Plant–virus–vector interactions: Tobacco rattle virus infection alters root volatile emissions to attract nematode vectors

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Abstract

Plant–virus–vector interactions mediate important ecological processes and can significantly reduce crop production. Almost all plant viruses require vectors for transmission and use sophisticated mechanisms to achieve this: they manipulate host plants to release volatile chemical that attract their vectors. This chemoattraction has been demonstrated in above-ground interactions but has never been studied in viruses transmitted by soil-dwelling nematodes. Tobacco rattle virus (TRV) is an important pest of potato, transmitted by trichodorid nematodes. This work uses a model plant system to investigate the effects of TRV infection on host root architecture, root volatile release and chemoattraction of its nematode vectors.

TRV infection alters root structure, producing a smaller and more compact root system. It modifies root volatile profiles compared to uninfected plants, which leads to trichodorids preferentially moving towards infected plants. The TRV genome contains genes known as 2b and 2c; implicated for nematode transmission. Mutations in these genes reduce the severity of root architecture symptoms, remove differences in the profile of volatiles released from infected roots compared with uninfected ones and, in the case of 2b, make plants less attractive to nematodes than plants infected with viruses without mutations. The release of the volatile 2-ethyl-1-hexanol significantly increased in roots infected by TRV. When added to uninfected plants, it made them more attractive to nematodes than untreated counterparts.

This work demonstrates TRV manipulates the production of host root volatiles, leading to increased attraction of trichodorid vectors. 2b and 2c are important in this interaction and 2-ethyl-1-hexanol is a strong component of the attractant signal. This new knowledge shows nematode transmitted viruses use similar mechanisms to attract their vectors as their better-studied aboveground counterparts and contributes to the study of volatile- mediated rhizosphere interactions with implications for agricultural pest control.

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Author's declaration

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Chapter 1 : General introduction

Plant–virus–vector interactions in ecology and agriculture

Plant viruses are arguably some of the most influential viruses on ecosystems, as plants comprise up to 80% of global biomass (Lefeuvre *et al.* 2019). The abundance and ubiquity of plant hosts allows for a broad diversity of plant viruses, with over 1,500 species currently described (Moreno and López-Moya 2020). Plant viruses manipulate not only the host plant but also the movements and feeding behaviour of viral vectors; those organisms that transport the virus from infected host to uninfected plants. Plant–virus interactions therefore form an important basis for communication between organisms within the ecosystem. These viral vectors are also an important food source for other trophic levels, both predators and parasitoids. By affecting the distribution of their prey, plant viruses affect these higher trophic levels.

Viruses make up 47% of emerging plant diseases and the majority of these are transmitted by vectors (Whitfield *et al.* 2015). Newly emerged diseases can be devastating for crops, particularly intensively farmed monocultures, and diseases caused by viruses increase with intensification (Roossinck and Garcia-Arenal 2015). For example, Rice yellow mottle virus, transmitted by beetles, has emerged as an economically important disease, particularly in East Africa, as a result of intensification (Rybicki 2015). Pathogens, including viruses, cause global crop losses of up to 16% annually, a figure which is increasing even while food crop yields also increase, as highlighted in the 2016 State of the World's Plants report (RBG Kew 2016).

Some agricultural plant viruses, such as Cucumber mosaic virus, are transmitted by multiple different vectors (Gallitelli 2000). Other virus–vector pairings are specific, and display complex interactions, suggesting a long history of co-evolution (Mauck et al. 2012). These long-term co-evolutionary interactions may determine the host range of the virus. A virus that relies upon generalist vectors that feed on a broad range of plants, and is therefore presented with a range of different hosts, is more likely to become a generalist themselves. Alternatively, viruses with vectors that are very selective in their feeding are likely to specialise. This pattern may be different for non-circulative viruses; for instance, aphid vectors of Cucumber mosaic quickly probe plants even if they do not feed on them. This allows non-circulative viruses held in aphid mouthparts

to quickly enter the plant, whereas circulative viruses require a longer feed for successful transmission.

Controlling viral diseases in agricultural systems is an acknowledged challenge for the 21st century. The begomovirus Africa cassava mosaic virus (ACMV) and other related viruses, transmitted by the whitefly *Bemisia tabaci* (McCallum *et al.* 2017), are a major threat to food security on the African continent with particular challenges for farming families who rely on cassava as a dietary staple (Rybicki and Pietersen 1999). The most widely adopted way of controlling these pathogens is the development of resistant varieties of cassava, rather than direct control of the vector or virus (McCallum *et al.* 2017). Cucumber mosaic virus, with its broad host range, is responsible for crop losses in several different crops, including tomato, melon, peppers and sunflowers (Gallitelli 2000). Cucumber mosaic virus is also challenging to control. Conventional measures are not very effective (Scholthof *et al.* 2011), as insecticides work too slowly to kill aphids before they are able to transmit the virus, due to its non-circulative transmission mechanism (Mauck *et al.* 2010).

This illustrates that in order to better manage agricultural viruses it is important to understand the various interactions between plants, viruses and their vectors. Transmission dynamics are mediated by complex interactions between all three parties, both in terms of attraction and in transmission mechanism between plant and vector (Mauck *et al.* 2014).

Plant virus–vector relationships

In order to survive, viruses need to propagate and be transmitted to new indivudals. A unique challenge for plant viruses is that their hosts are sessile, which makes direct contact between infected and uninfected individuals more difficult. Transmission can either be horizontal, from one individual to another, or vertical, from one individual to progeny. Some viruses can be transmitted through seeds and pollen, providing two sources of vertical transmission (Hamelin *et al.* 2016) and while rare examples of horizontal transmission by direct contact do exist (Lefeuvre *et al.* 2019), in the majority of cases this transfer is carried out by transmission vectors (Whitfied *et al.* 2015). Of those vectors, over 70% are insects (Dietzgen et al. 2016), primarily hemipterans such as aphids, whitefly, leafhoppers, planthoppers and thysanopterans (thrips) (Claflin *et al.* 2017, Moritz *et al.* 2004, Safari *et al.* 2019), which are well adapted to transmitting viruses due to their piercing and sucking mouthparts (Lefeuvre *et al.* 2019). A smaller number

of plant viruses are transmitted by mites (Rodrigues and Childers 2013) nematodes (Taylor and Brown, 1997), fungi (Campbell 1996) and protists (Tamada *et al.* 2013).

Relationships between virus and vector vary in length and specificity (Dáder *et al.* 2017, Whitfield *et al.* 2015). Some viruses occupy their vectors transiently, and are known as non-circulative, whereas circulative viruses enter the body of the vector, usually an insect. Non-circulative viruses can either be very short-lived and remain in the mouthparts of the vector, persisting for just minutes, or be semi-persistent and have the potential to remain within the insect until the next moult, with precise interactions between viral capsids and specific retention sites within the vector (Zhou *et al.* 2018). Circulative viruses enter the vector body and pass into the cells; of these some even propagate within the vector tissues (Whitfield *et al.* 2015). Therefore, it can be disingenuous to call some of these viruses purely "plant viruses" as they colonise and propagate in the non-plant vectors that transport them, and many potentially evolved from viruses that had non-plant hosts (Lefeuvre *et al.* 2019).

Typically, non-circulative viruses tend to be more generalist (Lefeuvre et al. 2019), while circulative viruses have higher virus–vector specificity. Gallet *et al.* (2018) suggested this to be due to an increased need for specificity of the protein–protein interactions between viral and vector proteins for circulative viruses. While circulative viruses must bind to epithelial cells in the gut before being endocytosed (Ammar et al. 2009), non-circulative viruses bind to cuticles in mouthparts (Webster et al. 2018) with the foregut as the primary target for semi-persistent viruses (Deshoux et al. 2018).

These interactions are not limited to purely viral–vector relationships. The multitrophic interactions of plant, virus and vector are co-evolved and complex (Pinheiro et al. 2019). Plants defend themselves against both virus and vector and it has been suggested that the virus/vector pair collaborate in response in a continuing evolutionary tug of war (Kersch-Becker and Thaler 2014).

Plant viruses affect plants in several ways and are often thought of as purely pathogenic, imposing a burden and producing purely negative consequences for the host. However, plants can be infected with multiple viruses at a time (Moreno et al. 2020) and not all have necessarily negative impacts on the host (Lefeuvre et al. 2019), although interactions between multiple viruses can often exacerbate symptoms of disease (Xu et al. 2003). It has been suggested that viruses may not be purely

antagonistic in their indigenous systems, causing little harm to their natural, uncultivated hosts, with suggestions that they may even lead to mutualisms (Roossinck 2015, Shates et al. 2019). Generally, viral infections still cause harm to their hosts, even if this may be reduced with less severe disease symptoms in unmanaged, stable populations (Fraile et al. 2017, Malmstrom and Alexander 2016). New diseases emerge when new ecological conditions are encountered and almost all studies of new disease emergence are centred on agriculture (Lefeuvre et al. 2019), where diseases move from wild hosts to managed cultures across borders between natural and managed ecosystems known as the "agro-ecological interface" (Alexander et al. 2014). Viral infection can cause many physiological changes within the host plant, often influencing the behaviour of their vectors, leading to greater dispersal rates. These have been reviewed extensively in the past decade (Carr et al. 2018, Dáder et al. 2017, Eigenbrode et al. 2018, Mauck et al. 2018), but these reviews have focused on insect vectors due to insufficient data for other vectors, such as nematodes. Viruses may indirectly affect their vectors by making host plants more attractive, leading to increased settling and feeding on infected plants (Eigenbrode et al. 2018), although specific feeding behaviours may be driven through different functional outcomes depending upon the circulatory nature of the virus (Mauck et al. 2012). Non-circulative viruses only persist in the vector for minutes but are quickly taken up; so making the plant unpalatable, or of lower nutritional value, to encourage the vector to find a different plant quickly may benefit transmission (Hodge and Powell 2008). Circulative viruses on the other hand are less easily taken up, and persist in the vector for longer, so increased palatability of the plant and improved survival rates through enhanced nutrition of the vector are preferable (Legarrea et al. 2015, Mauck et al. 2010). Circulative viruses that enter the tissues of their insect vectors can directly modify the behaviour of their vectors. This causes viruliferous vectors to prefer feeding on uninfected plants, while non-viruliferous vectors tend to prefer infected plants (Dáder et al. 2017).

There are two main mechanisms by which viruses can directly attract above-ground vectors to settle on host plants: colour and odour (Fereres and Moreno 2009). Aphids and other hemipteran insects are attracted to the colour yellow (Döring and Chittka 2007), and changes in leaf colour to lighter colours including yellow is a symptom of many different viral infections (Li *et al.* 2016). Preference tests carried out in light vs dark conditions confirm the importance of these visual cues (Eigenbrode *et al.* 2018). Olfactory attraction is mediated by volatiles released by infected plants. These

differ from healthy plants, apparently either by increasing the total concentration of emitted volatiles or by altering the concentration of a few compounds within the blend of volatiles emitted (Eigenbrode *et al.* 2018). The volatile cocktail emitted by virus-infected plants form crucial, long-range cues for vector behaviour in choosing hosts (Fereres *et al.* 2016). Understanding the volatile chemical cues that influence vectors is helpful to understand vector behaviour and could lead to options for better agricultural control. For example, volatile cues and vector preference from Barley yellow dwarf virus (BYDV) pathosystems have been well studied (Bosque-Pérez and Eigenbrode 2011, Ingwell *et al.* 2012), identifying (Z)-3-hexenyl-acetate as being present at significantly elevated concentrations, and overall volatile organic compound (VOC) concentrations being higher in the headspace of infected plants (Jiménez -Martinez *et al.* 2004). (Z)-3-hexenyl-acetate and several other compounds were found to be attractants for aphid vectors (Medina-Ortega *et al.* 2009). This may inform future integrated control methods for BYDV, although due to its highly effective dispersal, understanding the spatial and temporal dynamics is crucial (Van den Eynde *et al.* 2020) and identifying viral resistance in crop plants a critical focus (Choudhury *et al.* 2019). BYDV is transmitted by over 25 species of aphids and is probably the most economically important cereal virus (Walls *et al.* 2019). It can cause losses of 11-33% (Miller *et al.* 1997), and sometimes up to 80%, of spring cereals (Perry *et al.* 2000). Most of the work on identifying chemical cues from plants infected with viruses has been carried out on those transmitted by aphids. While these aboveground virus–vector systems, particularly aphids, have been well researched, little is known about chemical cues and vector attraction for viruses spread by non-insect, subsurface vectors such as nematode-transmitted viruses.

Nematode vectors of plant viruses

Nematodes, otherwise known as "roundworms", are a species-rich group of invertebrates that have spread to occupy incredibly diverse environments across the globe. They are ubiquitous in soils, freshwater, estuarine and marine sediments and exist at the extreme boundaries of life. Of these, it is estimated that there are over 41,000 species of plant parasitic nematodes, which constitutes about 15% of described nematode species (Quist *et al.* 2015). Plant parasitism evolved at least four times, forming four distinct lineages (Figure 1.1). It is likely that plant parasitism evolved from fungal feeding and each plant-parasitic clade contains basal species that are fungivores (Quist *et al.* 2015). While the majority of plant-parasitic nematodes feed on plant roots, a small number are aerial nematodes, which enter plants through the roots, travel upwards through the shoot and feed and reproduce on aerial plant tissue (Chin *et al.* 2018). Root parasitic nematodes can be endo- or ectoparasites; a few enter plant roots and feed and reproduce therein, while most feed externally and reproduce in the soil matrix.

