Exploring the Use of Plant Volatiles as a Sustainable Control Method for Glasshouse Whiteflies on Glasshouse-Grown Tomato

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Abstract

In the UK, tomato (*Solanum lycopersicum* L.) is cultivated in bespoke glasshouse systems which facilitate the use of sustainable pest control techniques such as integrated pest management (IPM). IPM focuses on the use of naturally occurring control agents and is globally endorsed as the future paradigm for crop protection. Despite this, important tomato pests such as glasshouse whiteflies (*Trialeurodes vaporariorum* Westwood) are often still managed with insecticides. These synthetic chemical sprays, to which whiteflies have shown resistance, are extremely detrimental to the environment and human health. Plant volatile organic compounds (VOCs) offer an environmentally benign alternative to insecticides and have great potential for incorporation into IPM systems. This thesis explored the use of VOCs to control glasshouse whiteflies on tomato in a commercial glasshouse setting.

In a series of intercropping experiments with whitefly non-host plants, we found French marigold to effectively 'push' whiteflies from a tomato crop through emission of airborne limonene. We then used repellent limonene dispensers in place of companion plants, and also in conjunction with methyl salicylate, a potent inducer of plant defence. This combined VOC-based protection method performed no better than the standalone limonene treatment, meaning there was no synergy between the two compounds. These experiments highlighted the defensive inadequacies of cultivated tomato and prompted us to analyze whitefly resistance mechanisms in *Solanum pimpenillefolium*, a wild tomato species more resistant to whiteflies. Superior defences in *S. pimpenillefolium* were characterized by greater quantity and diversity of VOC emissions than cultivated tomato. In olfactory preference experiments, *S. pimpenillefolium* VOCs were less preferred by whiteflies and more attractive to the whitefly parasitoid *Encarsia formosa*. Host plant resistance is a key component of IPM and these results illustrate the importance of tomato VOCs for whitefly resistance. The methods and concepts explored in this thesis could be used to support current IPM systems and help achieve sustainable whitefly control.

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Chapter 1. Introduction

1.1 Tomato: Origin and Domestication

As with many other *Solanaceous* crops, the cultivated tomato (*Solanum lycopersicum* L.) originates from South America (Mattoo and Razdan 2006; Jenkins 1948). As well as the cultivated tomato, there are a number of other tomato species which are found growing wild across the western Andes of Peru and Ecuador (Blanca et al. 2015). The diverse and isolated ecological habitats of the Andean region almost certainly contributed to the diversity of wild tomato species (Mattoo and Razdan 2006). The most likely ancestor of cultivated tomato is the cherry tomato (*S. lycopersicum* var. *cerasiforme*), a highly invasive species which still has a large natural range across South and Central America. This was likely domesticated from *Solanum pimpenillefolium* and first cultivated by the Incas in Peru followed by the Aztecs in Central America, over 1000 miles from its natural range (Blanca et al. 2012).

The tomato was brought to Europe by Spanish conquistadors after the conquest of Tenochtitlan (the Aztec capital known today as Mexico city) in the 16th century (Jenkins 1948). However, due to its association with botanically similar toxic *Solanum* species such as mandrake and belladonna, tomato was initially only used for ornamental purposes (Bergougnoux 2014). From the 17th century onwards tomato has been grown for human consumption across the European continent. Since then, domestication of tomato has been fuelled by improvements to fruit appearance, quality and quantity. This has led to cultivated tomato plants which produce much larger and/or more plentiful fruits than their wild ancestors (Schauer et al. 2005). Domestication of tomato was also accompanied by changes in plant architecture from an “unruly shrub” to a more erect, apical dominant and thick stemmed plant more suitable for cultivation (Paran and van der Knaap 2007).

1.2 Tomato in Modern Agriculture

Tomato is the 4th most consumed vegetable in the world and in 2017 global production reached over 180 million tons (FAOSTAT 2017). The global annual production of tomatoes increased by around 300% over the last four decades and growth is expected to continue in low-cost production areas such as China, which currently dominates global production (FAOSTAT 2017; Costa and Heuvelink 2007) (see Figure 1.1 for global distribution of tomato cultivation). In Europe, Spain and Italy are the largest producers of tomato and production is focused around outdoor systems and large plastic greenhouses/poly-tunnels. In these southern regions tomato production relies on favourable weather conditions characterised by long summers and relatively high light levels throughout the year (Heuvelink 2005). In northern European countries such as the UK, tomato production is predominantly conducted in large glasshouse units. In these capital intensive systems, the use of modern technologies such as hydroponics, climate control and integrated crop protection make glasshouse grown tomatoes one of the most advanced and innovative horticultural industries (Costa and Heuvelink 2007). Whilst the weather conditions of northern Europe are not favourable for tomato growth, the use of glasshouses can not only facilitate high yields, but also extend the growing season throughout most of the year (Heuvelink 2005). Glasshouse production can help prevent pest and pathogen infestations and uses as little as a quarter of the water required in outdoor production systems (Boulard et al. 2011). These factors contribute to the fact that northern countries such as the Netherlands, Belgium, Norway and the UK achieve the most efficient rates of tomato production (hectogram per hectare, Hg/Ha) anywhere in the world (Bergougnoux 2014).

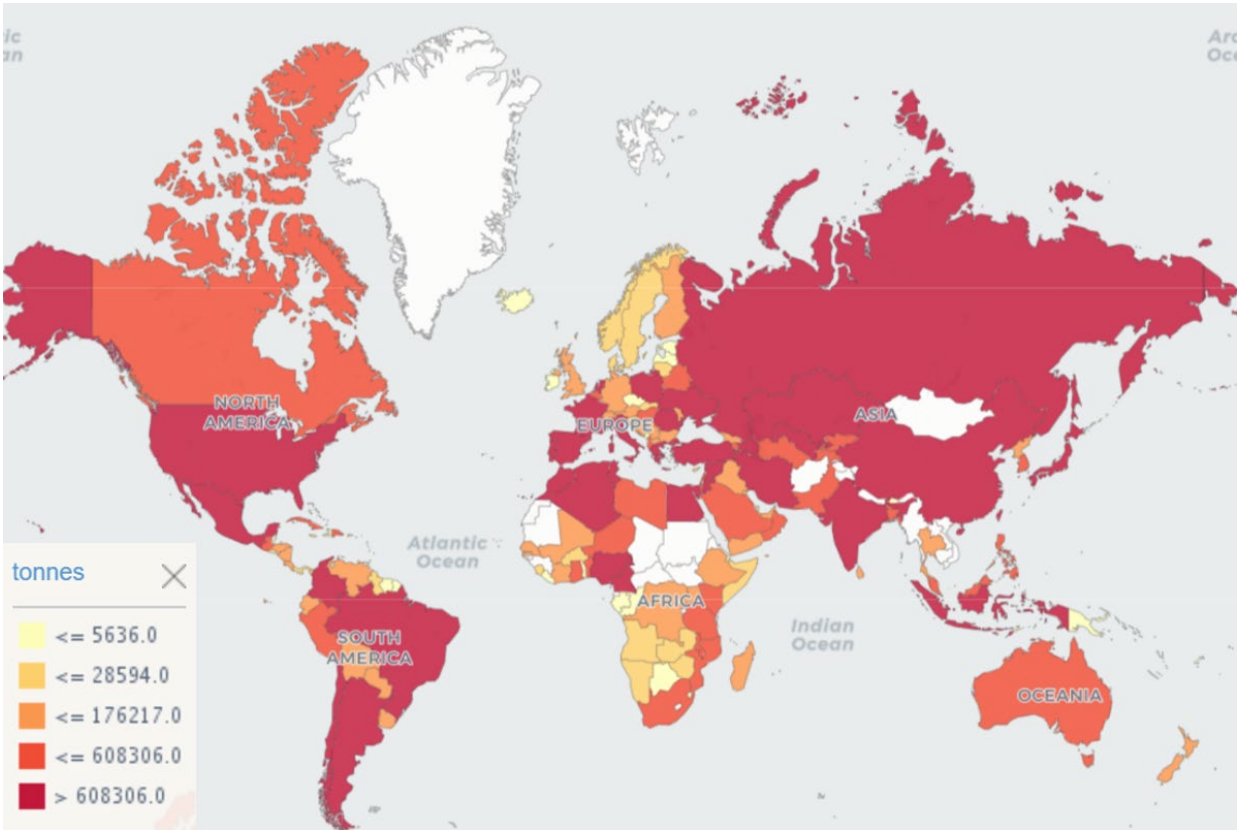

Figure 1.1: Total production quantity of tomatoes per country in 2017 (FAOSTAT 2017). This figure illustrates the global distribution of tomato production and highlights some of the top tomato producing countries such as China, USA and Spain.

1.3 Whiteflies

Whiteflies are small (~1-2 mm) hemipteran phloem-feeding insects of the family *Aleyrodidae.* This is split into two subfamilies: the *Aleurodicinae* that can be found in Central and South America, and the *Aleyrodinae*, which have a global distribution (Inbar and Gerling 2008; Byrne and Bellows 1991). Most whitefly species are found in warmer, tropical regions of the world but some can be found in cooler temperate regions such as northern Europe. In these areas whiteflies are usually present as pest insects which are restricted to protected cropping environments such as glasshouses (Martin et al. 2000). All whitefly species follow a similar life cycle, which begins with females that lay their eggs in epidermal cells on the abaxial side of leaves (see Figure 1.2, A for image of whitefly eggs). Unfertilized females lay haploid eggs that develop into males, whilst fertilized females lay diploid eggs which produce only females. This phenomenon is called arrhenotokous parthenogenesis and allows whiteflies to produce large

numbers of offspring, which contributes to their rapid population growth. From the eggs hatches a 1st instar nymph often referred to as a 'crawler' as it is the only mobile form of the whitefly larval instars. These larvae move around the leaf in order to locate an appropriate feeding site and from here it develops into the 2nd and then 3rd instar nymphs which are immobile (see Figure 1.2, B for image of whitefly nymphs). In the final stage, the pharate adults develop within the cuticle of the fourth instar, emerging as winged adults. Each female can live for up to two months and can lay over 400 eggs across their lifetime (Inbar and Gerling 2008; CABI 2018; Cranshaw 2013; Byrne and Bellows 1991). The life cycle is temperature dependent and can be completed in as little as 2 weeks at favourable temperatures (20-35 °C) or up to several months in colder conditions (<20 °C) (Hollis 1991).

Of the 1200 known species of whiteflies, only some 20 are considered pest species (van Lenteren and Martin 1999). The glasshouse whitefly (*Trialeurodes vaporariorum* Westwood) (Figure 1.2, A) and the tobacco whitefly (*Bemisia tabaci* Gennadius) of the *Aleyrodinae* subfamily are the two most economically important whitefly pest species and they cause damage to hundreds of agricultural and ornamental crops (van Vianen and van Lenteren 1986; Cock 1984). Whiteflies feed on plants by extracting tissue fluid from the phloem which can significantly reduce crop yields (Byrne and Bellows 1991; McKee et al. 2007). Similar to other phloem feeding insects, honeydew secretion from whitefly adults and larvae can limit transpiration, reduce photosynthetic capacity and promote sooty mould growth that can ultimately render fruit unmarketable (Jauset et al. 2000). As well as the direct feeding damage they cause, whiteflies are also transmitters of a number of plant viruses (Jones 2003), and these are often be more damaging to plants than the whiteflies themselves. Production losses attributed to whitefly-transmitted viruses are a major constraint on sweet potato production in Kenya, with uninfected sweet potatoes yielding 50% more production than plants infected with sweet potato chlorotic stunt virus (Wainaina et al. 2018).

1.4 Glasshouse Whiteflies in Agriculture

Originally native to South America, the glasshouse whitefly (*Trialeurodes vaporariorum*) has become established across Europe. However, in northern cooler climates such as Scandinavia and the British Isles, it is restricted to protected cropping environments (CABI 2018; Martin et al. 2000). The glasshouse whitefly first reached the UK around 160 years ago and population structure studies show there have been multiple introductions from nearby European countries since then (Kareem et al. 2019). Glasshouse whiteflies are one of the most important and widespread insect pests and are found on all continents except for Antarctica. They are highly polyphagous and have been reported to feed on approximately 859 plant species throughout the world (CABI 2018) with some of the most severely affected crops comprising members of the *Solanum* genus (Jones 2003). Along with the two-spotted spider mite it is one of the most important arthropod pests in the UK (Gorman et al. 2002) and is considered the main insect pest for glasshouse-grown tomatoes (Lange and Bronson 1981). Despite the fact that physical barriers with the surrounding environment offer a greater level of pest control, pest penetration in glasshouses is often significant and whiteflies are still an extremely prevalent pest in protected cropping environments (George et al. 2015).

Herbivory from glasshouse whiteflies is detrimental to plants (McKee et al. 2007) and in Chapter 3 of this thesis we report a 33% reduction in fruit yield from tomato plants not protected by repellent VOC dispensers. Glasshouse whiteflies transmit viruses of the genera *Crinivirus* and *Torradovirus* (Navas-Castillo et al. 2014), however it is members of the *Crinivirus* genera such as tomato chlorosis virus and 'tomato infectious chlorosis virus' which are the most damaging to tomatoes (Wainaina et al. 2018). As it is not possible to cure plants of infections from whitefly-transmitted viruses, efforts must be taken to prevent plants from becoming infected or to manage the rate, timing or severity of the infection to protect crop health (Gilbertson and Lucas 1996). Therefore, the most prudent method of whitefly management is often preventing whiteflies from ever infesting the target crop through preventative release of natural enemies and glasshouse sterilisation (Cranshaw 2013).

1.4.1 Current glasshouse whitefly control methods

Control methods for glasshouse whiteflies differ throughout the world but in protected cropping environments the use of biocontrol has proven to be an effective management method. In pioneering research from the early 20th century, entomologist Edward R. Speyer identified *Encarsia formosa* Gahan (Figure 1.2, D) as a whitefly parasitoid after a tomato grower noticed black pupae (see Figure 1.2, B) amongst the normally white scales of glasshouse whitefly larvae (Speyer 1927). Shortly after this discovery, a research station in England was supplying up to 1.5 million of these parasites annually to about 800 nurseries in Britain (Woets 1988). Use of *E. formosa* spread to other European countries but popularity of this method receded following World War II, primarily due to newly introduced insecticides which provided convenient and efficient pest control (Woets 1988). More recently, biological control of whiteflies has seen something of a resurgence and global use of *E. formosa* on greenhouse crops increased from 100 hectares in 1970 to 4800 hectares in 1993 (Hoddle et al. 1998) (Figure 1.2, C). *E. formosa*'s 'outstanding' success at controlling whiteflies on tomato (Hoddle et al. 1998) has contributed to its status as the world's most important parasitoid for the control of whiteflies on vegetable crops (Wang et al. 2019). Whilst the economic and environmental benefits of biocontrol are clear, there a number of drawbacks to biocontrol that mean it has not completely replaced the use of pesticides in the glasshouse sector as some predicted (George et al. 2015; van Lenteren 2000). Firstly, *E. formosa* are only active at temperatures above 17 °C, which can complicate their release in cooler temperatures early/late in the season (Gorman et al. 2007). Other issues such as delayed efficacy (Bale et al. 2008) and hyper- parasitism (Schooler et al. 2011) can result in biocontrol failure, meaning there is still a reliance on synthetic chemical sprays to control whiteflies (George et al. 2015; Gorman et al. 2002). Additionally, the import and release of these exotic biocontrol species could potentially cause issues for native fauna (Huang et al. 2009). Despite the fact that pesticides have caused a reduction in populations of beneficial insects (Geiger et al. 2011), conserving and utilising native natural enemy populations would be a more sustainable alternative to current biocontrol practices.

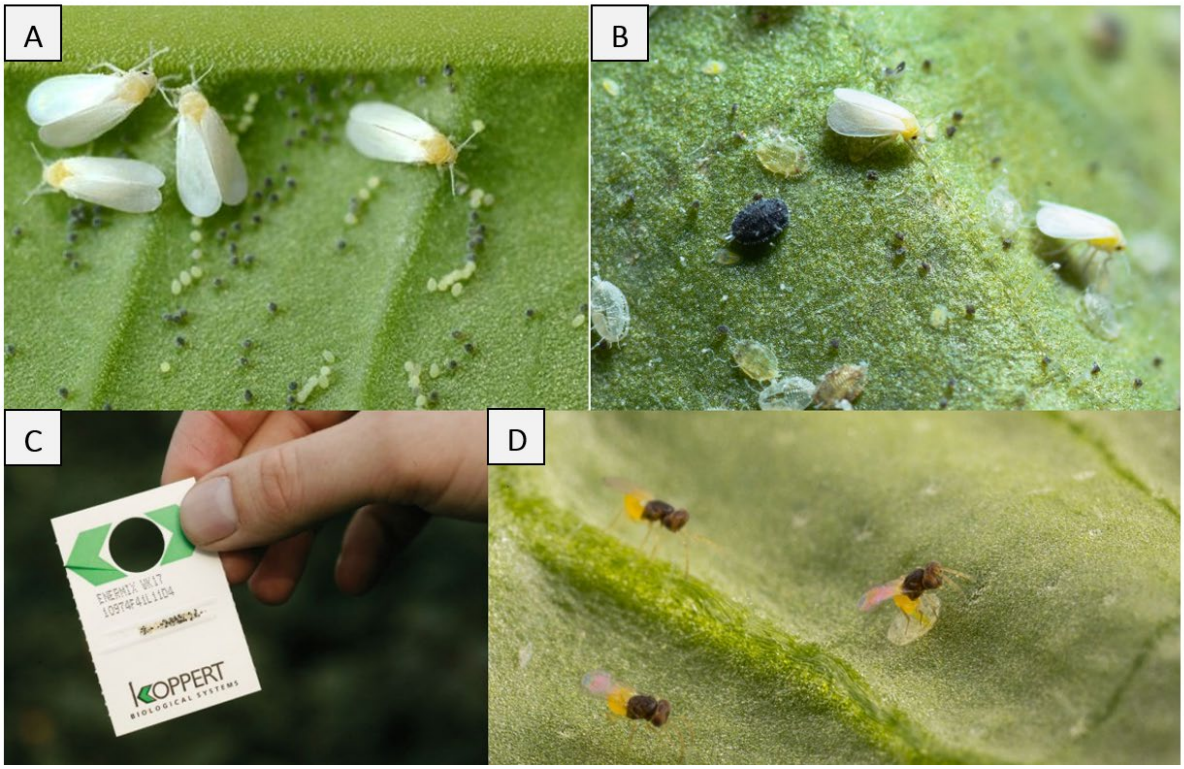

Figure 1.2: Life cycle images for Glasshouse whiteflies (*T. vaporariorum*) and *Encarsia formosa*. Image (A) shows several adult glasshouse whiteflies feeding on the abaxial side of a tomato leaf. Whitefly eggs are initially creamy-white when first laid, but turn brown within 24 hours, both forms can be viewed in (A). (B) Adult glasshouse whiteflies surrounded by eggs and nymphs, note the black whitefly nymph, which indicates it has been parasitized by *E. formosa*. (C) 'en-strip' biocontrol cards that represent the typical way *E. formosa* are deployed in glasshouses. The card has 300 whitefly eggs parasitized by *E. formosa*, which hatch and parasitize up to 95 whitefly nymphs across their 12-day life expectancy. (D) Adult *E. formosa*, the individual to the right of the image is parasitizing a whitefly nymph. Images A, C and D were obtained from Koppert biological systems (https://bit.ly/2PvxV4D) and image B was obtained from Anatis bioprotection (https://bit.ly/34sAA39).

Unlike other insect pests, control of whiteflies with insecticides is generally quite difficult due their morphological and autecological characteristics (Osborne and Landa 1992). Because whiteflies feed on the abaxial side of leaves, standard spraying of chemicals onto plants can often elude the target pest. Another consideration to make is non-target effects on natural enemies that may also be present, particularly in glasshouse set ups where biocontrol is commonplace. In these situations the use of insect growth regulators (IGRs) is preferred due to their high specificity (Simmonds et al. 2002), although glasshouse whiteflies have shown resistance to these chemicals (Gorman et al. 2002). Despite the difficulties associated with

insecticide use, they remain the most prominent method of whitefly control across the world (Schlaeger et al. 2018). Synthetic chemical sprays such as organophosphates, carbamates, ketoenols, pyrethroids and neonicotinoids are all used to control glasshouse whiteflies (Zanic et al. 2008; Gorman et al. 2007). Due to excessive use of insecticides glasshouse whiteflies from multiple regions worldwide have shown resistance to many different classes of insecticides including pyrethroids, neonicotinoids, organophosphates and ketoenols (Zanic et al. 2008; Gorman et al. 2007; Wardlow et al. 1976; Kapantaidaki et al. 2018). Further to this, the number of available insecticides is ever decreasing due to changes in legislation restricting development of synthetic chemicals (Hillocks 2012). By limiting the number of insecticides available we are inadvertently increasing the use of the select few which are still accessible, thus intensifying selection pressure and increasing the chance of resistant whitefly populations (Bruce et al. 2017). Due to their haplodiploid breeding system that encourages the rapid selection and fixation of resistance genes (Brun et al. 1995; Denholm 1998), combined with ever increasing selection pressure, it is prudent to assume that whitefly resistance to all conventional insecticides is somewhat inevitable. Clearly, our reliance on insecticides to control whitefly infestations is lacking in longevity and sustainability.

1.5 Volatile organic compounds (VOCs): background and basis for pest control

Volatile organic compounds (VOCs) are natural products of almost all plant taxa (Vivaldo et al. 2017) and are what humans would recognise as the smell of a plant. VOCs fall into several major classes based on their biosynthetic origin, which include terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives and amino acid derivatives (Dudareva et al. 2013). VOCs are amongst the most energetically expensive metabolites for plants to produce (Gershenzon 1994; Robert et al. 2013) and play an extremely important role in the way plants interact with their surrounding environment (Vivaldo et al. 2017). Plants produce VOCs constitutively but also in response to herbivore infestation, and these induced compounds are referred to as herbivore induced plant volatiles (HIPVs). Plants release VOCs/HIPVs which can act as defensive compounds against insects or pathogens (Penuelas and Llusia 2001; Brilli et al. 2019) and serve as attractants to beneficial insects such as parasitoids (Birkett et al. 2003) or pollinators (Dudareva et al. 2013). VOCs/HIPVs can enhance the efficiency of within-plant defence signalling and also influence the behaviour and

physiology of nearby plants, a phenomenon known as plant-plant signalling (Heil and Ton 2008). HIPVs can alert neighbouring plants to the presence of biotic stressors, causing these receiver plants to induce their own innate defence mechanisms (Brilli et al. 2019; Erb 2018). Plants can also release VOCs which negatively impact competitive plant species by impairing growth and reproduction (Arimura et al. 2010).

Understanding the complex cross-kingdom interactions VOCs can mediate has been the focus of a great deal of research attention, particularly with regard to the way plant VOCs interact with insect herbivores. VOCs offer an extremely versatile pest control option and have been shown to successfully manage insect pests in a number of different ways. VOCs can be used to repel insects away from the target crop (Du et al. 2016; Pickett et al. 1992), attract pests to sticky traps in a practice known as 'attract-and-kill' (Martel et al. 2005; Moreau and Isman 2011) and also to serve as attractants to natural enemies (Rodriguez-Saona et al. 2011a; James and Price 2004). VOCs offer a relatively inexpensive, environmentally benign alternative to pesticides and could prove to be extremely valuable to sustainable pest management practices (Schlaeger et al. 2018).

Whilst vision also plays a key role in whitefly host selection, whiteflies have been shown to use olfactory cues in their selection of a suitable host (Schlaeger et al. 2018). Whiteflies are extremely sensitive to subtle differences in plant VOCs and can differentiate between VOCs from different plant species (Zhao et al. 2014; Darshanee et al. 2017; Li et al. 2014) and even different cultivars (Darshanee et al. 2017) and accessions of the same species (Bleeker et al. 2009). Whiteflies also use plant VOCs as an indication of host quality and can distinguish plants that are infested with other herbivorous insects (Tan and Liu 2014) and a variety of different plant pathogens (Inbar and Gerling 2008). Due to way whiteflies interact with plant VOCs, there has been some effort to manipulate whitefly behaviour with VOCs in order to achieve whitefly control on agricultural crops (Zhao et al. 2014; Du et al. 2016; Li et al. 2014). Whilst the use of VOCs in agriculture and implementation of VOCs into IPM is still in its infancy, VOCs offer tremendous potential for use against whiteflies (Schlaeger et al. 2018) and could prove pivotal in our efforts to sustainably control whiteflies in the future.

1.5.1 Companion planting

Most agricultural crops are now produced in monoculture, a practice that renders cropping areas devoid of wildlife and essential 'ecosystem services' (Malezieux et al. 2009). These unbroken environments facilitate rapid population growth for many insect pests due to the unnatural abundance of available host plants. Companion planting, the practice of growing two or more plant species alongside the target crop, is a traditional farming practice which is still used by subsistence farmers and other small scale growers across the world (Altieri 1991). Companion planting has been shown to provide benefits such as nutrient exchange, reduced weed competition and pest/pathogen control (Vandermeer 1989). The use of multiple plant species provides a more natural environment that can also increase agro-ecosystem biodiversity (Gaba et al. 2015). Reinstating habitat diversity could return agricultural ecosystems to a state of ecological balance and reverse the negative effects of intensive agriculture (Nicholls et al. 2004). Whilst positive effects on yield are well documented (Jolliffe 1997), companion planting is generally under-utilized at the commercial farming level.

Intercropping with companion plants can provide greater control of insect pests either directly, by preventing pest establishment, or indirectly by attracting natural enemies (Parker 2013). Herbivorous insects use a series of visual and chemical cues in their selection of a suitable host and the presence of companion plants can disrupt this process in the following ways;

1) VOCs from companion plants can mask host plant odour, which disrupts host-finding behaviour of the herbivorous insect. (Ben-Issa et al. 2017; Togni et al. 2010). This process is known as "associational resistance" (Thiery and Visser 1986).
2) Repellent VOCs from companion plants can 'push' insects away from the target crop (Parker 2013; Ben-Issa et al. 2017).
3) Companion plants can alter host plant chemistry through allelopathic transfer of defence eliciting compounds (Ben-Issa et al. 2017). This has been shown to occur both above and below ground with VOCs and also with transfer of other specialized metabolites from plant root exudates (Semchenko et al. 2014).
4) Companion plants which are more preferred to the specific pest than the target crop can be effective. These 'trap plants' can be used as standalone features to draw pests away from the target crop (Ben-Issa et al. 2017), in combination with repellent companion plants in a 'push-pull' system (Pickett et al. 2014) or alongside sticky traps (Moreau and Isman 2011).

5) A diverse background of plant species can result in more 'inappropriate' landings, which is thought to disrupt insect feeding behaviour (Finch and Collier 2000).

Some success has already been achieved at controlling whiteflies with companion planting (Mutisya et al. 2016; Zhao et al. 2014; Gold et al. 1990) and in each of these examples the mode of action was inferred to be repellent volatile chemistry. This is unsurprising as whiteflies are extremely sensitive to changes in VOC composition/abundance and numerous VOCs have been shown to be repellent to whiteflies (Bleeker et al. 2009b; Du et al. 2016; Bleeker et al. 2012; Li et al. 2014). Despite these successes, there is still no research assessing the ability of companion plants to reduce glasshouse whitefly abundance on glasshouse-grown tomato in the UK. The potential to control whiteflies with VOCs is clear and companion plants could provide the medium by which these VOCs are delivered. The 'push-pull' method of companion planting is expected to work well for managing whiteflies in protected cropping environments due to their enclosed nature, which concentrates VOC abundance (Schlaeger et al. 2018; Cook et al. 2007). In the context of protecting glasshouse-grown tomatoes, which are almost exclusively cultivated in monoculture, intercropping with whitefly repellent companion plants could provide a much-needed influx of biodiversity to these ecologically sparse agro-ecosystems.

1.6 Integrated Pest Management (IPM)

Over-use and over-reliance on synthetic chemical sprays has led to the development of resistant pest populations and mortality to non-target organisms (Smith et al. 1976; Geiger et al. 2011). The negative environmental and human health related implications of pesticides (insecticides, fungicides and herbicides) (Geiger et al. 2011; Nicolopoulou-Stamati et al. 2016) has now urged governing bodies to implement measures to reduce pesticide use and place more focus on integrated pest management (IPM) (USDA-ARS 2018; Directive 128/EC 2009). IPM combines multidisciplinary methodologies to develop agro-ecosystem management strategies that are practical, effective, economical and protective for both public health and the environment (Smith et al. 1976). This holistic approach to pest management relies on the synergy of numerous pest management components such as the use of natural enemies, biopesticides, habitat manipulation, resistant plant varieties, crop rotation and

cultural/traditional management practices (Peterson et al. 2018; Perez-Hedo et al. 2017; Ellsworth and Martinez-Carrillo 2001; Stenberg 2017). IPM is based on ecological principles and places more focus on the use of naturally occurring control agents (Prokopy 2009) whilst reducing pesticide applications (Stenberg 2017) (see Figure 1.3 for IPM pyramid showing the most important pest management elements).

The transition to IPM requires a cultural change in the way we approach the control of insect pests. In most agricultural systems, growers use pesticides with the expectation of eliminating pest presence completely. However, the aim of IPM is not to eradicate pests but to maintain pest populations below economically injurious levels (EIL) (Stenberg 2017). Allowing some level of tolerance reduces selection pressure on pest populations and limits the chance of resistant pest genotypes (Peterson et al. 2018). The synergistic effects of multiple IPM components create a multi-faceted system that further reduces the likelihood of resistant pest genotypes. This synergy increases the durability of individual components and make the whole IPM package more sustainable, both environmentally and economically (Birch et al. 2011). Pesticides are still important tools in the integrated management of pests (Zanic et al. 2008) but as part of an IPM set up they are only to be used after all other IPM elements have been exhausted (Stenberg 2017) (Figure 1.3). There is great potential for IPM to be used against whiteflies (Schlaeger et al. 2018) and there are some successful examples of whitefly-focused IPM programs (Ellsworth and Martinez-Carrillo 2001; Van Driesche and Lyon 2003; Arnó 2009).

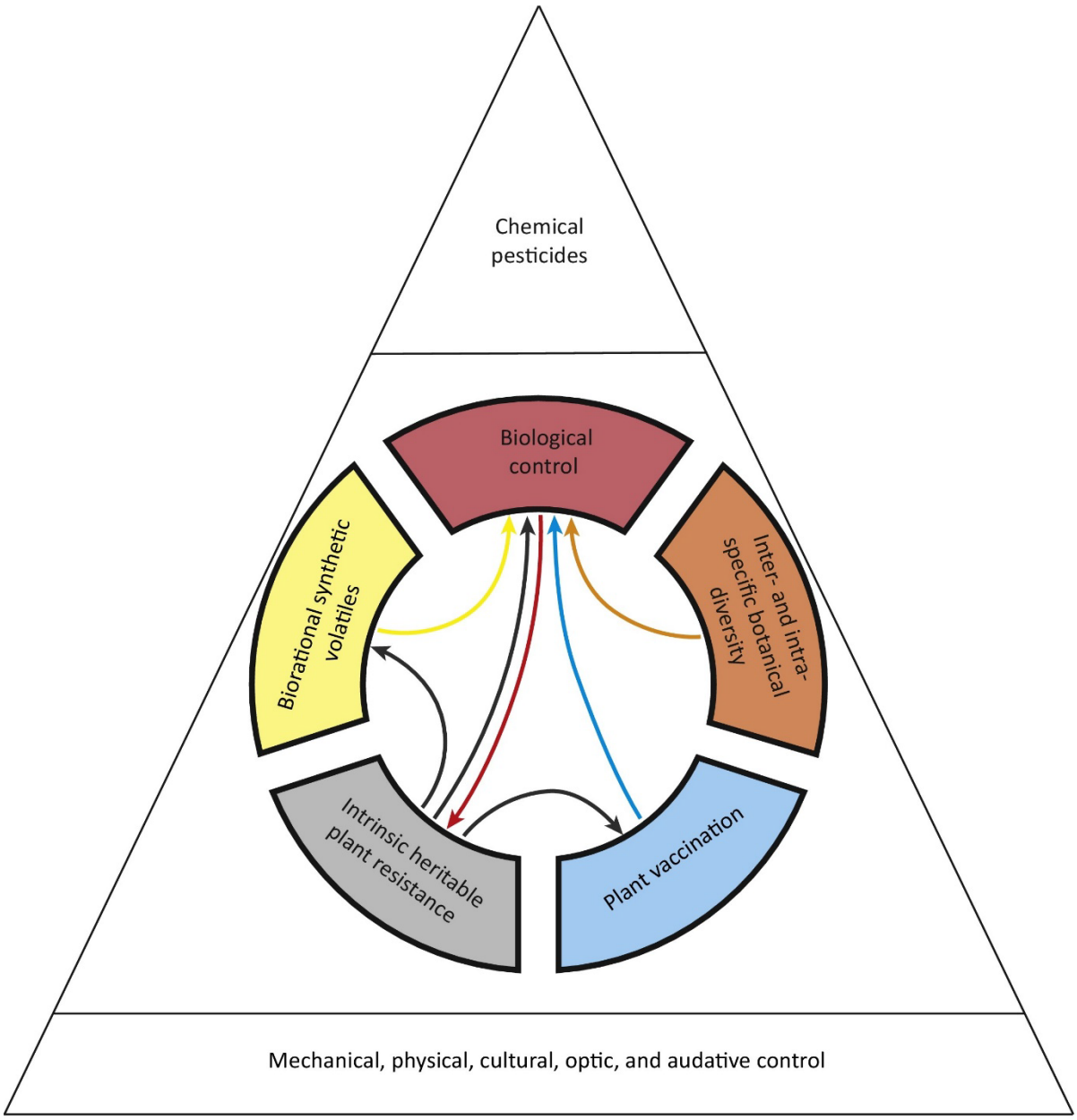

Figure 1.3: The integrated pest management (IPM) pyramid showing the most important pest management elements. The base tier of the IPM pyramid consists of abiotic actions that can be applied before or immediately after planting and may continue until harvesting. The circular tier shows important elements that can be classified as 'ecological' and form the basis for ecology-based IPM, arrows denote particularly important interactions between the ecological elements. All of these ecology-based IPM components are investigated in this research thesis. Note the presence of chemical pesticides at the top of the pyramid, indicating that they should only be used after all other IPM components have been explored. These seven interactions are key research areas that require elucidation to integrate the elements and realize the full potential of IPM. (Stenberg 2017).

IPM is globally endorsed by scientist and governing bodies as the future paradigm for crop protection (Stenberg 2017). Despite this, broad-spectrum pesticides, for which there is an ever growing market in excess of US$220.9 billion (Mordor Intelligence 2019), are consistently used as the sole means of pest protection in the majority of agricultural systems. There are several key factors contributing to low rates of IPM adoption amongst agriculturalists. Firstly, IPM is a complex pest management strategy that requires comprehensive training on a number of different methodologies for effective use (Piñero et al. 2018). Growers which take on IPM without sufficient training increase the chances of negative results (Trumble 1998), which could deter them from using IPM in the future. Whilst IPM can be a consistent pest management strategy on a national scale (Birch et al. 2011), operationally it is implemented and adapted under localised conditions. Thus, IPM is temporally and spatially contextual, which can frustrate attempts to implement large-scale IPM programs (Ellsworth and Martinez-Carrillo 2001). Even though individual adopters of IPM can expect to increase profits by significantly reducing their expenditure on pesticides (Taylor 1980; Petty 2005), IPM is still viewed as less effective than pesticides and it can be difficult to convince growers to take on these sustainable principles. Whilst the incentives to adopt IPM practices are clear at the societal level, these pressures often elude individual growers. Some have suggested that only a 'pest crisis', where widespread pesticide resistance renders chemical sprays ineffective, is enough to compel some growers to adopt IPM (Zalucki et al. 2009). In a recent survey of oil seed rape farmers from various European countries, 10-20% of all farmers from all countries would not even consider adopting IPM, even if it would improve profits and not involve any extra work compared with their normal practices, if at the same time there would be a higher number of pests on the crop than before (Menzler-Hokkanen and Hokkanen 2018). This highlights the nonsensical scepticism some farmers show towards IPM and emphasizes the cultural change that is needed in order to convince growers to deviate from current pest management strategies. Despite this, there has been an undoubted change in farmer perceptions and practices over the past 30 years (Zalucki et al. 2009; Arnó 2009). Particularly with high-input crops such as tomato, growers are keen to save as much money as possible and this has compelled growers to adopt significantly cheaper IPM systems for tomato in the South Eastern USA (Bauske et al. 1998). Ultimately, most growers will take the most cost-effective approach to pest management (Zalucki et al. 2009) and providing there is a market for cheaper IPM control strategies, we could continue to see growers adopt IPM. It is therefore essential that we continue research and development into novel IPM

components in order to provide sustainable pest management strategies and enhance current IPM systems (Birch et al. 2011).

Whilst many agricultural systems have been slow to adopt IPM, utilizing IPM in protected cropping environments is extremely popular. Due to greater environmental control in greenhouses/glasshouses, pesticides can be replaced with IPM much more easily compared with outdoor systems (Nicot and Baille 1996). As of 2016, over 80% of cultivated tomato surface in Almería (south-eastern Spain) is protected with IPM (Perez-Hedo et al. 2017) and in the Netherlands pesticides have been restricted to only occasional use in protected cropping environments (van der Velden 2012). IPM for glasshouse whiteflies is common across Europe (Arnó 2009) and typically incorporates components such as sticky traps, netting and biocontrol (Moreau and Isman 2011; George et al. 2015; Arnó 2009). Whist this can be effective, these IPM systems are relatively simple and usually incorporate only a handful of individual components. By integrating additional management elements to these already well-established systems, it is conceivable that we could gain greater control over whitefly infestations and further limit the need for synthetic insecticides.

1.7 Plant defence against insect herbivores

The first phytophagous insects evolved some 410 million years ago (Johnson 2011) and today almost all plant species face attack from herbivorous insects (Gatehouse 2002). Plants respond to herbivore attack through an intricate and dynamic defence system that has co-evolved alongside the equally complex mechanisms by which insects counter these defences. This evolutionary 'arms race' between plants and insects has led to the incredibly diverse array of morphological and biochemical defences that plants employ to resist insect herbivory (War et al. 2012; War et al. 2018). The mechanisms which plants use to defend themselves against insects can be divided into constitutive and induced defences. Constitutive or 'static' defences are invested in during the course of normal growth and development and are present irrespective of external stimuli (Gatehouse 2002). Structural traits such as leaf surface wax, thorns and trichomes are examples of constitutive defences (Hanley et al. 2007). This is in contrast to inducible or 'active' defences that are initiated in response to insect attack. Whilst constitutive and induced defences often thought to be distinct, the end-products of

the mechanisms and the defensive compounds themselves, are often the same in constitutive and induced defences (Gatehouse 2002). A good example of this is nicotine, a secondary metabolite found in tobacco leaves that has activity against a number of insect herbivores. Nicotine is synthesized by tobacco under normal conditions but nicotine production is drastically increased in response to insect attack (Kaplan et al. 2008). Both constitutive and induced defences can act upon herbivores directly (direct defence) by impacting survival and altering host susceptibility, or they can act indirectly (indirect defence) through the recruitment of beneficial insects (Gatehouse 2002). Most plant species exhibit a combination of direct and indirect defensive measures in order to have maximum efficacy against the attacking organism.

Investing metabolic energy in defensive measures incurs a fitness cost and plants must manage resources between growth and defence by eliciting anti-herbivore defence only when necessary (Karban 2011). This trade-off between growth and defence is illustrated by reduced growth in plants which have initiated their inducible defences when compared with un-induced plants (Agren and Schemske 1993). For example, induction of tomato defences was found to significantly reduce fruit and seed numbers (Redman et al. 2001). Despite this, it is still extremely beneficial for plants to employ inducible defence mechanisms because they offer a bespoke response to insect attack. Plants are able to detect and respond to specific insect feeding guilds that often require a different defensive response to effectively supress infestation. This is demonstrated by the fact that caterpillars, leaf miner flies and russet mites each induce different compositions of proteinase inhibitors, peroxidases, lipoxygenases and polyphenol oxidases in tomato plants (Chadwick and Goode 2008).

1.7.1 Plant defence signalling

Plant defence responses are regulated by a complex network of signalling pathways that are mediated by specific signalling molecules. Two key signal molecules, namely salicylic acid (SA) and jasmonic acid (JA), mediate expression of both specific as well as basal defence responses (Jalali et al. 2006). Leaf chewing insects and necrotrophic pathogens cause induction of the jasmonic acid (JA) signalling pathway. This begins with the proteolytic cleavage of prosystemin to form systemin, which interacts with the systemin receptor in plant cell walls. This then stimulates the release of linoleic acid from membrane lipids, which then goes on to create JA

via the octadecanoid pathway (Gatehouse 2002). JA is then transported throughout the plant to stimulate the synthesis of wound response products such as protease inhibitors (PI), polyphenol oxidases (PPO) (Gatehouse 2002) and various HIPVs (War et al. 2011). These secondary metabolites have activity against most insect feeding guilds and help defend the plant from insect attack. The importance of JA for plant defence against insect herbivores is best illustrated with experiments using the JA deficient tomato mutant *def-1*. This mutant has been shown to produce fewer PI's and HIPVs compared with wild type 'Castlemart', which makes these *def-1* plants more susceptible to insect attack (Gatehouse 2002; Perez-Hedo et al. 2018). Conversely, the salicylic acid (SA) signalling pathway does not lead to wound response products and has little effect on plant resistance to insect herbivores (Zhang et al. 2019; Gatehouse 2002). SA stimulates the synthesis of pathogenesis related (PR) proteins, which help the plant defend against a variety of viruses and biotrophic pathogens (Gatehouse 2002; Jalali et al. 2006). SA is an essential component for the creation of systemic acquired resistance (SAR), whereby the plant stimulates defensive measures systemically following detection of pathogen infestation at a localised infection point (Park et al. 2007).

The end products of the SA pathway are effective against pathogens but not insects, and end products of the JA pathway are effective against insects but not pathogens. The antagonistic effects of these two key signalling pathways can make it difficult for plants to combat simultaneous attack from both SA-inducing pathogens, and JA-inducing insects (Loake and Grant 2007). It is important for plants to induce the correct signalling pathways in response to insect attack because induction of inappropriate defensive measures can serve to increase insect performance (Zhang et al. 2019; Zhang et al. 2013).

1.7.2 Plant defence against whiteflies and other phloem feeding insects

Phloem tissue fluid consists of a nutritionally rich cocktail of carbohydrates, proteins and amino acids that makes it a primary feeding target for a variety of phytophagous insects (Jiang 2019). These phloem feeding insects, such as whiteflies, aphids and planthoppers etc, penetrate the phloem using adapted mouthparts known as 'stylets', which allow them to access the phloem sieve elements with minimal damage to plant cells (VanDoorn et al. 2015). To protect the integrity of the sieve elements, as well as their content, plants possess a diverse range of chemical and physical defence mechanisms (Will et al. 2013). Physical

defences primarily consist of callose deposition and protein plugging, which both result in occlusion from the phloem vessel (Will et al. 2013). Following insect feeding, a surge of Ca^{2+} ions in the sieve element stimulates the synthesis of callose, which forms a gel like substance that blocks the sieve plate. This blockage prevents tissue fluid from flowing through the sieve element and prevents insects from extracting phloem tissue fluid. Phloem proteins (P-proteins) are also used to block sieve elements. When the phloem is cut or chewed into, the release of pressure causes specific P-proteins to surge toward the sieve plate, where they become entangled and plug the sieve element. Protease inhibitors (PIs) can be found in the sieve tubes of tomato plants, and insect feeding has been shown to stimulate the synthesis of additional PI's in the sieve tubes (Gatehouse 2002). These proteins act as a feeding deterrent and are ingested by phloem feeding insects as they extract tissue fluid from the phloem. PI's then degrade essential proteins in the insect gut, which inhibits feeding and can impair growth and reproduction (Losvik et al. 2017; Gatehouse 2002).

