# Novel Integrated Pest Management components for the control of the glasshouse whitefly (*Trialeurodes vaporariorum*) on glasshouse-grown tomatoes (*Solanum lycopersicum*)

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## Abstract

The glasshouse whitefly (*Trialeurodes vaporariorum* Westwood*)* is one of the most important U.K. pests. This phloem-feeding insect is a particular threat to glasshouse-grown crops, including the tomato (*Solanum lycopersicum* L.). Integrated Pest Management (IPM) involves applying a range of biological, cultural, physical and chemical control measures, with monitoring, to reduce pest and pathogen numbers on commercial crops below acceptable economic thresholds, with minimal environmental damage. Different IPM tools are used together to achieve an acceptable control level. Whilst IPM has been effectively utilised to control the glasshouse whitefly, greater knowledge of individual IPM components is still needed to continue to effectively protect greenhouse-grown tomatoes in the future, particularly with increasing pesticide resistance levels in whitefly populations. Therefore, this PhD thesis sought to advance knowledge of existing and novel IPM components for whitefly control on tomatoes.

Several distinct IPM methods were investigated. A wild tomato species, *Lycopersicon pimpinellifolium* (L.) Mill, was assessed for enhanced whitefly resistance, with a novel dual method of resistance discovered, one pre- and one post-phloem penetration, which may be introduced into modern tomato cultivars to enhance whitefly resistance. The 'push-pull' method of intercropping tomatoes with whitefly-repellent species, and surrounding them with attractive host species, was investigated in a large scale glasshouse trial, with French marigolds revealed to be an effective intercrop plant to reduce whitefly numbers on tomatoes. The potential of whitefly-induced plant volatiles to enhance whitefly resistance in uninfested tomatoes was examined, with plant-plant communication shown to be an effective method at reducing settling and oviposition in volatile-exposed tomatoes, potentially by priming defences against a subsequent whitefly infestation.

It is anticipated that these IPM tools could be combined to achieve control of the glasshouse whitefly in glasshouse-grown tomatoes, contributing to environmentally sustainable food production and reduced synthetic pesticide use, whilst managing whitefly pesticide resistance.

## Acknowledgements

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# Chapter 1. General Introduction

## 1.1 Whitefly Taxonomy and Life Cycle

Whiteflies possess an uncertain taxonomy, due to a focus on the final puparial stage for their systematic determination (Martin and Mound 2007). This is problematic as the appropriateness of many puparial characters for taxonomic analysis remains unclear, as many species display large puparial variability dependent on environmental conditions (Manzari and Quicke 2006). Current thinking places whiteflies as hemipteran insects, comprising approximately 1500 species in the family Aleyrodidae, which is split into three subfamilies: the Aleyrodinae which are of global origin, the Aleurodicinae which are found mainly in Central and South America, and the Udamoselinae which comprises two South American species (Byrne and Bellows Jr 1991; Inbar and Gerling 2008; Martin and Mound 2007; Manzari and Quicke 2006).

The whitefly lifecycle is similar amongst all species: a small egg is inserted erect into a slit in a plant leaf made by the ovipositor of the adult, or into a stoma, which then hatches into a mobile first instar nymph usually termed a "crawler", which has functional legs that allow the crawler to locate a leaf vein to feed from (Byrne and Bellows Jr 1991; CABI 2013). The subsequent second, third and fourth instar nymphs are stationary, with the fourth instar entering a non-feeding puparial stage that then hatches into an adult whitefly via a T-shaped slit in the puparium (Byrne and Bellows Jr 1991). The adult is capable of flight after several hours during which time the wings dry and pigment is deposited during cuticle hardening (CABI 2013; Byrne and Bellows Jr 1991). The length of this lifecycle varies according to temperature and host plant (CABI 2013).

## 1.2 Whiteflies as Agricultural Pests

Whiteflies damage plants in three main ways. Whiteflies extract large quantities of sap from plant phloem via their proboscis, reducing the amount of energy and resources available to plants for growth and reproduction (Byrne and Bellows Jr 1991). Whiteflies also excrete a sticky honeydew which supports the growth of sooty mould (such as species of *Capnodium*) that not only reduces the photosynthetic potential of the plant, but makes fruits unsightly and therefore unsaleable (Byrne and Bellows Jr 1991; Inbar and Gerling 2008). Finally, whitefly act as vectors for many damaging plant viruses which have negative implications for plant health, such as the *Tomato chlorosis* and *Beet pseudoyellows* viruses (Jones 2003).

Of the approximately 1500 known species of whitefly, two species are acknowledged as being the most damaging pests: the tobacco whitefly *Bemisia tabaci* Gennadius and the glasshouse

whitefly *Trialeurodes vaporariorum* Westwood (Bleeker et al. 2009). *B. tabaci* possesses high genetic complexity and is a species complex with several biotypes identified, which are to a greater or lesser degree reproductively isolated, and possess different genetic and biological characteristics (Himler et al. 2011; Dinsdale et al. 2010). The B and Q biotypes in particular have caused widespread damage to crops and ornamentals globally, being highly invasive, adaptive to temperate regions and having a wide host range (Bleeker et al. 2009; Cui et al. 2008; Oliveira et al. 2001). Due to its global economic impact *B. tabaci* has attracted a great deal of research attention. *T. vaporariorum*, by contrast, has received relatively little attention, and as it is the main whitefly pest species found in the U.K. (*B. tabaci* has yet to be found in the U.K.; CABI (2017); Cuthbertson and Vänninen (2015)) and is considered the main insect pest of tomato in the U.K (Lange and Bronson 1981), it is the focus of this thesis. *T. vaporariorum* is a cosmopolitan pest of many crop plants including tomatoes, peppers, legumes and others, with the total number of potential host plants currently estimated to be approximately 859 species in 469 genera across 121 plant families (CABI 2013). The glasshouse whitefly is globally distributed, and is found in Africa, Oceania, South America, Central America, North America, Asia and Europe (see Figure 1.1; EPPO (2017)). *T. vaporariorum* is less important as a vector for plant viruses than *B. tabaci*, but in the European-Mediterranean region it is responsible for the transmission of important plant viruses in the *Closteroviridae* that include *Beet pseudoyellows* virus, *Tomato chlorosis* virus and *Tomato infectious chlorosis* virus (Jones 2003).

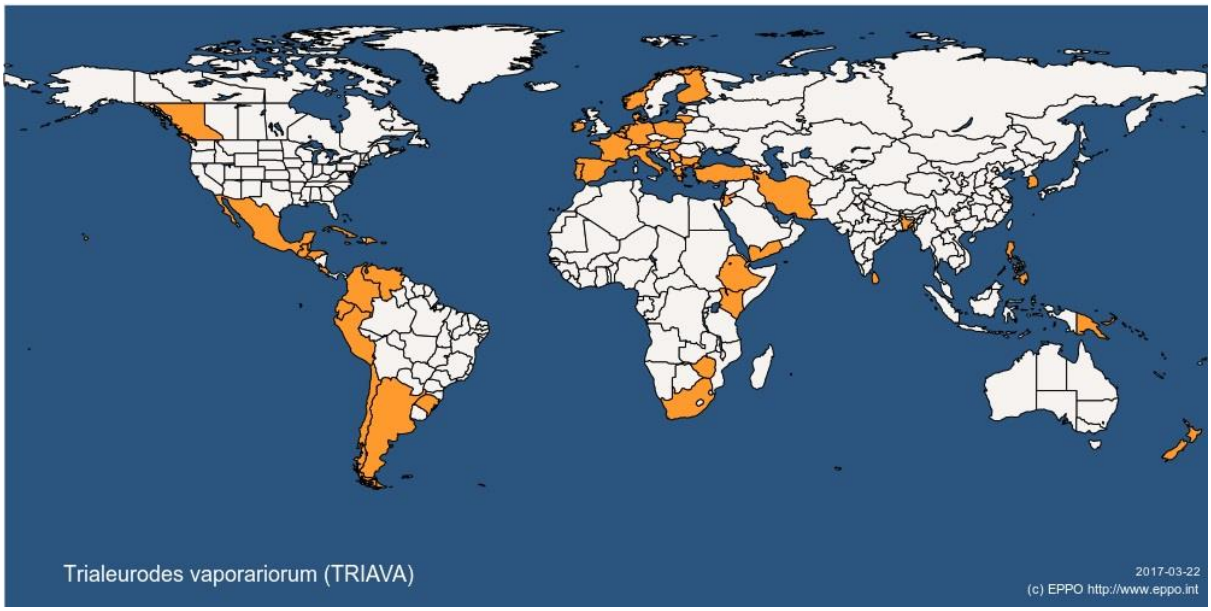


**Figure 1.1:** The global distribution (highlighted in orange) of the greenhouse whitefly (*Trialeurodes vaporariorum*), as defined by the EPPO (EPPO 2017). This phloem-feeding insect is one of the two most important whitefly pest species, and feeds on a wide range of

important crop plants. To date *T. vaporariorum* is the main whitefly pest in the U.K.


### 1.3 Tomato: Economic Importance and Scientific Relevance

The tomato (*Solanum lycopersicum*; Solanaceae) is thought to be native to the Andean region of South America (Lin et al. 2014) and its native distribution is across South America from Central Ecuador to northern Chile (Bauchet and Causse 2012). Tomatoes were introduced to Europe by the Conquistadors in the 16$^{th}$ Century (Jenkins 1948; Lin et al. 2014) and until the mid-20$^{th}$ century underwent further selection by growers using traditional breeding methods to develop heirloom varieties with relatively low but stable yields, and were open pollinated to allow seeds to be saved for subsistence farming (Bauchet and Causse 2012; Bai and Lindhout 2007). From the middle of the 20$^{th}$ Century, owing to the development of plant breeding programmes based on plant hybridisation techniques and selection theory (Bauchet and Causse 2012), extensive selective breeding occurred causing the development of the modern commercial tomato which is characterised by higher productivity, adaptation to different cultivation systems, resistance to biotic and abiotic stress, fruit quality and heterosis, and yield (Bai and Lindhout 2007; Bauchet and Causse 2012; The 100 Tomato Genome Sequencing et al. 2014). These improvements, along with the increasing importance of tomato as a food crop around the world, resulted in 2014 in an estimated 170,750,767 tonnes of tomatoes being produced worldwide, with 16,900,206 tonnes in the EU and 98,500 tonnes in the U.K. (FAOSTAT 2014). Tomatoes have been the subject of much research, becoming a model organism for fruit development and metabolite production (Bauchet and Causse 2012), seed weight variation (Doganlar et al. 2000), and fleshy fruit biology in general (Lin et al. 2014), with many genetic resources now available (The 100 Tomato Genome Sequencing et al. 2014; Labate et al. 2007). Tomato biochemistry, and in particular their defence responses, have become well described; for example the work by Ryan and Pearce (1998) in identifying systemin as a locally produced systemic defence signalling protein.

Tomatoes are affected by a wide range of pathogens and insect pests. Csizinszky et al. (2005) provide a comprehensive analysis of the agents which cause tomato crop loss worldwide. As many as 200 insect and mite pest species affect tomatoes across their whole range (Lange and Bronson 1981), which can cause large losses to crops. For example, in the state of Virginia in the U.S.A. untreated plots experienced a 34% decrease in yield on average compared to pesticide-treated fields (Nault and Speese 2002). Insects may be categorised on the basis of how they utilise plant tissue for nutrition and cause damage; either by chewing mouthparts or

by piercing and sucking mouthparts. Insect pests on tomato may also be categorised by the part of the tomato plant, and the plant growth stage, which they affect.

Whilst Table 1.1 is a comprehensive list of many of the possible tomato diseases and pests, not all causative agents will be present across the entire tomato geographical growth range. Pests and diseases that are particularly problematic in greenhouse-grown tomatoes (a common growth method in temperate climes such as the U.K.) include insects, such as whiteflies, aphids, thrips, mites, pinworms, leafminers, caterpillars, and psyllids (Peet and Welleds 2005). In particular *T. vaporariorum* and the two spotted spider mite *Tetranychus urticae* C.L.Koch are seen as the most important insect pests in tomatoes grown under glass (Lange and Bronson 1981). Fungal pathogens encountered in glasshouses include the *Pythium* sp., Botrytis grey mould, leaf mould, and Fusarium wilt (also known as Fusarium crown rot), but also include powdery mildews (*Erysiphe* sp.; Peet and Welleds (2005)) which produces round, white pustules on leaves and stems (Matsuda et al. 2001). Important viral diseases prevalent in glasshouses include the *tomato spotted wilt virus*, as well as the *beet pseudo-yellows virus* (vectored by *T. vaporariorum*, and which causes interveinal chlorosis in mature leaves and reduced fruit size and growth; Tzanetakis et al. (2013)), *tomato mosaic virus* (which causes mosaic pattern on, and elongation of, leaves; Broadbent (1976)), and *tobacco mosaic virus* (which also forms a mosaic pattern on leaf surfaces, stunted growth and potentially "mosaic burn" on lower leaves in dry weather (BSPP 2017; Peet and Welleds 2005)).

In the U.K., according to the Food Standards Agency, tomatoes are the most important glasshouse grown salad crop, with *T. vaporariorum* and the two spotted spider mite *Tetranychus urticae* being the two most important insect pests that require pesticide control on tomatoes, ranking joint first on the evaluation of the importance of protection for crop yield implications (Caspell et al. 2006). Other important insect pests include: the tomato leaf miner (*Liriomyza bryoniae*), mealy bugs (*Pseudococcus viburni*) which are phloem-feeders, and *Macrolophus caliginosus*, which is a control agent for whitefly that can also cause plant damage (Caspell et al. 2006). The most important fungal diseases are grey mould, powdery mildew, verticillium wilt and root rots caused by *Pythium*, *Phytopthera* and *Rhizoctonia solani* (Caspell et al. 2006). As can be seen, the pests found in the U.K. are a smaller subset of those found globally, with *T. vaporariorum* a highly important contributor to crop loss.

| Pest type | Organism common name | Scientific name | Damage type caused | Plant part damaged | Plant growth stage affected | Symptoms | Other information | Reference |
|---|---|---|---|---|---|---|---|---|
| Insect | Field cricket | *Gryllus assimilis* | Chewing | Stem base | Seedling | Chew at soil line | | (Csizinszky et al. 2005) |
| Insect | Mole cricket | *Scapteriscus vicinus* | Chewing | Underground tissue | Seedling | Attack plant from below | | (Csizinszky et al. 2005) |
| Insect | Cutworm e.g. variegated cutworm | e.g., *Peridroma saucia* | Chewing | Stem base | Seedling | Attack plants at or just above soil level | Cutworm refers to caterpillars of various spp | (Csizinszky et al. 2005) |
| Insect | Flea beetle | *Epitrix* and *Phyllotreta* spp | Chewing | Foliage | All | Chew pits in leaves | | (Csizinszky et al. 2005) |
| Insect | Leafminer e.g. vegetable leafminer | e.g *Liriomyza sativae*, | Chewing | Foliage | All | Maggots tunnel through leaf mesophyll | Adults lay eggs in leaves | (Csizinszky et al. 2005) |
| Insect | Looper e.g cabbage and tomato looper | e.g *Trichoplusia ni*, *Chrysodeixis chalcites* | Chewing | Foliage | All | Larvae chew “windows”, adults chew holes | | (Csizinszky et al. 2005) |

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|---|---|---|---|---|---|---|---|---|
| Insect | Armyworm e.g. beet armyworm, tomato moth | e.g. *Spodoptera exigua*, *Lacanobia oleracea* | Chewing | Fruit and foliage | All | Larvae feed on lower leaf surfaces | | (Csizinszky et al. 2005) |
| Insect | Fruit worm e.g. tomato fruitworm | e.g *Helicoverpa zea* | Chewing | Fruit | Fruit | Larvae bore into (mostly) unripe fruit | May cause 20% losses of tomatoes in the field | (Gianessi 2009) (Csizinszky et al. 2005) |
| Insect | Hornworm e.g. tomato and tobacco hornworms | e.g. *Manduca quinquemaculata* and *M. sexta* | Chewing | Foliage and fruit | All | Feed voraciously on leaves and fruit | | (Csizinszky et al. 2005) |
| Insect | Potato tuberworm | *Phthorimaea operculella* | Chewing | Fruit | Fruit | Larvae form blotch mines and bore intro fruit | | (Csizinszky et al. 2005) |
| Insect | Tomato pinworm | *Keiferia lycopersicella* | Chewing | Fruit | Fruit | Larvae form blotch mines and bore intro fruit | May reduce crop yield in California by 25% | (Csizinszky et al. 2005; Gianessi 2009) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Insect | Stink bug e.g. southern green stink bug | *Nezara viridula* | Piercing | Fruit | Fruit | Cause lightened blotches on unripe fruits | | (Csizinszky et al. 2005) |
| Insect | Coreid bug e.g. leaf-footed bug | *Coreidae* e.g. *Leptoglossus phyllopu*s | Piercing | Fruit | Fruit | Pierce deeper into fruits than stink bugs | | (Csizinszky et al. 2005) |
| Insect | Aphid e.g. green peach aphid | e.g. *Myzus persicae* | Sucking | Foliage and flowers | All | Penetrate plant phloem, leaf spotting and distortion, plant stunting and wilting | | (Csizinszky et al. 2005) |
| Insect | Tomato russet mite | *Aculops lycopersici* | Sucking | Foliage and flowers | All | Silvery and chlorotic appearance on upper leaf surfaces | | (Csizinszky et al. 2005) |
| Insect | Thrips e.g. western flower thrips, onion thrips | e.g. *Frankliniella occidentalis*, *Thrips tabaci* | Sucking | Foliage and flowers | All | Cause bloom abscission, necrotic lines and pitting on fruits | *Frankliniella* spp. mostly affect flowers, other spp. affect foliage | (Csizinszky et al. 2005) |

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|---|---|---|---|---|---|---|---|---|
| Insect | Whitefly e.g. tobacco whitefly, greenhouse whitefly | e.g. *Bemisia tabaci*, *Trialeuroides vaporariorum* | Sucking | Foliage and flowers | All | Wilting, honeydew supports mould growth, transmit viruses | Prior to insecticide use caused $25 million in crop losses in Florida tomatoes | (Csizinszky et al. 2005; Gianessi 2009) |
| Arachnids | Spider mite | *Tetranychus* genus e.g. *T. urticae* | Sucking | Foliage and flowers | All | Cover lower leaves in silk webbing, chlorotic spots on upper leaves | | (Csizinszky et al. 2005) |
| Nematode | e.g. Root-knot nematode | e.g *Meloidogyne* spp. | Sucking | Roots | All | Disrupt vascular system, reduce plant growth, chlorosis, susceptible to pathogens and drought | | (Csizinszky et al. 2005; Corbett et al. 2011) |
| Fungus | Anthracnose | *Colletotrichum* genus e.g. *C. coccodes* | Hemibiotroph | Mature fruits, leaves, roots | All | Sunken, dark, necrotic lesions on most plant parts | | (Csizinszky et al. 2005; O'Connell et al. 2012) |
| Fungus | Early blight | *Alternaria solani* | Necrotroph | Over ground plant parts | All | Expanding yellow-ringed black lesions on plant tissue | | (Csizinszky et al. 2005) |
| Fungus | Fusarium wilt | *Fusarium oxysporum*. f. sp. *lycopersici* | Necrotroph | Whole plant | | Turns vascular tissue black and causes wilting and yellowing in older leaves | Infects via vasculature, eventually affects whole plant | (Csizinszky et al. 2005; Swarupa et al. 2014) |
| Fungus | Grey leaf spot | *Stemphylium botryosum* f. sp. *lycopersici*, and *S. solani* | Necrotroph | Foliage | All | Infects leaf blades, causes black specks and yellowing when heavily affected | Caused by three pathogens | (Csizinszky et al. 2005) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Fungus | Grey mould | *Botrytis cinerea* | Necrotroph | All aerial plant parts | All | Causes necrotic lesions and soft rot on fruits | | (Csizinszky et al. 2005) |
| Fungus | Tomato leaf mould | *Passaflora fulva* | Biotroph | Leaves | All | Light to dark green mould, may cause leaves to drop off | | (Csizinszky et al. 2005; Collemare et al. 2014) |
| Fungus | Southern blight | *Sclerotium rolfsii* | Necrotroph | Stem base | All | Black or brown lesions on stem collar, causes wilting | Affects tomatoes in tropical areas | (Csizinszky et al. 2005; Dixit et al. 2016) |
| Fungus | Verticillium wilt | *Verticillium albo-atrum* and *V. dahlia* | Hemibiotroph | Vasculature | All | Infect the xylem, causes yellowing of leaves, wilting and dieback | | (Csizinszky et al. 2005; Ralhan et al. 2012) |
| Oomycete | Late blight | *Phytophthora infestans* | Hemibiotroph | All aerial plant parts | All | Water-soaked spots, develop brown lesions, foliage shrivels and dies | | (Csizinszky et al. 2005) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Oomycete | *Pythium* spp. | e.g. *P.*. *myriotylum* | Hemibiotroph | Roots, then other parts | All | Seed, stem and fruit rot, damping off both pre- and post-emergence | | (Csizinszky et al. 2005) |
| Bacteria | Bacterial canker | *Clavibacter michiganensis* subsp. *michiganensis* | | Leaves and fruit | All | Marginal necrosis, leaflet margins wilt and curl (occurs in lower leaves first). Yellow to red streaks appear in vasculature | Pathogen is often seed disseminated. | (Csizinszky et al. 2005; Tancos et al. 2013) |
| Bacteria | Bacterial speck | *Pseudomonas syringae* pv. *tomato* | | Over ground plant parts | All | Spotting occurs on fruits, and round, dark lesions appear on leaflets which develop a halo over time | Most destructive in wet and cold conditions | (Csizinszky et al. 2005; Uppalapati et al. 2008) |
| Bacteria | Bacterial leaf spot | *Xanthomonas campestris* pv. *vesicatori* | | Over ground plant parts | All | Circular dark spots appear on stems, leaves and fruits | | (Csizinszky et al. 2005) |
| Bacteria | Bacterial wilt | *Ralstonia solanacearum* | | Over ground plant parts | All | Younger leaves, then whole plant, become flaccid, with watery lesions | | (Csizinszky et al. 2005) |
| Virus | Cucumber mosaic virus | | | Whole plant | All | Yellow, stunted and bushy plants in early stages, shoestring leaves | Transmitted by aphids, affects temperate regions | (Csizinszky et al. 2005) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Virus | Curly top | Curly top virus | | Whole plant | All | Young plants may die; stunts and yellows old plants; thick, rolled leaves | Vectored by leafhoppers in arid regions | (Csizinszky et al. 2005) |
| Virus | Tobacco etch virus | | | Whole plant | All | Stunting, fruit mottling, and intense mottling and rugosity of leaves | Vectored by 10 aphid species in the Americas | (Csizinszky et al. 2005) |
| Virus | Tomato Spotted Wilt Virus | | | | | Bronzed younger leaves develop black spots, streaks on stems and petioles, less fruit, lower leaf veins purple, purple spots in interveinal tissue, downturned young leaves, main shoot yellow and stunted | Vectored by 9 thrips spp. Symptoms vary by plant age, genotype, and environment. | (Csizinszky et al. 2005; Roselló et al. 1996) |
| Virus | Tomato Yellow Leaf Curl Virus | | | Whole plant | All | Early growth stunted, abnormal leaflets, vein clearing, rosette shape, leaves downturned, hooked leaflets, interveinal and marginal chlorosis. | Vectored by *B. tabaci* and *B. argentifolii*. Virus worse in areas over 25$^{o}$C | (Csizinszky et al. 2005; Picó et al. 1996) |
| Virus | Pepino Mosaic Virus | | | Whole plant | All | Leaf bubbling, pointed leaflets, yellow mosaic on leaves and fruits | Vectored by plant-plant contact or worker handling | (Csizinszky et al. 2005) |

**Table 1.1:** The variety of insects, nematodes, fungi, bacteria and viruses that affect tomatoes across their whole geographic range. Such disease-causing organisms may be classified by the mode of the damage they cause plants, the plant part they damage, or the plant growth stage affected.

The popularity of tomato and its importance as a crop plant (Figure 1.2), its extensive research background, and its susceptibility to whitefly attack, make the tomato a suitable candidate for study in this body of work. Tomatoes are affected by whiteflies wherever they are grown, and in the U.K. the glasshouse whitefly is a particular problem as most tomato production occurs under glass (Caspell et al. 2006). Modern commercial tomatoes may be more susceptible to insect pests due to the development of a "domestication syndrome" (Hammer 1984), where a plant undergoes extensive selection for certain traits related to fruit size, attractiveness and ease of harvest, but experiences a reduction in genetic diversity of other gene classes, such as defence genes, as these are not selected for and therefore may be accidentally bred out over time (Bai and Lindhout 2007). This enhanced susceptibility is another reason that the present work is timely and necessary.

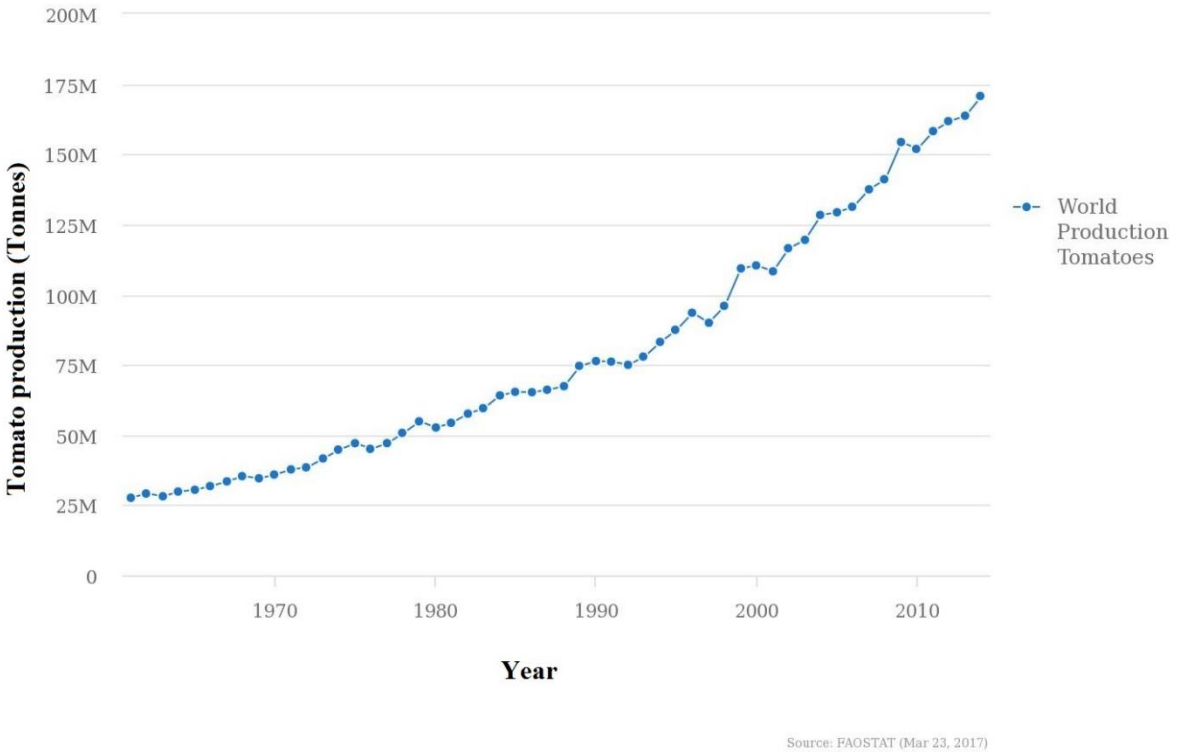


**Figure 1.2:** The total global production of tomatoes in tonnes for each year since 1961 to 2014. A clear positive trend shows the influence of targeted breeding programmes prioritising yield in commercial cultivars, as well as tomato's increasing popularity as a food crop amongst growers (FAOSTAT 2014). Presented under the FAOs web content Terms and Conditions.

### 1.4 Plant Defences and the Mechanistic Basis of Tomato Resistance to Whiteflies

Tomato plants possess a number of antixenosis- and antibiosis- based elements that can impact whitefly performance. Antixenosis refers to a repellent effect by the plant on an insect before it infests, and antibiosis refers to mechanisms employed after colonisation by an insect (Nombela et al. 2001). Trichomes are one of the most important mechanisms of antibiosis.

Trichomes are hair-like protuberances of the plant epidermis that may be multicellular or unicellular, and glandular or non-glandular (Kennedy 2003; Tissier 2012; Bergau et al. 2015). Trichomes in tomato have been classified into seven different classes (Luckwill 1943) with multiple classes being present in a single species of tomato (Bergau et al. 2015). The glandular trichomes are types I, IV, VI and VII, and have a 'head' that, on contact, releases sticky and/or toxic chemical exudates that may kill or entrap pests (Simmons and Gurr 2005). Non glandular trichomes are types II, III and V that affect pests by mechanical means, acting as a physical barrier to pest infestation by restricting insect movement (Simmons and Gurr 2005). Of these trichome types, type IV (due to acylsucrose production; Leckie et al. (2012)) and type VI (due to 2-tridecanone and 2-undecanone production; Muigai et al. (2002)) have been particularly implicated in tomato interactions with insects (Tissier 2012; Simmons and Gurr 2005). Type IV trichomes (which are found in wild tomato species only; Bergau et al. (2015)) have been shown in *Solanum pennellii* (Slocombe et al. 2008) to produce glucose esters of short chain fatty acids, whilst type VI trichomes (found in commercial as well as wild tomatoes; Bergau et al. (2015)) are thought to produce mono- and sesquiterpenes, and methyl ketones (Tissier 2012). Type VI trichomes may also contain phenolics such as rutin, caffeic acid conjugates, chlorogenic acid, and polyphenol oxidase and peroxidase (Kennedy 2003). Upon insect contact, the tip of type VI trichomes discharges, causing the components to mix and produce quinones via oxidation, which polymerise with proteins (reducing their nutritive value) and are toxic to the insect (Kennedy 2003). The level of quinone production varies between tomato species (Kennedy 2003). It has been shown that whiteflies have a preference for tomato plants with non-glandular trichomes over glabrous plants (Neal and Bentz 1999); such behaviour is thought to provide better physical protection for developing nymphs (Walling 2008).

The trichomes of wild tomato species have been shown to be more effective than those of the cultivated tomato at resisting whitefly infestation. Wild tomatoes that resist *T. vaporariorum* include *Solanum habrochaites* and *Lycopersicon hirsutum* f. *glabratum* (Bas et al. 1992). Those that are resistant to *B. tabaci* include *S. pennellii*, *L. hirsutum* f. *typicum* and *L. hirsutum* f. *glabratum* (Muigai et al. 2002; Nombela et al. 2000), *Solanum galapagense* (Lucatti et al. 2013; Firdaus et al. 2013), and the range of wild species examined by Firdaus et al. (2012) which varied in their resistance level. Crosses between wild and commercial tomato cultivars, and introgressions of trichome genes into cultivated tomatoes, have been made that successfully increase resistance to whitefly attack. Examples include enhanced resistance to *T. vaporariorum*, where resistance was introduced from *L. hirsutum* f. *glabratum* (Bas et al.

1992) and *Solanum pennellii* (Erb et al. 1994), and tomato species giving enhanced resistance to *B. tabaci*, where resistance was introgressed from *Solanum pennellii* (Maciel et al. 2017; Nombela et al. 2000) and *Solanum pimpinellifolium* (Silva et al. 2014)). Much of this increased resistance occurs as a result of elevated acylsugar content in the new crosses compared to commercial species (Maciel et al. 2017; Silva et al. 2014) which has been shown to have a role in the antibiosis resistance of tomato to whitefly (Lucatti et al. 2013), although only effective beyond a concentration of 37.8 µg $cm^2$ leaf (Nombela et al. 2000). The mode of action of acylsugars has not been fully elucidated but may be related to feeding deterrence (Leckie et al. 2012). Antixenotic effects have also been reported for acylsugars, with reduced oviposition being correlated with repellent effects of these compounds (Simmons and Gurr 2005).

The only Resistance (R) gene to have been discovered and implicated in tomato-whitefly interactions is the *Mi-1.2* gene, which confers resistance to root knot nematodes, aphids and whiteflies in the wild tomatoes *Solanum habrochaites* and *Solanum pennellii*, crosses between these species and commercial tomatoes, and in commercial tomatoes transformed with the *Mi-1.2* gene (Nombela et al. 2000, 2001; Nombela et al. 2003; Lucatti et al. 2010). *Mi-1.2* confers resistance to both *B. tabaci* and *T. vaporariorum* (Lucatti et al. 2010; Nombela et al. 2003; Rodríguez-Ãlvarez et al. 2015). Studies using whole plants have shown that salicylic acid is required for *Mi-1.2*-mediated resistance to root knot nematodes and whiteflies (Branch et al. 2004; Rodríguez-Ãlvarez et al. 2015), whilst other study systems suggest a role for jasmonic acid (Bhattarai et al. 2008). Resistance to both aphids and whiteflies is developmentally regulated, with plants up to 4-5 weeks of age being susceptible to insect attack despite *Mi-1*.2 expression (Bhattarai et al. 2007). *Mi-1.2* produces a 4kb transcript that encodes a 1,257 amino acid protein, which contains a nucleotide binding site and leucine-rich repeat (Lucatti et al. 2010). Proteins of this type are one of the largest known protein classes to be involved in resistance in plants to pests and pathogens (Milligan et al. 1998). The chaperone *Hsp90-1* is necessary for correct *Mi1.2* functioning (Bhattarai et al. 2007). Other genetic studies that investigate wild tomato resistance to whiteflies include work that identifies the *OR-5* locus, which confers reduced *B. tabaci* oviposition in crosses between *S. habrochaites* and commercial tomatoes (Lucatti et al. 2014).

Volatile organic compounds (VOCs) are gaseous chemicals synthesised and released by plants that mediate interactions between the plant and its environment (Dudareva et al. 2013). Much research attention has been afforded to the complex ways in which VOCs allow plants, as sessile organisms, to influence their surroundings. VOCs act as signalling molecules

between plants and a wide range of other agents, including insects, other animals, and other plants (Peñuelas and Llusià 2004) with interactions being either positive or negative for the receiver. VOC involvement in plant reproduction is well documented (Raguso 2008), with examples abundant for floral VOCs attracting pollinators including honeybees, which have well documented olfactory and learning abilities to differentiate between different VOCs (Robertson and Wanner 2006), moths, such as the noctuid moth *Hadena bicruris* attracted by white campion (Gupta et al. 2012), and bats, such as those in the genus *Glossophaga* which are attracted to pollinate flowers emitting sulphur containing compounds (von Helversen et al. 2000). Plant VOCs also play an important role in signalling within the plant, with plant parts able to communicate damage (Heil and Silva Bueno 2007) or herbivore attack (Heil and Silva Bueno 2007; Frost et al. 2007; Li and Blande 2017) to other plant areas that are spatially close, but poorly connected via plant vasculature, using VOCs that are likely small and highly volatile, and therefore disperse rapidly in the plant headspace (Baldwin et al. 2006). VOCs also have a role in plant-plant signalling, with VOCs produced after damage or an insect herbivore attack increasing the resistance of neighbouring plants to an attack by the same insect, which has been observed between wheat plants (Ton et al. 2007), tomato plants (Lopez et al. 2012), and sagebrush as a VOC emitter and tobacco as the receiver (Karban et al. 2000; Karban et al. 2003). These examples show that inter plant communication occurs between plants of the same and different species. VOCs may also be emitted by one plant, absorbed by another plant, then transformed into another compound for use in defence, as is observed in the transfer of the VOC (Z)-3-hexenol between tomato plants, where it is converted into a glycoside as a defence against cutworms (Sugimoto et al. 2016). VOCs also have a direct role in plant defence; they act directly against pathogens e.g. (E)-β-caryophyllene from *Arabidopsis thaliana* flowers against *Pseudomonas syringae* pv *tomato* (Huang et al. 2012), and green leaf volatiles against a range of microorganisms (Prost et al. 2005). Against insect pests, VOCs act indirectly by advertising the presence of insects to natural enemies (Dicke and Baldwin 2010), including (E)-β-caryophyllene attracting entomopathogenic nematodes that reduce western corn rootworm numbers from maize roots (Degenhardt et al. 2009). They can also repel insects, as seen in isoprene repelling caterpillars of *Manduca sexta* from feeding upon tobacco plants (Laothawornkitkul et al. 2008).