All nematode vectors of plant viruses belong to those two most basal clades, (Figure 1) within Dorylaimida (*Longidorus, Paralongidorus* and *Xiphinema* spp., Taylor and Brown 1997) and Triplonchida (*Paratrichodorus* and *Trichodorus* spp., Taylor and Brown 1997). They are all migratory ectoparasites, able to move between suitable host plants and feed on the roots. They are also all polyphagous (King *et al.* 2011), able to feed on a variety of host plants, and depending on the species they feed on different parts of the roots (Taylor and Brown 1997). Some feed on root tips (*Longidorus*, *Xiphinema*), others on the root elongation zone (1-3mm behind the root tips) (*Paratichodorus*, *Trichodorus*, *Nanidorus*) or other parts of the actively growing root (*Xiphinema*).

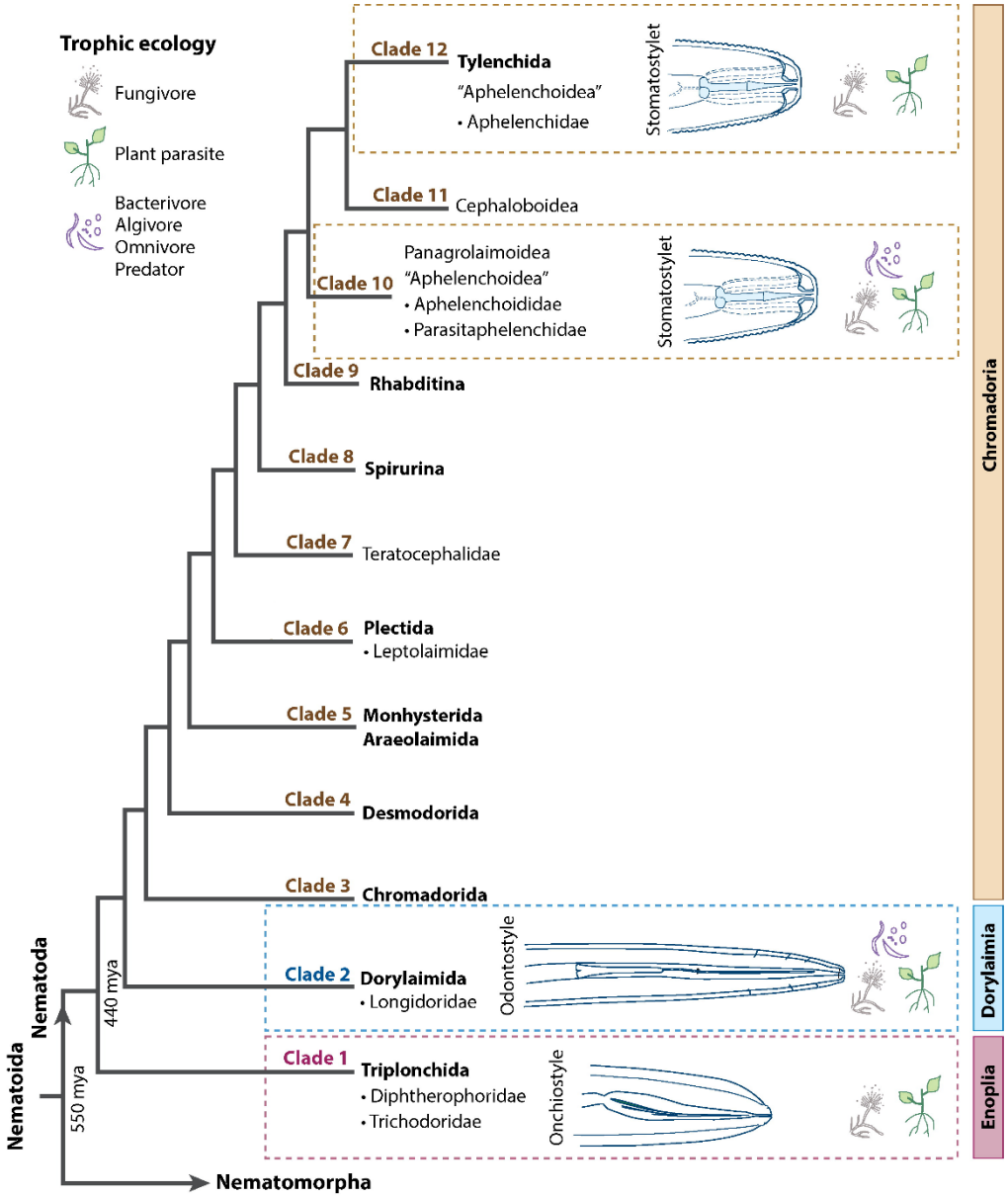

Figure 1.1 Taxonomy of phylum Nematoda highlighting the four clades of plant parasitic nematodes. (from Quist *et al.* 2015). The different mouthparts (onchiostyle, odontostyle and stomatostylet) are illustrated, although the morphology of stomatostylets in Clade 10 will differ from those pictured. All virus-vector nematodes are in Clades 1 and 2.

Trichodoridae: vectors of tobraviruses

There are currently 116 described species in the family Trichodoridae (Subbotin *et al.* 2020) and the didelphic genera of *Trichodorus, Paratrichodorus* and *Nanidorus* contain the 14 species known to act as vectors to the tobraviruses. The tobraviruses are a genus of three viruses—Tobacco rattle virus (TRV), Pea early browning virus (PEBV) and Pepper ringspot virus (PepRSV)—that are transmitted by trichodorid nematodes. The distribution of trichodorids is global (Taylor and Brown 1997), but particularly widespread in North America and Europe (Duarte *et al.* 2011), with the highest number of species per country (15) in Spain (Subbotin *et al.* 2020). In the UK, the most important and well distributed species are *Paratrichodorus pachydermus, Trichodorus primitivus* and *Trichodorus similis* (Boutsika *et al.* 2004) with high concentrations in eastern Scotland, Yorkshire and East Anglia. *P. anemones*, another vector species, is rare outside Yorkshire (Holeva *et al.* 2006). Trichodorids are almost exclusively found in free-draining sandy soils and rarely found in clay or silt-based soils, although exceptions exist (Taylor and Brown 1997). Small nematodes like trichodorids rely on films of water to move through soils and it has been suggested that densely packed soils of predominantly small particles such as silt and clay are impassable to trichodorids (Jones *et al.* 1969).

Feeding by trichodorids leads to swelling and galling of roots and can stunt root growth, eventually leading to root decline (Figure 1.2); hence the epithet "stubby root nematodes". When feeding, trichodorids initially explore the root surface with their lips to find a suitable feeding zone, then use their onchiostyle, a solid spear shaped stylet located in the oesophagus at the anterior end of the body, to pierce through the cell wall. A feeding tube is then formed around the onchiostyle, allowing for suction of cell contents into the nematode. After the initial penetration, the nematode continues thrusting the onchiostyle at a rate of 5-6 thrusts a second, which accompanies secretions that liquify the cell contents and allow them to pass down the feeding tube (Brown *et al.* 2003). Due to the shortness of the onchiostyle relative to other nematode feeding stylets like the longidorid odontostyle (see Figure 1.1), trichodorids are only able to feed on epidermal cells (Singh *et al.* 2020).

Figure 1.2 Effects of trichodorid feeding on onion plants. (photo by Dr Hajihassani, University of Georgia).

Virus particles are included in the liquified contents of cells that are ingested through the feeding tube and are retained in the feeding apparatus of nematodes, bound to the cuticular lining of the oesophagus in trichodorids (Taylor and Robertson 1970 and Figure 1.3). Retained virus is released during feeding when the initial gland secretions are released and wash over the virus; this is thought to occur as a result of changes in pH, which brings dissociation from retention sites (Taylor and Robertson 1977).

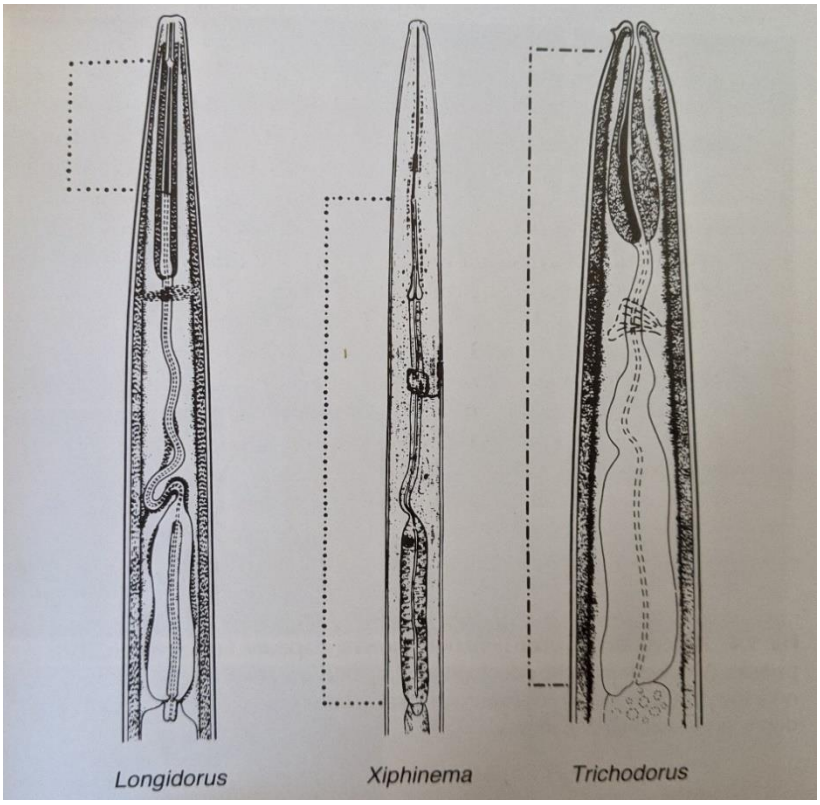

Figure 1.3 Figure 1.3 Virus retention sites in the feeding apparatus of vector nematodes. (from Taylor and Brown 1997).

Like most nematodes, trichodorids have six life stages: egg, four juvenile stages and adult. However, unlike endoparasites, of either plants or animals, all stages take place in soils. Trichodorids moult between juvenile instars and before becoming adults, in which the cuticle separates from the underlying layers, a new cuticle forms and the old one sheds. This shedding includes the oesophageal lining, including the odontostyle, which leads to loss of any retained virus during moulting (Singh *et al.* 2020). Apart from this loss, viruses can persist for a long time within their vector, and it has been demonstrated that trichodorids are capable of serially infecting multiple plants (van Hoof 1964).

Nematode chemotaxis is well documented in the literature, Nematode chemotaxis is well documented in the literature, with attractive compounds ranging from ketones and terpenes to organic acids, alcohols and carbon dioxide (Rasmann *et al.* 2012).. Chemotaxis has been shown to exist in at least three of up to seven taxonomically heterogeneous functional types: plant-parasitic (e.g. Ali *et al.* 2011, Wang *et al.* 2009, Farnier *et al.* 2012), entomopathogenic (e.g. O'Halloran and Burnell 2003, Hallem *et al.* 2011, Ali *et al.* 2011, Rasmann *et al.* 2005) and the bacteriovorous (Hallem *et al.* 2011). However, no published examples of trichodorid chemotaxis exist. Nematodes have

internal and cuticular sense organs; the internal sense organs are thought to respond to light and mechanical cues, while the cuticular respond to a wider range of cues including chemical (Rasmann *et al.* 2012). The most important sense organs for chemoattraction are the amphids, which are pairs of innervated depressions in the cuticle found at the anterior end of the nematode. Many nematodes, such as *Caenorhabditis elegans*, have further chemoreceptors at the posterior end called phasmids (Hilliard *et al.* 2002), but these are lacking in trichodorids (Holterman *et al.* 2006). There is well-documented evidence of root volatile organic compounds (root VOCs) being released as a result of plant wounding by herbivores, attracting entomopathogenic nematodes (reviewed in Turlings *et al.* 2012).

It would seem very likely that there exists a combination of these two systems, wherein trichodorid vectors respond to similar attracting signals from plant roots, which in this case are exaggerated by viral infection.

Global impact of tobraviruses

. The type virus, TRV, has a wide host range of over 50 plant families and is the cause of multiple diseases in crop plants: stem mottle and spraing disease in potato, rattle in tobacco (so named because infected leaves dried and rattled in the wind) , and yellow blotch in sugar beet, as well as diseases in horticultural plants and weeds (King *et al.* 2011). In some cultivars of potato, TRV infection can produce arcs, flecks and rings of necrotic material in the tubers (MacFarlane 2010), referred to as spraing (UK) or corky ringspot (USA) disease. These marks (Figure 1.4), combined with a reduction in size of the tubers, can make the crops unmarketable (Sahi *et al.* 2016).

