-Octanone in a Petri dish fumigation assay. Three larvae were used per replicate and wireworm were exposed to VOCs for both 1- and 3-hour time points before mechanical homogenisation.

were observed between both VOCs and untreated controls at 3 hours for caspase 3-7 and caspase 8, with caspase 9 showing the converse pairwise interactions.

Timepoint exhibited significant effects on caspase activity for both caspase 8 ($F_{(1,217)}$ = 14.78, p = 0.0002) and caspase 9 ($F_{(1,217)}$ = 15.07, p = 0.0001), though was considered insignificant for effects on caspase activity of 3-7 ($F_{(2,217)}$ = 0.025, p = 0.876). 1-octen-3-ol prompted higher caspase activity than 3-octanone at one hour exposure for caspase 3-7 (est = 39464.2, t = 9.209, p < 0.0001), caspase 8 (est =

391,901.7, t = 8.821, p < 0.0001) and caspase 9 (est = 436,228.8, t = 6.977, p < 0.0001). At 3 hours exposure, there was no significant difference between 1-octen-3-ol and 3-octanone for each caspase.

5.3.7 Stress and Kill – VOC and EPN Mortality Assay

Wireworm exposed to both VOCs and EPN in a stress-and-kill fumigation assay were affected primarily by the dose at which the VOC was applied concomitantly with the IJs of *H. bacteriophora* (χ2 = 12.29, df = 4, p = 0.015). AIC values were weighted evenly across dose and treatment and their additive effect but the simple effect of dose in modelling wireworm mortality was accepted as most appropriate (AIC = 342.6).

The most pathogenic dose was 10% 3-Octanone (Figure 5.12) applied with IJs, eliciting marginally lower survival (40%), than untreated EPN controls (60%) and significantly more than the VOC applied by itself at the same dose (5%). No dose of 1-octen-3-ol applied with EPN showed less than an 80% survival rate after 21 days. Untreated controls showed complete survival after the same time period.
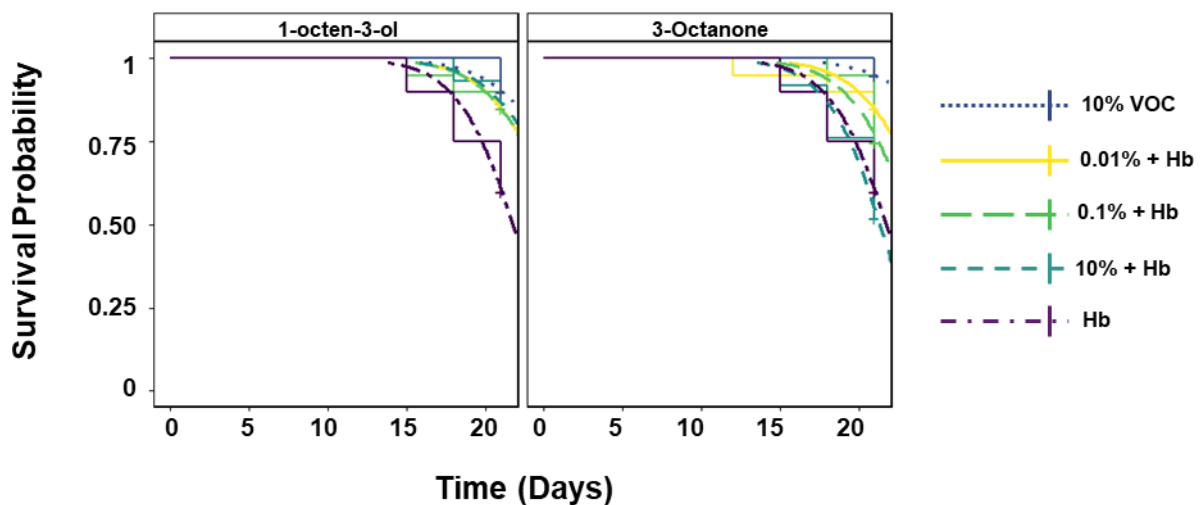
Fig. 5.12. Wireworm survival in a soil assay exposed to both concentrations of *M. brunneum* VOCs (1-octen-3-ol & 3-octanone) and EPN (*H. bacteriophora*, or Hb). Fitted survival regression curves are plotted for each treatment and controls (10% VOC only and Hb only). Ten replicates were used, and this was repeated twice.

5.4 Discussion

The chemical ecology of invertebrate pests and their relationship with known entomopathogens is an area of research that can be exploited to improve integrated management solutions. The defences of wireworm are particular robust against both EPF and EPN, but some repellent effects have been observed in response to high concentrations of the former (Kabaluk & Ericsson, 2007b; Eckard *et al.*, 2017). Additionally, *Metarhizium* spp. have been shown to exhibit some synergistic effects with EPN (Shapiro-Ilan *et al.*, 2004; Ansari *et al.*, 2010) towards other insect pests. Specific constituents from the volatile profiles of *Metarhizium* spp*.* may have significant bioactive effects on wireworm to enable greater levels of control with EPN, or manipulation of behaviours in a crop protectant application. A drawback of the use of EPF for wireworm control is the host species specificity of strains of *M. brunneum* & *anisopliae* (Eckard *et al.*, 2014)*.* Though this may be an advantage in avoiding knock-on effects to beneficials, the likelihood of stability of the most virulent strain and its suitability for mass production for a commercial application is much reduced (Kabaluk & Ericsson, 2007; Badjugar et al., 2009). Identifying specific VOCs or secondary metabolites might increase targeting of the elusive pest within or between the crop, or provide a fumigation effect that is more commercially viable.

Using the VOCs 1-octen-3-ol and 3-Octanone, identified through previous research (Hummadi *et al.*, 2021) there was clear bioactivity on wireworm, with both showing fumigant effects on the insects and on EPN. Some evidence was presented for improvement of a commercially available strain of *H. bacteriophora*, which may suggest a lower application rate and a cost saving measure for growers. This may be supported by the use of the identified VOCs at a lower concentration to exploit sub-lethal behavioural effects (Bourdon *et al.*, 2022). A directed behaviour or immobilisation may address issues in targeting the pest itself and allow a lower application of biological controls.

As an indication of viability of use within a crop neither 1-octen-3-ol and 3-Octanone exhibited any phytotoxic effects towards tomato plants at any dose tested, compared to untreated controls. There was no clear indication of biostimulation of *Metarhizium* but no suggestion of phytotoxicity in applications of the EPF (Chinniah *et al.*, 2016; Praprotnik *et al.*, 2021). These results suggest that the VOCs will mirror the environmentally friendly benefits of the naturally occurring entomopathogen towards crops (Sharma *et al.*, 2020), allowing for variability in timing and area of application.

There was a clear dose dependent mortality of wireworm exposed to both VOCs in fumigation assays. Surprisingly, although requiring three times as long to kill larvae in the absence of soil, 3-Octanone performed better in the soil, with complete mortality in half the time in dry soil compared to 1-octen-3-ol, and more than 50% mortality in wet soil. The buffering capacity of the soil was evident in the increase seen in both the time-to-kill and lethal dose required. In no-choice CT assays, the effect of the soil had minimal effect on larval behavioural responses with complete repellence from the tube and a suppression of their feeding response. Choice assays in a larger, semi-natural arena, support these conclusions but there was slightly more larval feeding, though in control sections, suggesting a limited diffusion through the soil profile. In Baverstock *et al's* review (2009), it is suggested that within soil insect behavioural responses to EPF are often much reduced or non-existent, highlighting another Coleopteran, the Colorado potato beetle (*Decemlineata leptinotarsa* L.), as an example when exposed to *B. bassiana* (Klinger *et al.*, 2006). Although some observations have been made of wireworm directional responses to soil applied *Metarhizium* (Kabaluk & Ericsson, 2007), this may be an as yet unexplained artefact of the specific soil biome, or a more complex ecological response to available food, abiotic factors or conspecifics.