VOCs are deployed by tomatoes as a defence against whitefly, either directly by having a toxic (Muigai et al. 2002) or antixenotic effect (Bleeker et al. 2009; Bleeker et al. 2011), or indirectly by attracting natural enemies of whiteflies, such as the parasitoid wasp *Encarsia formosa*, that then reduce the pest load of the plant (Cui et al. 2016; Walling 2008; Kessler

and Baldwin 2001). Wild tomatoes, again, possess more effective VOCs than commercial tomatoes to negatively affect whitefly behaviour e.g. *S. pennellii* and *S. habrochaites*, which produce mono- and sesquiterpenes such as *p*-cymene, zingiberene, and curcumene (produced from trichomes) that elicit a strongly repellent effect in *B. tabaci* (Simmons and Gurr 2005; Bleeker et al. 2009). Other examples of VOCs implicated in tomato defence include 2-undecanone, which has been shown to have fumigant toxicity to *B. tabaci* (Muigai et al. 2002), and limonene and *Z*-3-hexanol which strongly attract the parasitoid wasp *E. formosa* (Cui et al. 2016).

Other factors that influence plant resistance to whitefly include cuticle thickness, where thicker cuticles affect the ability of whitefly to access phloem (Rodríguez-López et al. 2012; Janssen et al. 1989), and plant age, with older plants proving more resistant to whitefly infestation (Bas et al. 1992).

### 1.5 Control of Whitefly on Commercial Crops

Whitefly control on commercial crops takes several forms. Biological control, where natural predators, parasites and parasitoids, and pathogens are used to control pest numbers (Bale et al. 2008), has been implemented very successfully to control whitefly (George et al. 2015), with parasitoid wasps such as *Encarsia formosa* routinely used to keep pest numbers below economic thresholds (Garthwaite et al. 2013; Gorman et al. 2007). Other commercially produced whitefly predators and parasitoids which have been deployed include *Chrysoperla rufilabris* (Legaspi et al. 1994), *Amitus bennetti* (Drost et al. 1999) and *Eretmocerus mundus* (Urbaneja et al. 2006). Evidence may also be found for the effective control of two pest species, western flower thrips and *T. vaporariorum*, by a single biocontrol agent, *Amblyseius swirskii*, indicating that such biocontrol agents may possess increased value as pest control agents due to their dual mode of action (Messelink et al. 2008). As well as insect predators, pathogenic biocontrol agents may be deployed, such as the hyphomycetes *Beauveria bassiana* and *Lecanicillium lecanii* which are deployed as mycoinsecticides to control *T. vaporariorum* on tomato (Fargues et al. 2003). Biocontrol agents which control for different pests have been shown to be compatible with each other, further demonstrating their importance in pest control strategies (Bardin et al. 2008). However, biological control agents depend upon a range of different factors such as grower experience and release timings, meaning a greater degree of accuracy and planning is required in their use when compared to other pest control techniques such as chemical pesticide sprays. The use of parasitoids also carries the risk of hyperparasitism occurring, which can reduce their efficiency (George et al. 2015).

Where biological control agents fail, chemical pesticides are usually deployed to reduce pest numbers on crops (George et al. 2015). In the past a wide range of insecticides such as pyrethroids, organochlorines, organophosphates, insect growth regulators and neonicotinoids were used to control whitefly, and were very effective (Sharaf 1986). Whilst in recent times neonicotinoids, pyrethroids, pyrethrins and spirocyclic phenyl-substituted tetronic acids still exert some measure of whitefly control (Fera 2015; Karatolos et al. 2010), the number of effective chemical pesticides that may be used has dramatically reduced due to legislative restrictions (NFU 2014), such as the restriction on three of the most widely used neonicotinoids by the European Commission (EC 2013), and, more importantly, the development of pesticide resistance in many whitefly populations collected globally (Gorman et al. 2007). *B. tabaci* has shown strong levels of cross resistance to neonicotinoids such as imidacloprid, thiamethoxam and acetamiprid in several countries (Nauen and Denholm 2005; Cahill et al. 2009; Ahmad and Khan 2017; Wang et al. 2017). *T.vaporariorum* has exhibited resistance to pyrethroids and organophosphates, with documentation available from the 1970's and 80's (Wardlow et al. 1976) and evidence still accumulating more recently (Karatolos et al. 2012). Evidence also exists for resistance in *T. vaporariorum* to insect growth regulators like teflubenzuron and buprofezin (Gorman et al. 2002). Resistance to the important neonicotinoid imidacloprid has also been shown for the glasshouse whitefly (Gorman et al. 2007), and resistance to this pesticide has also been shown to have a strong association with the unrelated pyridine azomethine pymetrozine in an age specific manner (Karatolos et al. 2010). The increasing incidence of resistance in whitefly populations to some of the most important pesticides is an issue that needs addressing, either through the development of new effective synthetic chemical pesticides (Brück et al. 2009), or (possibly more effectively) by the (re)adoption of a systematic Integrated Pest Management approach to pest control that encompasses a range of complementary techniques (Bale et al. (2008); Malézieux (2012)).

Biopesticides have also been utilised to control whitefly on crops. These are naturally derived products which repel, or have a pesticidal effect on, crop pests. Examples include natural plant extracts, that are often essential oils, such as: neem (Lynn et al. 2010), thyme and patchouli (Yang et al. 2010), and limonene (Du et al. 2016) amongst others. Ginger oil has been reported to be repellent to *B. tabaci* (Yang et al. 2010), whilst limonene has reported toxic effects on all whitefly life stages (Du et al. 2016).

Various cultural practices can be utilised to enhance control of whiteflies on crop plants. Early research looked to utilise banker plant systems to enhance the effectiveness of biological

control by *Encarsia formosa* on *T. vaporariorum*; whitefly-infested tomatoes were produced separately to a tomato crop and then exposed to *Encarsia formosa* in order to keep populations of the predator high (Stacey 1977; Frank 2010). Whiteflies may be physically separated from crops by the use of plastic sheets and other physical barriers (Cohen and Berlinger 1986). Pests may be temporally separated from hosts by measures such as crop-free years (Hilje et al. 2001). The behaviour of whitefly may be manipulated by trap crops and intercropping systems (Hilje et al. 2001).

Intercropping is a well-studied technique that has been relatively rarely applied to whitefly pest control, and then only in *B. tabaci*. Intercropping brings a range of benefits that may be seen in its use in other crop-insect systems, including enhanced soil organic carbon and nitrogen content, shown in intercrops between faba beans, and maize or wheat (Cong et al. 2015). Increased overall and per plant productivity has been observed in intercropping systems between watermelon, okra and peanut (Franco et al. 2015). Enhanced soil microbial community diversity has been detected in soil previously cultivated with legumes (Alvey et al. 2003) which is believed to have a beneficial effect on plant growth and productivity, particularly in early growth stages (Duchene et al. 2017). As well as these beneficial effects on plant growth, intercropping has been shown to reduce pest effects on crop species (Malézieux et al. 2009). Intercropped plants can directly influence pests by releasing toxic chemicals, as seen in reduced carrot fly attacks on carrots intercropped with onion due to deterrent onion volatiles (Uvah and Coaker 1984), or indirectly via biofumigant effects from decomposition of their residues in soils that deters soil-borne pests (Wezel et al. 2014). These negative effects on pests can be used to 'push' pests from a crop (Ratnadass et al. 2012). Other plants that are more attractive to pests than the crop may be intercropped to act as trap crops, to 'pull' pests from the crop, such as in the use of yellow rocket as a trap crop to 'pull' diamondback moth from a cabbage crop (Badenes-perez et al. 2005). These effects may be combined in a 'push-pull' strategy to protect crops by simultaneously driving pests from the crop plant using a 'push' plant, and 'pulling' them with an attractive plant, to achieve an enhanced level of pest control (Wezel et al. 2014). This strategy was effectively used by Khan et al. (1997b) to achieve control of stem borers on a maize crop using native grasses. Intercropping has been used to achieve control of whitefly on crop plants other than tomato. Zhao et al. (2014) intercropped cucumber with celery and Malabar spinach to achieve significantly lower settling numbers of *B. tabaci* on the cucumber crop, which was suggested to be due to production of repellent volatiles D-limonene and geranyl nitrate from the intercropped vegetables, respectively. Sharaby et al. (2015) reduced *B. tabaci* nymph numbers

by 62% and 69% on a potato crop over two seasons by intercropping with onions. Tomato crops have also been intercropped with other host plants in an attempt to control whitefly. Bird and Krüger (2007) intercropped two crop species, tomato and cucumber, to monitor *B. tabaci* preference, and observed fewer whitefly on tomato. Carvalho et al. (2017) intercropped tomato with coriander or greek basil, and observed significantly reduced adult and nymph densities on the tomato crop.

The use of naturally resistant cultivars may represent an effective method to control whitefly populations in crop plants (Broekgaarden et al. 2011) and the viruses they vector (Morales 2001). Whilst the modern commercial tomato is lacking in many defence genes due to selective breeding over time (Bai and Lindhout 2007), research has been completed evaluating the wild relatives of tomato for enhanced resistance mechanisms to whitefly that may be reintroduced into commercial tomatoes to reduce whitefly impact (Pilowsky and Cohen 2000; Firdaus et al. 2012; Silva et al. 2014) with Quantitative Trait Loci identified to aid the introgression of these advantageous genes into commercial tomatoes (Firdaus et al. 2013).

In the U.K. biological agents are by far the most used techniques to control tomato pests and pathogens, being applied to 12,049 hectares of tomato growth area and comprising approximately 83% of total control agents used on tomato in 2011 (Garthwaite et al. 2011). In 2013 the numbers were reduced to 4,172 hectares of tomato growth area and approximately 55% of the total control agents used (Garthwaite et al. 2013), but this reflects the decoupling of biological control agents from pollinators in the 2013 data. Of this biological control, deployment of *Encarsia formosa* to control *T. vaporariorum* was by far the most used control agent, comprising 48% of the biological control used and being deployed on 89% of all tomato growth area in 2011, and comprising 74% of used biological control and being used on 92% of all tomato growth area in 2013 (Garthwaite et al. 2011; Garthwaite et al. 2013). In 2011 *Eretmocerus eremicus* was also used to control *T. vaporariorum*, being 17% of all biological control used and the second most used control agent, but in 2013 was not in the top five biological control agents used (Garthwaite et al. 2011; Garthwaite et al. 2013). Other important biological control agents in 2013 were: *Diglyphus isaea* for leaf miner control (8% of total biological control), *Phytoseiulus persimilis* for two-spotted spider mite control (7% of biological control), *Macrolophus pygmaeus* for *T. vaporariorum* control (7% of total), and *Aphidius colemani* for aphid control (2% of total; Garthwaite et al. (2013)).

In terms of chemical pesticide use in the U.K., whitefly control was responsible for 13% of the total insecticide use on tomato in 2011 (Garthwaite et al. 2011), but in 2013 whitefly control was not cited as an important reason for chemical pesticide use, possibly accounted for by the much reduced use of Acetamiprid on tomatoes in 2013 compared to 2011 (36,876 spray sq m versus 140,809 spray sq m; Garthwaite et al. (2011); Garthwaite et al. (2013)).

### 1.6 Integrated Pest Management (IPM): History, Importance and Modern Usage

Integrated Pest Management (IPM) as a concept has existed since at least the 1960s (Stern et al. 1959) and is concerned with the integrated application of a range of biological, cultural, physical, and chemical control measures to reduce pest and pathogen numbers on commercial crops below acceptable economic thresholds, with minimal impact on the environment (Stern et al. 1959; Bale et al. 2008; Tang et al. 2005). IPM takes a systems approach to pest control, with different tools used together to achieve an acceptable control level (Tang et al. 2005; Van Lenteren and Woets 1988). Such tools include physical exclusion of pests, cultural practices to reduce pest incidence and survival between crops, genetically resistant crop cultivars, biopesticides that act in a specific manner to kill pests and, as a last resort, specific chemical pesticides to limit pest outbreaks (Bale et al. 2008). Whilst having been developed more than 50 years ago, IPM use has declined recently due to the extensive development of synthetic chemical pesticides and their use as "magic bullets", being relied upon as the sole method of pest control in many crops (Bale et al. 2008). For example, in the U.K. alone, a total area of 80,274,553 hectares was treated with chemical pesticides (calculated by multiplying the area treated by each active substance, then by the number of times the area was treated) in 2015, with a total weight of 17,817,809 kg applied (Fera 2017). Recent issues concerning overuse of chemical pesticides have included toxic and inhibitory effects on non-target organisms, environmental damage, and selection for resistance in pest species (Abd-Rabou and Simmons 2015; Tang et al. 2005; Bale et al. 2008). Therefore, there is a growing movement for IPM to be reintroduced into farming systems to achieve an acceptable level of pest control with fewer environmental consequences (Moreau and Isman 2012). IPM in the modern age faces a number of challenges, however. IPM is viewed as being less efficient than the application of broad spectrum pesticides, less robust, and more susceptible to environmental alterations. It is also knowledge intensive in its planning and execution, and therefore inspires risk aversion in end users. Finally, market entry barriers exist for IPM components: they have the same regulatory restrictions applied to them as chemical pesticides, with expensive (and often unnecessary) authorisation procedures required. The regulatory system also emphasises the weakness of IPM components (as usually no instant

death is seen, and comparisons are made based on chemical standards) which is inappropriate as IPM is a portfolio of different tools meant to be used together but which are often assessed on their own, meaning much of the effectiveness of the interactions between IPM components is overlooked. By contrast, chemical pesticides are seen as cheap, reliable and effective, but many of the costs of pesticides are experienced by society at large rather than the individual users, so the drive to reduce their use is much reduced. Therefore, regulatory restrictions are required to reduce pesticide use and increase IPM uptake, and some support for this has been seen with the EC1107/2009 regulation restricting some pesticide use (EC 2009b) and the sustainable use directive on pesticides which makes IPM compulsory for professional users and gives priority to non-chemical plant protection methods (EC 2009a). IPM has the potential to be the pest control strategy for the future, but its uptake will depend on reduced regulatory restrictions, and an increased knowledge base on IPM components, and their interactions.

Greenhouses are well suited to the application of IPM due to the enclosed growing environment allowing greater control of environmental conditions to favour natural enemies, physical enclosure of crops stopping mass influx of pests and efflux of control agents, the ability to cleanse the growing environment before cropping, the ready availability of resistant cultivars of greenhouse crops, and the compartmentalisation of crops allowing different control strategies in different areas (Van Lenteren 2003). However, constant cropping and warming in cold periods can lead to an all-year availability of food for pests and sustenance of pests from one year to the next (Van Lenteren 2003). Nevertheless, effective IPM strategies exist for whitefly control on greenhouse crops, such as for *T. vaporariorum* (Moreau and Isman 2012) and *B. tabaci* (Calvo et al. 2009) on sweet peppers. Whiteflies on greenhouse grown tomatoes may be effectively controlled using IPM (Van Lenteren and Woets 1988; Reddy 2016). Resistant cultivars (Van Giessen et al. 1995), physical exclusion, careful monitoring e.g. by the use of sticky traps, biocontrol agents such as parasitoids like *Encarsia formosa*, *Eretmocerus* sp. and predators such as *Macrolophus* (Van Lenteren 2003), and chemical pesticides (George et al. 2015) are all important elements of whitefly IPM (Reddy 2016). Still, management of the development of pesticide resistance (see above) and greater knowledge of individual components of IPM are needed in order to continue to effectively protect greenhouse-grown tomatoes in the future.

### 1.7 Aims, Objectives and Thesis Format

The aim of this thesis is to investigate novel, environmentally sustainable control methods for the glasshouse whitefly on glasshouse-grown tomatoes. The following data chapters have

been published in, or submitted to, peer reviewed journals, as indicated in each chapter. Each data chapter consists of its own Abstract, Introduction, Materials and Methods, Results and Discussion, and Conclusion. Below, the contribution of each author is indicated for each data chapter, as well as a brief summary of the work undertaken in each chapter.

**Chapter One** provides a general introduction.

**Chapter Two** details an investigation into the potential of a wild tomato species, *Lycopersicon pimpinellifolium* (=*Solanum pimpinellifolium*), to act as a source of resistance genes against the glasshouse whitefly, for introgression into commercial tomato species. The objectives of this work were to quantify the relative preference of whiteflies for both the commercial 'Elegance' and the wild *L. pimpinellifolium*, then to identify the source of any increased resistance based upon whitefly feeding data collected with the electrical penetration graph technique. This work represents one of the only studies to compare the resistance to *T. vaporariorum* of this wild tomato species, with that of the commercial cultivar *Solanum lycopersicum* 'Elegance'. As resistant cultivars are an important component of IPM, expanding knowledge of the level of resistance that may be found in closely related wild relatives, and the possible mechanism of these resistance factors, represents an important strategy for development of resistant cultivars in the future. This study was published in *Agronomy for Sustainable Development* (McDaniel et al. 2016). The study was conceived by Barry Brogan (BB), Colin Tosh (CT), Michelle Robson (MR) and Thomas McDaniel (TM), data collection was carried out by BB and MR, and data analysis and preparation of the manuscript was carried out by TM. All authors contributed to the preparation of the final manuscript.

**Chapter Three** describes a set of glasshouse experiments into the effect of intercropping tomato plants with both less preferred and more preferred host plants, to quantify the efficacy of a 'push-pull' strategy of controlling the glasshouse whitefly. It also quantifies the use of marigolds, and the plant volatile limonene, as emergency measures to control a high-density whitefly infestation. The objectives of this study were to assess the level of whitefly control that could be achieved with a low diversity or high diversity of 'push' plants. It was then assessed whether this level of control could be increased using low or high diversity of 'pull' plants in addition to the 'push' plants. The ability of marigolds and the volatile chemical limonene to reduce high-density whitefly pest populations was also investigated. The use of other plants to provide a protective effect on the primary crop plant, as well as the use of naturally-occurring plant products as biopesticides, are well-established tools of IPM, with

this study seeking to attach a scientific basis to the common assertion of home gardeners that marigolds are effective at reducing whitefly numbers on a tomato crop. This study also seeks to demonstrate that an increased level of plant diversity can be achieved in a commercial setting. The portion of this chapter describing the 'push-pull' glasshouse trials from 2016 has been submitted to *Agronomy for Sustainable Development*. This study was conceived by CT, TM and Niall Conboy (NC), the data collection was conducted by TM and NC, the data analysis was conducted by TM, and the preparation of the manuscript was completed by CT and TM. All authors had input into the final manuscript.

**Chapter Four** details a study into the potential of whitefly-induced tomato VOCs to increase the resistance of neighbouring, uninfested tomato plants to a subsequent whitefly infestation. The objectives of this work were to assess whether VOCs from infested tomatoes could increase the resistance of neighbouring plant to whiteflies. The most effective combination of infestation and exposure time was then sought. The mechanism of increased plant resistance, whether defence activation or defence priming, was then investigated. Recent studies into VOCs have revealed an important role in mediating how plants interact with their environment, and the potential for certain VOC mixes to allow infested plants to "communicate" their predicament to as-yet uninfested neighbours could be a powerful tool in the greenhouse to achieve pest control with minimal synthetic pesticide input. The mechanism by which any resistance occurs, which could have a large impact on the suitability of this process for commercial usage, was also investigated. TM conceived the study, and carried out data collection, analysis and manuscript preparation, and all authors aided with the final draft.

**Chapter Five** is a general discussion of all results obtained with respect to the literature, with the future directions, strategies, and challenges of and for IPM discussed.

# Chapter 2. Novel Resistance Mechanisms of a Wild Tomato against the Glasshouse Whitefly

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**Abstract**

The glasshouse whitefly, *Trialeurodes vaporariorum*, is an important pest of many crop plants including tomato, *Solanum lycopersicum*. Many wild tomato species exhibit a higher resistance to whiteflies. Therefore, locating the source of this enhanced resistance and breeding it into commercial tomato species is an important strategy to reduce the impact of pests on crops. Here, the pest resistance of *Lycopersicon pimpinellifolium* was assessed by comparing oviposition and feeding data from *T. vaporariorum* on this wild tomato species with data collected from a susceptible commercial tomato, *Solanum lycopersicum* var. 'Elegance'. The location of resistance factors was examined by use of electrical penetration graph (EPG) studies on these tomato species. Results show that whiteflies preferentially settled on the commercial tomato more often in 80% of the replicates when given free choice between the two tomato species, and laid significantly fewer eggs on *L. pimpinellifolium*. Whiteflies exhibited a shorter duration of the second feeding bout, reduced pathway phase probing, longer salivation in the phloem, and more non-probing activities in the early stages of the EPG on the wild tomato species compared to the commercial tomato. These findings evidence that a dual mode of resistance is present in this wild tomato against *T. vaporariorum*: a post-penetration, pre-phloem resistance mechanism, and a phloem-located factor, which to the best of the authors' knowledge is the first time that evidence for this has been presented. These findings can be used to inform future breeding strategies to increase the resistance of commercial tomato varieties against this important pest.

## 2.1 Introduction

**Figure 2.1:** *Trialeurodes vaporariorum* feeding on aubergine.

One of the foremost arthropod pests of glasshouse crops, and in particular tomatoes, is the glasshouse whitefly, *Trialeurodes vaporariorum* Westwood. This phloem-feeding, cosmopolitan, hemipteran pest damages plants in three ways. Whiteflies extract sap from phloem during feeding and reduce the nutrients available to the plant for growth and reproduction (Byrne and Bellows Jr 1991). They also produce a sticky excreta called honeydew which supports sooty mould growth on the plant, limiting its photosynthetic potential and causing aesthetic damage to fruits, reducing their commercial value (Inbar and Gerling 2008). Finally, whiteflies transmit damaging viruses via their saliva, such as the *Tomato chlorosis* and *Tomato infectious chlorosis viruses* (Jones 2003).

Under glasshouse production the foremost whitefly control method is the use of biocontrol agents, including *Encarsia formosa* Gahan. This parasitoid wasp oviposits into the immobile nymph stages of the whitefly, with the subsequent emerging larvae using the nymphs as a food source (Gorman et al. 2007). Whilst these agents are a moderately effective control measure, the method has several limitations. For one, multiple releases, often on a weekly basis, are typically required to manage whitefly numbers. Deployment of biocontrol is thus labour-intensive, also requiring that wasps are dispensed rapidly after arrival for maximum efficacy. Secondly, and perhaps more importantly, biocontrol agents alone are not always sufficient to reduce whitefly numbers below acceptable thresholds, with biocontrol often breaking down under extreme pest pressure, or in the face of natural movement of hyperparasitoids into the system. In these instances it is necessary to deploy chemical pesticides as a ‘second line of defence’ to redress balances between pest and parasitoid, or to replace biocontrol where this has failed to function due to the appearance of a 4$^{th}$ trophic

level. In this respect, pesticides remain a key component of current glasshouse crop production (George et al. 2015).

Several different synthetic pesticide classes are used to control whitefly, including neonicotinoids, pyrethroids, pyrethrins, and spirocyclic phenyl-substituted tetronic acids (Fera 2015). However, effective use of conventional chemical pesticides is becoming increasingly difficult due to whiteflies evolving resistance to active ingredients, tightening legislation restricting availability of approved products, and consumer concerns regarding chemical persistence in the environment, which may impact upon non-target and beneficial species (Karatolos et al. 2010). Owing to these shortcomings of current control methods, alternative methods of reducing whitefly impact on crop plants are currently being sought, with significant recent effort being directed to investigating the potential of biopesticidal and biorational products against this pest (George et al. 2015). Whilst such work has merit in potentially expanding pesticide availability, such 'reduced risk' products are not free from limitations, which, depending on product types, can include reduced residual activity, environmental sensitivity and variable efficacy (George et al. 2015). Free from such limitations, an alternative, complementary and at least equally promising approach is to increase crop resistance to whitefly pests through incorporation of genes from more resistant wild tomato species into commercial varieties.

Due to its status as a crop plant of global importance, the cultivated tomato, *Solanum lycopersicum* L., has undergone extensive selection to enhance its desirable traits (Bas et al. 1992). This selection process has potentially left the cultivated tomato bereft of the genetic variation required to allow it to cope with a range of environmental and biological stresses, including attack by *T. vaporariorum* (Sim et al. 2011). Therefore, attempts have been made to increase the innate genetic resistance of the cultivated tomato. Crossbreeding methods for breeding genes into commercial plants from wild relatives are an important means of increasing plant resistance to various pests, diseases and stresses: they are under no regulatory scrutiny and are generally well accepted by consumers, unlike genetic engineering methods. Wild relatives are often much more resistant to pest attack and have consequently been used in these interbreeding programmes, as well as in genetic engineering as gene sources. Several studies have demonstrated the success of this approach to increase plant resistance to the tobacco whitefly, *Bemisia tabaci* Gennadius (e.g. Morales (2001); Carabali et al. (2013) amongst others), though similar work on *T. vaporariorum* is less prevalent.

Relatively little is known about the molecular responses of plants to phloem-feeding insects, such as whitefly, compared to chewing insects. Whilst most work on whitefly resistance mechanisms has been conducted on *B. tabaci* and not *T. vaporariorum*, the mechanisms are thought to be similar between the two species (Toscano et al. 2002). Due to the highly specialised feeding method employed by this guild of insects, whereby the insect's stylet negotiates the intercellular space to penetrate the phloem, the defensive response of plants is more akin to that observed in response to pathogenic infection (Zarate et al. 2007). The signalling pathways involved in the plant's response to whitefly have been studied, with a complicated pattern emerging. The salicylic acid pathway has been shown to be strongly upregulated by whitefly attack (Zarate et al. 2007), but it has been suggested that this is a strategy used by whiteflies to aid their development by causing antagonism and subsequent downregulation of the jasmonate and ethylene-responsive signalling pathways, which have been proposed to be the key pathways for coordinating plant defences against whiteflies (Puthoff et al. 2010). The only R (resistance) gene in tomato which has been shown to interact with any whitefly species to date is the *Mi-1.2* gene (which encodes a protein with putative coiled-coil nucleotide-binding site and leucine-rich repeat motifs; Bhattarai et al. (2007)) which has been shown to confer resistance to *B. tabaci* in tomato, as well as the root-knot nematode and potato aphid (Nombela et al. 2003). RNA transcripts of jasmonate and ethylene-responsive pathogenesis-related (*PR)* proteins, such as the glucanase *GluB*, the chitinase *Chi9* and *Pathogenesis-related protein-1*, have been shown to accumulate in infested tomato leaves in response to feeding by *B. tabaci* and *T. vaporariorum* nymphs, indicating a role for these proteins in tomato whitefly resistance (Puthoff et al. 2010). Volatile organic compounds may reduce whitefly impact on tomato (Guo et al. 2013), mainly via repellence but also via potential toxic effects (Bleeker et al. 2011). Glandular trichomes on leaf surfaces, particularly type IV and type VI (Firdaus et al. 2013) have been shown to physically ensnare whitefly (Toscano et al. 2002) and exude deterrent or toxic chemicals such as acyl sugars (de Resende et al. 2009). Other physical methods are also important in determining host susceptibility, such as cuticle and epidermis thickness (Toscano et al. 2002). Proteins present in the phloem sap, of which a large proportion of those that have been characterised are predicted to be stress or defence-related, may also affect whitefly feeding behaviour (Kehr 2006).

*Lycopersicon pimpinellifolium* (L.) Miller is the most closely related wild species of tomato to the commercial tomato *S. lycopersicum*. It has been used previously as a source of genes for hybridising with *S. lycopersicum*, this being facilitated by *L. pimpinellifolium* producing red

fruit, and the relative ease with which the two species hybridise (Kazmi et al. 2012). *L. pimpinellifolium* has been used to improve several traits in the commercial tomato, including improved seed abiotic stress resistance (Kazmi et al. 2012) and increased vegetative tissue salt tolerance (Foolad and Chen (1999); Foolad (2007)). Many studies have investigated the ability of wild tomato species to resist *B. tabaci*, e.g. Firdaus et al. (2012), Rodríguez-López et al. (2011) (which both included accessions of the wild tomato species considered here), Firdaus et al. (2013) and de Resende et al. (2009). Work to elucidate the source of whitefly resistance in wild tomato species has also been attempted. Studies include comparing the strength of resistance to *T. vaporariorum* of wild accessions and commercial tomato species (Bas et al. (1992); Lei et al. (1999)) and comparing wild accessions with inter-specific hybrids of commercial and wild species (Rodríguez-López et al. 2011). Other studies have looked to compare resistance in commercial tomato varieties to other *T. vaporariorum* host species (Lei et al. 2001). However, to the best of the authors' knowledge, this work presents the only comparative data on *T. vaporariorum* resistance in *L. pimpinellifolium* compared to a commercial tomato variety, *S. lycopersicum* var. 'Elegance'.

With the above in mind, the aim of the current study was to assess *L. pimpinellifolium* for resistance to *T. vaporariorum* and to investigate the specific resistance mechanisms present in this wild tomato species. These data will add to the body of knowledge on wild tomato varieties that potentially possess enhanced resistance to *T. vaporariorum*, and provide some of the only evidence comparing the commercially used 'Elegance' line of tomato to the wild tomato, *L. pimpinellifolium*. In expanding current knowledge of whitefly behaviour on *L. pimpinellifolium*, this work further aims to provide insight into the mechanism of resistance in this wild species.

## 2.2 Materials and Methods

### *2.2.1 Insects*

*Trialeurodes vaporariorum* whiteflies were taken from a mixed-age colony maintained in a laboratory at Newcastle University (UK) on Aubergine (*Solanum melongena* 'Moneymaker') under 16 h light, 8 h dark cycle and constant 20°C temperature conditions (Figure 2.1). This colony was originally obtained from a laboratory culture at Rothamsted Research, first collected in 1960 in Kent originating on French bean plants.

### *2.2.2 Plants*

Commercial tomato seeds (*Solanum lycopersicum* Mill., 'Elegance' Cat. E/12/11, Batch 0113479253) were obtained from Monsanto and *Lycopersicon pimpinellifolium* seeds were

obtained from Magic Garden Seeds Ltd. (product code LYC09). All plants were grown from seed in Clover Multipurpose Compost (http://www.cloverpeat.co.uk/CLOVER-RETAIL-COMPOST-1.html) in 9-cm-diameter and 8.7-cm-deep pots, at a density of one plant per pot. All plants were grown at a distance of approximately 60 cm from a 400 W Son-T bulb housed in a Harrier HR400SH 400W lamp under a 16 h light, 8 h dark cycle and a temperature regime of 25 °C during the light period and 20 °C during the dark period, synchronised with the light regime that all other experiments were conducted under. Plants were liberally watered before and during the experimental period, and used for all assays at the 3-5 true leaf stage.

### *2.2.3 Free choice assays*

The settling preference of *T. vaporariorum* for the two tomato species was quantified, with whiteflies having free choice between the commercial 'Elegance' cultivar and *L. pimpinellifolium*. For each repeat of the experiment, six plants (three Elegance, three *L. pimpinellifolium*) were placed into a 20 L transparent Perspex tank with an open mesh top, and were spaced 3 cm apart. Whiteflies were caught using a mouth pipette (a length of rubber tubing with a pipette tip on the end) then placed in a small petri dish and anaesthetised with $CO_2$ for 90 seconds before the petri dish was placed in the cage. In this way, simultaneous release of whiteflies was achieved. Whiteflies of equal gender mix were placed into the tank and allowed free settling choice over the course of 24 hours; the number of whiteflies used was 15 males and 15 females for four replicates, and 40 males/40 females for the 5$^{th}$ replicate. Numbers were increased for the 5$^{th}$ replicate as high mortality had been observed in earlier runs. After 24 h the number of settled whiteflies on each plant was recorded. The experiment was conducted under a 16 h light, 8 h dark cycle and a constant temperature regime of 20 °C. The differences in settling behaviour were analysed using Pearson's Chi squared test, with expected values representing an even distribution between plant species after the number of dead whiteflies were removed.

### *2.2.4 No choice assays*

The second behavioural measure taken was the rate of oviposition of whiteflies in a no choice situation. A single female whitefly was placed on the second apical leaflet of a tomato plant in a small clip cage and left for 72 h, with the plant placed inside a small mesh cage. The clip cage consisted of two foam rings, with clear acetate over opposing sides of the rings, which could be closed over a leaf and secured using two staples. This allowed the whitefly in the experiment to move on and off the plant as well as between the abaxial and adaxial surfaces of the leaf (Figure 2.2). After 72 h the clip cage was removed and the leaf analysed at low

magnification (3x) to count the number of eggs laid by the whitefly on both sides of the leaf. Whitefly survival was also recorded, with 17 replicates completed for 'Elegance' and 19 for *L. pimpinellifolium*. The data were analysed using the Mann Whitney U test due to deviations from the normal distribution and lack of homogeneity of variances within the data.


**Figure 2.2: (A)** Clip cage used in no choice and video trials. **(B)** EPG equipment used for EPG studies. **(C)** Detail of volatile delivery box and positioning of plants for EPG studies.

### *2.2.5 Video trials*

To measure the impact of the different tomato species on the settling and movement behaviour of the whiteflies, video trials were conducted where a female whitefly was placed on the terminal leaflet of the tomato, and its movement was recorded using a high definition video camera (Sony HD Handycam, HDR-CX130). The whitefly was placed on the underside of the second apical leaf in a clip cage with a piece of transparent perforated plastic over the bottom, with the clip cage constructed so that the whitefly could leave the underside of the leaf, but was not able to reach the topside; only the sides and bottom of the clip cage. A recording of the whitefly was made for 65 minutes, which incorporated a 5 minute period of recovery from the mild $CO_2$ anaesthetisation used to capture and select the whitefly, and 60 minutes of data recording. This experiment was conducted for each of the tomato species under study, with 24 replicates per species. The data were analysed for four sets of behaviour over the 60 minutes: 1) time first present on the leaf (after 5 minute recovery period), 2) time

between the first and second stationary periods of > 5 s on the leaf, 3) time spent moving and 4) time spent on the leaf. Comparisons were made between both tomato species using the Mann Whitney U-test due to deviations from the normal distribution and lack of homogeneity of variances within the data.