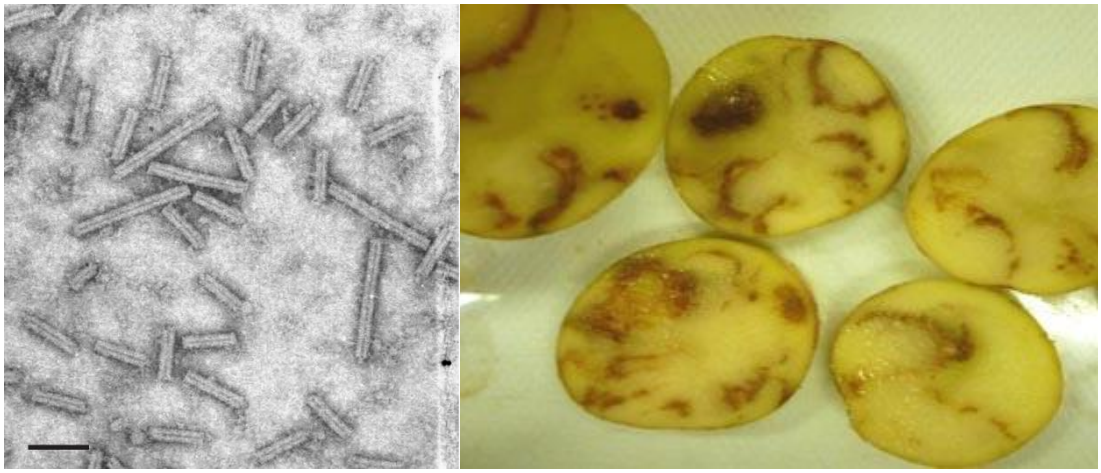

Figure 1.4 Electron micrograph of long and short Tobacco rattle virus particles and symptoms of spraing disease in cut potato tubers. (from MacFarlane 2008 and hutton.ac.uk). The bipartite Tobacco rattle virus genome is encapsidated separately in two particles of different sizes and can cause spraing disease when present in potato tubers. The scale in the micrograph=100nm.

Virus populations that exist now were probably established in the last 150 years, with movement via agricultural practice of infected potato seed tubers (Dale and Neilson 2006), a problem potentially exacerbated by the movement of infected symptomless tubers that were then a source of virus for transmission (Xenophontos *et al.* 1998). This has led to the virus becoming established in large, static nematode populations with common weed species acting as reservoirs for the virus in the field between potato harvests and during crop rotations. Current methods for controlling free living plant-parasitic nematodes such as trichodorids are inefficient and rely on nematicides such as aldicarb, which has been withdrawn in the UK (Dale and Nielson 2006). Combined costs of damage to potato crops and nematicide application have been conservatively estimated to be £2.2 million per annum in the UK (Dale and Nielson 2006).

The TRV genome is split into two single-stranded, positive-sense RNAs that are encapsidated separately in two rod shaped particles of different lengths (Figure 1.4, MacFarlane 1999). The larger (L) particle that contains RNA-1 is about 185 nm in length and carries a replicase gene, a movement protein (1a) gene and a cysteine-rich protein gene (1b), and is involved in replication, co-ordination and movement. The smaller (S) contains RNA-2 which carries the gene for the viral coat protein (CP) but

also encodes one or two non-structural proteins, 2b and 2c (Figure 1.5, MacFarlane 2010), depending on the isolate. The structure and size of these genes can vary between isolates (Vassilakos *et al.* 2001) which impacts the size of the particle; the particle can range from 46-115nm in length (MacFarlane 2008).

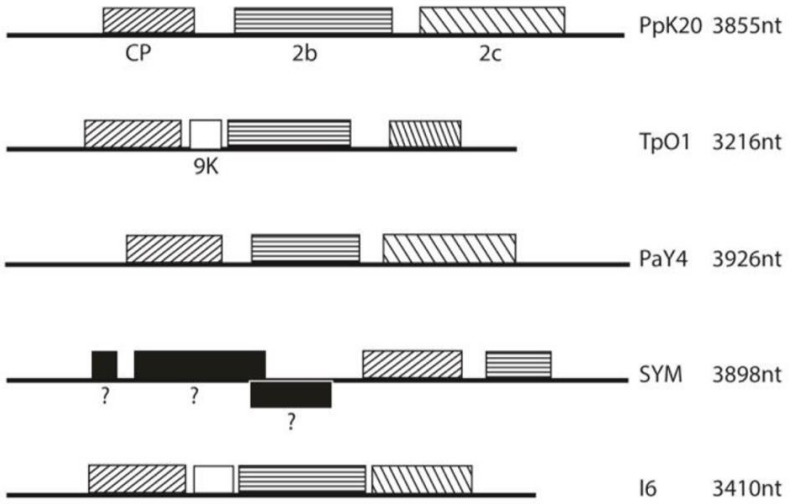

Figure 1.5 Genome organisation of various RNA-2 isolates showing the diversity and distribution of genes (from Sahi 2016). Isolate name and the RNA-2 size are shown at the right of the figure. The gene names are shown under the diagrams and boxes with the same fill pattern denote genes conserved across isolates. Expression of SYM genes shown with ? have been not demonstrated experimentally.

Early work on TRV identified two different types of infection: non-multiplying (NM) and multiplying, or particle producing (M) (Harrison and Robinson 1978), with the latter being a full infection with both RNA particles present. Non-multiplying infections occur when only RNA-1 is present and are non-transmissible by nematodes. Although they cannot form encapsidated particles due to the lack of CP gene they are able to spread systemically through plants and were reported to cause more severe symptoms in potato (MacFarlane 1999).

Potato cultivars differ in their ability to resist TRV infection and can be split into three categories depending on their response to infection (Dale and Neilson 2006). The first are fully resistant (TRV-resistant) to infection and do not exhibit spraing symptoms to any isolates, apart from to the PpO85 resistance-breaking isolate (Robinson 2004). The second are spraing reactant (TRV-sensitive) and exhibit an intermediate response to infection through production of spraing symptoms. This infection is usually a NM infection (MacFarlane 2010) and transmission is only possible vertically through daughter tubers. The third group are tolerant (TRV-susceptible). These are often infected with full (M) infection with both virus particles, although they do not exhibit

spraing symptoms, other than a reduction in number and size of tubers, affecting crop yield (Dale *et al.* 2000, 2004).

Different tobravirus isolates have virus–vector specificity with different trichodorid species e.g. TRV isolate PpK20 is transmitted by *Paratrichodorus pachydermus* and not *Trichodorus primitivus* while PEBV isolate TpA56 is transmitted by *T. primitivus* and not *P. pachydermus* (MacFarlane *et al.* 1999). TRV PaY4 can be transmitted by *P. pachydermus* and *Paratrichodorus anemones*, which can transmit PEBV but not TRV PpK20 (Vassilakos *et al.* 2001). It has been suggested that the selection of isolate–vector pairing is due to an interaction between the CP and 2b protein acting together.

2b has been implicated for nematode transmission, as a protein encoded for by the 2b gene specifically has been suggested to form a bridge between the nematode esophogeal surface and the virus particle (Bragard *et al.* 2013) and transmission cannot occur without it for TRV PpK20, PaY4 or PEBV TpA56 (Hernández *et al.* 1997, Vassilakos *et al.* 2001). It is also required for efficient movement of the virus into the root (Valentine *et al.* 2004). The 2c protein greatly increases efficiency of transmission in PEBV TpA56 (MacFarlane *et al.* 1996, Schmitt *et al.* 1998) but does not appear to be required for TRV PpK20 nematode transmission (Hernández *et al.* 1997). Some TRV isolates, which probably arose through deletion and recombination of RNA-2, are missing one or both 2b and 2c genes, making transmission by nematodes impossible.

Impact of plant volatiles

Plant volatiles, or volatile organic compounds (VOCs), are a subset of secondary metabolites produced within plants. Plant secondary metabolites (PSM) are specialised and specific substances that are not associated with essential functions of metabolism (Field *et al.* 2006) but contribute to plant fitness by responding to changes in the environment (Bourgaud *et al.* 2001). PSMs exuded by plant roots into the rhizosphere can help with foraging and uptake of scarce or biologically unavailable micronutrients (Metlen *et al.* 2009), such as through the release of carboxylates from cluster-roots to assist with phosphorus uptake (Li *et al.* 2007). They also have a role in plant protection, from abiotic stresses and herbivore defence to host pathogen resistance, including viruses (e.g. Berini *et al.* 2018, Joo *et al.* 2019, Lan *et al.* 2020, Landoni *et al.* 2020).

PSMs fulfil another important ecological role for plants: communicating with other organisms (Massalha *et al.* 2017), including signalling danger to other parts of the same

plant or other plants (Karban *et al.* 2006). Some PSMs are attractants for pollinators and seed dispersers (Midgley *et al.* 2015, Schiestl and Roubik 2003, Suinyuy and Johnson 2018) while others mediate interactions with beneficial microbes, such as flavonoids acting as chemoattractants for rhizobia in legumes or root exudates recruiting microbes to attack plant-parasitic nematodes (Cooper 2007, Topalović *et al.* 2020).

Roots tend to release a mixture of exudate, including low volatility, soluble compounds for short-distance communication and high volatility compounds that travel further and faster through gaseous diffusion than soluble compounds (Massalha *et al.* 2017). These root VOCs are often distinct from those released from leaves and the profiles show little or no overlap within species (Peñuelas *et al.* 2014). Root-derived VOCs are used for a variety of interactions with other organisms, including neighbouring plants (Ens *et al.* 2009, Jassbi *et al.* 2010) and herbivores (Hu *et al.* 2018). Volatiles can also be used to modify plant–herbivore interactions of neighbouring plants, making competitors more susceptible to attack (Huang *et al.* 2019). Root VOCs are incredibly diverse, and mixes can include fatty acid derivatives, terpenes, phenylpropanoids and benzenoids, as well as sulphur-containing compounds including glucosinolate-breakdown products (Peñuelas *et al.* 2014).

Viruses can induce changes in the volatiles produced from infected plants and use these to indirectly attract insect vectors (Claudel *et al.* 2018). While a majority of work has focused on aphid-transmitted viruses from the family *Luteoviridae* (Bosque-Pérez and Eigenbrode 2011), such as Potato leaf roll virus, Barley yellow dwarf virus, and Turnip yellows virus, this mechanism is found in other virus families and with different insect vectors. For example, two aphid-borne raspberry viruses, Black raspberry necrosis (*Secoviridae*) and Raspberry mottle virus (*Closteroviridae*), altered volatile emissions and increased the soluble amino acid concentrations in leaves (McMenemy *et al.* 2012). Whitefly has been shown to respond to volatiles produced by Tomato chlorosis virus and Tomato severe rugose virus (Fereres *et al.* 2016), a crinivirus and a begomovirus respectively, and Maize chlorotic mottle virus has been shown to attract vector thrips (Mwando *et al.* 2018).

It is possible that volatiles may also play a role in the preferences seen between viruliferous and non-viruliferous vectors of circulative plant viruses (Fereres *et al.* 2016, Medina-Ortega *et al.* 2009, Ngumbi *et al.* 2007), where viruliferous vectors prefer healthy plants and non-viruliferous, infected plants (Ingwell *et al.* 2012, Rajabaskar *et al.* 2014).

TRV infection has been shown to affect leaf secondary metabolites within plant tissues (Fernández-Calvino *et al.* 2014) but no study on volatile emissions either above or belowground has been carried out.

Purpose of thesis

This thesis sets out to explore the effects of TRV infection on root volatile emissions and how these affect the behaviour of vector trichodorids, in order to better understand the plant–virus–vector interactions around transmission to potentially inform pest management. With the knowledge that plant viruses use volatile cues to attract aboveground vectors for transmission, and that examples exist of nematodes responding and moving towards root-derived VOCs, we sought to affirm that TRV–trichodorid interactions make use of attractant volatile cues to aid transmission and to identify which chemical(s), if any, define this signal (Figure 1.6).

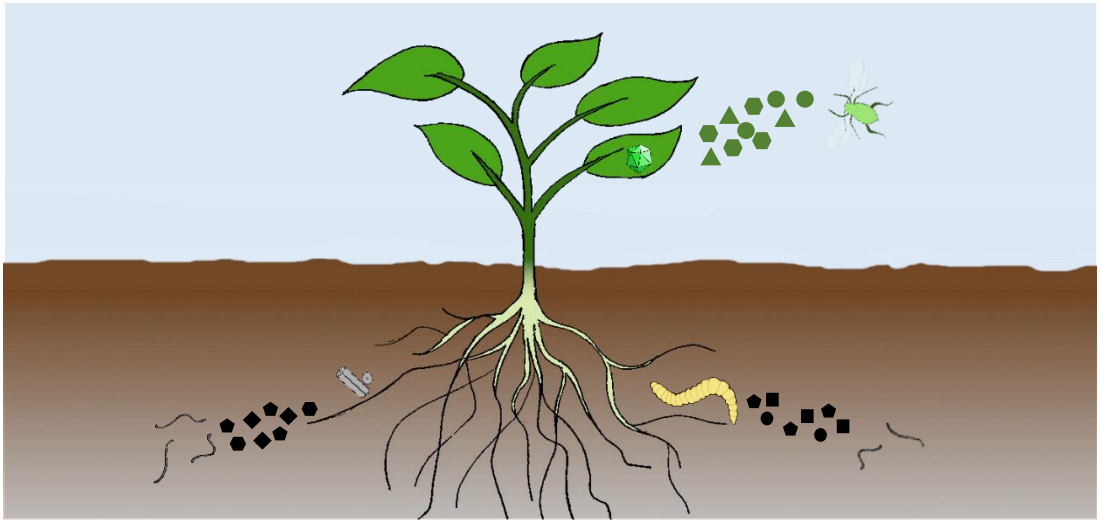

Figure 1.6 Examples of volatile-mediated interactions that provided the background for this thesis. Viruses mediate green leaf volatiles to attract vector arthropods, and belowground herbivory stimulates release of root VOCs which recruits entomopathogenic nematodes.