To counter these plant defence mechanisms, phloem feeding insects possess a range of feeding mechanisms that facilitate tissue fluid extraction. Firstly, phloem feeders use gustatory cues to determine the value of a plant as a feeding and oviposition host, this also allows them to "taste" the presence of defensive metabolites which could interfere with feeding (Hollis 1991). This helps insects avoid unsuitable hosts, and plants which may already have their defences activated due to the presence of other herbivorous insects (REF). Whiteflies have been shown to avoid 'unhealthy plants' that have induced SA defences (Ueda et al. 2019), a ubiquitous symptom of pathogen infestation. If a plant is deemed a suitable host, phloem feeders can make further attempts to subvert plant defences. En route to the phloem, whiteflies and aphids inject a rapidly gelling saliva to form a sheath that protects the stylet from apoplastic defences. The sheath also provides a route along which the stylet can move, which is thought to further limit the amount of damage to plant cells (Walling et al. 2008). This stealthy approach to feeding is especially clear with whiteflies, which rarely damage cells en route to the phloem (Janssen et al. 1989). Even aphids do not show this level of deception and often rupture cells when accessing the phloem. The gelling saliva is also used to cement the sheath to the sieve element. Quickly sealing the lesion site prevents a loss of pressure in the sieve element, which can prevent the plant from recognising the insects' presence and initiating occlusion mechanisms (Will et al. 2013).

Upon reaching the phloem sieve elements, phloem feeders can then use their stylets to deliver salivary chemicals and/or proteins to the sieve elements that influence wound healing, defense signalling pathways, and volatile emissions (Walling et al. 2008). A recent study has found that whitefly possess a salivary protein called ferritin BtFer1, which supresses callose deposition in tomato. Herbivory from mutant whiteflies, which did not exhibit the BtFer1 gene, caused increased callose deposition in tomato, which in turn reduced whitefly survival on tomato (Su et al. 2019). Whiteflies release 'effector' proteins that interfere with plant defences by suppressing JA related defences and inducing SA related defences. SA related defences are usually induced in response to pathogen infestation, so the end products of SA defence signalling are less effective at defending the plant from whitefly attack. By supressing the effective JA-related defences and inducing SA-related defences, whiteflies can nullify plant defences and significantly increase individual performance (Zhang et al. 2019; Zarate et al. 2007). Whilst the mechanisms by which whiteflies achieve this are still not fully understood, recent studies have discovered salivary effectors released by *B. tabaci* can inhibit supress JA related defences (Xu et al. 2018; Wang et al. 2019). There are a few other examples of this, which suggests that whiteflies probably release a variety of salivary effectors, with various different functions.

1.7.3 Tomato defence against whiteflies

The cultivated tomato is susceptible to whitefly herbivory and growers are forced to deploy chemical and/or biological control measures to manage whitefly infestations. However, numerous wild tomato species have been shown to resist whiteflies much more effectively than cultivated tomato (McDaniel et al. 2016; Rakha et al. 2017; Bleeker et al. 2012). The inferior defences of cultivated tomato are as a result of years of selective breeding, in which growers have selected for growth related traits at the expense of plant defence. Whilst some efforts have been made to identify whitefly-resistant traits in wild tomato, there are still only a limited number of studies. Intrinsic heritable plant resistance is a key component of IPM (Stenberg 2017; Bruce et al. 2017; Peterson et al. 2018) and by restoring whitefly-resistant traits to commercial tomato varieties we could sustainably improve whitefly control. Despite this, cultivated tomato plants do exhibit a range of defensive measures that can serve to negate the detrimental impacts of whitefly herbivory. Defensive enzymes, trichomes and volatile organic compounds (VOCs) are some of the most important components of tomato

defence and have all been shown to negatively impact whitefly performance. Here we detail the key mechanisms by which cultivated tomato can resist whitefly attack, and how this compares with resistant wild tomato species.

One of the main ways tomato plants resist insect pests is with trichomes, hair-like protrusions from leaf surfaces that are responsible for both antixenotic (affecting host-choice) and antibiotic methods of defence. They provide defence against a range of insect herbivores and can be particularly effective at protecting tomato from whiteflies (Dias et al. 2016; Bleeker et al. 2009). There are several different types of trichomes and they can be categorized as either glandular or non-glandular based on their secretion ability (Huchelmann et al. 2017). Tomato exhibit three types of non-glandular trichomes (type II, III and V) and their primary mode of action is to impair insect mobility (Simmons and Gurr 2005). Glandular trichomes take on four different morphological forms (type I, IV, VI and VII) and called glandular trichomes due to the presence of a glandular 'head' which can secrete, store, and release secondary metabolites. Amongst these secondary metabolites are methyl ketones, acyl sugars, flavonoids and sesquiterpenes that have all been associated with pest resistance (Freitas et al. 2002; Huchelmann et al. 2017). Type I and IV glandular trichomes produce a variety of acyl sugars that can prevent whitefly oviposition and feeding (Dias et al. 2016; Simmons and Gurr 2005). Terpenoids such as zingiberene are released from type IV trichomes and have both toxic and repellent effects on whiteflies (Bleeker et al. 2009; Freitas et al. 2002). In particular, these type IV glandular trichomes have been positively correlated with increased whitefly resistance (Erb et al. 1994; Dias et al. 2016; Rodriguez-Lopez et al. 2011).

Tomato VOCs can contribute to decreased whitefly performance by altering whitefly preference (antixenosis) and survival (antibiosis) (Bleeker et al. 2009). Numerous tomato VOCs have been shown to be directly repellent to whiteflies (Conboy et al. 2019; Bleeker et al. 2011; Du et al. 2016) and decreased emissions of VOCs has been shown to make tomato plants more attractive to whiteflies (Sanchez-Hernandez et al. 2006). Further to this, tomato plants also release a suite of herbivore induced plant volatiles (HIPVs) in response to whitefly infestation (Lopez et al. 2012; Bleeker et al. 2009; Zhang et al. 2019). Many of these HIPVs have been associated with indirect defensive measures and serve as attractants to beneficial insects. For example, methyl salicylate (MeSA) is released by tomato in response to whitefly

infestation (Lopez et al. 2012) and MeSA has been shown to be extremely attractive to natural enemies of whiteflies such as *E. formosa* (James and Price 2004).

Tomato plants also use a variety of defensive enzymes to resist attack from insect pests. Peroxidases (PODs) and polyphenol oxidases (PPOs) are defensive enzymes that are produced constitutively by tomato but are also induced in response to whitefly herbivory (Mayer et al. 2002; McKenzie et al. 2002; Su et al. 2015; Zhang and Cui 2012). Phloem feeding insects cause the release of reactive oxygen species (ROS) (Thompson and Goggin 2006) and both PODs and PPOs are part of the plants' antioxidant defence system which serves to reduce the degree of oxidative damage caused from insect herbivory (Mittler et al. 2004). High levels of PODs have been associated with increased resistance to various insect pests, including whiteflies (Taggar et al. 2012; Zhang et al. 2008). PODs also contribute to lignification of plant cell walls that has been correlated with increased resistance to phloem-feeding insects in a variety of plant species (Liu et al. 2018). PPOs catalyse the oxidation of phenolics to form *o*-quinones which serve to alkylate dietary protein during insect feeding, and to degrade essential amino acids in insect guts (Constabel and Barbehenn 2008). PPOs are also found in leaf trichomes which, upon stimulation from herbivorous insects, leads to rapid PPO-mediated oxidation and polymerization of phenolics to produce a sticky polymer which can entrap insects and impair feeding (Constabel and Barbehenn 2008).

Whilst most cultivated tomato varieties are susceptible to whitefly infestations, various wild tomato species have been shown to be resistant to whiteflies (McDaniel et al. 2016; Rakha et al. 2017; Bleeker et al. 2012). Studies have shown wild tomato species to have higher densities of trichomes, which has been positively correlated with increased resistance to whiteflies (Bergau et al. 2015; Firdaus et al. 2012; Rakha et al. 2017). The wild tomato species *Solanum pennellii* and *Solanum pimpenillefolium* both have been credited with high levels of acyl sugars that makes them a less attractive to whiteflies compared with cultivated tomato (Rodriguez-Lopez et al. 2011). VOCs from wild tomato species are less attractive to whiteflies compared with that of cultivated tomato (McDaniel et al. 2016; Bleeker et al. 2009; Lucatti et al. 2010) and engineering a sesquiterpene synthase gene from wild to cultivated tomato increased resistance to whiteflies (Bleeker et al. 2012). Several research groups have crossed cultivated tomato species with these more resistant wild tomato species in order to restore some of these whitefly resistant traits to cultivated tomato (Bas et al. 1992; Erb et al. 1994).

Particularly with the advent of precise gene editing technologies, restoration of whitefly resistant traits lost through the process of domestication could increase whitefly resistance in cultivated tomato. Whilst promising, this work relies on the identification of specific whitefly resistant traits in wild tomato, for which there is currently a lack of available information.

1.7.4 Defence elicitors and plant priming

As well as direct attack from biotic stressors, various other physical and chemical stimuli have been shown to elicit plant defences. Early studies into plant defence induction showed mechanical damage to leaf tissue can induce plant defences both locally and systemically (Green and Ryan 1972). Mechanical damage causes this response in plants as it mimics the cellular disruption caused by insect herbivory (Leon et al. 2001). Plants also recognize compounds from insect oral secretions as a sign of herbivore presence. For example, if caterpillar regurgitant is applied to tomato leaves, the plant will induce a defence response commensurate with caterpillar infestation (Zhang et al. 2019; Gatehouse 2002; Rowen et al. 2017). Phytohormones such as salicylic acid (SA) can also induce plant defences when applied to leaves exogenously (Loake and Grant 2007). This is thought to emulate how tissue fluid components (such as SA) are deposited onto plant leaves by phloem feeding insects (VanDoorn et al. 2015). Ultimately, plants use these signals as a sign of 'damaged-self recognition' and is one of the main ways plants recognize and respond to specific stressors (Heil 2009).

Another way in which plants respond to biotic stress is by perceiving the stress related signals released by neighbouring plants. HIPVs are the main method by which plants perceive the threat of impending herbivorous attack but rather than inducing defences, they are more often primed (Heil and Ton 2008). Defence priming is an adaptive, low-cost defensive measure because defence responses are only transiently activated by a priming stimulus (Martinez-Medina et al. 2016). Primed plants will then produce a more rapid and effective defence response upon subsequent challenge from the triggering stress (i.e. herbivorous insects). Defence induction is an energetically costly process for plants and it is thought that priming evolved as a means by which plants can account for 'false alarms', which are expected to be inevitable in a natural environment with a multitude of airborne VOCs.

Priming is a much more attractive method of defence induction to growers as there is little/no energy cost incurred during this primed state, meaning yield will not be impacted if the pest never successfully infests the crop (Balmer et al. 2015; Martinez-Medina et al. 2016). Further to this, priming agents do not have direct toxic effects on the target organism, are compatible with IPM and can even enhance a plants attractiveness to natural enemies (Bruce et al. 2017; Bruce 2010). Particularly when considering the protection of glasshouse-grown tomatoes, where IPM is often utilised alongside natural enemy release, incorporating plant priming to these systems could be both seamless and effective (Bruce et al. 2017). Research into the discovery and application of priming agents is very popular and VOCs have been successfully found to prime plants as either mixtures (Hu et al. 2018; Farag and Pare 2002) or individual compounds (Song and Ryu 2018; Erb et al. 2015). Specific priming agents will induce a defence response that is specific to an individual biotic stress (Zhang et al. 2019; Timilsena et al. 2019), which can make selecting defence-inducing compounds a complex process. Plants induce defences in response to specific airborne distress signals released by their neighbours, so research groups often select defence-inducing compounds from the suite of HIPVs the plant releases in response to infestation/infection from the particular biotic stress (Erb et al. 2015). In chapter 3 of this thesis, we conduct an analysis of HIPVs from whitefly-infested tomato plants in an attempt to identify defence-inducing compounds. We found that MeSA was the most prominent compound in tomato HIPV emissions and subsequent application of MeSA to un-infested tomato plants induced defensive enzyme activity.

1.8 Aims and Thesis format

The main aim of this thesis is to explore the use of VOCs to protect glasshouse-grown tomato from glasshouse whiteflies. We investigate several commercially applicable VOC-based whitefly management strategies, which all result in negative effects on whitefly performance in large-scale glasshouse trials. In a series of companion planting experiments, we found that intercropping tomato with marigold and other whitefly non-hosts reduced whitefly performance on tomato. Subsequent analysis of marigold VOCs revealed that limonene is a primary component of marigold VOC emissions, and in laboratory bioassays we confirm its repellent effect on whiteflies. Following this finding, we developed limonene slow-release

dispensers that were found to successfully repel whiteflies from a tomato crop, but were limited in their capacity to control a pre-existing whitefly infestation. We attempted to synergise repellent limonene dispensers and plant defence activation, although these two methods produced similar effects on whitefly performance as standalone limonene dispensers (i.e. they failed to synergise). We also endeavour to broaden understanding of host plant resistance in a wild (*S. pimpenillefolium*) and commercial tomato species (*S. lycopersium* var. 'Elegance'), with a focus on how these two species interface with their volatile environment. We find that *S. pimpenillefolium* and 'Elegance' release a different composition of constitutive VOCs and whitefly-induced HIPVs, which make *S. pimpenillefolium* odours repellent to whiteflies and attractive to *E. formosa*. We also find that *S. pimpenillefolium* primes a whitefly resistance gene (Mi-1.2) after exposure to HIPVs from whitefly-infested conspecifics, whereas 'Elegance' immediately induces defences after exposure to HIPVs. Other biochemical resistance mechanisms such as increased POD activity in *S. pimpenillefolium* could also contribute to increased resistance to whiteflies. An overview of each chapter, along with relevant author contributions is listed below.

Chapter 1: In this general introduction, we detail the key themes that underpin the research goals of this thesis. Firstly, information on the origins, domestication and current status of tomato as a global agricultural crop is discussed. Following this, the biology of glasshouse whiteflies is detailed, along with information on current whitefly management methods. We explain the potential for VOCs to be used in sustainable agricultural practices, and detail the numerous examples of how VOCs have been used to control agricultural pests. We explain the importance of IPM, and how VOCs could be used to support current whitefly-based IPM systems. Current understanding on the mechanisms of plant defence against insect herbivores, with a focus on how tomato plants can defend against whiteflies, is also discussed.

Chapter 2: Companion planting with French marigolds protects tomato from glasshouse whiteflies through the emission of airborne limonene.

This work was published in the peer-reviewed journal PLOS One on the 1st of March 2019. In this study we investigated the effect of intercropping tomato with French marigold and other whitefly non-host plants to control glasshouse whiteflies on glasshouse-grown tomato. Both

low diversity (French marigold) and high diversity (basil, nasturtium and Chinese cabbage) intercropping treatments significantly reduced whitefly performance on tomato. After finding that French marigold intercropping significantly reduced whitefly performance on tomato, we confirm the mode of action as repellent volatile chemistry. This was confirmed in laboratory experiments where we show that limonene, a major component of marigold VOC emissions, has a repellent effect on glasshouse whiteflies. In a second glasshouse trial, we assess the ability of French marigold and limonene slow-release dispensers to be used to control a heavy whitefly infestation. However, this was not as effective as when used from the beginning of the growing period. This study was devised by Niall J.A. Conboy, Colin Tosh and Thomas McDaniel with field sampling assistance from Rhiannon Curtis. VOC analysis was conducted by Niall J.A. Conboy and Paul Donohoe. Whitefly free-choice assays were conducted by Niall J.A. Conboy and whitefly olfaction experiments were conducted by Ellie Wharton. Colin Tosh, Thomas McDaniel and Niall J.A. Conboy wrote the initial draft that comprised work from the two glasshouse trials and leaf disc assays. Niall J.A Conboy wrote the final draft that added the VOC analysis, free-choice assays and whitefly olfaction experiments. All other authors contributed to the work by providing technical advice and editing the final draft.

Chapter 3: Volatile Organic Compounds as Insect Repellents and Plant Elicitors: An Integrated Pest Management (IPM) Strategy for Glasshouse Whitefly *(Trialeurodes vaporariorum).*

This work was submitted to the peer-reviewed Journal of Chemical Ecology on the 18th of December 2019. Combining multiple pest management components, which synergize for maximum efficacy, is one of the core principles of IPM. Here we attempt to synergize repellent volatile chemistry and plant defence elicitation to control whiteflies on glasshouse-grown tomato. Limonene, identified in marigold VOC emissions (Chapter 2), was used as the whitefly repellent component and MeSA was used as the plant defence elicitor after detection and quantification from tomato HIPVs. Whilst combination of these two methods did not produce any synergistic effects, this combined treatment did reduce whitefly performance on tomato. Importantly, the standalone limonene treatment was more effective than the combined treatment and significantly increased tomato fruit yield (g) by 33%. The standalone MeSA treatment also reduced whitefly performance on tomato and in subsequent laboratory experiments we characterized MeSA plant defence induction with defensive enzyme assays and assessment of plant defence related genes. We found that our application

of MeSA immediately induced defences, rather than priming them. This works illustrates the effectiveness of VOCs as a whitefly control method and could be used to supplement current whitefly-focused IPM systems. Our repellent limonene dispensers were extremely effective at deterring whiteflies and offer a low cost and easy to implement whitefly control option. This study was devised, planned and carried out by Niall J.A. Conboy with field sampling assistance from Colin Tosh and Thomas McDaniel. Technical assistance with GC-MS and qPCR assays was provided by Paul Donohoe and Martin Edwards respectively. Niall J.A. Conboy wrote the manuscript. All other authors contributed to the work by providing technical advice and editing the final draft.

Chapter 4: How a Cultivated and Wild Tomato Species and Interface with their Volatile Environment.

In this study we compared the way a wild and commercial tomato species respond to herbivory from glasshouse whiteflies, with a focus on how both species interface with their volatile environment. We find that *S. pimpenillefolium* and 'Elegance' release a different composition of constitutive VOCs and whitefly-induced HIPVs which make *S. pimpenillefolium* odours repellent to whiteflies and attractive to *E. formosa*. Both species were exposed to HIPVs from whitefly-infested conspecifics and defensive enzyme activity, defence related gene expression and phytohormone abundance is used to characterize plant defences. We find that *S. pimpenillefolium* primes a whitefly resistance gene (Mi-1.2) after exposure to HIPVs from whitefly infested conspecifics, whereas 'Elegance' immediately induces defences after exposure to HIPVs. Host plant resistance is a key component of IPM and this work provides fundamental insights into how tomato plants can resist whitefly infestation. This study was devised, planned and carried out by Niall J.A. Conboy with technical assistance from Colin Tosh, Paul Donohoe and Alex Charlton. Niall J.A. Conboy wrote the manuscript. All other authors contributed to the work by providing technical advice and editing the final draft.

Chapter 5: In this final chapter, the results of this research thesis are discussed within the wider context of VOCs as a potential pest management method for glasshouse whiteflies.

Chapter 2. Companion planting with French marigolds protects tomato from glasshouse whiteflies through the emission of airborne limonene.

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Abstract

Horticulturalists and gardeners in temperate regions often claim that planting marigolds next to tomato plants protects the tomatoes from the glasshouse whitefly (*Trialeurodes vaporariorum* Westwood). If shown to hold true, this technique could be used in larger-scale tomato production, protecting the crop and helping to introduce greater plant diversity into these agro-ecosystems. Here we present two large-scale glasshouse trials corresponding to the two main ways growers are likely to use marigolds to control whiteflies. In the first, marigolds are grown next to tomato throughout the growing period and we quantify whitefly population growth from the seedling stage over a 48-day infestation period. Here we show that association with marigolds significantly slows whitefly population development. Introducing additional whitefly-attractive 'pull' plants around the perimeter of plots has little effect, but reducing the proportion of marigolds and introducing other non-hosts of whiteflies (basil, nasturtium and Chinese cabbage) also reduces whitefly populations on tomato. The second experiment assesses the efficacy of marigolds when used as an 'emergency' measure. Here we allow whitefly populations to build to a high density on unprotected tomatoes then introduce marigolds and assess whitefly population over a further period. Following laboratory work showing limonene to be a major chemical component of French marigolds and a negative behaviour response of whiteflies to this compound, limonene dispensers are added as an additional treatment to this experiment. "Emergency" marigold companion

planting yielded minimal reductions in whitefly performance, but the use of limonene dispensers was more effective. Our work indicates that companion planting short vine tomatoes with French marigolds throughout the growing season will slow development of whitefly populations. Introducing marigolds to unprotected tomatoes after significant whitefly build-up will be less effective. The use of limonene dispensers placed near to tomato plants also shows promise. It is argued that this work supports the possibility of the development of a mixture of tomato companion plants that results in 'associational resistance' against many major invertebrate pests of tomato. Such a mixture, if comprising edible or ornamental plants, would reduce the need for additional chemical and biological control, and, if used outdoors, would generate plant-diverse agro-ecosystems that are better able to harbour invertebrate wildlife.

2.1 Introduction

Tomatoes (*Solanum lycopersicum* L.) are the second most important edible horticultural crop by production in developed nations (Rubatzky and Yamaguchi 2012). Under glass, pest infestations are dominated by the common glasshouse whitefly (*Trialeurodes vaporariorum*) (Lange and Bronson 1981) which is known to cause severe yield losses to an array of crops through transmission of a number of plant viruses (Nasruddin 2016; Wainaina et al. 2018). Direct feeding from both adults and larvae results in honeydew secretion which reduces the photosynthetic capacity of the plant and renders fruit unmarketable (Jauset et al. 2000). Biocontrol is widely used to manage *T. vaporariorum* infestations but limitations such as delayed efficacy (Bale et al. 2008) and hyperparasitism (Schooler et al. 2011) can lead to failure. Where biocontrol fails, there is still reliance on chemical control for tomato production and excessive use of chemical sprays has resulted in resistant *T. vaporariorum* genotypes, phytotoxicity and pesticide residue problems (Rezaei et al. 2015; Gorman et al. 2000; Gorman et al. 2007). As with most contemporary cropping systems, tomatoes are typically produced in monoculture, thus rendering crop areas of limited value to wildlife and largely devoid of "ecosystem services" (Malezieux 2012). Any alternative methods of whitefly control that can reduce pesticide use and introduce greater animal and plant diversity into agricultural and horticultural systems should therefore be welcomed. The insistence of temperate region gardeners that planting marigolds next to tomatoes protects the tomato

crop from whiteflies therefore merits further investigation. Companion planting is a well-researched (and implemented) pest control strategy and is believed to be founded on 'associational resistance' (Barbosa et al. 2009; Malézieux et al. 2009). Both French (*Tagetes patula* L.) and English marigold (*Calendula officinalis*) have been used as effective companion plants in a number of other pest/crop scenarios. They have been shown to reduce pest populations either directly through repellent volatile chemistry (Ben Issa et al. 2016) or indirectly through promoting beneficial arthropod populations (Jankowska 2009; Sujayanand et al. 2016; Zhao et al. 2017; Balzan 2017). Despite this, no research appears to be available that quantifies the potential for marigolds to control whiteflies on tomatoes.

Here, large-scale glasshouse trials in the United Kingdom are described investigating the potential of intercropping tomato plants with marigold and other plant species to repel the glasshouse whitefly, *T. vaporariorum.* One set of experiments investigated the effect of intercropping with other plant species for the duration of the tomato growth period. The following year, another experiment investigated the effect of introducing intercropped treatments into a tomato crop grown alone for the majority of the growing season, when a high density whitefly population had developed. This represents an "emergency" situation which horticulturists may experience if no previous control methods have been in place. These studies aimed to investigate: 1) Whether propagation of French marigolds (*Tagetes patula* L.) amongst tomato plants from the start of the growing period protects tomatoes from whitefly infestation by 'pushing' them from the tomato crop; 2) If supplementing marigolds over the whole growth period with other non-host species less preferred by whitefly, to 'push' whiteflies from tomatoes, increases the marigold protective effect; 3) Whether this protection may be enhanced by positioning preferred 'pull' whitefly host plants around the edge of the 'push' non-hosts (Cook et al. 2007), 4) Using laboratory studies, what is the main chemical component of marigold airborne volatiles and do they have behavioural activity against whiteflies? 5) Whether the introduction of marigolds and limonene (identified from 4 above) dispensers can protect tomatoes in an "emergency" situation where there is a heavy whitefly infestation on the tomato crop. The investigation into whether increased diversity achieves greater levels of whitefly control was included because, from an ecosystem health perspective, plant diversity is desirable, but also because plant diversity is known to be a fundamental determinant of invertebrate abundance (Knops et al. 1999; Landis et al. 2000). Plant diversity in agro-ecosystems is also important for natural enemies and marigolds have been found to increase populations of beneficial arthropods (Balzan 2017; Zhao et al. 2017).

In contrast, the current work was conducted in a closed glasshouse with no biocontrol present and little opportunity for natural enemies to enter. No natural enemies or parasitized pupae were observed in either glasshouse experiment and whilst they may have still been present in very low numbers, their effect on plant-whitefly interactions in both trials can be assumed to be minimal. Therefore, any effects on whitefly performance were most likely due to direct effects between the introduced non-host plants and whitefly. Non-host plants can act as a repellent to pests (Ben Issa et al. 2016), make host plants harder to find (disruptive crop hypothesis) (Finch and Collier 2012) and presence of multiple non-hosts have been found to induce 'restlessness' or 'confused' behaviour in the whitefly species *Bemisia tabaci* (Bernays 1999). The current work aimed to investigate if these effects from non-host companion planting translate to lower rates of whitefly population development on tomato.

2.2 Materials and Methods

2.2.1 Laboratory experiments

2.2.2 Whitefly

Whiteflies, *T. vaporariorum* (Westwood), originated from a lab culture at Rothamsted Research which was first collected in 1960 in Kent on French bean and had subsequently been maintained in a large laboratory population. The insects for both glasshouse and laboratory experiments were taken from a mixed age colony from a culture at Newcastle University (UK) where they were maintained on pre flowering aubergine (*Solanum melongena* "Moneymaker"- Marshalls Seeds Cat. 1020-2017) at 20 °C, 16:8 light/dark.

2.2.3 Plants

For all laboratory experiments (Air-entrainment, free-choice and root exudates assays), tomato seeds (*S. lycopersicum* Mill., 'Elegance' Cat. E/12/11, Batch 0113479253) were obtained from Monsanto and used for all experiments. In the laboratory assays, plants were grown from seed in J. Arthur Bowers John Innes no 2 in 9-cm-diameter and 8.7-cm-deep pots. All plants were grown at a distance of approximately 60 cm from a 400-W Son-T bulb housed in a Harrier HR400SH 400-W lamp (6000 lumens) under a 16 h light/8 h dark cycle and a temperature regime of 25 °C in the light and 20 °C during the dark period. Tomato and

marigold seedlings were grown in separate propagation units to prevent the transfer of volatile organic compounds between the two species. For air-entrainment, whitefly free-choice and root exudates assays, both tomato and marigold plants were at stage 14 on the Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (BBCH) scale (Klingauf 2001).

2.2.4 Air entrainment volatile analysis

Headspace volatiles of marigold plants were analysed by dynamic air entrainment (Pye volatile collection kit, Kings Walden, Herts, UK) in order to deduce compounds potentially repellent to *T. vaporariorum*. All equipment was washed with Teepol detergent (Sigma-Aldrich) and rinsed with acetone and distilled water twice before baking at 180°C for 2 hours. Porapak Q (60/80 mesh, 0.05 g) tubes were eluted with diethyl ether (Fischer Scientific, 12347103) and heated at 140°C for 2 hours under a stream of constant nitrogen to remove contaminants, this process was repeated twice for each tube. Either a single flower or leaflet of 3 week old tomato seedlings was partially enclosed in a glass bell cylinder, fresh weight (g) of the entrained section was recorded after use. The bottom of the cylinder was closed without pressure around the plant stem by using two semicircular aluminum plates with a hole in the centre to accommodate the stem. Charcoal filtered air was pumped in at 1L min^{-1} and drawn out at 800 ml min^{-1} through the porapak Q adsorbent tube in a 5-mm diameter glass tube. The difference in flow rates created a slight positive pressure to ensure that unfiltered air did not enter the system, thus removing the need for an airtight seal around the stem. Plants were entrained for 24 hours from midday onwards. The porapak Q filter tube was eluted with 0.75 ml of diethyl ether (Fisher Scientific, 12347103), providing a 500µl solution that contained the isolated volatile compounds. All samples were tightly sealed in GC vials and stored at -20°C until needed.

2.2.5 Gas Chromatography (GC)

GC-FID analyses were carried out using an Agilent 5890 GC. The injection port (280°C) was in the splitless mode and the flame ionization detector was heated to 300°C. The sample (1ul) in diethyl ether was injected by an HP7673 auto sampler and the split opened after 1 minute. Separation was performed on a fused silica capillary column (30m x 0.25mm i.d) coated with

0.25um dimethyl poly-siloxane (HP-5 phase). The GC was temperature programmed from 50°C-310°C at 5°C min and held at final temperature for 20 minutes with Hydrogen as the carrier gas (flow 1ml/min, pressure of 50kPa, split at 30 mls/min).

2.2.6 Gas Chromatography-mass spectrometry (GC-MS)

GC-MS analysis of the biological extracts was performed on a Agilent 7890A GC split/split less injector (280°C) linked to a Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1200V, interface temperature 310°C). The acquisition was controlled by a HP Compaq computer using Chemstation software, initially in full scan mode (50-600 amu/sec) or in selected ion mode (30ions 0.7cps 35ms dwell) for greater sensitivity. The sample (1ul) in diethyl ether was injected by an Agilent7683B auto sampler and the split opened after 1 minute. Separation was performed on an Agilent fused silica capillary column (30m x 0.25mm i.d) coated with 0.25um dimethyl polysiloxane (HP-5) phase. The GC was temperature programmed from 50-310°C at 5°C min and held at final temperature for 10 minutes with Helium as the carrier gas (flow rate of 1ml/min, initial pressure of 50kPa, split at 30 mls/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST05 library if > 90% fit or from their elution order from biochemical literature

2.2.7 Limonene dispensers

The use of limonene as a treatment to control whitefly infestation required the development of a slow-release dispenser that was comparable in its rate of emission to the quantity of limonene released by a marigold plant. To achieve this, the amount of limonene released over 24h from a single marigold plant of weight 26.37g (a typical size of a marigold grown in the growth rooms at Newcastle University) was quantified as 363.26 µg (using air entrainment/GC-FID). For the lab based whitefly preference assays (Figure 2.2), a single 30ml medicine vial (obtained from https://bit.ly/2q9Dzwh) with a 2mm hole drilled in the screw top lid was used. This was limonene dispenser “type 1”. Three millilitres of pure limonene (Sigma-Aldrich, 183164) was placed inside this vial and was found to release 652.86 µg over 24 hours after headspace analysis followed by GC-MS conducted in the same way as described for the

marigold seedlings. For the “heavy infestation” experiment (Figure 2.3), the comparable marigold plants in the glasshouse at Stockbridge Technology Centre were much larger (151.6g on average, equating to 2. 08mg of limonene per plant) than those found in the laboratory at Newcastle University and therefore limonene output needed to be increased accordingly. These same medicine vials with 3ml limonene were used, only the lid was replaced with a rectangular piece of muslin cloth secured with two with elastic bands, this was limonene dispenser “type 2”. Two limonene dispensers were found to release 2.96mg of limonene over 24 hours. Limonene in the bottles was replaced every two weeks, which was shown to give a constant level of limonene output. Exactly matching emission rates to that of respective marigold plants proved difficult with the slow-release dispenser system in place here. Nevertheless, comparative limonene emission rates were achieved and future studies could invest more time developing volatile slow-release systems that exactly match volatile emissions from other repellent non-host companion plants.

2.2.8 Limonene and root exudates free-choice assays

To confirm the repellent effect of marigold and limonene to adult *T. vaporariorum,* a series of laboratory bioassays were conducted. For each experiment 8 “Elegance” tomato seedlings were placed inside a 90x60x60cm mesh cage (Watkins and Doncaster, product code: E6098). These 8 plants were separated into two groups of four and placed at opposite ends of the cage, 50cm apart. A schematic overview of this setup can be viewed in Appendix A. Two-hundred whiteflies of mixed age and sex were released into the centre of the cage and total eggs and settling adults on every plant was recorded after exactly 24 hours. For the repellence assay, two marigold seedlings were placed in the two corners of the cage behind one of the two groups of tomato seedlings. Whitefly preference was assessed based on their location in the cage, i.e. at the side accompanied by marigold seedlings or not. The same procedure was conducted for “type 1” limonene dispensers and each experiment was repeated a total of 6 times.

To assess the effect of root exudate sharing between tomato and marigold, similar whitefly free-choice assays were conducted where both species were placed in communal drip trays (52x42cm) prior to assessment of whitefly settling and oviposition preference. Four tomato seedlings were placed in small plastic drip tray filled with water to make the pots roughly 2cm submerged, topping up when necessary. Two flowering marigold seedlings were introduced

to these drip trays for one or seven days, and for control replicates 2 tomato seedlings were added. Whilst plants in the glasshouse study shared root exudates for longer, difficulties in replicating this with the laboratory growth space available limited us to these time frames. Despite this, it was assumed that any morphological and/or physiological changes resulting from root exudate sharing would be apparent after the time frames implemented here. These plants remained in the growth rooms under the Harrier HR400SH 400W lamp described previously. Whitefly preference was then assessed against four other tomato seedlings (taken straight from the growth rooms described previously) and set up in the same 90x60x60 cm cage as described previously. For both experiments the Pearsons chi-squared test was used to compare settling distribution and expected values for the marigold and limonene assays were assumed as equal (50/50). For the root exudates assays, settling distribution was compared to the average across the 6 control replicates described above.

2.2.9 Glasshouse experiment 1: "Push", "Push-Pull" and "High Diversity" control strategies applied at the beginning of the infestation period

In order to establish a baseline measure of whitefly preference for different plant species, a laboratory assay was conducted using plant leaf discs in a no choice assay that quantified *T. vaporariorum* preference for each species individually. Whitefly preference was quantified by assessing settling on each leaf disc after a 21-hour period. Full details of this experiment can be viewed in Appendix B, with materials and methods for this experiment available in Appendix K. On the basis of the leaf disc results nasturtium, Chinese cabbage, basil, and marigold were designated as 'push' plants and pumpkin, melon, courgette, and sunflower as 'pull' plants in the glasshouse trial that followed. Appendix B displays the full results of the leaf disc assays with comparisons to similar experiments on *T. vaporariorum* preference from published literature.

Experiments were conducted in the mid-late growing season (started 10th August) 2016 in a 448m^3 glasshouse in the grounds of Stockbridge Technology Centre Ltd., Yorkshire, England (Grid Ref. SE 55988 36575). Experiments were supplemented with a population of *T. vaporariorum* consisting of 3 heavily infested aubergine (*Solanum melongena* "Moneymaker") plants from the culture described above, distributed in the centre of the greenhouse. The glasshouse represented a closed environment which limited the ability of natural pests to infest our experimental crop. However, the glasshouse was not screened and we expect that

some whiteflies from the surrounding environment did infest our experimental crop, but that the vast majority originated from the introduced cultures described. We did observe low numbers of Thrips and Spider mites (which entered the glasshouse naturally) in both glasshouse trials. Plants were grown from seed, one per compartment, in standard seed germination trays in glasshouses at Stockbridge Technology Centre (UK) for 5 weeks. At the point of replanting for both "push" and "push-pull" experiments, plants were at stage 13 on the BBCH scale and had the following number of fully expanded leaves: tomatoes 3-6 leaves, marigold 4-6 leaves, basil 3-4 leaves, nasturtium 4-5 leaves, cabbage 4-6 leaves, sunflower 3-5 leaves and courgette, pumpkin, and melon 2-4 leaves. Plants were replanted into 5 litre pots and placed into 110 x 55 x 4cm drip trays, 8 pots to a tray. Plants were replanted in Clover Multipurpose Compost (Dungannon, Co. Tyrone,N, Ireland BT71 4QR).

Two experiments were undertaken to investigate whether intercropping from the start of the growth period could assert control over whitefly populations, Appendix Figure C contains a schematic overview of these experiments. The first experiment sought to answer whether intercropping with marigolds could reduce whitefly numbers on tomato by 'pushing' whiteflies from tomato, and if so, whether this effect could be enhanced by the use of additional non-host, 'push' plants. The second experiment sought to identify whether the 'push' effect could be combined with the 'pull' effect of attractive host plants placed around the edge of the treatment, to attract whiteflies from tomatoes, and whether this effect could be further intensified by using a higher diversity of both 'push' and 'pull' plant species.

Single, fully-expanded leaves were selected by randomising both compound leaf selection and then individual leaflet from 8 tomato plants per replicate. These were then examined *in situ* for *T. vaporariorum* adults and adults of other insect pests. The sampling regime can be viewed in Figure 2.1 and covered a period of 48 days. These leaves were removed and placed in sealed plastic bags, then stored overnight at 4°C and examined the next day for whitefly (and other pest) larvae and eggs. The abundance of all pest insects present was recorded to test the effect of intercropping on other pest species as well as the target pest *T. vaporariorum*. *Thrips tabaci* were the only other pest to be consistently observed in the glasshouse, albeit at very low numbers. Other insects were encountered in very low abundances so were not included in analysis. Thrips population development in both experiments is presented as average number of pests per leaf and can be viewed in Appendix Figure D. Procedures were identical for the 'push-pull' experiment, the outer ring of pull plants was not sampled. At the end of the experimental period, each focal tomato plant was

destructively sampled and total fruit and total aboveground tissue weight measured (Appendix Figure E).

Data for insect abundance was non-normally distributed and was therefore (log + 1) transformed to meet normality and homogeneity of variance assumptions for statistical analysis. Whitefly and thrips abundance (adult, larvae and egg numbers were added together to give a single value for each insect) were analysed with repeated measures ANOVA's using the "lmerTest" package in R. Time (sampling date) was used as the repeated measure with treatment as the fixed factor. The Bonferroni post-hoc correction was used to analyse differences between treatments at individual sampling points, this was done by multiplying *p* values by the total number of comparisons made. Therefore, all *p* values reported are significant at $\alpha = 0.05$. Tomato plant and fruit weights were also non-normally distributed but were analysed with Mann Whitney U tests. The non-parametric effect size measure, Cliff's delta (*d*) was determined using the 'effsize' package for R in order to allow a standardised comparison of effects that takes into account different start times of experiments (Torchiano 2016). The Cliff's delta *(d)* measure was ascertained from the un-transformed non-parametric data.

2.2.10 Glasshouse Experiment 2: "Emergency" Intercropping to protect tomatoes from an established heavy whitefly infestation

These experiments were run in the same greenhouse at Stockbridge Technology centre from 13th July 2017 – 14th August 2017. The aim of the experiment was to build up a high density whitefly population on a tomato crop, then introduce either more tomatoes (control), French marigolds (*T. patula*; marigold treatment), or limonene dispensers (limonene treatment), and observe the level of whitefly control achieved. Marigolds and tomatoes for this experiment were planted as seeds in standard seed germination trays in a glasshouse at Stockbridge Technology Centre on 25th April 2017. On 6th June 2017, when the plants had reached stage 13 on the BBCH scale, plants were re-potted in the experimental glasshouse into 5L pots containing 5L of Clover multipurpose compost (details above). These plants were then divided into two groups: 192 tomato plants (hereafter referred to as the focal tomato plants) were placed in the experimental glasshouse in 110 x 55 x 4cm drip trays as described above, and were subdivided into three treatment blocks, replicated eight times. A schematic overview of both experiments in the glasshouse can be viewed in Appendix Figure C. These plants had

four heavily infested aubergine plants from the laboratory at Newcastle University placed amongst them to supplement naturally occurring whitefly pest populations (Appendix Figure C); the aim was to achieve a heavy whitefly infestation across the whole glasshouse. The second group of plants (comprising 64 tomato plants and 64 marigold plants) were placed in an adjacent greenhouse, of the same dimensions as the experimental greenhouse, and covered in porous white gauze. The aim was to keep these plants uninfested so as to not affect the whitefly population once they were introduced.

Both groups of plants were grown in their respective greenhouses for 13 days until 26th July 2017, at which point a high density whitefly population had been achieved (this density may be seen in the first sampling point of Figure 2.3, A). On this date, the uninfested plants from the second greenhouse were introduced into the infested greenhouse, and arranged amongst their respective treatments as follows. The experiment was arranged as for the 'Push' experiment outlined (see Appendix Figure C) above, with eight replicates of three treatments: a control, a marigold treatment and a limonene treatment (which was used in place of the HD treatment, see Appendix Figure C). The control, as before, comprised eight, whitefly-infested focal tomato plants, with eight uninfested tomato plants randomly distributed amongst them. The marigold treatment comprised eight focal tomato plants with eight marigold plants randomly distributed amongst them (identical to the LD 'push- treatment, see Appendix Figure C). The limonene treatment included eight tomato plants with 16 "type 2" limonene dispensers (described above) bottles randomly distributed amongst them, with limonene bottles placed two per 5L pot filled with 5L of soil.

After the introduction of the treatment plants, the experiment was continued for a further 29 days, with sampling being undertaken in the same way as for the 'push-pull' assay. The number of adult insects, including whitefly adults, which had settled on a single leaf from each of the focal tomato plants were counted each week, as well as the number of unripe tomatoes as they emerged. As before, adult insects were assessed on the day, and eggs and nymphs counted the next day under low power microscopy. Plants were sampled weekly over 29 days, after which the experiment was ended due to declining plant health in the greenhouse, possibly due to the heavy whitefly infestation. At the culmination of the experiment, after the insect assessment, each focal plant was destructively sampled and total fruit and total aboveground tissue weight measured. Very few tomatoes were ripe on each treatment, so tomato weights represent the weight of the green tomatoes on the plants at the time of harvesting. Abundance of all whitefly life stages was (log +1) transformed and

analysed with repeated measures ANOVA's with Bonferroni post-hoc correction in the instance of a significant interaction (as above in glasshouse trial 1). The final plant and fruit weight were compared using t-tests on untransformed data.

2.3 Results and Discussion

The first large scale glasshouse study sought to establish a scientific basis for the propagation of French marigolds amongst tomato plants from the start of the growing season to protect tomatoes from whitefly infestation. This work aimed to identify whether this practice achieved significant control of the important glasshouse pest *T. vaporariorum*, and whether this control could be enhanced by other aversive, non-host plants. A second treatment also sought to quantify whether this 'push' effect could be combined with the 'pull' of attractive host plants to increase whitefly control. French marigold was found to be an effective companion plant and subsequent headspace analysis followed by laboratory bioassays confirmed the volatile limonene to be the probable mechanistic basis of the repellent properties this plant possesses. In a second glasshouse trial, the potential for marigold plants and limonene dispensers to control a heavy infestation of whiteflies on tomato was assessed.