### *2.2.6 Electrical Penetration Graph studies*

To investigate the feeding behaviour of whitefly on *Solanum lycopersicum* Mill., 'Elegance' and *Lycopersicon pimpinellifolium,* the electrical penetration graph (EPG) technique was used, as developed by Tjallingii (1978) and used previously to investigate whitefly feeding behaviour on tomato (Tosh and Brogan 2015; Lei et al. 1997; Lei et al. 1999; Lei et al. 2001). The different waveforms produced by the completion of a partial electrical circuit between the plant and the whitefly's stylet when the whitefly probes the plant correspond to different feeding behaviours of the whitefly. An eight-channel DC EPG system supplied by EPG Systems (EPG-Systems, Dillenburg 12, 6703 CJ Wageningen, the Netherlands, http://www.epgsystems.eu/contact.htm; Tjallingii (1978)) was used. Tosh and Brogan (2015) developed a modified EPG apparatus (Figure 2.2 (B) and (C)) to monitor whitefly feeding under different conditions, and here the same experimental set-up and method for the EPG data collection were used for both tomato species. Briefly, a single female whitefly attached with gold wire to the EPG apparatus was placed on the terminal leaflet of a tomato plant and allowed to feed for 20 h. Four replicates were run simultaneously (with one whitefly per plant for each of four plants). EPG waveforms for *L. pimpinellifolium* were collected, analysed and compared with waveforms previously collected by Tosh and Brogan (2015) for whiteflies feeding on the commercial *Solanum lycopersicum* cultivar 'Elegance'. In total, waveforms from 20 whiteflies feeding on *L. pimpinellifolium* and 23 whiteflies feeding on 'Elegance' were analysed. To identify the waveforms produced, the waveform guide supplied by Giga 4/8 EPG systems manual (http://www.epgsystems.eu/files/aphid%20waveforms.pdf) as well as two studies investigating whitefly-specific waveforms (Lei et al. (1997) and Lei et al. (1999)) were used. The waveforms of interest which may be observed on a whitefly EPG recording are: C waveforms which indicate apoplastic stylet penetration and salivation, pd or potential drops indicating brief (4-12 s) intracellular probes and E waveforms indicating phloem penetration. The E waveforms may be divided into E1, indicating salivation into the phloem, and E2, indicating phloem sap ingestion. A probe is when an insect's stylet is inserted into the plant. A "non-probe period" is when no waveform is observed due to an insect's stylet being outside the plant (Rodríguez-López et al. 2011). Analysing the quantity, frequency and distribution of these waveforms during the EPG, both alone and in relation to each other,

generates a large number of parameters which may be analysed to dissect insect feeding patterns. The raw data from the waveform analysis were exported to and analysed using the spreadsheet devised by Sarria et al. (2009).

## 2.3 Results and Discussion

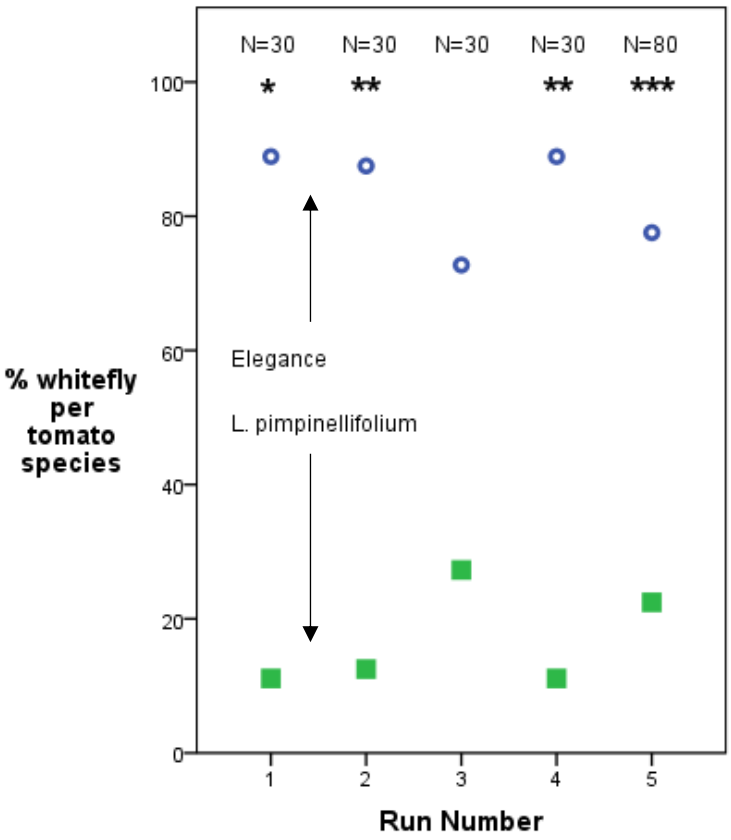
Commercial tomato species exhibit a reduced ability to cope with attack by a wide range of pests and pathogens due to extensive selection through history (Sim et al. 2011). An effective strategy for increasing the resistance of commercial food crops is the introduction of genes that confer enhanced resistance to a target pest, as has been achieved for several insect/crop systems such as *B. tabaci* and cassava (Carabali et al. 2013). With this in mind, the current work aimed to assess the wild tomato species, *Lycopersicon pimpinellifolium*, for resistance to *Trialeurodes vaporariorum*, and to attempt to identify the location of this resistance in the plant.

### *2.3.1 Free choice assays*

When 30 whiteflies were given free choice between the commercial 'Elegance' and the wild *L. pimpinellifolium* over 24 hours (Figure 2.3A), a significantly higher number of whiteflies were found to settle on the commercial 'Elegance' compared to the wild *L. pimpinellifolium* according to the Pearson's chi squared test. Similarly, when greater numbers of whitefly (80) were used, a highly significant preference ($p$<0.001) for the commercial tomato species was observed (Figure 2.3A). In all cases more whiteflies were found on the commercial species than the wild tomato, with between 73-89% of whitefly preferring to settle on the commercial vs the wild tomato over the 5 trials. These data suggest a preference by the whitefly for the commercial species, potentially because it represents a better food source due to a lack of resistance mechanisms that were present in the wild species. It deserves note that statistical analysis from the first replicate had an inflated probability of a type I error, due to more than a fifth of the expected values equalling <5. However, as statistical output matched that from the other 4 runs (where this assumption was not violated) results of replicate one were retained and included herein. Firdaus et al. (2012) examined a range of wild tomato relatives for resistance to *B. tabaci.* They found that *L. pimpinellifolium* showed little evidence of being less preferable to *B. tabaci* based upon free choice assays, which is in contrast to the findings presented here. This may be due to differences in experimental design or be indicative of subtle differences in the ecology of the two whitefly species. Rodríguez-López et al. (2011) also conducted free choice assays in their comparison of the commercial tomato 'Moneymaker' and the ABL14-8 tomato breeding line, formed by the introgression of a *Solanum pimpjnellifolium* L. accession into the 'Moneymaker' cultivar. They found that *B.*

*tabaci* showed a strong preference for the commercial ‘Moneymaker’, similar to the identification of a preference for ‘Elegance’ found in the current study, but only in older plants (10 leaf v. 4 leaf stage). The emergence of a stronger defensive response at an earlier stage in the present study may demonstrate a stronger presence of defensive traits in *L. pimpinellifolium* than in the ABL14-8 breeding line used by Rodríguez-López et al. (2011).

**A**




**B**

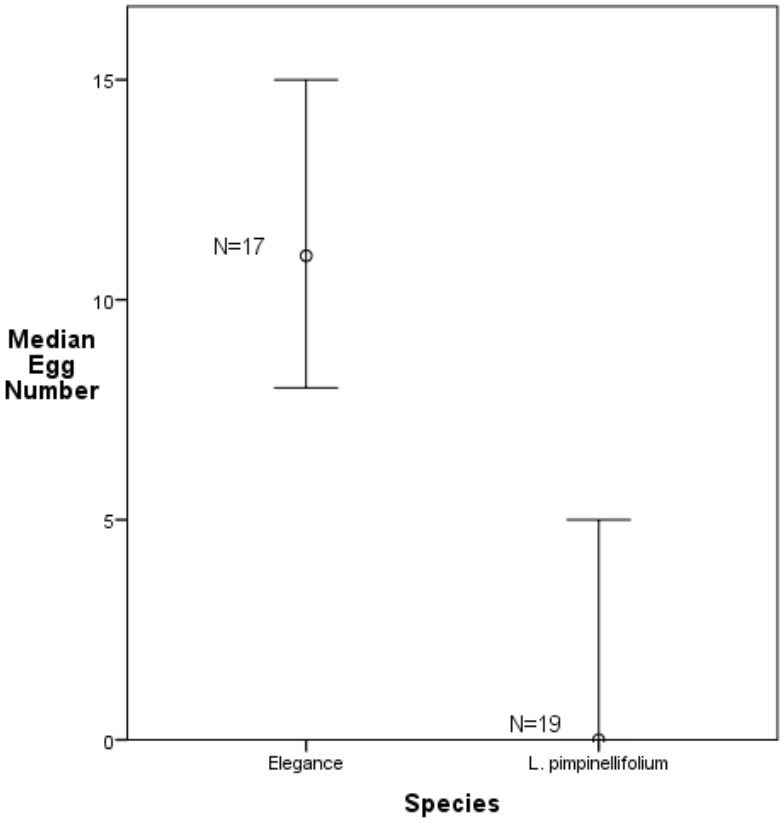



**Figure 2.3:** The results of free and no- choice assays to ascertain whitefly preference for *L. pimpinellifolium* or 'Elegance'. **(A)** The percentage of whiteflies settling on three plants of

the commercial tomato species 'Elegance' and three plants of the wild *L. pimpinellifolium* after 24 hours, repeated 5 times. Thirty whiteflies were used in Rep No. 1-4, 80 whiteflies were used in Rep No. 5. (*) indicates significance of $p < 0.05$, (**) shows significance of $p < 0.01$ and (***) shows significance of $p < 0.001$. No asterisk indicates a non-significant difference between the numbers of whitefly found on each species. Rep 1 $X^2 = 4.00$, $p < 0.05$; Rep 2 $X^2 = 7.56$, $p < 0.01$; Rep 3 $X^2 = 3.68$, $p > 0.05$; Rep 4 $X^2 = 9.39$, $p < 0.01$ and 80 whiteflies in rep 5 gave $X^2 = 13.80$, $p < 0.001$, $df = 1$ for all reps. **(B)** Median number of eggs laid by a single female whitefly after 72 hours on the second apical leaf of either 'Elegance' or *L. pimpinellifolium*. The difference is significant according to the Mann Whitney *U* test with a *p*-value <0.001. Ninety five percent confidence intervals are shown. Test statistic, $U = 28.5$, $df = 34$.

### *2.3.2 No choice assays to record whitefly oviposition*

The level of oviposition by the whitefly on 'Elegance' and *L. pimpinellifolium* after 72 h was analysed using the Mann Whitney U test (Figure 2.3B). A significantly greater number of eggs were laid on 'Elegance' compared to *L. pimpinellifolium* plants after 72 h, $p < 0.001$. These results demonstrate that the commercial tomato is a more preferred host for oviposition than the wild species. Bas et al. (1992) studied oviposition rates of *T. vaporariorum* on four genotypes of *Lycopersicon esculentum* varying in their resistance to the glasshouse whitefly, and on two wild tomato species. The wild tomato *Lycopersicon hirsutum* var. *glabratum* was found to be most resistant and experienced the lowest oviposition rate. Whilst no resistance mechanism was suggested, the presence of greater resistance in the wild tomato species is concordant with our findings. Bas et al. (1992) also found that older individuals of *L. hirsutum* var. *glabratum* used in the study (those tested at 14 weeks rather than 8) displayed enhanced resistance, which has interesting implications for the present study in that the apparent resistance observed in *L. pimpinellifolium* in the present work at the 3-4 leaf stage (~3 weeks old) may increase as the plants age. Erb et al. (1994) studied the potential of another wild tomato species, *Lycopersicon pennellii*, to act as a source of resistance traits against *T. vaporariorum*. Hybrids produced using this species supported the fewest eggs and were the least attractive host of the whitefly. Firdaus et al. (2012) also considered oviposition and found one accession of *L. pimpinellifolium* to be resistant on the basis of supporting low levels of egg-laying, as corroborated by the present work.

### *2.3.3 Whitefly movement trials*

The median time spent by whiteflies engaging in selected behaviours on the two tomato species is shown in Figure 2.4. No significant differences between 'Elegance' and *L. pimpinellifolium* for any of the selected behaviours were observed. A large proportion of whiteflies on each tomato species (66% for Elegance and 58% for *L. pimpinellifolium*) did not land on the tomato leaves at all during the hour long assay. This may represent a

methodological limitation of the current study and differences may have been detected had video capture periods been extended. Although these whiteflies were excluded from subsequent analysis, a Chi-squared analysis revealed that there was no significant difference in the number of whiteflies which avoided the 'Elegance' or the wild tomato leaves compared to those which chose to land on the leaf ($X^2 = 2$, $df = 1$, $p > 0.05$, $N = 24$ for both). Many whitefly resistance mechanisms in tomato have been found to be surface based, including type IV trichomes which reduce whitefly feeding efficiency (Firdaus et al. 2012). Rodríguez-López et al. (2011) monitored the feeding behaviour *B. tabaci* on the commercial 'Moneymaker' strain of tomato and the ABL14-8 tomato breeding line. This breeding line was formed by the introgression of accession TO-937 of *Solanum pimpjnellifolium* L. into the 'Moneymaker' cultivar and was backcrossed to exhibit a particularly high density of type IV trichomes and high acylsucrose production. This paper concluded that the presence of these surface-based resistance mechanisms deterred whitefly from landing and settling on the ABL14-8 breeding line. Lei et al. (2001) stated that the main resistance mechanism in the commercial 'Moneydor' species of tomato was the presence of very dense hairs on the leaf surfaces, physically preventing the whitefly from effectively probing. Erb et al. (1994) attributed the greater resistance of *L. pennellii* to *T. vaporariorum* to toxic exudates from glandular trichomes. Firdaus et al. (2012) also suggest that the resistance found in *L. pimpinellifolium* is based upon the presence of type IV trichomes. These studies contrast with the present work. The movement data presented here indicate that surface characteristics are not involved in the resistance of *L. pimpinellifolium* to whiteflies: the non-significant differences obtained for any whitefly behaviours on the leaf surface between the tomato species in this study would suggest that whiteflies easily navigate *L. pimpinellifolium* leaf surfaces. This discrepancy may be due to the methodological limitations mentioned above, with longer video capture periods possibly being needed to reveal the importance of trichomes on *L. pimpinellifolium.*

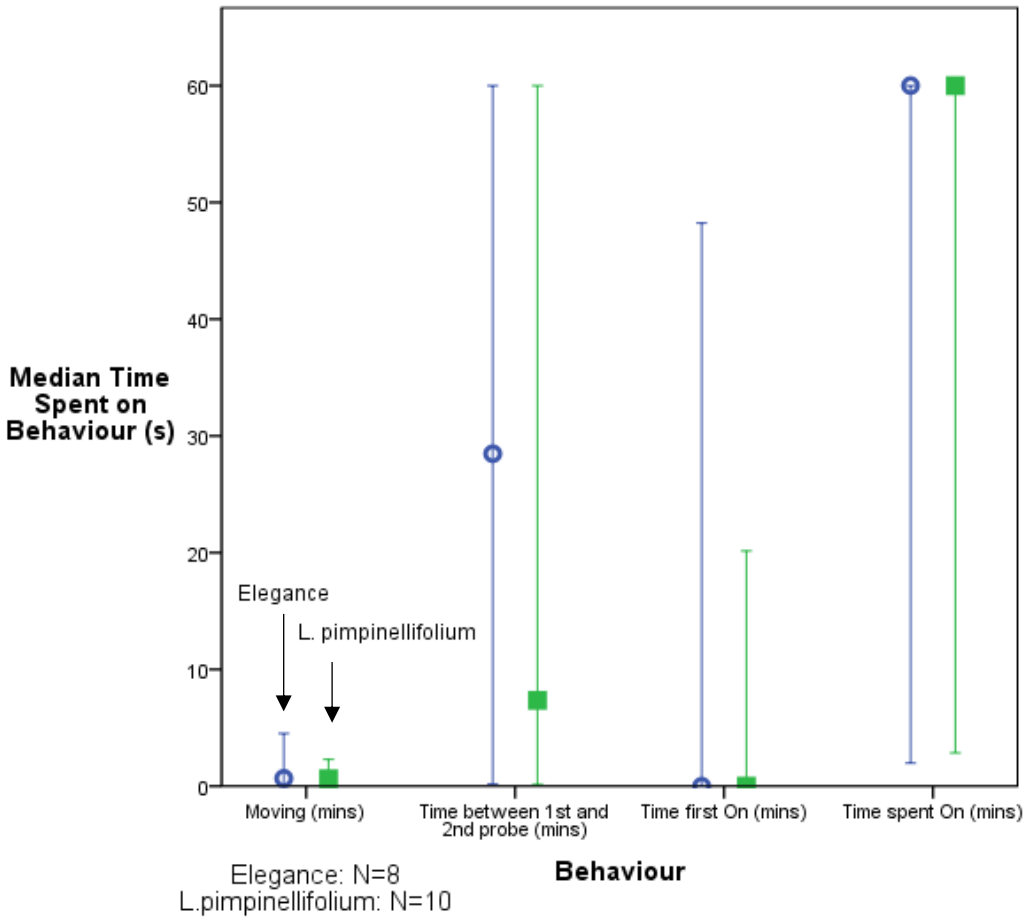



**Figure 2.4:** Different movement behaviours by a single whitefly on either the commercial 'Elegance' or the wild *L. pimpinellifolium* over one hour. The median and 95% confidence intervals are indicated. Differences between the two species are non-significant. Test statistics for: Time first on: *U*, (*df* = 34) = 32 (*p* = 0.368), Time between first and second probe: *U*, (*df* = 34) = 35.5 (*p* = 0.684), Time spent moving: *U*, (*df* = 34) = 38.5 (*p* = 0.892) and Time spent on: *U*, (*df* = 34) = 39.5 (*p* = 0.958).

### *2.3.4 EPG studies to monitor whitefly feeding behaviour*

Whitefly feeding behaviour was analysed using the EPG technique, with parameter selection based on prior study by Lei et al. (1997), Lei et al. (1999), Lei et al. (2001) and Rodríguez-López et al. (2011) . These parameters are detailed in Table 2.1, and are subdivided into those parameters which relate to pre-phloem probing behaviour and those which relate to phloem phase probing (similar to EPG work by Jiang et al. (2001)).

| | **Elegance** | | ***L. pimpinellifolium*** | | ***P*** |
|---|---|---|---|---|---|
| **Pre-phloem parameters** | | | | | |
| 1. Time to 1st probe from EPG start (min) | 12.6 ± 4.5 | N=23 | 90.9 ± 48.4 | N=20 | 0.527 |

| | | | | | |
|---|---|---|---|---|---|
| 2. Duration of 1st probe (min) | 14.1 ± 5.1 | N=23 | 2.0 ± 0.6 | N=20 | 0.080 |
| 3. Duration of 2nd probe (min) | 16.8 ± 4.8 | N=23 | 6.0 ± 3.7 | N=20 | 0.011 |
| 4. Total number of probes | 25.3 ± 5.8 | N=23 | 31.75 ± 6.78 | N=20 | 0.575 |
| 5. Total number of C[a] | 31.3 ± 6.3 | N=23 | 32.7 ± 6.8 | N=20 | 0.961 |
| 6. Total duration of C (min) | 410.2 ± 39.1 | N=23 | 193.8 ± 34.2 | N=20 | 0.001 |
| 7. Number of probes to the 1st E[b] | 10.4 ± 2.1 | N=19 | 17.8 ± 3.6 | N=13 | 0.092 |
| 8. Time from start of EPG to 1st E (min) | 215.9 ± 35.9 | N=19 | 281.7 ± 42.4 | N=13 | 0.147 |
| 9. Time from 1st probe to 1st E (min) | 206.0 ± 36.1 | N=19 | 243.8 ± 42.0 | N=13 | 0.270 |
| 10. Duration of np during 1st h (min) [c] | 29.7 ± 3.5 | N=23 | 46.1 ± 3.8 | N=20 | 0.002 |
| 11. Duration of np during 2nd h (min) | 29.4 ± 3.7 | N=23 | 40.3 ± 4.3 | N=20 | 0.062 |
| 12. Duration of np during 3rd h (min) | 19.8 ± 3.8 | N=23 | 35.8 ± 5.3 | N=20 | 0.024 |
| 13. Duration of np during 4th h (min) | 28.7 ± 5.0 | N=23 | 35.5 ± 5.6 | N=20 | 0.581 |
| 14. Duration of np during 5th h (min) | 30.1 ± 5.1 | N=23 | 31.6 ± 6.2 | N=20 | 0.911 |
| 15. Duration of np during 6th h (min) | 28.2 ± 5.4 | N=23 | 29.3 ± 6.0 | N=20 | 0.667 |
| **Phloem parameters** | | | | | |
| 16. Number of E | 6.4 ± 1.5 | N=23 | 8.5 ± 2.8 | N=20 | 0.796 |
| 17. Total duration of E (min) | 95 ± 31.4 | N=19 | 323.7 ± 59.6 | N=13 | 0.002 |
| 18. Duration of 1st E (min) | 22.0 ± 20.3 | N=19 | 26.0 ± 14.0 | N=13 | 0.024 |
| 19. Number of probes after 1st E | 18.4 ± 5.4 | N=19 | 20.8 ± 6.5 | N=13 | 0.999 |

| | | | | | |
|---|---|---|---|---|---|
| 20. Total duration of E1 (min) | 34.0 ± 9.0 | N=19 | 257.1 ± 57.9 | N=13 | 0.001 |
| 21. Number of E1 | 5.3 ± 1.2 | N=23 | 5.4 ± 1.7 | N=20 | 0.538 |
| 22. Total duration of E2 (min) | 144.9 ± 56.2 | N=8 | 96.3 ± 42.5 | N=9 | 0.370 |
| 23. Number of E2 | 1.1 ± 0.5 | N=23 | 3.1 ± 1.2 | N=23 | 0.269 |
| 24. % whitefly entering E phase | 82.6 | N=23 | 65.0 | N=20 | 0.186 |
| [a]C=pathway phase probing, [b]E=phloem phase probing, [c] np= not probing | | | | | |

**Table 2.1:** Mean values and standard errors of EPG parameters collected from *T. vaporariorum* probing the commercial tomato species 'Elegance' and the wild tomato species *Lycopersicon pimpinellifolium*. Replicate numbers (N) and *P* values according to the Mann Whitney *U* test (or Pearson's Chi square for % whitefly entering E (phloem phase)) are indicated.

The differences between Elegance and *L. pimpinellifolium* for most of the parameters measured were found to be non-significant by the Mann Whitney U test, although 'Duration of First Probe' ($p$=0.080), 'Number of probes to the First E' ($p$=0.092) and 'Duration of np during second hour' ($p$=0.062) approached statistical significance (Table 2.1). However, several parameters did show significant differences. Of the pre-phloem parameters the 'Duration of the Second Probe' ($p$=0.01) and 'Duration of C', or pathway, probing ($p$=0.001) were found to be significantly shorter in *L. pimpinellifolium* than in 'Elegance', and the 'Duration of non-probing behaviour' by the whitefly in the 1st and 3rd hours of the probe ($p$=0.002 and 0.024, respectively) were found to be significantly longer in the wild species compared to the commercial species. Of the phloem based parameters the 'Total Duration of E', or phloem, probing ($p$= 0.002), 'Duration of the first E probe' ($p$=0.024) and the 'Total Duration of E1', the waveform indicating sieve element salivation ($p$=0.001), were significantly longer in *L. pimpinellifolium* compared to 'Elegance'. These results indicate that whiteflies encounter difficulties when feeding on *L. pimpinellifolium* compared to 'Elegance'. EPG studies have also been conducted by other authors on tomato species differing in *T. vaporariorum* susceptibility. Two such studies are Lei et al. (1999) and (2001). In these experiments the commercial 'Moneymaker' and two resistant lines (produced using *Lycopersicon hirsutum glabratum* as a resistance source and named the 82216 and the 82207 resistant line) were compared. In the study by Lei et al. (1999) it was proposed that the

primary mechanism of resistance for line 82207 was located in the phloem sap. This was supported by the difference between the commercial and resistant lines in a number of EPG parameters, including a significantly higher total number of phloem phases, a shorter initial phloem phase, a longer phloem subphase 1 (E1) and a shorter subphase 2 (E2) that were found in the resistant 82207 line compared to the 'Moneymaker' line. The EPG data presented here support a longer E1 phloem subphase as being linked to resistance, though differences in other parameters identified as important by Lei et al. (1999) were not detected. Differences in alternative parameters were nevertheless detected in the current work ('Duration of second probe', 'Duration of C' and 'Duration of np in the 1st and 3rd hours') that may indicate the presence of a resistance mechanism in a different location to that found in line 82207. Rodríguez-López et al. (2011) showed that *B. tabaci* is less able to reach the phloem, spent more time in non-probing activities and displayed a reduced amount of probing on the ABL14-8 tomato breeding line than the commercial 'Moneymaker'. The present study supports the finding of more non-probing behaviour but only over the first three hours, after which the effect disappears. The results of the present study also indicate no difference in the ability of *T. vaporariorum* to access the phloem of either *L. pimpinellifolium* or 'Elegance'. These differences may occur as a result of differences in the whitefly species used, revealing subtle differences in the ecology of these two species, or as a result of the differences in the wild tomato species used.

### *2.3.5 Proposed location of resistance to T. vaporariorum in L. pimpinellifolium and comparison to existing studies*

Based upon these data, it is proposed that two separate resistance factors are present in *L. pimpinellifolium*: a resistance factor encountered early during *T. vaporariorum* feeding, and a phloem-based resistance factor. The first resistance factor is proposed to be epidermal/mesophyll based, encountered by the whitefly during labial dabbing as it assesses the tomato as a prospective host, and during pathway probing as the whitefly attempts to locate the phloem. It has long been known that phloem-feeding insects conduct shorter, gustatory sampling probes at the start of a feeding bout in order to assess the quality of the host plant (Tosh et al. 2002). When using the EPG technique to study aphid plant probing, gustatory sampling is indicated by an abundance of potential drops on the trace (Tjallingii 1985) which indicate the puncturing of host plant cells. However, whitefly have been shown to be less invasive in their feeding method, moving their stylet between cells rather than puncturing them to sample the internal contents (Lei et al. 1997). This indicates that any sub-epidermal resistance mechanism is unlikely to occur inside cells punctured *en route* to the plant phloem, and instead is located extracellularly in the mesophyll. This pre-phloem based

factor is further supported by the significantly shorter 'Duration of the second probe' and 'Duration of C waveforms' (pathway-phase probing) and the significantly higher level of non-probing behaviour in the early part of the EPG trace. It is suggested that the whitefly encounters this factor during its initial probe, and that this factor causes the whitefly to attempt to avoid this aversive element by reducing the length of its next probe, spending less time in the mesophyll of the plant and spending more time in non-probing behaviours e.g. resting or moving to avoid the resistance factor. This is also supported by the free choice data, where a significant proportion of the whiteflies had moved to the more palatable 'Elegance' tomato variety over the course of the experiment. Whilst the exact timing of this movement between the tomato species has not been observed, the increased restlessness of the whitefly in the first three hours of the EPG experiments (indicated by the significantly greater level of non-probing) suggests that the whitefly respond quickly upon exposure to this mesophyll based mechanism.

During the EPG experiment the whitefly were tethered and therefore forced to interact with and feed upon the wild tomato species to avoid starvation. This may account for the lack of a significant difference in the non-probing behaviours 3-6 hours after the start of the experiment. As can be seen by the duration of E2 probing, after 15h there was no significant difference in the amount of time spent ingesting the contents of the phloem, presumably because the whitefly must feed to avoid starvation due to this forced interaction. This pattern is repeated in the findings of studies into the mode of action of the *Mi-1.2* gene, which confers resistance to nematodes, aphids and whitefly in tomato. Jiang et al. (2001) suggested that the *Mi-1.2* gene was expressed in the mesophyll or epidermis of the plant and that when the whitefly had free choice they avoided tomatoes possessing *Mi-1.2*. When they were forced to interact with the plant, however, they were able to access the phloem in a similar manner to the control. Whilst it cannot be claimed that *Mi-1.2* has been discovered in *L. pimpinellifolium*, as this gene originated from a different wild tomato species (*Lycopersicon peruvianum*) the similar mode of action of the resistance mechanism described may suggest evolution of a similar gene to deter whitefly in *L. pimpinellifolium*. This lends credence to our suggestion that a similar factor may be present in this wild tomato species.

The second resistance factor proposed is a phloem-based factor. This is evidenced by the significantly higher level of E1 phase probing, or salivation, of the whitefly when accessing the phloem of *L. pimpinellifolium*. Salivation occurs in order to prepare the phloem for whitefly feeding, and as such takes up a relatively small proportion of the phloem phase when the whitefly is feeding on a susceptible host. The significantly greater amount of salivation,

and length of the first E probe (likely to be an E1 probe), observed when *T. vaporariorum* fed on *L. pimpinellifolium* is therefore indicative of a less favourable interaction between the insect and the host. It has long been hypothesised that aphids produce watery saliva during feeding to combat occlusion of the phloem sieve elements (e.g. Tjallingii and Esch (1993)) and evidence for this is provided by elegant work using legume forisomes (Will et al. 2007). That whiteflies utilise the same method has also been suggested in work by Liu et al. (2013). The authors reported that *B. tabaci* infected with *Tomato yellow leaf curl virus* showed extensive salivation into sieve elements, which was interpreted as the virus affecting the ability of the whitefly to prevent sieve element occlusion to enhance the virus' transmission in watery saliva. In both whitefly and aphids, increased or extended salivation has been correlated with feeding on resistant plants (Will et al. (2007); Jiang and Walker (2007)). Sieve element occlusion is a mechanism employed by most plants to prevent loss of sap from ruptures in the phloem. It is also employed as a resistance mechanism against phloem-feeding insects. Aphid watery saliva contains proteins which bind to calcium and prevent the signalling cascade leading to occlusion of sieve elements by the plant (Will et al. 2007). Recent analysis of whitefly salivary glands has revealed that these glands contain genes encoding several calcium-binding proteins which are hypothesised by the authors to fulfil the same function in whitefly saliva as in saliva of aphids (Su et al. 2012). It is therefore possible that whitefly saliva is able to prevent sieve element occlusion and allow continued access to the phloem. The greater amount of salivation observed in this work is therefore suggested to be a response by *T. vaporariorum* to a much stronger defensive effort by *L. pimpinellifolium* to plug the holes in the phloem than was exhibited by the commercial tomato. The significantly greater amount of salivation by *T. vaporariorum* when feeding on *L. pimpinellifolium* is proposed to account for both the greater "Total duration of E" (as E phase probing comprises total time of E1 and E2 waveforms, and E2 showed no significant difference between the tomato species) and the greater length of the first E probe (as greater salivation was required for a successful phloem-phase probe). The increased level of salivation required to successfully access the phloem of *L. pimpinellifolium* may indicate that the wild species possesses genes which allow it to mount this more effective response, which are attractive targets for incorporation into the 'Elegance' genome. Whilst there was no difference in the amount of E2 waveforms, showing that the glasshouse whitefly is able to ingest as much sap from *L. pimpinellifolium* as it does from 'Elegance', the increased effort and energetic expenditure required to do so may be sufficient to deter feeding in a situation where the whitefly has the choice to move onto a less resistant host. This evidence of a sub-

epidermal source of resistance and a phloem-based resistance mechanism provide interesting targets for breeding programmes to attempt to incorporate into commercial tomato species.

Future research should focus on the genes, or sets of genes, which confer the two resistance mechanisms suggested here. The introduction of these whitefly resistance genes could potentially aid the continued and more effective production of tomato plants in the future. Future work could also involve determining whether whiteflies definitely obtain gustatory information about a plant during an initial probe. This would confirm the suspected sub-epidermal location of the resistance mechanism of *L. pimpinellifolium*.

### *2.3.6 Conclusion*

The wild tomato species *Lycopersicon pimpinellifolium* represents a source of genetic resistance to *T. vaporariorum*, based on the oviposition and settling behaviour of whitefly on the wild species when compared to the commercial tomato variety *Solanum lycopersicum* 'Elegance'. The resistance in *L. pimpinelifolium* appears to be based upon a dual mechanism: a post-penetration but pre-phloem resistance mechanism similar to the *Mi1.2* gene previously discovered in other species of tomato, and a phloem-based mechanism which may be linked to sieve element occlusion. It is hoped that this work describing resistance in *L. pimpinelifolium* will inform future breeding programmes for the introduction of whitefly resistant genes into commercial varieties of this highly important crop plant.

## 2.4 Acknowledgements

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# Chapter 3. Intercropping Tomatoes with Marigolds Controls Whiteflies in a Formal Glasshouse Trial

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The majority of this chapter, detailing the 'push-pull' glasshouse trials in 2016, has been submitted to the journal *Agronomy for Sustainable Development*, and is reproduced here with the kind permission of my co-authors.

**Abstract**

Horticulturalists and gardeners in temperate regions often claim that planting marigolds next to tomato plants protects the tomatoes from whitefly pests. 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, large-scale glasshouse trials in the UK are presented, which demonstrate that companion planting with marigolds reduces numbers of the glasshouse whitefly, *Trialeurodes vaporariorum*, on tomato, and does not attract greater numbers of another major pest, the onion thrips, *Thrips tabaci*. 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. Introducing marigolds as an emergency control measure for heavy whitefly infestations yielded slight reductions in whitefly performance, but the use of slow-release bottles containing the plant volatile limonene was more effective. Marigolds are most effective when used as a whitefly deterrent grown concurrently with tomatoes, rather than being used as a treatment against high whitefly population densities. This is the first scientific study to prove the effectiveness of marigolds in reducing whitefly performance on tomato. It is argued that this work supports the possibility of the development of a mixture of tomato companion plants that infer 'associational resistance' against many major invertebrate pests of tomato. Such a mixture, if comprising edible or ornamental plants, would be economically viable, 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.

### 3.1 Introduction

Tomatoes (*Solanum lycopersicum* L.) are the second most important edible horticultural crop by production in developed nations (Rubatzky and Yamaguchi 2012) with a worldwide output of 160 million tonnes in 2013, valued at around 80 billion US dollars (FAOSTAT 2014). They are affected by up to 200 insect and arthropod pests (Lange and Bronson 1981), as well as viral and fungal pathogens. In temperate regions, such as the site of the present study, there are nine major insect pests of tomato: field crickets (several species, e.g. *Gryllus assimilis* Fabricius) tobacco and glasshouse whiteflies (*Bemisia tabaci* Gennadius and *Trialeurodes vaporariorum* Westwood, respectively), tomato mirids (*Nesidiocoris tenuis* Reuter), green stink bugs (*Chinavia hilaris* Say), tomato leafminers (*Tuta absoluta* Meyrick), tomato fruitworms (*Helicoverpa zea* Boddie), onion thrips (*Thrips tabaci* Lindeman), and spider mites (*Tetranychus* spp) (Hill 1987). Under glass (a common cultivation method in temperate regions and the method employed in this study) infestations are dominated by glasshouse whiteflies (*T. vaporariorum*) and two-spotted spider mites (*Tetranychus urticae*; Lange and Bronson (1981)). Losses to pests and pathogens can be substantial. Untreated outdoor-grown tomatoes in the state of Virginia in the U.S.A, for example, will commonly experience a 30% yield loss due to arthropod pests (Nault and Speese 2002). For this reason, a pest and pathogen management strategy is essential to any large-scale tomato growing facility.

In tomato production facilities, particularly under glass, arthropod pests may often be reduced to beneath commercial thresholds by one of a range of commercially available predatory or parasitic arthropods that attack other insects (Frank 2010). However, where biocontrol fails or is less utilised there is still reliance on chemical control for tomato production. As with most contemporary large-scale cropping systems, it is also the case that tomatoes are typically produced in monoculture, thus rendering cropped areas of limited value to wildlife and devoid of associated beneficial ‘ecosystem services’ (Malézieux 2012). Any pest control method that can reduce pesticide use and introduce greater animal and plant diversity into agricultural and horticultural systems should therefore be welcomed, particularly given the current climate of increasing pest resistance to synthetic chemicals and ever-reducing availability of active ingredients. One such method is mixed species cropping, which includes techniques such as companion planting. This method utilises one or more plant species planted alongside the focal crop to provide services to the crop, such as diverting pests or providing nutrients (Malézieux et al. 2009). If companion plant/s are edible or ornamental, this may allow the grower to profit from their cultivation. Although used relatively widely in developing countries, this cropping technique is not prevalent in modern large-scale intensive farming in

developed nations. However, as the crisis of reduced biodiversity and organismal abundance intensifies (Dirzo et al. 2014), with ever increasing losses of plant and animal species, it is envisaged that a greater effort to embrace plant diversity in agriculture and horticulture may appear. The advancement of precision agriculture may facilitate this movement in modern farming, with techniques such as precision seeding, spraying and harvesting making it far easier for farmers and growers to integrate companion planting into standard production techniques.