While the act of transmission of TRV by trichodorid nematodes is well understood, including the genetic differences between some of the different isolates, the presence of an attraction effect towards infected plants was unknown, as was any potential cause for that signal. In addition, the direct effects of TRV infection on root growth in isolation from trichodorid feeding were not well understood.

Chapter 2 explores the foundational elements of this research question. Three aspects were under investigation: 1) if the changes seen in root architecture after feeding by trichodorids (stubby root nematodes) are due entirely to the action of nematode feeding, or if TRV infection alters root architecture in a way to be partially responsible for these effects; 2) if TRV-infected plants release different volatiles, or volatiles at more elevated concentrations than uninfected plants, in line with previous

work on other plant viruses; and 3) if trichodorid nematodes preferentially move towards TRV-infected plants when offered a choice between infected and uninfected plants.

Using *Nicotiana benthamiana* (Solanaceae) as a model organism, plants were infected with TRV and measures of different root morphological traits were taken, and root volatiles analysed. This is the first study to have looked at the volatiles emitted from the roots of TRV-infected plants. Some plants were also infected with a TRV mutant missing two non-structural genes from RNA-2 that have been implicated in nematode transmission of TRV. Trichodorids were tested in olfactometers and given a choice between an uninfected plant and a plant infected with TRV. These initial experiments were necessary to show that these interactions were taking place, before trying to identify chemoattractant agents or the genetic underpinnings in viral infection responsible for this volatile manipulation.

The experiments in Chapter 2 were exploratory and proof of concepts to identify whether these effects were present. In Chapter 3, I refined our methodology and confirmed my original research questions. The hypothesis that the effects seen on root morphology are transient was tested by extending the length of the experiment, and plants infected with TRV mutants with loss of function mutations in just one of the RNA 2-genes were compared to uninfected and wild-type TRV-infected plants for root architecture and volatile emission. In order to see greater difference in root volatile emissions, the sampling time of the volatile experiments was increased. RNA-2 2b loss of function mutants were also tested against uninfected plants and wild-type infected plants in nematode preference trials.

In Chapter 4, the volatile found to be most expressed in the VOC profiles of infected roots, 2-ethyl-1-hexanol, was investigated to see if it was the causative agent of the attraction seen in previous chapters. The first hypothesis is that addition of 2-ethyl-1-hexanol to the rhizosphere of an uninfected plant makes it more attractive than a control uninfected plant. The second hypothesis is that addition of 2-ethyl-1-hexanol to a 2b mutant infected plant makes it as attractive to trichodorids as a wild-type TRV infected plant.

The final chapter is a discussion of the results presented in this thesis and the evidence they provide for suggesting that TRV–trichodorid interactions are mediated by volatile

cues. It draws together the evidence from the other chapters with regards to the presence of a volatile signal, how this seems to be affected by the presence of certain RNA-2 genes, how these may affect infection intensity and so the strength of the signal and the possibility of 2-ethyl-hexanol as the first identified compound of that signal. It reflects on the importance of developing understanding of virus–vector relationships in neglected belowground systems and how this information fits into the understanding of chemically mediated plant interactions. It explores the ecological context of this plant–virus–vector interaction, and the ramifications for TRV as an agricultural pest; how this information may be applicable within that context; and that root volatile emissions may be important when considering resistance for TRV for crops and as part of an integrated pest management solution.

Chapter 2

Introduction

Multitrophic interactions, in which plant viruses alter host plants to manipulate vectors in order to aid transmission to novel hosts, are common (Ziegler-Graff 2020). As previously discussed, the majority of these are spread by insects (Bragard *et al.* 2013, Dietzgen *et al.* 2016, Ziegler-Graff 2020), particularly hemipteran insects such as aphids, whitefly and mealybugs (Hogenhout *et al.* 2008), which are well adapted for virus transmission. Of the remainder, most are transmitted by soil borne vectors, predominantly nematodes (Bragard *et al.* 2013). Trichodorid nematodes are root feeding nematodes that are vectors for the tobraviruses: Tobacco rattle virus (TRV), Pea early browning virus (PEBV) and Pepper ringspot virus (PepRSV).

Nicotiana bemthamiana is a widely used model organism in plant virology (Bhaskar *et al.* 2009, Goodin *et al.* 2008, Hogenhout *et al.* 2009, Ma *et al.* 2012) due its susceptibility to pathogens, including viruses, compared to other model organisms such as *Arabidopsis thaliana* and continues to be used in a variety of host–pathogen research (e.g. Ibrahim *et al.* 2020, Nath *et al.* 2020, Prator *et al.* 2020). A reason for this hyper susceptibility to plant viruses is due to a naturally occurring mutation in an RNA-dependent RNA polymerase gene (Yang *et al.* 2004).

N. benthamiana is attractive as a model organism in this study as it is much easier to infect mechanically than potato; is quicker to grow and remains a manageable size for data collection; and it is susceptible to a variety of plant viruses, including TRV. *N. benthamiana* is easy to infect with TRV using agroinfiltration. In agroinfiltration, transfer DNA (T-DNA) of the tumour-inducing plasmid *Agrobacterium tumefaciens*, a bacterial pathogen of dicot plants with a broad host range, is transformed to carry binary vectors containing the viral genome (Du *et al* 2014, MacFarlane 2010). A suspension containing the transformed *Agrobacterium* is inoculated with a needleless syringe to the abaxial surface of leaves (Du *et al.* 2014, Goodin *et al.* 2008, Senthil-Kumar and Mysore 2014). This then enables viral particles to spread through the plant, including into the roots (Valentine *et al.* 2004). Mechanical infection of potato is unreliable, often leading to localised, low level infection (Ghazala and Varrelmann 2007, Sahi *et al.* 2016) and infrequent system spreading of TRV to other tissues (Sahi *et al.* 2016) including roots. Potato can be more reliably infected by the feeding action of viruliferous nematodes in

field conditions, but this is not satisfactory for this study, as it is impossible to know the time of infection. If effects of infection are transient, no comparisons can be made between plants. For these reasons, even though the sensitivity of *N. bethamiana* to plant viruses may increase the severity of symptoms of TRV infection compared to natural environments, it was an ideal candidate for these investigations.

Kollman and colleagues (2007) found differences in aboveground biomass of *Impatiens glandulifera* in continental Europe due to potential TRV infection. In potato, TRV infection affects plant emergence, crop yield and overall reduced biomass (Dale *et al.* 2000, 2004). However, infection has not been shown to have stronger or targeted effects on root allocation and biomass. TRV infection has been shown to be transient and viral load reduces 10 days post-infection (Valentine *et al.* 2004, Vassilikos *et al.* 2001). However, even a transient viral infection can impact biomass production and allocation, altering root morphology. There are many examples of plant–virus interactions where infection had a significant effect on growth (Chen *et al.* 2017, Dolenc *et al.* 2000), aboveground architecture and leaf size and colour (Bazzini *et al.* 2011, Zaitlin and Hull 1987).

Root architecture has been shown to be sensitive to a variety of different infections and attacks (e.g. Cordovez *et al.* 2017, Hetrick 1991, Hishi *et al.* 2017, Hodge 2004, Lu *et al.* 2020, Ma *et al.* 2013, Razavi *et al.* 2017, Treonis *et al.* 2007 Villordon and Clark 2014). These range from beneficial mycorrhizal colonisation to nematodes, fungal pathogens, and viruses, but no information exists in the literature on the impact of TRV infection on this trait. Trichodorid feeding can result in decline of root growth (Pitcher 1967) and development of root swelling and galling in certain plant species (Taylor and Brown 1997), but there have been no studies to confirm if this effect is exacerbated by TRV infection of the host plants, or if viral infection has no effect. If this reduction in root growth is partially due to TRV infection, decreases in plant biomass may be greater for root systems than above ground.

For successful vector-borne transmission, the virus must be able to enter the vector from the host, persist long enough within the vector to reach another host and be able to leave the vector and successfully infect the new host. These processes are well understood for TRV virus–vector interaction (MacFarlane 2010). When trichodorid juveniles and adults feed on roots, they disrupt and destroy cells around the feeding site with injected enzymes delivered by a hollow stylet-shaped mouthpart and the resulting

plant tissues, including viral material, are ingested. The viral particles can remain in the nematode for up to two years but are non-persistent (Ng and Falk 2006), which means they do not enter the body of the nematode; they remain in the nematode oesophagus (Taylor and Robertson 1970) before being regurgitated with the digestive enzymes at a novel feeding site.

However, at present little is known about the mechanism with which TRV attracts potential vectors to infected plants, or if such a mechanism exists. There are multiple ways in which viruses can alter their host to manipulate their vectors. Alterations to host physiology to attract vectors with sensory cues and changes to feeding behaviour are two of these mechanisms and are covered in multiple recent reviews, which focus on aboveground insect–vector interactions (Blanc and Michalakis 2016, Carr *et al.* 2018, Dáder *et al.* 2017, Eigenbrode *et al.* 2018, Mauck 2016). The translation of these well-known mechanisms in aboveground systems to this virus–vector relationship is important for understanding drivers of transmission of TRV.

A possible explanation for attraction to infected plants is the production of sensory volatiles, which are released into the rhizosphere and detected by trichodorids. The manipulation of plant volatiles by viruses to attract vectors is well documented and reviewed (Groen *et al.* 2016, Hammerbacher *et al.* 2019, Roossinck 2015), as is nematode response to chemical cues, including volatiles (Rasmann *et al.* 2012), but the response of nematode vectors to virus-mediated volatiles has not been studied.

There are many common techniques for sampling and analysing volatiles and other compounds produced and released by plants. The most suitable method will depend on the biological questions asked, the conditions the plant is in and what part of the plant is being sampled (Tholl *et al.* 2006). Some techniques involve destructive sampling of plant tissues (Rasmann *et al.* 2005), which are easier to carry out and can have a greater accuracy in collection of volatiles produced within tissues, but they are not necessarily an accurate measure of what is released and detectable in the environment. They may also produce additional volatiles from a wounding response. Non-destructive sampling techniques present the challenge of isolating the plant material being tested (e.g roots, leaves, flowers) from the rest of the plant and only sampling compounds produced and emitted from those areas, while simultaneously avoiding loss of sample (REF). Whether the sampling is destructive or non-destructive, there are then multiple options of collection technique, and different collection

techniques can create bias towards different classes of volatile or semi-volatile compounds (Rering *et al.* 2020).

These collection methods can be separated into static and dynamic headspace techniques. In static techniques, the plant or section being sampled are fully enclosed in a chamber and emitted volatiles are allowed to accumulate within the chamber before being sampled. These volatiles can either be trapped on an adsorbent, such as solid phase microextraction (SPME) fibres, or a direct subsample of the air removed for analysis. SPME is an incredibly popular sampling technique that relies on the collection of volatiles or semi-volatiles on a fibre and the subsequent thermal desorption to a gas chromatograph. Part of the power of this analysis method is the ability to switch between different fibre types to collect different classes of volatiles and semi-volatiles (Alborn *et al.* 2021, Pontes *et al.* 2012, Rering *et al.* 2018), giving excellent ability to collect different compounds. In both cases, there is no contamination or dilution of the sampled volatiles due to a continuous air stream. Conversely, this also results in accumulation of moisture and heat, particularly if the plant is being sampled under illumination, either during daylight in the field or in lab conditions in a light cycle, which can affect the volatiles collected (Tholl *et al.* 2006). A further limitation of SPME static sampling is that the resultant volatile profiles may not be true to the gas phase composition and may bias the resulting chromatogram patterns towards the compounds the fibre is better at ab/adsorbing (Alborn *et al.* 2021). Direct headspace subsampling with air that is subsequently cryofocused on-column before injection to a gas chromatograph does not suffer from these drawbacks but can have poor effectiveness and miss compounds at low concentrations (Rering *et al.* 2020).

Dynamic headspace techniques have a continuous flow of air flowing through the sampling chamber as a carrier gas, which is often air filtered through a trap to scrub impurities and contaminants, such as activated charcoal. The air leaving the chamber passes through an ad/absorbent trap filled with polymers such as Porapak Q/Super Q/Hayasep Q that are preconditioned before use with a solvent (e.g. Jassbi *et al.* 2010, Steen *et al.* 2019, Thöming *et al.* 2014). The compounds are then eluted with a solvent, and an internal standard may be added, which helps with quantification of the total amount of compounds collected and injected onto a gas chromatograph. Instead of solvent extraction, adsorbent materials such as Tenax can be used for thermal desorption rather than the polymers mentioned above. Solvent extraction has

drawbacks in terms of dilution of the sample in the solvent and reduced extraction efficiency compared to thermal desorption. On the other hand, it does allow repeated sample injections and avoids artifacts due to the breakdown of thermally instable compounds (Tholl *et al.* 2006). Integration of SPME fibre methods with dynamic headspace technique in solventless volatile collection methods have been very popular and different methods have been compared extensively (Alborn *et al.* 2021, Rering *et al.* 2020, Yuang *et al.* 2013). Dynamic headspace techniques hold advantages over many static headspace methods in being able to pre-concentrate the collection of volatiles and semi-volatiles onto a trap and to compare volatile emissions at different time points. Static headspace cannot do this effectively as not all compounds are removed from the chamber in sampling, so accumulation of compounds of interest interferes with potential changes in emission rates over time. However, dynamic headspace sampling requires sampling times lasting multiple hours (Steen *et al.* 2019), which subjects plants to considerable water stress when sampling root tissues.