2.3.1 Glasshouse trial 1: "Push", "Push-Pull" and "High Diversity" control strategies applied at the beginning of the infestation period

Figure 2.1 shows levels of whitefly infestation on the tomato crop through time. In the 'push' experiment (Figure 2.1, A) whitefly numbers on the control (tomato plants only), low diversity (LD, tomato intercropped with marigold) and high diversity (HD, tomato intercropped with marigold and other *T. vaporariorum* non-hosts) treatments began to diverge after 34 days. There was a significant effect of treatment (Control, LD and HD)(repeated measures (rm) ANOVA $F_{(2,126)} = 18.85$, $p =< 0.001$) and treatment *x* time (each sampling point, n=6) (rm ANOVA $F_{(10,126)} = 2.14$, $p = 0.025$) in the "push" experiment (Figure 2.1, A). There was no difference in whitefly abundance between the treatments until day 34 where there were significantly fewer whiteflies on tomatoes in LD ($t = 2.89$, $df = 126$, $p = 0.013$) and HD ($t = 3.61$, $df = 126$, $p = 0.001$) plots. This trend continued until the final sampling point at day 48. Statistical comparisons were made between whitefly population size in the HD and LD

treatments but there were no significant differences observed. This indicates that increasing the diversity of non-hosts does not improve the repellent effect (conversely, and importantly, it does not reduce the potency of the marigold-only effect). In the 'push-pull' experiment (Figure 2.1, B), was a significant effect of treatment (rm ANOVA $F_{(2,105)} = 3.73$, $p = 0.027$) but no significant interaction between treatment *x* time (rm ANOVA $F_{(8,105)} = 1.49$, $p = 0.166$). Average whitefly numbers on tomato were fewer on LD and HD treatments relative to the control after around 20 days and there were significantly fewer whiteflies in the HD treatment at day 43 ($t = 3.018$, $df = 105$, $p = 0.009$). It is important to note that relatively low levels of whitefly numbers were present in the greenhouse. Figure 2.1, C shows that while the 'push-pull' experiment reached its maximum effect sooner than the 'push' experiment, it did not produce a greater maximum effect. Additionally, there was no significant difference in the effect of LD and HD treatments within experiments. It is, therefore, doubtful that growers could be persuaded to make the extra effort to propagate 'pull' plants on the basis of these results. Figure 2.1, C should be viewed with caution as experiments were terminated at the end of the commercial growing season and effects may have diverged further (or disappeared) if planting had begun earlier.

While some studies have shown a positive relationship between plant species richness and insect abundance, this is thought to be related to plant productivity (Knops et al. 1999). The artificial way in which glasshouse plants are propagated, i.e. with physically separated roots, as well as the short term nature of the experiments, may preclude such effects. In contrast, it was hypothesised that increased plant diversity (the HD treatment) would depress whitefly population growth on tomato, because a previous study showed increased plant diversity causes whiteflies to become 'restless' and move around diversified cultures more than simple, single species ones (Bernays 1999), presumably reducing time available for reproductive output. However, this hypothesis was not upheld, and increased plant diversity appeared relatively neutral with regard to whitefly performance on tomato in the current work. While these effects produced by marigold and other non-hosts are exciting, they are of even more relevance if the planting regime does not attract other pests to tomato. The only other pest to infest the experiment in significant numbers was the onion thrips (*Thrips tabaci*) there were no significant differences in larvae and adult abundance between treatments in either the "push" or "push-pull" experiment (Appendix D).

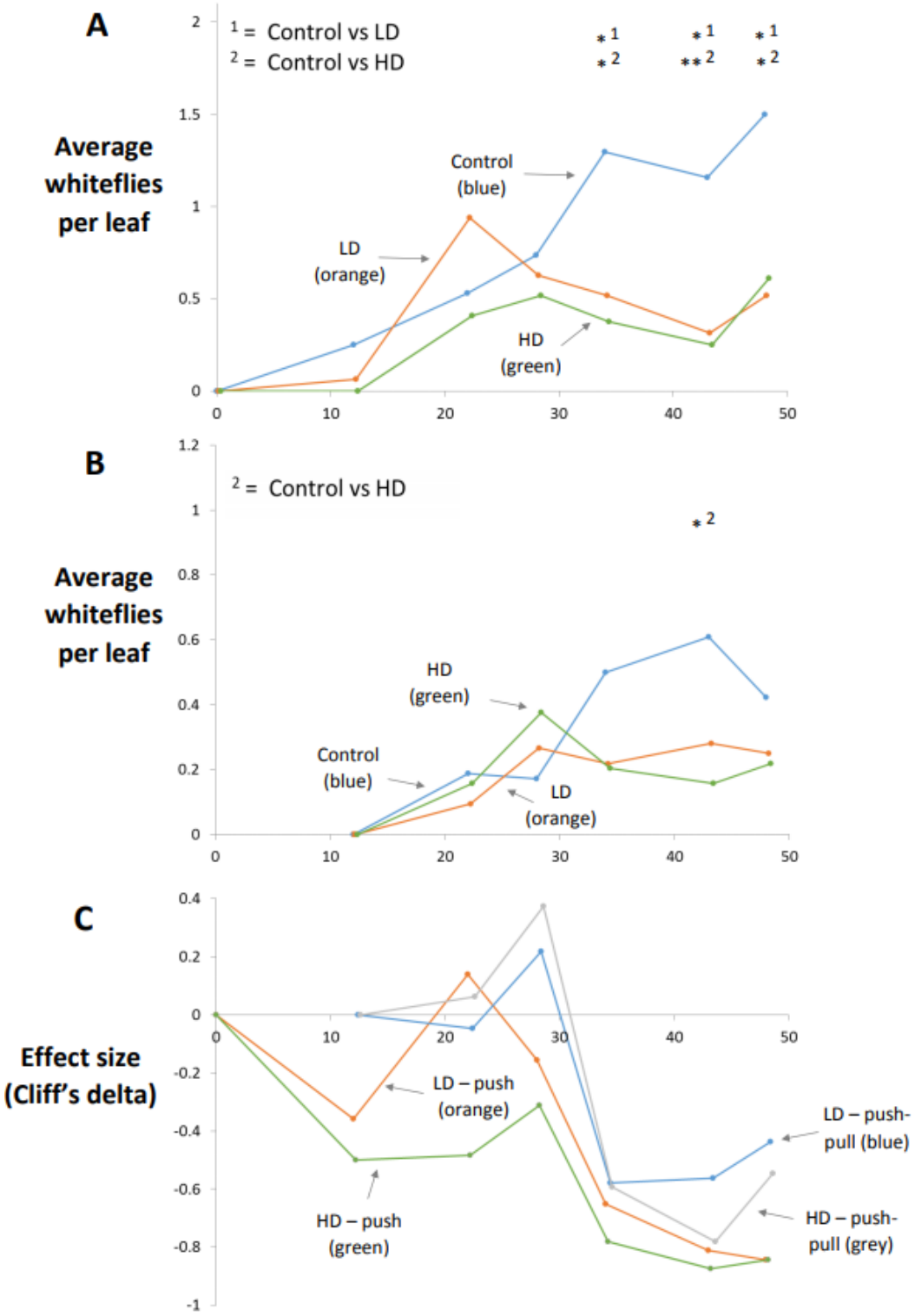

Figure 2.1: Population development of the whitefly, *T. vaporariorum* on tomato in the glasshouse, with all whitefly life stages (adult, eggs and nymphs) contributing to the average number of whitefly/leaf (*n*= 8). Day 0 is 10th August 2016 and the 'push-pull' was started 12 days after the "push" experiment. Whitefly abundance data from Figure 2.1, A and B was (log +1) transformed and analysed over time with repeated measures ANOVA's and Bonferroni corrected post-hoc comparisons, in which the *p* values were multiplied by the total number of

comparisons (*n*= 3). Significant observations between treatments at individual sampling points are annotated onto the graphs with asterisks, "*" indicates a *p* value of <0.05, "**" *p*<0.001. All other *p* values were non-significant at α = 0.05. Figure 2.1, A shows the "push" experiment in which repellent plants such as marigold (LD) and marigold plus non-hosts (HD) are intercropped amongst among tomato plants. There was a significant effect of the treatment (rm ANOVA $F_{(2,126)} = 18.85$, *p*< 0.001) and treatment x time (rm ANOVA $F_{(10,126)} = 2.14$, *p*= 0.025) across the sampling period. Figure 2.1, B Shows the 'push-pull' experiment which is the same as the 'push' experiment but additionally a single (LD) and several (HD) host plant species surround the repellent hosts and tomato. There was a significant effect of treatment ($F_{(2,105)} = 3.73$, *p*= 0.027) but no significant effect of treatment x time ($F_{(8,105)} = 1.49$, *p*= 0.166) following repeated measures ANOVA's. Post-hoc comparisons from Figure 2.1, A showed there were significantly fewer whiteflies in LD and HD plots at day 34 (LD, *t*= 2.89, *df* = 126, *p*= 0.013. HD, *t* = 3.61, *df* = 126, *p*= 0.001), day 43 (LD, *t*= 3.59, *df*= 126, *p*= 0.001. HD, *t*= 3.92, *df* = 126, *p*<0.001) and day 48 (LD, *t*= 3.66, *df*= 126, *p*= 0.001. HD, *t*= 3.29, *df*= 126, *p*= 0.003). For Figure 2.1, B, there were significantly fewer whiteflies on the HD treatment at day 43 (*t*= 3.018, *df*= 105, *p*= 0.009) but not on the LD treatment (*t*= 2.20, *df*= 105, *p*= 0.088). Figure 2.1, C shows the data in Figure 2.1, A and B expressed as effect size (relative to control). The Cliff's d measure is used as this is suitable for non-normal data of the type observed in experiments. Values of 1 or -1 (the sign shows the direction of effects relative to control) indicate complete non-overlap between the groups under consideration and a value of 0 indicates complete overlap. Ninety-five percent confidence intervals have been calculated and are available in the Appendix (Table A and B), but to aid visualisation they have been removed from the figure.

Other pests were found during the trial, but in much lower numbers, so were not included in the analysis. Plants were selected for the experiment specifically with glasshouse whiteflies in mind and it was not predicted that they would also repel other pests; therefore the lack of any significant effect on other pest insects is encouraging for the future development of this method. Total above ground fresh weight and fruit production was assessed at the end of the experimental period for every tomato plant in each of the three treatments, although no significant differences were observed between any of the treatments (Appendix Figure E).

2.3.2 Mechanistic basis of French marigold intercropping

Whilst repellent volatile chemistry is thought to be a major mode of action of companion plants (Ben Issa et al. 2016), in the glasshouse experiment described above plants were kept in communal drip trays which would have allowed the sharing of root exudates between species. This phenomenon has been shown to trigger complex behavioural changes in

Deschampsia caespitosa and *Arabidopsis thaliana* plants (Semchenko et al. 2014; Biedrzycki et al. 2010). Therefore, a series of bioassays were conducted to see if this cross-species sharing of exudates could have influenced whitefly population development on tomato. No change in *T. vaporariorum* preference or performance (oviposition) was observed when tomato seedlings were previously exposed to marigold root exudates (Appendix Figure F). This indicates that repellent volatile organic compounds from marigold are the probable cause of the reduction in whitefly performance on tomato intercropped with marigold (Figure 2.1). Following this finding, headspace analysis of marigold seedlings was conducted in order to identify the repellent volatiles in operation. Limonene, a compound known to have insecticidal activities against a number of arthropods (Ibrahim et al. 2001) including the Silverleaf whitefly (*B. tabaci*) (Shi et al. 2016; Du et al. 2016; Tu and Qin 2017), was found to be the most abundant volatile released from both flowers (24.01% of volatile output) and leaf tissue (21.04%) of marigold seedlings (Appendix Figure G).

Whilst controlling insect pests by increasing plant diversity in agro-ecosystems would have greater environmental benefit, the development of "low-risk" plant-based crop protection products, with favourable environmental credentials is still favourable to the use of synthetic chemical sprays. Being based on naturally occurring chemistry, such products are generally considered to offer a more sustainable alternative to conventional synthetic chemistry, particularly when considering their strong potential use in IPM programmes. Whilst issues such as phytotoxicity can arise from over-use of repellent volatile sprays, volatile dispensers alleviate this issue by releasing the compounds of interest more slowly, and this method of delivery was actually found to be more effective at controlling whitefly settling (Du et al. 2016). Additionally, volatile dispensers take up less production space in the glasshouse and do not require the upkeep/husbandry costs that could be associated with companion plants. Considering this, the use of repellent volatile dispensers may be a more attractive alternative for some horticulturalists. We therefore developed limonene dispensers similar to that used by Du et al. (2016) in an attempt to "push" whitefly from the target crop in a similar manner to that observed with marigold intercropping. In laboratory assays, we found that the limonene dispensers repelled whitefly from tomato and to only a slightly lesser extent than marigold seedlings (Figure 2.2), with 36.12% of whitefly choosing limonene intercropped tomato and 26.93% choosing marigold intercropped tomato (comparison of distribution between both treatments, $X^2 = 2.016$, $p = 0.156$, $df = 1$). Subsequent 4-way olfactometry

experiments presented odours to whitefly from marigold flowers, marigold leaf tissue and the limonene dispensers. All of these treatments showed a significant level of repellence to *T. vaporariorum* (Appendix Figure B). Marigold flowers were found to release approximately double the amount of limonene than that of marigold leaf tissue per gram of fresh weight (Appendix Figure G) with the level of repellence observed being proportional to the respective tissue area (Appendix Figure B). These experiments confirmed the effectiveness of limonene as a repellent to *T. vaporariorum* and led to limonene dispensers being used along with marigold plants in the following glasshouse trial.

From the glasshouse trial and subsequent laboratory experiments we conclude that the reduced whitefly populations observed on marigold intercropped tomato was due to repellent volatile chemistry. Other marigold species have been shown to be repellent to tobacco whitefly (*Bemisia tabaci*) (Sujayanand et al. 2016), green peach aphid (*Myzus persicae* Sulzer) (Ben Issa et al. 2016), cabbage aphid (*Brevicoryne brassicae* L.), flea beatles (*Phyllotreta*), small and large white butterly (*Pieris rapae* L., *Pieris brassicae* L.) and the diamondback moth (*Plutella xylostella* L.) (Jankowska 2009).

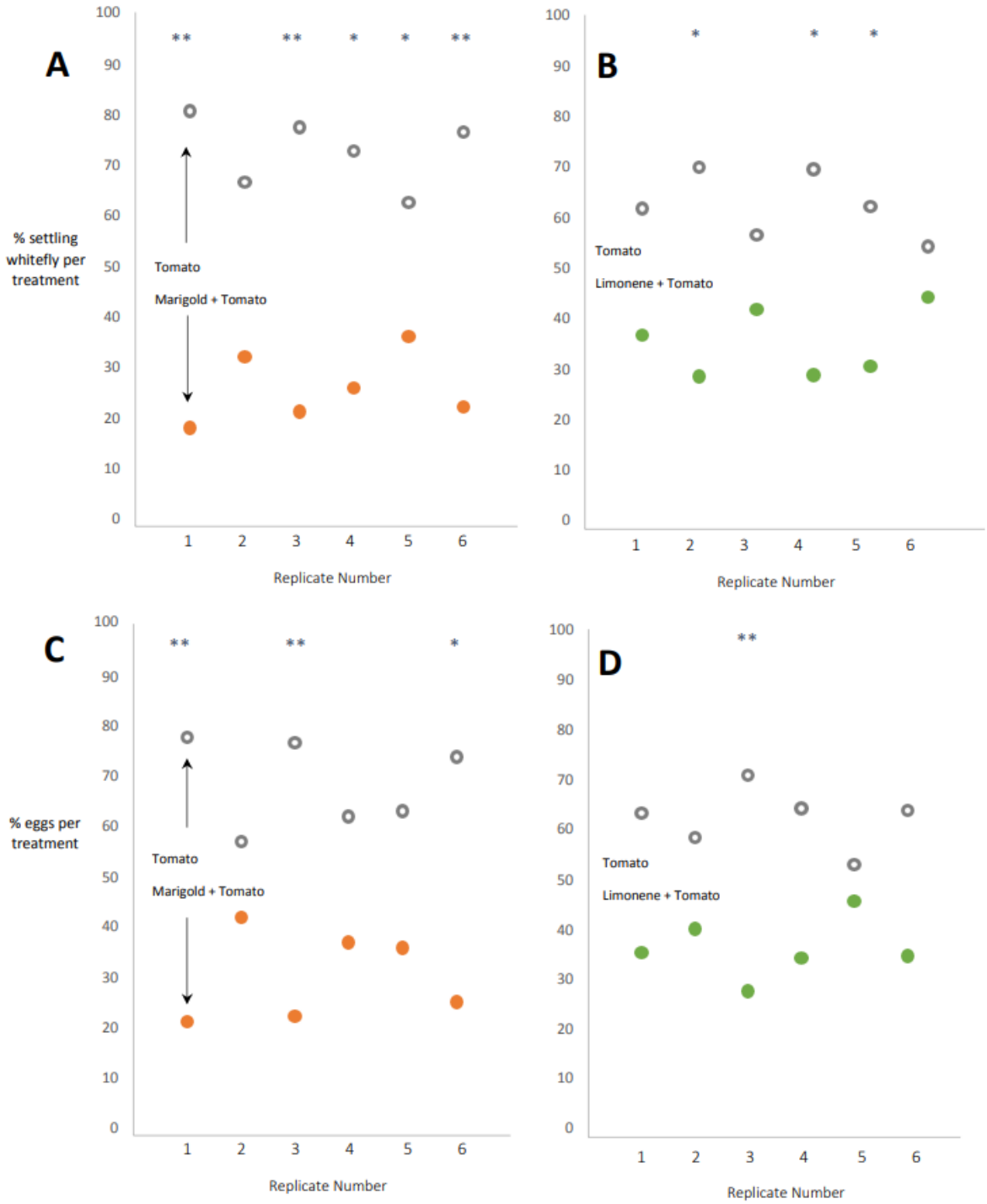

Figure 2.2: The percentage of whiteflies on tomato (*n*= 200) when given the choice between four un-accompanied "Elegance" tomato seedlings or four "Elegance" seedlings intercropped with either flowering marigold plants (Figure 2.2, A) or limonene dispensers (Figure 2.2, B). Whitefly eggs were also recorded for each replicate (Figure 2.2, C and D). Values were transformed to percentages and the Pearson's chi-squared test was used to test differences in settling and oviposition behaviour. Significant differences are annotated onto the graph for each treatment with the following format; "*" indicates a *p* value of <0.05, "**" *p*<0.001. Comparisons between individual replicates and controls for all four graphs (Figure 2.2, A, B, C and D) are as follows. *df*= 1 for all reps, Figure 2.2, A, rep 1 X^2 = 21.26, *p*<0.001; rep 2 X^2 =

3.43, p= 0.064; rep 3 X^2 = 17.01, p<0.000; rep 4 X^2 = 11.17, p= 0.001; rep 5 X^2 = 5.95, p= 0.015; rep 6 X^2 = 15.72, p<0.001. Figure 2.2, B, rep 1 X^2 = 2.92, p= 0.087; rep 2 X^2 = 8.33, p= 0.004; rep 3 X^2 = 0.985, p= 0.321; rep 4 X^2 = 8.33, p= 0.004; rep 5 X^2 = 5.32, p= 0.021; rep 6 X^2 = 0.501, p= 0.479. Fig 2C, rep 1 X^2 = 13.82, p<0.001; rep 2 X^2 = 0.323, p= 0.570; rep 3 X^2 = 12.65, p<0.000; rep 4 X^2 = 1.65, p= 0.198; rep 5 X^2 = 2.05, p= 0.152; rep 6 X^2 = 9.51, p= 0.002. Figure 2.2, C, rep 1 X^2 = 2.31, p= 0.128; rep 2 X^2 = 0.731, p= 0.398; rep 3 X^2 = 6.876, p= 0.009; rep 4 X^2 = 2.97, p= 0.084; rep 5 X^2 = 0.02, p= 0.887; rep 6 X^2 = 2.49, p= 0.114. On average over the 6 replicates, 21.93% of whiteflies settled on tomato intercropped with marigold and 36.12% whiteflies settled on tomato intercropped with limonene dispensers. 31.44% of eggs were laid on marigold intercropped tomato and 37.24% were laid on limonene intercropped tomato. Average distribution (n= 6) of whiteflies in limonene and marigold treatments were compared to ascertain whether marigold control was significantly more effective at repelling whitefly, however this was not the case (X^2 = 2.016, p= 0.156, df= 1).

2.3.3 Glasshouse trial 2: "Emergency" Intercropping to protect tomatoes from an established heavy whitefly infestation

Having shown that marigolds repel whiteflies from tomatoes during the early stages of plant growth at relatively low whitefly density (Figure 2.1), and that limonene is a major volatile component of marigolds (Appendix Figure G) and repellent to whiteflies (Figure 2.2), a further treatment with the limonene dispensers was tested. Here they were introduced to heavily infested tomato plots in the same way as marigold plants. Heavy infestations are likely to occur if no protective measures are deployed against whiteflies in tomato growing facilities and home gardens. At this stage, even if more environmentally sound measures such as biocontrol are utilised, they are notoriously slow to take effect (Bale et al. 2008) and introduction of repellent plants or volatile dispensers could have a more immediate impact on whitefly infestations. If found to be capable of reducing the impact of a heavy whitefly infestation, then marigolds and/or limonene could be used instead of a chemical pesticide application, reducing the environmental impact of synthetic, conventional chemical whitefly control.

The effect of these treatments on settling adult whiteflies, egg and nymph numbers can be seen in Figure 2.3, A, B and C. Across the sampling period, there was no significant effects observed on settling whitefly adults (Figure 2.3, A) between treatment (rm ANOVA $F_{(2,105)}$ = 1.71, p= 0.185) and treatment x time (rm ANOVA $F_{(8.105)}$ = 0.896, p= 0.522) according to repeated measures ANOVA's. Average settling whitefly numbers were lower throughout

the course of the experiment on the limonene treatment (Figure 2.3, A) whereas settling adults on the marigold treatment were actually higher than the control at day 22. This suggests that any protective effect provided by limonene dispensers is longer lasting than that provided by marigold plants. Figure 2.3, B shows that no significant effect was observed on whitefly egg abundance between treatment (rm ANOVA $F_{(2,105)} = 0.477$, $p = 0.625$) or treatment x time (rm ANOVA $F_{(8,105)} = 0.351$, $p = 0.943$), meaning that adults were not deterred from laying eggs on the intercropped tomatoes. Whilst no significant differences were observed, average settling adults and eggs were lower on marigold and limonene intercropped plots at the second (day 7) and third (day 14) sampling point after introduction. This implies that the treatments did initially repel whitefly settling and oviposition, albeit to a non-significant extent. These results from Figure 2.3, A and B would suggest that our treatments had minimal effect on whitefly behaviour and that any effects were only apparent shortly after control measures were introduced. However, there was a significant effect of treatment *x* time on whitefly nymph abundance Figure 2.3, C across the sampling period (rm ANOVA $F_{(8,105)} = 2.62$, $p = 0.011$). Tomato plants from the limonene treatment had significantly fewer nymphs than the control on day 29 (*t = 4.18, df = 105, p=< 0.001*) and neared significance at day 22 (*t = 2.17, df = 105, p= 0.095*). The limonene treatment also had significantly fewer whiteflies than the marigold treatment at day 29 (*t = -2.61, df = 105, p= 0.030*). The results from Figure 2.3, C show that the limonene treatment had significant long-lasting effects on whitefly nymph abundance over the course of the experiment.

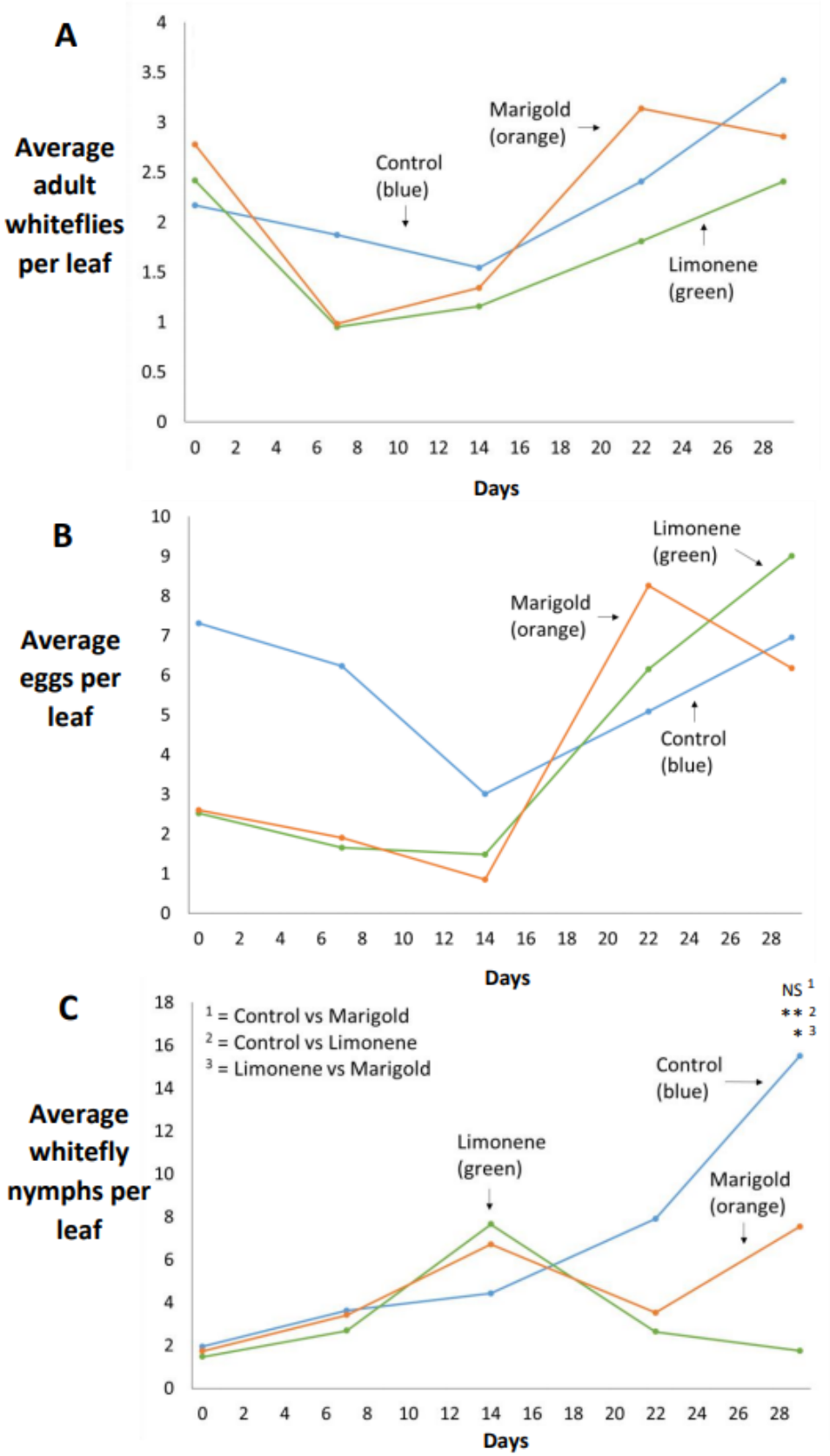

Figure 2.3: Whitefly population development in the second glasshouse trial with marigolds and limonene "emergency" intercropping treatments to control an established whitefly population (*n*= 8). Treatments comprised intercropping eight heavily whitefly-infested tomato

plants with eight further tomato plants (control), eight French marigold plants (marigold treatment), or eight limonene dispensers (limonene treatment). The first data point in Figure 2.3, A, B and C represents whitefly abundance (adults/eggs/nymphs) on the focal tomato plants immediately before treatment plants were introduced. '*' indicates a *p* value of <0.05, '**' indicates a *p* value of <0.001, NS = not significant. Figure 2.3, A displays the average number of adult whitefly settled on each focal tomato plant in the three treatments over the course of the experiment (*n*= 8). No significant effect on settling adult whiteflies was observed between treatment (rm ANOVA $F_{(2,105)} = 1.71$, *p*= 0.185) and treatment x time (rm ANOVA $F_{(8.105)} = 0.896$, *p*= 0.522) across the sampling period. Figure 2.3, B displays the average number of eggs laid on each focal tomato plant over the course of the experiment (*n*= 8). No significant effect on whitefly egg abundance was observed between treatment (rm ANOVA $F_{(2,105)} = 0.477$, *p*= 0.625) and treatment x time (rm ANOVA $F_{(8,105)} = 0.351$, *p*= 0.943) across the sampling period. Figure 2.3, C displays the average number of whitefly nymphs of all stages counted on each focal tomato plant over the experiment (*n*= 8). There no significant effect on treatment (rm ANOVA $F_{(2,105)} = 2.72$, *p*= 0.070) but there was a significant effect on treatment x time (rm ANOVA $F_{(8,105)} = 2.62$, *p*= 0.011). The limonene treatment had significantly fewer nymphs than the control on day 29 (*t*= 4.18, *df*= 105, *p*< 0.001) and neared significance at day 22 (*t*= 2.17, *df*= 105, *p*= 0.095). The limonene treatment also had significantly fewer whiteflies than the marigold treatment at day 29 (t = -2.61, df = 105, p= 0.030). Ninety-five percent confidence intervals have been calculated and are available in the Appendix (Table A and B), but to aid visualisation they have been removed from the figure.

Taken altogether, despite the low number of significant effects between the treatments for the various whitefly life stages, these results are suggestive of a mildly suppressive effect on adult whiteflies of both marigolds and limonene (with limonene having the slightly stronger effect), and more pronounced effects on whitefly nymphs, with the limonene treatment being more effective. The fact that no significant effect was observed on settling whiteflies and egg numbers between treatments was interesting, as in previous experiments we found whiteflies to be repelled from both marigold plants and limonene dispensers. In a similar study, Du et al. (2016) detected no significant mortality on different whitefly life stages, whilst the present study identified significantly fewer nymphs on tomato plants presented with limonene dispensers, which would suggest an effect on nymph mortality or egg hatch. The disparity observed in the results, of minimal effects on settling adults and eggs, but a significant effect on nymph mortality in the limonene treatment presents an interesting pattern, with several potential explanations. This may be evidence of the repellent effect being insufficiently strong to repel whiteflies completely, as they are still capable of laying as many eggs on the treated tomatoes. Toxicity of limonene against eggs or nymphs may be another explanation: limonene has been shown to be toxic to the whitefly species *Aleurodicus disperses*, and *A. antidesmae*, and the inability of eggs and nymphs to move away from this

chemical could make them more susceptible to any toxic effects (Hollingsworth 2005). However, in the aforementioned study from Hollingsworth (2005) toxicity was not life stage specific, but achieved an equally significant effect on egg numbers. In addition to this, Hollingsworth (2005) used limonene as a concentrated aqueous solution, so similar toxic effects might not necessarily be seen with airborne limonene.

Figure 2.4 shows the effect of the treatments on tomato fruit production over the course of glasshouse trial 2 (Figure 2.4, B), and the aboveground plant tissue weight (excluding tomatoes, Figure 2.4, A), per plant at the conclusion of the study, when plants were destructively sampled. In Figure 2.4 (A), tomato vegetative tissue in the limonene treatment was significantly heavier per plant than both the control ($p = 0.05$) and the marigold treatment ($p = 0.005$). This indicates that as a result of the limonene treatment, tomato plants were able to produce more vegetative tissue, possibly as a result of the reduction in whitefly performance on these plants. Despite this, there were no significant differences between the limonene treatment and the control in terms of total fruit weight per plant (Figure 2.4, B). By contrast, marigold treated tomatoes experienced a near-significant reduction in fruit weight per plant compared to the control ($p = 0.100$) and a significantly lighter fruit weight than the limonene treatment ($p = 0.014$). There appears to be a difference in the effect that marigold and limonene treatments had on the plants, with limonene enhancing vegetative growth and possibly increasing fruit number, and marigold treatment potentially resulting in lighter plants with less fruit weight. An explanation for this may be found in the density of the plants in the treatments: based on observations of the plant growth in the different treatments, the introduction of marigolds (with a very bushy growth habit) may have restricted access to sunlight of the lower parts of tomato plants in that treatment, possibly inhibiting growth. The limonene treatment, by contrast, had a very low planting density, with the introduction of the limonene dispensers not restricting access to sunlight. Whilst limonene may be providing benefits to the tomato plants of reduced whitefly pest load, resulting in greater plant growth, it is necessary to see whether this advantage over controls persists in future studies that control for planting densities.

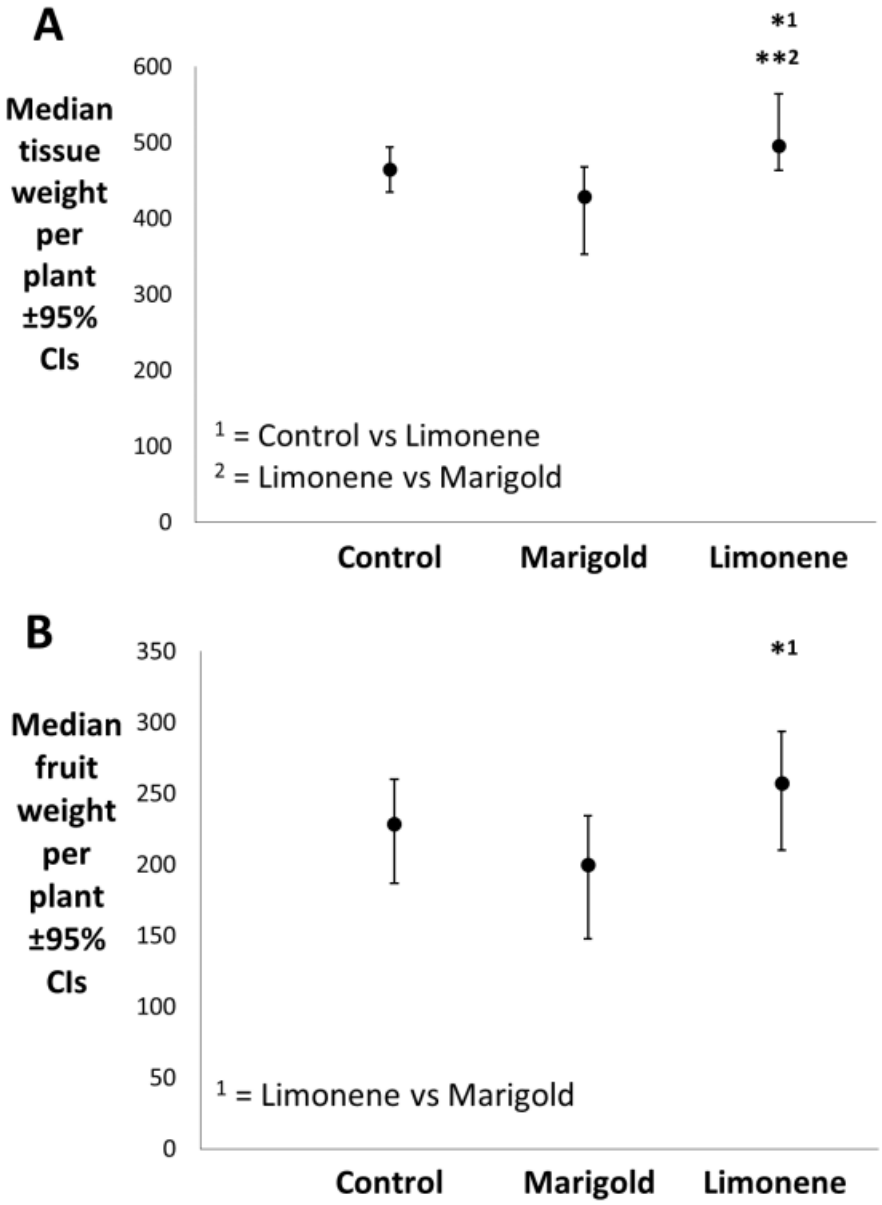

Figure 2.4: Plant development characteristics at the end of the 2017 assay into the efficacy of marigolds and limonene as an emergency treatment for the control of established whitefly populations (*n*= 8). Figure 2.4, A displays the median aboveground tissue weight of each focal tomato plant, excluding tomatoes, at the end of the 29-day experiment (*n*= 8). Tomato plants from the marigold treatment were near-significantly lighter than tomato plants from the control (*t*= -1.86, *df*= 14, *p*= 0.085) and significantly lighter than tomato plants from the limonene treatment (*t*= -3.3, *df*= 14, *p*= 0.005) according to *t*-tests. Tomato plants from the limonene treatment were significantly heavier than those from the control according to a *t*-test (*t*= 2.15, *df*= 14, *p*= 0.05). Figure 2.4, B displays the median weight of all tomatoes from each focal tomato plant at the end of the 29-day experiment (*n*= 8). The total tomato weight from plants in the marigold treatment was near-significantly lower than plants in the control

(t= -1.76, df= 14, p= 0.100) and significantly lower than the total tomato weight of plants from the limonene treatment (t= -2.8, df= 14, p= 0.014). There was no significant difference in tomato weight between the control and limonene treatment.

From these experiments it would appear that marigolds have a less pronounced influence on whitefly populations when introduced as an emergency control measure to reduce the impact of a heavy whitefly infestation. By contrast, application of limonene dispensers achieved a greater control over whitefly populations, giving reductions in average adult settling throughout the experiment and achieving significant reductions in nymph survival, despite similar numbers of eggs being laid on limonene-treated plants as the control. This translated to significant impacts on plant size (above ground tissue weight) and marginal impacts on fruit yield (Figure 2.4), showing limonene dispensers to have some degree of efficacy when deployed in this "emergency" scenario. This volatile based system involving limonene could be developed into a highly effective control agent of whitefly and since the efficacy of repellent volatiles is thought to be dose dependant (Ben Issa et al. 2016), increasing limonene release could have a more pronounced disruptive effect. Future experiments should look to find optimum delivery levels of limonene, whilst ensuring phytotoxicity and other non-target effects do not occur.

2.3.4 Conclusions and Prospects

The efficacy of a method popular amongst domestic gardeners, of intercropping tomato with French marigolds for pest control, appears to have been supported in the present study. Significant control of whitefly was achieved when marigolds were intercropped amongst tomatoes from the beginning of the growth period. However, introducing marigolds as a replacement for chemical control methods after significant whitefly infestation produced minimal effects. In the "emergency" situation, the limonene dispensers were more effective at reducing whitefly performance than whole plants, and this method warrants further experiments for optimisation of its deployment. Future experiments should look to ascertain how repellent volatiles affect whitefly behaviour in a no-choice situation without control plots present, which could have acted as refuge areas for the whitefly. Despite this, we predict that repellent volatiles would still reduce whitefly performance by invoking restlessness and/or

abnormal behaviour, as has been shown previously (Bernays 1999). Effects on natural enemies and other prominent pest species should be explored before implementing our methods in a commercial glasshouse setting. Nevertheless, the results obtained from this work are extremely promising for the application of repellent volatile chemistry for the protection of glasshouse-grown tomatoes.

Increasing plant diversity further in the 'push-pull' assay in the first glasshouse trial did not result in enhanced pest control. Thrips, another important pest of glasshouse grown tomato, showed no level of preference to any of the treatments employed in both the "push" and "push-pull" experiments in this first glasshouse trial. In one respect this is positive and strongly suggests a future direction for this research, as it appears that other non-host plants can be added to the mixture alongside marigolds and still produce a negative effect on whiteflies on tomato, whilst having a neutral effect on other insect pests. It is envisaged that a mixture of plant species may even be developed that can be intercropped with tomato and will repel a number of the major invertebrate pests of tomato. This will be a challenge and will become more difficult as the number of pests considered increases, as each plant species must be a non-host of the focal pest, but also of the other pests the mix aims to repel (introducing a plant species that repels one pest but is suitable to another risks reducing the effectiveness of the technique). For example, both marigold (Price 2013) and tomato (Zanin et al. 2018) are susceptible to the two-spotted spider mite (*Tetranychus urticae* Koch) and therefore as part of this mix a plant would need to be introduced to alleviate negative effects of this pest. We believe that through careful planning and constructing models of known plant-insect interactions it would be possible to create a mix of plants which mitigate arthropod pest damage to agricultural crops. Such a mix, if comprising edible or ornamental species, could be very attractive to growers and provide numerous societal benefits such as reducing pesticide use, diversifying horticultural production (Du et al. 2016), increasing the diversity of invertebrate fauna within agroecosystems (Knops et al. 1999; Malézieux et al. 2009), and increasing the diversity of produce on market shelves in a world increasingly dominated by fewer food types.

Chapter 3. Volatile Organic Compounds as Insect Repellents and Plant Elicitors: An Integrated Pest Management (IPM) Strategy for Glasshouse Whitefly (*Trialeurodes vaporariorum*)

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Abstract

The glasshouse whitefly (*Trialeurodes vaporariorum* Westwood) is a polyphagous arthropod pest that is of particular detriment to glasshouse-grown tomato (*Solanum lycopersicum*) across temperate regions of the world. Control of whiteflies with synthetic pesticides has resulted in the evolution of resistant genotypes and a reduction in natural enemies, thus highlighting the need for environmentally sound control strategies. Volatile organic compounds (VOCs) offer an environmentally benign alternative to synthetic chemical sprays and this study explored the use of VOCs as insect repellents and plant defence elicitors to control whiteflies on tomato in a commercial glasshouse setting. Limonene in the form of a volatile dispenser system was found to successfully repel whitefly from the target crop and increased fruit yield by 32% during a heavy whitefly infestation. Analysis of tomato herbivore induced plant volatiles (HIPVs) led us to select methyl salicylate (MeSA) as the plant elicitor and application of MeSA to un-infested tomato plants was found to successfully reduce whitefly population development and increase yield by 15%, although this was not statistically significant at $\alpha = 0.05$ ($p = 0.085$). Combination of these two methods was also effective but whitefly abundance in combined plots was similar to the standalone limonene treatment across the course of the experiment. All of the VOC based control methods we used had a negative impact on whitefly performance, with more pronounced effects during the first few weeks of infestation. In subsequent laboratory experiments, we found elevated peroxidase (POD) activity and a significant increase in TPX-1 and PR-1 transcripts in MeSA treated plants.

This led us to deduce that MeSA immediately induced plant defences, rather than priming them. We did however see evidence for residual priming, as plants treated with MeSA and infested with whiteflies produced significantly higher levels of POD activity than whitefly infestation alone. Despite the fact that our treatments failed to synergise, our methods can be optimised further and the effectiveness of the standalone treatments is promising for future studies. In particular, our repellent limonene dispensers were extremely effective at deterring whiteflies and offer a low cost and easy to implement whitefly control option. The methods we have used here could be incorporated into current integrated pest management (IPM) systems, a sustainable approach to pest control that will be central to our efforts to manage whitefly populations under glass in the future.

3.1 Introduction

The glasshouse whitefly (*Trialeurodes vaporariorum* Westwood*)* is a widespread and persistent pest species of many horticultural and ornamental crops, including tomato (*Solanum lycopersicum*). In commercial glasshouses whitefly populations are often managed with biocontrol, typically the parasitoid *Encarsia formosa* (Hanan et al. 2017). However, issues such as delayed efficacy and hyper-parasitism can result in failure, meaning there is still a reliance on synthetic chemical sprays (George et al. 2015). Whiteflies have shown resistance to some of our most important insecticides (Gorman et al. 2007; Wardlow et al. 1976) and the EU has placed restrictions on development of new chemical sprays (Hillocks 2012). Therefore, the need for alternative control methods is clear. Additionally, the human health related impacts of pesticides are widely recognised and major legislative advisory bodies are urging increased use of integrated pest management (IPM) (USDA-ARS 2018; Directive 128/EC 2009). IPM systems operate by limiting applications of chemical pesticides (Stenberg 2017) and placing more focus on the use of naturally occurring control agents (Prokopy 2009). Whilst still in its infancy at the commercial level, IPM is widely regarded as the future of sustainable agricultural pest management and to meet the challenge of ultimately replacing chemical pesticides in the future, novel control components must constantly be developed (Stenberg 2017). Volatile organic compounds (VOCs) are natural products of almost all plant taxa (Vivaldo et al. 2017) and offer an extremely versatile option for incorporation into IPM systems. VOCs are considered environmentally benign alternatives to insecticides and have a proven action against some of our most important insect pests, including whiteflies

(Schlaeger et al. 2018). The current study seeks to explore the use of VOCs in the form of an IPM system to control whiteflies on glasshouse-grown tomatoes.