With the above in mind, it is critical that science identifies optimal companion plant species for use in crop production. The insistence of temperate region gardeners that planting marigolds (e.g. those in the genus *Tagetes*) next to tomatoes protects the tomato crop from whiteflies therefore merits further investigation. Although the pest control potential of companion planting *per se* has been well researched and is believed to be founded on 'associational resistance' (Barbosa et al. 2009; Malézieux et al. 2009), no research appears to be available that quantifies this potential for controlling whiteflies on tomatoes using marigolds. Nevertheless, there does appear to be some interest in the insecticidal properties of marigolds and their extracts on other insects and pests, and a limited number of studies have examined oviposition behaviour of *Bemisia tabaci* Gennadius confined to marigolds (Liu et al. 1994) and the insecticidal properties of marigold extracts on this whitefly (Baldin et al. 2013). One study indicated that *B. tabaci* occurs on okra in lower numbers with marigold intercropping (Sujayanand et al. 2016). Therefore, some evidence exists in the literature to suggest that marigolds are a scientifically valid potential companion plant for controlling whitefly on tomato crops, possibly due to their repellent volatile chemistry.

Here, large-scale glasshouse trials in the United Kingdom are described (Figure 3.1A and B), investigating the potential of intercropping tomato plants with other plant species to repel the glasshouse whitefly, *T. vaporariorum* (Figure 3.1C). 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, after a high-density whitefly population had developed. 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. 2006), and 4) whether marigolds, and the chemical limonene, may be introduced into an established tomato crop as effective whitefly control measures when pest population levels are high. To achieve these aims, the numbers of whitefly (and other insect pest) adults, larvae and eggs found on a tomato crop protected by marigolds alone, marigolds and other non-hosts, and attractive ‘pull’ host plants in addition to the ‘push’ plants, were counted, and the effect of these intercropping measures on tomato plant productivity (measured by final above-ground plant, and fruit, weight; Figure 3.1D) was quantified. An additional study investigating the effect of marigolds, and the plant volatile limonene, on a high-density population of whiteflies under glasshouse conditions is also described. 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). More specifically, multiple plant species can induce ‘restlessness’ or ‘confused’ behaviour in the whitefly, *B. tabaci* (Bernays 1999) and the current work aimed to investigate if this translates into lower rates of whitefly population development.

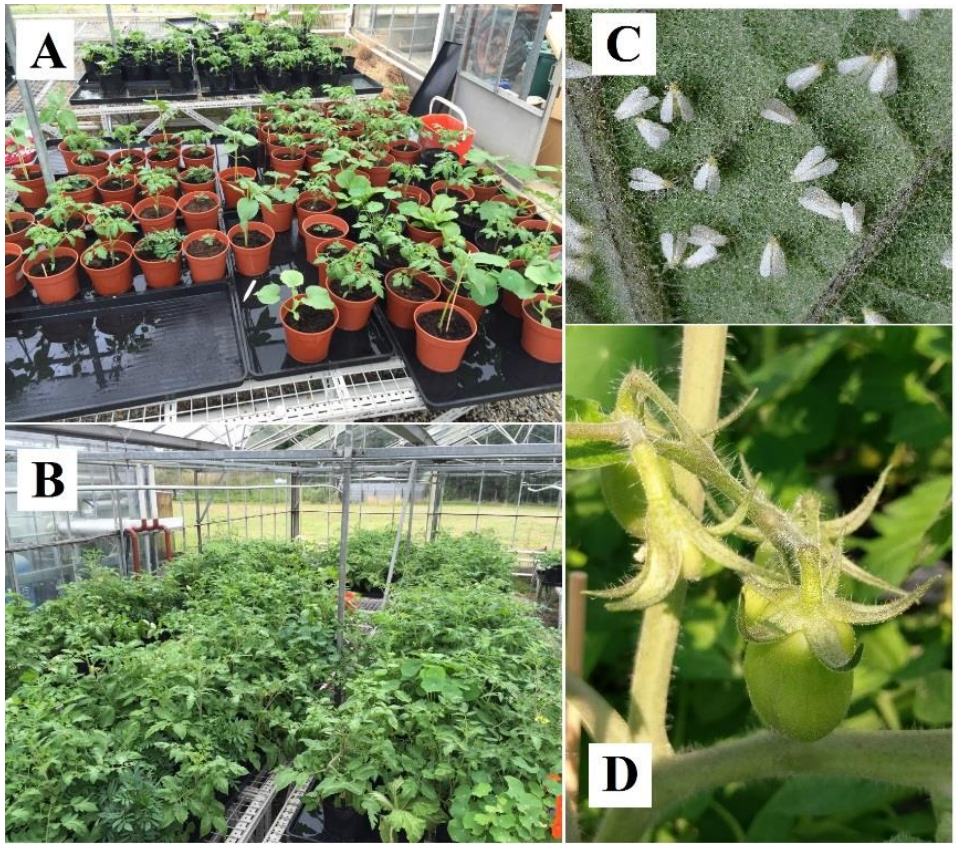



**Figure 3.1:** The experimental design in a glasshouse at Stockbridge Technology Centre (UK) in August-September 2016. **(A)** shows one replicate of the ‘push-pull’ experiment at three weeks old, with the Control, Low Diversity and High Diversity treatments shown (anti-

clockwise from the top). This design was replicated eight times. **(B)** shows the plant growth in the 'push' experiment after one month. **(C)** shows the glasshouse whitefly (*Trialeurodes vaporariorum*) on aubergine. **(D)** shows the level of fruit development at one month in the 'push' experiment.

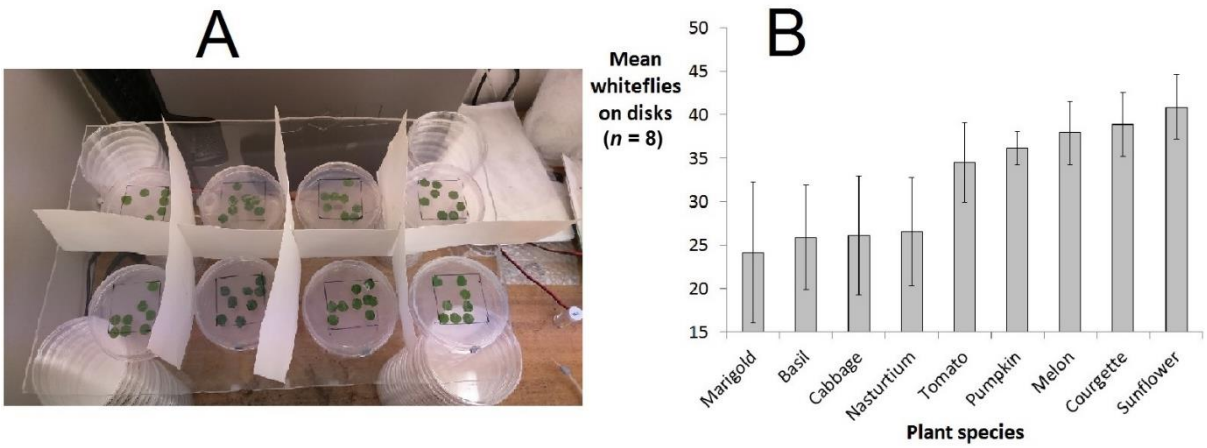
## 3.2 Methods and Materials

### *3.2.1 Laboratory assays of plant preference*

The glasshouse experiments described in the next section required sets of plants that are less and more preferred by *T. vaporariorum* than tomato. To quantify whitefly preference for these plants, a laboratory leaf disk assay (shown in Figure 3.2A) was used to examine whitefly preference in microcosm, by monitoring how readily whiteflies colonised leaf disks of the different plant species in a no-choice situation and comparing these numbers to whitefly numbers on tomato leaf disks (Figure 3.2B). Findings were compared to previous surveys of *T. vaporariorum* host range (CABI 2013; Mound and Halsey 1978; Roditakis 1990). 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, *Trapoleum 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 disk 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 at a distance of approximately 60 cm from a 400-W Son-T bulb housed in a Harrier HR400SH 400-W lamp under a 16 h light/ 8 h dark cycle and a temperature regime of 25 °C during the light period and 20 °C during the dark period, synchronised with the light regime that the leaf disk experiments were conducted under. Plants had the following number of fully expanded leaves (not including the cotyledons) when used, which approximated to stage 13 on the BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) 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.

One cm diameter leaf disks cut with a cork borer were secured in the bottom of 90 mm diameter 16 mm height petri dishes by pressing them top-side down into just-setting, 1% agar in the base of the dish. Eight disks from a single plant species were randomly arranged in a 50mm$^2$ square in the centre of the dish (Figure 3.2A). The petri dish was turned over such that the leaf disks were correctly orientated (abaxial side facing down) with the lid secured, and 50

adult whiteflies were introduced via a small hole which was then sealed. Eight replicates of this design were completed for each plant species and were completed simultaneously, with one plant supplying 16 disks for two dishes, so that four plants provided the disks for the full eight replicates. Dishes were left for 21 h at 20 $^{o}$C, 16 h light / 8 h 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 eight replicated dishes. This leaf disk assay was modified from a previous study (Frei et al. 2003). A 21 h leaf disk assay *in situ* is shown in Figure 3.2(A). Whiteflies used in the leaf disk experiments were taken from a culture of several thousand individuals maintained on aubergine (*Solanum melongena* "Moneymaker") under the same 16 h light, 8 h dark cycle and constant 20°C temperature conditions that the leaf disk assay was conducted under. This colony was originally obtained from a laboratory culture at Rothamsted Research, first collected in 1960 in Kent and originating on French bean plants (*Phaseolis vulgaris* L.).




C

| | M | B | C | N | **T** | P | Me | Co | S |
|---|---|---|---|---|---|---|---|---|---|
| | Marigold | Basil | Chinese cabbage | Nasturtium | **Tomato** | Pumpkin | Melon | Courgette | Sunflower |
| CABI, 2013 | + | - | + | - | + | + | + | + | + |
| Roditakis, 1990 | 0 | + | 0 | 0 | 0 | 0 | + | 0 | + |
| Mound and Halsey, 1978 | - | - | - | + | + | + | + | + | + |

Low — Whitefly plant preference — High

**Figure 3.2:** Photograph and results from the no-choice assay to assess whitefly preference for different plant species. The no-choice plant tissue preference assay may be seen in **(A)**, with eight disks of tomato in each dish. Fifty whiteflies were added and the mean number of whiteflies settled on plant tissue after 21 h was calculated. The mean whiteflies settled ($n$ = 8) on each plant species can be seen in **(B)**, with 95% confidence intervals 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 (2013), Roditakis (1990) and Mound and Halsey (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.

### *3.2.2 Glasshouse experiments: Push-pull assay*

Experiments were conducted in the mid-late growing season (started 10th August) 2016 in a 448 $m^3$ glasshouse in the grounds of Stockbridge Technology Centre Ltd., Yorkshire, England (Grid Ref. SE 55977 36605). Experiments were supplemented with a population of *T. vaporariorum* consisting of three heavily infested aubergine (*Solanum melongena* "Moneymaker") plants from the culture described above, distributed in the centre of the greenhouse (see Figure 3.3). The principle focus of the experiments was *T. vaporariorum* and it was anticipated that opportunistic infestation could not be relied upon to produce suitable pest numbers for useful experimental results. Plants were grown from seed, one per compartment, in standard seed germination trays in glasshouses at Stockbridge Technology Centre (UK) for five weeks (temperature: 12.6°C minimum - 36.22 °C maximum, mean 21.8 °C; Spectral flux density: 0MFU - 973.39MFU, mean 176.1MFU). At the point of replanting for experiment 1, plants were at stage 13 on the BBCH scale and had the following number of fully expanded leaves (not including the cotyledon): tomatoes 3-6 leaves, marigold 4-6 leaves, basil 3-4 leaves, nasturtium 4-5 leaves and cabbage 4-6 leaves. For experiment 2 plants were again used at BBCH scale 13, and had the following number of fully expanded leaves (not counting cotyledons): tomato plants 3-5 leaves, marigold 4-6 leaves, basil 3-4 leaves, nasturtium 4-6 leaves, cabbage 3-6 leaves, sunflower 3-5 leaves and courgette, pumpkin, and melon had 2-4 leaves. Plants were replanted into 5 litre pots and placed into 110 x 55 x 4 cm drip trays, eight pots to a tray. Plants were mostly replanted in Clover Multipurpose Compost (Dungannon, Co. Tyrone,N, Ireland BT71 4QR), though replicate four of the 'push' experiment was replanted also using Richmoor Multipurpose compost (Richmoor Seerys, Lewis Drove, Panborough, Wells, Somerset, BA5 1PT) and Bulrush Crop Specific Substrate (Bulrush Horticulture Ltd, Newferry Road, Bellaghy, Magherafelt, County Londonderry, BT45 8ND). These extra substrates were used in replicate four only, due to insufficient supply of the Clover compost. Within this replicate, the substrates were evenly distributed such that the same number of plants of each species were grown in this alternative media, so that any impacts on plant growth etc. would be evenly distributed across treatments.

Two experiments were used to investigate whether intercropping from the start of the growth period could assert control over whitefly populations (Figure 3.3 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. Experiment 1 (the 'push' experiment) comprised three treatments: a control treatment of eight tomatoes, and eight additional tomatoes (as any effects seen in other treatments must be more effective than simply providing a greater number of targets for whitefly to infest). The second treatment was a low diversity (LD) treatment of eight tomatoes mixed with eight French marigold plants. The third treatment was a high diversity (HD) treatment made up of eight tomatoes intercropped with eight non-host plants: two marigolds, two basil plants, two nasturtium plants, and two Chinese cabbage plants. Experiment 2 consisted of the same design as experiment 1, but also with eight further, attractive 'pull' plants randomly placed around the 16 plants of each treatment. For the control, eight additional tomato plants surrounded the 16 tomato plants in the centre. For the LD treatment, eight sunflower plants were placed around the tomatoes and marigolds. In the HD treatment, 'pull' plants of four species (two plants per species) were used to surround the treatments: sunflower, courgette, pumpkin and melon plants. These two experiments took place in opposing halves of a single greenhouse, with experiment 2 commencing 12 days after experiment 1. Each treatment was replicated eight times, and arranged in a randomised block design (see Figure 3.3).

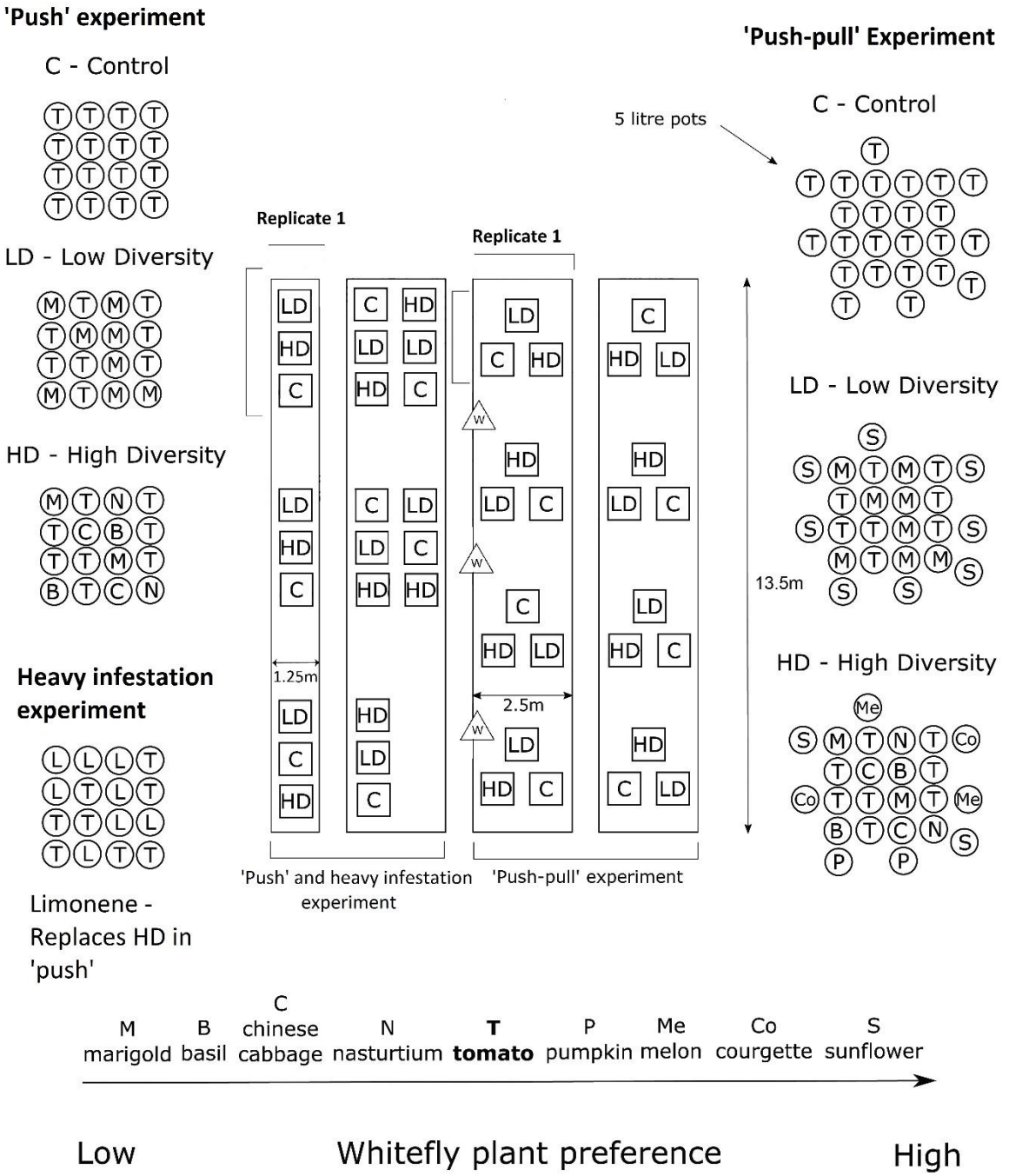



**Figure 3.3:** Layout of experiments to test the efficacy of 'push' and 'push-pull' strategies against the glasshouse whitefly on tomato, and the efficacy of intercropping at reducing large whitefly population sizes. A randomised block design was used, with each of the eight replicates containing all three 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 disk experiments 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 slow-release bottles placed in compost replacing the non-tomato plant species in the HD treatment that were used in the 'push' assay.

Sampling intervals can be seen in Figure 3.5, and sampling was identical for each experiment. For the first two sampling periods, single, fully-expanded leaves were randomly selected from four, randomly selected tomato plants per replicate of each treatment, and were examined *in situ* for *T. vaporariorum* adults and adults of other insect pests. These leaves were then removed and placed in sealed plastic bags, then stored overnight at 4 °C for examination under low power microscopy (4x magnification) 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*. Other insects were encountered in very low abundances so were not included in the analysis. These data on other pest insects can be viewed in Appendix A. After the first two observation periods it was decided to increase sampling to single leaves taken from eight tomato plants per replicate of each treatment to allow greater representative sampling of each treatment and provide a greater ability to detect changes in whitefly number. This procedure was repeated for each of the eight replicates. The mean number of pests per leaf was calculated for each replicate of each treatment, with the median pest number calculated from these means for each treatment. Procedures were identical for the 'push-pull' experiment; the outer ring of pull plants was not sampled. As pest numbers were expressed 'per leaf', the change in sampling procedure (from four to eight leaves per treatment replicate) was not considered to have affected results.

After the final insect observation period, eight tomato plants from every replicate of every treatment were destructively sampled and weight of above ground plant tissue (minus tomatoes) and tomato weight were measured. Tomato fruits had begun to form only in the 'push' experiment and were not ripe at the time of sampling, but as the plant growth season was at an end it was necessary to terminate the experiment before conditions became sub-optimal for plant growth. The mean above ground plant and fruit weight were expressed per plant for each treatment replicate, and expressed as the median for each experimental treatment.

Data were non-normally distributed so controls were compared to LD and HD using the non-parametric Mann-Whitney *U* test for the insect numbers (adult, larvae and egg numbers were added together to give a single value for each insect) and tomato plant and fruit weights. The non-parametric effect size measure, Cliff's delta (*d*) (Cliff 1993) 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).

### *3.2.3 Glasshouse experiments: Heavy infestation assay*

Upon completion of the experiment described above, additional analysis of the effect of marigolds on tomato was undertaken by other members of the lab group. In particular, the volatile output of marigold plants was ascertained (see Figure 3.4A) and the volatile chemical (R)-(+)-limonene was identified by Gas chromatography–mass spectrometry (GC-MS) and GC-FID analysis as a major constituent, comprising 25.9% of the volatile output of a single marigold flower (Conboy (2017) pers. comm.). Limonene has been identified as an important constituent of the insect repellent essential oil citronella (Maia and Moore 2011), to be released by plants upon insect damage (Fürstenberg-Hägg et al. 2013), and to repel the whitefly *B. tabaci* (Du et al. 2016). Therefore, it was hypothesised that limonene was being released by marigolds and exerting control over *T. vaporariorum* in the assays described above, and was incorporated into experiments to identify whether marigolds could control a high-density whitefly infestation.

These experiments occurred in the same greenhouse at Stockbridge Technology Centre from 6$^{th}$ June 2017 – 14$^{th}$ 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 in slow release bottles (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 25$^{th}$ April 2017. On 6$^{th}$ June 2017, when the plants had reached stage 13 on the BBCH scale, plants were repotted in the experimental glasshouse into 5 L pots containing 5 L of Clovers 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 4 cm drip trays as described above, and were subdivided into three treatment blocks, replicated eight times (see Figure 3.3). These plants had four heavily infested aubergine plants from the laboratory at Newcastle University placed amongst them to supplement naturally occurring whitefly pest populations; the aim was to achieve a heavy whitefly infestation, similar to those that may be experienced on commercial tomato crops. 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 sheeting; the aim was to keep these plants uninfested so as to not affect the whitefly population number 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 3.7A). 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 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). 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). The limonene treatment was eight tomato plants with 16 slow-release limonene bottles (described below) randomly distributed amongst them, with limonene bottles placed two per 5 L pot filled with 5 L of soil. The soil and pot design was to reduce the differences between the limonene and the plant treatments as much as possible.

The use of limonene as a treatment to control a high-density whitefly infestation required the development of a slow release bottle that mimicked the quantity of limonene released by a marigold plant. To do this, the amount of limonene released over 24 h from a single marigold of weight 47.8 g (a typical size of a marigold grown in the growth rooms at Newcastle University) was quantified using air entrainment, GC-MS and GC-FID. This was calculated as 258.58 µg and, using the mean plant weight of marigolds grown in the experimental greenhouse at Stockbridge Technology Centre (which was 151.6 g), the amount of limonene produced by a marigold plant of this weight was calculated as 820.1 µg. Slow-release bottles were then designed that would release limonene at a constant rate over the course of a week. This design involved a 30 ml medicine bottle (obtained from https://www.ampulla.co.uk/) containing 3 ml of limonene (obtained from Sigma Aldritch https://www.sigmaaldrich.com/united-kingdom.html), with a hole drilled through the plastic screw-top of the bottle, and covered in a rectangle of muslin cloth secured with two elastic bands. The quantity of limonene released from this bottle over the course of a week was measured as roughly half the 820.1 µg released by a marigold plant grown in the greenhouse. Therefore, two limonene bottles were equivalent to a single marigold plant, which was verified using GC analysis (see Figure 3.4B). Limonene in the bottles was replaced every two weeks, which was shown to give a constant level of limonene output.

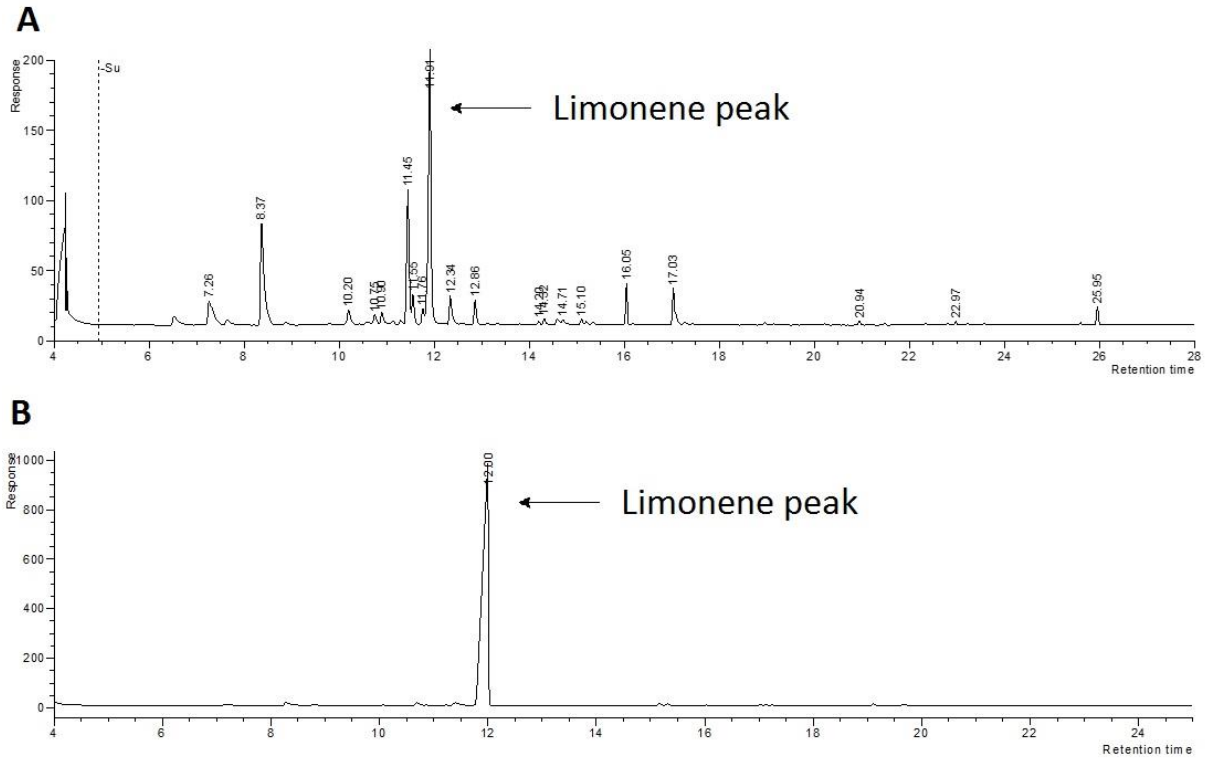



**Figure 3.4:** GC-MS analysis outputs showing the quantity of limonene released from a single French marigold and a limonene slow release bottle. **(A)** Output from a GC-MS trace showing the volatile output from a single French marigold over 24 h. The peak at a retention time of 11.91 is (R)-(+)-limonene, and forms 25.9% of the volatile output of the marigold flower over 24 h. Based on this finding, an experiment was designed to assess the ability of limonene to control a heavy whitefly infestation of a tomato crop. **(B)** Output from a GC-MS trace of the slow-release limonene bottle designed for use in the experiments to ascertain the effect of marigolds and limonene on heavy whitefly infestations. The peak shown is limonene, with no other contaminating volatiles detected from the slow release system, and shows that an amount of limonene is released from the bottle over 24 h that is equivalent to half that given off by a marigold plant grown in the glasshouse at Stockbridge. Traces used with the kind permission of Niall Conboy.

After the introduction of the treatment plants, the experiment was continued for a further 29 days, with sampling occurring in the same way as used in 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 the number of open and unopen flowers on each focal tomato plant, and the number of unripe tomatoes. As before, adult insects were assessed on the day, and eggs and nymphs counted the next day under low power microscopy. The mean number of each whitefly life stage per leaf was calculated for each treatment in every replicate, with the median whitefly number calculated from these means for each treatment, in the same way that whitefly numbers were quantified in the 'push-pull' assay. 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 above-ground 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. Differences amongst the treatments for these metrics of whitefly and plant performance were analysed using Mann-Whitney U tests, as above, with the exception of the final plant and fruit weight, which were compared using *t*-tests as the normal distribution of the data and homogeneity of the variances allowed the use of parametric statistics.

## 3.3 Results and Discussion

This large-scale glasshouse study aimed to assert a scientific basis for the propagation of French marigolds *(Tagetes patula* L.) amongst tomato plants 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 further assay also sought to quantify whether this 'push' effect could be combined with the 'pull' of attractive host plants to increase whitefly control. Finally, the potential for marigold plants, and the plant volatile chemical limonene, to be introduced to tomato crops to control a heavy whitefly infestation was assessed.

### *3.3.1 Laboratory assay of T. vaporariorum plant preference*

In order to establish a baseline measure of whitefly preference for different plant species, a laboratory assay was conducted using plant leaf disks in a no choice assay that quantified whitefly preference for each species individually. *T. vaporariorum* preference for a range of plant species was compared to the preference for tomato, with this pest found to have a similar or slightly greater preference for the plant tissue of pumpkin, melon, courgette, and sunflower relative to tomato (Figure 3.2B). There was a clear discontinuity in whitefly preference for tomato plants and the remaining plants (nasturtium, Chinese cabbage, basil, and marigold), which were all less preferred than tomato. Previous surveys of *T. vaporariorum* hosts (CABI 2013; Mound and Halsey 1978; Roditakis 1990) are generally in agreement with these findings: all the plants that were found to be equally or more preferred than tomato are consistently listed as hosts in these surveys. The plants that were found to be less preferred in the leaf disk assay are less consistent in their designation, sometimes being listed as hosts and sometimes listed as non-hosts (Figure 3.2B). On the basis of the leaf disk results nasturtium, Chinese cabbage, basil, and marigold were designated as 'push' plants and pumpkin, melon, courgette, and sunflower as 'pull' plants.

### *3.3.2 Glasshouse trials of 'push' and 'push-pull' control strategies*

Figure 3.5 shows the level of whitefly infestation on the tomato crop through time. In the 'push' experiment (Figure 3.5A) whitefly numbers on the control and the LD and HD treatments began to diverge after around 35 days with significantly fewer whiteflies on LD and HD relative to the control. This divergence was maintained until the final observation at around 50 days. There was no significant difference between whitefly numbers on tomato in the LD and HD treatments indicating 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 3.5B), whitefly numbers on tomato were fewer on LD and HD treatments relative to the control after around 20 days. Weakly significant values at or around the classic $\alpha = 0.05$ 'significance' level were obtained over time. The fall in control numbers on the 'push-pull' experiment in the final observation should be viewed with caution as symptoms of late season blight, *Phytophthora infestans*, began to appear on tomatoes, particularly in some replicates of the 'push-pull' experiment, and therefore affected tomato suitability as a whitefly host. It is also important to note that relatively low levels of whitefly numbers were present in the greenhouse. However, as all treatments experienced this low infestation level, comparisons between treatments are valid. This assay most closely simulates early season whitefly infestation, at population levels that would be experienced in practice were companion plants to be adopted into commercial production.

Figure 3.5(C) summarises the comparison between the control and the LD and HD treatments in experiments 1 and 2, and shows the data expressed as the non-parametric Cliff's delta effect size measure. This gives a standardised measure of overlap between experimental group and control, which is useful as absolute numbers are not directly comparable between the two experiments because they were started at different times and sited in different sections of the glasshouse. This analysis 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 appeared to be little 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 3.5(C) should be viewed with caution as experiments were terminated at the end of the commercial growing season and effects may have diverged further if planting had begun earlier.

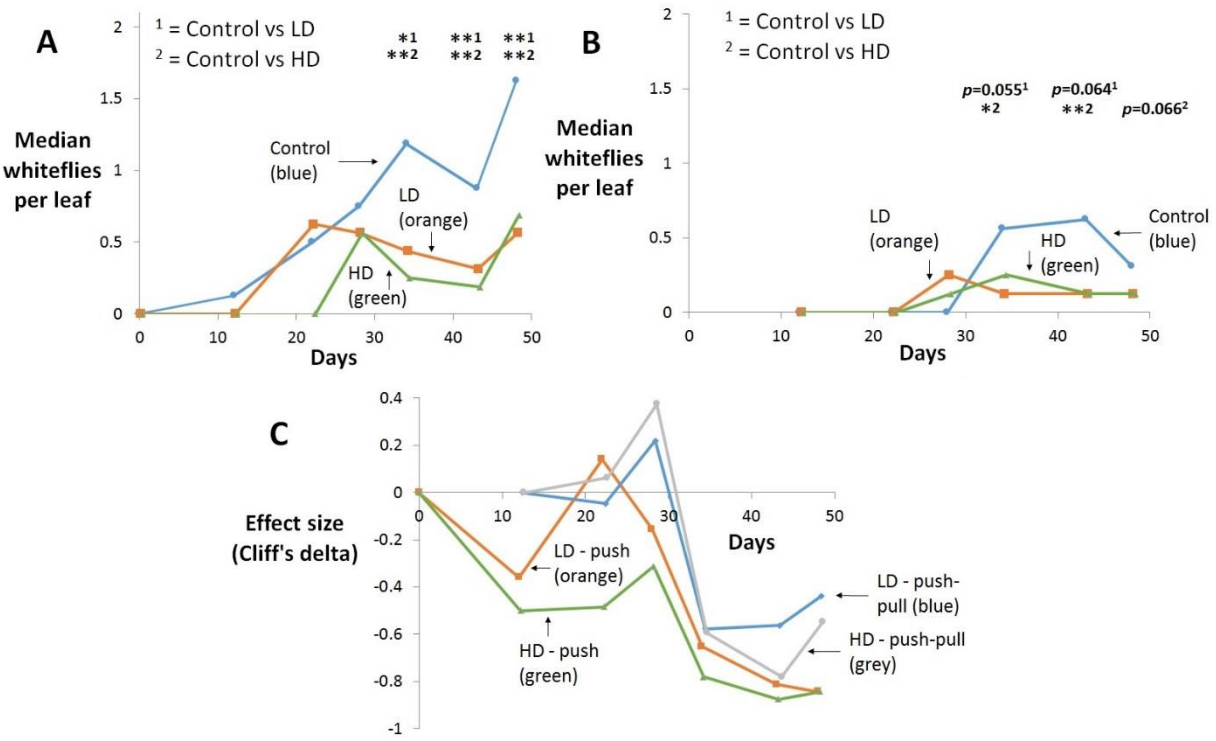



**Figure 3.5:** Population development of the whitefly, *T. vaporariorum* on tomato in the glasshouse, with all whitefly life stages (adult, scale and egg) contributing to the median number of whitefly/leaf (*n*=8). Day 0 is 10th August 2016 and the 'push-pull' was started 12 days after the 'push' experiment. The Mann Whitney *U* test was used to compare the control to both low and high diversity treatments for each sampling point. '*' indicates a *p* value of <0.05, '**' indicates a *p* value of ≤ 0.01, with those of marginal significance shown as actual probability values on the graphs and all other *p* values non-significant. Ninety five percent confidence intervals have been calculated and are available by contacting the authors, but to aid visualisation they have been removed from the figure. **(A)** shows the 'push' experiment in which repellent plants such as marigold (LD) and marigold and other non-hosts (HD) are intercropped amongst among tomato plants. The *p* values for the control vs LD for the sampling points with significant differences in the number of whiteflies (days 34, 43 and 48) were $p = 0.031$, 0.007 and 0.0049, respectively ($w = 89$, 94 and 95 respectively, df = 14 for all). For control vs HD, the *p* values for days 34, 43 and 48 were $p = 0.01$, 0.0037 and 0.0053, respectively ($w = 93$, 96, and 96, respectively, df = 14 for all). **(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. The *p* values for the control vs LD were mostly non-significant, or near significance (shown as actual probability values on the graph). For control vs HD, the *p* values for the sampling points significantly differing in the number of whitefly per leaf (days 34 and 43) were $p = 0.049$ and 0.0092, respectively ($w = 87$ and 93, respectively, df = 14 for both). **(C)** shows the data in (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.