Here, *Nicotiana benthamiana* is employed as a model organism to provide an initial overview of the effects of TRV infection on plant biomass and root allocation; the differences in root volatiles emitted from intact root systems between infected and uninfected plants; and to test whether TRV-infected plants are more attractive to trichodorid nematodes than uninfected plants. The potential hypersensitivity to viral infections reported in *N. benthamiana* may be of benefit in this initial study to try to identify novel effects of TRV infection.

Many different isolates of TRV exist, and these are specific to different trichodorid species, with some specific isolate–species pairings (Asfaq *et al.* 2011, Boutsika *et al.* 2004, Crosslin *et al.* 2003, Holeva *et al.* 2006, MacFarlane and Brown 1995, Ploeg and Brown 1997, Ploeg *et al.* 1992). This presents a complication because while the genome of RNA-1 is relatively conserved across isolates, different isolates of TRV RNA-2 do not have conserved sequences, and as such there is no standardised RT-qPCR test for all isolates (Sahi *et al.* 2016). Therefore, in order to accurately compare levels of infection, all plants must be infected with the same RNA-2 isolate.

The RNA-2 isolate used in this study was TRV-PpK20. This isolate is one of the earliest TRV isolates to have a well-characterised virus–vector interaction (Hernández *et al.* 1995) and is one of the best understood. It is also transmitted by *Paratrichodorus pachydermus,* one of the most economically important and widespread

trichodorid nematodes in the UK (Boutsika *et al.* 2004). The trichodorids used in this work are bulk populations from agricultural soil samples from across the UK, but particularly from Scotland, where this isolate was first identified. TRV-PpK20 has three genes present on RNA-2, the coat protein gene (CP) and two non-structural genes, 2b and 2c (Hernández *et al.* 1995, Hernández *et al.* 1996, MacFarlane 1999). 2b is necessary for nematode transmission (Hernández *et al.*1995, MacFarlane *et al.* 1996), possibly due to the formation of bridges linking the 2b protein to sites within the nematode oesophagus, enabling retention within the vector (MacFarlane 1999).

This study investigated root morphology, root volatile emissions and nematode attraction, all of which relied on whole root systems and non-destructive sampling in the case of volatile capture, and non-disruptive sampling for testing nematode attractiveness. Growing plants in soil or within substrate poses additional complications for non-destructive sampling of roots compared to hydroponic systems due to the increased potential of damage to roots.

Work on root architecture is challenging, due to the difficulty of observing roots in soils and harvesting plants without causing damage to fine roots. Techniques like microrhizotrons (Bates 1937, Bragg *et al.* 1983, Faget *et al.* 2010, Lu *et al.* 2019, Lu *et al.* 2020, Svane *et al.* 2019) allow collected measurements over time *in situ*, avoiding the risk of destruction of the finer architecture. However, image analysis is time consuming, especially when analysing entire root systems, and these approaches can lead to underestimates in root length (Vamerali *et al.* 2012). Without extracting the roots, it is also impossible to remove some material to test for successful transmission of the virus to the roots. Computerised analysis of scanned images of whole extracted root systems can give fast and accurate measures of root morphological traits (Bouma *et al.* 2000, Himmelbauer 2004, Pierret *et al.* 2013, Songsri *et al.* 2008). Downsides of this method include the lack of 3D information, as the roots must be scanned flat, and loss of finer sensitivity when harvesting, although this can be reduced by growing plants in a substrate that requires less destructive harvesting than soil, such as sand. Use of hydroponics or aeroponics, where plants are grown with roots immersed in nutrient solution or exposed to nutrient mist in a closed system (Chen *et al.* 2011, Liu *et al.* 2018, Kratsch *et al.* 2006), minimises disturbance and removes destructive sampling, but both methods have been shown to produce significantly altered root systems to plants grown in substrate (Graves 1992, Liu *et al.* 2018, Sankhalkar *et al.* 2019) and may confound

observations and wider conclusions on the effect of TRV infection on root morphology.

The removal of stressful harvesting does make hydroponics attractive to study root volatile emissions from intact roots. Plant stress can lead to changes in root volatiles (Copolovici *et al.* 2012, Holopainen and Gershenzon 2010, Loreto and Schnitzler 2010, Timmusk *et al.* 2014) and mitigating root stress, both through physical disturbance and water stress from a time-consuming extraction, are worth the trade-off of a more artificial environment. Two types of volatile extraction were tested in this investigation, in order to try to identify as many compounds of interest as possible.

As volatiles and semi-volatiles of interest released from TRV infected roots are currently unknown, direct headspace sampling was chosen as the first sampling method. This avoids any biases of SPME fibre or adsorbent polymer choice on the types of volatiles and semi-volatiles collected (Arban *et al.* 2021, Tholl *et al.* 2006) and allows the broadest capture in terms of analysis. Unlike other static-headspace sampling techniques, it also allows quantification of concentration within the gaseous phase (Redeker *et al.* 2018). A dynamic headspace method using solvent extraction was also used to trial a methodology that allowed repeated sample injections and reduced manual handling at analysis, allowing a greater number of samples to be tested than with direct headspace sampling.

Here, the effects of infection on root architecture and plant biomass, whole root volatile emissions and nematode attraction were compared. This allowed testing of the following hypotheses:

> There is a cost to infection that will show as a significant decrease in above and belowground biomass and differences in the root architecture in TRV-infected plants compared to uninfected plants. Significant differences in root architecture between uninfected and infected plants exist.
>
> There will be a significant reduction in the root to total plant weight ratio in TRV-infected plants.
>
> TRV-infected plants will show different root volatile emission profiles than uninfected plants.

Trichodorids will preferentially move towards TRV-infected plants in olfactometer assays.

There will be a positive relationship between level of infection and attractiveness to nematodes, and between level of infection and emission levels of VOCs elevated under infection.

.

Methods

Plant growth and infection

Seeds of *N. benthamiana* were sterilised by exposure to 100% ethanol in Eppendorf tubes for 2 minutes, followed by centrifugation, removal of the ethanol and addition of 50% commercial bleach solution. The bleach and seed solution were left for 15 minutes then centrifuged, the bleach removed, and the seeds rinsed five times with distilled water. Seeds were germinated on moist filter paper in a Petri dish at 20°C in a controlled growth room with 16h/8h light and dark intervals, then transferred to trays of the 90:10 mix of autoclaved sand:John Innes no2 compost. Seedlings were watered with ½ strength Hoagland's nutrient solution (Table 2.1) until two true leaves had expanded, and were then potted on to different media, depending on the experiment. Plants for volatile experiments went to 1L pots filled with nutrient solution (see Root VOC Sampling, Figure 2.1a); plants for root morphology were grown in 1L pots filled with autoclaved sand (see Root Morphology); and plants used in trichodorid assays were planted directly in the external chambers of the olfactometers (see Trichodorid Response).

Table 2.1 Composition and concentration of Hoagland's nutrient solution

Compound	Formula	Concentration in full strength solution (µM)
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	1079.14
Calcium nitrate tetrahydrate	$Ca(NO_3)_2.4H_2O$	6034.30
Potassium nitrate	KNO_3	4131.68
Potassium phosphate	KH_2PO_4	1906.25
Managnous sulphate tetrahydrate	$MnSO_4.4H_2O$	3.63
Boric acid	H_3BO_3	48.03
Sodium molybdate dihydrate	$Na_2MoO_4.2H_2O.$	0.50
Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	2.02
Cupric sulphate pentahydrate	$CuSO_4.5H_2O$	0.36
Fe-EDTA	$C_{10}H_{12}N_2NaFeO_8$	64.73

Seven days prior to testing, plants were infected with *Agrobacterium tumefaciens* strain GV3101 (Holsters *et al.* 1980) cultures that were transformed with TRV RNA-1 or RNA-2 isolates. TRV RNA-1 is isolate Pp085 and TRV RNA-2 is PpK20, cloned by S. MacFarlane. All infections used the same TRV RNA-1 culture, but two different TRV RNA-2 cultures were used. One was transformed with wild-type TRV RNA-2 and the other with a mutant where the 2b and 2c genes were removed and replaced with a Green Fluorescent Protein (GFP) gene. Wild-type RNA-2 (TRV wild-type) was used in all experiments and the mutant (TRV-Δ2) was used as an additional treatment in the root volatile experiments. Infection solutions were prepared by making overnight stocks of each culture from frozen glycerol stocks pricked into 2ml of 25g/L LB liquid medium (Sigma Aldrich LB Broth [Miller]) with added kanamycin (50mg/ml) and rifampicin (10mg/ml). These were incubated at 28°C at 120rpm for 10-12 hours, then 0.5ml of the overnight stock were subcultured into 10ml of fresh LB liquid medium containing kanamycin (50mg/ml) and rifampicin (10mg/ml) and incubated again for 6 hours in the same conditions. The absorbance of the cultures was measured with a spectrophotometer (Biochrom) at OD_{600} using LB liquid medium with added antibiotics as a blank. If an OD_{600} of 0.5-0.6 was reached, the cells were used.

The resulting cells were spun down at 3000g for 5 minutes and re-suspended in 10ml of an induction buffer of 195mg (N-morpholino)ethanesulfonic acid (MES) dissolved in 100ml dH_2O with 200µl of 200µM acetosyringone added. These were then incubated at 20°C at 50rpm for 3 hours. The cells were spun down again and resuspended in 10ml infiltration buffer of 97.6mg of MES dissolved in 100ml dH_2O. and checked by spectrophotometer, using infiltration buffer as a blank, for a desired OD_{600} of 03-0.5.

The separate cultures were combined to give a culture containing equal concentrations of RNA-1 and RNA-2, based on the spectrophotometer readings. 0.5ml of the combined culture was inoculated onto the apoplast of each leaf using a needleless syringe (Senthil-Kumar and Mysore 2014). Four leaves were inoculated per plant. "Uninfected" plants were inoculated with untransformed *Agrobacterium*, incubated in the same conditions, to control for any side effects of *Agrobacterium* infection.

After sampling, successful infection with end-point reverse transcription polymerase chain reactions (d-RT-PCR) on leaf and root tissue from infected plants which quantified expression of virus genes. Total RNA was extracted using TRIzol (ThermoFisher) and chloroform according to the manufacturer's protocol to reduce contamination with genomic DNA. Extracting RNA rather than DNA identifies the genes that are being actively expressed, rather than merely present (Liang and Pardee 1992). DNA synthesis was carried out using SuperScript Reverse Transcriptase (Invitrogen), following the manufacturer's instructions using Random Hexamers (Invitrogen) as primersbut using half volumes to give a 10 µl reaction volume. The cDNA was stored at -80ºC until required.

PCR amplification was carried out in 25µl containing 1µl of 1:10 diluted cDNA, 5 µl of 5x GoTaq buffer (Promega), 0.5 µl of 10mM dNTP mix (Promega), 1 µl of each primer at 10 µM concentration and 0.125 µl GoTaq DNA polymerase (Promega), made up to 25 µl wth RNAse-free water. The primers used were previously described primers for RNA-1 and RNA-2 (Robinson 1992, Boutsika *et al.* 2004) (Table 2.2). The primers for RNA-1 amplify a section of the RNA-1 1b gene while the RNA-2 primers amplify part of the coat protein (CP) gene on RNA-2. PCRs were run on a Techne TC-512 thermo cycler (Bibby Scientific Ltd. UK). Initial denaturation was 94°C for 1 minute with heated lid, followed by 30 cycles of 94°C for 10 seconds, 58°C for 30 seconds and 72°C for 60 seconds. Final extension was 10 minutes at 72°C. Samples were held at 15°C before being stored at -85°C. End point PCR gels were run on 2% agarose gels to check for bands present in infected plants.

Table 2.2 TRV-1 and TRV-2 primers used to test successful infection of plants.
Modified from Boutsika et al. 2004

Primer	Length (bp)	Primer sequence (5'-3')
RNA-1 F	20	GACGTGTGTACTCAAGGGTT
RNA-1 R	21	CAGTCTATACACAGAAACAGA
CP RNA-2 F	19	CGCGGTAGAACGTACTTAT
CP RNA-2-R	17	GGACCGCCCGACTTGTC

RT-qPCR was used for quantification of infection. *N. benthamiana* reference genes were chosen from housekeeping genes that are vital to cellular metabolism and so ubiquitously expressed in all cells of the plant to act as internal controls. The internal standard reference genes *APR* and *EF1a* were selected due to their stability in *N. benthamiana* under TRV infection (Liu *et al.* 2012) (Table 2.3). *APR* codes for an adenine phosphoribosyltransferase-like enzyme that recycles adenine into adenylate nucleotides using cytokinins as substrates (Allen *et al.* 2002, Mok and Mok 2001). *EF1a* is the alpha subunit of elongation factor-1 and plays a central role in polypeptide chain elongation in all eukaryotes, including plants (Pokalsky *et al.* 1989). *EF1a* promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis. qPCR primers were used for the replicase gene on TRV RNA-1 and the coat protein on TRV RNA-2 (Sahi *et al.* 2016) (Table 2.3). qPCR was carried out in 10 µl reactions, prepared over ice. Each reaction mix contained: 0.2 µl cDNA diluted in 4.4 µl RNAse-free water, 5 µl of 2X SYBR Green Power mix (Applied Biosystems) and 0.2 µl of each primer. Each cDNA sample was run with 2 primer pairs (RNA1 and RNA2) and 2 housekeeping genes (*APR* and *EF1a*) and in triplicate, so 12 reactions were run per cDNA sample. These were run and analysed on a StepOnePlus real-time PCR system (Applied Biosystems). The initial denaturation was 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Table 2.3 RT-qPCR primers for the replicase gene on RNA1 and the coat protein gene on RNA2 of TRV with two N. benthamiana reference genes. TRV primers from Sahi et al. 2016 and N. benthamiana primers from Liu et al. 2012.