Whiteflies are known to respond to subtle changes in their olfactory environment and recognition of volatiles is used in their selection of a suitable host (Darshanee et al. 2017; Bleeker et al. 2009a; Shi et al. 2016). Whilst some studies have identified individual VOCs that are repellent to whitefly (Li et al. 2014; Bleeker et al. 2009a; Sacchetti et al. 2015), work implementing whitefly repellent VOCs into a commercial IPM set up are extremely limited (Schlaeger et al. 2018). Despite this, we recently had success deterring whiteflies from a tomato crop using limonene, a compound which we identified from the whitefly non-host French marigold (*Tagetes patula L.*) (Conboy et al. 2019). Limonene dispensers were effective under laboratory conditions but were limited in their ability to curb a large pre-existing whitefly infestation in a commercial glasshouse setting. These results indicated that using limonene from the beginning of the growing period could be more effective. In the present study, we decided to use limonene dispensers from the beginning of the growth period to assess whether this could "push" whiteflies from the tomato crop and subsequently increase plant performance. We decided to utilise limonene in the form of a slow-release bottle (as has been demonstrated previously (Conboy et al. 2019; Du et al. 2016)) to allow a constant dispersal rate of the compound. This odour based method of pest control leads to very little environmental impact and was actually found to be more effective than direct repellent spray application (Du et al. 2016).

VOCs can also be used to induce or optimise plant immune systems; a term often referred to as plant vaccination (Brilli et al. 2019). Plant vaccination is relatively cheap, uses a low amount of generally benign plant compounds and can increase a plant's attractiveness to biocontrol (Bruce et al. 2017). Despite the lack of applied research in a commercial agricultural setting (Martinez-Medina et al. 2016), plant vaccination still offers tremendous potential for incorporation into IPM systems (Bruce et al. 2017). In response to defence eliciting compounds, plants can either directly induce or prime their innate defensive measures (van Hulten et al. 2006; Heil and Ton 2008). Plant priming is a phenomenon whereby the plant enters an induced state of readiness in which the plant will respond more rapidly and effectively to insect attack (Martinez-Medina et al. 2016; Frost et al. 2008). This is a much more attractive method of defence induction to growers as there is little or no energy cost

incurred during this primed state, meaning yield will not be impacted if the pest never successfully infests the crop. VOCs have been found to prime plant defences in the form of mixtures (Farag and Pare 2002; Hu et al. 2018) or individual compounds (Song and Ryu 2018; Erb et al. 2015). Other research groups have successfully selected defence-inducing VOCs based on release from infested conspecifics (Erb et al. 2015) and involvement in the plants' defence signalling pathways (Shulaev et al. 1997; Tang et al. 2015). For tomato, methyl salicylate (MeSA) is a compound that fulfils both of these criteria. MeSA is released by tomato in response to herbivory (Lopez et al. 2012; Ament et al. 2004; Takayama et al. 2012) and is a potent inducer of plant defence (Heil and Ton 2008). It has also been proposed that MeSA could serve as a long distance signalling molecule for the plant (Heil and Ton 2008), as has been previously shown in tobacco (Park et al. 2007). We therefore hypothesised that exogenous application of MeSA to un-infested tomato plants, at concentrations similar to that released by whitefly-infested tomato, would induce a defence response that would confer resistance to the glasshouse whitefly. We therefore undertook headspace analysis of whitefly infested tomato plants to confirm presence of MeSA and quantify emission rates for exogenous application to naïve tomato plants.

Considering how repellent volatile chemistry and plant defence induction place performance pressure on pests in completely different ways, we sought to synergize these two methods for maximum efficacy against glasshouse whiteflies. We assessed the efficacy of combined and standalone treatments at reducing whitefly abundance on tomato in a commercial glasshouse setting. MeSA was selected as the plant elicitor and was sprayed onto plants before introduction to the experimental glasshouse. Limonene was used as the whitefly repellent VOC and was deployed in the form of a slow-release dispenser that was placed alongside plants for the duration of the infestation period. Plants were grown from seed through to harvest in order to assess if our VOC based protection methods could improve yield during a heavy whitefly infestation. Following the glasshouse trial, activity of tomato defence related enzymes and expression of defence related gene transcripts were analysed to characterise resistance shown in the MeSA treated plants. In order to deduce effects on tomato defence signalling, we analysed expression of genes that related to salicylic acid (SA) and jasmonic acid (JA) signalling pathways. In summary, this experiment aimed to assert the potential for VOCs to be used in commercial tomato production systems and to elucidate any performance effects on the plants and insects involved.

3.2 Materials and Methods

3.2.1 Whitefly

Whiteflies, *T. vaporariorum* (Westwood), originated from a lab culture at Rothamsted Research that was first collected in 1960 in Kent on French bean and had subsequently been maintained in a large laboratory population. The insects for both glasshouse and laboratory experiments were taken from a mixed age colony maintained on pre flowering aubergine (*Solanum melongena* "Moneymaker"- Marshalls Seeds Cat. 1020-2017) at 20 °C, 16:8 light/dark.

3.2.2 Plants used for enzyme and genetic assays

Tomato seeds (*S. lycopersicum* Mill. var. 'Elegance' Cat. E/12/11, Batch 0160685360) were obtained from Monsanto and used for all experiments. Plants were grown from seed in J. Arthur Bowers John Innes no 2 compost in 9-cm-diameter and 8.7-cm-deep pots. 'Elegance' seedlings used for the enzyme and genetic assays were grown at a distance of approximately 60 cm from a 400-W Son-T bulb (6000 lumens) housed in a Harrier HR400SH 400-W lamp under a 16 h light/8 h dark cycle, the temperature regime was 25 °C in the light and 20 °C during the dark period. Treatments applied for the enzyme and genetic assays were identical, and preparation of treatments was carried out under ambient lighting (16 h light/8 hr dark) at 25 °C in the light and 20 °C during the dark period. Treatments for enzyme and genetic assays consisted of 1) Controls (C): naïve 'Elegance' seedlings at stage 14 on the BBCH scale (Klingauf 2001). 2) Methyl salicylate treated (MeSA): as first true leaves emerged, 'Elegance' seedlings were sprayed with MeSA at the same frequency and concentration as in the glasshouse experiment (see 'Glasshouse experiments' subheading in materials and methods) and subsequently left for 10 days. After this treatment course, plants were at stage 14 on the BBCH scale. 3) Whitefly infested (Tv): 'Elegance' seedlings at stage 14 on the BBCH scale were placed inside a 30x40cm cylindrical mesh cage (Watkins and Doncaster, product code: E6090) and infested with 50 adult whiteflies taken from the laboratory culture described under the materials and methods subheading 'Whitefly'. These plants were infested with whiteflies for 24 hours. 4) MeSA treated and whitefly infested (MeTv): This treatment was a combination of MeSA and Tv treatments. After MeSA application, plants were left for 10 days and then infested with 50 whiteflies for 24 hours. Following this treatment course, plants were at stage 14 on the BBCH scale. We also assessed enzyme activity in plants exposed to limonene

dispensers to see whether our repellent volatile IPM method had any non-target effects on tomato defences. Eight 'Elegance' tomato seedlings at stage 11 on the BBCH scale along with 5 limonene dispensers, arranged in the same way as in the glasshouse experiment (see Appendix Fig L, A for image), were placed inside a 90x60x60cm mesh cage (Watkins and Doncaster, product code: E6098). These plants were left for 10 days until leaf tissue was harvested for enzyme assays. These plants comprised the limonene (L) treatment and were at stage 14 on the BBCH scale.

3.2.3 Glasshouse experiments

For the glasshouse experiments, 'Elegance' tomato plants were grown from seed in standard germination trays in a pest-free propagation glasshouse at Stockbridge Technology Centre (UK) from 1st July -22nd of July 2017. As the first leaves began to appear, half of the 288 seedlings were sprayed with MeSA (Sigma-Aldrich, M6752) dissolved in 50% ethanol at a concentration commensurate with the amount of MeSA we found to be released by whitefly infested tomato plants on each day of infestation (Appendix Table C). On day 1 each plant was sprayed with 67.76µg of MeSA, day two; 60.80µg, day three; 66.32µg, day four; 52.84µg and on day five 143.96µg. On each day of MeSA application, plants which comprised the Control and Limonene treatments were sprayed with 50% ethanol only. This 5-day spraying regime was based on a previous study from our lab group which found that tomato plants exposed to herbivore induced plant volatiles (HIPVs) from whitefly infested conspecifics for 5 days were more resistant to whiteflies (Appendix Figure J). We aimed to prime plant defences and to do this we considered that for a plant to be primed, the defence response of the plant must return to basal levels before second stimulation from the triggering stress, at which point the plant will respond more effectively to insect attack (Martinez-Medina et al. 2016). Therefore, following this 5-day MeSA application period, the plants were left in the same pest free glasshouse for a further 10 days before being introduced to the experimental glasshouse and infested with whiteflies.

At the 3-4 leaf stage (22nd July, day 21 from seed, roughly stage 14 on the BBCH scale) all plants from all treatments were introduced to a 448m^3 glasshouse (Figure 3.1) containing 10 aubergine (*Solanum melongena* "Moneymaker") plants heavily infested with *T. vaporariorum* (approximately 1000 insects per plant) which were taken from the laboratory culture described in under the materials and methods sub heading 'Whitefly'. Whilst whitefly were

the main focus of our experiment, we allowed natural pest populations to develop without control. *Thrips tabaci* were the only other pest observed in the glasshouse, albeit at insignificant numbers. Throughout the course of the experiment, we observed no natural enemies in the glasshouse so any effects on whitefly performance cannot be attributed to parasitism or predation. The plants were arranged into blocks of four different treatments with 8 plants in each treatment and this four treatment block was replicated 9 times (Figure 3.1). The control treatment (C) had 8 untreated tomato per block, the limonene treatment (L) had 5 limonene dispensers (see Conboy et al. (2019) for details on design) placed along the centre line of 8 untreated tomato (see Appendix Fig L, A for image), the MeSA treatment (MeSA) had 8 MeSA treated plants, and the combined treatment (ML) had 8 MeSA treated plants with 5 limonene slow release bottles also. During observations of whitefly development, single fully-expanded leaves were selected by randomising both compound leaf selection and then individual leaflet from a tomato plant (selection of which was also randomised) in each treatment block from each of the 9 replicates. These were then examined *in situ* for whitefly adults and adults of any other insect pests. These leaves were removed and placed in sealed plastic bags, then stored overnight at 4°C and examined under low power microscopy the next day for whitefly (and other pests) larvae and eggs. The abundance of whitefly adults, eggs and nymphs were analysed separately and used as a measure of whitefly performance. Sampling was conducted over a 53-day infestation period (22nd July-13th September) and sampling frequency can be viewed across the x axis of Figure 3.2 A, B and C.

At the end of the 53-day infestation period, fruit count per plant and fresh weight of total fruits (g) per plant was recorded and average fruit count and weight per plant in each treatment was calculated (*n= 72*) (see Appendix Figure I, B for image of tomatoes at point of harvest). Percentage difference in yield between treatments and controls was calculated with the formula ((Average Treatment Yield – Average Control Yield)/Average Control Yield) x 100. To give indication of fruit quality, one tomato was selected from the lowest fruit cluster of 4 randomly selected plants from each individual treatment block (*n*= 36 total for each treatment) and immediately placed in a plastic bag which was then sealed. These tomatoes were left to ripen in a dark room at ~21°C for 14 days before being frozen at -20°C until needed. At which point they were defrosted (12hr), homogenised with a razor blade and assessed for brix soluble solids content using a portable refractometer (PCE-032, PCE-Instruments) (Appendix Table D).

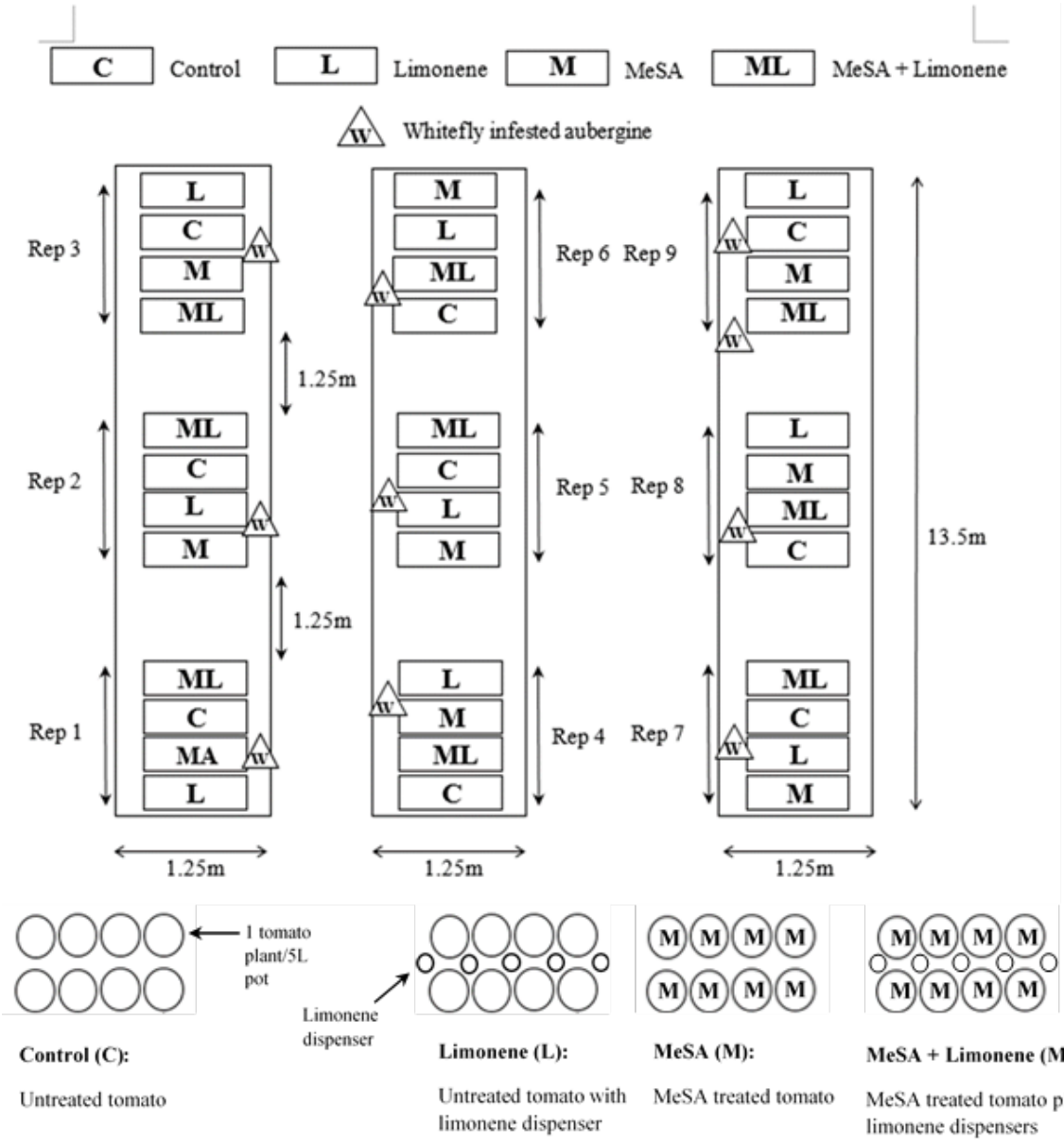


Figure 3.1: Layout of the glasshouse experiment assessing the impact of repellent volatiles and plant elicitation on whitefly performance. Treatments were arranged in a randomised block design with 9 replicates containing 4 treatments in a random order. The treatments were arranged exactly as shown in the figure above with the heavily infested aubergine plants placed around the glasshouse and labelled with the letter "W".

3.2.4 Enzyme assays

Peroxidases (POD) and polyphenol oxidases (PPO) are defence related enzymes which are often used as chemical markers of induced resistance in members of the *Solanaceae* family

(Karban et al. 2003). Both of these enzymes are produced constitutively by tomato but are also induced in response to whitefly feeding (Mayer et al. 2002; McKenzie et al. 2002; Su et al. 2015). We sought to assess activity of these defensive enzymes to characterise any induction of plant defences that may have occurred during the glasshouse trial. All five treatments for this experiment (C, L, MeSA, Tv and MeTv) were implemented as described under the materials and methods subheading 'Plants used for enzyme and genetic assays'. PPO and POD activities were measured using modified methods from Thaler et al. (1996) and (Rowen et al. 2017) . For all treatments, plant tissue apical to the dicotyledonous leaves was removed and flash frozen in liquid nitrogen. This tissue, which ranged from 150-300 mg, was homogenised in 1.25 ml of ice-cold K-phosphate buffer (0.1 M, pH 7) containing 2% (*w*/*v*) polyvinylpolyprolidine (Sigma-Aldrich). Subsequently 0.4 ml of 10% Triton X-100 (Sigma-Aldrich) was added to the homogenate, vortexed and centrifuged at 6000 rpm at 4 °C for 15 min. For the analysis of PPO activity, 50 µL of the supernatant was added to 200 µl of 29.2 mM caffeic acid in K-phosphate buffer (0.1 M, pH 8). For the analysis of POD activity, 30 µl of the supernatant was added to 220 µl of 0.3% guaiacol and 0.1% H_2O_2 in K-phosphate buffer (0.1 M, pH 8). PPO and POD activities were determined by tracking change in absorbance at 450 nm over 10 min. Activities are presented as change in optical density per minute per gram of fresh weight.

3.2.5 Genetic analysis

In order to understand the resistance to whitefly shown by MeSA sprayed plants, we undertook genetic analysis of three tomato defence related genes LOX-1, PR-1 and TPX-1. We assessed activity of PR-1 and LOX-1 due to their close association with plant defence signalling pathways. LOX-1 encodes a JA regulated enzyme which catalyses the production of 13-hydroperoxy-linolenic acid from linoleic acid (Heitz et al. 1997; Akram 2008). Analysing the transcription rates of this gene will allow us to identify whether JA defences are being induced or supressed by whitefly feeding and/or MeSA application. The PR-1 gene encodes a pathogenesis related (PR) protein and is commonly used as a marker of systemic acquired resistance (SAR) across a wide range of higher plants (Medeiros et al. 2017; Zarate 2007; Chinnasri et al. 2016). We assessed activity of PR-1 due to its close association with the SA defence-signalling pathway (Riviere et al. 2008). After observing high POD activity in MeSA treated plants, we assessed activity of the known POD related gene TPX-1. Whilst peroxidases

can have numerous beneficial functions for plants, this particular peroxidase gene is known to be encode a POD specific to cell wall lignification (Medeiros et al. 2017). By analysing activity of this specific peroxidase gene, we hope to further characterise the defence related responses in MeSA sprayed plants. All treatments for this experiment (C, L, MeSA, Tv and MeTv) were prepared as described under the materials and methods subheading 'Plants used for enzyme and genetic assays'.

Total RNA was isolated with Trizol Reagent (Invitrogen, Thermo Fisher Scientific) and purified with PureLink RNA Mini Kit (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. Concentrations of the RNA preparations were determined photometrically using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The RNA preparations were stored at −80℃ until use. One µg of RNA was reverse transcribed using a reverse transcriptase kit to synthesize cDNA. Primers sequences for actin (reference gene), LOX-1, PR-1 and TPX-1 can all be viewed in Appendix Table E, along with the referenced paper that we acquired the sequences from. qPCR was performed with a Rotor-Gene Q (Qiagen), using SensiFAST SYBR No-ROX Kit (Bioline) for 5 min at 95 °C, followed by 35 cycles consisting of 20 s at 95 °C, 30 s at 57 °C and 30 s at 72 °C, then 10 min at 72 °C. All quantifications were normalized to the reference gene actin using the delta ct method. The qPCR reactions were performed using three independent RNA preparations from independently grown plants and each RNA sample was run in triplicate for each qPCR run.

3.2.6 Data analysis

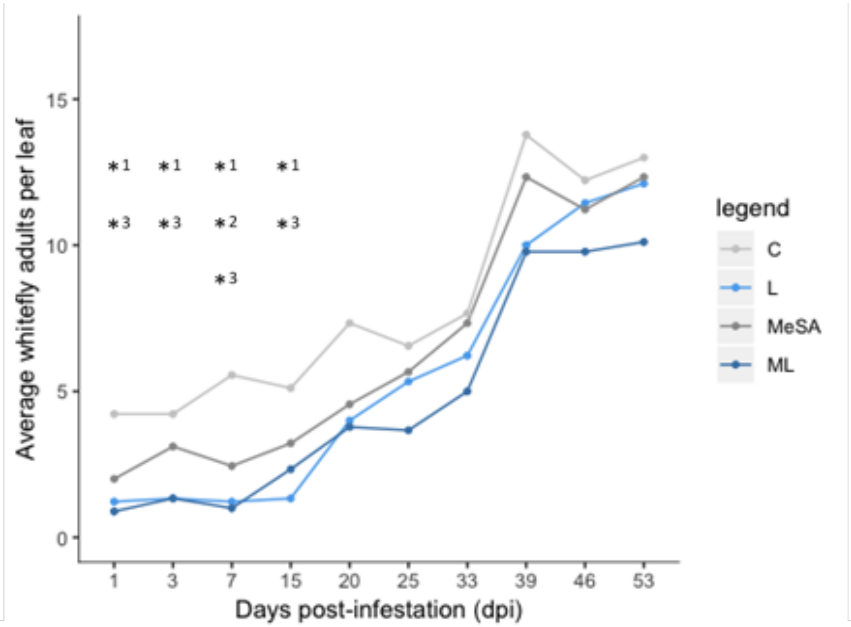
Data for whitefly abundance from the glasshouse trial was (log + 1) transformed to meet normality and homogeneity of variance assumptions for statistical analysis. Whitefly abundance at each individual life cycle stage was analysed with repeated measures ANOVA's using the "lmerTest" package in R. Time (sampling date) was used as the repeated measure and treatment was used as the fixed factor. The Tukey HSD post-hoc correction was used to analyse differences between treatments at individual sampling points. Fruit weights/counts, OD values from the enzyme assays and Dcq values from genetic assays were analysed using one-way ANOVAs with the base statistics package in R. Tukey HSD post-hoc tests were then used to compare differences between individual treatments. In order to meet normality and homogeneity of variance assumptions, data for POD activity was log transformed.

3.3 Results

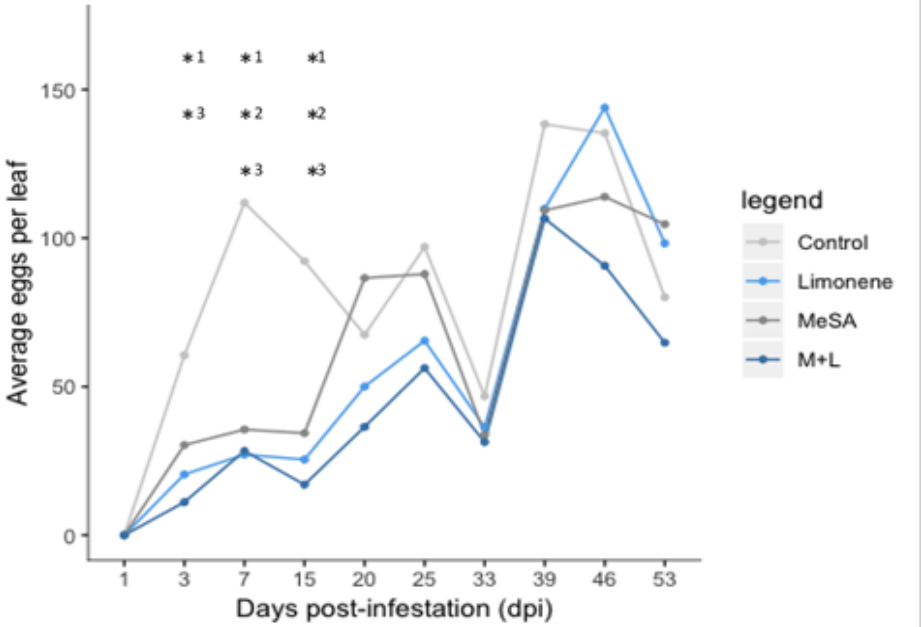
3.3.1 Whitefly Performance in the Glasshouse Experiment

All treatments had a negative impact on whitefly performance at some stage of the 53-day infestation period (Figure 3.2). The strongest effects were seen in the early stages of the experiment (first 15 days) where settling rates (Figure 3.2, A) and oviposition (Figure 3.2, B) were generally higher on the control plots compared to the other treatments. There were significantly fewer settling whiteflies in ML and limonene plots at 1dpi, 3dpi, 7dpi and 15 dpi whereas settling on MeSA plots was only significantly reduced at 7dpi. MeSA plots had significantly fewer whitefly eggs (B) than control plots at day 7dpi and 15dpi whilst both limonene and ML plots had significantly fewer eggs at 3dpi, 7dpi and 15 dpi. Whilst average settling and oviposition was generally lower (see Figure 3.2, B at 20dpi and 46dpi for exceptions to this) on protected treatments throughout the experiment, no significant differences were seen between any treatment and control post 20 dpi. Interestingly, the early reduction in oviposition seen between 1dpi and 15 dpi translated to a reduction in nymphs formed after 7 dpi (Figure 3.2, C), showing longer term effects on the whitefly life cycle after this opening 15-day period. The subsequent reduction in nymph abundance was only seen at 7 dpi for MeSA, whilst the limonene and ML plots had significantly fewer nymphs than controls at 7 dpi and 20 dpi. Similar to adult and egg abundance, there were consistently fewer nymphs on all three treated plots across the course of the experiment (Figure 3.2, C). In summary, the MeSA plots did not reduce whitefly performance on tomato as effectively or for as long a period as the plots accompanied by limonene slow-release bottles.

A


Average whitefly adults per leaf
Days post-infestation (dpi)
legend
C
L
MeSA
ML

B


Average eggs per leaf
Days post-infestation (dpi)
legend
Control
Limonene
MeSA
M+L

C

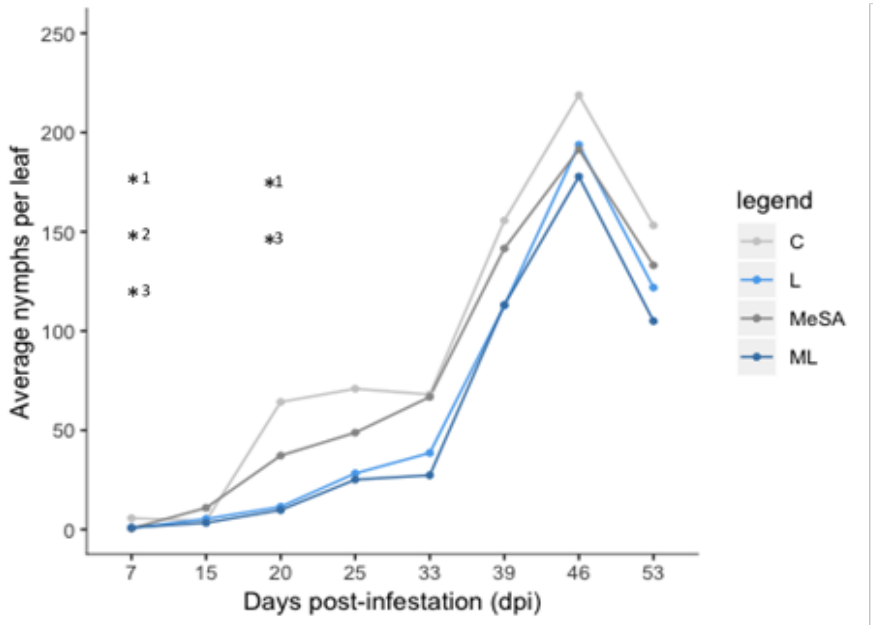

Average nymphs per leaf
Days post-infestation (dpi)
legend
C
L
MeSA
ML

Figure 3.2: Population development of whiteflies on tomato at all life cycle stages (Settling adults= A, Eggs= B and Nymphs=C) is expressed as average whitefly per leaf (*n*= 9) across the following treatments: Control= Light grey, Limonene = light blue, MeSA= grey and ML= dark blue. Data was (log+1) transformed and analysed using repeated measures ANOVA's with Tukey HSD post-hoc tests to compare treatments at individual sampling points (days post infestation (dpi)). Significant observations at individual sampling points are annotated onto the graphs above the sampling point this corresponds to. The subscript numbers accompanying each significance measure indicate which two treatments are being compared. [1] = Control vs Limonene, [2] = Control vs MeSA and [3] = Control vs ML. (A) Shows the average number of settling whitefly adults per leaf, there was no significant effect of treatment * time (ANOVA $F_{(27,320)} = 1.36$, $p= 0.111$) but there was a significant effect of treatment (ANOVA $F_{(3,320)} = 21.40$, $p<0.001$) following repeated measures ANOVA's. Post-hoc comparisons revealed there were significantly *fewer* whiteflies on Limonene and ML plots at 1dpi (Limonene, $t= 2.67$, $df= 320$, $p= 0.039$, ML, $t= 3.32$, $df= 320$, $p= 0.005$), 3dpi (Limonene, $t = 3.88$, $df= 320$, $p<0.001$, M+L, $t= 3.61$, $df= 320$, $p= 0.002$), 7dpi (Limonene, $t= 4.01$, $df= 320$, $p<0.001$, M+L, $t= 4.97$, $df= 320$, $p<0.001$), and 15dpi (Limonene, $t= 4.52$, $df= 320$, $p<0.001$, M+L, $t= 3.25$, $df= 320$, $p= 0.006$). Whereas there were significantly fewer whiteflies on MeSA plots only at 7dpi ($t= 2.76$, $df= 320$, $p= 0.030$). There were also significantly fewer whiteflies on Limonene plots compared with MeSA plots at 15 dpi ($t= -2.06$, $df= 320$, $p= 0.046$). (B) Shows the average eggs laid per leaf, there was no significant effect of treatment * time (ANOVA $F_{(24,288)} = 1.49$, $p= 0.066$) but there was a significant effect between treatments (ANOVA $F_{(3,288)} = 10.42$, $p<0.001$) following repeated measures ANOVA's. Limonene and ML treatments had significantly fewer eggs than control plots at 3dpi (Limonene, $t= 3.49$, $df= 288$, $p= 0.003$, ML, $t= 3.51$, $df= 288$, $p= 0.002$), 7dpi (Limonene, $t= 3.89$, $df= 288$, $p<0.001$, M+L, $t= 3.58$, $df= 288$, $p= 0.002$) and 15dpi (Limonene, $t= 4.05$, $df= 288$, $p<0.001$, M+L, $t= 4.19$, $df= 288$, $p<0.001$). MeSA plots had significantly fewer eggs than control plots at 7dpi ($t= 3.06$, $df= 288$, $p= 0.012$) and 15dpi ($t= 3.44$, $df= 288$, $p= 0.003$). (C) Shows the average nymphs (all larval stages) per leaf, there was a significant effect of treatment * time (ANOVA $F_{(21,256)} = 2.02$, $p= 0.005$) and a significant effect between treatments (ANOVA $F_{(3,256)} = 12.91$, $p<0.001$) following repeated measures ANOVA's. There were significantly fewer nymphs on all treated plots at 7dpi (Limonene, $t= 3.45$, $df= 256$, $p= 0.003$, MeSA, $t= 3.29$, $df= 256$, $p= 0.006$ and ML, $t= 2.68$, $df= 256$, $p= 0.038$). At 20 dpi there were significantly fewer nymphs on Limonene ($t= 4.30$, $df= 256$, $p<0.001$) and ML ($t= 5.32$, $df= 256$, $p<0.001$) plots compared with controls and significantly fewer nymphs on ML plots compared with MeSA ($t= 2.88$, $df= 256$, $p= 0.021$). Ninety-five percent confidence intervals have been calculated and are available in the Appendix (Table F), but to aid visualisation they have been removed from the figure.

3.3.2 Yield

There was a significant difference in average fruit weight per plant between the treatments (ANOVA, $F_{(3,268)} = 9.85$, $p < 0.001$) and all three protected treatments produced more tomatoes than the control (Figure 3.3, A). Plants from the limonene and ML treatments produced significantly higher fruit weight than the control with average fruit weight (g) per plant increasing by 32% and 24% respectively. Average fruit weight per plant increased by 15% from the MeSA plots, although this difference was not significant ($p = 0.085$) following Tukey HSD post-hoc tests. There was also a significant difference between the treatments when measuring fruit count per plant (ANOVA, $F_{(3,268)} = 9.99$, $p < 0.001$) (Figure 3.3, B). Tukey HSD tests showed that both limonene and ML treatments produced significantly more tomatoes per plant on average compared with controls, increasing by 28% and 14% respectively. There was only a small increase in tomatoes per plant from the MeSA treatment (3%) and this was not statistically significant. Assessment of Brix percentage soluble solids in tomato fruits from the four treatments produced no discernible differences following a one-way ANOVA ($F_{(3,140)} = 1.15$, $p = 0.329$, Appendix Table D)

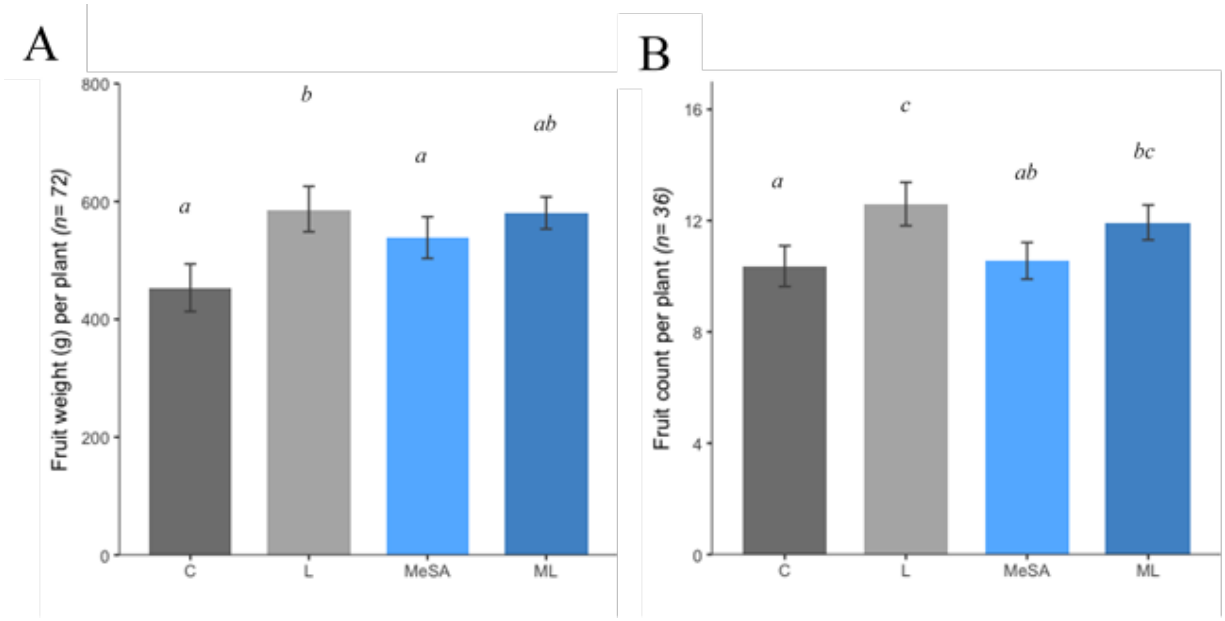


Figure 3.3: To assess the effectiveness of our whitefly control measures, plant performance was assessed by quantifying fruit weight and fruit count per plant at the end of the 52-day infestation period for each of the four treatments (Control, Limonene, MeSA and ML). Total fruit weight per plant (g) (A) was measured and collated into average fruit weight per plant in each of the four treatments (n= 76). A one-way ANOVA ($F_{(3,268)}$ = 9.85, p< 0.001) followed by Tukey HSD post-hoc tests were used to compare differences between the treatments. Both limonene (t= 155.80, df= 268, p< 0.001) and ML (t= 116.48, df= 268, p< 0.001) plots had significantly greater weight of tomatoes per plant than in the control plots. MeSA plots had a greater weight of tomatoes per plant than the control, although this difference was not significant (t= 72.69, df= 268, p= 0.058). There was also no significant difference seen between the Limonene and ML treatments (t= 39.31, df= 268, p= 0.577). On average, the limonene treatment produced a 24% greater weight of tomatoes per plant than the control, the MeSA and ML treatments produced 14% and 19% greater weight of tomatoes per plant than the control respectively. A one-way ANOVA test showed a significant difference in fruit count per plant (B) across the four treatments ($F_{(3,268)}$ = 9.99, p< 0.001). Limonene (t= 2.85, df= 268, p< 0.001) and ML (t= 1.51, df= 268, p= 0.027) treatments produced significantly more tomatoes than controls but there was no significant difference in tomato numbers between limonene and ML treatments (t= 1.33, df= 268, p= 0.095). There was no significant difference in tomato numbers between control and MeSA treatments (t= 0.33, df= 268, p= 0.926) but there was significantly more tomatoes in the limonene treatment compared with the MeSA treatment (t= 2.51, df= 268, p< 0.001). Error bars on both graphs (A and B) represent 95% confidence intervals. Significant differences between treatments are annotated onto the graph, bars with different letters (*a, b* or *c*) denote a significant difference between treatments.

3.3.3 Enzyme assays

Activity of the defence related enzymes PPO and POD were assessed in tomato leaf tissue following exposure to limonene dispensers (L), MeSA application (MeSA), herbivory from whiteflies (Tv) and a combination of MeSA application and herbivory from whiteflies (MeTv) (Figure 3.4). Enzyme activity is expressed as change in optical density per minute per gram of fresh weight and statistical analysis compared activity in each of the treatments to control (C) levels. There was a significant difference in POD activity (Figure 3.4, A) between the treatments and there was significantly higher POD activity after spraying with MeSA. POD activity after MeSA application was similar to that of a plant subject to whitefly herbivory (Tv), yet MeSA plus herbivory (MeTv) produced a significantly higher level of POD activity. The limonene (L) treatment had a similar POD activity to controls and there was no significant difference between the two. Levels of PPO activity (Figure 3.4, B) showed no significant change between the treatments (ANOVA, $F_{(4,35)} = 0.47$, $p = 0.752$).

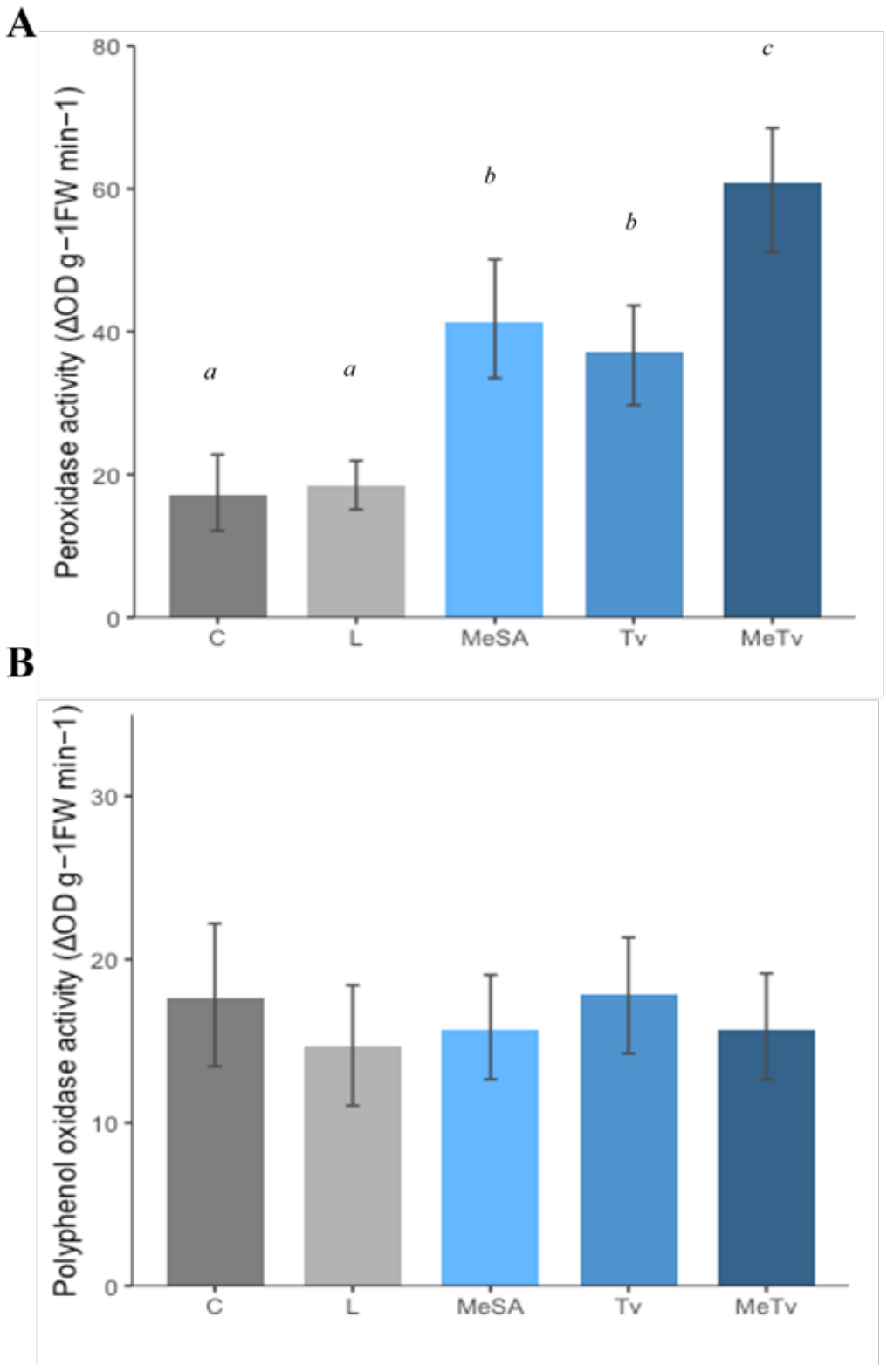


Figure 3.4: Activity of POD (A) and PPO (B) in control tomato leaf tissue (C) and after exposure to limonene slow release bottles (L), MeSA treatment (MeSA), herbivory from *T. vaporariorum* (Tv) and MeSA treatment + *T. vaporariorum* (MeTv) with error bars displaying standard error. Enzyme activity was analysed with one-way ANOVAs followed by Tukey HSD post-hoc tests. There was a significant difference in POD activity (Figure 3.4, A) between the treatments ($F_{(4,35)} = 23.19$, $p < 0.001$) but no significant effect was observed in PPO activity (Figure 3.4, B) ($F_{(4,35)} = 0.47$, $p = 0.752$). Tukey HSD post-hoc tests on POD activity revealed no significant difference between Control and Limonene treatments ($t = 0.06$, $df = 35$, $p = 0.91$)

but significant differences between Control and MeSA (t= 0.41, df= 35, p< 0.001), Tv (t= 0.36, df= 35, p< 0.001) and MeTv (t= 0.63, df= 35, p< 0.001). There was also a significant difference in POD activity between MeTV and MeSA (t= 0.22, df= 35, p= 0.044) and between MeTv and Tv (t= 0.27, df= 35, p= 0.008). Significant differences between treatments are annotated onto the graph, bars with different letters (*a, b* or *c*) denote a significant difference between treatments.