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 cultures more (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, *T. tabaci*. Other pests were found on the trial, but in much lower number so they were not included in the analysis, and these can be viewed in Appendix A. Figure 3.6 shows that the treatments did not attract significantly more thrips to tomato relative to the control. The lower absolute numbers of thrips on the 'push-pull' experiment does not necessarily indicate a protective effect, however, as the two experiments were started at different times and occurred in different areas of the glasshouse. Plants were selected for the experiment specifically with glasshouse whiteflies in mind and no predictions were made that they would also repel other pests; therefore the lack of any beneficial effect on other pest insects is encouraging for the future development of this method.

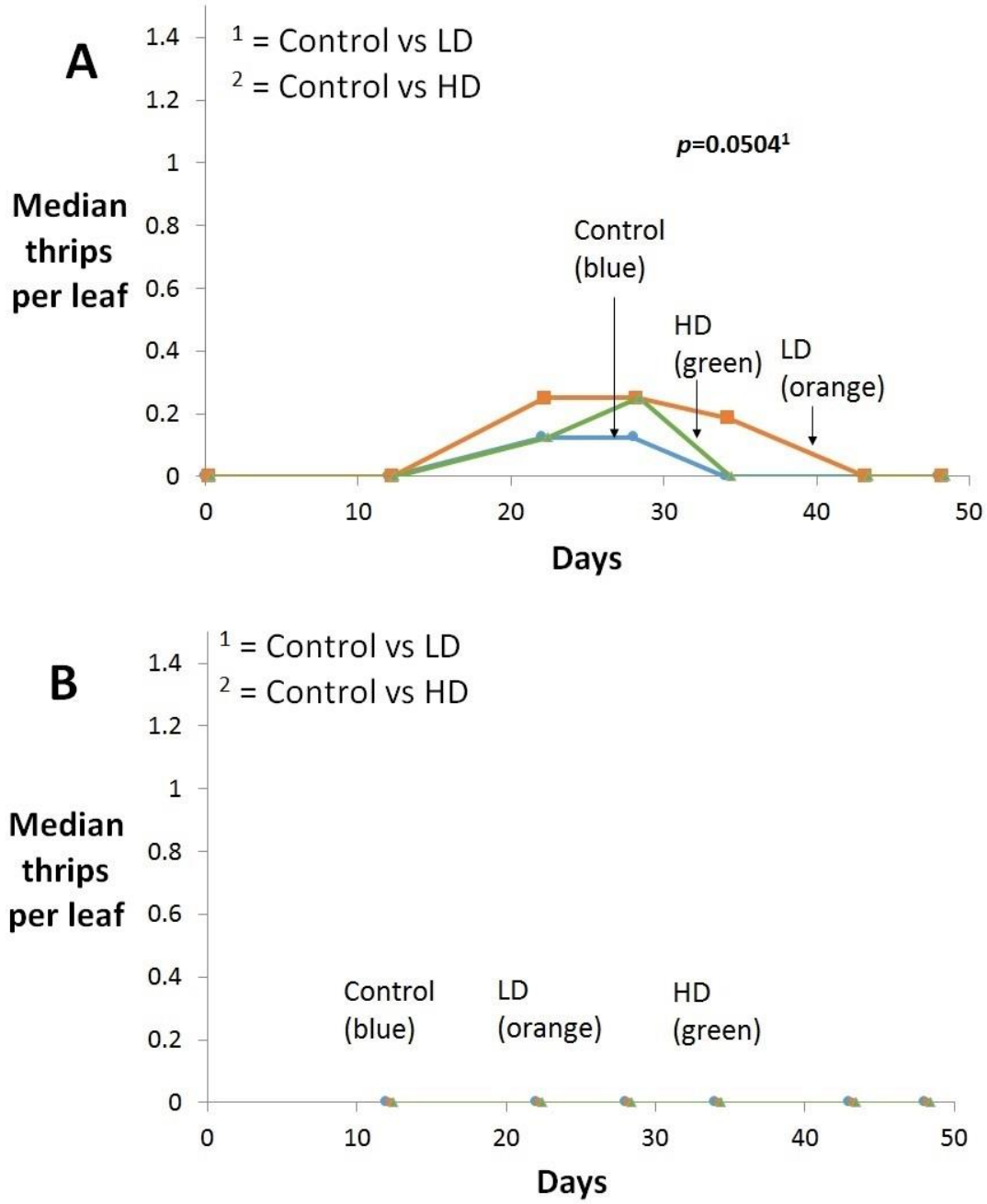



**Figure 3.6:** Population development of thrips, *T. tabaci* on tomato in the glasshouse, with all thrips life stages (adult and larvae) contributing to the number of thrips/leaf. The Mann Whitney *U* test was used to compare the control to both low and high diversity treatments for each sampling point. All *p* values are non-significant, with those of marginal significance shown as actual probability values on the graphs. Ninety five percent confidence intervals have been calculated and are available by contacting the authors, but to aid visualisation they have been removed from the figure. **(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. Whilst all medians are 0 in (B), not all replicates were 0 and most data points have highly skewed CI's for the median.

Neither marigold companion planting (LD) nor companion planting with marigold and a variety of other plants (HD) had a significant impact on tomato plant productivity in the 'push' experiment, when compared with the Mann Whitney *U* test. The total above ground plant and tomato weight figures calculated per plant did not differ significantly between Control and LD or Control and HD treatment for either fruit weight (LD: $p = 0.8335$, $df = 14$, $W = 70.5$; HD: $p = 0.4948$, $df = 14$, $W = 61.0$) or above ground plant weight (LD: $p = 0.4309$, $df = 14$, $W = 60.0$; HD: $p = 0.9581$, $df = 14$, $W = 67.0$). Results were similar in the 'push-pull' experiment, where plants were sampled after 35 days of growth and above ground plant weight did not differ significantly between the control and the LD or HD 'push-pull' treatments (LD: $p = 0.4005$, $df = 14$, $W = 59.5$; HD: $p = 0.8748$, $df = 14$, $W = 66.0$). As fruit had not yet appeared, marketable yield could not be sampled for this trial.

### *3.3.3 Potential mechanistic basis of push-pull pest control*

'Associational resistance' provided to pest host plants by nearby non-hosts is relatively well established as a concept (Barbosa et al. 2009), though multiple hypotheses exist to explain how associational resistance functions. The Natural Enemies Hypothesis (Root 1973) suggests that increased plant diversity encourages greater numbers of pest predators and parasitoids, though as the current study was conducted under glasshouse conditions without natural enemy release and no predators or parasitoids were observed on or around plants, this is unlikely to explain the results seen. Alternatively, non-host plants may 'protect' host plants according to the Resource Concentration Hypothesis (Root 1973), which itself could be explained by a range of pest responses to increased plant diversity, or to positioning of certain 'companion' plants in the vicinity of pest host plants. For example, increased plant diversity may deter pests from crops because the constant contact sampling of non-hosts by pests as they move around the plant mixture disrupts the normal processes of plant acceptance (Finch and Collier 2000). It is assumed that the 'appropriate/inappropriate landings' (Finch and Collier 2000) mechanism, or some component of it, is in operation here, through negative signals provided to adult whitefly on contact with marigolds or other non-host plant leaves, or through volatile signals that repel whitefly before landing. The observation that volatiles released from marigold essential oils repel the whitefly *B. tabaci* and several species of mosquito emphasises the potential role of non-host volatiles in the effects demonstrated in this work (Baldin et al. 2013; Gillij et al. 2008). However, pots containing plants were placed in large communal drip trays, so the washings of marigold-containing pots will have drained into these trays and may potentially have been taken up by pots containing tomato plants. It is known that root exudates can impact the biology of neighbouring plants (Bais et al. 2003) and

potentially their suitability for certain pests (Dahlin and Ninkovic 2013), but studies to investigate this possibility are scarce. Some plant species are also known to adsorb leaf volatiles from neighbouring non-hosts and re-release them to a sufficient degree that they become disagreeable to their insect pests (Himanen et al. 2010), while others will adsorb, modify, and re-release leaf volatiles to produce new defensive compounds (Sugimoto et al. 2016). Certain non-host plants are also known to produce volatile chemistry that may confuse/repel pest insects searching for hosts in their vicinity, and the possibility that non-hosts operate by volatile repellence should not be discounted here, particularly for 'aromatic' species such as marigold (Baldin et al. 2013; Gillij et al. 2008).

### *3.3.4 Glasshouse trial of intercropping to control high density whitefly populations*

Having identified that marigolds may be used to deter whitefly from a tomato crop when grown concurrently with tomatoes from seedlings, it was of interest to discover whether introducing mature marigold plants, which had not been grown alongside tomato plants, was effective against a heavy whitefly infestation of a tomato crop. Heavy infestations are likely to occur if no protective measures are deployed against whiteflies in tomato growing facilities and home gardens. If found to be capable of reducing the impact of a heavy whitefly infestation, then marigolds could be used instead of a chemical pesticide application, reducing the environmental impact of synthetic chemical whitefly control. A further aim was to attempt to identify the mode of action of marigold intercropping to control whitefly populations, whether based on volatile production or root exudates. Studies completed by other lab members had identified the volatile chemical limonene to be a considerable constituent of marigold flower volatile output, comprising 25.9% of floral volatiles from a single flower (Conboy (2017), pers. comm.). It was therefore hypothesised that if volatile chemistry was important in the control of whitefly by the use of intercropped marigolds, limonene could be a key volatile driving this effect. Limonene is an important component of the essential oil citronella (Maia and Moore 2011) and has been shown to be released by plants in response to herbivore damage (Fürstenberg-Hägg et al. 2013; Pare and Tumlinson 1997), to repel insects such as the twig beetle *Pityophthorus pubescens* (López et al. 2013), and the tobacco whitefly, *B. tabaci*, with this study showing limonene to be effective on its own or as part of a mixture of other volatiles (Du et al. 2016). Limonene has also been shown to be toxic to insects including mealy bugs, the Spiraling whitefly, *Aleurodicus disperses*, and *A. antidesmae*, in which it caused 99% mortality (Hollingsworth 2005). Therefore, limonene was included in the present study to identify any effect on *T. vaporariorum*.

Other studies investigating the use of plant volatiles to control insect pests have utilised slow release systems to ensure constant release of plant volatiles at similar rates and concentrations to those achieved by plants e.g. (Du et al. 2016). It was decided that a similar system should be used in the present study. To this end, a slow release system for limonene was developed in the Tosh lab to mimic the quantity of limonene released over a week by a marigold plant that had been grown in the glasshouse at Stockbridge. This system was deployed as a treatment in a glasshouse experiment similar to the 'push-pull' assay described above, in the same glasshouse the following year. This experiment comprised tomato plants intercropped with other tomato plants (control), marigolds (marigold treatment) and slow-release limonene bottles (limonene treatment) (see Figure 3.3 for experimental layout). The effect of these treatments on whitefly adult, egg and nymph numbers may be seen in Figure 3.7. Seven days after the treatments were applied to the whitefly infested crop, a near-significant effect on adult settling was observed on both marigold and limonene treatments, of $p = 0.092$ and 0.082 respectively. Whilst not a significant difference, this is suggestive of a repellent effect on adult whiteflies immediately after the deployment of both marigolds and limonene, although this effect disappears in later time points for the marigold treatment. The limonene treatment, however, displays another near-significant difference 22 days after the slow release bottles were deployed ($p = 0.170$), and whitefly numbers were lower throughout the course of the experiment on this treatment, which suggests that any protective effect provided by limonene is longer lasting than that provided by marigold plants. Whilst the work of Du et al. (2016) suggest that slow-release bottles become ineffective after 29 days, the shape of the curve for the limonene treatment from the present study remains flat, whilst that of the control appears to be undergoing a near exponential increase, so had the experiment been run for a longer time period, significant differences between these treatments may have appeared. Further studies to test the longevity of the effect of limonene need to be undertaken. Figure 3.7 (B) shows that no significant effect was exerted by either marigolds or limonene on whitefly egg numbers, meaning that adults were not deterred from laying eggs on the intercropped tomatoes. However, Figure 3.7 (C) shows that after 14 days a near-significant reduction in nymph numbers was achieved by the limonene treatment when compared to the control ($p = 0.093$), and that this effect became significant at both 22 and 29 days ($p = 0.040$ and 0.004, respectively). By contrast, the marigold treatment achieved a near-significant reduction in nymphs at 22 days ($p = 0.103$), but this effect disappeared at 29 days, and in fact there were significantly fewer nymphs on the limonene treatment than on the marigold treatment after 29 days ($p = 0.009$).

Taken altogether, despite the low number of significant differences between the treatments for the various whitefly life stages, these results are suggestive of a mildly repellent effect on adult whiteflies of both marigolds and limonene (with limonene having the slightly stronger effect), and strong effects on whitefly nymphs, with the limonene treatment being much stronger than the marigold treatment. The fact that no effect was observed on whitefly egg numbers between treatments was interesting, as if adults are repelled from a plant it would be expected that they would not lay eggs on that host. This finding agrees with that of Du et al. (2016), who also found limonene to repel adult *B. tabaci* whiteflies from tomatoes, but not to affect oviposition. However, Du et al. (2016) detected no significant mortality on other whitefly life stages, whilst the present study identified significantly fewer nymphs on tomato plants. Experimental differences and the different whitefly species used may account for these differences.

The disparity observed in the results, of slight adult repellence, no effect on egg lay, but a significant effect on nymph mortality from 14 days onwards in the limonene treatment, and a lesser effect in the marigold 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 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 nymphs to move away from this chemical could make them more susceptible to any toxic effects (Hollingsworth 2005). However, in the above study, toxicity was not life stage specific, but achieved an equally significant effect on egg numbers. This pattern of effects could also be evidence of defence activation in the treated plants, where whiteflies are able to settle and lay eggs, but are subsequently repelled by activated defences. Evidence exists of oviposition acting as a signal to induce defences in plants, in a manner that is distinct from the perception of adults: *Tuta absoluta* oviposition on tomato elicits a distinct bouquet of volatiles to that elicited by adults, indicating a different signalling process occurring in the plant in response to oviposition (Anastasaki et al. 2015). A similar mechanism may be in operation here: limonene may be acting as a priming agent for tomato defences, which become rapidly and strongly activated upon whitefly oviposition, resulting in significant toxicity in the nymph whitefly stage, or of reduced hatching rates in whitefly eggs. The outcome of any of these scenarios would have long lasting implications on whitefly populations on crops treated with limonene, as adult numbers would decline due to the inability of the whiteflies to effectively

reproduce. Further studies would be necessary to elucidate the exact mode of action on different whitefly life stages.

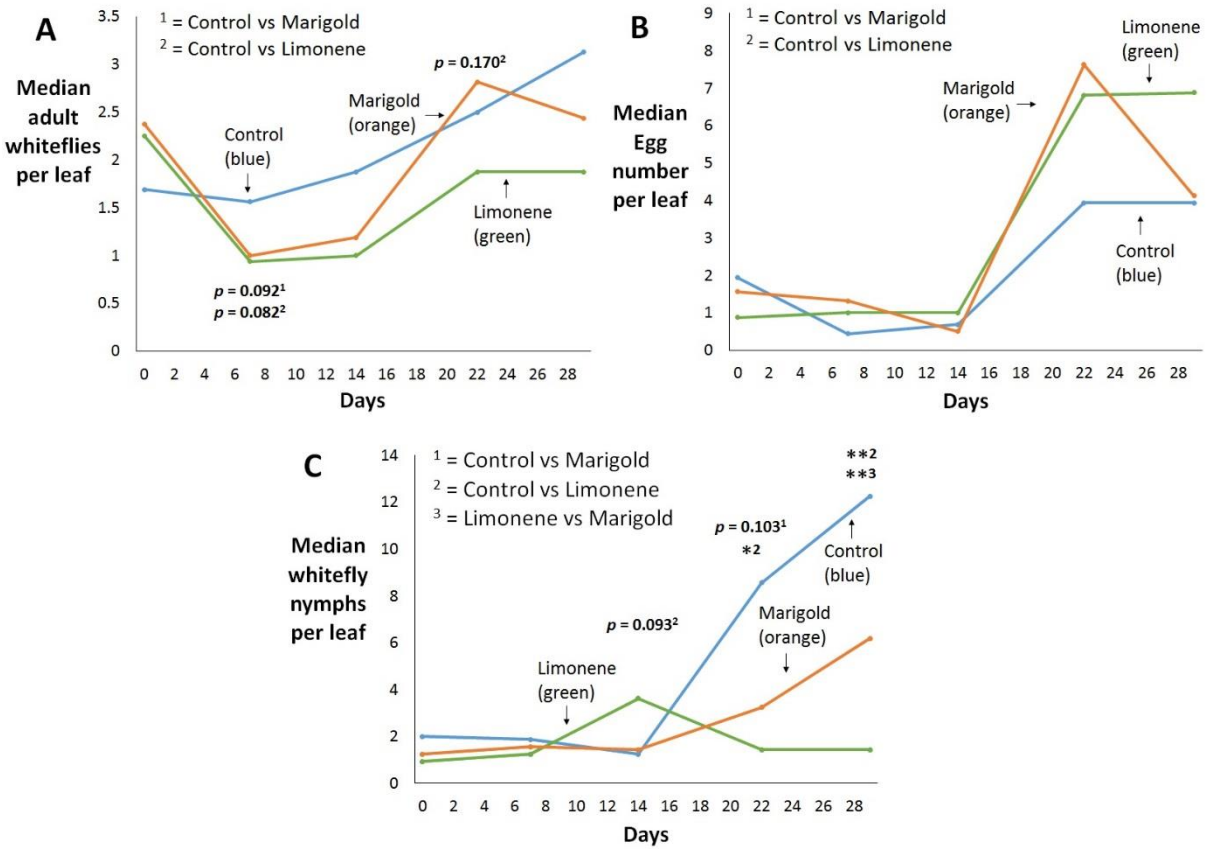



**Figure 3.7:** Whitefly population development in the 2017 assay into the efficacy of marigolds and limonene as an emergency treatment to control established whitefly population numbers ($n$ = 8). Treatments comprised intercropping eight heavily whitefly-infested tomato plants with eight further tomato plants (control), eight French marigold plants (marigold treatment), or eight pots each containing two limonene slow release bottles (limonene treatment). The first data point in **(A-C)** represents the number of whitefly settled on the focal tomato plants immediately before treatment plants were introduced. '*' indicates a $p$ value of <0.05, '**' indicates a $p$ value of ≤ 0.01, with those of marginal significance shown as actual probability values on the graphs, and all other $p$ values non-significant. **(A)** The median number of adult whitefly settled on each focal tomato plant in the three treatments over the course of the experiment ($n$ = 8). Whitefly numbers decreased near-significantly according to Mann Whitney $U$ tests between the control, and marigold ($p$ = 0.092, $W$ = 84.5, $df$ = 14) and limonene treatments ($p$ = 0.082, $W$ = 85, $df$ = 14) respectively after 7 days, and again between control and limonene after 22 days ($p$ = 0.170, $W$ = 81.5, $df$ = 14). **(B)** The median number of eggs laid on each focal tomato plant over the course of the experiment ($n$ = 8). No significant differences were observed between treatments in egg numbers at any point over the experiment. **(C)** The median number of whitefly nymphs of all stages counted on each focal tomato plant over the experiment ($n$ = 8). Nymph numbers were near-significantly lower on the marigold treatment compared to the control according to Mann Whitney $U$ tests after 22 days ($p$ = 0.103, $W$ = 84, $df$ = 14). Nymph numbers were near-significantly lower on the limonene treatment when compared to the control after 14 days according to Mann Whitney $U$ tests ($p$ = 0.093, $W$ = 51.5, $df$ = 14) and significantly lower after 22 ($p$ = 0.040, $W$ = 88, $df$ = 14) and 29 days ($p$ = 0.004, $W$ = 96, $df$ = 14), respectively. Nymph numbers were also

significantly lower on the limonene treatment when compared to the marigold treatment using Mann Whitney *U* tests after 29 days ($p = 0.009$, $W = 42.5$, $df = 14$).

Other insect pests were not detected in any great numbers on the crop, with *T. tabaci* being encountered in much lower numbers than on the 'push-pull' assay. Two-spotted spider mites, *Tetranychus urticae*, were observed in larger numbers on this study compared to the 'push-pull' assay, but still at very low rates (adults and eggs combined appeared at a maximum of 0.5 mites per leaf, and a median of 0.063 mites per leaf from all treatments in the study) and no significant differences between the controls and the other treatments for any time points were observed. Additional pest data are not presented as *T. urticae* and *T. tabaci* were the only other insects besides whiteflies to be detected on this experiment. It is encouraging for the future development of this technique as a pest control agent that non-target pests such as thrips and spider mites are not attracted to the tomato crop as a result of intercropping. Evidence has been presented that limonene from potato plants is neutral with regards *T. tabaci* attraction to different potato cultivars (Wilson et al. 2017). Whilst this is a different plant species, with differences in how the insect and plant will interact compared to the present study, it provides evidence of a neutral effect of this volatile in a closely related plant species, which is promising for the future use of limonene in an intercropping system, where limonene may be utilised as an emergency measure.

Figure 3.8 shows the effect of the treatments on tomato fruit production over the course of the experiment, and the above-ground plant tissue weight (excluding tomatoes), and the total unripe tomato weight, per plant at the conclusion of the study, when plants were destructively sampled. Figure 3.8 (A) shows that tomato plant vegetative tissue in the marigold treatment was near-significantly lighter per plant than in the control ($p = 0.085$, $t = -1.86$, $df = 14$), and in the limonene treatment was significantly heavier per plant than both the control ($p = 0.05$, $t = 2.15$, $df = 14$) and the marigold treatment (($p = 0.005$, $t = -3.3$, df = 14). 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. Whilst there were no significant differences between the limonene treatment and the control in terms of total fruit weight per plant, there was a near-significantly greater number of tomato fruits produced on the limonene-treated tomato plants compared to the control ($p = 0.115$, $W = 52.5$, $df = 14$) according to a Mann Whitney U test. By contrast, marigold treated tomatoes experienced a near-significant reduction in fruit weight per plant compared to the control ($p = 0.100$, $t = -1.76$, $df = 14$) and a significantly lighter fruit weight than the limonene treatment ($p = 0.014$, $t = -2.8$, $df = 14$). 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 slow release bottles 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 the controls persists in future studies that control for planting densities.

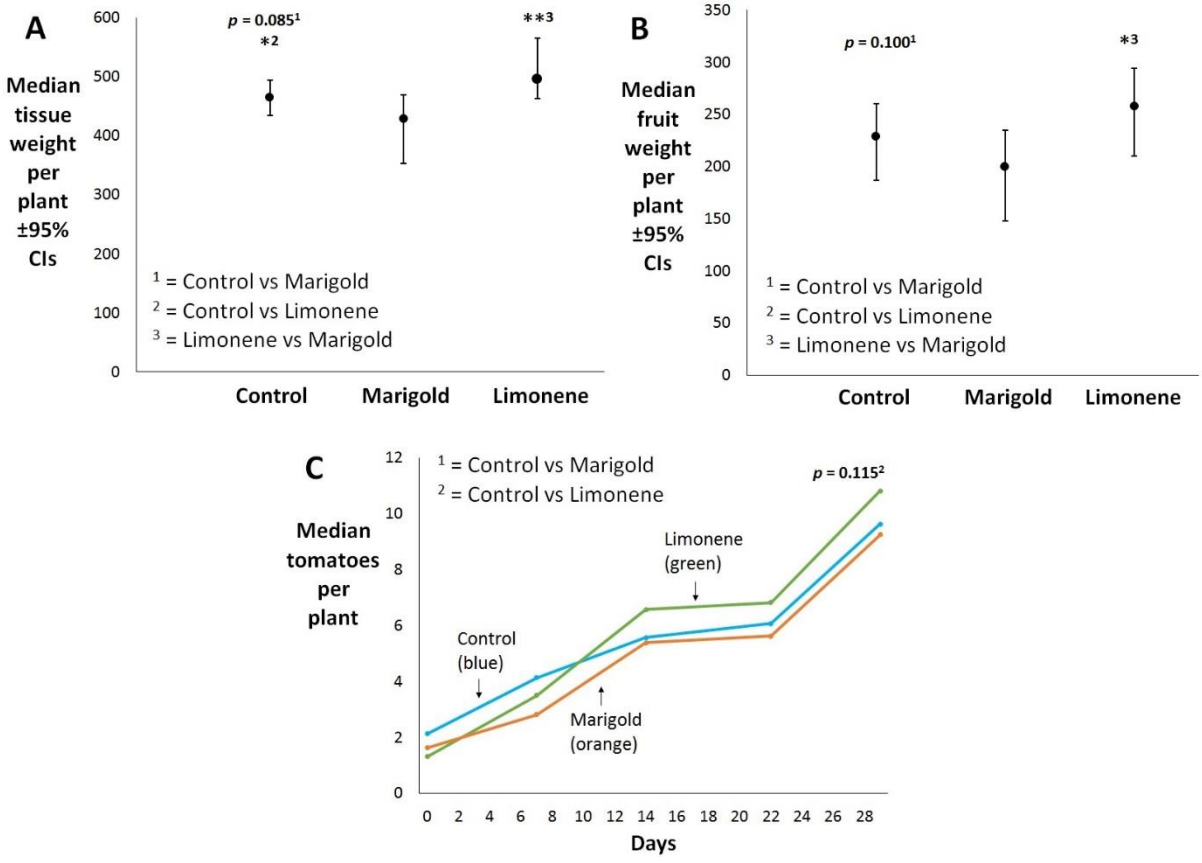



**Figure 3.8:** Plant development characteristics at the end of the 2017 assay into the efficacy of marigolds and limonene as an emergency treatment for the control of established whitefly populations ($n$ = 8). Treatments comprised intercropping eight heavily whitefly-infested tomato plants with eight further tomato plants (control), eight French marigold plants (marigold treatment), or eight pots each containing two limonene slow release bottles (limonene treatment). The first data point in **(C)** represents the number of tomatoes on the focal tomato plants immediately before treatment plants were introduced. '*' indicates a $p$ value of <0.05, '**' indicates a $p$ value of ≤ 0.01, with those of marginal significance shown

as actual probability values on the graphs and all other *p* values non-significant. **(A)** The median above-ground tissue weight of each focal tomato plant, excluding tomatoes, at the end of the 29 day experiment ($n = 8$). **(B)** The median weight of all tomatoes from each focal tomato plant at the end of the 29 day experiment ($n = 8$). **(C)** The median number of tomatoes produced by focal tomato plants in each treatment over the course of the experiment ($n = 8$).

From these results it would appear that marigolds have a limited influence on whitefly populations when introduced as an emergency control measure to reduce the impact of a heavy whitefly infestation. Marigolds caused reductions in adult settling that approached significance seven days after the introduction of the adult plants, and caused near-significant reductions in nymph numbers after 22 days. However, vegetative tissue and fruit weights were also near-significantly lower in plants grown alongside marigolds for 29 days, though this was possibly due to the growth habit of the marigolds limiting access to sunlight, and could be avoided with more careful spacing of the tomato plants. By contrast, application of limonene slow-release bottles achieved a greater control over whitefly populations, giving near-significant reductions in adult settling seven and 22 days after application (which is predicted to continue to be effective over longer time periods based on the shape of the whitefly population development curves on the limonene treatment) and achieving significant reductions in nymph survival, despite the same number of eggs being laid on limonene-treated plants as the control tomatoes. This volatile based system involving limonene could be developed to be a highly effective control agent of whitefly. The efficacy of limonene at reducing whitefly performance when applied to a tomato crop is suggestive that marigold-induced whitefly control in the 'push-pull' study is volatile based, and could involve limonene. However, from the results of these experiments, it is unclear whether limonene is the sole basis of the whitefly control observed in the 'push-pull' assay. As limonene was used at the same concentration and in the same quantity as would be released from a marigold plant grown at Stockbridge, if limonene was the cause of reduced whitefly performance in the 'push-pull' assay, or in the marigold treatment of the heavy infestation assay, it would be expected that any impact observed on whitefly would be the same in both the limonene and marigold treatments. However, limonene proved more effective than marigolds at controlling whitefly in the heavy infestation assay, and this non-equivalence of effects makes it unclear whether limonene is the main source of whitefly control from the 'push-pull' assay. It may be that other volatiles produced by marigolds diluted the effect of marigold-derived limonene, explaining these differences. Also, the issues with planting density described above may have caused reduced plant health in the marigold treatment, cancelling out any positive effects of reduced whitefly performance. Such planting density issues should be avoidable with refinements of this technique for use in commercial glasshouses, which would allow for

adequate spacing between plants. Further studies are necessary to better understand whether limonene is the main component of marigold-induced whitefly control in tomato crops, or whether other factors, such as other plant volatiles, are involved.

### *3.3.5 Domestic and commercial application of intercropping*

Results from the experiments performed here may be most applicable to domestic gardeners growing tomatoes in small glasshouses or outdoors, and potentially to larger outdoor tomato production facilities, where tomato plants do not grow to a great height and marigolds and other non-hosts will be in close proximity to the crop across most of the tomato plants' height. Many large-scale glasshouse growers use hydroponic systems where roots and plants can be raised off the ground and plants grow to head heights of up to 10ft. In these systems it may be necessary to conduct further studies to adapt the methods used here in order to make them amenable for commercial use. However, with the suggestion that plant volatiles from marigold plants are playing a role in reducing whitefly numbers on tomato, then results could be directly applicable, for example by extracting/synthesizing marigold volatiles for deployment in irrigation/ventilation of these systems. Similarly, identification of any repellent/deterrent volatile chemistry from the companion plants could also form the basis of an extracted or synthesized commercial plant protection product. Limonene has shown significant negative impacts on whitefly performance at high whitefly population densities, and has the potential to be applied as a spray to reduce a heavy whitefly infestation to below acceptable thresholds. Gardeners or growers who might consider applying these results to their own gardens, plots, or glasshouses should ensure that tomatoes and marigolds are planted in close proximity, as volatile-based control has shown to occur over short distances only (Frost et al. 2008b), which for the effect of limonene on whiteflies has been shown to be 0.8–1.2 m (Du et al. 2016). As is mentioned above, whitefly infestation levels in the 'push-pull' experiment were low and the experiment most closely simulated early season colonisation population growth by whiteflies. It may be argued that if early growth can be depressed then populations on tomato are unlikely to become heavily infested. From the results presented here for the heavy infestation assay, it would appear that marigolds are more effectively used as a whitefly deterrent mechanism deployed when tomato plants are first introduced, rather than being used as an emergency whitefly control tool when whitefly numbers become numerous. Near-significant impacts on whitefly adult settling and nymph numbers were observed, so there may be some benefit to introducing marigolds at a late stage of whitefly infestation, but this contrasts with significant reductions in whitefly performance when marigolds were intercropped amongst tomatoes from the start of plant growth. It can

therefore be recommended that growers looking to utilise the protective effects of marigolds should deploy them at the start of the growing season to assert the most effective control over whitefly populations. Limonene shows promise as an emergency measure to assert control over heavy whitefly infestations, and the slow-release bottles described herein appear to be a valid delivery method of this volatile chemical.

### *3.3.6 Conclusions and Future Work*

The efficacy of a method popular amongst domestic gardeners, of intercropping tomato with French marigolds, 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. Introducing marigolds as a replacement for chemical control methods produced weaker effects, although some measure of control was achieved. More effective at reducing whitefly performance was the use of limonene in slow release bottles, and this method warrants further experiments as to the mode of action of this volatile, as well as further optimisation of its deployment, for example whether it can be used at the start of a growth season to deter whitefly populations. Increasing plant diversity further in the 'push-pull' assay did not result in enhanced pest control, but achieved similar levels to marigolds alone. Neither thrips nor spider mites were attracted significantly more to the treated crops in either experiment described here. In one respect this is positive and strongly suggests a future direction for this research, as it appears that other 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 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 repel the focal pest, but also not attract other pests that the mix overall aims to repel (introducing a plant species that repels one pest but is attractive to another risks reducing the effectiveness of the technique). 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 (Kremen and Miles 2012), 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.

## 3.4 Acknowledgements

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# Chapter 4. The Temporal Dynamics of VOC-Induced Tomato Defence Priming

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**Abstract**

The role of volatile organic compounds (VOCs) in plant-plant communication has been the subject of much recent research. Differing bouquets of VOCs are emitted by plants when damaged or infested with insect pests. These may be detected by uninfested plants that can respond by activating or priming defences, in preparation for an imminent infestation. This work seeks to assess the presence and efficacy of VOC-based communication between tomato plants (*Solanum lycopersicum*) as a method of controlling the glasshouse whitefly (*Trialeurodes vaporariorum*), a highly damaging crop pest. This study sought to characterise: 1) the ability of non-infested tomato plants to use VOCs from whitefly-infested neighbours to increase their whitefly resistance levels, 2) whether an optimal combination exists of whitefly infestation time to produce defence-inducing VOCs, and time of exposure to VOCs, to maximally increase tomato resistance against whitefly, and 3) the mechanism of any VOC-induced resistance. To achieve this, 24 combinations of different infestation and exposure times were tested, with neighbouring tomatoes assessed for whitefly resistance by monitoring whitefly settling preference and oviposition on these plants. Results indicate the presence of VOC-based plant-plant communication between tomato plants, with the most promising combination (one day infestation time and six days exposure of plants to the resultant VOCs) achieving a 66% reduction in oviposition and a moderate reduction in settling of 0.4*d* (Cohen's *d*, effect size). To identify the basis of this VOC-induced resistance, whitefly settling rates on different plant treatments were compared using non-linear regression, the results of which suggest that defence priming, rather than direct defence activation, is the mode of action that best explains the responses seen. This work has the potential to provide an efficient and environmentally sustainable method of whitefly control in glasshouses, and is the first study to examine the temporal dynamics of VOC-induced whitefly defence in tomato.

## 4.1 Introduction

Whiteflies are some of the most important global arthropod crop pests, with approximately 1500 species in two sub-families: the Aleyrodinae and the Aleyrodicinae (Inbar and Gerling 2008). These polyphagous pests damage plants by penetrating host phloem and extracting large quantities of sap, depriving the plant of resources (Byrne and Bellows Jr 1991). They also produce honeydew, a sticky excreta, which covers leaves and supports sooty mould growth. This reduces the ability of the plant to photosynthesise and impacts fruit aesthetics (Inbar and Gerling 2008). Whiteflies also vector important plant viruses, such as the tomato yellow leaf curl and tomato chlorosis viruses (Jones 2003). The glasshouse whitefly (Figure 4.1A), *Trialeurodes vaporariorum* Westwood, is of particular importance in the U.K. (CABI 2013), affecting a wide range of glasshouse crops. Here and elsewhere, biological control agents such as the parasitoid wasps *Encarsia formosa* Gahan and *Eretmocerus eremicus* Rose and Zolnerowich (the two most common agents in use in the U.K.; Garthwaite et al. (2011)) are used in glasshouses to control whitefly numbers to acceptable levels (Gorman et al. 2007). Despite widespread use of biological control, however, pesticides remain an important component of whitefly IPM (George et al. 2015), and in 2011 *T. vaporariorum* management alone accounted for 13% of U.K. pesticide usage on glasshouse-grown tomatoes (Garthwaite et al. 2011). However, increasing global use of pesticides has led to the development of pesticide resistance in the glasshouse whitefly, with resistance reported to pyrethroids, organophosphates and the insect growth regulator buprofezin, as well as neonicotinoids such as imidacloprid (Gorman et al. 2007). This increasing incidence of pesticide resistance, combined with the recent restrictions on the use of three of the most common neonicotinoids by the European commission (EC 2013), highlights the need to develop alternative methods of crop protection, particularly where these are compatible with biological control approaches.