Primer	Length of amplified fragment	Primer sequence (5'-3')
RT-TRV RNA-1-F	89	TACCAAGGGAATGTG TTCTA
RT-TRV RNA1-R		CTCGGAACTCCAGCT ATC
RT-TRV RNA2 K20 CP-F	114	CAGTGCTCTTGGTGT GAT
RT-TRV RNA2 K20 CP-R		GTCGTAACCGTTGTG TTTG
RT-APR-F	108	CATCAGTGTCGTTGC AGGTATT
RT-APR- R		GCAACTTCTTGGGTT TCCTCAT
RT-EF1α- F	116	AGCTTTACCTCCCAAG TCATC
RT-EF1α- R		AGAACGCCTGTCAAT CTTGG

Growth and root morphology

Once two true leaves had formed on each, 30 *N. benthamiana* plants were potted into 1L pots of autoclaved sand. They were infected when 10 true leaves had formed (TRV infection or *Agrobacterium* control) to test infection impacts on plant growth and

root morphology. Sand was used as the solid growth medium to allow extraction of clean whole root systems for accurate root scans.

Plants were fed with 250ml ½ strength Hoaglands twice weekly and watered with dH_2O as needed. Five plants per treatment (TRV-infected and *Agrobacterium* controls) were harvested at 4-, 7- and 14-days post infection (dpi); weighed; and parameters measured following Gamalero *et al.* (2002): root and shoot fresh weight, total root length, total root surface area, total root volume, number of tips and degree of root branching (tip number divided by total root length). These were collected by carefully spreading the root system in a thin layer of water on a transparent tray and scanning them. These images were then analysed using WINRhizo software (Regent Instruments, Canada) that pulls out the parameters from the image using a skeletonization method (Himmelbauer 2004). Small samples of the root and shoot tissue were removed after this procedure, flash frozen and stored at -80°C before RNA extraction to test for successful infection and viral load.

Root VOC sampling

Direct headspace semi-static sampling

For the direct headspace sampling, 18 *N. benthamiana* plants were grown in individual hydroponic pots (Figure 2.1a) in ½ strength Hoagland's that was changed weekly so that plants could be instantly removed from their pots and placed in the sampling chamber. Once 10 true leaves had formed, plants were either infected with wild-type TRV ("infected-wild-type"), a TRV mutant that was missing genes 2b and 2c from RNA-2 ("infected-mutant") or untransformed *Agrobacterium,* and sampled 7 dpi (Figure 2.1b).

Figure 2.1 Hydroponic plants and volatile sampling. a: Hydroponic plants in individual pots filled with nutrient solution connected to an air pump for aeration. b: Plants being sampled. Stainless steel line leads through a CO_2 and moisture trap to stainless steel sampling canisters (seen behind). Samples were taken at 0mins and 20mins after plant was placed in the box. These plants were used for preliminary work, data not shown.

Plants were placed in a sampling chamber that isolated the plant roots in a sealed space (Figure 2.2) from the rest of the plant. The chamber (38cm x 25cm x 12cm = 11.4L) allowed plants of variable widths to be placed in the centre and plant roots to be enclosed. A stainless-steel line out connected to an ascarite trap to filter CO_2 and water from the sample before connecting to a sampling canister. A small hole at the back of the chamber allowed ambient air to replace the air taken at each sample, avoiding depressurization of the plant roots in the chamber. Between samples, the valve connecting to the ascarite trap was closed to prevent air escaping the chamber through the stainless-steel line. This valve was also used to pull some air through the line just prior to a sample being taken, to ensure that the sample was well mixed.

The seal between the above and belowground portions of the plant was made with a pair of silicon discs and high vacuum silicon grease (Dow Corning GMBH, Wiesbaden). The volatiles were captured in pre-evacuated stainless-steel electropolished canisters (Figure 2.1b, background). Samples were analysed within the two-week period that these canisters have been demonstrated to be stable for reactive compounds (Low *et al.* 2003). Volatiles within the samples were condensed onto a liquid nitrogen condensation trap; this cryofocused the samples as the majority of nitrogen, oxygen, helium and argon are removed while compounds with freezing points above -170°C are frozen onto the trap (Redeker *et al.* 2007, Redeker *et al.* 2018). Samples were then

transferred to an Agilent DB-5 column or Agilent PoraPlot Q column, depending on method (see below) and analysed by gas chromatography-mass spectrometry (GC:MS).

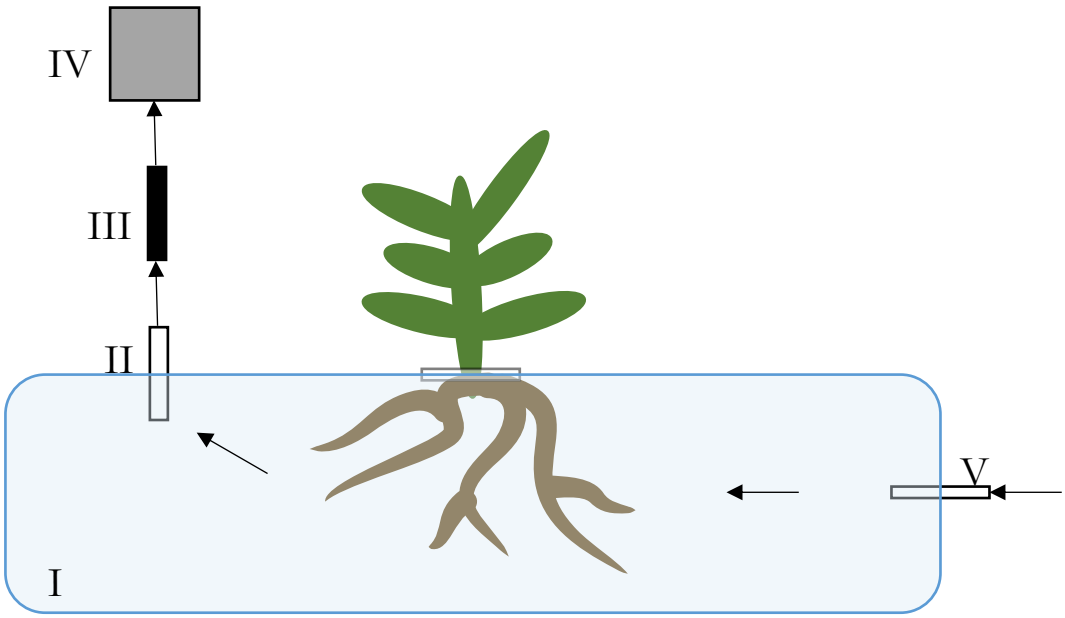

Figure 2.2 Semi-static headspace sampling with stainless steel canisters. I) Sampling chamber: The stainless-steel line out (II) connects to an ascarite trap (III) before connecting to a sampling canister (IV) via a valve. A small hole at the back of the chamber allows ambient air to replace the air taken at each sample, avoiding depressurization of the plant roots in the chamber (V).

Samples were taken within 1 minute (time=0), providing a baseline sample of background air, and 20 minutes (time=20) after the first sample was collected. Compared to other studies (Durenne *et al.* 2018, Movafeghi *et al.* 2010, Souza Silva *et al.* 2017), 20 minutes is a shorter length of time for collecting plant-emitted volatiles, but this short time was chosen to minimise the effects of water stress on the plants, which may affect volatile emissions (Salerno *et al.* 2017, Griñán *et al.* 2019). Most head-space sampling of intact plants have focused on whole plant or aboveground portions of plants (e.g. Aharoni *et al.* 2003, Durenne *et al.* 2019, Gross *et al.* 2019, Rid *et al.* 2016) which allows reduction of plant stress through the use of pots or roots placed in beakers of water. However, as root volatiles were the target of this investigation, this was not possible.

Samples were randomly allocated to one of two analytical methods, each of which targeted different molecular sizes of compounds. One method gave greater sensitivity for smaller compounds (<C6), both in terms of the GC column used and a slower initial ramp and masses up to 150 atomic mass units (amu). They were run on a

25m PoraPlotQ column (Agilent) with 0.32ID and 5μm wall thickness with an initial oven temperature of 30°C, held for 5 minutes, then a first ramp of 10°Cmin^{-1} to 90°C (no hold), a second ramp of 2°C min^{-1} to 150°C (no hold) and a final ramp and bake-out of 10°C min^{-1} to 220°C with a 5 minute final hold for a total runtime of 53 minutes (Redeker *et al.* 2018).

The second method targeted larger molecular weight compounds (<C20), with masses up to 450amu. Samples were run on a 30m DB-5 column (Agilent) with an initial oven temperature of 30°C, held for 5 minutes, first ramp of 4°C min^{-1} to 250°C (no hold) and a second ramp and bake-out of 40°C min^{-1} to 325°C with a 5 minute final hold (total runtime of 66 minutes). For both methods, 46 was the smallest mass examined, in order to avoid CO_2 contamination, which was likely to saturate the detector. Peak areas were calculated using Agilent ChemStation Data Analysis software, which also gave a putative identification of compounds.

Peak areas were initially converted to concentration in parts per trillion (pptr) using a calibration slope derived from internal standard testing and the number of moles of gas injected, divided by the molar volume of gas (22.4L mol^{-1}) i.e. the space 1 mole of gas occupies at standard temperature and pressure.

$$conc.(pptr) = \frac{10^{12}(peak\ area\ *\ calibration\ slope)}{moles\ in\ sample/22.4}$$

The number of moles in the sample are calculated using the combined volume of the canister and condensation line (1000ml or 500ml depending on the size of canister used + 33.35ml), the pressure of the injected sample and the conversion factor from torr to atmospheres:

$$moles\ in\ sample = (canister\ vol\ + line\ volume)\frac{pressure\ of\ sample}{760}$$

These concentrations were then converted to fluxes of each compound in μg min^{-1}g dry root weight^{-1}:

$$flux\ (\mu g\ min^{-1}g^{-1})$$
$$= \frac{(conc.at\ t = 20\ - conc.at\ t = 0) * \left(\frac{11.4}{22.4}\right) * molecular\ weight\ of\ compound * 10^6}{time\ difference * dry\ weight\ of\ roots}$$

The concentration at 0 minutes, as a baseline estimate of the ambient air, was subtracted from the concentration at 20 minutes to give the change in concentration. This was multiplied by molar amount of air in the sampling chamber (11.4L / 22.4L mol^{-1}). This is the number of moles of compound generated or consumed during the time between samples. This value was multiplied by the molecular weight of each compound (g mol^{-1}) and by 10^6 to convert from grams to micrograms. Finally, it was divided by the time between samples and the dry weight of the plant's roots to give the flux per minute and per gram of dry root weight. Means and standard errors of these fluxes were calculated.

After sampling, the plants were harvested and separated into above and belowground portions. This cutoff was where the plant entered the growth medium if in substrate or where the opaque foam bung that held the plant in the hydroponic pots was attached to the plant. Fresh weight was recorded for both above and belowground biomass and samples of ~100mg (90-150mg) were flash frozen in liquid N_2 then stored at -80°C for RNA extraction. After 3 days drying at 70°C, above and belowground dry weights were recorded and corrected for the weight of tissue removed for RNA extraction.

Dynamic solvent-based headspace sampling

36 *N. benthamiania* plants were grown in the same conditions as the static headspace sampling mentioned above in individual hydroponic pots. They were also infected when 10 true leaves had formed, either with wild-type TRV ("infected-wild-type") or untransformed *Agrobacterium,* and sampled at 7, 10 and 14 dpi. This was changed from the static headspace sampling in order to be able to directly compare with that technique at 7 dpi, but also to take advantage of the ability to collect and analyse a greater number of samples.

The sampling setup was similar to that used in semi-static headspace sampling, but slightly altered (Figure 2.3). Instead of the outlet from the sampling chamber leading to a canister, the air passed over a glass tube filled with 70mg Porapak Q adsorbent (80-100 mesh, Sigma). Incoming air was passed through activated charcoal to filter it (de Moras *et al.* 1998) and strip it of any volatiles already present in the lab air. The incoming air was pumped into the chamber at a higher rate (700ml min^{-1}) than the

air pulled out over the trap (400ml min^{-1}) in order to create a positive pressure difference in the sampling box and prevent air entering that had not passed through the charcoal filter (Jassbi *et al.* 2010). Plant roots were kept in the chamber for 45 minutes as a tradeoff between capturing enough emitted compounds and putting the plants under excessive stress and the chamber was left empty for 20 minutes between samples to allow any residual plant released volatiles from the previous sample to pass through the system. Lab air blanks were taken for 45 minutes at the beginning of each sampling day. The traps were eluted with three washes of 200µl dichloromethane (DCM). Each wash was sampled to ascertain how many washes were required to elute all compounds of interest. Before and after use, traps were rinsed twice with DCM and dried under a helium stream. Traps were stored at 115°C when not used.