3.3.4 Defence gene expression

Figure 3.5 displays fold change gene expression relative to control expression levels of each gene. Defence related genes comprised LOX-1, TPX-1 and PR-1 and relative fold change for MeSA treated plants (MeSA), whitefly infested plants (Tv) and MeSA treated plus whitefly infested plants (MeTv) is displayed for each gene. All statistical analysis was performed on the Dcq values with one-way ANOVA's followed by Tukey HSD post-hoc tests. For all three genes, there was a significant difference in gene expression between the four treatments (C, MeSA, Tv and MeTv) following one-way ANOVA's. Whitefly infestation (Tv) significantly increased transcript levels of all three genes compared with respective control levels. Combination of both MeSA and whitefly infestation (MeTV) also produced significantly higher transcript levels than controls but did not result in any significant increases compared with either standalone treatments (Tv or MeSA). Our application of MeSA significantly down regulated the JA related LOX-1 and significantly up regulated the SA related (PR-1). The POD encoding TPX-1 was also significantly up-regulated following MeSA application, which is consistent with our experiments on POD activity (Figure 3.4, A).

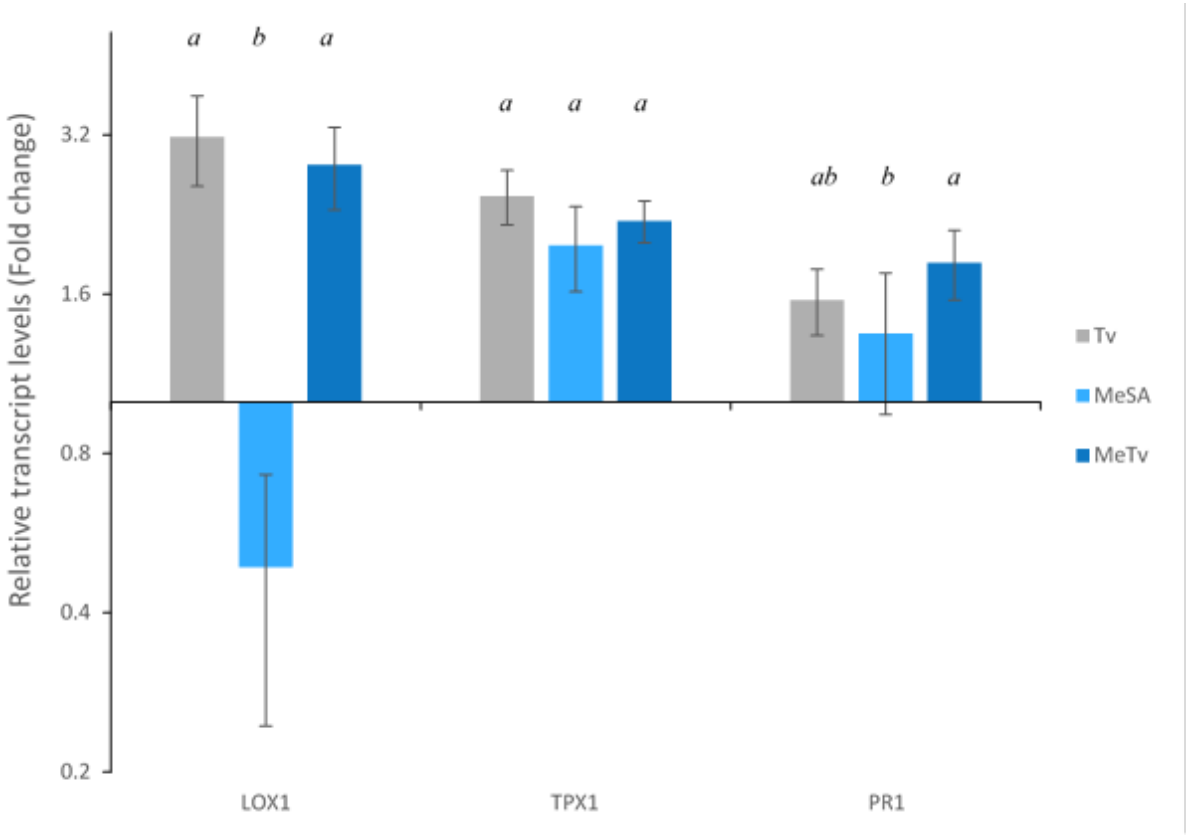


Figure 3.5: Relative fold change in expression levels of three tomato defence related genes (LOX-1, TPX-1 and PR-1) after MeSA application (MeSA), whitefly infestation (Tv) and MeSA plus whitefly infestation (MeTv). Data for each of the three genes is presented as average (*n*= 3) relative fold change compared with control transcript levels for each gene. Data analysis was performed on Dcq values and there were significant differences amongst treatments for all three genes following one-way ANOVA's (LOX-1 = $F_{(3, 32)}$ = 79.26, *p*< 0.001. TPX-1 = $F_{(3, 32)}$ = 21.37, *p*< 0.001. PR-1 = $F_{(3, 32)}$ = 10.91, *p*< 0.001). The Tukey HSD post-hoc test was used to test differences between the treatments. There were significantly more LOX-1 transcripts in the Tv (*t*= -1.66, *df*= 32, *p*<0.001) and MeTv (*t*= -1.49, *df*= 32, *p*<0.001) treatments but significantly fewer transcripts in the MeSA treatment (*t*= 1.03, *df*= 32, *p*<0.001) compared with controls. There was also significantly more LOX-1 transcripts in the Tv (*t*= -2.70, *df*= 32, *p*<0.001) and MeTv (*t*= -2.52, *df*= 32, *p*<0.001) treatments compared with the MeSA treatment. All three treatments had significantly higher amounts of TPX-1 (Tv, *t*= -1.29, *p*<0.001, MeSA, *t*= -0.98, *p*<0.001, MeTV, *t*= -1.13, *p*<0.001, *df*= 32 for all) and PR-1 (Tv, *t*= -0.64, *p*= 0.002, MeSA, *t*= -0.43, *p*= 0.048, MeTv, *t*= -0.87, *p*<0.001, *df*= 32 for all) transcripts compared with controls. There was also a significantly more PR-1 transcripts in the MeTv treatment compared with the standalone MeSA treatment (*t*= -0.44, *df*= 32, *p*= 0.042). Error bars display ninety-five percent confidence intervals; this was constructed using Dcq values. Significant differences between treatments are annotated onto the graph, bars with different letters (*a, b* or *c*) denote a significant difference between treatments.

3.4 Discussion

In a large-scale glasshouse trial, we assessed the efficacy of repellent limonene dispensers and plant elicitation with MeSA as combined and standalone control methods for glasshouse whiteflies. All treatments had a negative impact on all three whitefly life cycle stages at some point across the 53-day infestation period, with most of the significant effects between treatment plots and controls seen in the early stages of the experiment (Figure 3.2). We propose the insignificant differences seen for settling (Figure 3.2, A) and oviposition (Figure 3.2, B) post 20 dpi were most likely due to the comparatively large size of the plants, resulting in the limonene dispensers having less effect on whitefly behaviour as the compound diffused to lower concentrations throughout glasshouse. Although previous studies have shown volatiles to have efficacy against insects at up to 1.2 metres (Du et al. 2016), the nature of their repellence is understood to be dose dependant with higher concentrations having more of an effect (Ben Issa et al. 2016). Maintaining high concentrations of limonene throughout the canopy may prove key in deterring whitefly in a commercial glasshouse environment, where tomato plants can reach up to 4 metres in height. Similar to the plots with limonene dispensers, all significant effects on whitefly life cycle stages in MeSA plots were observed in the early stages of the experiment. Defence induction has been shown to be long lasting, even trans-generational (Rasmann et al. 2012), so the longevity of induced resistance is probably not the cause of these short-lived effects on whitefly performance. A potential explanation for this is that the whitefly population in the glasshouse reached a threshold which masked any effects of these defence induced plants. It is unlikely that growers would introduce plants into a glasshouse with such a large pre-existing pest population and the effects of our treatments may have been revealed more clearly with fewer whiteflies in the glasshouse. The inferior efficacy of MeSA treated plants could also highlight the inadequacies of the innate defence responses of this commercial tomato variety, which is known to be inferior to its wild cousins (McDaniel et al. 2016). Identifying aspects of defence that are found wanting in these commercial varieties could prove to be key in optimising the defence responses elicited by MeSA. Limonene and ML treatments produced a longer lasting effect on whitefly performance compared with MeSA plots with significantly fewer adults at 15dpi Figure 3.2, A) and significantly fewer nymphs at 20dpi (Figure 3.2, C) compared with control plots. Considering how plant defences and repellent volatiles challenge whiteflies in different ways, we expected the combined ML treatment to be more effective at reducing whitefly

performance than either standalone treatment. However whitefly abundance was similar on both ML and limonene treatments across the course of the experiment and there were no significant differences observed between the two treatments. Interestingly, limonene plots produced a slightly higher yield (g) and more tomatoes per plant than the ML plots (Figure 3.3), but these differences were not statistically significant. Whilst we cannot attribute any direct fitness costs associated with MeSA application, we can assert that addition of MeSA does not improve yield or decrease whitefly performance when combined with repellent volatile dispensers. Despite this, the MeSA standalone treatment still produced a greater yield than control plots (Figure 3.3, A), proving that there is still value in applying MeSA to elicit tomato defences. Whitefly feeding is known to result in a loss of yield (McKee et al. 2007) and these results show that if left unprotected and exposed to relatively high numbers of insects, tomato can be susceptible to yield loss. The increase in yield (g) from treated plots (Figure 3.3) is presumably a consequence of the whitefly performance reduction seen in Figure3.2 and illustrates the effectiveness of our control measures used here. It is possible that the untreated plots could have acted as a refuge for the whiteflies as they were "pushed" from the plots containing the repellent volatile dispensers and/or defence induced plants. Even if this was the case, this still demonstrates the ability of our VOC control measures to manipulate whitefly behaviour and illustrates their potential for incorporation to other IPM systems. In a no-choice situation we predict that limonene would still inhibit whitefly performance as previous groups have shown whitefly to become "restless" when exposed to high concentrations of VOCs (Bernays 1999).

Plants from each of our treatments were assayed for activity of the defensive enzymes PPOs and PODs in order to characterise the resistance to whitefly shown in MeSA treated plants and to ascertain whether limonene dispensers had any non-target effects on plant defence. Plants grown alongside limonene dispensers had levels of PPO and POD activity similar to controls (Figure 3.4), suggesting that direct repellent effects of this chemical are responsible for the decreased whitefly performance, as we have shown previously (Conboy et al. 2019). Other groups have found tomato PPOs to be induced by insect herbivory (Bhonwong et al. 2009; Rowen et al. 2017) however we found no significant changes in PPO activity between our treatments (Figure 3.4, B). Unlike PPO, we found significant differences in POD activity across our treatments and POD activity was significantly increased following MeSA application (Figure 3.4, A). Plant PODs are salicylic acid (SA) related pathogenesis proteins and their ability to scavenge reactive oxygen species (ROS) is known to contribute to increased plant fitness

(Nath et al. 2016). Other groups have correlated plant PODs with resistance to whiteflies (Zhang et al. 2008; Taggar et al. 2012) and the increase in POD activity observed here could contribute to the increased resistance to whitefly shown in these plants. We cannot claim that our application of MeSA has a priming effect on POD activity as one of the key stipulations of a primed plant is that defences are only transiently or partially induced following the priming stimulus (Martinez-Medina et al. 2016). This is an example of direct defence induction and MeSA in the form of a spray application was found to have a similar effect on POD activity in poplar (Tang et al. 2015). Despite this, it seems that MeSA has increased the capacity of the plant to produce more of this defensive protein as POD activity in MeTv plants was significantly higher than whitefly infested plants (Figure 3.4, A). Whilst plant defence priming is often associated with preparing for future attack, a primed state may also persist as a residual effect following initial exposure to a stress (Frost et al. 2008). This could be an explanation for the results we have observed here, with MeSA inducing defence responses to an elevated level, followed by stimulation of defences by whitefly infestation resulting in an elevated response. We hypothesised that these enhanced levels of POD in the MeTv treatment could be due to accumulation of peroxidase transcripts following MeSA application, leading to an augmented response upon whitefly infestation. We therefore undertook qPCR of a known peroxidase gene, TPX-1, and observed a significant increase in TPX-1 transcripts following MeSA application (Figure 3.5). Whilst we did not observe the same combined effect on TPX-1 transcript levels in the MeTV treatment, it is pertinent to consider that multiple genes contribute to peroxidase activity. This particular peroxidase gene is specific to cell wall lignification, but a more thorough analyses of peroxidase genes could potentially reveal similar expression patterns in other areas of antioxidant defence. More importantly, these results provide insight into how MeSA elicitation influences tomato antioxidant defences that could be responsible for increased resistance to whiteflies.
Further gene expression analysis was conducted to decipher how MeSA impacts tomato defence signalling pathways (Figure 3.5). TPX-1 and PR-1 transcript levels significantly increased (compared with control levels) following MeSA application. Importantly, there was no significant difference in TPX-1 and PR-1 transcript levels when comparing whitefly infested transcript levels (Tv) with the combined treatment (MeTv). This shows that MeSA does not prime the accumulation of these defence related genes. Whilst plant priming has previously been characterised by mRNA accumulation (Martinez-Medina et al. 2016), mRNA levels during the priming phase have been shown to be small in comparison to mRNA levels induced

by the triggering stimulus (Balmer et al. 2015). The fact that transcript levels are similar in both MeSA and MeTv treatments would suggest that priming has not occurred and that defences have activated with MeSA. Our glasshouse trial illustrates the protective effect of MeSA application and these genetic assays indicate that this increased resistance to whiteflies could be caused by induction of SA related genes, which have been shown to be induced by phloem feeding insects (Smith and Boyko 2007). We propose that spraying of exogenous MeSA has induced this signalling pathway, similar to how methyl jasmonate (MeJA) has been shown to influence the JA signalling pathway (Wu et al. 2008). There is currently a disparity amongst published literature with some groups showing MeSA to induce JA related defences (Rowen et al. 2017) and other groups showing induction of SA related defences (Park 2008). Whilst we can associate MeSA with induction of SA related defences, which may correlate with increased resistance to whiteflies, the way in which MeSA influences plant signalling pathways remains unclear. MeSA caused down regulation of the JA related LOX-1, however both Tv and MeTv treatments had significantly higher levels of LOX-1 transcripts than controls. JA and SA related defences are often thought to be antagonistic, whereas here we see both SA and JA related genes induced by whitefly feeding. Cross talk between these two pathways may be responsible for the results seen here but this cannot be ascertained from the 3 genes we analysed. Genomic analyses of tomato subject to MeSA and/or whiteflies would fully elucidate the effect whitefly herbivory and defence elicitation with MeSA have on tomato defence. This is an essential next step to this research as successful plant elicitation relies on the stimulation of the pertinent metabolic processes.

We set out to prime tomato with our application of MeSA, however our subsequent enzyme and genetic assays have revealed that defences were immediately induced with MeSA. It has been previously reported that lower concentrations of defence eliciting compounds can induce priming, whereas larger concentrations immediately activate defences (van Hulten et al. 2006). By decreasing the amount of the MeSA it could still be possible to induce priming in tomato, although we predict that this precise nature of delivery could make plant elicitors difficult to use, especially for unexperienced home growers. Despite this, our work does demonstrate that if growers were to introduce plants to a glasshouse with significant pest pressure, application of MeSA could still increase yield. We only tested efficacy of MeSA for protection against whiteflies but other groups have shown MeSA to confer resistance to pathogens (Shulaev et al. 1997) and even caterpillars (Rowen et al. 2017). This broad-spectrum resistance could be very attractive to horticulturalists and MeSA could provide an

environmentally benign alternative to current synthetic defence elicitors such as benzothiadiazole's, which have been shown to have limitations (Kouzai et al. 2018).

Our work highlights the efficacy of repellent volatile chemistry as a protection method against whiteflies on glasshouse grown tomato and direct repellence of whiteflies using slow-release limonene dispensers is the most attractive whitefly control method investigated here. Limonene dispensers were extremely effective at deterring whitefly from the target crop and offer a cheap, safe, and environmentally benign control method for glasshouse grown tomatoes that could be very attractive to both commercial and domestic horticulturalists. We show that limonene dispensers can effectively "push" whiteflies from a target crop that translates to a more productive yield from these protected plots. Limonene could also be incorporated into a push-pull system with other volatile dispensers containing compounds attractive to whiteflies, similar to what has been demonstrated with companion plants (Pickett et al. 2014). Considering that other pests such as mealy bugs (Hollingsworth 2005), mites (Ibrahim et al. 2001) and beetles (Raffa et al. 1985) are repelled by limonene, the potential uses of this control measure could far exceed that of whitefly management. Further studies will be needed to deduce its physiological effect on whiteflies, but the fact that limonene does not seem to cause mortality could be seen as another positive factor. Tolerating and repelling pests rather than completely eradicating them is a more sustainable method of pest control and something which we should look to endorse in our efforts to limit resistant pest genotypes and grow our food sustainably (Peterson et al. 2018).

Chapter 4. How a Cultivated and Wild Tomato Species Interface with their Volatile Environment

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Abstract

Wild ancestors of cultivated crops are often more resistant to insect pests and in previous work we found a wild tomato species (*Solanum pimpenillefolium* L.*)* to be more resistant to glasshouse whiteflies (*Trialeurodes vaporariorum,* Westwood) than a cultivated tomato variety (*Solanum lycopersicum var.* 'Elegance'). In this study, we attempt to determine the constitutive and whitefly-induced resistance mechanisms in 'Elegance' and *S. pimpenillefolium,* with a focus on how each species produces and responds to volatile organic compounds (VOCs). VOCs are an essential element of plant defence and here we show that the wild *S. pimpenillefolium* and cultivated 'Elegance' release a different composition of constitutive and whitefly induced VOCs. In olfactory preference assays, whiteflies were repelled by constitutive VOCs from *S. pimpenillefolium* and we propose that increased emissions of whitefly repellent terpenes such as limonene could be responsible for this antixenotic effect. Both constitutive and whitefly induced VOCs from *S. pimpenillefolium* make these plants more attractive to *Encarsia formosa* (Gahan), a commonly used biocontrol organism for suppression of whitefly infestations. We observed significantly higher constitutive and whitefly induced Peroxidase (POD) activity in *S. pimpenillefolium*, along with significantly higher levels of whitefly-induced Polyphenol oxidases (PPO). These superior constitutive and inducible biochemical defences in *S. pimpenillefolium* contributing to increased resistance to whiteflies. Our phytohormone analysis and genetic assays presents evidence to suggest that whiteflies induce SA related defences more strongly in 'Elegance'. Whilst we do not see any significant effects on the antagonistic JA related defences, which are known to be detrimental to whitefly performance, the distinct differences in whitefly-induced defence signalling could have wider implications for the way these plants defend against whiteflies. Previous exposure to herbivore induced plant volatiles (HIPVs) from whitefly-

infested conspecifics had no effect on defensive enzyme activity or phytohormone abundance for either tomato species. However, previous exposure to HIPVs was found to prime a whitefly resistance gene (Mi-1.2) in *S. pimpenillefolium*. This same effect was not observed in 'Elegance', which significantly increased Mi-1.2 transcript abundance after exposure to HIPVs (i.e. defences were directly induced). In summary, we found that *S. pimpenillefolium* interface with their volatile environment more effectively than 'Elegance', highlighting another essential evolutionary trait that has been lost through domestication. The resistance mechanisms elucidated in this study could be used to inform future breeding programs to enhance whitefly resistance in cultivated tomato.

4.1 Introduction

Centuries of selective breeding has led to agricultural crops that invest a disproportionately large amount of energy in growth related traits (Huot et al. 2014). This unbalanced resource allocation has come at the expense of innate resistance mechanisms and can make cultivated crops vulnerable to insect pests (Chaudhary 2013). Numerous studies have demonstrated that the re-introduction of resistant traits from wild ancestors of commercially grown crops can improve resistance to insect pests (Degenhardt et al. 2009; Zsogon et al. 2018; Bleeker et al. 2012; Escobar-Bravo et al. 2016). This can be achieved with conventional selective breeding techniques (Vidavski 2007; Erb et al. 1994) or, more precisely, with gene editing technologies (Bleeker et al. 2012; Zsogon et al. 2018). Whilst genetically modified crops are still prohibited in Europe, genetic engineering is expected to be fundamentally important for future sustainable pest management practices (Kennedy 2008). Accessing this source of resistant traits in wild ancestors of cultivated crops could be crucial in our efforts to control insect pests and grow crops more sustainably (Mammadov et al. 2018).

The glasshouse whitefly (*Trialeurodes vaporariorum*) is a phloem-feeding pest of global importance that causes yield losses through direct feeding damage (Byrne et al. 1990) and transmission of numerous plant viruses (Wainaina et al. 2018). Whiteflies are one of the most important pests of glasshouse-grown tomato in the UK (Gorman et al. 2007; Lange and Bronson 1981) and horticulturalists employ numerous chemical and/or biological control

methods to protect their crops. Whilst whitefly control with the parasitoid *Encarsia formosa* (Gahan) can be effective, issues such as delayed efficacy and hyper-parasitism mean there is still a reliance on synthetic chemical sprays (George et al. 2015), which have various negative environmental and human health related implications (Hillocks 2012; Nicolopoulou-Stamati et al. 2016). The need for alternative control methods has led some to explore the transfer of whitefly resistant traits from wild ancestors to cultivated tomato varieties (Bleeker et al. 2012; Zsogon et al. 2018; Escobar-Bravo et al. 2016). Intrinsic heritable plant resistance is a key component of sustainable pest management practices such as integrated pest management (IPM) (Stenberg 2017; Bruce et al. 2017; Peterson et al. 2018) and restoring whitefly resistant traits to cultivated tomato could help improve whitefly control. Whilst whitefly resistance has been documented in numerous wild tomato species (Firdaus et al. 2012; McDaniel et al. 2016; Zsogon et al. 2018), the mechanisms by which these wild plants resist whitefly herbivory are still not fully understood.

Tomato plants have a variety of constitutive and inducible defence mechanisms that can serve to negate the detrimental effects of whitefly herbivory. Defensive enzymes, volatile organic compounds (VOCs), trichomes and phenolic compounds are all present in tomato and have been shown to have activity against whiteflies (Dowd and Lagrimini 2006; Bleeker et al. 2009; Zhang et al. 2017; Rakha et al. 2017). Wild tomato species have been shown to exhibit higher levels of constitutive defences such as VOCs and trichomes, which make these plants more resistant to whiteflies (Rakha et al. 2017; Bleeker et al. 2009). Whilst some efforts have been made to restore whitefly-resistant traits to cultivated tomato varieties (Escobar-Bravo et al. 2016; Bleeker et al. 2012; Leckie et al. 2012), these examples have focused on *Bemisia tabaci*, a whitefly species not currently established in the UK. Whilst morphologically similar to the glasshouse whitefly, the feeding methods and associated plant defences induced by these two species are distinct (Jiang et al. 1999; Zhang et al. 2017). In order to inform the direction of future breeding programs, it is essential that we characterise the mechanisms by which wild tomato resist herbivory from glasshouse whiteflies (*Trialeurodes vaporariorum*). In previous work, we found the wild tomato species *Solanum pimpenillefolium* to be more resistant to glasshouse whiteflies than a cultivated tomato variety (*Solanum lycopersicum* var. 'Elegance') (McDaniel et al. 2016). The present study will compare glasshouse whitefly resistance mechanisms in wild *S. pimpenillefolium* and the domesticated 'Elegance' tomato.

Volatile organic compounds (VOCs) are an important element of plant defence (Heil 2014; Vivaldo et al. 2017) and tomato VOCs have been found to repel whiteflies (Conboy et al. 2019; Bleeker et al. 2009) and attract beneficial insects (Kost and Heil 2006). Decreased VOC output in commercially grown crops has been correlated with susceptibility to insect pests (Degenhardt et al. 2009; Sanchez-Hernandez et al. 2006) and can also make plants less attractive to beneficial insects (Gols et al. 2011; Degenhardt et al. 2009). Tomato species are known to vary in leaf volatile composition (Bleeker et al. 2009b; Lundgren et al. 1985) and some research groups have shown that wild tomato species produce a greater quantity of VOCs compared to cultivated tomato (Bleeker et al. 2009; Firdaus et al. 2012). Some groups have made attempts to restore pest resistance to cultivated crops by introducing VOCs which repel insect pests (Bleeker et al. 2012) or attract beneficial insects (Degenhardt et al. 2009). Analysis of *S. pimpenillefolium* VOCs could be used to characterise the antixenotic effects of this plant species on whiteflies observed previously (McDaniel et al. 2016). In addition to the synthesis and release of VOCs, plants can interpret changes in environmental VOCs to warn of herbivore attack, anticipate drought and recognise the presence of other plant species (Erb 2018; Erb et al. 2015; Arimura et al. 2010). Plants perceive herbivore induced plant volatiles (HIPVs) as a warning of impending insect attack and respond by either directly inducing their innate defensive measures, or priming them (Heil and Ton 2008). Plant priming is a phenomenon where the plant undergoes an induced state of readiness and will respond more effectively to subsequent insect attack (Martinez-Medina et al. 2016). Plant priming is seen as a viable plant protection method that could be compatible with current whitefly-focused IPM systems (Bruce et al. 2017). In much the same way that domesticated plants have been shown to produce fewer VOCs than wild counterparts, the ability to interpret and respond to airborne distress signals (HIPVs) may have been impaired through centuries of domestication. This is an issue yet to be addressed in published literature and is something that we investigate in this study.

Restoring pest resistant traits to commercially grown crops from their wild ancestors is the focus of a great deal of research attention (Zsogon et al. 2018; Rakha et al. 2017; Escobar-Bravo et al. 2016; Rodriguez-Lopez et al. 2011). To even consider this possibility, the biochemical and genetic mechanisms by which wild plants resist insect herbivory must be determined. In this study, we conduct a comprehensive analysis of how the wild *S. pimpenillefolium* and commercially grown tomato variety exhibit constitutive and whitefly-

induced defence mechanisms. We assess how each of these tomato species contribute to their volatile environment, with a focus on how endogenous (originating from the plant) airborne signals interact with whiteflies and *E. formosa*. Domestication has led to inferior defences in cultivated crops and we also sought to determine whether the ability to interpret and respond to airborne distress signals has also been impaired. To this end, we exposed 'Elegance' and *S. pimpenillefolium* to HIPVs from whitefly-infested conspecifics and measured defensive enzyme activity, phytohormone abundance and defence related gene expression to characterize any elicitation of plant defences.

4.2 Materials and Methods

4.2.1 Insects

Glasshouse whiteflies (*Trialeurodes vaporariorum* Westwood) originated from a lab culture at Rothamsted Research which was first collected in 1960 in Kent on French bean. Whiteflies were taken from a mixed age colony maintained on pre flowering aubergine (*Solanum melongena* "Moneymaker"- Marshalls Seeds Cat. 1020-2017) at 20 °C, 16:8 light/dark. *Encarsia formosa* (Gahan) were obtained from Koppert biological systems in the form of an 'En-strip' (https://www.koppert.co.uk/en-strip/) which comprised a small card (6 x 3 cm) with ~300 whitefly pupae parasitized with *E. formosa*. 'En-strip' cards were used for olfactory experiments on the day of arrival.

4.2.2 Plants

Commercial tomato seeds (*S. lycopersicum*, 'Elegance' Cat. Batch 0160685360) were obtained from Monsanto and wild tomato *S. pimpenillefolium* seeds were obtained from Magic Garden Seeds Ltd. (product code LYC09). In the laboratory assays, plants were grown from seed in J. Arthur Bowers John Innes no 2 compost in square seed tray inserts (https://amzn.to/2Gj8rlm). All plants were grown at a distance of approximately 60 cm from a 400-W Son-T bulb housed in a Harrier HR400SH 400-W lamp (6000 lumens) under a 16 h light/8 h dark cycle and the temperature regime was 25 °C in the light and 20 °C during the dark period. Plants used for all experiments were between 2 and 3 weeks old and at stage 12 on the BBCH scale (Klingauf 2001).

4.2.3 Air entrainment

Headspace volatiles of un-infested and whitefly infested tomato plants were collected by dynamic air entrainment (Pye Volatile Collection Kit, Kings Walden, Herts, UK). All equipment was washed with Teepol detergent (Herts County Supplies, Herts, UK) and rinsed with acetone and distilled water twice before baking at 180°C for 2 hours. Porapak Q (60/80 mesh, 0.05 g) tubes were eluted with hexane and heated at 140°C for 2 hr under a stream of constant nitrogen to remove contaminants, this process was repeated twice for each tube. 'Elegance' and *S. pimpenillefolium* seedlings were enclosed in a glass bell cylinder, the bottom of the cylinder was closed without pressure around the plant stem by using two semicircular aluminum plates with a hole in the centre to accommodate the stem. Due to the slight differences in plant size, fresh weight (g) of the entrained plant section was recorded after use. Charcoal filtered air was pumped in at 1L min^{-1} and drawn out at 800 ml min^{-1} through the porapak Q adsorbent tube in a 5-mm diameter glass tube. The difference in flow rates created a slight positive pressure to ensure that unfiltered air did not enter the system, thus removing the need for an airtight seal around the stem. Plants were entrained for 12 hours. The porapak Q filter tube was eluted with 0.75 ml of hexane (Fisher Scientific, 12347103), providing a 500µl solution that contained the isolated volatile compounds. All samples were tightly sealed in GC vials and stored at -20°C until needed.

4.2.4 Gas Chromatography (GC)

GC-FID analyses were carried out using a Agilent 5890 GC. The injection port (280°C) was in the splitless mode and the flame ionization detector was heated to 300°C. The sample (1ul in hexane) was injected with a HP7673 auto sampler and the split opened after 1 minute. Separation was performed on a fused silica capillary column (30m x 0.25mm i.d) coated with 0.25um dimethyl poly-siloxane (HP-5 phase). The GC was temperature programmed from 50°C-310°C at 5°C min and held at final temperature for 20 minutes with Hydrogen as the carrier gas (flow 1ml/min, pressure of 50kPa, split at 30 mls/min).

Gas Chromatography-mass spectrometry (GC-MS)

GC-MS analysis of the biological extracts was performed on a Agilent 7890A GC split/split less injector (280°C) linked to a Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1200V, interface temperature 310°C). The

acquisition was controlled by a HP Compaq computer using Chemstation software, initially in full scan mode (50-600 amu/sec) or in selected ion mode (30ions 0.7cps 35ms dwell) for greater sensitivity. The sample (1ul) in hexane was injected by an Agilent7683B auto sampler and the split opened after 1 minute. Separation was performed on an Agilent fused silica capillary column (30m x 0.25mm i.d) coated with 0.25um dimethyl polysiloxane (HP-5) phase. The GC was temperature programmed from 50-310°C at 5°C min and held at final temperature for 10 minutes with Helium as the carrier gas (flow rate of 1ml/min, initial pressure of 50kPa, split at 30 mls/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST library if > 90% fit or from their elution order from biochemical literature. Presence of compounds was confirmed by comparison with authentic standards and subsequently semi-quantified using the single point external standard method.

4.2.5 Olfaction assays

Whitefly and *E. formosa* response to volatile odours from 'Elegance' and *S. pimpenillefolium* was assessed using a 4-arm olfactometer as described in (Pettersson 1970). This consisted of an enclosed, airtight Perspex arena with four 'arms' which directed airflow into the central arena. At the end of each of the four arms, a 'partial non-return' glass bulb was fitted which limited the chance of insects returning to the central arena after entering one of the four arms of the olfactometer. An airstream of 400 ml per min was created by removing air from the centre of the arena with a vacuum pump. The scent of the odour sources was added to the airflow by connecting four transparent, sealable plastic boxes (odour source chambers) to each of the four arms with odourless polytetrafluoroethylene (PTFE) tubing. The charcoal filtered air flowed over the odour source into each of the four arms towards the central arena. For 'un-infested' assays, insects were presented with odours from 'Elegance' and *S. pimpenllefolium* seedlings at stage 12 on the BBCH scale. We used two 'Elegance' seedlings, along with two *S. pimenillefolium* seedlings for each assay (four plants in total), and a single plant was placed in each odour source chamber. This meant insects had the option of odours from two different individual plants from each species. To avoid directional bias, the position of the plants was rotated 90° after each assay, although plants of the same species were always placed at a 180° angle from each other. For the 'Whitefly infested' assays, the set up was exactly the same as the un-infested assay, only each of the four plants was infested with

100 whiteflies. To assess whitefly preference, we released 100 adult whiteflies into the central arena and were left for 12 hours. For analysis of *E. formosa* plant preference, we placed four 'en-strip' cards in the central arena and allowed *E. formosa* adults to emerge and respond to odour sources over a period of 72 hours. After the allotted time period, any insects which were found in the 'partial non-return' glass bulbs were adjudged to have made a choice towards the corresponding odour source. The entire apparatus was cleaned between each experiment and covered with an opaque sheet during experimental procedures to ensure the light intensity was approximately equal. The experiment was repeated six times per treatment.

4.2.6 Preparation of treatments for enzyme, phytohormone and genetic assays

All four treatments (un-infested plants (C), HIPV exposed plants (VE), whitefly infested plants (Tv) and whitefly infested + HIPV exposed plants (TvE)) used in the enzyme, phytohormone and genetic assays were prepared in the same way. We modified a four-arm olfactometer by replacing the central chamber with a transparent, airtight plastic box which was large enough to fit several tomato seedlings (See Figure 4.1 for image). By placing tomato seedlings in this central chamber, along with tomato seedlings in the odour source chambers, we were able to expose tomato seedlings to volatile odours from either un-infested or whitefly infested conspecifics in a controlled and precise manner. This central chamber was connected to the four odour source chambers with odourless PTFE tubing only. An airstream of 400 ml per min was created by removing air from the centre of the arena with a vacuum pump. The scent of the odour sources was added to the airflow by connecting four transparent, sealable plastic boxes (odour source chambers) to each of the four arms with odourless PTFE tubing. For both 'Elegance' and *S. pimpenillefolium*, the four treatments were prepared in exactly the same way. For the control treatment, three un-infested tomato seedlings at stage 12 on the BBCH scale were placed inside the central chamber, along with a single un-infested tomato seedling in each of the four odour source chambers. Plants in the central chamber were exposed to odours from these four un-infested tomato seedlings for a period of 12 hours. For the VE treatment, the experiment was set up in the same way only each tomato seedling in the odour source chambers was infested with 100 whiteflies. For the Tv treatment, the experiment was run in the exact same way as for the control treatment, but after 12 hours the plants in the central chamber were removed and placed inside cylindrical mesh cages

(Watkins and Doncaster, product code: E6090). These plants were then infested with 100 whiteflies for 24 hours. The TvE treatment was a combination of the VE and Tv treatments. Plants were exposed to odours from whitefly-infested conspecifics for 12 hours, then placed inside cylindrical mesh cages and infested with whiteflies for 24 hours. For each of the four treatments, phytohormone abundance, enzyme activity and gene expression was measured using the second compound leaf of each tomato seedling.

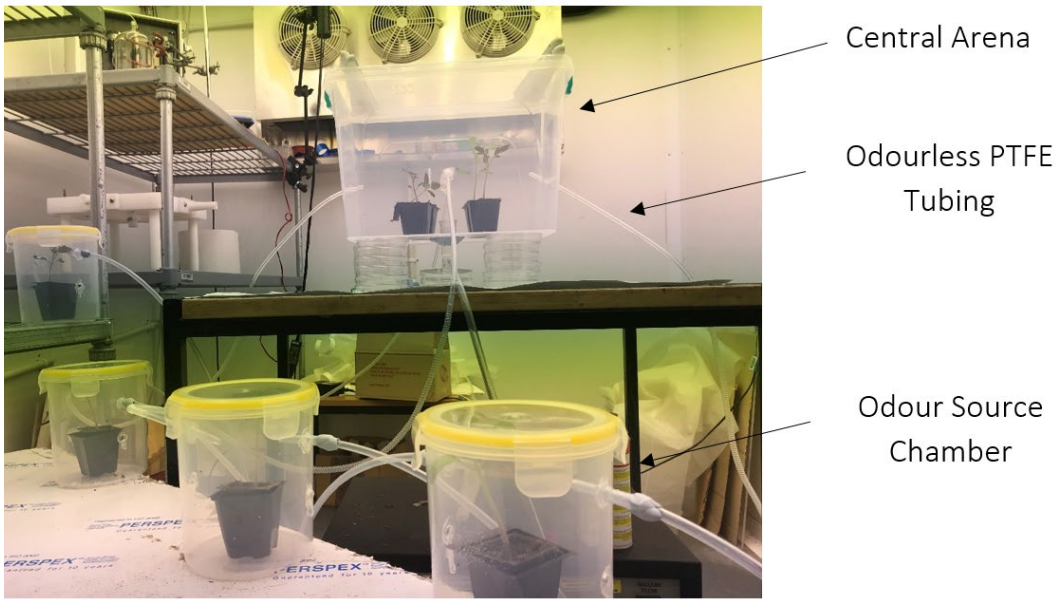

Figure 2.1: Experimental set-up used to present 'Elegance' and *S. pimpenillefolium* seedlings to odours from un-infested or whitefly-infested conspecifics. Full description of the experimental set-up can be viewed under the materials and methods subheading 'Preparation of treatments for enzyme, phytohormone and genetic assays'.

4.2.7 Phytohormone analysis

Analysis of plant phytohormones was carried out as described in (Forcat et al. 2008), with some minor alterations. For each of the four treatments (C, VE, Tv, TvE), the whole 2nd compound leaf of each plant was harvested into liquid nitrogen and freeze dried. Samples (*n*= 3) were next placed in a 2 ml microfuge tube and ground in a bead beater (Qiagen or equivalent) with 3 mm tungsten beads at 25 Hz/s for 3 min. The powdered tissue was weighed and then transferred a new 2 ml microfuge tube and extracted with 400 µl of 10% methanol containing 1% acetic acid to which internal standards had been added (1 ng of $^{2}H_{6}$ ABA, 10 ng of $^{2}H_{2}$ JA and 13.8 ng $^{2}H_{4}$ SA). Each treatment also included an extraction

control containing no plant material. A 3 mm tungsten bead was placed in each microfuge tube and samples were extracted in the bead beater for 2 min at 25 Hz/s, placed on ice for 30 min then centrifuged at 13,000 g for 10 min at 4°C. The supernatant was carefully removed and the pellet re-extracted with 400 µl of 10% methanol containing 1% acetic acid. Following a further 30 min incubation on ice, the extract was centrifuged and the supernatants pooled. Samples (10 µl) were then analysed by HPLC-electrospray ionisation/MS-MS using an Agilent 1100 HPLC coupled to an Applied Biosystems Q-TRAP 2000 (Applied Biosystems, California, USA). Chromatographic separation was carried out on a Phenomenex Luna 3 µm C18(2) 100 mm × 2.0 mm column, at 35°C. The solvent gradient used was 100%A (94.9% H_2O: 5% CH_3CN: 0.1% CHOOH) to 100%B (5% H_2O: 94.9% CH_3CN: 0.1% CHOOH) over 20 min. Solvent B was held at 100% for 5 min then the solvent returned to 100% A for 10 min equilibration prior to the next injection. The solvent flow rate was 200 µl/min. To reduce contamination of the MS, the first 2 min of the run was directed to waste using the inbuilt Valco valve.

4.2.8 Enzyme assays

Peroxidases (POD) and polyphenol oxidases (PPO) are defence related enzymes which are often used as chemical markers of induced resistance in members of the *Solanaceae* family (Karban et al. 2003; Karban 2011). Both of these enzymes are produced constitutively by tomato but are also induced in response to whitefly feeding (Mayer et al. 2002; McKenzie et al. 2002; Su et al. 2015). We sought to assess activity of these defensive enzymes in 'Elegance' and *S. pimpenillefolium* to characterise the way in which these two species respond to whiteflies and/or HIPVs. All four treatments for this experiment (C, VE, Tv and TvE) were implemented as described under the materials and methods subheading 'Preparation of treatments for enzyme, phytohormone and genetic assays'. PPO and POD activities were measured using modified methods Thaler et al. (1996) and (Rowen et al. 2017). For all treatments, the whole 2nd compound leaf was removed and flash frozen in liquid nitrogen. This tissue was homogenised in 1.25 ml of ice-cold K-phosphate buffer (0.1 M, pH 7) containing 2% (*w*/*v*) polyvinylpolyprolidine (Sigma-Aldrich). Subsequently 0.4 ml of 10% Triton X-100 (Sigma-Aldrich) was added to the homogenate, vortexed and centrifuged at 6000 rpm at 4 °C for 15 min. For the analysis of PPO activity, 50 µL of the supernatant was added to 200 µl of 29.2 mM caffeic acid in K-phosphate buffer (0.1 M, pH 8). For the analysis of POD activity, 30 µl of the supernatant was added to 220 µl of 0.3% guaiacol and 0.1% H_2O_2 in K-

phosphate buffer (0.1 M, pH 8). PPO and POD activities were determined by tracking change in absorbance at 450 nm over 10 min. Activities are presented as change in optical density per minute per gram of fresh weight (*n*= 6) (Figure 4.4).

4.2.9 Genetic analysis

In order to understand the resistance to whitefly shown by *S. pimpenillefolium*, we undertook genetic analysis of two tomato defence related genes Mi-1.2 and PR-1. The Mi-1.2 gene is positively correlated with resistance to a range of phloem feeding insects, including whiteflies (Lavrova et al. 2016). Whilst the associated protein is yet to be fully characterised, it is thought to act by blocking the establishment of feeding sites in vascular tissues of the plant (Pallipparambil et al. 2015). The PR-1 gene encodes a pathogenesis related (PR) protein and is commonly used as a marker of systemic acquired resistance (SAR) in across a wide range of higher plants (Medeiros et al. 2017; Chinnasri et al. 2016). We assessed activity of PR-1 due to its close association with the SA defence-signalling pathway (Riviere et al. 2008). All treatments for this experiment (C, VE, Tv and TvE) were prepared as described under the materials and methods subheading 'Preparation of treatments for enzyme, phytohormone and genetic assays'.

Total RNA was isolated with Trizol Reagent (Invitrogen, Thermo Fisher Scientific) and purified with PureLink RNA Mini Kit (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. Concentrations of the RNA preparations were determined photometrically using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The RNA preparations were stored at −80℃ until use. One µg of RNA was reverse transcribed using a reverse transcriptase kit to synthesize cDNA. qPCR was performed with a Rotor-Gene Q (Qiagen), using SensiFAST SYBR No-ROX Kit (Bioline) for 5 min at 95 °C, followed by 35 cycles consisting of 20 s at 95 °C, 30 s at 57 °C and 30 s at 72 °C, then 10 min at 72 °C. All quantifications were normalized to the reference gene actin using the delta ct method. The qPCR reactions were performed using three independent RNA preparations from independently grown plants and each RNA sample was run in triplicate for each qPCR run. Primer sequences can be viewed in Appendix Table E.