Plants are masters of secondary metabolite production, including production of gaseous compounds that are synthesised and released into the environment, known as volatile organic compounds (VOCs). These compounds have a range of roles, including long range signalling within a plant, and indirect defence, for example where natural enemies of a pest are attracted to VOCs omitted in response to infestation (Baldwin 2010). Recently, evidence has also been mounting for the use of VOCs as inter-plant communication molecules. Often this is presented as “eavesdropping” (Karban et al. 2006), where plants pick up on the VOCs released by their neighbours as a result of a stimulus, such as attack by herbivores. In the case of a neighbour being infested with a pathogen or pest, uninfested plants may not only respond by directly expressing defensive compounds, but may also become “primed” by producing

gene transcripts and precursor proteins required to mount a defensive response without excessive energetic investment (van Hulten et al. 2006). Priming has been the focus of an increasing body of research in recent years, with evidence accumulating for inter-plant communication leading to receiver plants becoming more resistant to a subsequent pest infestation. Plants damaged or infested by insect pests may communicate their status to other, uninfested plants of the same (Baldwin and Schultz 1983; Karban et al. 2006; Ton et al. 2007) or different species (Kessler et al. 2006). Communication may also occur between undamaged plants, leading to neighbours becoming better able to resist an attacking agent; this has been interpreted as a side-effect of the receiver plant preparing to engage in competition with the VOC-producer (Ninkovic et al. 2013). Evidence even exists for cross-kingdom interactions, such as the infestation of VOC-producing plants by whiteflies leading to greater resistance of the eavesdropper against a bacterial pathogen (Lopez et al. 2012). This volatile-based inter-plant communication method could potentially be exploited to achieve pest control in agricultural systems without affecting yield; priming results in minimal energetic expenditure if a pest does not infest a plant.

In their work on cross-kingdom VOC use, Lopez et al. (2012) describe the release of suites of VOCs by tomato after three days of glasshouse whitefly infestation, which had the effect of priming neighbouring uninfested plants, exposed to VOCS for six days, against subsequent inoculation with the bacterial pathogen *Pseudomonas syringae* pv tomato. This work links with a previous study published by our laboratory (Tosh and Brogan 2015) where the potential of non-host plant VOCs to confuse whitefly feeding on tomato plants was investigated, to attempt to reduce whitefly foraging efficiency. The current study builds on the findings of both Lopez et al. (2012) and Tosh and Brogan (2015) to test the following hypotheses: 1) whether exposure to VOCs from whitefly infested plants can increase tomato resistance to a subsequent whitefly infestation, 2) if an optimal combination of infestation time of the VOC-producing plant and exposure time of the receiver plant to VOCs, to maximally induce defences, exists, and 3) whether direct defence induction or defence priming is the probable mechanism of any VOC-based resistance observed. It is hoped that this work will contribute to the development of a novel biopesticide, based on the combinatorial effect of multiple VOC-based mechanisms, to provide tomato producers with a new product to effectively and sustainably control *T. vaporariorum*, in a manner that could be expected to be highly complementary, and potentially synergistic, with biological control.

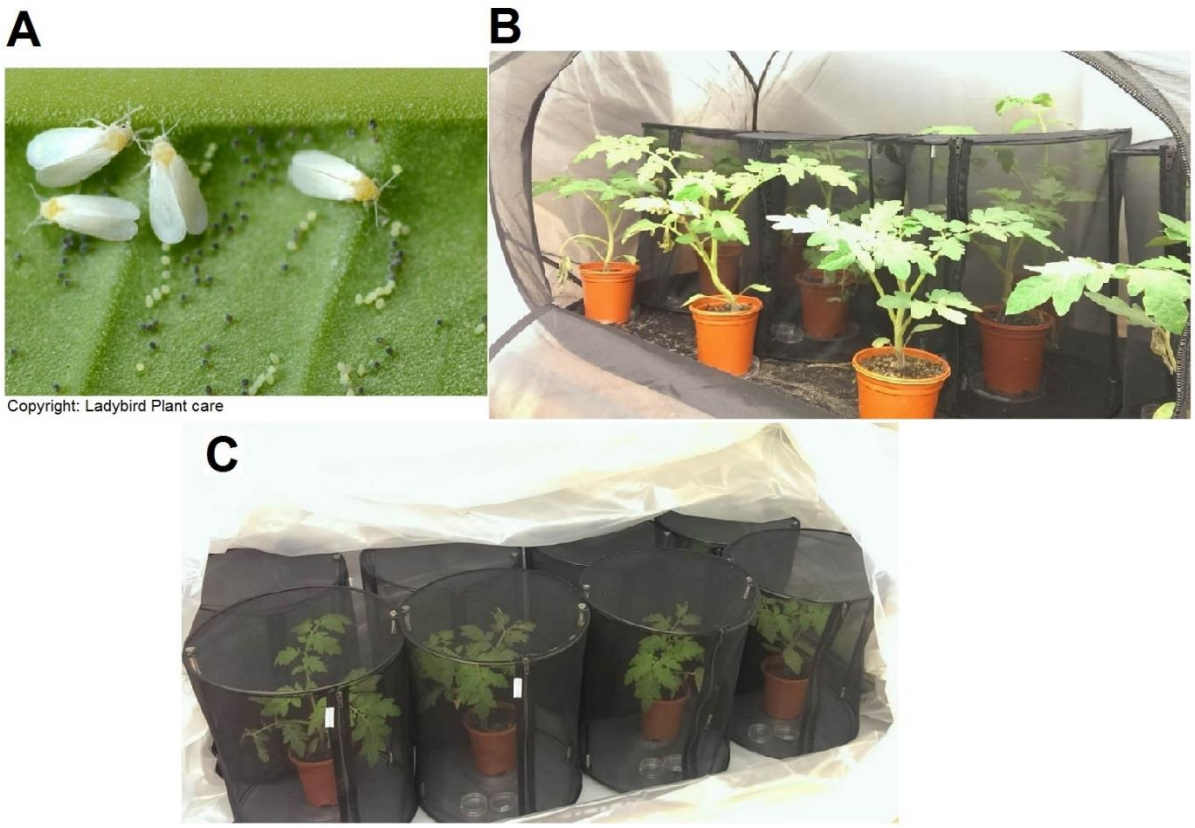


**Figure 4.1:** Experimental set-up of the infestation-exposure assay to assess resistance-increase in tomato following VOC-exposure from infested tomatoes. **(A)** Glasshouse whitefly adults feeding on tomato, with eggs of various ages visible. **(B)** Four whitefly-infested tomato plants in small cages produce volatile organic compounds (VOCs) to increase the resistance of the eight uninfested tomato plants surrounding them. Infestation and exposure time was varied to identify the most effective combination. **(C)** The eight VOC-exposed tomato plants were subsequently infested with whitefly to detect any increased resistance, by monitoring whitefly settling and egg lay.

## 4.2 Materials and Methods

### *4.2.1 Plants, insects, equipment and chemicals*

*Trialeurodes vaporariorum* were obtained from a colony maintained on aubergine (*Solanum melongena* L. 'Moneymaker') on a 16:8 h light/dark cycle and at constant 20 $^{o}$C. This colony was originally obtained from Rothamsted Research, from a naturally occurring colony found on French bean in Kent in 1960. *Solanum lycopersicum* Mill. 'Elegance' tomato plants were grown from seed obtained from Monsanto (Cat. E/12/11, Batch 0113479253), grown in John Innes No.2 compost in 9-cm-diameter and 8.7-cm-deep pots, at a density of one plant/pot, approximately 60 cm from a 400 W Son-T bulb housed in a Harrier HR400SH 400 W lamp under a 16:8 h light/dark cycle and at 25 °C during the light period and 20 °C during the dark period. This cycle was synchronised with the light regime that all experiments were conducted under. Plants were used at the 3-4 true leaf stage for all experiments, which

approximated to stage 13 on the BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) scale for tomato. Insect breeding cages of different sizes were obtained from Watkins and Doncaster insect breeding supplies (http://www.watdon.co.uk/): two large, net-lined, pop-up cages (1200 x 550 x 550 mm) to hold all plants and insects during the infestation/exposure period of the experiment, and sixteen smaller cages (approx. 250 mm high and 125 mm diameter) to contain the whitefly-infested plants within the larger cages, and for use in the settling experiments (see Figure 4.1B and C). Cages were lined with netting to stop movement of whitefly into or out of the cages. The large cages were also loosely encased in large translucent polythene bags (1250 x 2300 x 1600 mm high) to contain any VOCs produced in accordance with treatment within the cage (but with some airflow still present), and were obtained from Polybags Ltd (http://www.polybags.co.uk/index.htm). Acibenzolar – *S* – Methyl, used for determining the mechanism of resistance, was obtained from Sigma-Aldrich (http://www.sigmaaldrich.com/united-kingdom.html).

### *4.2.2 Effect of exposure to VOCs on subsequent whitefly infestation*

An assay was designed to determine the resistance against *T. vaporariorum* of uninfested tomato plants exposed for varying time points to VOCs from plants infested with whitefly for different durations. Four tomato plants were placed individually into four small cages lined with netting (to prevent whitefly escape) and infested with 100 whiteflies (50:50 male:female) randomly chosen from the whitefly colony. To infest plants, whiteflies were collected from the main aubergine colony using a mouth pipette, transferred to a small Petri dish (100/dish, 400 whitefly in total), then temporarily anaesthetised with $CO_2$ gas for 90 seconds. Petri dishes were then placed in the small experimental cages with the plants and opened; this allowed simultaneous exposure of whiteflies to the plants, and ensured no whiteflies escaped. The small cages, each containing an infested plant, were placed into large net-lined cages, and the large cage was encased with a large, translucent polythene bag. Plants were exposed to whiteflies for 0, 1, 3, 6, 9 or 12 days, after which eight free-standing uninfested tomato plants were evenly distributed around the small cages inside the large cage (Figure 4.1B). The large cage was closed and re-covered with the plastic bag, and the eight plants were left exposed to the VOCs for 0, 1, 3, 6 or 9 days, with cages only opened to water plants. Plastic bags were arranged to loosely cover the large cage to allow some airflow, but still contain VOCs. All possible combinations of the above infestation and exposure times were completed (24 in all).

After VOC exposure, the eight VOC-exposed plants were removed from the large cage, placed into individual small cages and infested with 50 whitefly (Figure 4.1C) of equal sex ratio chosen at random from the whitefly colony. These small cages were then all covered

loosely with a large plastic bag. The number of whiteflies settled on the tomato plant, and the number of eggs laid by these whitefly on the plant, were recorded after 24 h. Settling number was measured by visual observation. Egg number was counted by cutting all leaves from the plant and examining them under a light microscope at low magnification (3x). Controls constituted the same procedures described above, but without whitefly on the VOC-producing plant in the first part of the infestation-exposure assay. This experiment was based on work by Lopez et al. (2012), who investigated increased resistance to bacterial infection in tomato plants exposed to whitefly-induced tomato VOCs. Their experimental set-up differed as their work was conducted in glasshouses with a plastic arena, only considered one infestation and exposure time combination, and challenged plants with bacteria after VOC exposure.

Due to both egg and settling data having a poor fit to the normal distribution, according to normal probability plots, general linear models were discounted as an analysis method. Therefore both whitefly eggs and settling were analysed in IBM SPSS Statistics 22 using multi-way generalised linear models (GLMs) with pairwise testing with a Poisson distribution and loglinear scale, to identify if the treatment had caused a difference in the number of eggs laid, or whiteflies settled, on the VOC exposed plants compared to the control. A full model was initially fitted, with explanatory variables of study type (whether control or treatment), infestation time, exposure time and all possible 2- and 3-way interactions. The full model was then simplified using the Finite Sample Corrected Akaike's Information Criterion ($AIC_c$) to compare between potential models, to identify the model which best represented the data without overfitting. The $AIC_c$ refinement of the basic Akaike's Information Criterion was used, as the sample size divided by the number of parameters was less than 40, indicating the $AIC_c$ was the more appropriate method for the small sample size (Burnham and Anderson 2004). The $AIC_c$ was compared between models containing the main effects and just the interactions of interest, and full models containing all interaction terms, for both models of both whitefly settling and oviposition. The model giving the lowest $AIC_c$ score was selected as the most appropriate model.

### *4.2.3 Determination of optimum combination of exposure time and infestation time*

Whitefly settling and egg numbers were also analysed with *t*-tests between the control and treatment for each combination of infestation and exposure time, completed using the IBM SPSS Statistics 22 package, in order to identify the most effective infestation/exposure combinations. To control for the effect of multiple testing when using the *t*-tests, which brings an inflated risk of type I error, a Bonferroni correction (dividing α by the number of comparisons to be made) was applied to reduce the *p*-value at which the null hypothesis (in

this case no difference between treatment and control) was rejected. Effect size calculations were also implemented to compare treatment and control for all combinations, as it provides a standardised measure of effect that can be compared across exposure and infestation times. Cohen's *d* was used as the measure of effect size (equation 1) for the settling and oviposition results (Nakagawa and Cuthill 2007). The most promising time combinations (determined by the effect size on egg numbers, and by the overall length of the combination of exposure and infestation time) were then repeated to ensure the effect was replicable.

Cohen's *d* is determined by:

$$d = \frac{m_2 - m_1}{s(pooled)} \qquad s(pooled) = \sqrt{\frac{(n_2-1)s_2^2 + (n_1-1)s_1^2}{n_1+n_2-2}} \qquad (1)$$

where $m_1$ and $m_2$ are mean whitefly or egg number of the treatment and the control respectively, *s(pooled)* is pooled standard deviation, *n* is the sample size, and $s^2$ is variance.

### *4.2.4 Determination of mechanism of plant resistance*

A behavioural assay was designed to test whether the differences obtained in the infestation/exposure assay were due to direct activation of receiver plant defences by VOCs, or due to priming of receiver plant defences and subsequent activation upon whitefly infestation during the settling experiments. The settling behaviour of 100 whiteflies on a single tomato plant subjected to different treatments was monitored over 7.5 hours, replicated eight times per treatment. Experiments occurred in separate small mesh cages for each tomato plant, and treatments comprised: control (untreated) plants, plants infested with whitefly for a week prior to the assay and then the whitefly subsequently removed, plants sprayed with a chemical defence elicitor, or plants which had undergone VOC exposure in the one day infestation/six days exposure combination. The chemical defence elicitor used was Acibenzolar – *S* – Methyl (ASM), a benzothiadiazole derivative and the active ingredient in the commercial plant protection products Bion and Actigard, and was included as a positive control. ASM has been shown to elicit a defensive response in a number of plants against a range of pathogens and pests, including tomato against the whitefly pest species *Bemisia tabaci* (Nombela et al. 2005). The efficacy of ASM on inducing tomato defences against whitefly, which has not previously been shown, was demonstrated using no- and free-choice assays similar to those used by McDaniel et al. (2016) (results of these tests may be seen in Appendix B). 0.5 mM ASM was sprayed with a spray bottle to run off onto the second leaf of each plant used.

These experiments took place under the same conditions as the infestation/exposure assay (16 hours light/8 hours dark, 20 $^{o}$C). The number of whitefly settled on the plant was monitored at 5 minute intervals for the first 30mins, 10min intervals for the next 20 mins, 15 min intervals for the next 30 mins, 30 min intervals for the next hour and then hourly until the conclusion of the experiment. Experiments started at the same time each day. A 4 W LED work lamp was used to help visually assess whitefly numbers on plants. Four replicates were collected in a single session, with each treatment totalling eight replicates. It was expected that clear differences in settling numbers would be observed between, at least, the control and the other treatments; however this was not the case and patterns of settling appeared visually similar through time. These data were initially analysed using GLMs with a Poisson distribution, but non-significant differences were obtained. However, upon inspection of the variances produced by the four data sets, the volatile exposed treatment appeared to have much greater variances that suggested this treatment was influencing whitefly numbers, which was expected from the previous assay. It was therefore decided to use non-linear regression to attempt to differentiate these subtle effects, comparing the means and confidence intervals of the parameters (that describe the models) of the model of best fit. This model was identified by fitting all models listed on the Mathworks curve fitting webpage (Mathworks 2017) as available in MatLab 8.5 (R2015a) to the data using the model fitting toolbox, utilising the 'Trust-Region' algorithm. A total of 55 models were fitted (some model types such as Rational models did not have all possible iterations fitted once it was identified the model was inappropriate for the data). The best fitting model, identified by selecting the model with the highest adjusted R-squared value and with a minimum of overfitting judged by eye, was a Gaussian third order model (equation 2) which was further refined by constraining the 'a' parameters to positive integers, in order to produce more accurate fits (as recommended on the Mathworks webpage for Gaussian models; MathWorks (2016)). This is valid as these parameters will never be negative. Other models with higher R-squared values (such as a Fourier 4$^{th}$ order model) were rejected due to being overfitted: detecting an inappropriate amount of 'noise' in the sample that obscures the 'signal' (Lever et al. 2016), which was identified by examining the shape of the curves by eye. It was hypothesised that model parameters of the two defence activated treatments (ASM and previously whitefly-infested) should be similar, and consistently different from that of the VOC Exposed treatment, and the control, if volatile exposure induces priming, but the same if volatile exposure directly activates plant defences.

The Gaussian third order equation used was:

$$f(x) = a1 * exp\left(-\left(\frac{x-b1}{c1}\right)^2\right) + a2 * exp\left(-\left(\frac{x-b2}{c2}\right)^2\right) + a3 * exp\left(-\left(\frac{x-b3}{c3}\right)^2\right) \quad (2)$$

Where *f(x)* is the number of whiteflies settled, *x* is time in minutes, *a, b* and $c_{1\text{-}3}$ are model parameters, evolved to produce the best fit to the data.

### 4.3 Results and Discussion

This study sought to investigate the role of volatile organic compounds (VOCs) in increasing tomato defences against the glasshouse whitefly. The aims were to identify: 1) whether exposure to VOCs from whitefly infested plants can increase tomato resistance to a subsequent whitefly infestation, and what the strength of any observed effect was, 2) if an optimal combination of infestation time of the VOC-producing plant and exposure time of the receiver plant to these VOCs exists to maximally induce defences, and 3) what the probable mechanism underlying this maximum effect would be. This study represents the first in depth investigation into the multidimensional temporal dynamics of VOC-based resistance involving the glasshouse whitefly and tomato, with both the effect of infestation time of the VOC-producing tomato and exposure time of the "receiver" plant considered. This is also some of the first work to show inter-plant communication between tomatoes via VOCs (Zebelo et al. 2012) and the first to study the temporal dynamics of tomato-tomato communication with naturally derived compounds.

#### *4.3.1 Effect of exposure to VOCs on subsequent whitefly infestation*

Figure 4.2 shows the number of whiteflies that settled, and the number of eggs laid by those whiteflies, on plants exposed to VOCs produced from either whitefly-infested (treatment), or uninfested (control), plants. The length of exposure of the receiver plants to the VOCs varied between 1-9 days and the length of infestation of the volatile-producing plants varied from 0-12 days. The egg numbers on the treatment combinations ranged from a mean of 208.75 on 12days infested/9days exposed (12I/9E), to a mean of 51.125 on 1I/6E. The egg numbers on the control combinations ranged from 234 on 6I/3E to 95.5 on 9I/9E. The settled numbers on the treatment combinations ranged from 47.25 on 1I/3E to 35.125 on 0I/3E, and on the control varied from 48.75 on 12I/9E to 44.875 on both 0I/1E and 0I/3E. The largest decrease in egg number from control to treatment was -150.5 which occurred on 0I/3E, with the largest decrease in settling number being -9.75 on 0I/3E. The largest increase in egg number was +92.125 on 12I/9E, with the largest increase in settling being +1.5 on 1I/3E. The mean egg number from all combinations on the control was 165.00, and on the treatment was 134.79. The mean settled number, calculated from the mean values of all combinations, was 46.68 on

the control, and on the treatment was 43.13. Ten combinations had significantly fewer eggs laid on the treatment than the control according to *t*-tests, which reduced to five combinations being lower on the treatment when a Bonferroni correction, to reduce the threshold of significance to allow for repeated *t*-tests, was applied. Four combinations had significantly more eggs on the treatment than control plants according to *t*-tests, which dropped to two when the Bonferroni correction was applied. Fifteen combinations had significantly fewer whiteflies settling on the treatment than the control according to t-tests, reducing to five with the application of a Bonferroni correction. No combinations had significantly more whiteflies on the treatment.

The egg and settling data were analysed using GLMs. As shown in Table 4.1, a highly significant difference in study type ($p = 0.001$) was returned for both egg number and settled number, confirming a significant difference between treatment and control, as a result of VOC exposure, on both oviposition and settling. However, differences in the significance of the other terms upon these variables also existed. For egg number, both infestation and exposure time exerted a significant effect ($p = 0.001$) between treatment and control. The interactions of study type * infestation and study type * exposure time were also both highly significant ($p = 0.001$), meaning the difference between treatment and control depends on the levels of exposure time. This provides evidence that certain infestation and exposure times were more effective at reducing whitefly oviposition, as supported by the raw data in Figure 4.2.

For settled numbers, a significant effect was obtained for infestation time ($p = 0.013$), but not exposure time ($p = 0.141$), supporting that the infestation time of the VOC-producing plant is the main driver of settling differences in VOC-receiving plants. The interaction between study type and infestation time was also significant (at $p = 0.013$), showing that differences between treatment and control in settling vary for different infestation times, indicating again that certain infestation times are more effective than others for reducing settling numbers. The insignificant interaction between study type and exposure time ($p = 0.952$) supports that differences in settling numbers are the same across different levels of exposure time, again indicating that exposure time is not a driver of settling number change. These results are of interest as it may be concluded that infestation time of VOC-producing plants may be a more important factor than how long plants are exposed to these VOCs in reducing settling of whitefly. This indicates that it is the specific suite of VOCs that are produced that is more important at increasing plant resistance than how long the plant experiences these VOCs. This is an important finding, as these VOCs may be identified and synthetic analogues tested and developed for inclusion in pest control strategies.

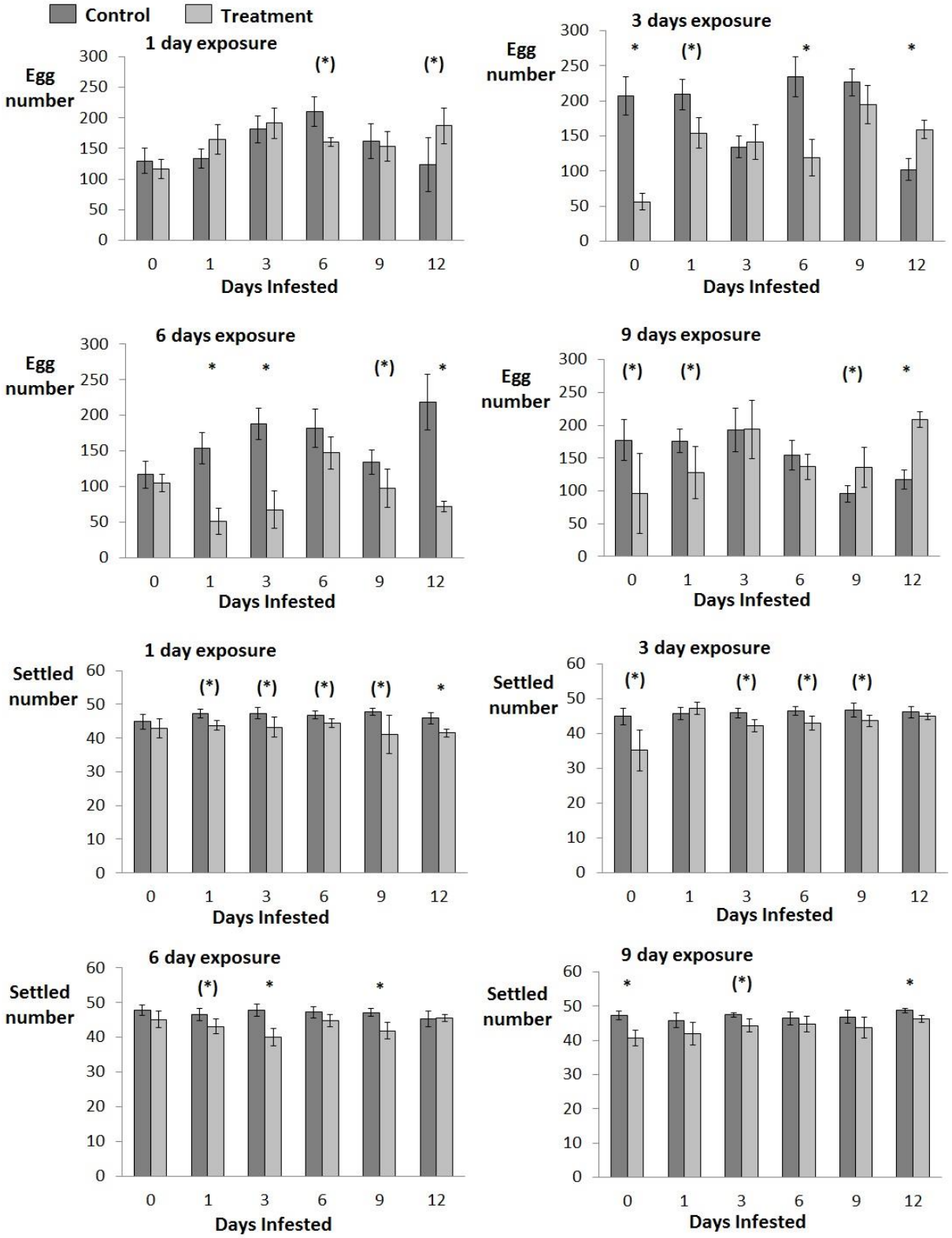


**Figure 4.2:** Raw data of egg and settling numbers from the infestation-exposure assay to assess increased tomato resistance after VOC-exposure from infested tomato plants. The graphs show the number of whiteflies settling and number of eggs laid on tomato plants exposed (for either 1, 3, 6 or 9 days) to volatiles produced from either non-infested (control; dark grey) or whitefly-infested (treatment; light grey) tomato plants, which had been infested for one of 0, 1, 3, 6, 9 or 12 days. An asterisk in parentheses '(*)' indicates there is a significant difference between control and treatment for that combination for the number of eggs laid according to a *t*-test, but that this difference becomes insignificant on application of

a Bonferroni correction. An asterisk without parentheses indicates a significant difference that holds with a Bonferroni correction.

| Egg number | Type III | | | Settled number | Type III | | |
|---|---|---|---|---|---|---|---|
| | Wald Chi-Square | df | Sig. | | Wald Chi-Square | df | Sig. |
| (Intercept) | 126350.722 | 1 | <0.0005 | (Intercept) | 926395.755 | 1 | <0.0005 |
| Study Type | 72.732 | 1 | <0.0005 | Study Type | 101.257 | 1 | <0.0005 |
| Infest time | 53.423 | 5 | <0.0005 | Infest time | 14.425 | 5 | .013 |
| Expose time | 58.896 | 3 | <0.0005 | Expose time | 5.455 | 3 | .141 |
| Study Type * Infest time | 51.081 | 5 | <0.0005 | Study Type * Infest time | 14.450 | 5 | .013 |
| Study Type * Expose time | 89.247 | 3 | <0.0005 | Study Type * Expose time | .340 | 3 | .952 |
| Infest time * Expose time | 116.172 | 15 | <0.0005 | | | | |
| Study Type * Infest time * Expose time | 152.008 | 15 | <0.0005 | | | | |
| Dependent Variable: Egg number<br>Model: (Intercept), Study Type, Infest time, Expose time, Study Type * Infest time, Study Type * Expose time, Infest time * Expose time, Study Type * Infest time * Expose time | | | | Dependent Variable: Settled Number<br>Model: (Intercept), Study Type, Infest time, Expose time, Study Type * Infest time, Study Type * Expose time | | | |

**Table 4.1:** The table shows generalised linear model output showing the statistically significant difference ($p = 0.001$, df = 1, $n = 48$) between the egg and settling numbers laid on tomato plants under the treatment and control conditions of the infestation/exposure assay.

### *4.3.2 Use of Effect Sizes to Interpret Infestation/Exposure Assay Results*

An objective of this study was to determine the infestation-exposure time combination that induces the greatest whitefly resistance in VOC-exposed plants. It is difficult to judge the size of treatment effects from inspecting raw data alone (as in Figure 4.2) however, whilst a large difference between treatment and control mean values may superficially indicate a large effect, within-treatment variation may be large. Effect size calculations such as Cohen's *d* (Nakagawa and Cuthill 2007) can remedy this issue by providing a standardised measure of effect, that incorporates both mean and standard deviation, that is comparable across different treatments and studies. Effect size calculations were therefore carried out on the egg and settling number data to help identify the infestation-exposure assay with the greatest effect on these measures of plant resistance to whitefly. The number of combinations of infestation and exposure time which yielded an increased resistance in the "receiver" plant was surprisingly high. All but two combinations resulted in reduced settling on the VOC-exposed plants (see Figure 4.3), with nine combinations giving a "weak" effect of around -0.2, six combinations giving a medium effect of around -0.5 and seven giving a strong effect of around -0.8. Interpretations of strength of effect are taken from Nakagawa and Cuthill (2007). The combinations giving a strong effect (I1/E1 I6/E1, I12/E1, I3/E3, I3/E6, I0/E9 and I12/E9) corresponded with the *t*-tests, which showed that treatment and control differed significantly for I1/E1, I6/E1, and I3/E3 before Bonferroni correction, and I12/E1, I3/E6, I0/E9, and I12/E9 after Bonferroni correction. The strongest combinations are also distributed widely throughout the times considered in the experiment, with variation both in terms of infestation and exposure time, but also in total length of time taken for the assay (which is 2, 7, 13, 6, 9, 12 and 21 days for each of the combinations respectively). This means that resistance of the

plants is not increasing simply as a function of increasing plant age. The most effective combination at reducing settling was I12/E9, the combination which was infested and exposed for the longest. This combination, however, also had a strongly positive effect on the number of eggs laid on the treatment. This may have resulted from the plant being in a stressful environment (exposed to defence activating VOCs communicating an impending whitefly attack) for a relatively long time, with the outcome of increasing plant suitability as a host for whitefly eggs. This is clearly not desirable in a pest control strategy, notwithstanding the effect on settling, with this result warranting further study.

For the oviposition data, 16 combinations reduced eggs on the treatment, seven increased eggs on the treatment and one exerted no detectable effect. Effect sizes were generally weaker, with the mean effect on oviposition (when discounting the disproportionally large positive effects of I12/E3 and I12/E9) being -0.03. This is classed as a very weak effect, compared to the effect on settling which can be considered as 'medium' (averaging -0.45). The strongest negative effects on oviposition were found for I0/E3, I1/E6, I3/E6, I3/E6, and I12/E6, which corresponded with *t*-tests showing that for all these combinations, significant differences were observed between treatment and control, despite a Bonferroni correction. I1/E6 gave the most effective (66%) reduction in whitefly oviposition, from 153 eggs on the control to 51 on the treatment (albeit with a weak effect size indicating high variability between replicates). By comparison, the same combination only provided a 10% reduction in settling, from 47 whiteflies to 43.

The effect size achieved for whitefly settling numbers far exceeded those for oviposition. The most likely explanation for this is that this difference in effect size is a function of the effect size equation. Such equations consider standard deviation and, even if separation of means is very slight, small standard deviations can result in high effect sizes, as can be seen here with the settling effect. This may have occurred as a result of the no-choice environment in the infection/exposure assay. Under these conditions whiteflies have to feed or starve, meaning the standard deviations may be artificially low for whitefly settling. An alternative, biological explanation for this observed disparity in effect strength may be that VOC-induced defences are more effective at reducing adult settling than deterring whitefly egg laying. It has been shown that the only R gene effective against whitefly, the *Mi-1.2* gene, deters whitefly settling to feed, but with those whiteflies that did settle on *Mi-1.2* plants feeding as effectively as on plants without *Mi1.2* (Jiang et al. 2001). A similar mechanism may be operating here, but more work is required to dissect the exact molecular consequences of whitefly-induced VOC exposure on tomatoes.

With the above in mind, it is worth considering both effect sizes and actual numbers when identifying the most effective combinations of infestation and exposure time on VOC-induced whitefly resistance of receiver plants. In doing so, the I1/E6 combination was taken forward for further experiments in this study, due to this treatment producing a large reduction in egg numbers, the lowest numbers of eggs overall, and a medium strength effect on settling (-0.43). In addition, the speed with which I1/E6 plants could be generated facilitated further experimentation. This combination also proved replicable, as similar effect sizes to the original experiment were observed in repeated I1/E6 treatments. In contrast, the I0/E3 combination (which also gave very good results for VOC receiver resistance parameters) proved non-replicable.

As well as being used in further experimentation here, the I1/6E combination can be recommended as the optimal infestation and exposure time for future VOC trials on whitefly and tomato. This optimal combination was not utilised by Lopez et al. (2012) (whose study inspired the present work) where a 6I/3E treatment was selected for VOC priming before inoculating with *P. syringae* pv tomato, with no evidence presented as to whether this combination would be most effective at enhancing VOC-receiver plant resistance. According to the current study, this combination would be only moderately effective at priming against a whitefly infestation, though the fact that the two studies focused on highly distinct plant antagonists (one a pest, the other a pathogen) means that comparisons can only be cursorily made. It is hoped that the present study can help inform future investigations into VOC-based priming in tomato, to indicate the appropriate time point for infestation and exposure needed to maximally activate plant defences.

The study by Lopez et al. (2012) included a time course analysis of the VOCs which were released by *T. vaporariorum* infested tomatoes. As the present study was conducted over a similar timeframe, it may be possible to infer from Lopez et al. (2012) the key VOCs which induced defences against whitefly in the current work. Lopez et al. (2012) identified suites of early-and late-release VOCs that are effective at inducing plant resistance, which may explain the finding here that receiver plants over the full range of infestation and exposure times exhibited enhanced whitefly resistance: defence-activating VOCs released throughout the infestation period would account for the wide number of combinations observed to be defensively activated. However, another possible explanation may be that defence priming from early exposure to priming VOCs may cause the plant to be defensively primed for the duration of the experiment, and therefore be capable of responding to a subsequent whitefly attack. Lopez et al. (2012) identified methyl salicylate (MeSA) as a key VOC, present only in

the volatile emission profile of tomato when infested with whitefly. MeSA emission commenced 5 days post whitefly infestation, which if true in the current study would explain increased resistance in many, but not all, of the infestation/exposure combinations tested. Thus, it seems likely that (effective) treatments with sub- 5 day infestation times must be driven by a different VOC emission. Candidates for these VOCs include decane (which is only released in the early period of infestation), the monoterpene (*E*/*Z*)-β-ocimene, the sesquiterpenes α-gurjunene, α-muurolene, δ-selinene, δ-cadinene, caryophyllene oxide and aromadendrene, and two unidentified VOCs designated NI 5 and 7, all of which are present in significantly higher quantities in the earliest infestation period investigated by Lopez et al. (2012). Further work needs to be completed to confirm these VOCs are active in inducing plant defences against whitefly (as Lopez et al. (2012) considered the effect on inducing defences against a bacterial attack), as well as identifying which individual VOCs, or mixes of compounds, are increasing tomato resistance to whitefly attack.