Some attraction of both EPN and PPN to VOCs of *Metarhizium brunneum* has been recorded in the soil (Khoja *et al.*, 2019; Hummadi *et al.*, 2021). Also, there is some evidence to suggest that implementation of an EPF formulation may have an adverse effects in promoting PPN density and plant damage in potatoes (Mwaura *et al.*, 2017). The timing and application location of EPF or its derivatives is crucial in avoiding knock on trophic effects. Here it was found that both VOCs were clearly nematocidal towards commercial strain of *H. bacteriophora*, even at lowest doses. Any potential solutions exploiting synergies between the VOCs and EPN towards wireworm must use the compounds at lower doses.

To further imply that both 1-octen-3-ol and 3-Octanone may lower wireworm defences for EPN infection, caspase activity shows stress cause by both VOCs compared to controls for caspases 3-7, 8 & 9. There was a stronger response to 1-octen-3-ol at 1 hour exposure, compared to other treatments and time points. This may be indicative of a faster onset of mortality, with cells too far beyond apoptosis to pick up the caspases (Li & Yuan, 2008).

Assays to examine synergy between VOCs and EPN revealed no compatibility between 1-octen-3-ol and *H. bacteriophora*. However, results were more promising for 3-Octanone with a marginal improvement over controls using nematodes alone. There

was not a significant level of control compared to assays carried out with EPF (Ansari *et al.*, 2009; Kabaluk *et al.*, 2015; Razinger *et al.*, 2018, Chapter 3), but comparable to research carried out with an attract-an-kill approach with potato extracts and strains of EPN by La Forgia *et al.* (2020).

There are previous examples of compatibility between both EPF & EPN in controlling invertebrate pests (Shapiro-Ilan *et al.*, 2004; Ansari *et al.*, 2010). Specific VOCs of *M. brunneum* here, both 1-octen-3-ol and 3-Octanone, appear to inhibit more than stimulate the efficacy of EPN, concomitant with findings in Hummadi *et al.* (2021), However, 3-Octanone shows some promise in co-applications, in conjunction with a commercial strain of *H. bacteriophora*, against wireworm. The strong repellent and fumigant effects of 3-Octanone in particular suggest potential for use as a direct application or within a push-pull strategy with either trap crops or other attractants.

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Chapter 6: Discussion

6.1 Overview

As discussed throughout this thesis, there is a clear need to improve control methods for wireworm in potato crops due to the ongoing deregistration of conventional pesticides (la Forgia & Verheggen, 2019; Poggi *et al.*, 2021). The efficacy of biological controls is not yet sufficient within a field setting, and faces clear hurdles in terms of targeting a heterogeneous subterranean pest population; overcoming heavy infestations and stability of the controls within a soil matrix (Kabaluk & Ericsson, 2007a; Eckard *et al.*, 2014; Morton & Garcia-del-Pino, 2017). The overall aim of this project was to determine novel strategies for the control of wireworm within potato crops with a multi-faceted approach. Firstly, identifying and evaluating environmentally-friendly bioactive compounds for direct control of the insect, and secondly, assessing their compatibility with existing entomopathogens.

The first section of the project focused on the insecticidal and semiochemical properties that botanical extracts held towards wireworm. The bioactivity of tea tree, rosemary and cedarwood oils against *Agriotes* spp. larvae were carried further as candidates with the most promise. It was found that both tea tree and rosemary exhibited repellent effects on wireworm, positively correlating with mortality for each, with a suppression of feeding response in laboratory assays. The converse was found for Cedarwood, with strong attractant effects observed, and no insecticidal effects against the larvae, and evidence of phagostimulation. These properties were reduced within the field, though comparable effects were observed in areas of lower infestation. The use of botanicals within integrated pest management is not a new concept (Tripathi *et al.*, 2005; Campolo *et al.*, 2018; Cai *et al.*, 2020), though their use against subterranean pests is understudied and, as posited here, underutilised. We suggest that there is strong potential for development of a novel control solution for wireworm using these botanicals, with formulations taking advantage of the specific fumigant constituents or through development of push-pull systems benefitting from their behavioural effects (Eigenborde *et al.*, 2016). Martel *et al.*, (2005) looked at use of the push-pull, or 'stimulo-deterrent' strategy, in a greenhouse study of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Use of both an antifeedant and the attractant properties of synthetic host plant volatile blends, combinations of (Z)-3-hexenyl acetate; (+/–)-linalool and methyl salicylate showed successful behavioural manipulation of emerged and older adults. This would build on findings within this thesis that such a strategy might be employed belowground as a

larval management strategy but also that it may be used effectively in the adult stages as an oviposition deterrent, or as a male capture to reduce breeding in conjunction with identified sex pheromones (Hicks & Blackshaw, 2008; Sufyan *et al.* 2011).

The second section of the project sought to address issues raised through scaling experiments up to field trials, with subterranean invertebrate taxis in response to external stimuli often unpredictable when scaling up from laboratory studies. Behavioural responses of wireworm were categorised in a substrate filled, semi-natural arena allowing omnidirectional movement of the organism in response to introduced stimuli, here botanical extracts or whole plants. Past research has highlighted the need for innovation in evaluation of wireworm behaviours (van Herk *et al.*, 2008; Barsics *et al.*, 2014; Johnson *et al.*, 2018), with classic entomological techniques, such as y-tube olfactometry, not applicable to subterranean larvae. The presence or absence of organisms within the CT, and proximity in relation to it, can give a range of information such as feeding preferences, speed of movement, and chemotactic response. Here we were able to concisely demonstrate the preference of maize over other crop types, concomitant with effects observed in the field (Staudacher *et al.*, 2013; Saussure & Plantegenest, 2015; la Forgia *et al.*, 2020). Additionally, behavioural responses to botanicals identified in chapter 2 were further explored in a semi-natural setting. The repellent effects of tea tree were re-affirmed. Rosemary exhibited weaker repellent effects, though CT studies corroborated the feeding suppression and random movements observed in earlier work. Cedarwood continued to suggest an attraction and phagostimulation response. Incorporation of lemon oil revealed a previously identified repellent compound may be more accurately classified as an antifeedant agent. This methodology may provide a bridge between laboratory and field evaluated natural products to evaluate subterranean invertebrate responses in a more natural arena. However, with results from field trials in chapter 1 inconclusive in heavier infestations, it is clear that this remains a hurdle to novel bioprotectants and thus formulation and application of products is key to future work. Vemmer et al., (2013) review a variety of encapsulation materials and matrices, primarily for containment of entomopathogens, that might suggest a viable route forward for application of novel biopesticides. Attracap®, mentioned in Chapter 1, would be a pertinent example of a formulated biocontrol that was robust enough for commercial use (Hermann *et al.*, 2017).