4.2.10 Data analysis

Data for individual VOCs (*n*= 3) were analysed with one-way ANOVA's followed by Tukey HSD post-hoc tests. Data for the emission of ß- ocimene and humulene (both VOCs released by tomato) was (log +1) transformed in order to meet normality and homogeneity of variance assumptions. Data from olfactory preference experiments was transformed to percentages and average percentages were compared between each plant species with *t* tests (*n*= 6). Data for enzyme activity (*n*= 6) and phytohormone abundance (*n*= 3) was analysed using two-way ANOVA's, with species and treatment as factors. Due to the large amount of post-hoc tests for phytohormone abundance and enzyme activity (28 in total), the Bonferroni post-hoc correction was used account for the higher degree of experiment wise error. This was done by multiplying *p* values by the total number of comparisons made (28), therefore all *p* values reported are significant at α = 0.05. Data for IND, JA and SA was (log) transformed to meet normality and homogeneity of variance assumptions. Statistical analysis of gene expression assays (*n*= 3) was performed using the Dcq values using one-way ANOVA's followed by Tukey HSD post-hoc tests. All statistical analysis was performed using the base stats package in R.

4.3 Results

In order to compare how 'Elegance' and *S. pimpenillefolium* respond to whitefly infestation, VOCs from un-infested plants (C) and HIPVs from whitefly-infested plants (Tv) were quantified using dynamic air entrainment (Figure 4.2). In nine of the thirteen compounds identified, there was a significant difference in emissions across all four treatments ('Elegance C', Elegance Tv, *S. pimpenillefolium* C, *S. pimpenillefolium* Tv) following one-way ANOVA's (see figure legend for ANOVA results). Both tomato species responded to whitefly infestation by significantly increasing emission of 2-hexenal ('Elegance': t= 0.94, df= 3 p< 0.001, *S. pimpenillefolium; t*= 0.63, *df= 3*, *p*< 0.001) and MeSA ('Elegance': t= 0.54, df= 9, p= 0.015, *S. pimpenillefolium; t*= 0.74, *df*= 3, *p*= 0.002). In addition to this, *S. pimpenillefolium* significantly increased emissions of α–pinene (*t*= 0.76, *df*= 8, *p*= 0.007), ß- myrcene (*t*= 1.04, *df*= 3, *p*= 0.001) and ß- ocimene (*t*= 1.04, *df*= 3, *p*= 0.021) in response to whiteflies. 'Elegance' significantly increased emissions of 2-carene (*t*= 0.66, *df*= 3, *p*< 0.001), ß-caryophyllene (*t*= 3.24, *df*= 3, *p*< 0.001) and humulene (*t*= 1.31, *df*= 3, *p*< 0.001) in response to whiteflies.

Compared with 'Elegance', *S. pimpenillefolium* had significantly greater constitutive (C) emissions of 2-hexenal (t= 1.09, df= 3, p< 0.001), limonene (t= 0.428, df= 3, p= 0.020), 2-carene (t= 0.56, df= 3, p= 0.002), ß-caryophyllene (t= 2.18, df= 3, p= 0.002) and humulene (t= 1.02, df= 3, p< 0.001), along with significantly higher emissions of 2-hexenal (t= 0.75, df= 3, p< 0.001), α–pinene (t= 1.09, df= 3, p< 0.001), ß- myrcene (t= 1.10, df= 3, p< 0.001) and ß-ocimene (t= 1.04, df= 3, p= 0.021) from whitefly infested plants (Tv). Conversely, 'Elegance' had significantly higher emissions of MeSA (t= 0.63, df= 3, p= 0.006) from un-infested plants (C) and significantly higher levels of MeSA (t= 0.43, df= 3, p= 0.048), ß-caryophyllene (t= 1.98, df= 3, p= 0.003) and humulene (t= 1.31, df= 3, p< 0.001) from whitefly infested plants (Tv). In summary, 'Elegance' responded to whiteflies primarily with release of MeSA, whereas *S. pimpenillefolium* released relatively large amounts of 2-hexenal, α–pinene, ß- myrcene and MeSA.

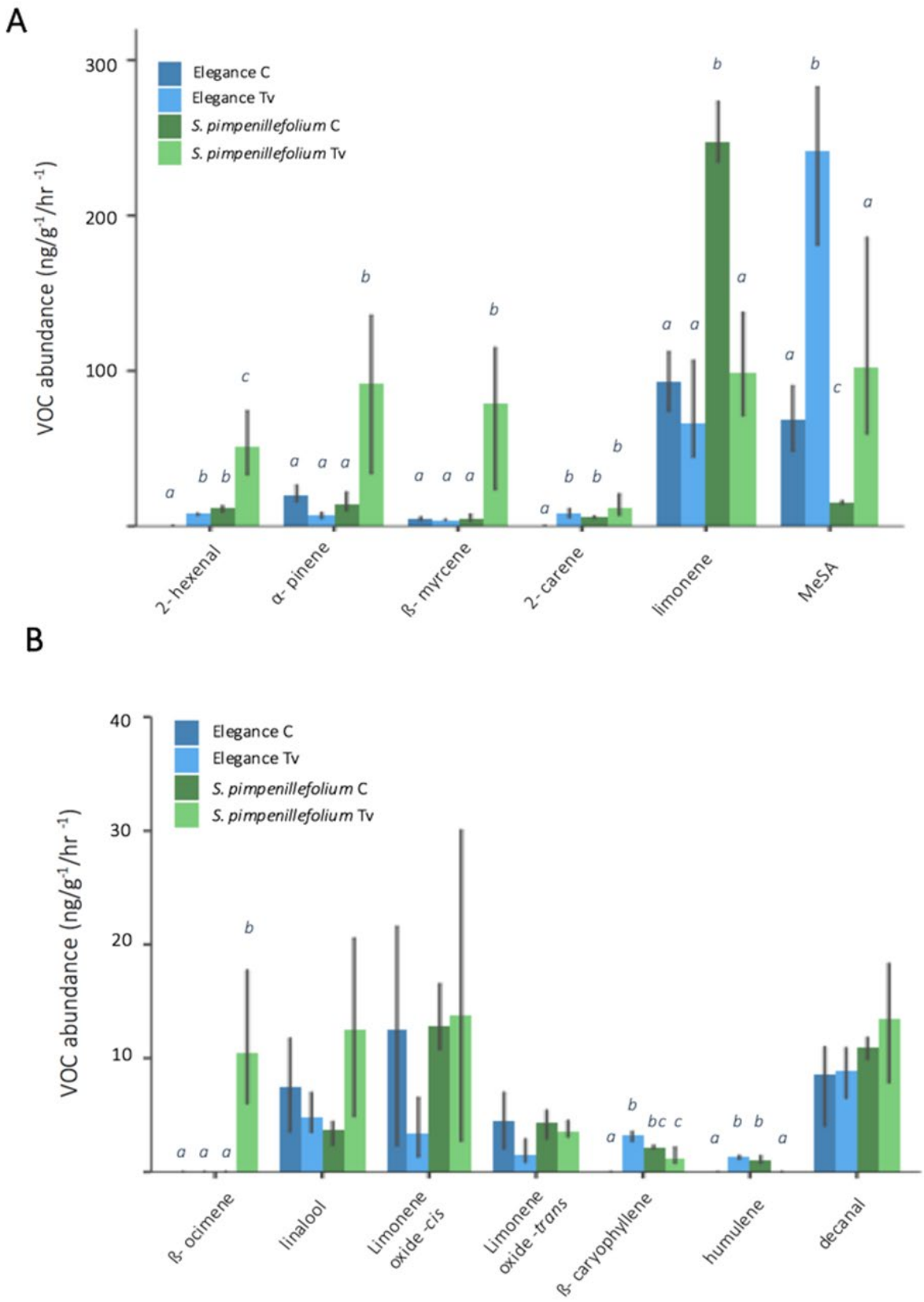

Figure 4.2: Airborne VOCs and HIPVs released from 'Elegance' and *S. pimpenillefolium*. Abundance of each compound detected from un-infested 'Elegance' (C) is displayed in dark blue, whitefly infested 'Elegance' (Tv) = light blue, un-infested *S. pimpenillefolium* (C) = dark green, whitefly infested *S. pimpenillefolium* (Tv) = light green. VOCs/HIPVs were analysed by

dynamic air entrainment and GC-MS over 12 hours and data is presented as ng per gram of fresh weight per hour (*n*= 3), with error bars displaying 95% confidence intervals. Compounds with relatively high abundance are shown in graph A, whereas less abundant compounds are shown in graph B. Data for each individual compound was analysed with one-way ANOVA's followed by Tukey HSD post-hoc tests. Letters (*a, b, c*) denote statistically significant differences (at α= 0.05) between treatments across both species for each individual compound. All compounds except decanal, limonene oxide-cis, limonene oxide-trans and linalool had a significant difference between all four treatments ('Elegance C', Elegance Tv, *S. pimpenillefolium* C, *S. pimpenillefolium* Tv) following one-way ANOVA's. Statistical results for each compound are as follows: 2- hexenal, ANOVA $F_{(3,8)} = 170.9$, $p< 0.001$; α - pinene: ANOVA $F_{(3,8)} = 15.61$, $p= 0.001$; ß- myrcene: ANOVA $F_{(3,8)} = 21.25$, $p< 0.001$; 2- carene: ANOVA $F_{(3,8)} = 24.92$, $p< 0.001$; limonene: ANOVA $F_{(3,8)} = 10.59$, $p= 0.003$; MeSA: ANOVA $F_{(3,8)} = 25.99$, $p< 0.001$; ß- ocimene: ANOVA $F_{(3,8)} = 7.25$, $p= 0.011$; linalool: ANOVA $F_{(3,8)} = 2.45$, $p= 0.138$; limonene oxide-cis: ANOVA $F_{(3,8)} = 0.88$, $p= 0.488$; limonene oxide-trans: ANOVA $F_{(3,8)} = 2.29$, $p= 0.154$; ß-caryophyllene: ANOVA $F_{(3,8)} = 25.77$, $p< 0.001$; decanal: ANOVA $F_{(3,8)} = 1.25$, $p= 0.352$; humulene: ANOVA $F_{(3,8)} = 38.38$, $p< 0.001$.

Considering the differences in VOCs and HIPVs released by 'Elegance' and *S. pimpenillefolium* (Figure 4.2), we set out to ascertain how volatile odours from both species influence whitefly and *E. formosa* behaviour (Figure 4.3). We assessed olfactory preference to constitutively produced VOCs and whitefly induced HIPVs from 'Elegance' and *S. pimpenillefolium*. Whiteflies (Figure 4.3, A) significantly preferred volatile odours from un-infested 'Elegance' relative to *S. pimpenillefolium* (*t*= 4.47, *df*= 10, *p*= 0.001) but showed no preference for odours from whitefly infested 'Elegance' and *S. pimpenillefolium* (*t*= 1.66, *df*= 10, *p*= 0.126). *E. formosa* (Figure 4.3, B) significantly preferred volatile odours from un-infested *S. pimpenillefolium* compared to 'Elegance' (*t*= 8.82, *df*= 10, *p*< 0.001) and also preferred odours from whitefly infested *S. pimpenillefolium* compared to 'Elegance' (*t*= 7.36, *df*= 10, *p*< 0.001).

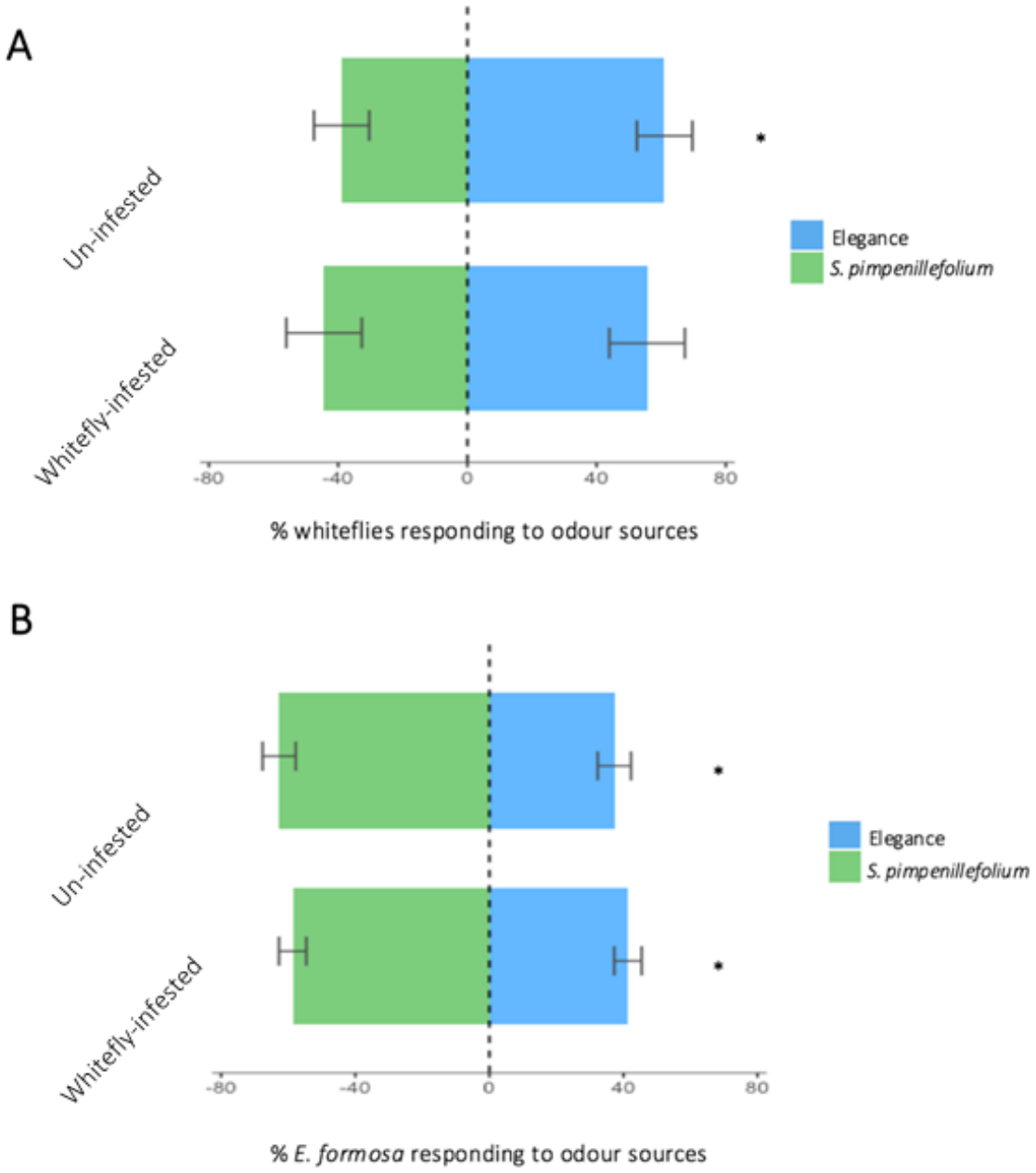

Figure 4.3: Whiteflies (A) and *E. formosa* (B) were presented with odour sources from un-infested 'Elegance' and *S. pimpenillefolium* plants (un-infested) and also to HIPVs from whitefly infested 'Elegance' and *S. pimpenillefolium* (Whitefly Infested) plants. Data is presented as the average (n= 6) percentage of insects which made a choice, with error bars displaying standard deviation. For each treatment, percentage means for each species were compared with t-tests and statistically significant results (at α= 0.05) are annotated onto the figure with '*'.

PPOs and PODs are defence related enzymes that are often used as chemical markers of induced resistance in members of the *Solanaceae* family (Karban et al. 2003; Karban 2011). We assessed activity of PPO and POD in leaf tissue of un-infested (C) and whitefly infested (Tv) plants in order to compare how 'Elegance' and *S. pimpenillefolium* respond to whitefly infestation. We also assessed enzyme activity in HIPV exposed plants (VE), and plants that were infested with whiteflies after previous exposure to HIPVs (TvE) in order to ascertain whether airborne signals can influence biochemical defences. Both tomato species had significantly higher POD levels (Figure 4.4, A) in whitefly infested plants (Tv) compared with respective controls (C) ('Elegance', t= 21.66, df= 40, p= 0.014, *S. pimpenillefolium*, t= 20.63, df= 40, p= 0.028). *S. pimpenillefolium* had significantly higher constitutive (C) (t= 25.48, df= 40, p< 0.001) and whitefly induced (Tv) (t= 24.45, df= 40, p= 0.002) POD levels compared with the equivalent treatments for 'Elegance'. There was a significantly higher activity of PPO (Figure 4.4, B) in *S. pimpenillefolium* ($F_{(1,40)}$ = 41.04, p< 0.001), but there were no significant differences between the treatments of either species following post-hoc tests.

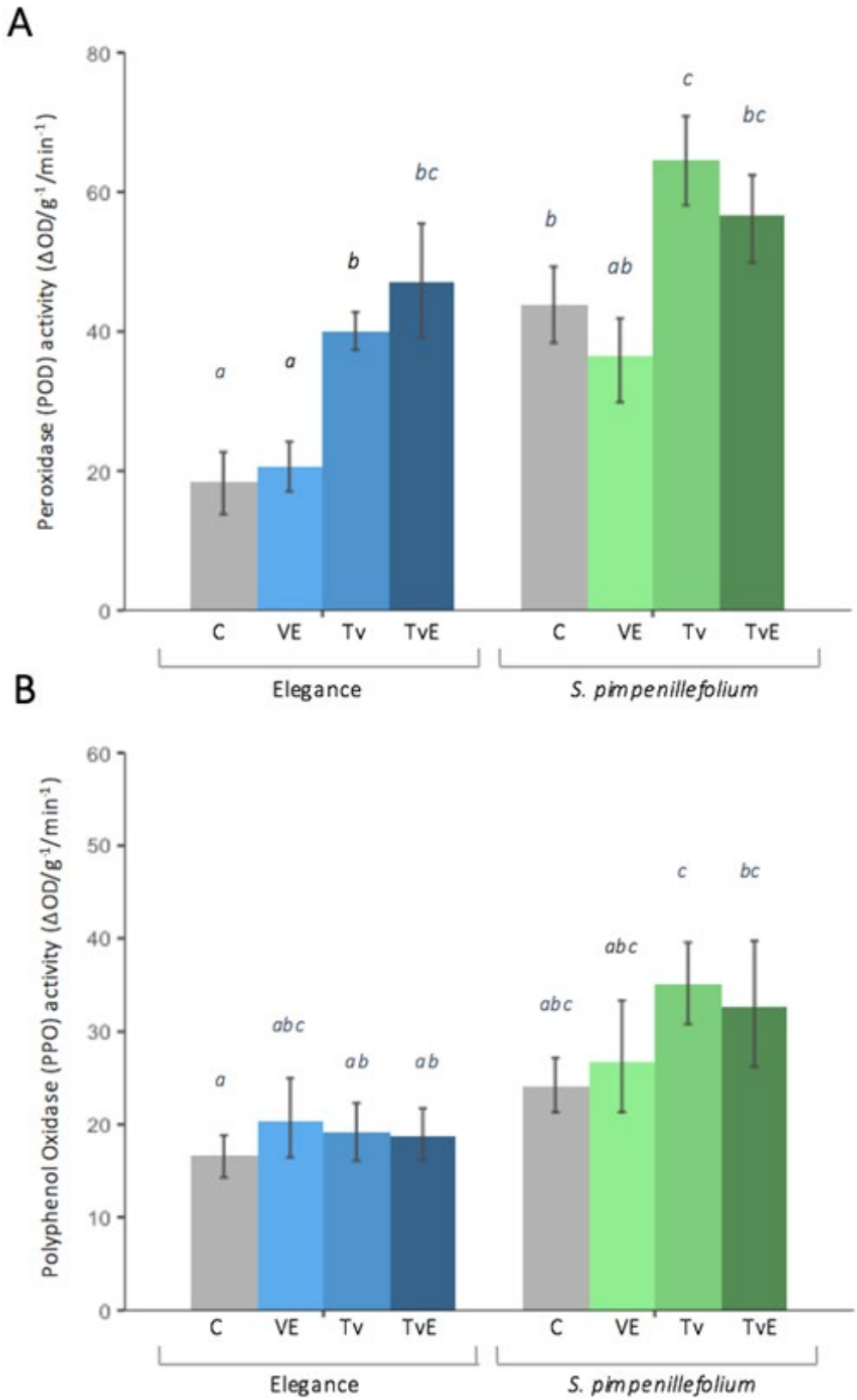

Figure 4.4: In order to ascertain how whiteflies and HIPVs influence tomato defence, activity of two defence related enzymes (POD and PPO) in leaf tissue of 'Elegance' and *S. pimpenillefolium* was assessed. For both species, treatments comprised un-infested plants

(C), HIPV exposed plants (VE), whitefly infested plants (Tv) and HIPV exposed + whitefly infested plants (TvE). Data for each enzyme is presented as change in optical density per gram of fresh weight per minute (n= 6), with error bars displaying 95% confidence intervals. Data for each enzyme was analysed with two-way ANOVA's, with species and treatment as factors, followed by Bonferroni corrected post-hoc comparisons. Significant differences (at α= 0.05) between the treatments, which can be compared across both species, are indicated with different letters (*a, b, c, d*). For POD activity (A), there was a significant difference between the two species (ANOVA $F_{(1, 40)} = 70.50$, $p< 0.001$) and between the treatments across both species (ANOVA $F_{(3, 40)} = 32.96$, $p< 0.001$), but no significant interaction between species x treatment (ANOVA $F_{(3, 40)} = 2.79$, $p= 0.052$) following a two-way ANOVA. There was a significant difference in PPO activity (B) between the two species (ANOVA $F_{(1, 40)} = 41.04$, $p< 0.001$) but no significant difference between the treatments (ANOVA $F_{(3, 40)} = 2.55$, $p= 0.068$) or species x treatment (ANOVA $F_{(3, 40)} = 1.23$, $p= 0.310$) following a two-way ANOVA.

Four key phytohormones (ABA, IND, SA and JA), all responsible for regulation of plant defence signalling pathways, were quantified in leaf tissue of 'Elegance' and *S. pimpenillefolium* (Figure 4.5). We assessed phytohormone abundance in un-infested (C) and whitefly infested (Tv) plants in order to ascertain how whiteflies influence tomato defence signalling. In addition to this, we also assessed phytohormone abundance in HIPV exposed plants (VE) and plants that were infested with whiteflies after previous exposure to HIPVs (TvE). By quantifying phytohormone abundance in VE and TvE plants, we aimed to elucidate whether airborne signals can influence plant defence signalling. There were no significant effects on ABA abundance between the two species (Figure 4.5, A), but there was a significantly higher abundance of IND (Figure 4.5, B) in *S. pimpenillefolium*. However, subsequent post-hoc tests found no significant differences in IND abundance between any of the treatments. 'Elegance' had a significantly higher abundance of JA than *S. pimpenillefolium* (Figure 4.5, C). However, post-hoc tests showed no significant differences between the treatments for 'Elegance' or *S. pimpenillefolium*. When infested with whiteflies, 'Elegance' significantly increased SA levels (Figure 4.5, D) (t= 0.88, df= 16, $p< 0.001$) whereas *S. pimpenillefolium* decreased SA levels, although this was not statistically significant (t= 0.33, df= 16, p= 1.000).

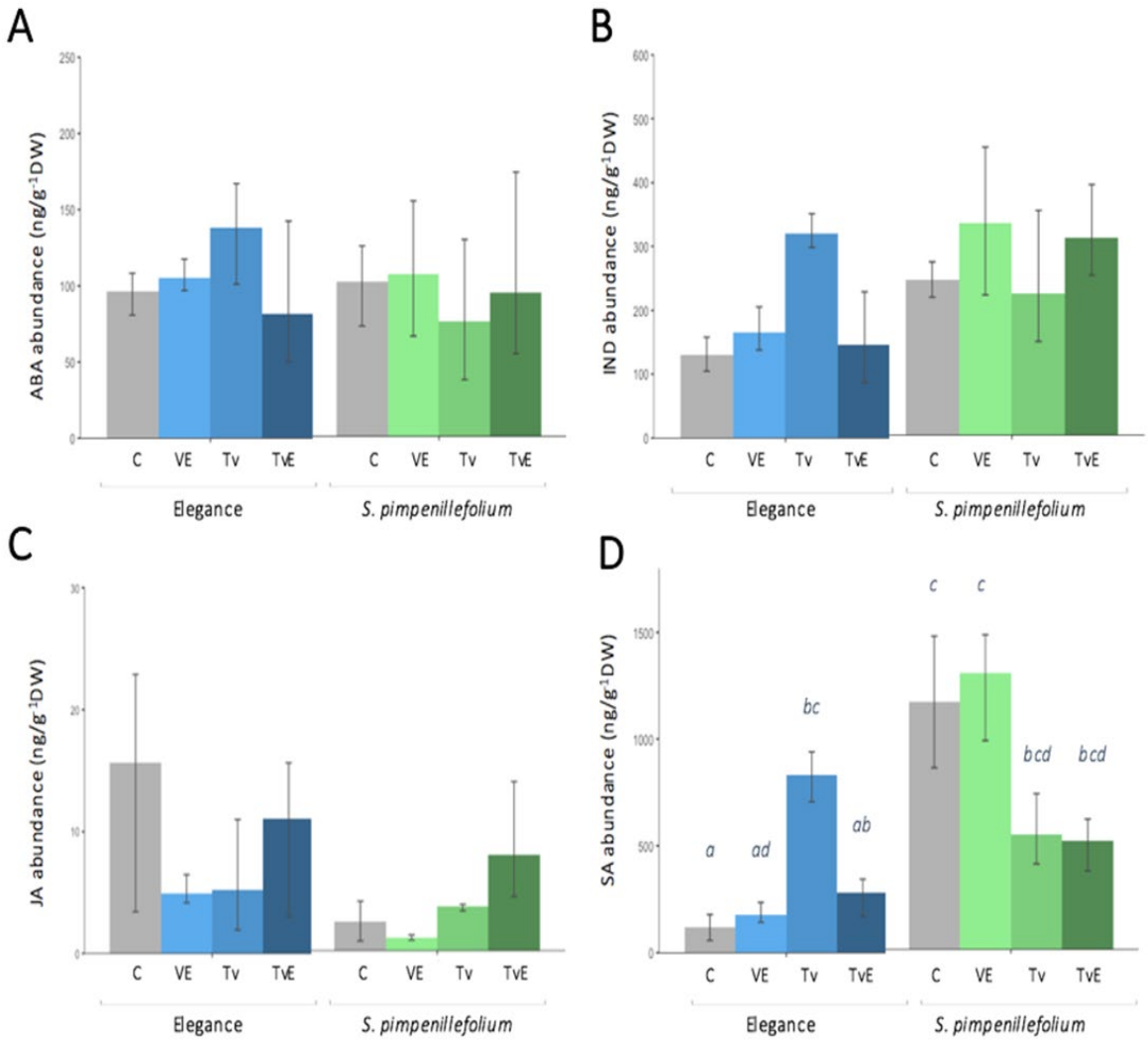

Figure 4.5: Abundance of four key phytohormones (abscisic acid (ABA), indole-3-acetic acid (IND), salicylic acid (SA) and jasmonic acid (JA)) in leaf tissue of 'Elegance' and *S. pimpenillefolium*. Abundance of each compound was assessed across four treatments that comprised un-infested plants (C), HIPV exposed plants (VE), whitefly infested plants (Tv) and HIPV exposed + whitefly infested plants (TvE). Graph A displays abundance of ABA, graph B displays abundance of IND, graph C displays abundance of JA and graph D displays abundance of SA. Data for each compound is presented as ng per gram of dry weight (n= 3), with error bars displaying 95% confidence intervals. Data for each compound was analysed with two-way ANOVA's, with species and treatment as factors, followed by Bonferroni corrected post-hoc comparisons. Significant differences (at α= 0.05) between the treatments, which can be compared across both species, are indicated with different letters (*a, b, c, d*). For ABA abundance (A), there was no significant difference between the two species (ANOVA $F_{(1,16)} = 0.40$, p= 0.535), between the treatments (ANOVA $F_{(3,16)} = 0.25$, p= 0.858) or species x treatment (ANOVA $F_{(3,16)} = 1.05$, p= 0.397). For IND (B), there was a significant difference between the two species (ANOVA $F_{(1,16)} = 0.30$, p= 0.005) and a significant interaction between species x treatment (ANOVA $F_{(3,16)} = 4.27$, p= 0.021), but there was not a significant difference between the treatments overall (ANOVA $F_{(3,16)} = 0.38$, p= 0.764). For JA abundance (C), there was a significant difference between the species (ANOVA $F_{(1,16)} = 8.76$, p= 0.009), but no significant difference between the treatments

(ANOVA $F_{(3,16)} = 2.87$, $p = 0.069$) or species x treatment (ANOVA $F_{(3,16)} = 1.79$, $p = 0.188$). For SA abundance (D), there was a significant difference between the species (ANOVA $F_{(1,16)} = 70.57$, $p < 0.001$), the treatments (ANOVA $F_{(3,16)} = 4.60$, $p = 0.016$) and also a significant interaction between species x treatment (ANOVA $F_{(3,16)} = 23.10$, $p < 0.001$).

We quantified expression of two defence related genes in 'Elegance' (Figure 4.6, A) and *S. pimpenillefolium* (Figure 4.6, B) in order to characterize the way these two plants respond to whitefly infestation, and HIPVs from whitefly infested conspecifics. Mi-1.2 expression levels for both 'Elegance' and *S. pimpenillefolium* were significantly higher in whitefly infested plants (Tv) compared with controls ('Elegance'; $t = 0.75$, $df = 32$, $p < 0.001$, *S. pimpenillefolium*; $t = 0.44$, $df = 32$, $p = 0.034$). 'Elegance' also had significantly higher Mi-1.2 transcript levels in HIPV exposed plants (VE) compared with controls ($t = 0.53$, $df = 32$, $p = 0.014$). Interestingly, there was a priming effect on Mi-1.2 expression in *S. pimpenillefolium*, with the TvE treatment displaying significantly higher transcript levels compared with Tv ($t = 0.50$, $df = 32$, $p = 0.013$). Conversely, there was no significant difference in Mi-1.2 transcript levels between TvE and Tv in 'Elegance' ($t = 0.37$, $df = 32$, $p = 0.128$), and also a significant difference between C and VE treatments ($t = 0.53$, $df = 32$, $p = 0.014$). After obtaining interesting results for SA abundance (Figure 4, D), we decided to quantify expression of PR-1, a defence related gene which is strongly associated with the SA signalling pathway. Both 'Elegance' and *S. pimpenillefolium* had significantly higher PR-1 transcript levels following whitefly infestation (Tv) ('Elegance'; $t = 0.98$, $df = 32$, $p < 0.001$, *S. pimpenillefolium*; $t = 0.49$, $df = 32$, $p = 0.008$). For both species, there was no significant difference between controls and VE treatments ('Elegance'; $t = 0.36$, $df = 32$, $p = 0.213$, *S. pimpenillefolium*; $t = 0.19$, $df = 32$, $p = 0.552$). There was also no significant difference in PR-1 expression between Tv and TvE for 'Elegance' or *S. pimpenillefolium* ('Elegance'; $t = 0.41$, $df = 32$, $p = 0.132$, *S. pimpenillefolium*; $t = 0.20$, $df = 32$, $p = 0.482$).

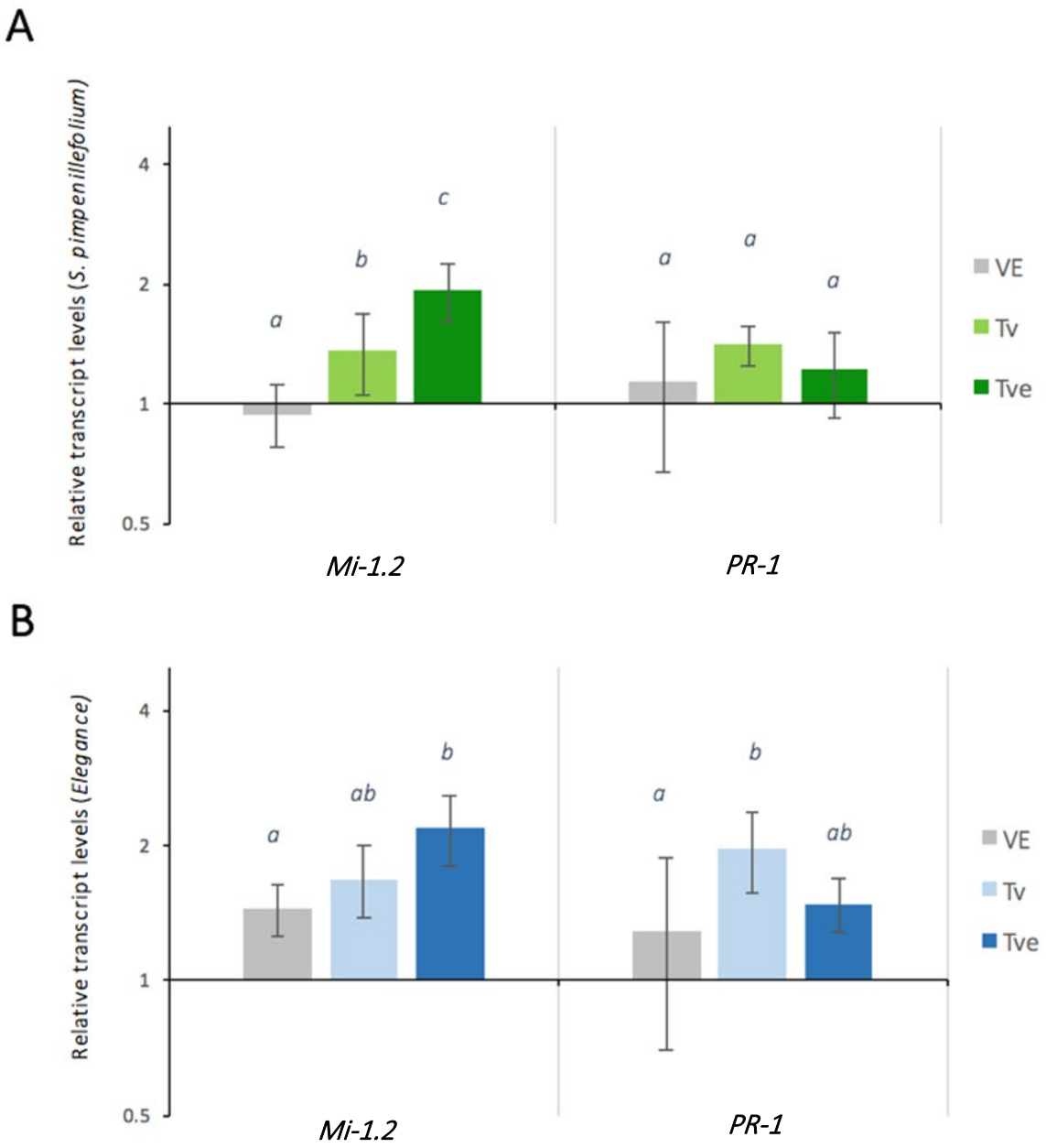

Figure 4.6: Relative fold change in expression levels of two tomato defence related genes (Mi-1.2 and PR-1) in *S. pimpenillefolium* (A) and 'Elegance' (B). Mi-1.2 and PR-1 transcript levels were quantified in tomato leaf tissue after HIPV exposure (VE), whitefly infestation (Tv) and HIPV exposure + whitefly infestation (TvE). Data is presented as average (n= 3) relative fold change compared with control transcript levels for each gene. Data analysis was performed on Dcq values using one-way ANOVA's followed by Tukey HSD post-hoc tests, bars with different letters (*a, b, c*) denote significant differences (at α= 0.05) between the treatments. Error bars represent standard deviation that was calculated using Dcq values. There was a significant difference amongst treatments for Mi-1.2 (ANOVA $F_{(3,32)} = 16.16$, $p< 0.001$) and PR-1 levels (ANOVA $F_{(3,32)} = 9.95$, $p< 0.001$) in 'Elegance' (A) following one-way ANOVA's. For *S. pimpenillefolium* (B), there was also a significant difference between the treatments for Mi-1.2 (ANOVA $F_{(3,32)} = 18.74$, $p< 0.001$) and PR-1 (ANOVA $F_{(3,32)} = 4.13$, $p= 0.013$).

4.4 Discussion

VOCs are an important component of plant defence and have an essential role in the way plants interact with insects. We characterized constitutive VOCs and whitefly induced HIPVs from 'Elegance' (a commercial variety of tomato) and *S. pimpenillefolium* (a wild species of tomato) over a 12-hour period (Figure 4.2). Across the two species, we observed significant differences in emission rates for nine of the thirteen compounds identified. Limonene was the most prominent VOC constitutively produced by both species but limonene abundance was significantly higher in *S. pimpenillefolium* (Figure 4.2, A). Limonene is known to be repellent to whiteflies (Conboy et al. 2019; Du et al. 2016; Zhao et al. 2014) and could contribute to their decreased preference for *S. pimpenillefolium* observed in our olfactory preference assays (Figure 4.3, A) and also by McDaniel et al. (2016). Un-infested *S. pimpenillefolium* constitutively produced 2-hexenal, 2-carene, ß-caryophyllene and humulene, but these compounds were not detected in un-infested 'Elegance' plants. Whiteflies have been shown to be more attracted to plants with decreased VOC emissions (Sanchez-Hernandez et al. 2006) and these additional VOCs produced by *S. pimpenillefolium* could contribute to the antixenotic effects on whitefly preference observed in Figure 4.3 (A). VOC production has been shown to require significant resource allocation (Robert et al. 2013; Gershenzon 1994) and here we show that *S. pimpenillefolium* invests metabolic energy in producing these compounds irrespective of external stimuli. Similar effects were observed with *Solanum pennellii* and *Solanum habrocaites*, two wild tomato species that were found to produce several terpenoids not detected in cultivated tomato (Bleeker et al. 2009). Tomato plants that are infested with whiteflies are known to produce compounds which are attractive to beneficial insects (Birkett et al. 2003), but here we see constitutive volatiles from *S. pimpenillefolium* attracting *E. formosa* (Figure 4.3, B). Whilst not observed before in tomato, constitutive VOCs have been shown to be attractive to beneficial insects (Ali et al. 2011). It is possible that constitutively produced ß-caryophyllene and humulene could have caused the attractive effect. Both of these compounds are attractive to *E. formosa* (Liu et al. 2017) and are not detected in VOC emissions of un-infested 'Elegance' plants (Figure 4.2, B).

Whilst whitefly infestation caused the release of several HIPVs from 'Elegance', methyl salicylate (MeSA) was by far the most abundant HIPV detected (Figure 4.2, A). *S.*

pimpenillefolium also releases MeSA in response to whitefly infestation, but emission rates were significantly less than observed in 'Elegance'. MeSA is a potent inducer of plant defence (Heil and Ton 2008) and has been shown to be attractive to a variety of beneficial insects, including *E. formosa* (James and Price 2004). Despite this, *E. formosa* were still more attracted to *S. pimpenillefolium* HIPVs (Figure 4.3, B). This would suggest that the composition of *S. pimpenillefolium* HIPVs, which primarily comprised 2-hexenal, α–pinene, ß- myrcene and MeSA are more attractive to *E. formosa*. A similar phenomenon was observed when assessing olfactory preference of the predatory mite *Phytoseiulus persimilis*, which was found to be more attracted to a mixture of HIPVs than any single compound from the mixture (van Wijk et al. 2011). Interestingly, whiteflies showed no preference for whitefly induced HIPVs from either species (Figure 4.3, A). Limonene release from *S. pimpenillefolium*, which may have been responsible for the repellent effect on whiteflies in control assays (Figure 4.3, A), was significantly reduced in whitefly infested *S. pimpenillefolium*. This has been observed in tomato before (Lopez et al. 2012), and it could be the case that repellent VOCs are less important for plants once infested with herbivores. Whilst not present in un-infested 'Elegance' emissions, whitefly infestation induced the release of 2-hexenal, 2-carene and ß-caryophyllene in 'Elegance'. Interestingly, *S. pimpenillefolium* released these compounds constitutively, at similar levels to what was released by 'Elegance' upon infestation from whiteflies. Whitefly infested 'Elegance' released similar a similar amount of limonene as un infested *S. pimpenillefolium*, which could explain why whiteflies showed no preference to HIPVs from either species. In addition to this, the relatively large amount of MeSA released by 'Elegance' could act as a repellent to whiteflies, as has been shown before with *B. tabaci* (Shi et al. 2016). Whiteflies are known to avoid plants which harbour predators (Nomikou et al. 2003) and may recognise MeSA as an indicator of increased indirect defences (Walling 2008), therefore reducing a plants' suitability as a host. VOCs offer great potential to be used in IPM systems (Schlaeger et al. 2018) and identification of whitefly repellent volatiles, or *E. formosa* attractants, could help provide sustainable crop protection.

In response to whitefly infestation, both tomato species significantly increased POD activity in vegetative tissue (Figure 4.4, A). PODs have been correlated with increased resistance to whiteflies (Dowd and Lagrimini 2006; Taggar et al. 2012) and these results indicate that both tomato species respond to whiteflies by synthesising more PODs. Importantly, *S. pimpenillefolium* exhibited significantly higher activity of constitutive and whitefly induced

PODs compared with 'Elegance' (Figure 4.4, A) which could contribute to increased resistance to whiteflies. Whilst average PPO activity increased in response to whitefly infestation for both plant species, these differences were not statistically significant (Figure 4.4, B). Constitutive PPO levels were similar between the two species but whitefly induced PPO activity in *S. pimpenillefolium* was significantly higher than whitefly induced PPO activity in 'Elegance'. We also assessed POD and PPO activity in plants exposed to HIPVs from whitefly-infested conspecifics, but observed no significant effects when compared with unexposed plants. In summary, these results provide evidence for superior constitutive and inducible defensive enzyme activity in *S. pimpenillefolium*. Producing proteins is one of the most energetically costly processes for plants (Ishihara et al. 2015) and selecting for enhanced growth related traits in the domesticated 'Elegance' may have come at the expense of these essential defensive proteins.

SA and JA signalling pathways are known to be antagonistic (Gatehouse 2002), if SA related defences are induced then JA related defences are supressed (and vice versa). Whiteflies can manipulate plant defence signalling by inducing SA related defences and supressing JA related defences. This makes plants more susceptible to whitefly herbivory and can increase whitefly performance (Zarate et al. 2007; Zhang et al. 2013a; Zhang et al. 2019). We observed an increase in SA abundance (Figure 4.5, D) and a reduction in JA abundance (Figure 4.5, C) in whitefly infested 'Elegance', although the latter was not statistically significant. In contrast, we found no significant difference in SA abundance between control and whitefly infested *S. pimpenillefolium*. Following this finding, we decided to quantify PR-1 transcript levels in 'Elegance' and *S. pimpenillefolium* in an attempt to elucidate the way whiteflies influence SA defence signalling. Both plant species significantly increased PR-1 transcript abundance following whitefly infestation (Figure 4.5), but the average relative fold change in whitefly infested plants was higher in 'Elegance' (1.97) compared with *S. pimpenillefolium* (1.40). Taken together with the data for SA abundance, this would suggest that whiteflies induce SA related defences more strongly in 'Elegance', which could make them more susceptible to whitefly attack. Whiteflies induce manipulate plant defence signalling through the release of salivary chemicals/proteins (Walling 2008) and it is conceivable that *S. pimpenillefolium* is less susceptible to whiteflies' attempts to induce SA related defences. Importantly, previous work that has found increased whitefly performance on SA-induced plants have also observed significant reductions in the antagonistic JA related defences. Our experiments did not reveal

any significant effects on JA abundance, so we cannot assert this claim. However, we believe that our work could have unveiled a distinct difference in the way these two species induce defence signalling in response to whitefly infestation, which could be extremely important in our attempts to elucidate the way whiteflies induce defences in *S. pimpenillefolium*. It is important to note that our experiments analysed defensive components after 12 hours of infestation, but defensive enzymes, phytohormones and VOCs are known to fluctuate over several days of infestation (Lopez et al. 2012; Zhang et al. 2019). By analysing these defensive components over a longer, incremental time course, we could see further changes in phytohormone abundance that could help elucidate the whitefly-induced defence response produced by 'Elegance' and *S. pimpenillefolium*. Defence elicitation with HIPVs has been shown to induce physiological changes within the first 12 hours of exposure (Erb et al. 2015), but we did not observe any significant effects on defensive enzyme activity or phytohormone abundance after exposure to HIPVs for 12 hours. We could potentially find latent priming effects of HIPV exposure which only become apparent after several days of HIPV exposure, as has been shown previously (Martinez-Medina et al. 2016; Zhang et al. 2019). An obvious next step to our research would be to test some of the individual HIPVs from 'Elegance' and *S. pimpenillefolium* for defence inducing properties, and extend the assessed period post HIPV exposure to elucidate any latent effects on plant defence.