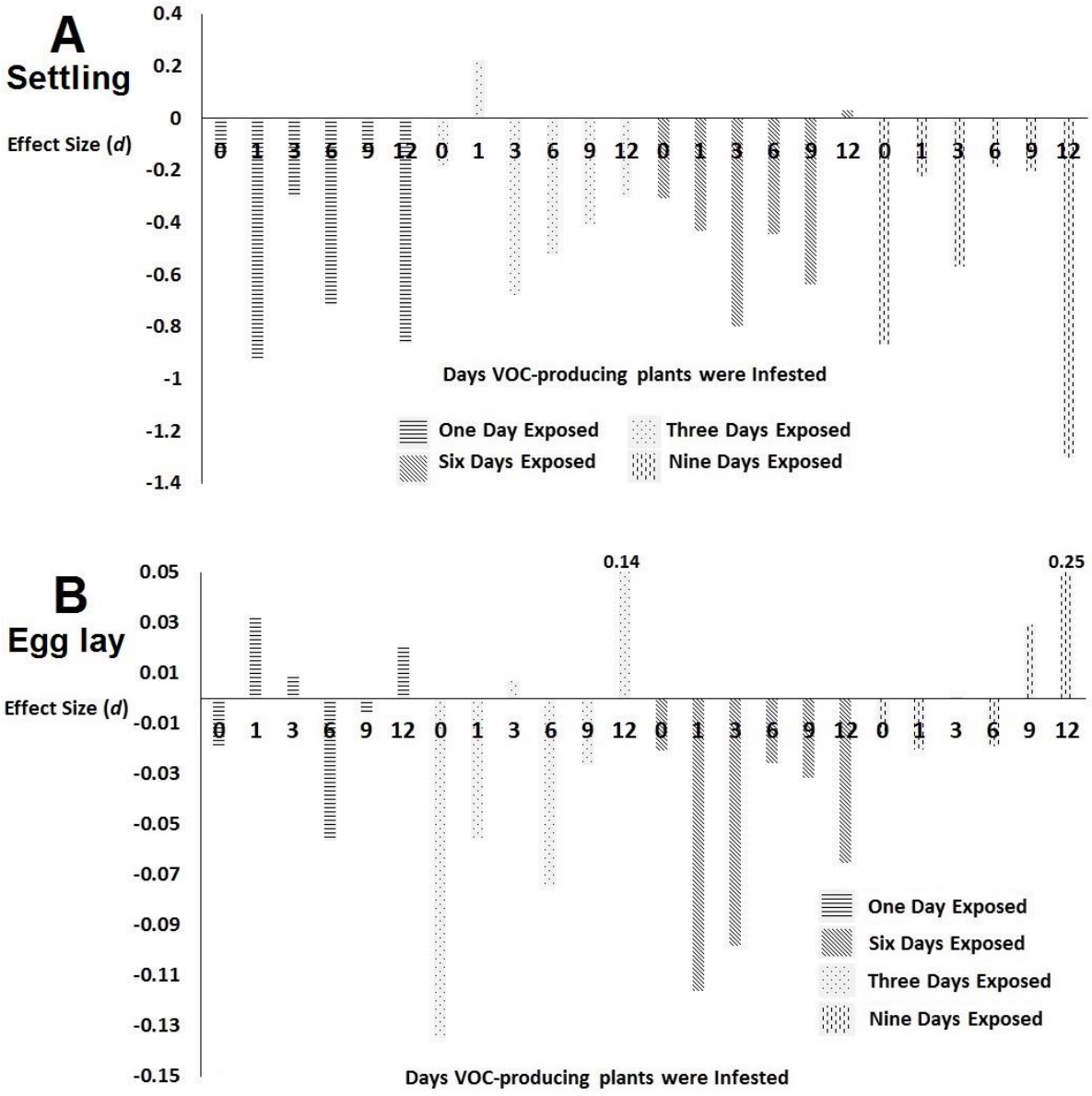


**Figure 4.3:** The effect size (Cohen's *d*) of different combinations of time whiteflies infested tomato plants to produce defence-inducing VOCs, and time other plants were exposed to these VOCs, on subsequent whitefly settling **(A)** and oviposition **(B)** after 24h on these VOC-exposed plants. Infestation time varies between 0 – 12 days, exposure time varies from 1 – 9 days. Effect size was calculated after Nakagawa and Cuthill (2007), with effect strength estimates taken from the same author: a weak effect ≤ -0.2, medium effect ≤ -0.5 and a strong effect ≤ -0.8.

### *4.3.3 Whitefly preference assays to determine VOC mode of action*

The number of whiteflies settling on differentially treated plants over time was analysed, in order to identify the mode of action of increased whitefly resistance in VOC-exposed tomato plants. Treatments comprised: control (untreated) plants, plants infested with whitefly for a week prior to the assay, plants sprayed with a chemical defence elicitor, or plants which had undergone VOC exposure in the I1/E6 combination. The plants infested for a week should have had defences already activated against whitefly, and so should have been relatively and

immediately unacceptable to further whitefly, as should the ASM-sprayed plants. If the VOC-exposed plants were defence activated, they too should have showed a pattern of whitefly settling different from the controls with this immediate effect. If primed alone, however, plants would have no defences activated, and so early in a whitefly infestation should display a pattern of whitefly settling similar to the controls. This would reflect the differences in physiological processes underlying direct defence activation and priming; namely that defence activation involves the immediate activation of defences, and priming involves no difference in defence activation until challenged by a specific pest, whereupon defences are strongly and swiftly switched on (van Hulten et al. 2006; Bruce et al. 2007).

Curve fitting analysis was used to differentiate between these treatments, based on increased variances observed in the whitefly settling on the volatile exposed treatment. A Gaussian third order model best fitted all 4 treatments, based on the R-squared value and visual inspection of the curves (Figure 4.4), and the parameters of the model were plotted with 95% confidence intervals. Non-overlapping confidence intervals were then taken to indicate significant differences between treatments (Figure 4.5). Of nine parameters in the model, five failed to deliver significant differences between the treatments, and one (a2) provided a difference between the ASM treatment and the control and volatile-exposed treatment, but no difference from the defence activated plants, which were in turn not different from the control and volatile exposed treatment. This may be due to a difference in the mode of action of the ASM when compared to the defence activated plants, and is difficult to explain without further studies.

Three other parameters (b1, b2 and b3) also provided significant differences between the treatments, with the same pattern for each: the control and volatile exposed plants had values with overlapping confidence intervals and are therefore seen as the same in value, and these values differed significantly from the defence activated and ASM sprayed plants based on non-overlapping confidence intervals, which themselves had parameter values with overlapping CIs and are therefore also seen as the same. Based on this grouping of the treatments, it is possible to suggest that the VOC-exposed plants have been primed as opposed to defence activated, as the grouping of the treatments fits that described above for priming.

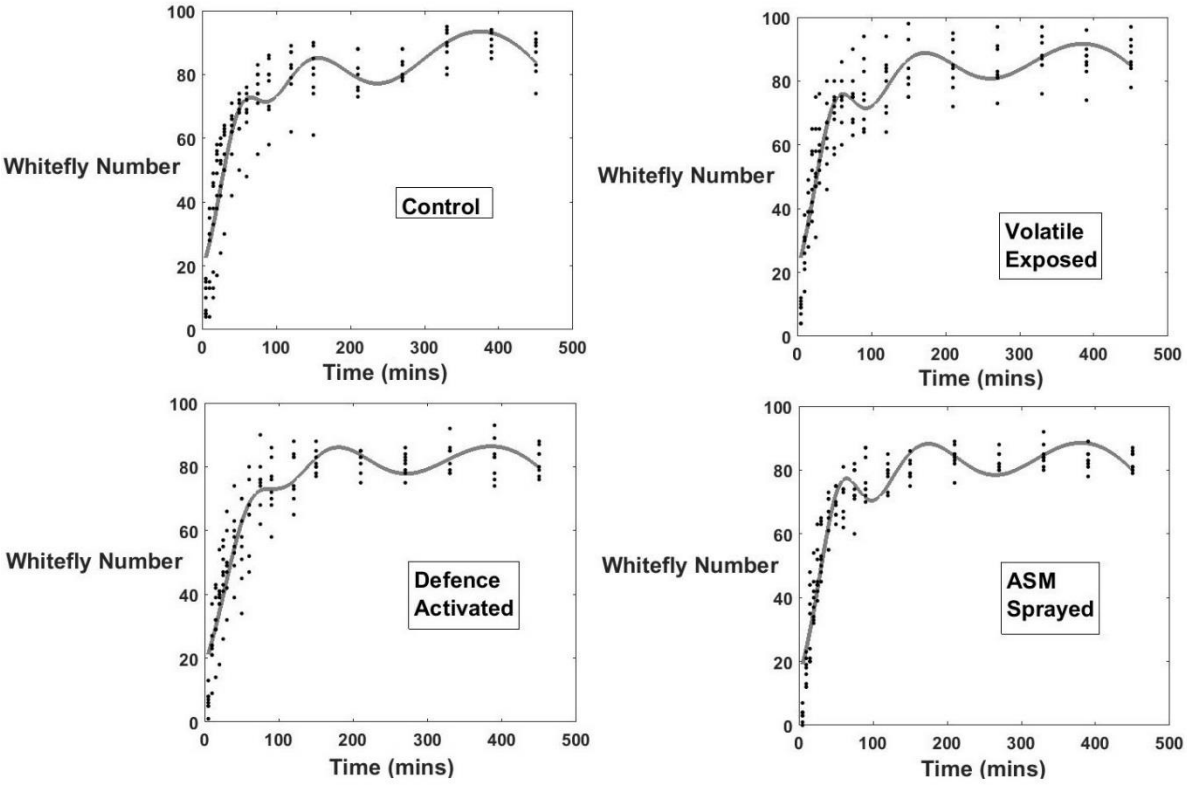


**Figure 4.4:** The number of whitefly that settled over 450 mins on tomato plants that were: untreated controls, defence activated by being infested with whitefly for a week, sprayed with the chemical defence elicitor ASM, and exposed for six days to VOCs from tomato plants that had been infested with whiteflies 24h previously (the I1/E6 combination identified earlier as the optimal infestation/exposure combination to enhance tomato resistance to whiteflies. ). In order to compare whitefly settling over time, these data were fitted with a range of models to identify the most appropriate fit, with a third order Gaussian model subsequently selected. Further analysis to compare these fits was conducted by comparing model parameters (see Figure 4.5).

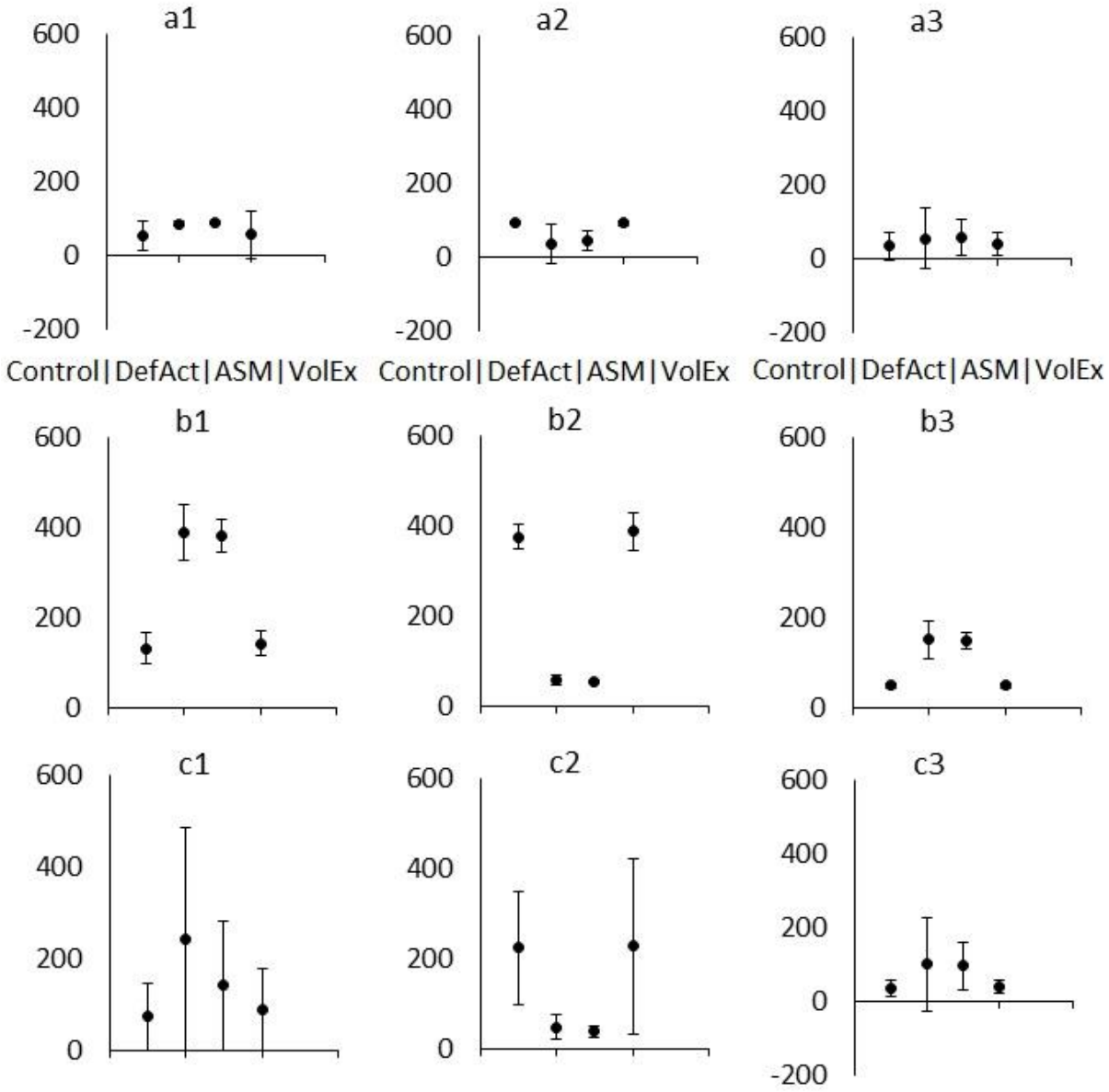


**Figure 4.5:** Gaussian third order model parameters from the whitefly preference assay to determine the mode of action of VOC-induced resistance in tomato, plotted with 95% confidence intervals. The value of these parameters are responsible for the exact form of the fitted Gaussian model. 'Control' indicates the control treatment, 'DefAct' the whitefly infested treatment, 'ASM' the chemical defence elicitor treatment and 'VolEx' the volatile exposed treatment. Non-overlapping CIs are taken to indicate significant differences between treatments. Parameters a2, b1, b2 and b3 are the only parameters showing differences between treatments. A2 indicates a complicated effect where the ASM treatment differs from the control and volatile exposed treatments, but not the defence activated treatment. B1, b2 and b3 all show the same pattern of the control and volatile exposed (VolEx) plants being the same as each other, but different from the defence activated (DefAct) and ASM treatments.

Priming has been shown to be the mode of action in increasing the resistance of neighbouring plants following VOC exposure in *P. syringae* infested lima bean (Yi et al. 2009), *S. littoralis* infested maize (Ton et al. 2007) and *P. brassicae* infested cabbage (Peng et al. 2011). This is in contrast with other studies where alternative mechanisms have been found to increase plant

resistance, such as direct defence induction (Arimura et al. 2000) or utilisation of the VOC as a defence molecule directly (Mescher and De Moraes 2014). Priming is thought to be particularly advantageous to the plant, as it involves relatively little energetic investment when compared to direct defence activation, and therefore imposes less of a fitness cost in the event of the plant not experiencing an attack in the short term, or at all (van Hulten et al. 2006). This also make priming especially attractive as a pest management tool for growers, as a primed crop would not inappropriately invest energy into defence at the expense of growth in the event of the plant not being attacked. It is therefore promising that priming seems to have been detected in the present study, though definitively confirming its operation will require ongoing molecular genetic studies.

VOCs possess several advantages as a pest management tool, which include the potential compatibility with existing whitefly control systems. As well as providing effective control, functioning of current biocontrol agents used to reduce whitefly population numbers, such as parasitoid wasps, could have their activity enhanced by the use of VOCs, as VOCS may strengthen the ability of the biocontrol agent to locate whitefly infested plants, although future work would be needed to confirm this. VOCs may also be used in parallel with other Integrated Pest Management components, such as intercropping, to increase the effectiveness of these components beyond that which they would achieve individually, e.g. with trap crops providing an alternative host for whitefly that would be repelled from VOC-primed tomato plants. It is for these reasons, related to environmental sustainability and favourable interaction with existing methods, that VOC-based control systems are being pursued, in this and other studies (Tosh and Brogan 2015; Ton et al. 2007).

### *4.3.4 Conclusions and future studies*

The current study has demonstrated that whitefly-induced tomato VOCs enhance the resistance of neighbouring tomato plants to a subsequent infestation. This work adds to the body of knowledge on VOCs, which have been shown elsewhere to facilitate inter-plant communication relating to pest infestation (Baldwin and Schultz 1983; Ton et al. 2007; Lopez et al. 2012). The work conducted here also provides greater insight into the dynamics of the VOC interaction: a wide range of infestation and exposure times of the VOC-producing and –receiving plant were considered, whereas in most studies a single infestation and exposure time are selected, often with little justification. It is hoped that this work may inform future studies on the effect of VOC-based plant communication. It illustrates that many combinations of infestation and exposure produce a beneficial response to the “receiver” plant, but some combinations give a stronger effect than others. Clearly, not all combinations

are equal in their effect and this should be considered in the design of VOC experiments. It is suggested that this enhanced resistance is driven by defence priming in the receiver plant, with further studies into the VOC profiles released from infested tomatoes, and work into the molecular impact of VOC exposure, planned to provide support for this hypothesis. It is hoped that this work will add to the work by Tosh and Brogan (2015) and McDaniel et al. (2016) to create a programme of whitefly control methods based around VOC-induced effects, which encompasses the confusion effect, defence priming, repellence and genetic improvements to crop plants to enable the environmentally sustainable production of tomatoes in the future.

### 4.4 Acknowledgements

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# Chapter 5. General Discussion

The current work investigated novel Integrated Pest Management (IPM) components to control the glasshouse whitefly on glasshouse grown tomatoes. IPM, first postulated nearly 60 years ago (Stern et al. 1959), encompasses a range of techniques that may be combined to achieve pest control greater than the sum of the individual components (Tang et al. 2005). These techniques may be broadly grouped into 'Avoidance', 'Sampling' and 'Effective Chemical Use', and approaches used should proceed in that order to achieve the most sustainable pest control (Fleischer et al. 2014). Avoidance involves taking steps to prevent pest invasion, and includes shaping the cropping landscape to preclude pest influx, for example by enclosing crops to exclude pests (Vincent et al. 2008). Cultural control measures, such as intercropping, that stop pests entering crops or prevent their build up to beyond economic injury levels (All 2008), are also key avoidance strategies. The use of crop types that possess genetic resistance to the pest, either through plant breeding e.g. tomatoes resistant to *Tomato Mosaic Virus* and *Tomato Spotted Wilt Virus* (García-Martínez et al. 2014), or through genetic engineering techniques, e.g. the development of *Bt* maize to control the European corn borer (Koziel et al. 1993), can avoid pest populations becoming problematic. Using knowledge of pest biology to reduce pest impact on crops is another key component of avoidance, for example by using the most effective available parasitoid against a certain pest (Conlong and Rutherford 2009). Another example is the utilisation of selective biological control agents to keep pest numbers low, e.g. by using natural predators that have an alternative host to maintain control agent population numbers year round (Bale et al. 2008). Sampling involves monitoring crops to maintain an awareness of pest numbers so that appropriate action may be taken when predetermined critical thresholds are reached (Tang et al. 2005) and includes having suitable detection systems in place, such as yellow sticky traps to catch whitefly and aid in population size monitoring (Gerling and Horowitz 1984), with an action plan to be followed once pests reach certain numbers that is based on existing knowledge of pest ecology (Mitchell and Hutchison 2008). Effective chemical pesticide use is a last resort, and only occurs once critical thresholds of pest numbers have been reached to protect crops from excessive damage (Barzman et al. 2015). Biopesticides, which are commercially available pest control agents derived from naturally produced chemicals, or from living micro-organisms (Chandler et al. 2011), should be used as a first instance (Rahioui et al. 2014). If chemical pesticides are needed, consideration must be given to using selective chemistries to protect beneficial insects and the environment (Naranjo and Ellsworth 2009), as well as avoiding the development of resistance in the pest, by using suitable

concentrations, application numbers and timings, and combinations of chemical control to ensure pests are effectively killed (Barzman et al. 2015).

The over-arching aim of the present study was to aid in the development of IPM by identifying novel mechanisms that may be deployed in the control of the glasshouse whitefly in glasshouse grown tomatoes. The mechanisms investigated fit into different areas of the IPM model. The investigation of novel whitefly resistance mechanisms in the wild tomato *Lycopersicon pimpinellifolium* is the start of a process to introduce these resistance mechanisms into commercial tomato species. This is an example of host plant genetic resistance, one of the tenets of avoidance in the list described above. The novelty of this mechanism is that this wild tomato has never before been assessed for its resistance to the glasshouse whitefly, and the mechanisms that are potentially detected have not been previously discovered or characterised. The investigation into the potential for the 'push-pull' mechanism to control whitefly on glasshouse grown tomatoes is another avoidance strategy, using intercropping as a cultural control mechanism to deter whiteflies from entering a crop, and trapping them on more preferred hosts. This also has the potential to feed into the monitoring level of IPM, with whiteflies likely to appear first on more preferred plants such as courgette, and so may be detected early in an infestation by monitoring these plants for whiteflies. This method is novel in that the 'push-pull' method of pest control has not been assessed for tomatoes against whitefly before, and this is the first time a scientific basis has been asserted for the use of French marigolds as part of a 'push-pull' strategy to control whitefly. This work also assessed marigold plants and limonene, the volatile chemical suggested as the mechanism of marigold-based whitefly control, for their ability to achieve control over a heavy whitefly infestation. This fits into the final section of IPM, where interventions are necessary to reduce pest populations below economic injury levels, and are proposed as novel alternatives to chemical pesticide sprays. The investigation of plant volatiles that prime tomato defences against whitefly is another example that may contribute to different IPM categories. Depending on how this technology is developed, it could be used as part of a strategy to enhance crop genetic resistance by priming tomatoes for a potential whitefly infestation. It may also be used as an emergency measure, for instance if volatile organic compounds (VOCs) are introduced as a biopesticide control mechanism to make plants that have been infested more resistant to their attackers. The use of VOCs in this way would also allow them to be incorporated into other ways of controlling whitefly, such as repellence or odour masking. The novelty of this technique lies in the fact that inter-plant communication between tomatoes to activate defences against whitefly has not been shown

before. These IPM components could contribute to the future environmentally sustainable production of tomatoes, as their use should allow for the control of whitefly populations below economic injury levels, and therefore reduce the need to spray crops with synthetic chemical pesticides that have well-documented effects on the environment (Woodcock et al. 2017) and human health (Alavanja et al. 2013). This reduction in pesticide use could alleviate the selection pressure that has led to the evolution of pesticide resistance in a wide range of insect pests (Ferguson et al. 2010) including whitefly (Gorman et al. 2007; Cahill et al. 2009). The use of intercropping to control whitefly may also have other effects that could aid the environment. Increasing the diversity of plants on farms may boost ecosystem services by attracting pollinators (Kremen and Miles 2012) and naturally occurring enemies of pests to perform biological control services (Power 2010), as well as aiding introduced biocontrol agents by providing floral resources and cover to maintain populations (Heimpel and Jervis 2005). It is hoped that using these mechanisms together may provide enhanced whitefly control beyond the sum of the individual parts, and integrating these measures into a coherent IPM strategy, alongside existing measures, is an important avenue for future research.

### 5.1 Investigating Genetic Resistance Mechanisms in a Wild Tomato Species for Future Incorporation into Commercial Tomato Cultivars

As is described above, host plant genetic resistance to pests is an important part of the IPM strategy. If plants can resist pests without any additional inputs from farmers and growers, food production may be more environmentally sustainable and cost effective. Increasing plant genetic resistance has been the focus of much research in recent years. Pest resistance has been introduced into commercial crops by conventional plant breeding, such as maize resistance to western corn rootworm (Ivezić et al. 2009). Genetic engineering (GE) techniques have also been used, such as the formation of transgenic rice and maize containing the fusion protein BtRB, which consists of the δ-endotoxin Cry1Ac fused with the galactose-binding domain of the nontoxic ricin B-chain (Mehlo et al. 2005). Tomato, as an important crop plant, has experienced a great deal of attention in terms of increasing its genetic resistance to a range of pests (Fernandes et al. 2014; Sim et al. 2012) including *B. tabaci* (Leckie et al. 2012; Firdaus et al. 2012; Firdaus et al. 2013) and *T. vaporariorum* (Bas et al. 1992). The only R gene known in tomato against whitefly is the *Mi1.2* gene which confers resistance to nematodes, aphids and whitefly (Nombela et al. 2003). This introduction of genetic resistance is made necessary by the "domestication syndrome" exhibited by modern crop plants (Hammer 1984), where certain traits have been selected for at the expense of a range of other traits. In the case of tomato, fruit morphology, and various growth traits including self-pruning, plant height and earliness, have been the main targets of improvement (The 100

Tomato Genome Sequencing et al. 2014), and traits such as pest resistance have been bred out of tomato species over time (Bevan et al. 2017). Wild and ancestral relatives and landraces of crop plants are potentially highly useful reservoirs of these genes which have been lost over time (Tanksley and McCouch 1997). In Chapter 2, one such wild relative of tomato, *Lycopersicon pimpinellifolium*, was assessed for enhanced resistance to the glasshouse whitefly. Using whitefly settling and oviposition assays, movement recordings, and the electrical penetration graph (EPG) technique to monitor whitefly feeding, a dual mode of resistance to *T. vaporariorum* was identified. *L. pimpinellifolium* is a less preferred host than the commercial *S. lycopersicum* 'Elegance' based on settling and oviposition assays, although movement assays revealed no difference in the ability of whitefly to traverse leaf surfaces of each species. EPG studies suggested this difference in host acceptance was due to two resistance factors present in the wild tomato that were absent in 'Elegance'. The first is suggested as a pre-phloem penetration mechanism, possibly located in the epidermis or mesophyll of *L. pimpinellifolium* leaf tissues, which deters whitefly from continuing to feed once it has been encountered. This is based on the EPG results, which revealed significantly shorter 'duration of the second probe' and 'duration of C waveforms' and significantly higher levels of non-probing behaviour in the early part of the EPG trace recorded on *L. pimpinellifolium*. The shorter second probe indicates that whiteflies encounter a repellent resistance factor during their first gustatory probe, which is undertaken in many phloem feeding insects to ascertain host quality (Tosh et al. 2002). The shorter C waveforms indicates that whiteflies spend less time in the mesophyll of leaf tissues, and suggests that this is the location of any pre-phloem resistance factor. As whiteflies move their stylets between cells, in a fashion stealthier than even aphids (which tend to puncture cells; Lei et al. (1997)) then the resistance factor is likely to be extracellular, either freely in the mesophyll space or on the surface of cells. This resistance factor, detected early in whitefly feeding and before phloem penetration, is likely to result in whitefly seeking an alternative host under glasshouse conditions, although the whitefly association with the plant continued in the EPG studies due to the artificial tethering of the insect to the plant. Such a mechanism would have the advantage of preventing plant virus transfer into plant phloem, as whitefly would be repelled before having the opportunity to salivate into these vessels and transmit the virus. The second resistance factor suggested in Chapter 2 is a post-phloem penetration mechanism, and is based on the observation of significantly higher levels of E1 phase probing, or salivation, of whiteflies when accessing *L. pimpinellifolium* phloem. Whiteflies salivate to allow phloem feeding, and this represents a small proportion of the phloem phase when feeding on a susceptible host. Increased salivation has been observed elsewhere in incompatible host/pest

interactions (Will et al. 2007; Jiang and Walker 2007) and is believed to be an attempt by the pest to overcome host defences such as vascular occlusion, possibly by the use of effectors in the saliva to block calcium ion signalling. This increased level of energy expenditure in order to feed would likely result in whitefly leaving the host in greenhouses. These resistance mechanisms have not been demonstrated before for this tomato species against the glasshouse whitefly, and are attractive targets for introgression into commercial tomatoes. Future work to facilitate this should focus on definitively identifying the mechanisms in *L. pimpinellifolium*, and the genes underlying them.

Assessing wild relatives of crop species for advantageous plant traits is a technique that has great potential for introducing novel or rediscovered genes into plants that will assist in the resistance of a range of biotic and abiotic stresses, such as breeding resistance to brown planthopper (Wei et al. 2009) or tolerance to flooding (Xu et al. 2004), in rice. Genetic engineering (GE) is a technique that will greatly aid in this endeavour by allowing the introduction of genes from unrelated plant species e.g. the introduction of bacterial RNA chaperones into maize to confer increased grain yield under drought stress (Castiglioni et al. 2008). One of the best examples of GE in the context of introducing pest resistance is the use of Bt toxins from *Bacillus thuringiensis,* which have been introduced into a range of crop plants to achieve control of chewing and boring insects (Pardo-López et al. 2013). However, GE has obvious constraints in terms of its uptake, with many geopolitical regions having strict restrictions on the use of GE techniques. Despite this, GE/biotech crops remain one of the brightest prospects of producing greater amounts of food over the next 30 years, to meet the target set by the FAO of increasing food production by 70% by 2050 (FAO 2009). Work must be done by the scientific community to aid in the acceptance of new techniques such as the CRISPR-Cas9 system, the newly emerging technique for site-directed genome modifications (Doudna and Charpentier 2014), such that they can fulfil their full potential and help avert a humanitarian catastrophe that has been avoided in the past by the use of new technologies and genetic manipulation of organisms (Evenson and Gollin 2003) .

### 5.2 Investigation into the use of Intercropping to Control Whiteflies in Glasshouses

In Chapter 3, the efficacy of intercropping tomato plants with less preferred hosts amongst the tomato crop (‘push’ treatment), and additionally placing more preferred hosts around the crop edge (‘push-pull’ treatment), were assessed for their ability to reduce whitefly performance on the tomato crop. Also, the effect of having low- and high-diversity in the plant species used to make up both the ‘push’ and ‘pull’ plants was investigated, with the number of species for both ‘push’ and ‘pull’ plants varying between a single species (low diversity, LD) and four

species (high diversity, HD). Finally, the ability of marigolds, as well as the volatile chemical limonene, to reduce heavy whitefly infestations of tomatoes was assessed. Significantly fewer whiteflies were observed on the LD and HD 'push' treatments compared to the control. The LD 'push' treatment, composed of tomatoes intercropped with French marigolds, was as effective as the HD 'push' treatment, which consisted of tomatoes intercropped with marigolds, basil, nasturtium and Chinese cabbage, at reducing whitefly numbers. Whitefly numbers were reduced on the LD and HD 'push-pull' treatments compared to the control, with significance values at or around the classic 0.05 α value. No difference in whitefly numbers between LD and HD 'push-pull' treatments was observed. This study is the first to provide scientific evidence for the commonly held assertion that French marigolds may be used as a companion plant to protect tomatoes from whitefly attack. Marigold plants had a slight effect on the heavy whitefly infestation, reducing adult numbers near-significantly after one week, and nymph number near-significantly after 22 days. Limonene proved much more effective, reducing adult numbers on treated tomato leaves near-significantly after one week and 22 days, as well as causing a significant reduction on nymph numbers in the last two sampling points in the experiment. Marigolds are recommended as a companion plant with tomatoes from the start of the growing period, with limonene suggested as a possible biopesticide to reduce heavy infestations. This study developed a novel slow release system for limonene that could be developed further in the future.

The 'push-pull' technique has been developed elsewhere for use in controlling different pest species on crop plants. In one of the more successful examples, Khan et al. (1997a) demonstrated in a Kenyan field trial that intercropping maize plants with a non-host molasses grass, *Melinis minutiflora*, achieved significant reductions in stem borer incidence on the crop by 'pushing' them from the crop, as well as attracting natural enemies to the crop. This system was rapidly followed by the use of Sudan grass, *Sorghum vulgare sudanense*, in addition to the molasses grass, to 'pull' stemborer from the maize crop, thus increasing maize yield (Khan et al. 1997b). Further work then found that a different intercropping plant, the leguminous forage plant *Desmodium uncinatum*, functioned as an effective repellent agent of stemborers, but also achieved significant control of the parasitic witchweed *Striga hermonthica* (Khan et al. 2000) by an allelopathic mechanism (Khan et al. 2002). Further development of this system now seeks to incorporate drought-tolerant plants to increase resilience to climate change (Khan et al. 2014). This highly effective system has been taken up by 68,689 farmers in sub-Saharan Africa, with the potential for many millions more to benefit (Khan et al. 2014). A 'push-pull' system was utilised by Gomes et al. (2012) to protect

tomato from thrips, using coriander and marigold amongst the tomato crop, and sorghum surrounding the crop. Intercropping tomato with maize plants achieved reduced *B. tabaci* and virus incidence on the tomato crop (Abd-Rabou and Simmons 2015), and intercropping tomatoes with coriander and with basil achieved reductions in *B. tabaci* populations compared to the control of 84 and 79%, respectively (Carvalho et al. 2017). However, no studies have considered the impact of the 'push-pull' method on *T. vaporariorum* numbers on tomato.

The example above of the control of stemborer on maize in sub-Saharan Africa illustrates many of the advantages that intercropping has for crop production, and has some parallels with the study in Chapter 3, as well as highlighting some of the future prospects for this work. The 'push-pull' system developed by Khan et al. (2014) is relatively simple in its design, but achieves substantial results. This was the aim for the study in Chapter 3, with significant whitefly control obtained by the simple spatial reorganisation of plants. Whilst the African 'push-pull' system is designed for use on smallholdings in Africa utilising native plants from that ecosystem, the tomato-based system was designed to be utilised by both commercial growers (once further refinements have been made to the system) and small scale growers, using plants that may be present already. The intercropping method developed by Khan et al. (2014) utilises plants that have another use beyond pest control, which is a major advantage of intercropping. In their system, *Desmodium*, which is a legume, may help in nitrogen fixation, and both 'push' and 'pull' plants are forage plants. In the whitefly control system, all plants used were either ornamental or edible, and so may provide alternative produce for growers. The stemborer control method also attracted natural enemies of the insect in greater numbers, whilst an aim of the whitefly study was that the greater diversity in the cropping system would provide benefits such as floral resources for natural predators, although this is yet to be quantified. The mode of action in the *Striga* control plants was via allelopathic exudates into the soil (Khan et al. 2002). The method of control achieved by French marigolds in the whitefly control system is thought to be based on the volatile chemistry of the marigold plants, repelling whitefly by the use of volatiles that may include limonene. Certainly, limonene is a significant component of marigold volatile output, and in the heavy infestation assay limonene proved effective at reducing large whitefly numbers on treated tomatoes. Further work is necessary to elucidate the exact mode of action of marigolds. Furthermore, Khan et al. (2014) demonstrated that their intercropping method, whilst giving a lower density of crop plants, resulted in a higher yield per unit area due to the quality of produce. It is hoped that such a situation will arise from the whitefly 'push-pull' system, but further work needs to be done to quantify impacts on crop yield. The use of marigolds as an emergency treatment

actually resulted in near-significantly lighter tomatoes in that treatment. This is suggested to be as a result of shading of the tomatoes by the introduction of the marigold plants, and could be avoided with more careful placement of the marigold treatment. By contrast, the limonene treatment resulted in near-significantly heavier plants, which holds promise for the use of this chemical as a biopesticide. The work by Khan et al. (2014) also targeted multiple pest species, with *Desmodium* controlling *Striga* weeds, and the combination of *Desmodium* and molasses grasses controlling stemborer. The future goal of the whitefly control 'push-pull' system is to achieve control over several pests damaging to glasshouse-grown tomatoes, by combining multiple less attractive 'push' plants and more attractive 'pull' plants. The control system will become more complex as each pest species is added, as each plant used will need to be at least neutral in its level of attraction to the other pests to be controlled. However, it may emerge that one plant will assert control over multiple pest species, and the fact that the work in Chapter 3 showed the high diversity treatments to be neutral in attractiveness to the onion thrips, *Thrips tabaci,* and there were no greater numbers of *T. tabaci* or the two-spotted spider mite, *Tetranychus urticae,* on the heavy infestation assay indicates that it may be possible to develop this 'push-pull' system to control multiple pests.