Perhaps the most promising route for a control solution to bridge the gap of conventional pesticide losses is the augmentation of existing entomopathogens with compatible or synergistic compounds (Gfeller *et al.*, 2013; Eckard *et al.*, 2014; La Forgia *et al.*, 2020). Research has turned its focus to improving the efficacy of EPF by manipulating

wireworm behaviours, or reducing their pathogenic defences, through inclusion of bioactive compounds, such as botanical extracts, in partnership with the fungi (Kabaluk & Ericsson, 2007b; Eckard *et al.*, 2017; Bourdon *et al.*, 2021). Here we showed that previously identified botanicals, tea tree, rosemary and cedarwood do exhibit fungicidal and fungistatic effects on strains of *Metarhizium brunneum*, but at lower concentrations can improve mortality and rate of pathogenicity. The fungitoxicity of botanicals is well studied (Daferera *et al.*, 2000; Tripathi *et al.*, 2008; Raveau *et al.*, 2020), though here present evidence that the two may be used concurrently in a wireworm control solution. The minimum inhibitory concentration of cedarwood oil towards *M. brunneum* strain ARSEF4556 was considerably higher than that of rosemary and tea tree, and no adverse effects towards wireworm were observed in fumigation assays. With previous chapters demonstrating a phagostimulant effect elicited by cedarwood, it may be that feeding behaviours provide an entry way for the EPF to more easily penetration into the insect. This phenomenon could be a side-effect of the attract-and-kill system Attracap®, in which larvae have been observed to feed on the granules themselves (Hermann *et al.*, 2017). This is perhaps a side-effect of the VOCs of the incorporated yeast alongside the long-range attractant effects of produced CO_2. There several instances of plant or microbe VOCs being suggested to take on this role in the rhizosphere of determining wireworm food suitability at a closer range (Barsics *et al.*, 2014, 2016; la Forgia *et al.*, 2020). As such, the incorporation of cedarwood in an attract-and-kill control solution for wireworm may augment a simple CO_2 gradient and provide a greater effect than a stress-and-kill approach with a negatively bioactive compound (Eckard *et al.*, 2017). With field evaluation of an existing EPF biocontrol (Attracap®) we further highlight the need to improve pest targeting with short-range cues in the soil, in addition to CO_2. The variability in behavioural effects of the three botanicals may allow for implementation of a push-pull system of control, but the hurdles of cost to growers in application and multiple products may hamper its implementation.

Progressing work from synergies with EPF took advantage of two keys findings within the literature. Firstly, that larvae have exhibited repulsion behaviours when exposed to high concentrations of the EPF within a soil matrix (Vernon *et al.*, 2000; Wraight *et al.*, 2008; Eckard *et al.*, 2017). Secondly, that evidence of synergy exists between EPF and EPN in the control of other invertebrate pests (Shapiro-Ilan *et al.*, 2004; Ansari *et al.*, 2010). In previous research conducted alongside the main project, the constituent composition of *Metarhizium sp.* yielded specific VOCs which can affect nematode and insect behaviours (Hummadi *et al.*, 2021; Khoja *et al.*, 2021). It was thus suggested that the discovered VOCs of *M. brunneum*, 1-octen-3-ol and 3-Octanone, may elicit

behavioural or deleterious effects on wireworm and improve the efficacy of EPN applications, previously demonstrated to have limited effects on wireworm control (Rahatkhah *et al.*, 2015; Morton & Garcia-del-Pino, 2017; La Forgia *et al.*, 2020). Here we demonstrated the fumigant properties of 1-octen-3-ol and 3-Octanone*,* towards both wireworm and a commercial strain of *Heterorhabditis bacteriophora*, and a potential for compatibility between the two in the control of the former. Results indicated some direct interaction between low concentrations of 3-Octanone and the EPN. However, repulsion effects of the VOC on wireworm in behavioural studies indicate the suitability of a push-pull system to more accurately target the larvae with the entomopathogen. Indeed, the most promising results were the direct bioactivity of the VOCs on the wireworm themselves, suggesting a fumigant or repellent application would be most suitable within a potato cropping system.

6.2 Conclusions and Future Work

It's clear that identified bioactive compounds, here botanicals and VOCs of EPF, can elicit behaviours from wireworm within a soil environment that can contribute to a crop protection program. The bioprotectant capabilities of the botanicals are reduced in areas of high infestation but patterns in areas of reduced population corroborate behavioural studies in laboratory and semi-natural arenas. Strong synergies were observed between a stable strain of *M. brunneum* (ARSEF4556), and the existence of commercialised EPF products strongly indicate their viability as natural products. Reduced synergies were observed between the Metarhizium VOCs 1-octen-3-ol and 3-Octanone and a strain of *H. bacteriophora*. However, as higher concentrations of 3-Octanone marginally improved mortality and caused obvious repellent effects against wireworm in the soil, it should be considered as having a foundation for push-pull or fumigant effects in potato crops.

Referring back to Furlan's (2005) comments on the lack of fundamental knowledge within wireworm ecology, it is clear that we are still some way off a holistic management solution for the pest. The three key points raised were related to species-specific knowledge, more accurate monitoring and an updated economic threshold for different crop. The outcomes of this thesis lend themselves most heavily to the second point, with behavioural manipulations of the pest and methods for more accurate classifications of those behaviours possibly able to contribute as much to monitoring as much as they are to a larval management solution within the crop. Updated behavioural methodologies, in

conjunction was modern molecular methods targeting species specific responses to identified compounds (Jankowska *et al.,* 2017), may suggest an improved approach to wireworm and adult responses at the species level, quickly and affordably. It is clear that the focus within biological control of wireworm focuses predominantly on entomopathogens and biorational synthetic volatiles or plant metabolites and VOCs (LaForgia & Verheggen 2019; Poggi *et al.* 2021), though compatibilities and relationships between the two is underexplored. If Stenberg's (2017) holistic framework of an interconnected biological control element of the IPM pyramid is the aim then the current state of research into agriculturally relevant Elaterids is a limited at identifying interactions between the proposed elements. More may be done to explore the botanical aspects of wireworm ecology, touched on only briefly with trap crop assays in the thesis, but the essential oil elements of the research presented here point towards a possible need for greater emphasis on inter- and intra- specific botanical diversity in particular. This in turn would lead to improvement of cultural controls in informing rotation practices, or more localised intercropping or trap-crops within a single season. This work is heavily focused on the larval stage, and as a result is by nature a curative solution to a pest already established within the soil. A complete IPM strategy must incorporate the preventative aspect of adult control in ultimately reducing oviposition to stop the replenishing of pest populations in field rotations.

Future research should focus on the mode of action of these identified bioactives. Behavioural work pointed towards neuroinhibition effects in Rosemary but direct effects on wireworm physiology are understudied. Jankowska et al. (2018) review a series of possible molecular targets for essential oils within insects, concentration on enzyme inhibition in the nervous system, specifically acetylcholinesterase and the octopaminergic system. Similar approaches may be appropriate for identified fungal volatiles, though their mode of action is less studied. Insect immune responses to fungal infection however might provide a solution to indicate the mode-of-action of isolated volatiles in their insecticidal or stress effects (Butt *et al.* 2016; McNamara *et al.* 2018). Further work investigating aspects such as detoxification enzymes may reveal a clearer picture alongside applied experiments and reveal any inclination towards habituation.. An immediate approach to habituation would be to focus on a lure-and-kill strategy to suppress populations, rather than relying on push-pull alone. Use of controls within only necessary crops within the rotation would guard against this behaviour in a long-lived pest species. This could be a crucial area of understanding for novel biopesticides to be used against such a robust semivoltine pest species. Also, formulation of compounds for application in the field should be a key area of future work. Factors affecting efficacy

range from control of release rate, stability when exposed to abiotic factors and robustness in storage and delivery through machinery.

6.3 References

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Appendices - Supplementary Figures

Appendix 1: Supplementary Figures for Chapter 2.