Plants are known to prime defences following exposure to HIPVs (Heil and Ton 2008; Erb et al. 2015) but we observed no priming effects on defensive enzyme activity (Figure 4.4) or phytohormone abundance (Figure 4.5) following exposure to HIPVs from whitefly infested conspecifics. However, we did observe a priming effect on the whitefly resistance gene Mi-1.2 in *S. pimpenillefolium*, with significantly higher transcript levels in TvE plants compared with Tv (Figure 4.6, B). Interestingly, we did not see the same effect on Mi-1.2 transcript levels in 'Elegance' (Figure 4.6, A). Priming is an adaptive, low-cost response to the threat of potential herbivore attack which mitigates the detection of 'false alarms' (Heil and Ton 2008), which are somewhat inevitable in a natural environment with numerous airborne VOCs. The fact that *S. pimpenillefolium* primes defences after just 12 hours of HIPV exposure is undoubtedly beneficial for plant fitness. Not only does 'Elegance' not prime Mi-1.2 transcript levels, we also observed a significant increase in Mi-1.2 transcript levels in HIPV exposed (VE) plants (Figure 4.6, A). Immediately inducing defences upon detection of HIPVs could be interpreted as effective recognition and response to the threat of whitefly infestation. However, we

would argue that this is an inefficient response to airborne signals which, combined with the fact we do not see a priming effect on Mi-1.2 transcripts in 'Elegance', is an inferior response to HIPVs. Even cultivated crops must be able to effectively interpret fluctuations in environmental VOCs and this could highlight an inferior ability to respond to airborne distress signals. Whilst we only observed this phenomenon in a single defence related gene, a genome wide assessment could reveal more of the same expression patterns we have observed here.

This study shows that *S. pimpenillefolium* produces superior levels of both constitutive and inducible defence mechanisms compared with 'Elegance', which may contribute to increased resistance to whiteflies. Importantly, many of the defensive components identified in *S. pimpenillefolium* were also present in 'Elegance', albeit at lower levels. The fact that these traits are still present in cultivated tomato facilitates future genetic engineering endeavours, as comparatively simple overexpression experiments would be feasible. Overexpression of a POD gene in tobacco was found to significantly decrease whitefly performance (Dowd and Lagrimini 2006) and similar effects could be achieved by overexpressing POD genes in 'Elegance'. Our olfactory experiments showed that odours from *S. pimpenillefolium* are repellent to whiteflies and attractive to *E. formosa*. Future work should be directed to identify the compounds responsible for this attractive/repellent effect. These compounds could be deployed as odour sources to influence insect behaviour (Conboy et al. 2019; Du et al. 2016), or alternatively these traits could be transferred to cultivated tomato, as has been attempted previously (Bleeker et al. 2012). Our phytohormone and genetic analysis revealed distinct differences in the way 'Elegance' and *S. pimpenillefolium* respond to whiteflies and HIPVs. Future work should look to expand on the research we have conducted here in an attempt to characterise defence signalling and plant priming in *S. pimpenillefolium*. Understanding complex evolutionary defence mechanisms such as these could prove key in our efforts to improve defences in cultivated tomato.

Chapter 5. Discussion

This thesis set out to explore the use of plant VOCs as a sustainable whitefly control method for glasshouse-grown tomatoes in the UK. VOCs provide an environmentally benign alternative to insecticides and offer great potential for use against whiteflies in integrated pest management (IPM) (Schlaeger et al. 2018). In three large-scale glasshouse trials, we deployed VOCs in the form of aromatic companion plants, repellent VOC dispensers and plant defence elicitors. All of our VOC based protection methods produced negative effects on whitefly performance and show promise for use in commercial agriculture. Intrinsic heritable plant resistance is a key component of IPM (Bruce et al. 2017; Stenberg 2017; Peterson et al. 2018) and re-introducing whitefly resistant traits from wild ancestors to cultivated tomato could help improve whitefly control. In line with this, we conducted a comprehensive analysis comparing the way a cultivated and wild tomato species defend against whiteflies. We analysed the way these two species employ constitutive and inducible defence mechanisms, with a focus on how these plants produce and respond to airborne volatiles. In this final chapter, we discuss the underlying mechanisms and applied potential of the VOC based control methods investigated in this research thesis. We also discuss the results of our experiments with wild tomatoes and how the defensive components we identified could be used to improve whitefly control in tomato production systems.

5.1 Companion planting

Increasing plant diversity in agro-ecosystems is known to increase arthropod diversity (Siemann et al. 1998), and we predict that by manipulating plant diversity to include non-hosts of a target pest, we could conceivably create agro-ecosystems that harbour greater levels of neutral and beneficial arthropods, with lower abundance of economically important pests. Plant diversity in agro-ecosystems can be increased with a practice known as companion planting, where multiple plant species are grown alongside the target crop. Companion planting is a sustainable and effective pest control practice that can increase agro-ecosystem biodiversity and reduce the need for synthetic chemical sprays (Parker 2013;

Malezieux et al. 2009). In large-scale glasshouse trials, we sought to test the claim from home growers that companion planting with French marigolds protects tomato from whiteflies. We found French marigolds to 'push' whiteflies from a tomato crop, with subsequent laboratory experiments revealing that emissions of airborne limonene are likely responsible for this repellent effect (Chapter 2). Companion planting with French marigold has been used to control a number of insect pests (Jankowska 2009; Ben-Issa et al. 2017; Sujayanand et al. 2016), although this was the first study to assert the effectiveness of French marigolds as a whitefly deterrent. Whilst we deduced the mode of action to be repellent volatile chemistry, other marigold species have been shown to provide a floral resource for beneficial insects (Zhao et al. 2017; Balzan 2017). Zhao et al. (2017) found that *Orius sauteri* (Poppius), a generalist predator that also feeds upon whiteflies (Kajita 1982; Wang et al. 2013), had significantly higher abundance in plots containing English marigold (*Calendula officinalis L.*), which resulted in reduced aphid and thrips abundance on tomato. Whilst *O. sauteri* is not currently used in the UK, *Eretmocerus mundus*, a whitefly parasitoid commonly used to control whiteflies in the UK, has also been shown to benefit from floral resources (Araj et al. 2019). Similar to what was observed for the closely related English marigold, French marigold could potentially provide a floral resource for beneficial insects such as *E. mundus*. This, combined with the repellent effects on whiteflies could represent a dual mode of action for French marigold companion planting, potentially making this pest management practice more effective and robust. Additionally, marigolds could provide a nectar resource for bumblebees, which are commonly used in glasshouses to pollinate tomato plants (Banda and Paxton 1991). Marigolds are attractive to a variety of different bee species (Dicks et al. 2010) and presence of flowering marigold could support bumblebee colonies and potentially increase pollination of tomato in glasshouses. Clearly, French marigolds offer fantastic potential for use in sustainable agriculture and could be used to protect tomato from whiteflies in a commercial glasshouse setting.

As with most contemporary cropping systems, tomatoes are typically produced in monoculture, a practice which renders crop areas of limited value to wildlife and largely devoid of "ecosystem services" (Malezieux et al. 2009). Enhancing plant diversity in agro-ecosystems has been shown to improve pest control and facilitate the growth of natural enemy populations (Landis et al. 2000; Knops et al. 1999). Additionally, Bernays (1999) found that *B. tabaci* become 'restless' when exposed to a complex composition of VOCs from

multiple plant species. Bernays (1999) concluded that due to the restless behaviour induced by the presence of multiple plant species, monocultures could potentially harbour more pests than polycultures. We therefore decided to assess the efficacy of a high diversity intercropping system to control whiteflies on tomato. We selected basil, Chinese cabbage and nasturtium for the high diversity intercropping treatment after discovering these plants are of low preference to whiteflies in leaf disc preference assays. High diversity plots reduced whitefly performance on tomato after 34 days of infestation, similar to what was observed in the low diversity marigold only plots. Whilst our work shows the benefits of high diversity cropping systems for controlling whiteflies, it is important to note that we selected plants for our intercropping mixture based on their suitability as a whitefly host. As we mention in our discussion of Chapter 2, future work should look to ascertain how other tomato pest species interact with our intercropping mixture. Enhancing biodiversity in agro-ecosystems is thought to promote natural enemy populations (Altieri 1991), although simply increasing plant diversity does not necessarily improve pest control. Introducing more plant species increases the complexity of the agro-ecosystem, which can introduce additional insect species that can positively or negatively impact the efficacy of introduced natural enemies. Only "the right kind of diversity" will increase natural enemy predation/parasitism of the target pest (Barbosa and Castellanos 2005). Assessing the way whitefly biocontrol organisms respond to our intercropping mixture is an essential next step to this research.

Glasshouses represent an artificial growing environment with low biodiversity (Enkegaard and Brødsgaard 2006) and we hope our work will encourage others to increase plant diversity in protected cropping environments. Enclosed cropping systems allow for greater pest control (Nicot and Baille 1996) and can also increase the effectiveness of companion plants due to the localisation of VOC abundance (Cook et al. 2007; Schlaeger et al. 2018). Despite these pest control benefits in protected cropping environments, physical barriers restrict interactions with the surrounding native ecosystem. Intercropping in outdoor systems with aromatic companion plants has been shown to reduce pest insect abundance and provide habitat for native wildlife (Afrin et al. 2017; Matintarangson 2018). We believe our intercropping set-up would also be applicable to outdoor tomato production systems, where it could potentially be more beneficial for native wildlife.

5.2 Limonene slow-release dispensers

Our experiments with limonene slow-release dispensers illustrate the potential for repellent VOCs to be used in commercial agriculture. Whilst limonene dispensers were limited in their capability to control a large pre-existing whitefly infestation (Chapter 2), when placed alongside tomato from the start of the growing period they were extremely effective (Chapter 3). Tomato plants accompanied by limonene dispensers had a 33% greater fruit yield (g) compared with control plots in what we believe to be one of the most impressive examples of repellent VOCs as a control method for agricultural pests. Whitefly adult settling in control plots was significantly higher than limonene plots for the first 15 days of infestation, and average settling adults per leaf across this 15-day period were 4.77 and 1.27 for control and limonene plots respectively. Importantly, this increased settling on control plots translated to a significantly higher abundance of nymphs on control plots until day 20 of infestation. Whitefly nymphs are also known to be detrimental to plant fitness (Riley and Palumbo 1995) and could contribute to the decrease in fruit yield from control plots. Our work demonstrates that reducing whitefly abundance on tomato with repellent VOC dispensers, over relatively short period of time, can translate to a significant increase in plant fitness. Importantly, during this initial 15-day infestation period, tomato plants were still at a relatively early developmental stage (21 days old at the beginning of the infestation period, stage 14 on the BBCH scale). Previous work has found that young tomato plants are more susceptible to whiteflies than mature plants (Bas et al. 1992) and this could be due to the plant vigour hypothesis, which states that rapidly growing/young developing plant tissues are more vulnerable to herbivory (Price 1991). This hypothesis can be applied to whiteflies, which are known to prefer young developing leaves for feeding and oviposition (Van Lenteren 1990). The combination of young susceptible plants and a high abundance of whiteflies could contribute to the reduced yield from control plots, despite the fact that whitefly abundance was greater on control plots for just a short period at the beginning of the 53-day infestation period. It is important to note that our experiments included control plots that gave whiteflies the option of naïve tomato plants with no additional performance pressure placed upon them. We predict that whiteflies were ‘pushed’ by limonene dispensers onto these control plots, which concentrated whitefly abundance and caused the decrease in plant performance.

In a real-world agricultural setting without these control plots present, the beneficial effects of limonene dispensers may not be as pronounced.

It is important to note that we used limonene dispensers that released a similar amount of limonene as a large marigold plant. Increasing the amount of limonene released, or using multiple limonene dispensers placed and/or hung around the target crop could also increase this protective effect. In addition to this, limonene dispensers could be used to supplement current whitefly-based IPM systems. Sticky traps are commonplace in glasshouses across the UK (George et al. 2015) and limonene dispensers could be used to 'push' whiteflies away from tomato and onto sticky traps. In an effort to emulate a push-pull farming system, sticky traps could be accompanied by whitely attractive VOC dispensers. This would alleviate the reservations some commercial growers may have with push-pull systems, notably that the additional plant species would occupy space in the glasshouse that could otherwise be used for tomato plants. Attractive/repellent VOC dispensers could be placed/hung around the target crop and occupy minimal growing space. Despite this, we would still encourage growers to increase plant diversity in agro-ecosystems. As a compromise, limonene dispensers could be used in conjunction with attractive 'trap' plants, thereby harnessing the space saving benefits of limonene dispensers whilst also increasing plant diversity. This strategy has been demonstrated previously by Lamy et al. (2018), the combination of repellent Z-3-hexenyl-acetate dispensers and an attractive 'trap' plant (Chinese cabbage) was found to successfully reduce cabbage root fly (*Delia radicum*) oviposition on broccoli . In summary, we believe that limonene dispensers could be used for a range of sustainable whitefly control strategies. Whilst what we have achieved with limonene dispensers is extremely promising, we believe there is room for optimisation of our methods that could improve the protective effect.

Another advantage VOC dispensers have over companion planting is the precise way that particular VOCs can be selected for activity against particular pest species. Whilst we found marigold to repel whiteflies, we did not observe any repellent activity against two-spotted spider mites (*Tetranychus urticae*) or onion thrips (*Thrips tabaci*). VOCs repellent to these respective pests could be added to limonene dispensers to potentially repel all three pest species. For example, limonene dispensers could be combined with terpinen-4-ol, repellent to two-spotted spider mites (Isman 2000), and β-elemene, repellent to onion thrips (Wilson et

al. 2017). This process would get more complicated as the number of pests/number of VOCs increases because insects are known to respond to individual VOCs and mixtures of VOCs in different ways (Szendrei and Rodriguez-Saona 2010). For example, the black bean aphid (*Aphis fabae*) is attracted to the composition of VOCs from *Vicia faba*, but 10 of the 15 VOCs from the mixture were found to have repellent effects when presented to the aphids as a single compound (Webster et al. 2010). Conversely, Du et al. (2016) found that increasing the complexity of VOC mixtures had a more of a repellent effect on whiteflies. Due to the relatively easy way with which VOCs can be added/removed from the VOC dispensers, we anticipate that with further research, VOC dispensers that confer repellence to a number of tomato pests could be developed.

5.3 Methyl salicylate (MeSA) as a plant defence elicitor

A previous study from our lab group found that tomato plants were more resistant to whiteflies after exposure to HIPVs from whitefly-infested conspecifics for a period of 5 days (appendix). Subsequent air-entrainment experiments revealed that MeSA, a potent inducer of plant defence (Heil and Ton 2008), is the most prominent HIPV released by whitefly-infested tomato. Following this finding, we used MeSA to induce defences in tomato to increase resistance to glasshouse whiteflies. In a large-scale glasshouse trial, tomato plants that were sprayed with MeSA had significantly fewer whitefly adults, eggs and nymphs at several sampling points across the 53 day infestation period (Chapter 3). Whilst not as effective as the plots accompanied by limonene dispensers, our study illustrates the potential for MeSA to be used to elicit defences in tomato that confer resistance to glasshouse whiteflies. Our experiments suggests that MeSA induces defences in tomato that are effective against whiteflies. This is important information for future work that could seek to optimise the methods we investigate here because defence elicitation with HIPVs has been shown to induce defences that are specific to individual herbivore species (Timilsena et al. 2019; Zhang et al. 2019). Subsequent laboratory experiments confirmed that our MeSA spray regime immediately induced defences in tomato, rather than priming them. Priming is a much more attractive method of defence induction for growers because primed plants will only transiently induce defences during the primed state, meaning yield will not be impacted if the pest never successfully infests the crop (Martinez-Medina et al. 2016). Low concentrations of

HIPVs are thought to induce priming, whereas large concentrations are thought to immediately induce plant defences (Heil and Ton 2008; van Hulten et al. 2006). This is in accordance with the 'likelihood' of a future herbivore attack occurring, with high concentrations of HIPVs indicating that the plant tissue under attack is located more closely to the perceiving plant tissue. Our direct spray application of MeSA probably delivered a relatively high concentration of MeSA to the plants, which caused the direct induction of plant defences. Future work should endeavour to induce a priming response in tomato, and this could potentially be achieved by reducing the amount of MeSA applied. We would still advise multiple applications of MeSA over a similar period of time because a previous study showed that multiple applications of MeSA produces a more robust priming response than single point applications (Song and Ryu 2018). We are confident that a similar time course application, with lower amounts of MeSA, could induce priming in tomato and confer resistance to glasshouse whiteflies. Ideally, we would support the use of HIPVs as an odour based application in order to ensure minimal environmental impact. However, this could be difficult to achieve due to the precise nature of plant priming with HIPVs. Even if priming can be achieved with a lower concentration of MeSA, we doubt that priming alone would be enough to effectively protect tomato from whiteflies because cultivated tomato varieties are known to exhibit poor levels of innate defence mechanisms (McDaniel et al. 2016; Rakha et al. 2017; Firdaus et al. 2012). Despite this, plant priming is expected to be a key component for future IPM strategies (Bruce et al. 2017) and would potentially be more effective if used in conjunction with a variety of other IPM components. Unfortunately, there is still a severe lack research combining plant priming/defence induction with other pest management components. Whilst our attempts to combine plant defence induction and repellent volatile chemistry did not produce any synergistic effects, we would still urge other research groups to integrate plant vaccination with additional IPM components.

5.4 Wild and cultivated tomato VOCs and HIPVs

VOCs are an important element of plant defence and studies have shown that wild tomato species release a greater quantity and diversity of VOCs compared with cultivated tomato (Proffit et al. 2011; Bleeker et al. 2009). Previous work from our lab group found that whiteflies preferentially settle on the commercially grown 'Elegance' in free-choice assays

with the wild *S. pimpenillefolium* (McDaniel et al. 2016). We speculated that this could be due to different volatile bouquets emitted from these two tomato species. We observed a significant difference in the way 'Elegance' and *S. pimpenillefolium* release constitutive VOCs and whitefly induced HIPVs (Chapter 4). Subsequent olfactory preference assays showed whiteflies were repelled by constitutive VOCs from *S. pimpenillefolium*, similar to what was observed by McDaniel et al. (2016). 2-hexenal, 2-carene, β-caryophyllene and humulene were released constitutively by *S. pimpenillefolium* but were not present in constitutive VOC emissions from 'Elegance'. VOCs have been shown to require significant resource allocation (Gershenzon 1994; Robert et al. 2013) and production of these compounds could have been sacrificed at the expense of growth related traits after centuries of domestication. A similar effect was observed in domesticated cranberry (*Vaccinium macrocarpon*), where breeding for yield and desirable fruit traits reduced VOC emissions and negatively impacted resistance to gypsy moth (*Lymantria dispar*) (Rodriguez-Saona et al. 2011). Whilst 2-hexenal, 2-carene, β-caryophyllene and humulene were not detected in constitutive VOC emissions from 'Elegance', they were released by 'Elegance' in response to whitefly infestation. The fact that these compounds are detected in 'Elegance' HIPV emissions confirms that the biosynthetic pathways involved in producing these compounds are still present in 'Elegance'. This is valuable information for future metabolic engineering attempts to enhance VOC/HIPV emissions from cultivated tomato. Other research groups have claimed that cultivated tomato lack certain VOCs that are still present in wild ancestors (Proffit et al. 2011; Bleeker et al. 2009). Importantly, these studies did not analyse HIPV emissions and this could mean that the VOCs that are apparently absent from cultivated tomato, could still be released in response to herbivory. Our work highlights the importance of HIPV analysis when comparing the way wild and commercial crops produce airborne compounds and we believe HIPV analysis should be a prerequisite of any future work comparing airborne defences.

Whiteflies preferred odours from naïve 'Elegance' over *S. pimpenillefolium* but showed no differential preference for odours from whitefly infested 'Elegance' or *S. pimpenillefolium*. This suggests that 'Elegance' produces a HIPV based response that has an antixenotic effect on whiteflies that reduces their preference for 'Elegance' as a host. MeSA was the most prominent HIPV released by 'Elegance' and MeSA emissions were significantly higher in 'Elegance' compared with *S. pimpenillefolium*. MeSA is an important HIPV that has been shown to be repellent to whiteflies (Shi et al. 2016) and elicit defences in tomato (Rowen et

al. 2017; Shulaev et al. 1997). We therefore expected *S. pimpenillefolium*, which is more resistant to whiteflies, to release more MeSA than 'Elegance', but this was not the case. Whilst numerous studies have shown that domesticated crops produce fewer VOCs than wild ancestors (Degenhardt et al. 2009; Bleeker et al. 2009; Rodriguez-Saona et al. 2011), a recent meta-analysis from Rowen and Kaplan (2016) found that domesticated crops from the families *Solanaceae* and *Brassicaceae* produce a larger amount of HIPVs than wild ancestors. The authors suggest that increased herbivore damage on the more susceptible domesticated plants could induce the release of more HIPVs. This assumes that the amount of HIPVs released is in accordance with the amount of damage inflicted. The elevated release of MeSA could be due to increased herbivory on the more susceptible 'Elegance' (McDaniel et al. 2016). Contrary to this, we also found that *S. pimpenillefolium* releases significantly larger amounts of 2-hexenal, α-pinene, β-myrcene and β-ocimene in response to whiteflies, so increased herbivory is probably not the sole explanation for the disparity in MeSA emissions between the two species. The mechanisms by which plants release certain HIPVs in response to specific biotic stressors is still not fully understood (Heil 2014), but specific types of feeding behaviour have been found to induce different HIPVs (Zhang et al. 2019). McDaniel et al. (2016) found that whiteflies probe 'Elegance' significantly more than *S. pimpenillefolium*, and it is possible that this behaviour could induce the release of more MeSA from 'Elegance'.

Interestingly, VOCs and HIPVs from *S. pimpenillefolium* made these plants more attractive to *E. Formosa*, a commonly used biocontrol organism for suppression of whitefly populations. In our discussion of Chapter 4, we putatively attribute this phenomenon to the presence of constitutive VOCs that are absent from 'Elegance', along with a more complex mixture of whitefly induced HIPVs from *S. pimpenillefolium*. Clearly, superior attraction to natural enemies is beneficial for *S. pimpenillefolium* and it would be pertinent to assess whether this translates to increased parasitism of whitefly pupae. Quantifying the parasitism of whitefly pupae on *S. pimpenillefolium* and 'Elegance' in a free choice experiment with *E. formosa* would help ascertain the value of the VOC/HIPV blend released by *S. pimpenillefolium*. MeSA was by far the most abundant compound detected in 'Elegance' HIPV emissions and has previously been shown to be attractive to *E. Formosa* (James and Price 2004). In our discussion of Chapter 4, we argued that the significantly higher amount of MeSA released by 'Elegance' could make these plants more attractive to *E. Formosa*, but this was not the case. Volatile mixtures can be perceived by insects in a manner that is qualitatively different from

the perception of single compounds (Szendrei and Rodriguez-Saona 2010), which may contribute to the fact that *E. Formosa* are more attracted to the more complex mixture of VOCs/HIPVs from *S. pimpenillefolium.* Current work developing odour based natural enemy attractors has focused on using single compounds (James and Price 2004; Yu et al. 2008) but our work would suggest that using specific mixtures of HIPVs could provide a more attractive effect. Further experiments assessing the attractiveness of *S. pimpenillefolium* HIPV mixtures, and individual HIPVs, could be used to develop attractive lures for *E. formosa*.

5.5 Wild tomato resistance mechanisms

Our analysis of within-plant biochemical defences revealed distinct differences in the way *S. pimpenillefolium* and 'Elegance' produce leaf tissue PODs. Both constitutive and whitefly induced POD levels were significantly higher in *S. pimpenillefolium* and this elevated POD activity could contribute to resistance to whiteflies. PODs are positively correlated with resistance to a wide range of biotic and abiotic stressors (Taggar et al. 2012; Hammerschmidt et al. 1982; Dowd and Lagrimini 2006) and numerous resistant plant varieties have been attributed with high POD activity (Mohammadi and Kazemi 2002; Bashan et al. 1987; Gorovits et al. 2007). In our discussion of Chapter 4, we propose that genetic engineering technologies could be used to up-regulate POD expression in 'Elegance' to increase resistance to whiteflies. This has been done previously using tobacco and the resulting transgenic plants were more resistant to glasshouse whiteflies, aphids and caterpillars (Dowd and Lagrimini 2006). This broad spectrum resistance should be appealing to growers because glasshouse grown tomatoes often face attack from multiple insect herbivores (Lange and Bronson 1981). It is important to note that over expressing POD activity can impair plant root growth and development (Lagrimini et al. 1997; El Mansouri et al. 1999).

Phytohormones are an essential component of plant defence (Bari and Jones 2009) and deciphering the way whiteflies induce plant defence signalling could help characterise the defence mechanisms of resistant plant varieties. We found distinct differences in the way 'Elegance' and *S. pimpenillefolium* induce defence signalling in response to whitefly infestation. In our discussion of Chapter 4, we focus on the results for SA abundance, a phytohormone responsible for regulation of the SA defence-signalling pathway. Whiteflies

have been shown to manipulate plant defence signalling by inducing SA related defences that facilitate whitefly population growth (Zhang et al. 2019; Zhang et al. 2013). We found that whitefly infestation significantly increased the amount of SA in 'Elegance' leaf tissue, but we observed no significant change in SA abundance for *S. pimpenillefolium*. We argue that this, combined with a higher average fold change in PR-1 transcript abundance, represents a more intense induction of SA related defences in 'Elegance'. This could make 'Elegance' more susceptible to whiteflies because induction of SA related defences results in end-products which are ineffectual against whiteflies (Zhang et al. 2019; Zhang et al. 2013). Clearly, this work needs to be extended to include a range of defence related genes and a longer, incremental time course in order to fully assert this claim. Despite this, our study is the first to assess phytohormone abundance in *S. pimpenillefolium* and we would urge other research groups to consider the importance of plant defence signalling when assessing resistance mechanisms in wild tomato species. Whilst we have focused on the way 'Elegance' and *S. pimpenillefolium* defend against whiteflies, our phytohormone results indicate that *S. pimpenillefolium* could be resistant to other biotic stressors. For example, the large amount of constitutively produced SA in *S. pimpenillefolium* could assist in producing an effective defence response against pathogens. SA is crucial for the induction of systemic acquired resistance (SAR) following pathogen infection (Gatehouse 2002) and plants with high levels of endogenous SA have been shown to be more resistant to pathogens (Verberne et al. 2000).

Tomato have been shown to induce defensive measures after exposure to HIPVs (Lopez et al. 2012; Sugimoto et al. 2014) and we sought to compare the way 'Elegance' and *S. pimpenillefolium* respond to airborne distress signals. We found that *S. pimpenillefolium* primes Mi-1.2 transcript abundance after exposure to HIPVs from whitefly-infested conspecifics, whereas 'Elegance' immediately induces Mi-1.2 transcript abundance after exposure to HIPVs. This work, which to our knowledge is the first of its kind, suggests that *S. pimpenillefolium* responds to airborne distress signals more efficiently than 'Elegance'. The mechanisms by which plants perceive HIPVs is still not fully understood (Heil 2014), but it is possible that these VOC/HIPV perception mechanisms may be less efficient in cultivated tomato, bred out at the expense of growth related traits. Most published work assessing the resistance mechanisms of wild tomato species, limited as it is, has focused on the analysis of biochemical or structural traits (Zsogon et al. 2018; Bleeker et al. 2009; Rakha et al. 2017). However, we would argue the importance of understanding the way resistant plant species

respond to HIPVs because recognising and responding to airborne signals is an integral component of plant defence (Heil 2014; Heil and Ton 2008). Future work should look to characterise HIPV defence induction on a genome wide level to ascertain which specific defensive components induced and/or primed in 'Elegance' and *S. pimpenillefolium*.

It is important to note that the observed priming effect in *S. pimpenillefolium* is intrinsically linked to the mixture of HIPVs that *S. pimpenillefolium* releases in response to whiteflies. It is possible that exposing *S. pimpenillefolium* to HIPVs from whitefly-infested 'Elegance' could cause an immediate induction of Mi-1.2 transcript abundance, similar to what was observed in 'Elegance'. Future work should look to assess the way these two plant species respond to specific HIPV blends to ascertain whether priming of defences in *S. pimpenillefolium* is specific to the suite of HIPVs that conspecifics release in response to whiteflies. It is possible that our work could represent a more general response to HIPVs, whereby *S. pimpenillefolium* primes defensive measures more readily than 'Elegance'. This would be extremely important because even domesticated crops must be able to effectively respond airborne distress signals. Immediate induction of plant defences can result in a loss of yield (van Hulten et al. 2006) and if 'Elegance' are unable to prime defensive measures, this could impact the productivity of 'Elegance' cultivation systems. Our work shows that *S. pimpenillefolium* are more sensitive to airborne distress signals and also produce a more effective defence response against whiteflies. Other groups have proposed that stress-sensitive wild plants could be intercropped alongside cultivated crops to act as "sentinel plants" that signal the occurrence of an imminent stressful situation to neighboring crops (Brilli et al. 2019). If *S. pimpenillefolium* HIPVs also prime defences in 'Elegance', *S. pimpenillefolium* could be used as sentinel plants to improve the way cultivated tomato respond to whitefly infestation. Introducing *S. pimpenillefolium* to tomato cultivation systems would also increase plant diversity and potentially provide a marketable crop, as the fruits of *S. pimpenillefolium* are edible.

5.6 Conclusion

Although there is great potential for VOCs to control whiteflies in commercial agriculture, there is still a lack of field-based research assessing the efficacy of VOCs as a whitefly control

method (Schlaeger et al. 2018). This thesis explored the use of plant VOCs as a sustainable whitefly control method and our work confirms the potential for VOCs to be used to control whiteflies on glasshouse-grown tomato. Our intercropping experiments highlight the efficacy of repellent companion plants as a whitefly management strategy and should be appealing for those who seek to increase agro-ecosystem diversity and reduce the need for pesticide applications. Whilst we assert the potential for companion plants to be used in commercial agriculture, we believe this work will also be valuable to home-growers who seek to grow tomatoes more sustainably. Our work with whitefly-repellent limonene dispensers produced positive effects on tomato yield and was our most attractive VOC based control method. The few studies that have sought to use repellent VOC dispensers in field-based experiments have also produced positive results (Lamy et al. 2018; Du et al. 2016; Alquézar et al. 2017) and we hope that our work will galvanise the scientific community to continue the development of repellent VOC dispensers to control insect pests. It is anticipated that genetic engineering will be fundamentally important for future IPM programmes (Kennedy 2008) and our analysis of *S. pimpenillefolium* resistance mechanisms highlights several defensive components that could be transferred to cultivated tomato to increase resistance to whiteflies. Our comparison of *S. pimpenillefolium* and 'Elegance' defensive components highlights the importance of VOCs/HIPVs for defence against whiteflies and attraction to natural enemies. We propose that *S. pimpenillefolium* VOCs/HIPVs could be used to develop *E. formosa* attractive lures, which could be used in conjunction with our limonene dispensers in a push-pull system. In summary, this thesis presents a range of applied and theoretical VOC based control methods for glasshouse whiteflies that could be used to alleviate our reliance on synthetic chemical sprays and grow our food more sustainably.

Appendix

Appendix A: Free choice assays experimental layout

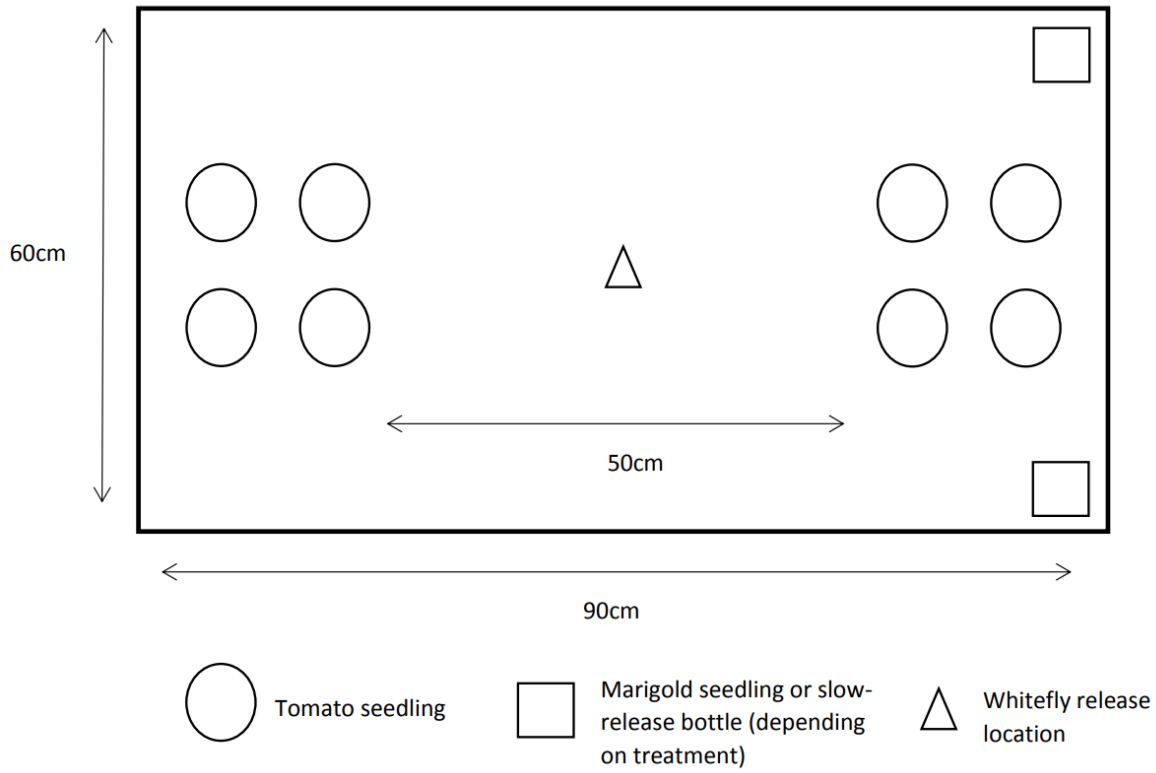

Figure A: This diagram shows a plan view of the experimental design used for the whitefly free-choice assays.

Appendix B: Leaf disc assays

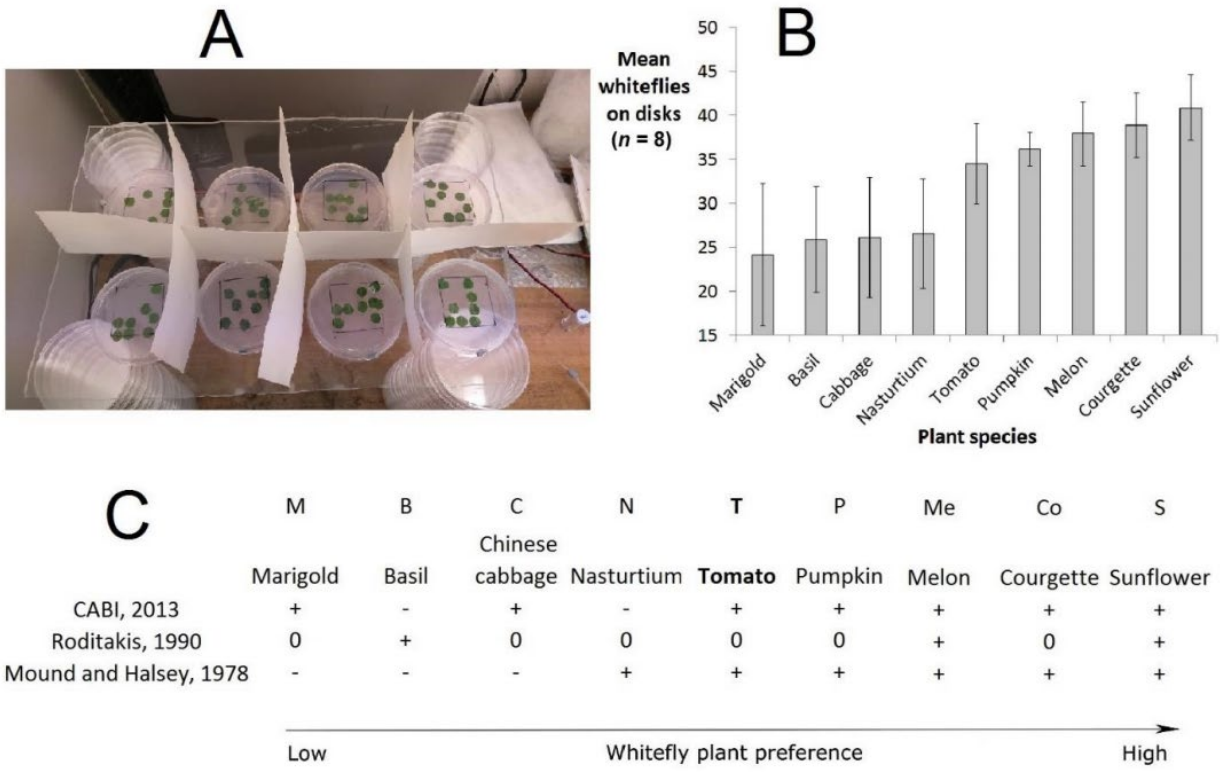

Figure B: The non-choice plant tissue preference assay may be seen in (A), with 8 discs of tomato in each dish. Fifty whiteflies were added and the average number of whiteflies settled on plant tissue after 21h was calculated. Average whiteflies settled (n = 8) on each plant species can be seen in (B), with 95% confidence intervals for the mean plotted, and the species ordered in order of preference. This quantification of preference agreed with previous broad surveys of T. vaporariorum plant range (C) from CABI (2018), Roditakis (1990) and Mound (1978), respectively, with non-hosts being less preferred and hosts more preferred. '+' indicates 'host', '-' indicates 'non-host' and '0' indicates that this plant was not considered. Methods for this experiment can be found in Appendix K.

Appendix C: Glasshouse trial experimental layout

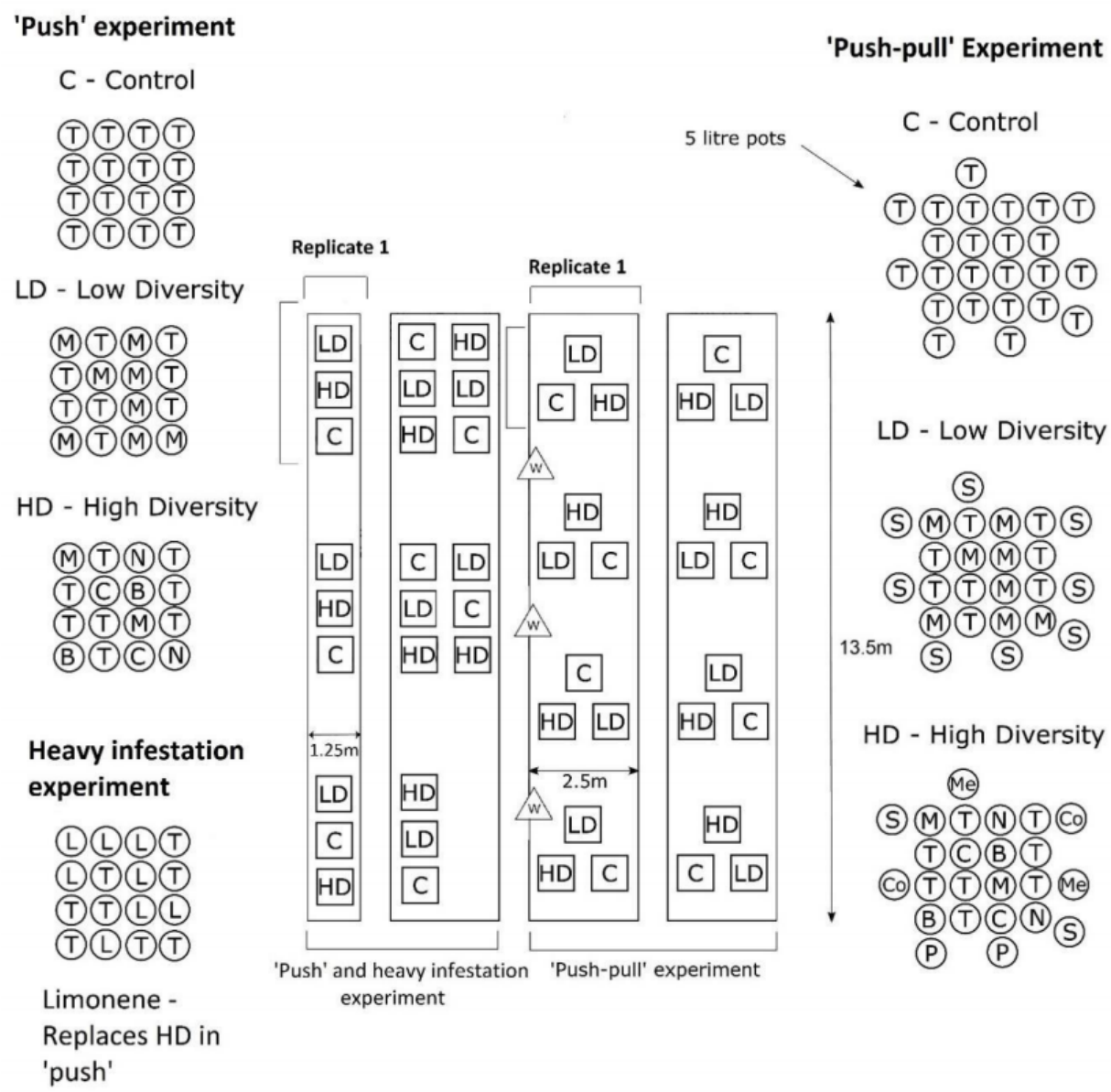

Figure C: Layout of experiments to test the efficacy of 'push' and 'push-pull' strategies against the glasshouse whitefly on tomato, and the efficacy of intercropping at reducing large whitefly population sizes. A randomised block design was used, with each of the 8 replicates containing all 3 treatments for each study in a random order. The 'push' experiment involved intercropping tomato with non-hosts. The 'push-pull' experiment was similar but additionally had attractive host plants around the perimeter. Whitefly plant preference for the various hosts was determined in laboratory leaf disc experiments (Appendix B) and confirmed by literature surveys. The location of heavily infested aubergine plants used to supplement natural whitefly populations are shown with triangles containing the letter 'W'. The experiment to test the introduction of plants during an advanced whitefly infestation was nearly identical in layout to the 'Push' experiment, but with limonene dispensers placed in

compost replacing the non-tomato plant species in the HD treatment used the 'push' assay. Acronyms for the treatments used are as follows; C—control, LD—low diversity, HD—high diversity. For the individual plants within each treatment; T—tomato, M—marigold, N—nasturtium, B—Basil, C–Chinese cabbage, Me–Melon, Co–courgette, S–sunflower.