If the whitefly control method demonstrated in Chapter 3 is to become useful at the commercial level, further work will need to be completed. The exact mode of action will need to be quantified, with further work needed to identify how limonene achieves control over whitefly, and whether it is the key driver of marigold-induced whitefly control. The impact on crop yield will need to be assessed, with the placement of plants optimised to achieve high yields whilst still achieving pest control. The system will likely need to be developed for use in commercial systems, where hydroponics rather than solid substrates are more prevalent, and vines may grow to a height of 10m or more (Resh 2016). If VOCs are the mode of action, then efforts will need to be made to ensure that chemicals are applied over the length of the cropping system to achieve whitefly control. This work has the potential to introduce greater diversity into cropping systems, which may achieve enhanced whitefly pest control, and may potentially introduce greater resilience into farming systems (Malézieux 2012). This work may also reduce reliance on chemical pesticides (Hassanali et al. 2008), which in turn could lead to the reduced development of pesticide resistance in important crop pests (Pickett et al. 2014).

### 5.3 Priming Tomato Defences against Whitefly using Plant-derived VOCs

In Chapter 4, the potential for VOCs from whitefly-infested tomatoes to increase the resistance of uninfested tomatoes to a subsequent whitefly infestation was investigated. This

having been demonstrated, the optimal combination of whitefly infestation time of tomato plants to produce resistance-inducing VOCs, and exposure time of other tomato plants to these VOCs, was sought, with the aim of identifying the combination to give the maximum effect on resistance. The mode of action of this resistance was also investigated, to ascertain whether it is achieved through direct defence induction or by defence priming.

The role of plant volatiles in a range of ecological functions, including pollinator attraction (Raguso 2008), and indirect defence against herbivores by natural enemy attraction (Dicke and Baldwin 2010) is well established, but VOC involvement as a plant resistance-inducing agent was initially highly controversial (Fowler and Lawton 1985). However, evidence is accumulating for the presence of VOC communication between plants, resulting in enhanced resistance to attack by insect pests in the receiver plant for alder and willow (Rhoades 1983), poplar and maple (Baldwin and Schultz 1983), sagebrush and tobacco (Karban et al. 2003), lima bean (Arimura et al. 2000), maize (Ton et al. 2007), and tomato (Sugimoto et al. 2016). The aim of the work in Chapter 4 was to identify whether this VOC-based system of defence induction could be identified in tomato in response to whitefly infestation. Tomato was selected as, if VOC-induced defence induction could be identified, the wealth of knowledge of tomato phytochemistry (e.g  Ryan and Pearce (1998)) could be taken advantage of to better understand this phenomenon. A wide range of infestation and exposure time combinations were used as it was observed that other studies into herbivore-induced VOC based defence activation often provided no justification for the time periods used in their studies (e.g. Lopez et al. (2012)) and it was felt that an analysis of a range of possible combinations would aid future research in this field. The present investigation revealed for the first time that tomato resistance to whitefly could be increased by exposure to whitefly-induced VOCs. Furthermore, the number of combinations of infestation and exposure time to give an effect on plant resistance was surprisingly high, with whitefly settling significantly reduced in 15 combinations, and whitefly oviposition significantly reduced in 10 combinations, according to *t*-tests between the treatment and control. This number was reduced to five combinations for both settling and oviposition after the application of a Bonferroni correction to allow for the inflated risk of a type I error that comes with the application of multiple *t*-tests, but this correction is acknowledged to be a very conservative measure. Therefore, the true value of the number of significantly different combinations likely lies between that given by the two measures of significance. It appears that tomatoes are capable of responding to VOCs produced by infested tomatoes irrespective of how long VOCs have been produced for or how long they are exposed, as more resistant plants were obtained across the whole spectrum of

infestation and exposure, although some combinations gave stronger defence induction than others. An assessment of the strength of the tomato defensive response was made by the use of effect size calculations from the difference between treatment and control, to allow comparisons between time points. The strength of the effect of VOC exposure was much higher on whitefly settling compared to whitefly egg lay, although this is likely a result of the very low variances for the whitefly settling which may have unduly affected the effect size calculations. The combination that is recommended for use in further whitefly-induced VOC defence activation studies in tomato is one day infestation of VOC-producing plants and six days exposure of tomato plants to these VOCs, based on the numerical decrease in egg numbers, the strength of the effect on whitefly oviposition and settling, and the practical consideration of how long these plants take to be produced (which was one week, whilst many of the other combinations took longer times to produce). This contrasts with the work of Lopez et al. (2012), whose work is most relevant for comparison to the current study as it investigates *T. vaporariorum*- induced VOC activation of tomato defences (although against the bacterial pathogen *P. syringae pv tomato*, and it is acknowledged that differences in the target pest used may make specific comparisons between these studies difficult). Lopez et al. (2012) utilised a six day infestation/three day exposure treatment for VOC priming before bacterial inoculation. From the study in Chapter 4, 6I/3E was a candidate for being recommended as a combination for further study, as it would have elicited a similar effect on whitefly settling, and would have actually given a stronger effect on oviposition. However, the actual egg levels achieved were a mean of 119 eggs per plant, as opposed to the 51 eggs per plant achieved by the 1I/6E treatment recommended here, and this combination also takes more time to generate plant material for experimental purposes.

Efforts were also made to attempt to identify the mechanism of this increased resistance observed in tomato upon whitefly-induced VOC exposure, whether direct defence induction or defence priming. Direct defence induction would represent the immediate switching on of plant defences to counter whitefly (Farag et al. 2005), whereas defence priming would involve the plant entering a readied state without full investment in anti-herbivorous defences, which would allow it to respond rapidly and more strongly to an attack by whitefly (Heil and Karban 2010). This primed state is advantageous to a plant as it allows an effective response to herbivores without undue energetic investment (Kessler et al. 2006), and from an agronomic viewpoint it would allow a tomato plant to continue to invest fully in fruit production, without wasting resources on whitefly defences if such an attack did not materialise. Studies are available to suggest that VOC exposure results in defence activation in the receiver plant

(Arimura et al. 2000; Farag et al. 2005), whilst others suggest that priming is the mode of action (Ton et al. 2007; Kessler et al. 2006; Engelberth et al. 2004). Curve fitting analysis was used to attempt to differentiate between these scenarios for the present study system; models were fitted to settling data from whiteflies feeding on control, VOC-exposed, chemical defence elicitor (Acibenzolar – *S* – methyl; ASM) sprayed, and previously-infested tomato plants. The parameters that described these models were then compared between the model fits, and the VOC-exposed and control plants were found to group separately from the ASM-sprayed and previously infested tomato plants for three of the nine parameters, with the other six parameters not differing between treatments. This grouping suggests the presence of priming in the VOC-exposed plants, as whiteflies did not differentiate between VOC-exposed and control plants, indicating defences had not been activated in these plants. This study is suggestive of priming, but more work is needed to better elucidate the exact mechanism of induced resistance, and exactly what defences are activated by VOC-exposure. An interesting interpretation of the work of Farag et al. (2005) by Frost et al. (2008a) was that the less energetically expensive defensive measures, such as VOC production to attract natural enemies, are induced by VOC exposure, but more energetically expensive ones, such as proteinase inhibitors, are primed. It would be interesting to see what proportion of tomato defences against whitefly are activated upon VOC exposure: VOC release to attract natural enemies forms a large part of tomato resistance to whitefly (Walling 2008), but as whitefly settling and oviposition were reduced in the absence of parasitoids other mechanisms may be activated to reduce whitefly performance on tomato, although it is possible the whiteflies are responding to changes in VOC output. Further work needs to be done to elucidate this.

Other work should focus on how long this priming effect can last. Frost et al. (2008a) argue that, according to Optimal Defence Theory (ODT; Stamp (2003)), defences should reduce over time, and therefore priming should also reduce over a certain time period. However, as priming involves very little energetic investment, it is unclear what this time period should be. Frost et al. (2008a) link priming with a previously observed phenomenon, delayed inducible resistance, where herbivore stress in one growth season results in reduced herbivore performance in the next (Zvereva et al. 1997) which suggests that priming may last at least a year before declining in its effectiveness. Slaughter et al. (2012) go further, providing evidence for transgenerational defence priming in BABA-primed Arabidopsis plants, where the offspring of plants exposed to this chemical defence-priming agent were more resistant to *P. syringae* and had more rapid accumulation of defence gene transcripts. Rasmann et al. (2012) also found evidence of transgenerational defence priming, in both Arabidopsis and

tomato, which casued a 50% reduction in caterpillar growth compared to controls. This priming effect was shown to last for two generations in in Arabidopsis, and to be dependent upon jasmonate perception and the ability to produce small interfering RNA (Rasmann et al. 2012). Further work needs to be done to identify how long the primed response lasts for in tomato in response to whitefly, and whether transgenerational priming can be identified in this study system. Other important work that needs to be completed is the elucidation of the identities of VOCs that induce tomato defence priming, and the composition of the VOC mix and dose leading to priming. It is envisaged that, if identified, synthetic VOC sprays based on these chemicals could be utilised in greenhouses to prime tomato plants against whitefly attack. Of other studies that have used VOCs to prime plant defences, several have proposed VOCs that are responsible for the priming effect. Lopez et al. (2012) identified methyl salicylate (MeSA) as an important VOC produced by tomato under whitefly infestation, present in the VOC emission profile five days post-infestation. Other candidates from that study that were differentially expressed in infested plants at earlier time points include decane, (E/Z)-β-ocimene, sesquiterpenes such as α-gurjunene, α-muurolene, δ-selinene, δ-cadinene, caryophyllene oxide and aromadendrene, as well as 2 unidentified VOCs (Lopez et al. 2012). These VOCs may be important in priming tomato resistance to *T. vaporariorum* infestation. Sugimoto et al. (2014) showed that the green leaf volatile (Z)-3-hexenol is released by cutworm-infested tomatoes, which is taken up by neighbouring uninfested tomatoes, transformed into the glycoside (Z)-3-hexenylvicianoside, and used as a defensive compound against a subsequent infestation of cutworm larvae. It would be interesting to see if this mechanism of uptake, transformation, and utilisation, involving this chemical or another compound, is implicated in the increased resistance to whitefly observed in VOC-exposed plants in the present study. In work by Worrall et al. (2012), tomato seeds treated with jasmonic acid were primed to respond to caterpillars, aphids, spider mites and the necrotrophic fungus *Botrytis cinerea*, and those treated with β-aminobutryric acid (BABA) were primed against the powdery mildew *Oidium neolycopersici*, with priming responses lasting for a minimum of 8 weeks. Whilst not VOCs, both these priming agents are present in plants, and, for jasmonic acid at least, can be volatilised in the form of methyl jasmonate (MeJA). Therefore, jasmonates may also be a candidate for the priming agent in the present study. Other volatile priming agents include a range of green leaf volatiles, which may prime maize (Engelberth et al. 2004), poplar (Frost et al. 2008a), lima bean (Kost and Heil 2006), and tobacco (Kessler et al. 2006). This class of VOCs has received much research attention, and was implicated in tomato defence in the study by Sugimoto et al. (2014) above, and so warrants further investigation in tomato as defence priming agent. These VOCs found in other

studies may be candidates for key priming agents that prime whitefly-specific defences in tomato, but further investigations are needed to verify this.

### 5.4 Ongoing Investigation into the Changes Induced in the Tomato Transcriptome by Whitefly-induced VOCs

A further investigation into the VOC-induced changes in the tomato transcriptome is underway which it had been hoped would be included in the present work. This investigation aimed to identify the whitefly-specific defences and signalling pathways activated by VOC-exposure, as well as to differentiate between changes at the transcriptomic level caused by whitefly infestation, ASM-exposure, and VOC-exposure, of tomato plants. This investigation was unable to be included in the present work due to time constraints. The study involved extracting RNA from tomatoes treated with the one day infestation/ six days exposure VOC combination described above, sprayed with 0.05mM ASM on the second tomato leaf, infested with whiteflies on the second leaf for a week, or VOC-exposed and then whitefly-infested, as well as two untreated controls which differed only in the bagging of the second leaf (to control for physical enclosure of this leaf in the whitefly-infested treatments). Leaf samples were taken from both the apical leaf, and the second leaf, of plants in each treatment, to allow systemic and local effects of treatments to be identified. These RNA extractions were completed and sent for analysis using microarray gene chips, the results of which are still awaited. This study will shed light on the transcriptional changes that are undergone in each of these differentially treated tomatoes, to increase the understanding of VOC-induced defence priming.

### 5.5 Integrating Novel Whitefly Control Measures into an IPM Strategy

It is hoped that the present study will contribute to the future effective management of *T. vaporariorum* as an important glasshouse pest of glasshouse-grown tomatoes. The strategies outlined herein represent different facets of the IPM paradigm, and as such there is the potential for these techniques to be used together to achieve greater whitefly control than could be achieved by using these strategies individually. It is anticipated that these control methods would synergise well. Assuming that the genetic basis for the enhanced whitefly resistance from *L pimpinellifolium* can be identified, and introduced into commercial tomato species, then this enhanced genetic resistance could underpin a whitefly control mechanism. This enhanced baseline level of protection would then be enhanced by the addition of plant VOCs, potentially as a synthetic spray, or as a slow release volatile system achieving both defence priming and whitefly repellence. This synergism would allow effective tomato defences bred into commercial lines to be more rapidly and strongly activated, as a result of

the defence priming. In addition to this, the 'push-pull' system would help achieve greater pest control, as defence primed plants would be even more unpalatable to whitefly, making the difference between the 'pull' plants and the tomato crop even greater, and presumably exerting a greater pressure for whiteflies to move to these 'pull' trap crops. It may also be the case that tomatoes can be primed against multiple pests concurrently, as well as building up a set of 'push-pull' plants that control a range of tomato pests, the synergism between which would achieve a greater level of control over not just whiteflies, but other insect pests such as thrips and spider mites. Finally, the identification of limonene as an effective plant protection compound could be developed further, along with the slow release method, to allow control over acute whitefly infestations.

There may potentially be risks of combining these mechanisms together in the same greenhouse. When introducing genetic resistance into commercial tomatoes, efforts would have to be taken not to reduce plant yield, as this would be unlikely to be acceptable to growers. The same is true of priming with VOCs: rigorous testing would have to ensure that plants are truly primed, and that the minimum energy is invested in defence responses, as this energy is wasted in the event a pest infestation does not occur. Also, there have been limited studies into the costs of defence priming (Frost et al. 2008a). Such studies would have to be undertaken in order for a VOC system to be fully accepted in greenhouses. In terms of the 'push-pull' system, future studies should identify the effect of intercropping on overall yield quantity and quality, as growers are unlikely to accept a reduction in produce quality, with the placement of plants needing to be optimised to different growth systems. Care would have to be taken to ensure that other pests are not attracted into greenhouses by the diversity of plants, although the study in Chapter 3 is promising in that no increase in other tomato pest numbers was observed on the treatments. For all these IPM components, the interactions with other components of IPM would have to be investigated, with particular care given to ensuring that existing whitefly control mechanisms are not compromised. The impact of any introduced intercropped plants, and VOC sprays, on biocontrol agents such as *Encarsia formosa* would have to be quantified, although there is the potential here for an increase in the effectiveness of these biocontrol agents, as VOCs may aid in the location of their whitefly hosts, as well as increased biodiversity providing floral resources, as is discussed above. The compatibility of these measures with existing chemical control agents would also have to be ascertained, as these chemical control agents are an important last resort for IPM, and any decrease in their effectiveness could have consequences for the whole crop. However, by using these novel IPM components, the use of these chemical control agents should be kept to a minimum.

To further investigate how these IPM components might fit into existing IPM systems, it is worthwhile considering, as a case study, the work of Naranjo and Ellsworth (2009). They describe the effective implementation of an IPM strategy to control *B.tabaci* on an Arizona cotton system. This was developed in the face of an invasion of *B. tabaci* biotype B into Arizona in 1992, which was initially countered with pesticide usage, namely fenpropathrin with acephate, and bifenthrin with endosulfan (Naranjo and Ellsworth 2009). However, amidst concern for the usage of these broad spectrum pesticides, an IPM programme was devised and implemented in that system to achieve control over whitefly populations. This IPM programme was predicated on a robust monitoring system, with a defined protocol for assessing whitefly pest densities, by counting nymphs and adults on the main cotton leaves at the fifth node below the terminal leaf (Naranjo and Ellsworth 2009). An Economic Injury level (EIL) was also set, to dictate the point at which intervention is necessary to maintain crop profitability. This also allowed the deployment of chemical control agents available at the time (mostly insect growth regulators; IGRs) at the point of maximum effect, with 3-5 adults per leaf and 0.5-1 nymphs of the $3^{rd}$-$4^{th}$ instar being adjudged the limit for when intervention was needed, based on tests completed in-field (Naranjo and Ellsworth 2009). When chemical pesticides were required, pesticides were used in order of selectivity, with the most selective such as buprofezin and pyriproxyfen used first and broad spectrum pesticides used as a last resort. Pesticides were used at the lowest effective dose and at the most effective time point of the whitefly lifecycle, reducing the impact of even broad spectrum pesticides on non-target insects. In this way, ecosystem services were protected as much as possible, with the greater survival rate of natural enemies of *B. tabaci* allowing greater long term pest control at a lower cost to the grower by the use of selective chemistries which balanced the predator:prey ratio in favour of the predator (Naranjo and Ellsworth 2009). This use of selective pesticides reduced the incidence of pesticide resistance developing in *B. tabaci* populations. It was also compatible with the control of other pests of cotton, including *Bt* cotton used to control pink bollworm (Naranjo and Ellsworth 2009). The application of IPM principles resulted in a reduction in the chemical sprays necessary in the cotton growing season from 4.1 sprays/season to 1.25 sprays/season in the 10 year post-IGR introduction period, a 70% reduction in foliar insecticide use, and saved $201,599,000 over 14 years from costs of insect control and reduced yield loss (Naranjo and Ellsworth 2009; Ellsworth et al. 2010). The case study provided by Naranjo and Ellsworth (2009) has several important lessons for how the work in the present study could be applied into an IPM system. The 'push-pull' work may aid in a monitoring system for whitefly pest density detection: whitefly are likely to accumulate in higher numbers on the 'pull' plants, and so early detection of a

pest influx may be made possible. The selectivity of the system described by Naranjo and Ellsworth (2009) was the key to its success. Based on this, the IPM components described herein should be compatible with this selective strategy. Increased genetic resistance will be highly selective to *T. vaporariorum* control, and VOC application should prime plants specifically against a whitefly attack, although the impacts of both these applications on plant interactions with other pests and natural predators should be explored. Finally, the potential impact of an effective IPM strategy is clear: the vast decreases in chemical pesticide application by the use of EILs and selective chemistries resulted in large financial savings, as well as untold effects on local insect biodiversity and human health (Naranjo and Ellsworth 2009), and is an example of what IPM can achieve when sufficient research attention is applied to its individual components and the interactions between them.

### 5.6 Barriers to IPM Uptake and Recommendations for Barrier Removal

A major challenge to the success of IPM is the rate of uptake by farmers and growers, with uptake rates low in European farming systems (Freier and Boller 2009). Attempts to quantify the main barriers to IPM uptake in farming systems around the world have been made, with several recurring issues identified. These include a failure to provide the correct support to growers once IPM system have been implemented (Parsa et al. 2014), lack of research investment (Wynn et al. 2014), and the dominance, and ease of use, of chemical pesticides for certain agricultural sectors (Lamichhane et al. 2015). Market forces are reported as a driver of both rapid and slow IPM adoption. In horticultural crops, low tolerances for pesticide residues have driven the use of IPM components, as they achieve pest control without excessive chemical pesticide inputs (Wynn et al. 2014). However, arable crops have a very low IPM uptake rate due to stringent specifications for crop quality, leading to growers and agronomists being risk averse in their use of, and recommendations for, pesticide spraying. This leads to growers being unwilling to forego a pesticide spray for fear of risking deteriorating crop quality that may not meet contract requirements for a crop (Doonan 2017). The availability of pesticides for use on a certain pest in a given crop is also a driver of IPM uptake: where pesticides are limited in number or in application, IPM uptake is more rapid e.g. in the amenity sector, where weeds and pests are controlled on public spaces and therefore the use of broad-spectrum pesticides is severely limited due to public health concerns (Wynn et al. 2014).

In the example above by Naranjo and Ellsworth (2009), adoption of the whitefly IPM strategy was driven by a large influx of pests that threatened the cotton industry in Arizona, concern over the use of non-specific pesticides at various levels in science, industry and government

driving collaboration between these bodies to form a response, and a rapid and directed investment in research to provide tools that could both overcome the pest influx and assuage concerns over the use of pesticides (Naranjo and Ellsworth 2009). With this in mind, and considering the barriers to IPM uptake outlined above, it may be possible to suggest courses of action that may be taken to improve IPM uptake across various agricultural sectors. Knowledge is clearly a limiting factor in IPM uptake, in terms of knowledge of how to use IPM components, in the awareness of the potential of IPM to control crop pests, and of what level of control is necessary to meet crop requirements. There is a pervasive attitude that IPM is a less effective alternative to pesticide applications, which in turn are seen as 'magic bullets' to control crop pests (Doonan 2017). To change this attitude, it is necessary for farmers and growers to be aware of the potential of IPM to achieve effective pest control, whilst also being made aware of the damage that is being caused by modern intensive, synthetic pesticide-based pest control. The work of Naranjo and Ellsworth (2009) highlights the importance of knowledge of the biological system to achieve pest control, with the example of the provision of the exact dose of pesticide to use such that pests are controlled but beneficial insects are not killed, which would not be possible without an in-depth knowledge of the growing system. Another recommendation is that support for growers and farmers in implementing IPM needs to be increased. Farmers and growers should not be left to themselves to apply what can be complicated pest management systems; help should be provided to allow these systems to be put in place and run correctly, and to allow farmers to have faith in the IPM method. A common issue with IPM uptake is that the system is often very specific to the farming environment in which IPM is to be deployed: what works in one environment will not work in another, and the exact suite of IPM components to be deployed needs to be selected carefully for the growing system. This is where grower support can be the most useful, in tailoring IPM systems to particular scenarios. Finally, governmental involvement has a large part to play in ensuring IPM is adopted. In the Arizona IPM example, governmental involvement allowed much more rapid IPM development, due to provision of funding and knowledge exchange necessary for whitefly to be controlled e.g. producing the Silverleaf Whitefly National Research, Action, and Technology Transfer Plan (Henneberry et al. 2002). Governmental regulation of synthetic chemical pesticides can reduce the availability of certain damaging pesticides, and increase the economic pressure to use alternatives that may be provided by IPM. The regulatory process for IPM components could also be made more appropriate, to allow IPM components to be adjudged as a suite of control measures, rather than being considered individually. This could also allow for more rapid and easy regulatory approval for IPM components, to increase their availability to end users. By

implementing these recommendations it is hoped that IPM use will increase in prevalence in the future.

### 5.7 Conclusions

Integrated Pest Management holds great promise for development as a crop protection strategy of the future. It is based, not on short-term or chemical-orientated solutions, but on a deeper understanding of the ecological context in which crops and therefore crop pests exist. IPM will become ever more important as existing pesticides become less effective due to increasing levels of pest resistance (Whalon et al. 2008), both as a means of managing this resistance, and for maintaining efficient crop production. This holistic approach to crop production may also ease some of the antagonisms which exist between large scale agriculture and the natural world, with greater ecological considerations given to the production of crops being to the benefit of both systems (Malézieux 2012; Malézieux et al. 2009). It is hoped that the work in this thesis will help develop the integrated management of whitefly pest populations in the future, to avoid *T. vaporariorum* reaching the same devastating pest status as *B. tabaci* (CABI 2017), and provide techniques that may be transferable to this whitefly pest. The future prospects of IPM are likely to include increased incorporation of transgenesis techniques, which may increase the potential for increased genetic resistance of crop plants (Fleischer et al. 2014). Genetic engineering is likely to be highly compatible with IPM, due to the specificity of pest control components incorporated into crops (Schünemann et al. 2014), and the subsequent reductions in chemical pesticide use which has benefits for natural predators and other beneficial insects. Techniques such as RNAi may be incorporated in the future to add to the techniques with which whitefly may be controlled. Recent examples exist of the development of this powerful tool to achieve control of *B. tabaci* (Ibrahim et al. 2017), and this represents an exciting advance in the field of whitefly control. In conclusion, the development of environmentally sustainable control measures to control a range of insect pests, including the glasshouse whitefly, will be necessary to continue the effective production of crops to support an ever-expanding world population.

# Appendix A Additional Pest Information from 'Push-pull' Glasshouse Trial

| **Date: 22/08** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LD | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| HD | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Date: 01/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
| Control | 2 | 0 | 8 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LD | 3 | 1 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HD | 3 | 1 | 6 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Date: 07/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
| Control | 8 | 0 | 17 | 7 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| LD | 3 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HD | 4 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| **Date: 13/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
| Control | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LD | 3 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| HD | 6 | 0 | 8 | 1 | 0 | 10 | 5 | 0 | 0 | 0 | 0 |
| **Date: 22/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
| Control | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LD | 2 | 3 | 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HD | 6 | 0 | 20 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| **Date: 27/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
| Control | 2 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| LD | 7 | 0 | 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HD | 8 | 0 | 17 | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A1:** Raw pest data collected from the 'push' experiment of the intercropping study conducted at Stockbridge Technology Centre in 2016 (see Chapter 3). These pest data are the total number of each pest adult, nymph or egg found on all eight replicates of each treatment, separated into the dates indicated. Clear-bodied spider mites were not successfully identified, and it was not possible to differentiate between the eggs of these clear-bodied mites and those of *T. urticae*.

| **Date: 01/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| **Date: 07/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LD | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HD | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| **Date: 13/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HD | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| **Date: 22/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HD | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| **Date: 27/09** | **Adult *Tetranychus urticae*** | **Clear spider mite** | **Spider mite egg** | **Adult *Myzus persicae*** | **Nymph *M. persicae*** | **Adult *Aphis gossypii*** | **Nymph *A. gossypii*** | **Adult *Macrosiphum euphorbiae*** | ***Sphinx ligustri* adult** | ***Pieris rapae* adult** | ***Helicoverpa armigera* egg** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| HD | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A2:** Raw pest data collected from the ‘push-pull’ experiment of the intercropping study in Chapter 3. These pest data are the total number of each pest adult, nymph or egg found on all eight replicates of each treatment, divided into the dates indicated. Clear-bodied spider mites were not successfully identified, and it was not possible to differentiate between the eggs of these clear-bodied mites and those of *T. urticae*.

# Appendix B Determining the Efficacy of Acibenzolar – S – Methyl (ASM) as an Activator of Tomato Plant Defences

## No Choice Assay Determining ASM Efficacy

The efficacy of acibenzolar – *S* – methyl (ASM) as an activator of tomato plant defences was completed using no- and free- choice assays similar to those used in Chapter 2. Briefly, for the no choice assays, one female whitefly was placed on the second apical leaflet of a 3-4 week old tomato plant that had been either untreated, or sprayed to run off with 0.5 mM ASM. Whiteflies were placed in a small clip cage, as described in Chapter 2, and left for 72 h, with the plant placed inside a small mesh cage. After 72 h the clip cage was removed and the leaf analysed at low magnification (3x) to count egg numbers laid by the whitefly. Eight replicates were completed for the control plants (due to one whitefly dying), and nine were completed for the ASM-sprayed treatments. The results can be seen in Figure B1; significantly more eggs were laid on the control plants than the ASM-sprayed plants according to a *t*- test ($p = 0.001$).

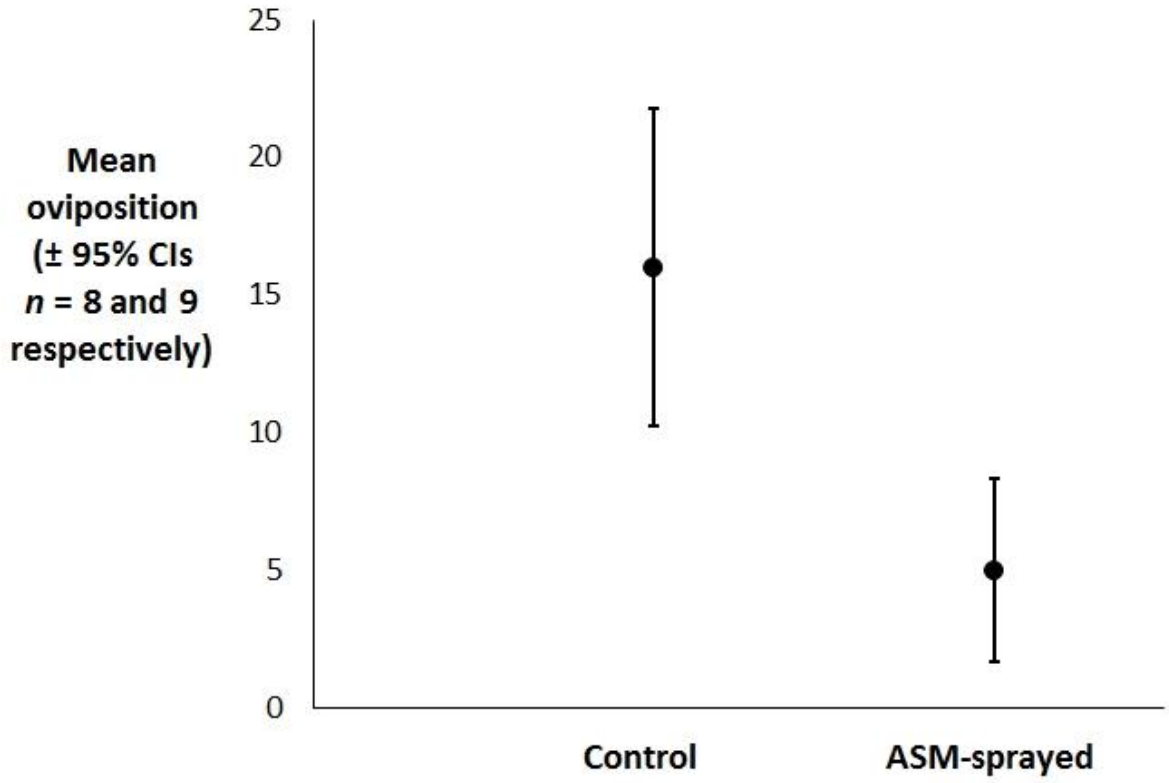


**Figure B1:** The mean number of eggs laid on either control tomato plants, or plants sprayed to run-off with 0.5 mM acibenzolar – *S* – methyl, with 95% confidence intervals ($n = 8$ and 9 for control and ASM-sprayed, respectively). The difference is significant according to a *t* – test ($p = 0.001$, $t = -3.99$, $df = 15$).

**Free Choice Assay Determining ASM Efficacy**

The method for the free choice assay was similar to that deployed in Chapter 2. Briefly, 90 whiteflies of equal gender mix were caught and placed in a 20 L Perspex tank containing three control tomato plants at the 3-4 leaf stage, and three tomato plants sprayed to run off with 0.5 mM ASM, equally spaced. Whiteflies were caught, anaesthetised, and deployed as previously described in Chapters 2 and 4, to achieve simultaneous whitefly release. Whiteflies had free settling choice over the course of 24 h, after which the number of settled whiteflies on each plant was recorded. The number of whitefly landing on control or ASM plants in each replicate was totalled and compared using Pearson's Chi squared test. Expected values were an even distribution between plant treatments. Significantly more whitefly settled on the control than the ASM-sprayed plants after 24 h in three of five replicates, and fewer whitefly were always observed on the ASM treatment. When all five values for each treatment were pooled and analysed using a *t* test, significantly fewer whiteflies settled on the ASM-sprayed plants over the whole assay than on the control ($p = 0.002$, $t = 4.44$ $df = 8$).

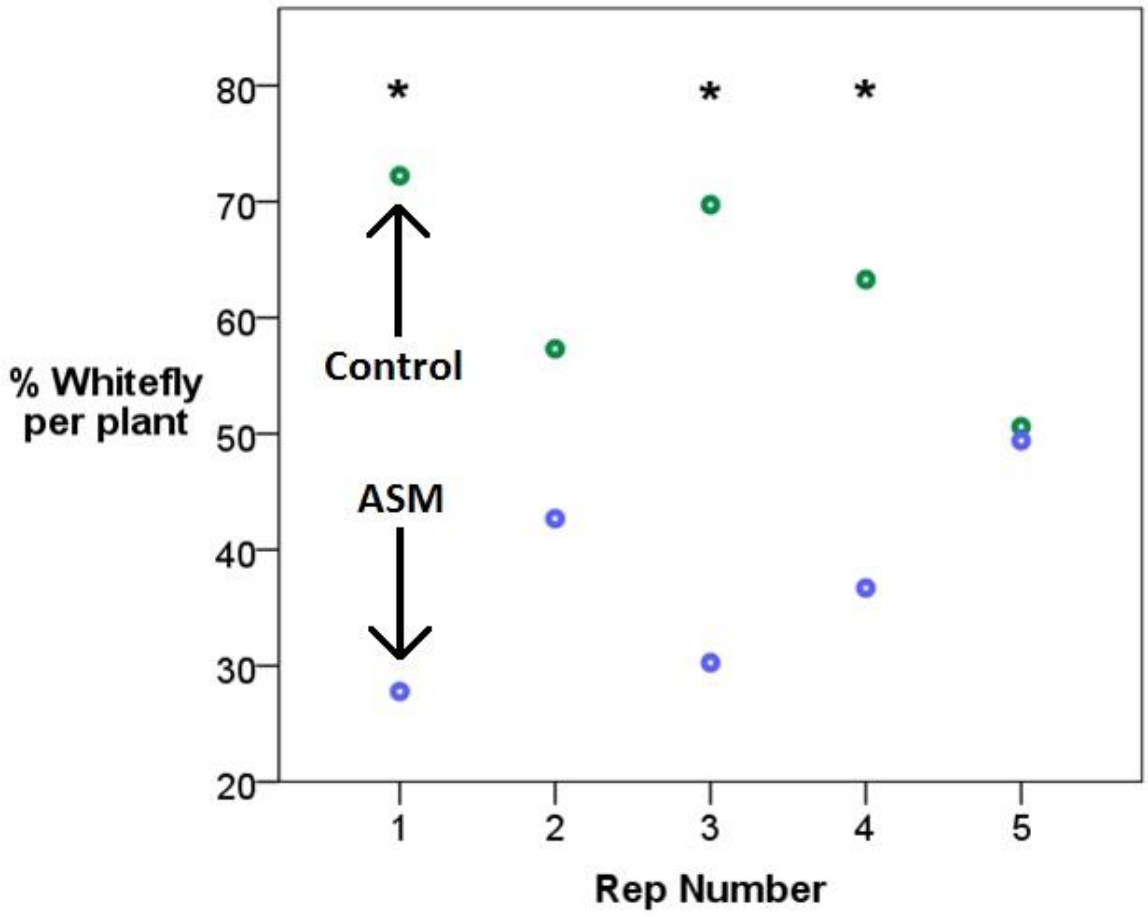


**Figure B2:** The percentage of 100 whitefly distributed amongst six tomato plants, half control and half sprayed to run-off with 0.5 mM acibenzolar – *S* – methyl, replicated five times. Differences for each replicate were analysed using Pearson's Chi squared test, with significantly more whitefly settling on the control than the ASM-sprayed plants after 24 h in three of five replicates, and fewer whitefly always observed on the ASM treatment. (*)

indicates significance of $p$ <0.05. Rep 1 $X^2$ = 14.22, $p$ < 0.001; Rep 2 $X^2$ = 1.76, $p$ = 0.19; Rep 3 $X^2$ = 11.84, $p$ < 0.001; Rep 4 $X^2$ = 5.58, $p$ = <0.05; Rep 5 $X^2$ = 0.012, $p$ = 0.92, $df$ = 1 for all reps.

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