Table A1. Constituent composition of five essential oils used in bioassays, obtained through GC/MS chromatogram profiles. Constituents with > 0.05% relative abundance (by peak area) were included.

Constituent (> 0.05% relative abundance)	Essential Oil				
	Lemon	Citronella	Cedarwood	Rosemary	Tea Tree
(Z,Z)-α-Farnesene			✓		
4-Terpineol					✓
Acetic acid				✓	
Artemiseole					✓
Bergaptene	✓				
Bisabolol			✓		
Camphene				✓	
Camphor, (+)-				✓	
Caryophyllene				✓	
Caryophyllene oxide			✓		
Citral	✓	✓			
Citronellol	✓	✓			
Eucalyptol				✓	✓
Eugenol		✓			
Farnesol		✓			
Fuocoumarins	✓				
Geraniol	✓	✓			
Germacrone			✓		
Himalchalene-1			✓		
Isoborneol				✓	
Isolongifolene			✓		
Isopinocarveol					✓
L-borneol				✓	
Limonene	✓	✓		✓	✓
Linalool	✓	✓		✓	
Longifolene-(V4)			✓		
Perillol			✓		
p-menth-1-en-8-ol				✓	
Terpinolene				✓	✓
Tumerone			✓		
Valencene			✓		✓
Vestitenone			✓		
Viridiflorol			✓		
α-Caryophyllene			✓		
α-Himachalene			✓		
α-Longipinene			✓		
α-Phellandrene				✓	
α-Pinene				✓	✓
γ-Gurjunene					✓
γ-Terpinene				✓	
γ-Terpineol				✓	
o-Cymene			✓	✓	✓

Table A2. Model selection table based on Akaike's Information Criteria (AIC) and relevant metrics for variables for both Petri dish and soil fumigation assays. T = Treatment, D = Dose. K = number of estimated parameters, $_\Delta$AIC = relative differences between selected model and subsequent AIC values, AIC wt. = relative likelihood weighting for the relevant model, Cum. wt. = cumulative AIC weight values, LL = log likelihood (maximum likelihood estimation).

Fumigation Arena	Model Interactions	K	AIC	$_\Delta$AIC	AIC wt	Cum. wt.	LL
Petri-dish	T x D	13	1271.41	0	1	1	-624.41
	T + D	8	1308.90	37.49	0	1	-647.32
	T	7	1613.65	342.23	0	1	-800.73
	D	3	1669.84	398.43	0	1	-872.37
In Soil	T + D	5	545.78	0	0.78	0.78	-267.66
	T x D	7	549.53	3.75	0.12	0.9	-267.32
	T	4	549.83	4.05	0.10	1	-270.76
	D	3	565.90	20.12	0	1	-279.86

III
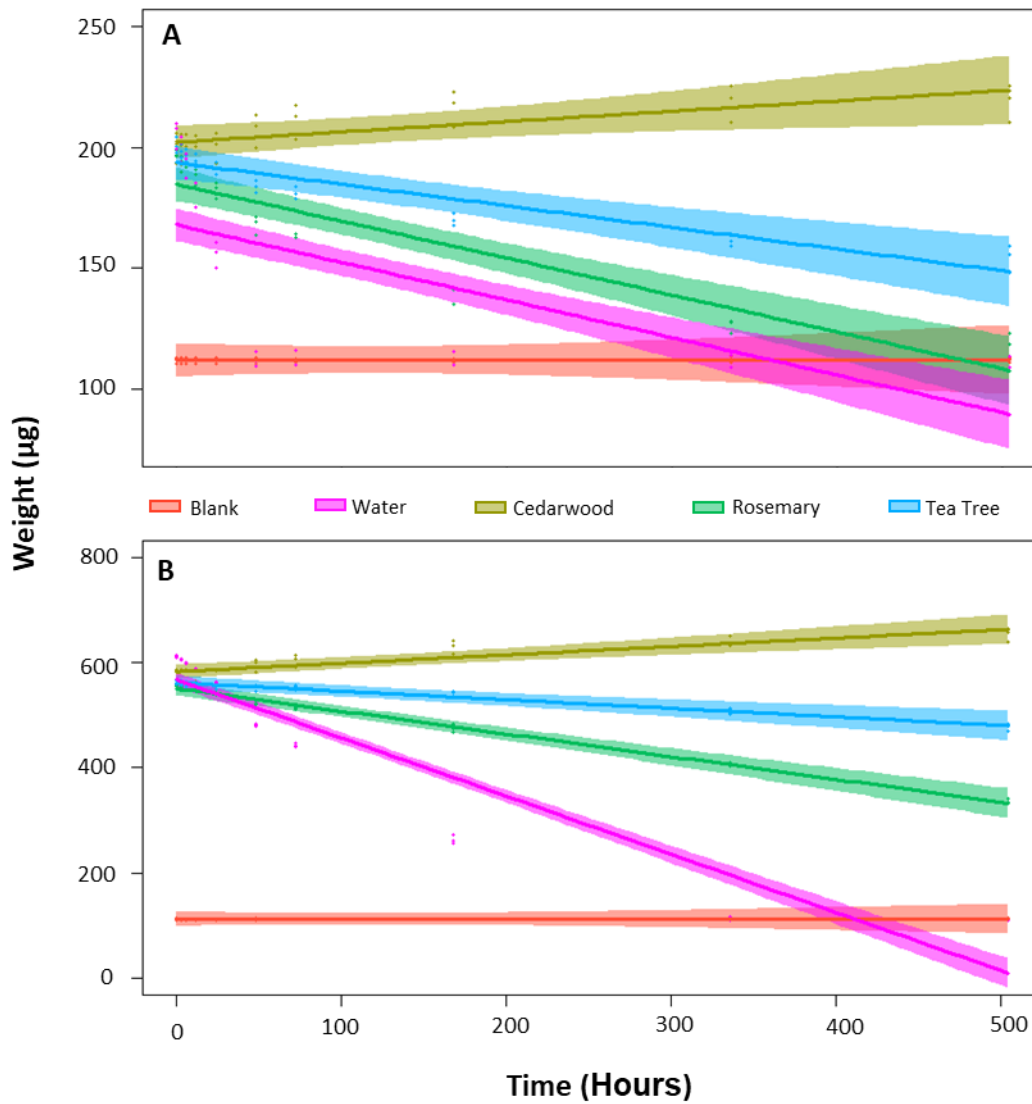
Fig. A1. Gravimetric release rates of essential oils and controls from filter tips. For A, 100 µl of oils were pipetted onto filter tips left in controlled conditions up to a period totalling three weeks. For B, 500 µl was used to examine the effect of loading capacity under the same conditions. Regressions are plotted with 95% CIs. Individual replicates are included.

Table A3. Aggregation and cluster indices for soil-filled terraria assays with associated statistical tests.

I_a = index of aggregation (> 1 indicates aggregate count, = 1 indicate random dispersion, < 1 = counts at regularity. *P_a = associated statistical test.)*

J_a = index of singular or multiple clusters (J_a > 1 = singular cluster, J_a < 1 = multiple clusters). Q_a = associated statistical test.

V_i & V_j = patch & gap cluster indices (V_i > 1.5 indicates strong clustering, V_j < -1.5 indicates sparse patches. P_{vi} & P_{vj} = associated statistical test.