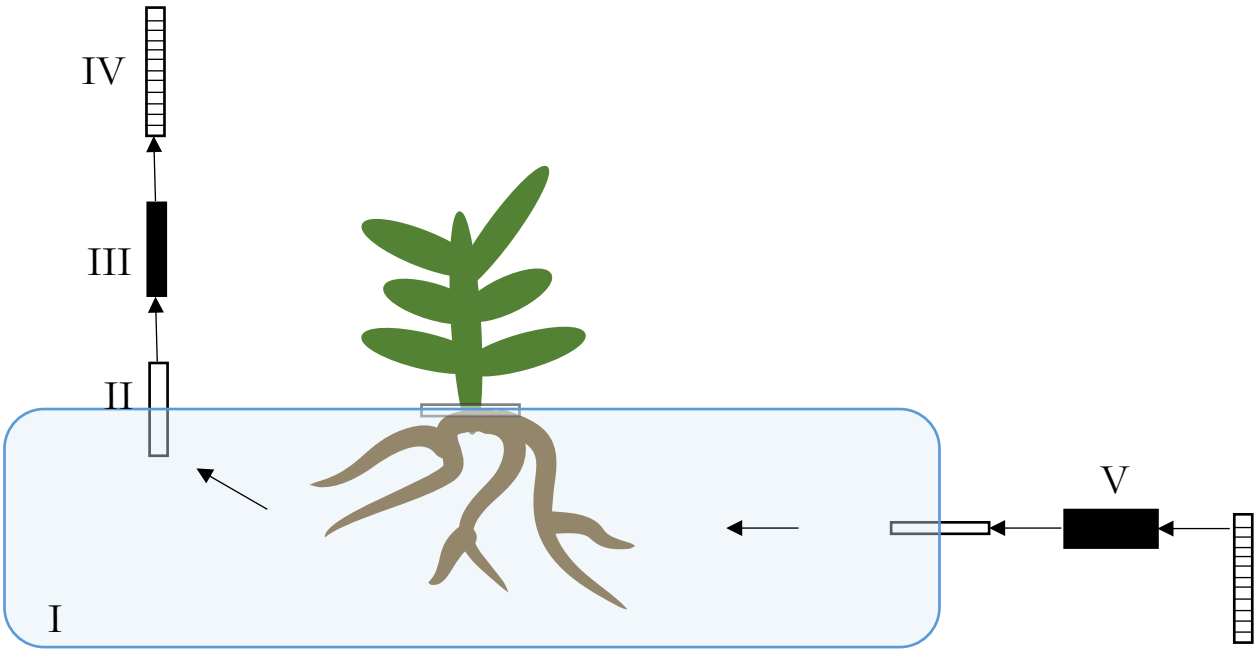

Figure 2.3 Dynamic headspace sampling with adsorbent volatile collection traps. I) Sampling chamber: The stainless-steel line out (II) connects to a volatile collection trap filled with Porapak Q (III) before connecting to a flowmeter (IV) and pump with flow rate set to 400ml min^{-1}. Air coming into the chamber was pumped in through an activated charcoal trap (V), with a flowmeter ensuring the flow rate stayed at 700ml min^{-1}.

Measurement of the samples were carried out using the same GC:MS combination as described above, using the second method for larger compounds. Injection was carried out using an autosampler and the chromatograph was operated in splitless mode with an injection volume of 1µl. In addition to the lab air blanks from the start of each day, DCM blanks were also run to ensure no contamination from the carrier solvent.

Standards of known nematode chemoattractants β-caryophellene (Hammack 2001, Rasmann *et al.* 2005, Sadeh *et al.* 2017), α-pinene (Hallem *et al.* 2011, Zaka *et al.* 2015, Zhao *et al.* 2007), limonene (Sadeh *et al.* 2017) and linalool (Hallem *et al.* 2011, Hammack 2001) were run on the GC:MS using the same injection method and the same analysis method as both the canister samples and the volatile collection trap samples. These were run at a range of concentrations from 1ng ml^{-1} to 5μg ml^{-1}. This was to ensure that the analysis method could detect these compounds, to confirm that if they were absent from the sample chromatograms, it was not due to inadequacies of the GC:MS analysis method.

After sampling, the plants were harvested as in the direct headspace method and tested for successful infection.

Trichodorid choice experiments

Olfactometers were built using cut 50ml falcon tubes and PTFE tubing. The PTFE "arms" leading to the outer pots were 80mm long in total. The longest section of the arm was 50mm, leading from the central pot towards the outer pots, before being blocked by a 2 micron mesh (TWP Inc. CA, USA). A 20mm piece then connected the 30mm portion of the arm to the outer pots. Each olfactometer had two outer pots; one contained a plant infected with wild-type TRV, the other with untransformed

Figure 2.4 Olfactometers with *N. benthamiana* plants. Plants were grown in different tubes and moved to the olfactometer one day before testing.

Agrobacterium controls that were moved to the pots 24 hours before the trials. The olfactometers were filled with washed and autoclaved sand that was kept at 20% moisture by volume until the start of the experiment. The pots were not handled during the trial to avoid disturbance.

Nematodes used in experimental work were extracted from a 200 g subsample (Wiesel *et al.* 2015) from agricultural soil samples using a sieving and decanting modified Baermann funnel extraction method (as detailed in Brown and Boag 1988). After c. 48 h, extracted nematodes were collected in 20 ml of water and left to settle for c. 2 hours and excess water decanted. Trichodorids were hand-picked into distilled water in 2ml Eppendorf tubes under low-powered microscopy and stored at 4ºC until required. Seven days post infection, 50 nematodes were added to the central pot. Four 50 nematode replicates were run (Figure 2.4) and the direction of the treatments (left or right pot) was randomly allocated for each replicate. The experiment was repeated two more times to give 12 replicate data points. The clear plastic pots were wrapped in aluminium foil to exclude light and the experiment was kept at 23-24°C for 24 hours.

Nematodes were extracted after the assay using a simplified version of the Brown and Boag Baermann funnel method where only the final separation step was required. The sand from each section (infected arm, control arm and central pot) was placed in individual Baermann extraction funnels filled with dH_2O (Figure 2.5) such that three funnels were required per olfactometer. After c. 48 hours, the nematodes had collected in the water at the bottom of the funnel and 5-10ml of this water, including the nematodes, was collected in 50ml plastic tubes and stored at 4ºC until counted.

Figure 2.5 Baermann extraction funnels. The funnels are filled with water and nematodes placed within the mesh lined pots. The mesh (opening diameter of 250µm; Sefar AG, Thal CH) is wide enough to allow trichodorids to pass through, while retaining sand. When the nematodes pass through the filter, they fall and collect in the tube at the bottom of the funnel. After 48hrs, approx. 10ml of water containing the nematodes was collected from the bottom of the funnel. This method will only collect nematodes that were alive at the end of the assay, as they will not fall through the mesh passively.

Nematodes in the extract were counted with a stereo microscope. Number of nematodes was converted to number of nematodes per cm^3 of sand ($0.5cm^3$ for the arms and $15cm^3$ for the central pot).

Statistical analysis

Data was analysed using R 4.0.3 (R Core Team, 2020). Count data from all three repeats of the trichodorid choice experiment were pooled and analysed with a generalised linear model (GLM) with Poisson regression. The assumptions of the model were checked, and a deviance goodness-of-fit test was conducted to ensure the appropriate GLM family was used, which was only accepted if the result was greater than 0.8 (Wood *et al.* 2020). Overdispersion was also tested using the package AER (Kleiber and Zeileis 2008). Nematode count was set as the dependent variable while the pot (infected, uninfected and no movement) and the repeat were set as the independent variables. Models with only pot as the independent variable had a better AIC, so pot was the only variable included in the final model. An analysis of deviance was performed on

the model with Tukey HSD post hoc tests with p value adjustment to investigate significance.

Comparisons between nematode numbers and expression of viral RNA was carried out with linear regression. All other data were analysed using analysis of variance, or Kruskal-Wallis test by ranks if the assumptions for analysis of variance were not met and the data could not be transformed (Kruskal and Wallis 1952). Analysis of variance model assumptions were checked with Shapiro-Wilk normality tests and Levene's test for homogeneity of variance. Tukey HSD post hoc tests were used when two or more levels of an interaction were significant. Several other R packages were used: arm () was used to visualise output from the GLM, emmeans (Lenth 2021) to carry out post hoc comparisons on the GLM and car (Fox and Weisberg 2019) to carry out Levene's test for homogeneity of variance.

Results

Growth and root morphology

In root scans of infected plants (Figure 2.6), there was a significant difference in root and shoot dry weight between treatments (root: ANOVA $F_{(1, 30)}$=7.5, p<0.05, shoot: ANOVA $F_{(1, 30)}$12.7, p<0.01) and day (root: ANOVA $F_{(2,30)}$=30.3, p<0.001, shoot: ANOVA $F_{(2, 30)}$=3.5, p<0.05). The cost of infection being apparent from 7 days post infection (dpi), with uninfected plants having significantly (p<0.05) more root biomass than infected plants. There was also a trend towards more shoot biomass, although this was not significant. These patterns were mirrored with fresh weight data (not shown). These effects were both more significant and more severe for both above and belowground biomass 14 dpi (p<0.001 for shoot and p<0.01 for root). Shoot dry weight at 14 dpi was 0.397g±0.019 and 0.165g±0.018 for uninfected and infected plants respectively; compared to 0.235±0.032 and 0.204±0.021 for uninfected and infected plants at 7 dpi. Belowground, root biomass at 14 dpi was 0.066±0.001 (uninfected) and 0.043±0.001 (infected) and at 7 dpi: 0.030±0.002 (uninfected) and 0.017±0.002 (infected). The interaction between infection/control and day was significant as the relationship between treatments changes between the days, particularly for root biomass (Figure 2.6).

There was a significant interaction between the effects of treatment and sampling day (ANOVA $F_{(2, 30)}$=5.0, p<0.05) in root weight to total weight ratio (Figure 2.7) between control and wild-type infected plants. At 7 dpi , the ratio of root to total plant weight was significantly greater for control plants than uninfected plants(p<0.05). This ratio reverses strongly by 14 dpi, with infected plants having a significantly greater proportion of total weight allocated to roots (p<0.001).

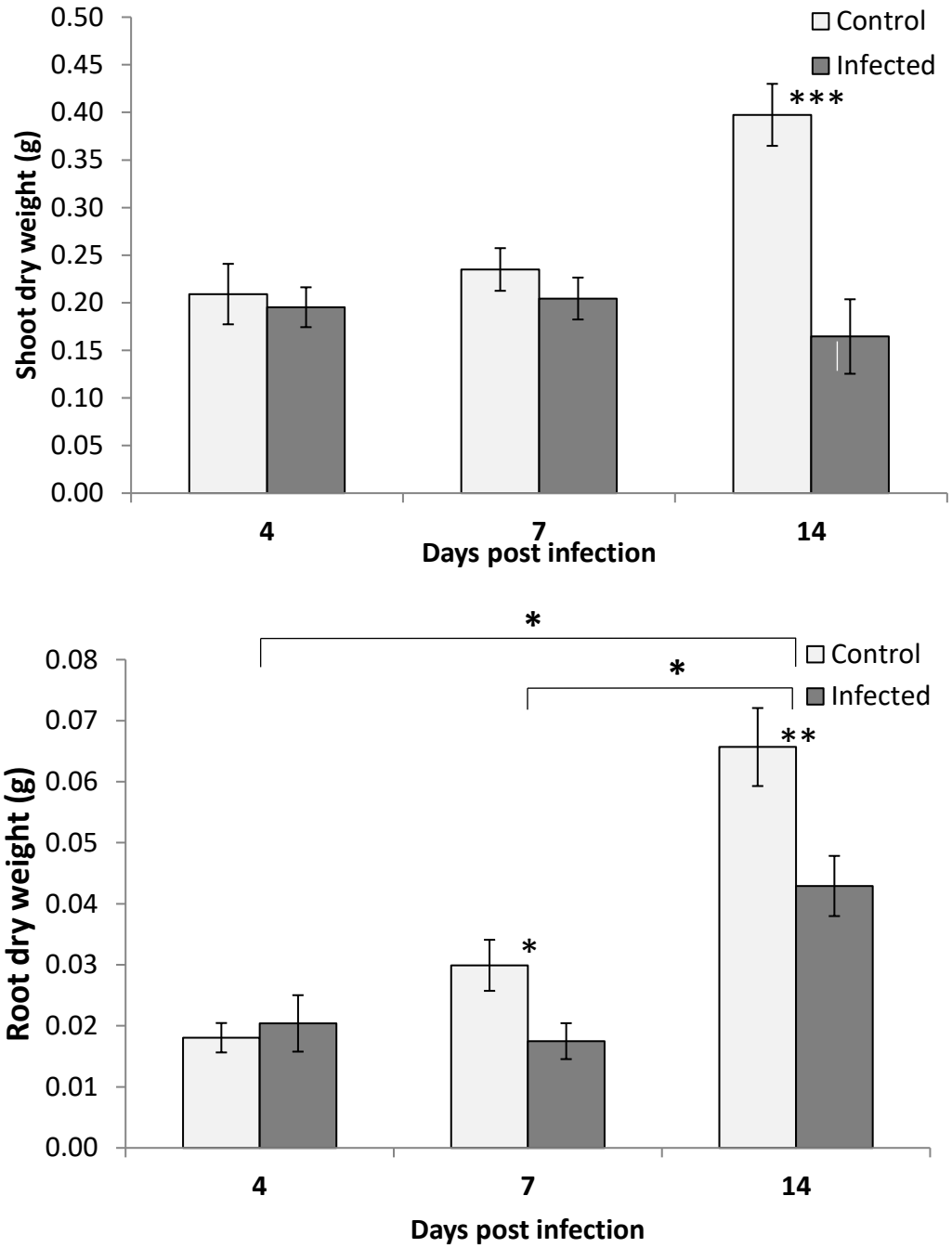

Figure 2.6 Mean dry weights for above and belowground portions of N. benthamiana plants grown in sand for root scanning different days post initial infection. Control plants were infected with Agrobacterium only, infected plants infected with Agrobacterium transformed with wild-type TRV. Error bars are standard error, n=5 for all treatments. Number of * denotes significance between treatments within and between days, * 0.05, ** 0.01, ***0.001. Only significant interactions are shown.