Appendix D: Population development of Thrips

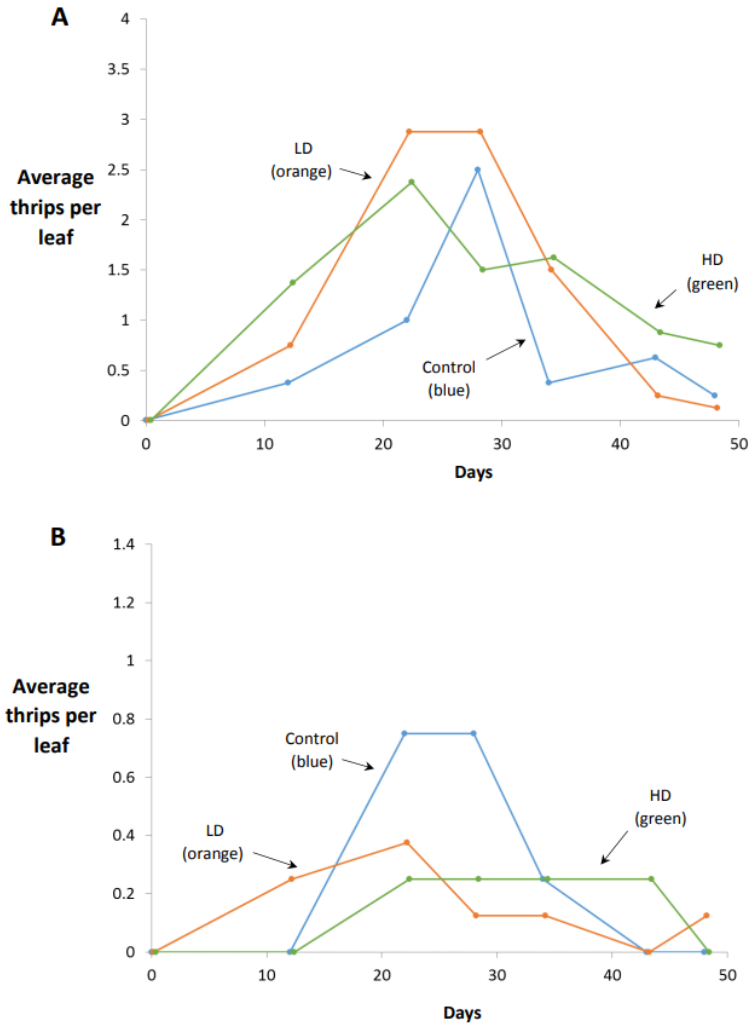

Figure D: *Population development of thrips (T. tabaci) on tomato in the glasshouse, with all thrips life stages (adult and larvae) contributing to the average number of thrips/leaf. Data was (log +1) transformed and repeated measures ANOVA's were used to assess the effect of treatment and treatment x time on thrips abundance across the experimental period. No significant interactions were observed between treatments (rm ANOVA $F_{(2,126)} = 0.95$, $p = 0.388$) or treatment x time (rm ANOVA $F_{(10,126)} = 0.570$, $p = 0.835$) for the "push" experiment (A). For the "push-pull" experiment (B), no significant effects were observed between treatment (rm ANOVA $F_{(2,105)} = 0.333$, $p = 0.717$) and treatment x time (rm ANOVA $F_{(8,105)} = 0.420$, $p = 0.906$). (A) shows the "push" experiment in which repellent plants such as marigold (LD) and marigold and other non-hosts (HD) are distributed amongst tomato plants. (B) shows the "push-pull" experiment which is the same as the "push" experiment but additionally a*

single (LD) and several (HD) host plant species are placed around the perimeter of the mixture of repellent hosts and tomato.

Appendix E: Plant development characteristics at the end of the 2016 "push" and "push-pull" glasshouse trials

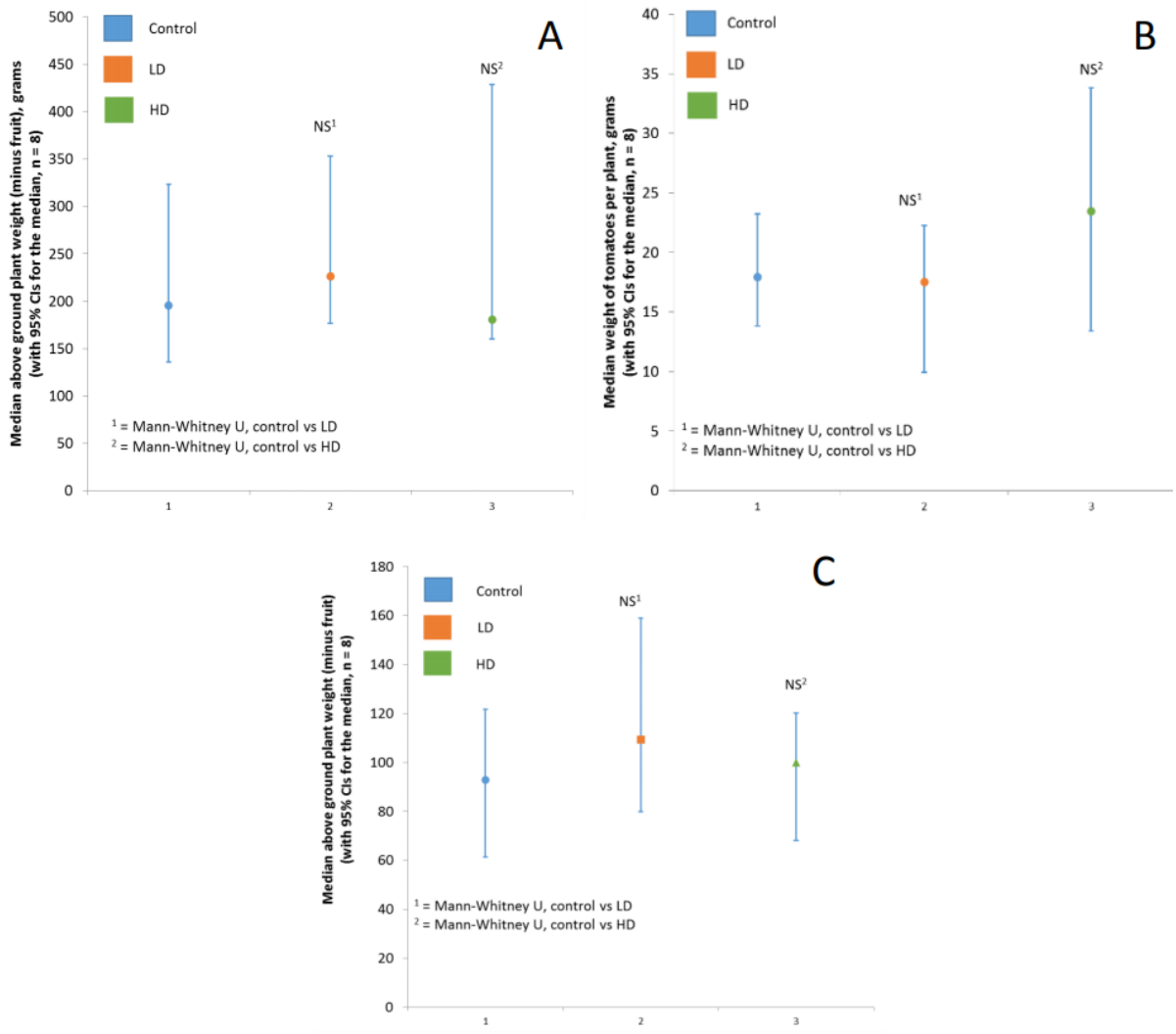

Figure E: *Plant development characteristics at the end of the 2016 "push" and "push-pull" glasshouse trials (n = 8). (A) shows the median above ground plant weight for each of the three treatments (control, low diversity and high diversity) from the "push" experiment with 95% confidence intervals annotated as error bars. (B) shows the median weight of tomatoes per plant per treatment also from the "push" experiment, 95% confidence intervals are annotated as error bars. (C) shows the median above ground plant weight for each treatment in the "push-pull" experiment with 95% confidence intervals annotated as error bars. There is no tomato yield data for the "push-pull" experiment as tomatoes had not yet formed due to this experiment starting later than the "push" experiment and being subsequently cut-short by the onset of late season blight. The Mann Whitney U test was used to compare differences*

between the treatments as the data was found to be non-normally distributed. For all three graphs, there was no significant differences observed between any of the treatments.

Appendix F: Root exudates assays

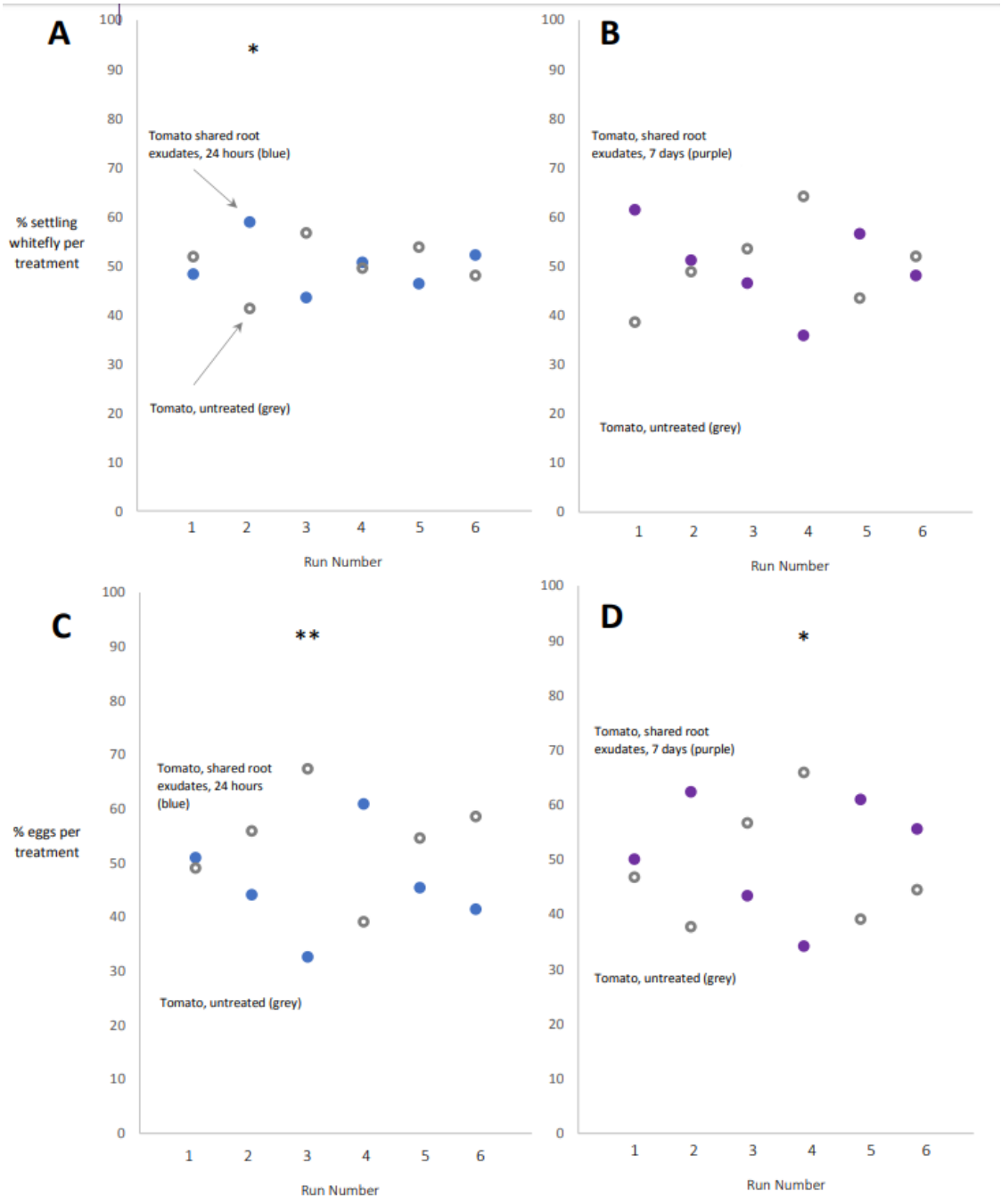

Figure F: *The percentage of settling whiteflies (n = 200) when given the choice between four "Elegance" tomato seedlings or four "Elegance" seedlings previously cultivated in communal drip trays with flowering T. patula seedlings for either 24 hours or 7 days (A and B). The tomato plants which were grown in this way are referred to with the acronym SRE (shared root exudates). Whitefly eggs were also recorded for each replicate (C and D) and the Pearson's chi-squared test was used to test differences in settling and oviposition behaviour*

*compared to control distributions. Significant differences are annotated onto the graph for each treatment with the following format; *p < 0.05 significance; **p < 0.01 significance, df = 1 for all groups. For (A), rep 1 X^2 = 0.18, p = 0.671; rep 2 X^2 = 3.92, p = 0.048; rep 3 X^2 = 0.08, p = 0.776; rep 4 X^2 = 0.72, p = 0.396; rep 5 X^2 = 0.02, p = 0.887; rep 6 X^2 = 0.98, p = 0.332. (B), rep 1 X^2 = 1.64, p = 0.199; rep 2 X^2 = 0.02, p = 0.887; rep 3 X^2 = 0.500, p = 0.479; rep 4 X^2 = 0.20, p = 0.887; rep 5 X^2 = 0.504, p = 0.478; rep 6 X^2 = 0.320, p = 0.572. (C), rep 1 X^2 = 1.62, p = 0.202; rep 2 X^2 = 0.082, p = 0.775; rep 3 X^2 = 0.02, p = 0.886; rep 4 X^2 = 7.22, p = 0.007; rep 5 X^2 = 0.325, p = 0.569; rep 6 X^2 = 0.021, p = 0.886. (D), rep 1 X^2 = 0.08, p = 0.777; rep 2 X^2 = 2.04, p = 0.153; rep 3 X^2 = 1.64, p = 0.203; rep 4 X^2 = 6.61, p = 0.010; rep 5 X^2 = 1.64, p = 0.199; rep 6 X^2 = 0.322, p = 0.570. On average over the 6 replicates, 49.89% of whiteflies settled on SRE tomato in the 24 hour treatment and 49.91% whiteflies settled on SRE tomato in the 7 day treatment.*

Appendix G: Limonene detection and quantification

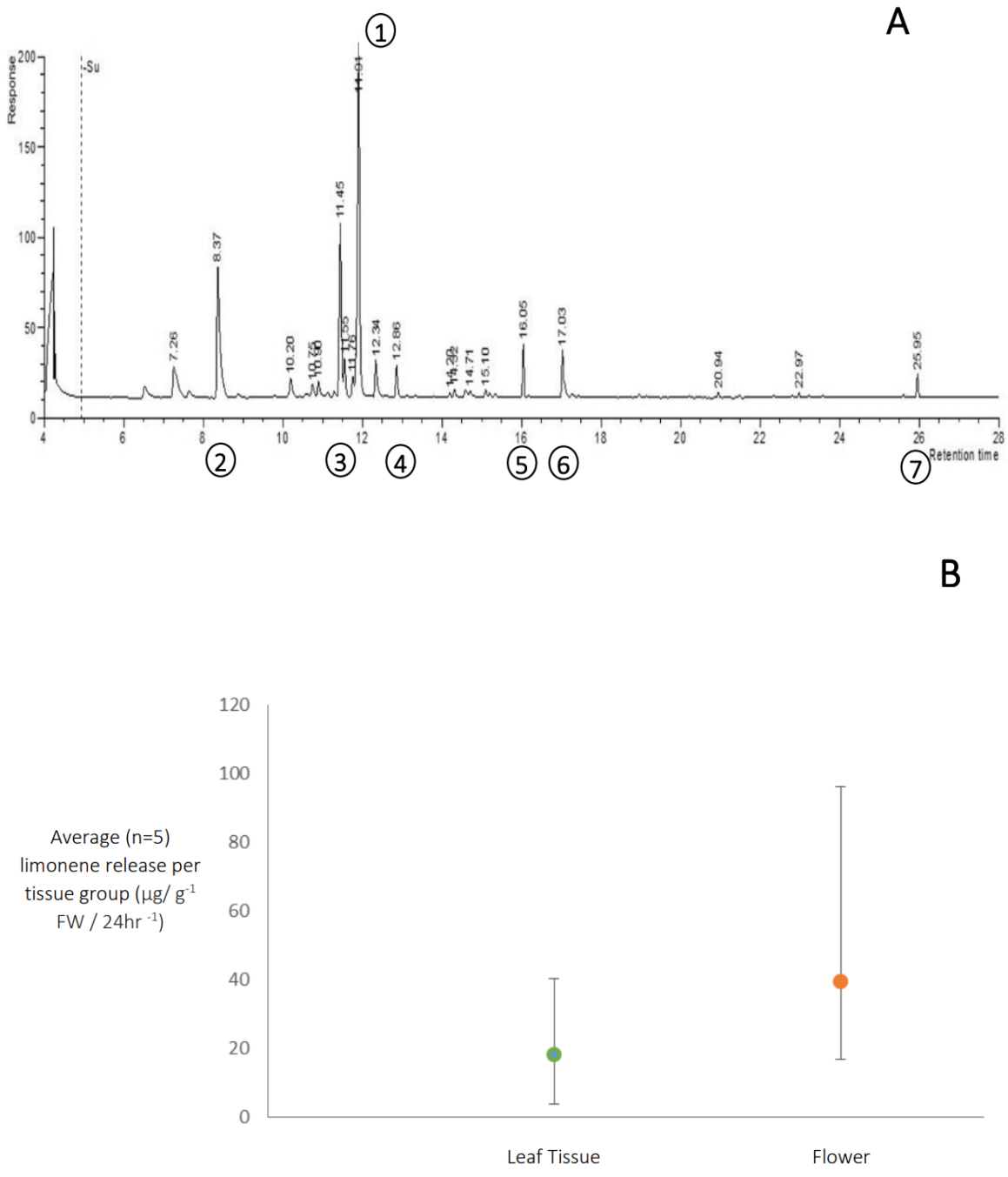

Figure G: *(A) displays a GC-FID profile showing the volatile output from a single French marigold flower over 24h. For both marigold flowers and leaf tissue, limonene was the most prominent volatile and the GC trace shown was typical across all the replicates. Limonene formed 24.01% and 21.04% of the volatile output from both flowers and leaf tissue respectively. Whilst other prominent volatiles were detected in marigold headspace samples, none matched the emission rates and cost effective appeal which was offered through the use*

*of limonene. It was therefore decided that whitefly repellence to this individual chemical would be assessed. Other identified volatiles from the GC-FID trace are labelled concurrently, "^" indicates confirmation of presence with authentic standards, where available; 1 = limonene^, RT 11.91; 2 = α-pinene^, RT 8.37; 3 = isobutyric acid, RT 11.45; 4 = (E/Z)-β-ocimene^, RT 12.66; 5 = Siloxane contaminant (originating from the GC column), RT 16.05; 6 = terpinolene^, RT 17.03; 7 = Butylated hydroxytoluene (stabilising agent from the diethyl ether), RT 25.95. (B)*http://www.plosone.org/article/info:doi/10.1371/journal.pone.0213071 - pone.0213071.s004 *displays average limonene release from marigold flowers and leaf tissue was quantified and displayed as µg of limonene per gram of fresh weight over 24 hours. Error bars display upper and lower bound 95% confidence intervals, each tissue group was replicated 5 times.*

Appendix H: Olfactory experiments

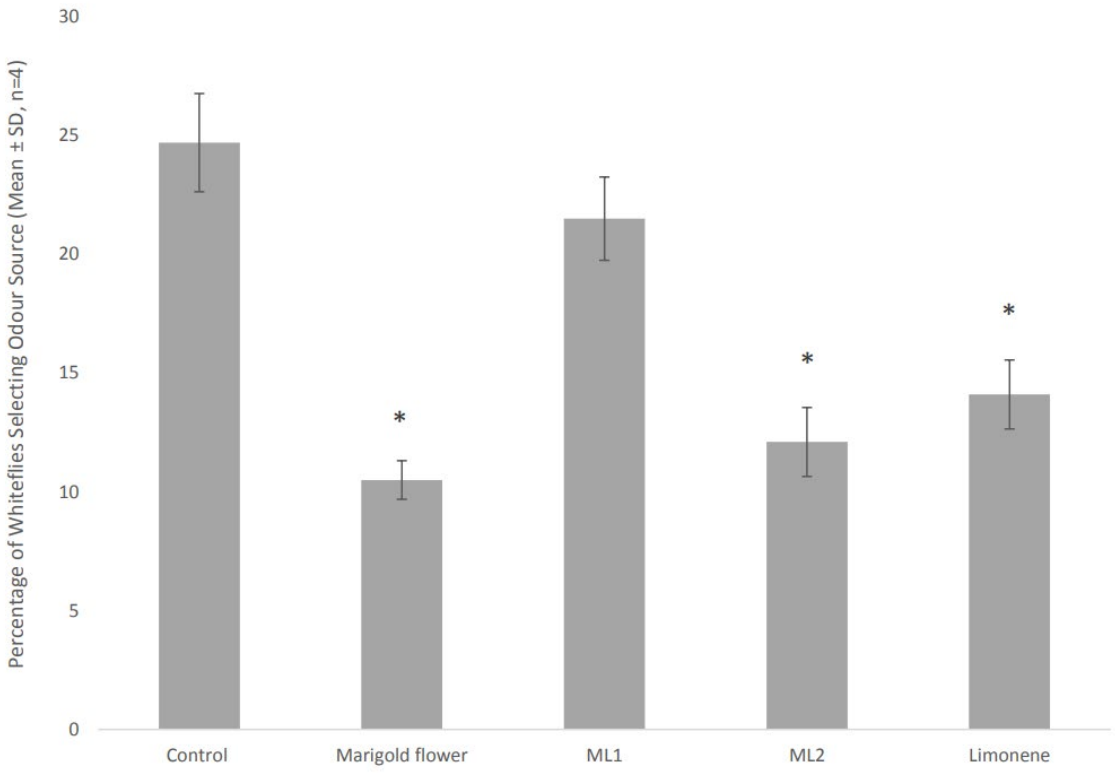

Figure H: *Whitefly response to marigold plant tissue and limonene was tested using a 4-way olfactometer with four different treatments; one marigold flower, marigold leaves that were the same weight as one marigold flower (1.713g) (ML1), marigold leaves that were double the weight of one marigold flower (3.426g) (ML2) and a "type 1" limonene dispenser. The two different amounts of leaf tissue were chosen considering the amount of limonene released from flowers was approximately double that of leaf tissue per gram of fresh weight (Figure G, B). For each treatment, 100 whiteflies of mixed sex were introduced to the olfactometer and*

average (n = 4) percentage of whiteflies which selected the wing of the olfactometer containing the marigold plant tissue or a limonene dispenser is displayed. The Pearson's chi-squared test was used to test if distribution of whiteflies differed significantly from the average settling distribution across 4 control replicates where only tomato was present in all four wings of the olfactometer. Significant differences are annotated onto the graph with "". For each of the treatments; marigold flower = X^2 = 11.21, p = 0.001; ML1 treatment = X^2 = 0.653, p = 0.419; ML2 = X^2 = 8.87, p = 0.001; limonene dispenser = X^2 = 6.33, p = 0.050, df = 3 for all. Average settling percentages across the four experimental wings of the olfactometer in the control was 24.70%. For each of the treatments, average settling in wings containing individual treatment materials were as follows; marigold flower = 10.5%, ML1 = 21.5%. ML2 = 12.1%, limonene = 14.1%. Methods for this experiment can be Appendix K.*

Appendix I: Images from Glasshouse trial (Chapter 3)

Figure I: *Overview of how limonene dispensers were distributed amongst tomato plants in the Limonene and ML treatments. B = Typical set of tomato fruits at the point of harvesting. C = Tomato seedlings at day one of MeSA application. D= View across the glasshouse at the final day of sampling.*

Appendix J: HIPV exposure experiment conducted by Dr Thomas McDaniel

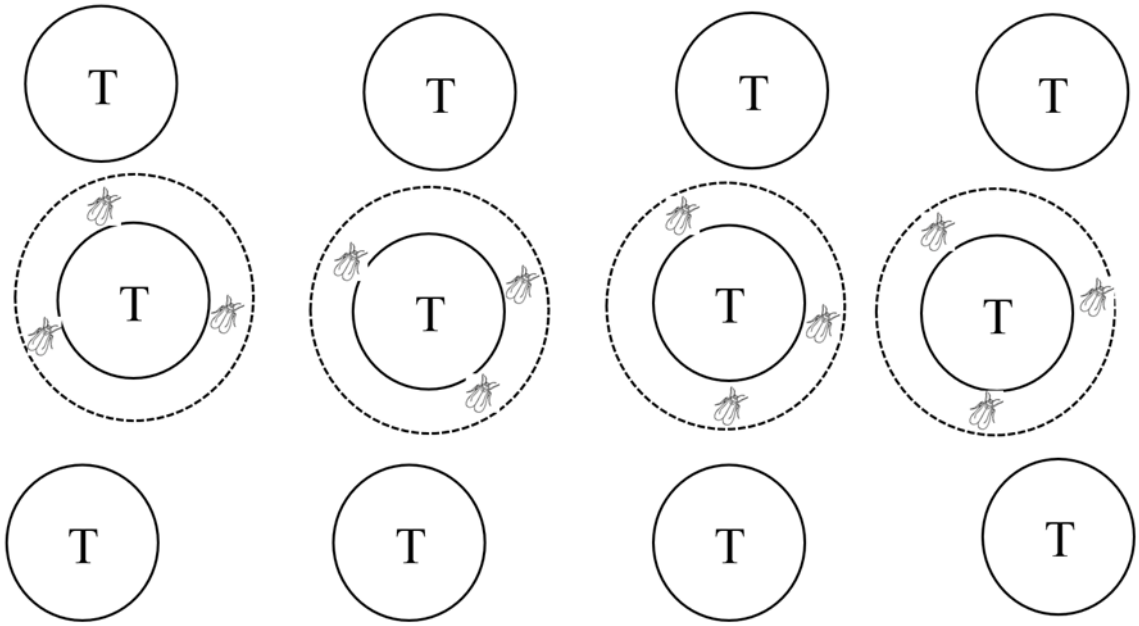

Figure J: *Schematic overview of the experiment conducted by Dr Thomas McDaniel to assess how tomato respond to volatiles from whitefly infested conspecifics. Each 'T' represents a tomato seedling, the tomatoes in the centre of the diagram surrounded by a dotted circle indicate plants which were infested with whiteflies. This dotted circle represents a mesh cage which allowed volatiles from these plants to interact with plants outside the cages, whilst also preventing whitefly escape.*

Appendix K: Materials and methods (Chapter 2)

Whitefly response to marigold plants and limonene was tested using a 4-way olfactometer, this consisted of an enclosed Perspex arena of 30cm in diameter with four side wings, as described in (Pettersson 1970). This was chosen as it provides a constant flow of air which was at a rate of 0.2l/min through each wing. At the end of each of the four arms a 'partial non-return' glass bulb was fitted. The scent of the odour sources was added to the airflow by connecting four plastic airtight boxes to each wing of the olfactometer. For the control, a single "elegance" tomato leaflet from seedlings at stage 13 on the BBCH scale was placed in a 5ml vial filled with water inside each plastic box on each of the four wings of the olfactometer. One hundred whiteflies of mixed sex (obtained from the laboratory culture described in the main text materials and methods) were released into the centre of the olfactometer and were left for 24 hours, at which point the number of adult whiteflies in each of the four wings were recorded. For each of the four treatments (marigold flower, ML1, ML2 and limonene) one of the tomato leaflets in the olfactometer was replaced with a marigold flower, marigold leaves with a weight of approximately 1.7g (ML 1 treatment), marigold leaves with a weight of approximately 3.4g (ML2 treatment) and a "type 1" limonene dispenser (limonene treatment). The specific weights for ML1 and ML2 were based on the finding that marigold flowers (with an average weight of 1.7g) produce on average roughly twice the amount of limonene as marigold leaf tissue (see Appendix D). The entire apparatus was cleaned between each experiment and covered with an opaque sheet during experimental procedures to ensure the light intensity was approximately equal. The experiment was repeated four times per treatment. The Pearson's chi-squared test was used to test if distribution of whiteflies differed significantly from the average settling distribution across 4 control replicates where only tomato was present in all four wings of the olfactometer.

To quantify whitefly preference for these plants, a laboratory leaf disc assay (shown Appendix B) was used to examine whitefly preference in microcosm, by monitoring how readily whiteflies colonised leaf discs of the different plant species in a no-choice situation and comparing these numbers to whitefly numbers on tomato leaf discs. This assay was modified from a previous study (Frei et al. 2003) and findings were compared to previous surveys of *T. vaporariorum* host range (CABI 2018; Roditakis 1990; Mound 1978). Plant species and

varieties used in the laboratory assay and in glasshouse experiments were as follows: French marigold, *Tagetes patula* 'honeycomb'; basil, *Ocimum basilicum* 'sweet'; Chinese cabbage, *Brassica rapa* 'Blues F1'; nasturtium, *Tropaeolum majus* 'jewel mixed'; tomato, *Solanum lycopersicum* (plum) 'Roma VF'; pumpkin, *Cucurbita pepo* 'Racer F1'; melon, *Cucumis melo* 'Antalya F1'; courgette, *Cucurbita pepo* (cylindrica) 'All green bush'; sunflower, *Helianthus annuus* 'Giant single'. For the leaf disc assays plants were grown in John Innes No. 2 compost in 9-cm-diameter and 8.7-cm-deep pots, at a density of one plant per pot, with plants watered liberally. All plants were grown under the same conditions as previously described in the "plants" section of the materials and methods of the main text. Plants had the following number of fully expanded leaves (not including the cotyledons) when used, which approximated to stage 13 on the BBCH scale for tomato, Chinese cabbage and sunflower: marigold 4-6 leaves, basil 2-4 leaves, Chinese cabbage 4-5 leaves, nasturtium 4-5 leaves, tomato 4 leaves, pumpkin 2-3 leaves, melon 3 leaves, courgette 1-2 leaves, and sunflower 4 leaves.

Sixteen 1cm diameter leaf discs were removed from each plant using a cork borer, with a total of 4 plants used per plant species. Hot 1% agar was poured into 90mm diameter 16mm height petri dishes to the point where it covered the bottom of the dish and 8 newly-cut leaf discs were pressed into the agar (once it had cooled and was on the point of setting) adaxial side down in random positions in a 50mm^2 square in the centre of the dish. The lid was secured and the petri dish was then turned over. Fifty mixed sex adult whiteflies were removed from the laboratory culture described in the main text materials and methods and introduced into the dish through a side hole which was subsequently sealed. A 21h leaf disc assay *in situ* is shown in Appendix B. Eight replicates of this design were completed for each plant species and were completed simultaneously, with 1 plant supplying 16 discs for 2 dishes so that 4 plants provided the discs for the full 8 replicates. Dishes were left for 21h at 20^{o}C, 16h light / 8h dark, synchronized with cultures and plant propagation facilities, after which the total number of whiteflies settled on plant tissue within each dish was counted, with the mean whitefly colonisation number calculated from the 8 replicated dishes.

Appendix L: Materials and Methods (Chapter 3)

Air entrainment

Headspace volatiles of *T. vaporariorum* infested 'Elegance' tomato plants were analysed by dynamic air entrainment (Pye Volatile Collection Kit, Kings Walden, Herts, UK) in order to identify potential defence inducing compounds and confirm presence of MeSA. All equipment was washed with Teepol detergent (Herts County Supplies, Herts, UK) and rinsed with acetone and distilled water twice before baking at 180°C for 2 hours. Porapak Q (60/80 mesh, 0.05 g) tubes were eluted with diethyl ether and heated at 140°C for 2 hr under a stream of constant nitrogen to remove contaminants, this process was repeated twice for each tube. Leaflet one of 2-3 week old tomato seedlings was partially enclosed in a glass bell cylinder, due to the slight differences in leaf size fresh weight (g) of the entrained leaf section was recorded after use. The bottom of the cylinder was closed without pressure around the plant stem by using two semicircular aluminum plates with a hole in the centre to accommodate the stem. Charcoal filtered air was pumped in at 1L min^{-1} and drawn out at 800 ml min^{-1} through the porapak Q adsorbent tube in a 5-mm diameter glass tube. The difference in flow rates created a slight positive pressure to ensure that unfiltered air did not enter the system, thus removing the need for an airtight seal around the stem. Plants were entrained for 24 hours from midday onwards. The porapak Q filter tube was eluted with 0.75 ml of diethyl ether (Fisher Scientific, 12347103), providing a 500µl solution that contained the isolated volatile compounds. All samples were sealed in GC vials and stored at -20°C until needed.

Gas Chromatography (GC)

GC-FID analyses were carried out using an Agilent 5890 GC. The injection port (280°C) was in the splitless mode and the flame ionization detector was heated to 300°C. The sample (1ul) in diethyl ether was injected by an HP7673 auto sampler and the split opened after 1 minute. Separation was performed on a fused silica capillary column (30m x 0.25mm i.d) coated with 0.25um dimethyl poly-siloxane (HP-5 phase). The GC was temperature programmed from 50°C-310°C at 5°C min and held at final temperature for 20 minutes with Hydrogen as the carrier gas (flow 1ml/min, pressure of 50kPa, split at 30 mls/min).

Gas Chromatography-mass spectrometry (GC-MS)

GC-MS analysis of the biological extracts was performed on a Agilent 7890A GC split/split less injector (280°C) linked to a Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1200V, interface temperature 310°C). The acquisition was controlled by a HP Compaq computer using Chemstation software, initially in full scan mode (50-600 amu/sec) or in selected ion mode (30ions 0.7cps 35ms dwell) for greater sensitivity. The sample (1ul) in diethyl ether was injected by an Agilent7683B auto sampler and the split opened after 1 minute. Separation was performed on an Agilent fused silica capillary column (30m x 0.25mm i.d) coated with 0.25um dimethyl polysiloxane (HP-5) phase. The GC was temperature programmed from 50-310°C at 5°C min and held at final temperature for 10 minutes with Helium as the carrier gas (flow rate of 1ml/min, initial pressure of 50kPa, split at 30 mls/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST library if > 90% fit or from their elution order from biochemical literature. Presence of compounds was confirmed by comparison with authentic standards and subsequently semi-quantified using the single point external standard method.

Appendix Table A: Confidence intervals from whitefly abundance in 'push' trial

Sampling point	1	2	3	4	5	6	7
C lower CI	0	0.125	0.266	0.391	0.5865	0.375	0.425
C upper CI	0	0.407	0.5	0.29	0.8525	0.935	0.145
LD lower CI	0	0	0.391	0.4375	0.1955	0.3125	0.1955
LD upper CI	0	0.0322	1.425	0.4775	0.4535	0.3285	0.0625
HD lower CI	0	0	0	0.4455	0.125	0.1875	0.3285
HD upper CI	0	0	0.629	0.1105	0.532	0.2195	0.1955

Appendix Table B: Confidence intervals from whitefly abundance in 'push-pull' trial

Sampling point	1	2	3	4	5	6
C lower CI	0	0	0	0.4375	0.383	0.0705
C upper CI	0	0.532	0.5	0.1955	0.375	0.4535
LD lower CI	0	0	0.25	0.125	0.125	0.125
LD upper CI	0	0.266	0.375	0.5	0.516	0.298
HD lower CI	0	0	0.125	0.25	0.125	0.008
HD upper CI	0	0.5	0.657	0.125	0.149	0.383

Appendix Table C: Amount of MeSA released per plant at each day of infestation with whiteflies. Data for MeSA µg/g^{-1} FW was calculated by multiplying the ng/µl/g^{-1} FW value by 500 (total volume of sample in µl) and then dividing by 1000 to give to express the value as µg. MeSA per plant was calculated by multiplying the µg/g^{-1} FW value by the total fresh weight of the plant and an average was taken across the three replicates to give a final value of MeSA released per plant (± standard deviation) at each day of whitefly infestation (*n*=3).

Days per infestation (dpi)	**1dpi**			**2dpi**			**3dpi**			**4dpi**			**5dpi**		
MeSA ng/µl/g^{-1} FW	10.400	17.562	23.376	24.239	8.4149	12.727	11.729	23.660	12.045	5.631	13.189	18.432	38.378	27.645	31.661
MeSA µg/g^{-1} FW	5.200	8.781	11.688	12.119	4.207	6.363	5.864	11.830	6.022	2.815	6.594	9.216	19.189	13.822	15.830
Total plant FW (g)	1.97	1.96	2.00	2.01	2.03	2.00	2.21	1.96	2.22	2.11	2.15	2.12	2.22	2.20	2.21
MeSA per plant	10.244	17.211	23.376	24.360	8.541	12.727	12.961	23.423	13.370	5.940	14.178	19.538	42.600	30.409	34.985
Average MeSA per plant (µg)	**16.94 (±) 6.57**			**15.20 (±) 8.19**			**16.58 (±) 5.92**			**13.21 (±) 6.84**			**35.99 (±) 6.15**		

This experiment utilised information from a previous experiment from our lab group which showed that tomato seedlings exhibited increased resistance to whiteflies after exposure to HIPVs from 4 whitefly infested conspecifics for a period of 5 days (layout of this experiment is shown in Appendix Table C). We therefore tried to replicate this by applying MeSA to tomato seedlings at concentrations similar to that released by whitefly infested tomato plants. The final value from Appendix Table C (average MeSA per plant) was multiplied by four (analogous to HIPV exposure from four plants) and this amount of MeSA was sprayed onto each tomato seedling on the corresponding day. On the first day, 67.76µg of MeSA was applied to each tomato seedling, on day two 60.80µg was applied, day three 66.32µg, day four 52.84µg and on day five 143.96µg was applied. Materials and methods for these experiments can be viewed in Appendix L.

Appendix Table D: Data for average fruit weight per plant (g), average fruit count per plant and average Brix (%) soluble solids across the four treatments from the glasshouse trial.

Yield parameter	***Treatment***			
	Control	*Limonene*	*MeSA*	*ML*
Total fruit weight per plant (g)	483.56 (±) 199.17 *a*	638.44 (±) 177.97 *b*	556.25 (±) 167.96 *a*	600.04 (±) 138.18 *ab*
Fruit count per plant	10.48 (±) 3.41 *a*	13.43 (±) 3.72 *c*	10.81 (±) 3.20 *ab*	12.00 (±) 2.83 *bc*
Brix (%)	3.58 (±) 0.02	3.54 (±) 0.02	3.60 (±) 0.02	3.58 (±) 0.02

Appendix Table E: Primer sequences used for gene expression (qPCR) analysis.

Name	**Sequences (5' → 3')**	**Gene**	**Reference**
PR1-*fw1*	CCTCAAGATTATCTTAACGCTC	Pathogenesis-related (*PR1*)	(Medeiros et al. 2017)
PR1-*rev1*	TACCATTGCTTCTCATCAACC		
LOX *fw*	GCCTCTCTTCTTGATGGA	Lipoxygenase (*LOX1*)	(Medeiros et al. 2017)
LOX *rev*	GTAGTGAGCCACTTCTCCAA		
TPX1-*fw*	GCTTTGTCAGGGGTTGTGAT	Peroxidase (*TPX1*)	(Medeiros et al. 2017)
TPX1-*rev*	TGCATCTCTAGCAACCAACG		
Mi-1.2-*fw*	TCCGAGCTAGATGAGGATGAACA	MI-1.2	N/a
Mi-1.2-*rev*	TATCCGGTGAGGTTTCCAGG		
Actin-fw	CACCACTGCTGAACGGGAA	Actin (*ACT*)	(Medeiros et al. 2017)
Actin-rev	GGAGCTGCTCCTGGCACTTT		

Appendix Table F: Mean and standard deviation for whitefly abundance

Column1	Settling	Column2	Column3	Column4	Eggs	Column5	Column6	Column7	Nymphs	Column8	Column9	Column10
	C	L	MeSA	ML	C	L	MeSA	ML	C	L	MeSA	ML
1 dpi Mean	4.2222222	1.2222222	2	0.8888889	0	0	0	0	0	0	0	0
1dpi Stdev	3.929942	0.8333333	1.7320508	0.781736	0	0	0	0	0	0	0	0
3dpi Mean	4.2222222	1.3333333	3.1111111	1.3333333	60.555556	20.444444	30.333333	11.111111	0	0	0	0
3dpi Stdev	1.3944334	1.7320508	2.5221243	1.3228757	48.252231	27.486866	23.329166	8.0381037	0	0	0	0
7dpi Mean	5.5555556	1.2222222	2.4444444	1	111.88889	27.111111	35.555556	28.333333	0	0	0	0
7dpi Stdev	3.8765678	0.8333333	2.0069324	1.6583124	93.369487	25.315235	34.666667	21.41845	0	0	0	0
15dpi Mean	5.1111111	1.3333333	3.2222222	2.3333333	92.222222	25.444444	34.333333	17	4.444444	5.3333333	10.888889	3.33333333
15dpi Stdev	1.6914819	1.6583124	2.3863035	2.3979158	54.579707	23.195426	26.953664	10.210289	7.090682	7.3654599	14.793955	8.18535277
20dpi Mean	7.3333333	4	4.5555556	3.7777778	67.444444	50	86.555556	36.444444	64.22222	11.444444	37.222222	9.77777778
20dpi Stdev	4.1231056	2.236068	3.3208098	1.9860625	53.244979	41.039615	122.9015	29.090854	40.561	6.5404723	31.905242	6.7966495
25dpi Mean	6.5555556	5.3333333	5.6666667	3.6666667	97	65.444444	87.888889	56.222222	70.88889	28.222222	48.777778	25.1111111
25dpi Stdev	5.2704628	1.5811388	3.6742346	2.5	102.51341	36.905661	62.648712	23.847315	45.12329	20.795299	29.063628	13.2329555
33dpi Mean	7.6666667	6.2222222	7.3333333	5	46.777778	36.333333	33.777778	31.333333	68	38.555556	66.777778	27.3333333
33dpi Stdev	2.5495098	3.8005848	3.4278273	2.5980762	54.416858	23.900837	19.194907	16.992645	51.82422	28.018347	52.232121	28.6923335
39dpi Mean	13.777778	10	12.333333	9.7777778	138.33333	109.88889	109.33333	106.55556	155.6667	112.77778	141.55556	113.111111
39dpi Stdev	5.4949472	3.2403703	3.9370039	3.34581	106.1261	50.377188	45.359674	42.006283	79.15491	47.071164	94.634971	41.9626156
46dpi Mean	12.222222	11.444444	11.222222	9.7777778	135.33333	143.88889	113.88889	90.666667	218.7778	193.77778	191.44444	177.777778
46dpi Stdev	5.6740148	4.6127842	3.9616214	6.3792197	43.860575	83.659196	77.031559	53.874855	79.65673	60.369234	91.62302	65.7262843
53dpi Mean	13	12.111111	12.333333	10.111111	80.111111	98.222222	104.66667	64.777778	153.2222	121.88889	133.11111	105
53dpi Stdev	6.041523	4.8333333	4.8989795	3.982601	50.976574	67.117021	88.314778	28.955905	63.32215	62.604801	31.782245	53.5770473

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