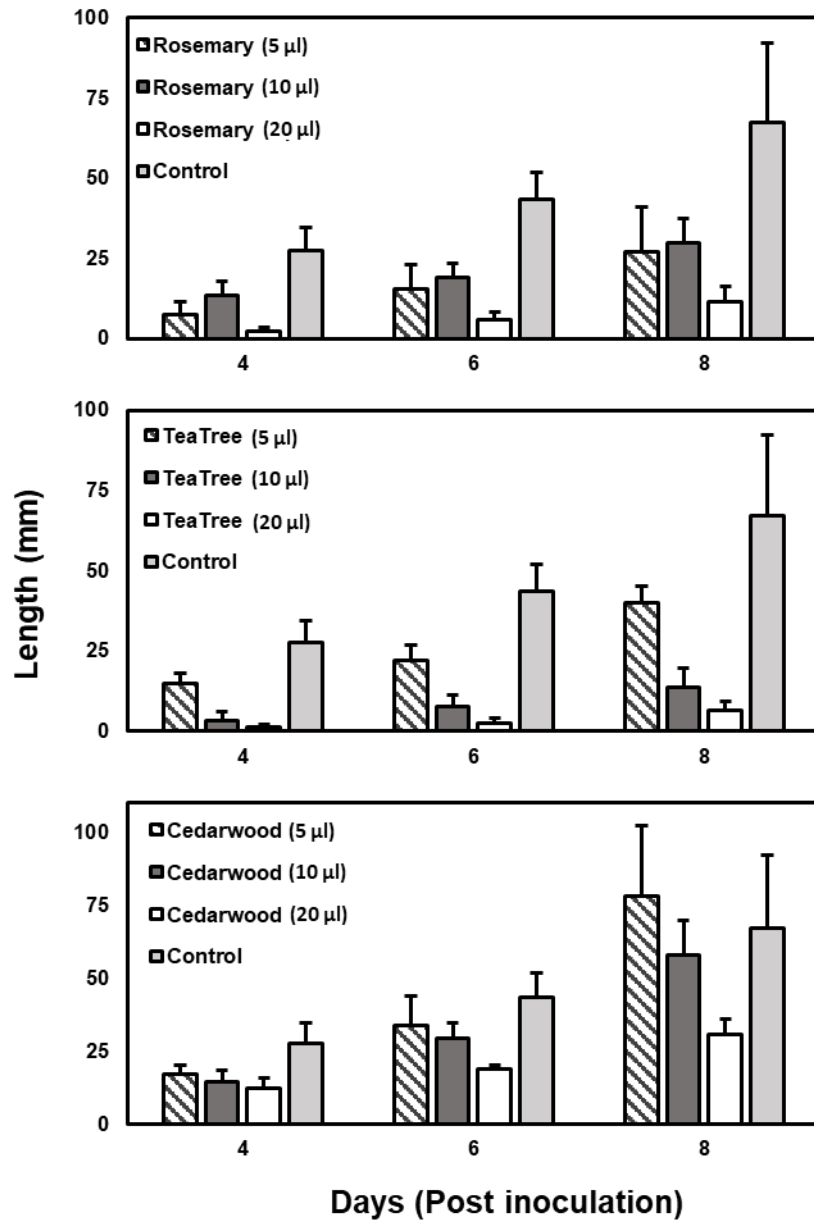| Treatment	I_a (P_a)	J_a (Q_a)	V_i (P_{vi})	V_j (P_{vj})
Rosemary	2.21 (< 0.001 ***)	1.04 (0.26)	2.23 (< 0.001***)	- 2.21 (<0.001***)
Tea Tree	2.41 (< 0.001 ***)	1.33 (< 0.001 ***)	2.53 (< 0.001***)	- 2.42 (< 0.001***)
Cedarwood	1.62 (0.0042 *)	1.44 (< 0.001 ***)	1.64 (0.003 **)	- 1.63 (0.002 **)
Control (Seed)	2.26 (< 0.001 ***)	1.60 (< 0.001 ***)	1.92 (< 0.001 ***)	- 2.24 (< 0.001 ***)
Control (No Seed)	1.31 (0.0463 *)	0.96 (0.59)	1.18 (0.108)	- 1.31 (0.044 *)

V

Fig. A2. Radicle length of germinated maize seeds in Petri dish fumigation assay with varying doses of oils. Untreated control included.
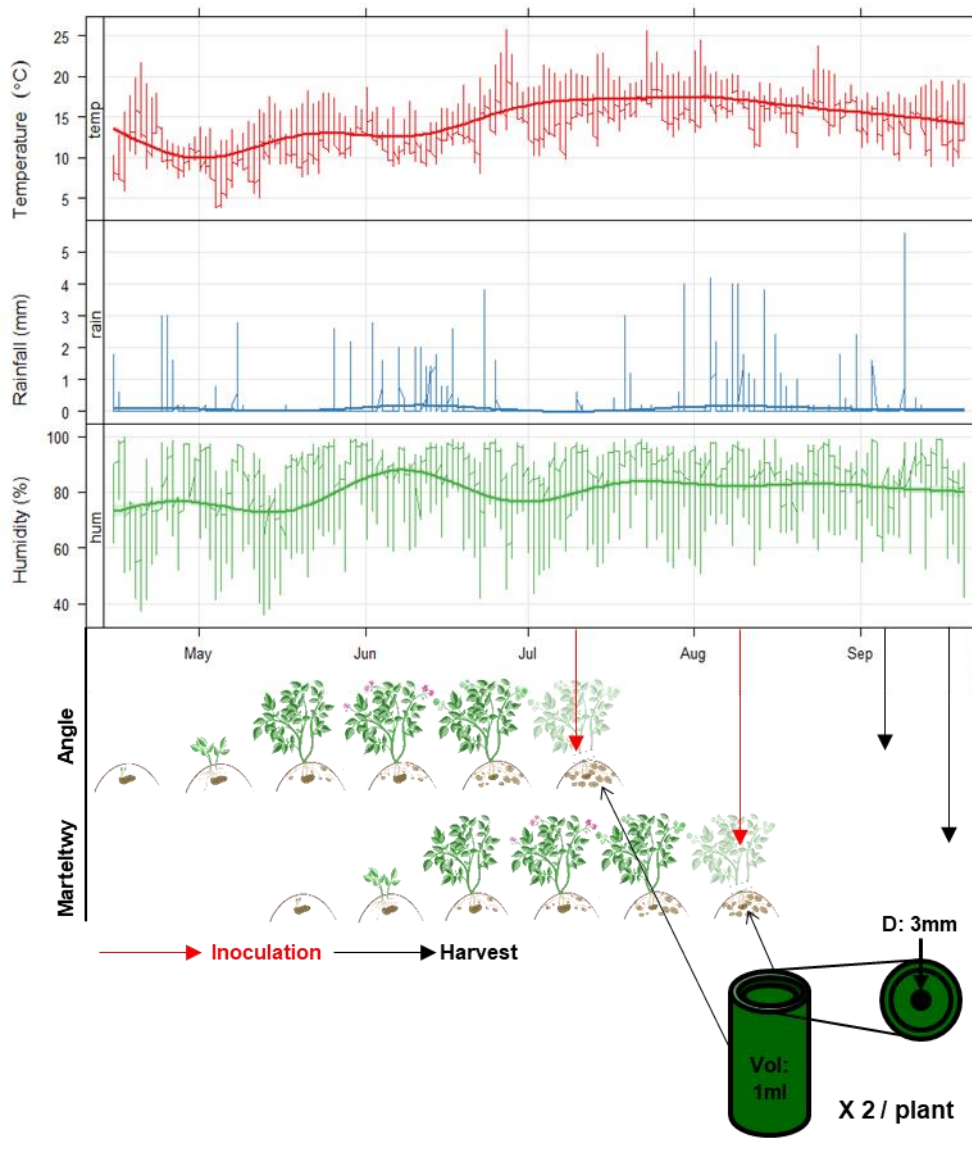
Fig. A4. Timeline of planting and haulm topping for each site overlaid with inoculation dates and harvest for each plot. Abiotic factors for the growth season are given from a weather station equidistant between the two sites. Biovials for treatment inoculation are indicated.
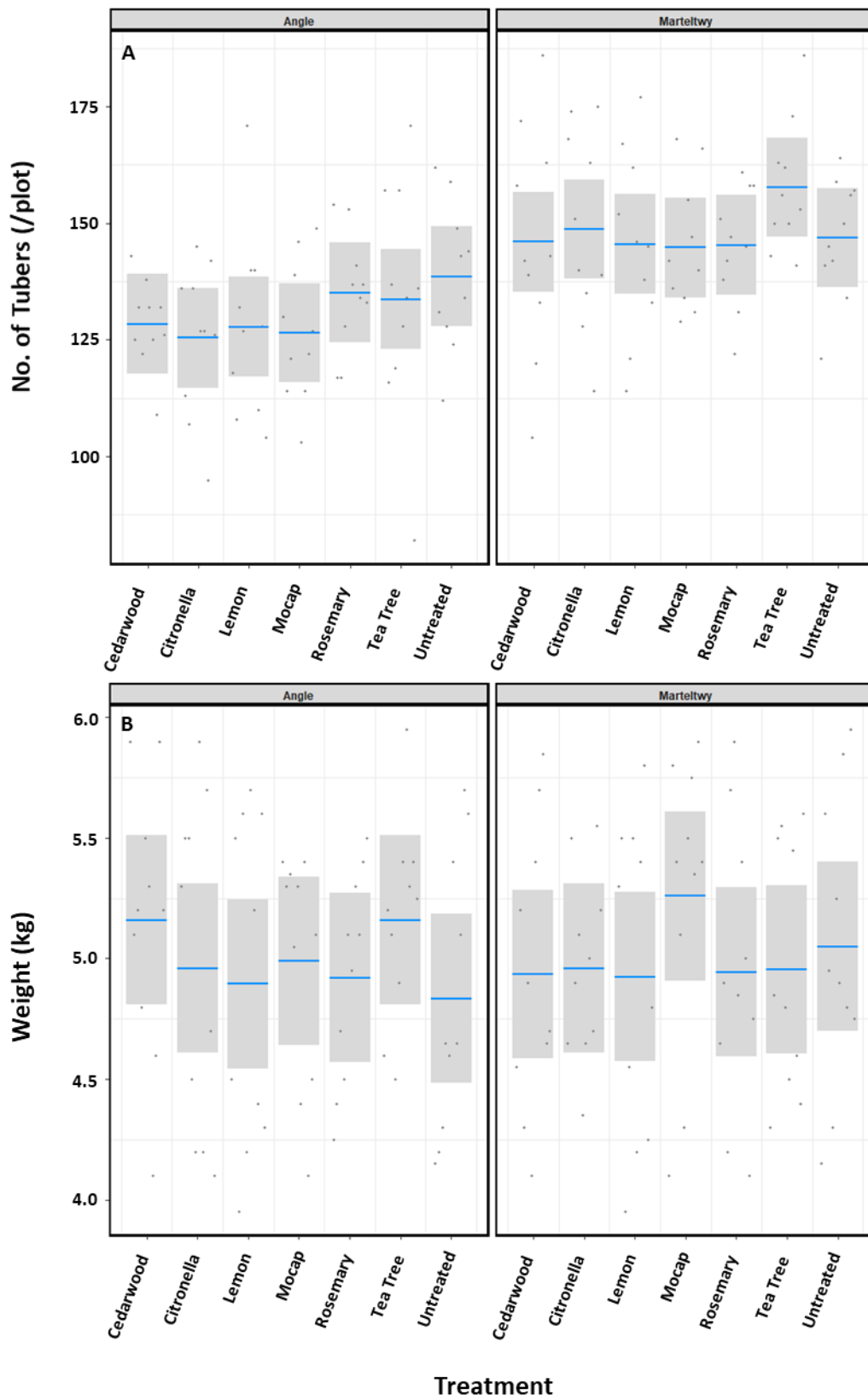
Fig. A6. Tuber number and weight results (average / plot) for harvested tubers across both field sites for botanicals trials. Means are presented with 95% CIs.
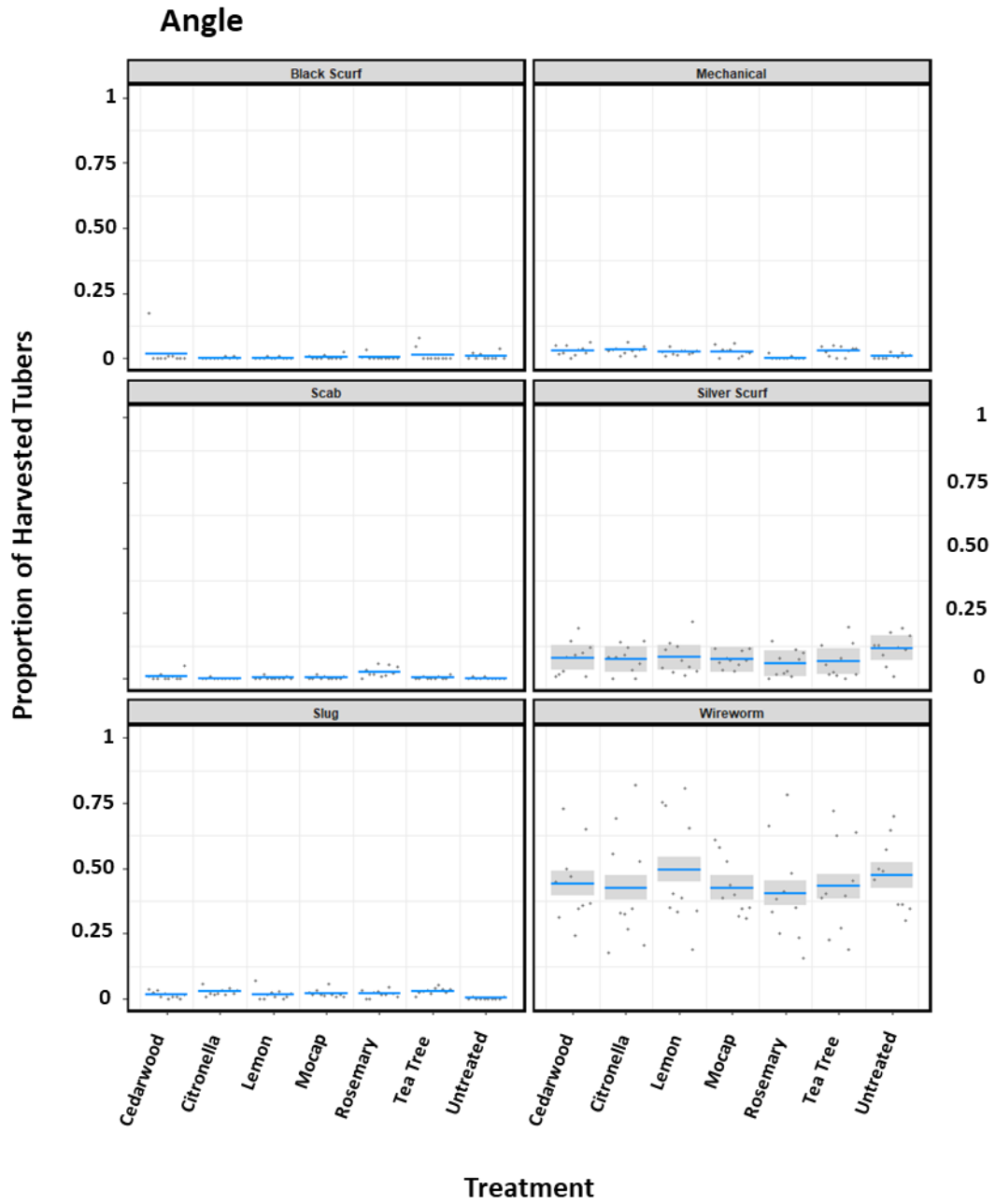
Fig. A7. Proportion of damaged tubers for the Angle field site within botanicals field trials. Means presented with 95% CIs for comparison.
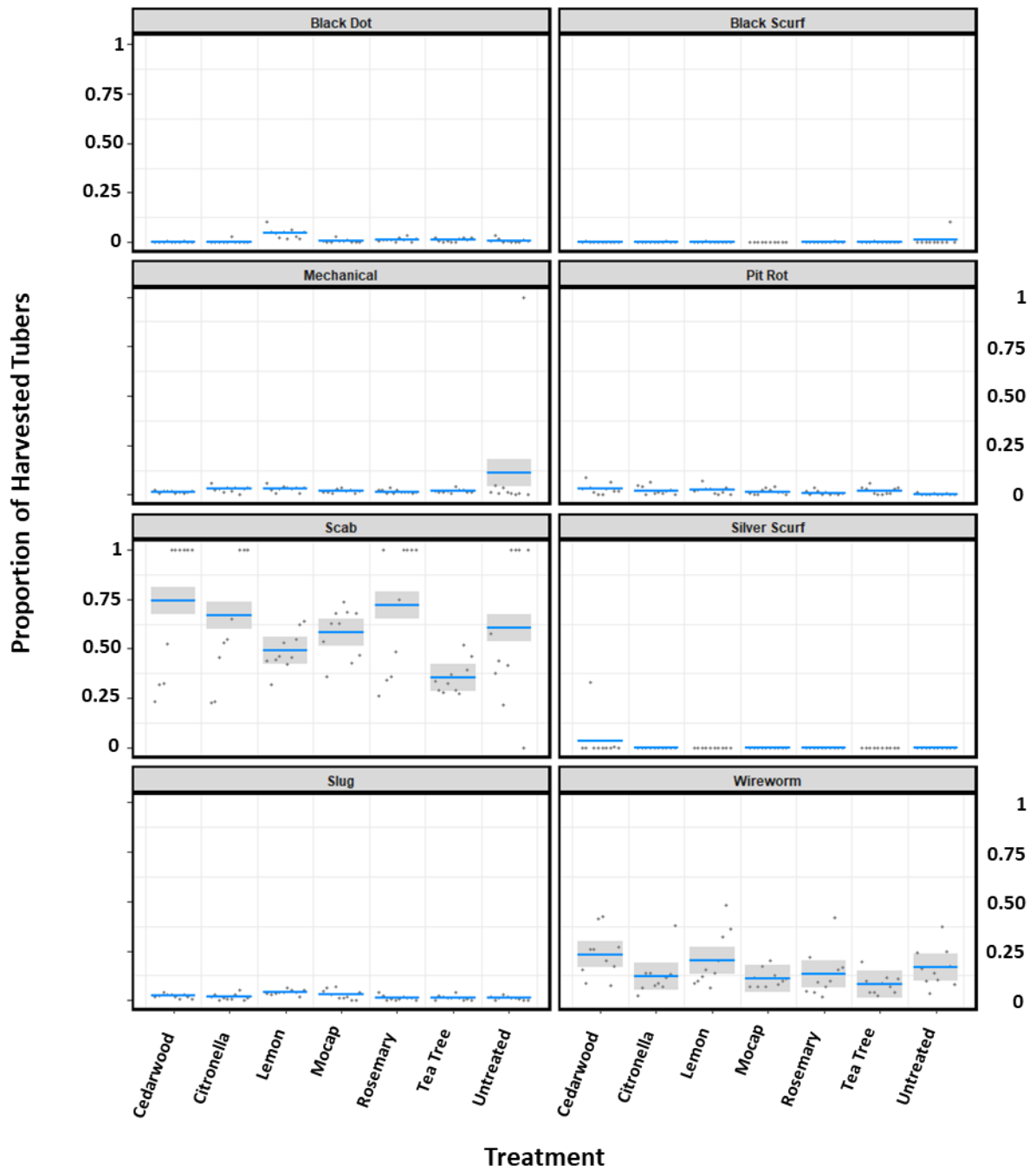
Fig. A8. Proportion of damaged tubers for the Marteltwy field site within botanicals field trials. Means presented with 95% CIs for comparison.

Table A4. Aggregation and cluster indices for wireworm damage and presence at each site with associated statistical tests.

I_a = index of aggregation (> 1 indicates aggregate count, = 1 indicate random dispersion, < 1 = counts at regularity. P_a = associated statistical test.)

J_a = index of singular or multiple clusters (J_a > 1 = singular cluster, J_a < 1 = multiple clusters). Q_a = associated statistical test.

V_i & V_j = patch & gap cluster indices (V_i > 1.5 indicates strong clustering, V_j < -1.5 indicates sparse patches. P_{vi} & P_{vj} = associated statistical test.

Site	Wireworm	Aggregation and Cluster Indices			
		I_a (P_a)	J_a (Q_a)	V_i (P_{vi})	V_j (P_{vj})
Angle	Damage	2.08 (0.003 **)	1.02 (0.151)	1.77 (0.002 **)	-2.12 (<0.001 ***)
Angle	Presence	1.45 (0.03 *)	0.99 (0.531)	1.32 (0.055 *[1])	-1.42 (0.032*)
Marteltwy	Damage	1.83 (0.002 **)	1.05 (0.076)	1.61 (0.007 **)	-1.72 (0.004 **)
Marteltwy	Presence	1.55 (0.013 *)	1.07 (0.14)	1.41 (0.026 *)	-1.48 (0.023 *)

[1] Where p is on the threshold of <0.05 being considered significant, this result was included to avoid ignoring the result based on marginal arbitrary cut offs. Other factors were considered in its effects on interpretations, as with all significant results regardless.

Appendix 2: Supplementary Figures for Chapter 3

**Table 2A1. Significant post-hoc pairwise interactions between treatments and larval location for capture tube choice assay to evaluate behavioural response to botanicals in the presence of maize seedlings.** The estimate indicates directional responses and degree of the significance of the relationship is given.

Pairwise Interaction			Est	SE	Z	p
Cedarwood (Control)	-	Tea Tree (Treatment)	-1.625	0.41	-3.965	0.0073***
Cedarwood (Control: Tube)	-	Rosemary (Treatment)	-1.75	0.41	-4.27	0.0021**
Cedarwood (Control: Tube)	-	Tea Tree (Control)	-2	0.41	-4.88	0.0001***
Cedarwood (Control: Tube)	-	Untreated (Control)	-1.5	0.41	-3.66	0.0228*
Cedarwood (Treatment)	-	Rosemary (Control: Tube)	1.75	0.41	4.27	0.0021**
Cedarwood (Treatment)	-	Rosemary (Treat: Tube)	2	0.41	4.88	0.0001***
Cedarwood (Treatment)	-	Tea Tree (Treatment)	1.625	0.41	3.965	0.0073**
Cedarwood (Treatment)	-	Tea Tree (Treat: Tube)	2.125	0.41	5.185	<.0001***
Rosemary (Control: Tube)	-	Tea Tree (Control)	-1.625	0.41	-3.965	0.0073**
Rosemary (Treatment)	-	Tea Tree (Treat: Tube)	1.75	0.41	4.27	0.0021**
Tea Tree (Treat: Tube)	-	Untreated (Control)	-1.5	0.41	-3.66	0.0228*
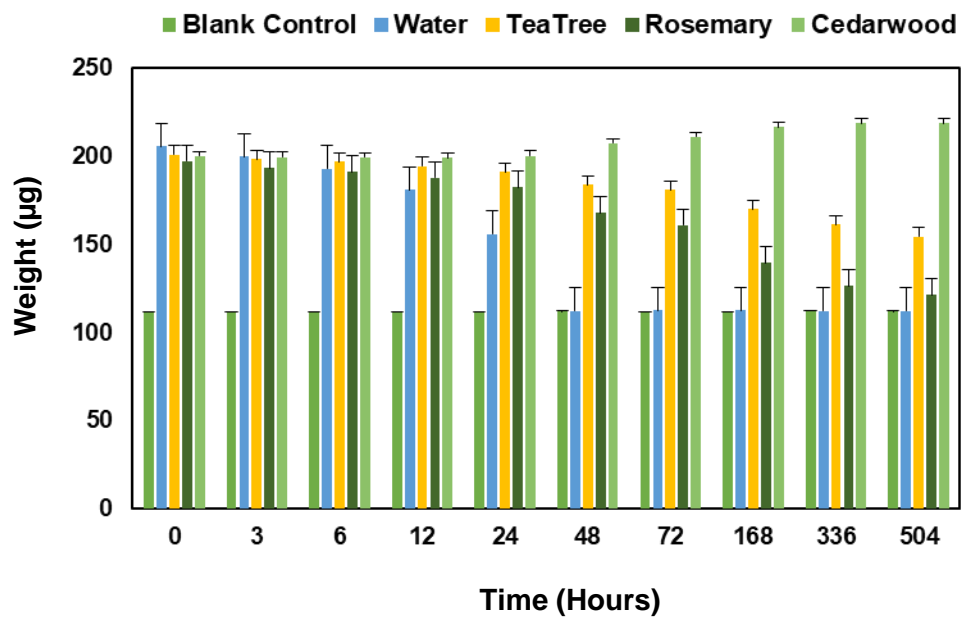
Fig. 2A1. Gravimetric release rates of oils inoculated into filter tip over a three-week period. Inoculated filters were left in glass dishes in controlled conditions (24°C, 65% RH) and weighed at 3, 6, 12, 24, 48 and 72 hours and then every week for three weeks thereafter. Water and a blank filter tip were used as controls.
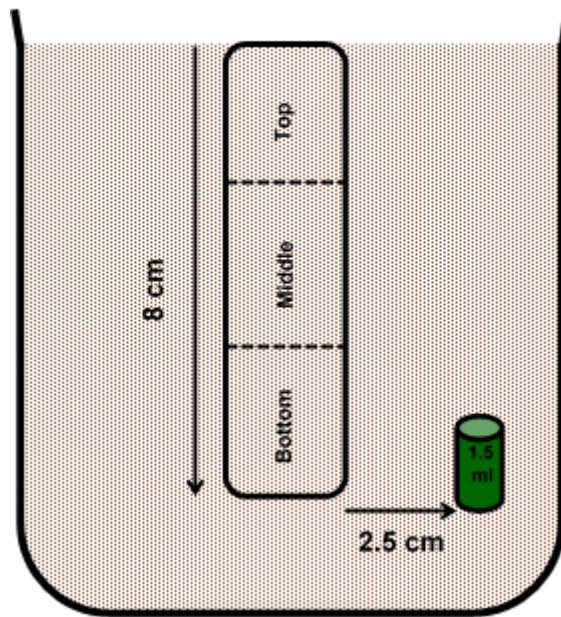
Fig. 2A2. Experimental arena for soil cores taken to examine volatile movement of essential oils through soil and into capture tubes. 1L pots filled with soil (mixed compost, multipurpose with John Innes) with a filled biovial of one of three oils placed on a layer of soil toward the base. Sections of capture tubes were taken at 3- & 7-day timepoints and split into three even section for headspace analysis.

Appendix 3: Supplementary Figures for Chapter 4

Table 3A1. Model selection table based on Akaike's Information Criteria (AIC) and relevant metrics for survival regression in a stress-and-kill assay exposing wireworm to both EPF and concentrations of botanical. M = Month, **T** = Treatment; **K** = number of estimated parameters, $_\Delta$**AIC** = relative differences between selected model and subsequent AIC values, **AIC wt** = relative likelihood weighting for the relevant model, **Cum. wt**. = cumulative AIC wt values, **LL** = log likelihood (maximum likelihood estimation.

Model Interactions	K	AIC	$_\Delta$AIC	AIC wt	Cum. wt.	LL
T x C	13	1060.78	0.00	0.92	0.92	-516.49
T + C	7	1066.58	5.80	0.05	0.97	-526.02
T	5	1067.98	7.20	0.03	0.99	-528.85
C	4	1070.41	9.63	0.01	1.00	-531.11

Appendix 4: Supplementary Figures for Chapter 5

Table 4A1 Model selection table based on Akaike's Information Criteria (AIC) and relevant metrics for fumigation assays against wireworm using VOCs of *M. brunneum*. T = Treatment, **D** = Dose; **K** = number of estimated parameters, $_\Delta$**AIC** = relative differences between selected model and subsequent AIC values, **AIC wt** = relative likelihood weighting for the relevant model, **Cum wt** = cumulative AIC wt values, **LL** = log likelihood (maximum likelihood estimation).

Fumigation	Model Interactions	K	AIC	$_\Delta$AIC	AIC wt	Cum. wt.	LL
No Soil	T + D	4	521.29	0	0.7	0.7	-256.41
	T x D	5	522.97	1.68	0.3	1	-256.13
	T	3	545.18	23.89	0	1	-269.45
	D	3	559.45	38.16	0	1	-276.58
Dry Soil	T x D	5	787.14	0	0.53	0.53	-388.36
	T + D	4	787.71	0.57	0.4	0.93	-389.72
	D	3	791.07	3.93	0.07	1	-392.45
	T	3	897.45	110.31	0	1	-445.64
Wet Soil	T + D	4	287.14	0	0.43	0.43	-139.33
	D	3	287.16	0.02	0.43	0.86	-140.44
	T x D	5	289.33	2.19	0.14	1	-139.31
	T	3	304.73	17.59	0	1	-149.23

Table 4A2. Model selection table based on Akaike's Information Criteria (AIC) and relevant metrics for stress-and-kill assay of wireworm using EPN and VOCs of *M. brunneum*, 1-octen-3-ol & 3-Octanone. For model parameter definitions, see Table 1(s). T = Treatment, D = Dose

Model Interactions	K	AIC	$_\Delta$AIC	AIC wt	Cum. wt.	LL
D	6	342.6	0	0.35	0.35	-165.07
T + D	8	342.75	0.16	0.33	0.68	-162.99
T	4	342.78	0.18	0.32	1	-167.28
T x D	16	356.94	14.35	0	1	-160.94