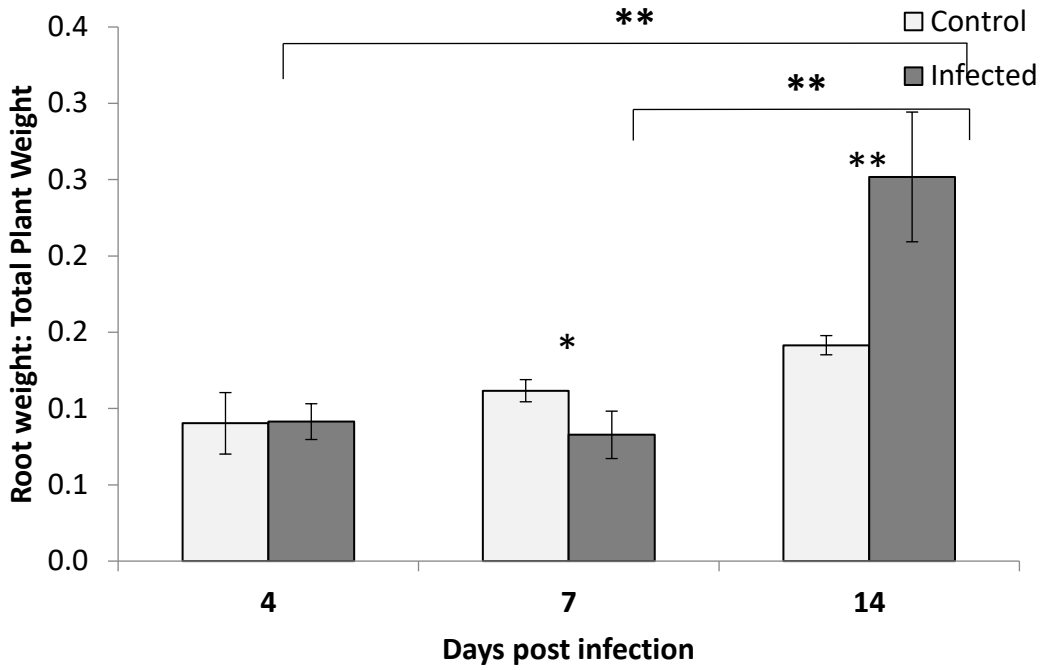

Figure 2.7 Ratio of root dry weight (g) to total plant weight (g) of N. benthamiana plants grown in sand for root scanning different days post initial infection. Control plants were infected with Agrobacterium only, infected plants infected with Agrobacterium transformed with wild-type TRV. Error bars are standard error, n=5 for all treatments. Number of * denotes significance between treatments, * 0.05. ** 0.01.

Of the root architecture parameters analysed, total root length gave the strongest differences between control and infected plants within days (7 dpi, uninfected 237cm ± 27 vs infected 132cm± 27, 14 dpi, uninfected 240cm ± 65 vs infected 79cm ±16, both comparisons significant at the $p<0.001$). While infected plants increased in root biomass over the time course, the total root length reduced between days 7 and 14 (Figure 2.8), making infected plants bushier with stunted roots. Root surface area to volume ratio did not change significantly between sampling days (Figure 2.8) but does show a significant difference between treatments at 7 and 14 dpi, with uninfected plants having a larger surface area to volume ratio than TRV infected plants. Differences between treatments increased in severity, apart from the number of root tips, where the significant difference between treatments was lost by 14 dpi. This was due to the wide range of values for both treatments at day 14 (uninfected: 1051 ± 290 compared to infected: 663 ±240). Root branching was not included; due to the size and compact structure of some of the samples, accurate readings could not be taken.

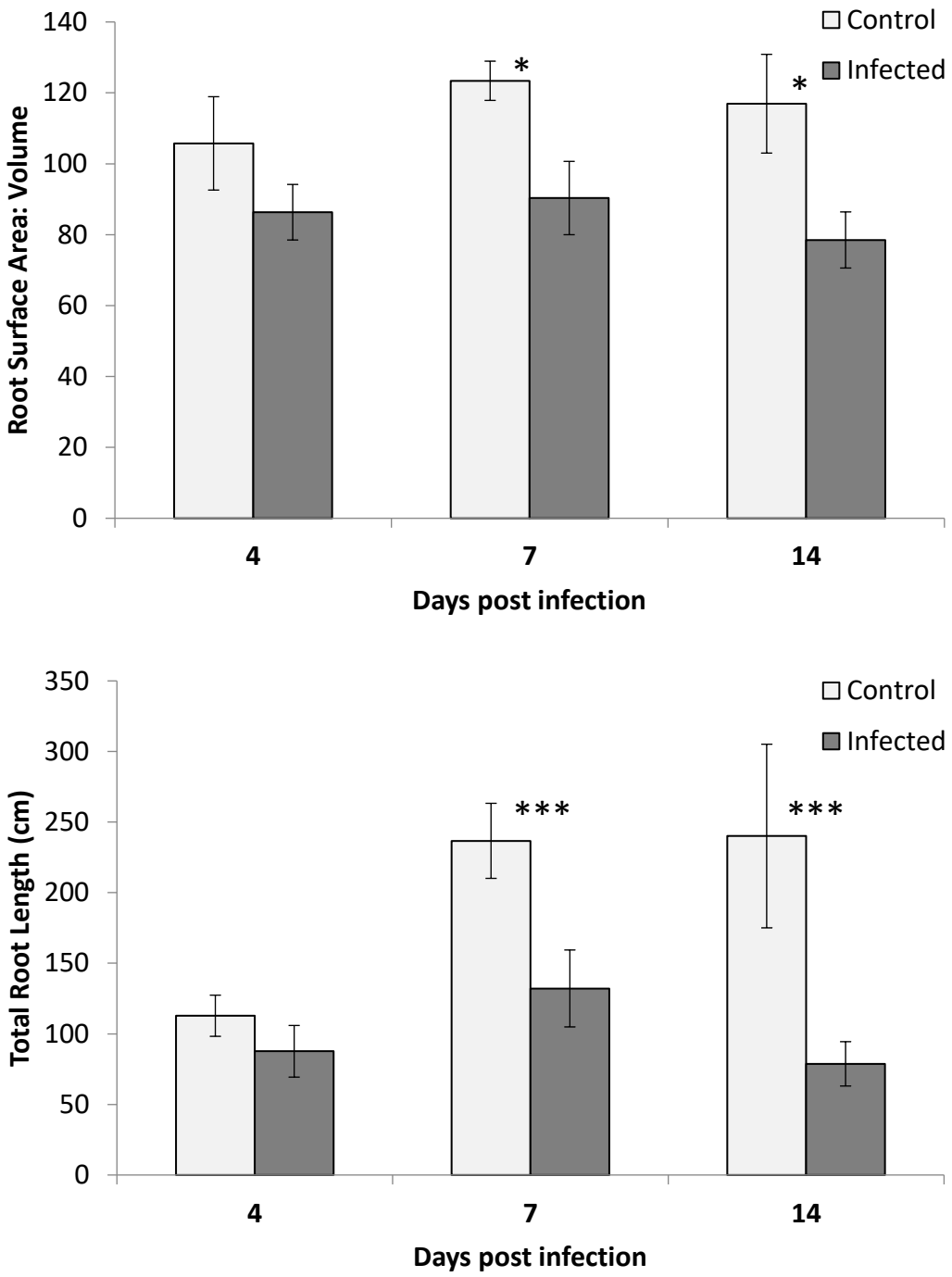

Figure 2.8 Root surface area:volume ratio and total root length (cm) of *N. benthamiana* plants grown in sand for root scanning different days post initial infection. These show the strongest deleterious effects of TRV infection out of the root architecture parameters studied. Error bars are standard error, n=5 for all treatments. Number of * denotes significance between treatments within days, * 0.05. ** 0.01, ***0.001. Data analysed with ANOVAs applied to linear models. No significant differences found between days.

Root VOC sampling

Direct headspace semi-static sampling

Hydroponically grown plant biomass showed significant reduction in both above and belowground biomass in wild-type TRV infected plants (0.78g±0.16 aboveground and 0.09g±0.02 belowground) compared to *Agrobacterium* infected controls (1.85g±0.44 aboveground and 0.36g±0.11 belowground) (Figure 2.9). Average dry shoot and dry weight of wild-type TRV-infected plants and control plants differed significantly from each other (both $p < 0.01$). Infection with TRV RNA-2 mutants missing 2b and 2c showed an intermediate effect of infection with no significant difference between mutant-infected plants (1.22g±0.47) and either control (1.85g±0.44) or wild-type infected plants (0.78g±0.16) for dry shoot weight. For dry root weight, there was a significant difference between control (0.36g ±0.11) and mutant infected plants (0.15g±0.05, Kruskal-Wallis $\chi^2=11.8$, df=2, $p<0.01$), but none between mutant-infected plants and wild-type infected plants. Other root architecture parameters were not recorded, but on visual observation, root length was reduced and root diameter larger than plants grown in sand.

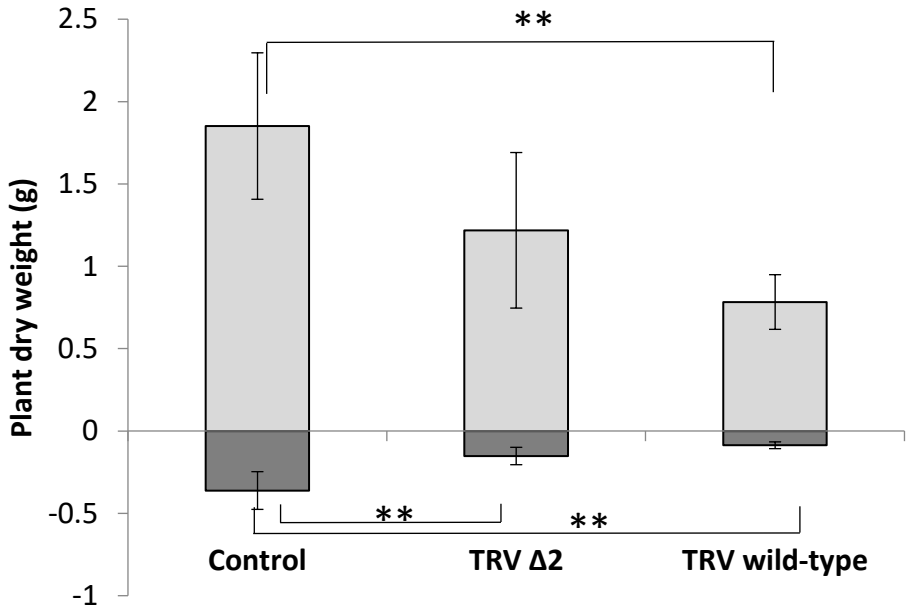

Figure 2.9 Mean dry weights for above and belowground portions of *N. benthamiana* plants grown in hydroponics 7 days post infection. Aboveground biomass is shown along the positive axis, with belowground biomass along the negative. Control plants were infected with *Agrobacterium* only, while both TRV treatments were infected with *Agrobacterium* transformed with TRV. Central bar shows data from mutant RNA-2 where 2b and 2c have been removed whilst the right bar shows a wild-type RNA-2 genome. RNA-1 was untransformed in both cases. Error bars are standard error, n=6 for all treatments. * $p<0.05$, ** $p<0.01$.

The canister samples were analysed by comparing profiles of t=20 chromatograms to the t=0 baselines. Peaks that were present in t=20 samples but missing or reduced at least twofold in the lab air were flagged up as potential plant volatile candidates for infection-dependent compounds. Nine compounds of potential interest were flagged up from the method looking at <C20 compounds, but none were found from the <C6 compound assay. Peak areas for selected compounds were recorded and their mass spectra were putatively identified using the inbuilt NIST database (Agilent ChemStation Data Analysis). Peak areas were converted fluxes of μg min^{-1} g dry root $weight^{-1}$ as described in Chapter 2 Methods. Of the nine compounds selected, four compounds of interest (Figure 2.10) showed significant differences between treatments; putatively identified as 2-ethyl-1-hexanol (K-W, chi=6.22, d.f.=2, $p<0.05$), m-ditertbutylbenzene (K-W, chi=8.3158, d.f=2, $p<0.05$), 2,4, ditertbutylphenol (K-W, chi=6.86, d.f.=2, $p<0.05$) and 2,6, ditertbutylquinone (K-W, chi=9.32, d.f.=2, $p<0.01$). For all compounds, significant differences were found between wild-type TRV-infected plants and the other treatments (all significant at $p<0.05$), with no differences between mutant-infected plants and *Agrobacterium* controls.

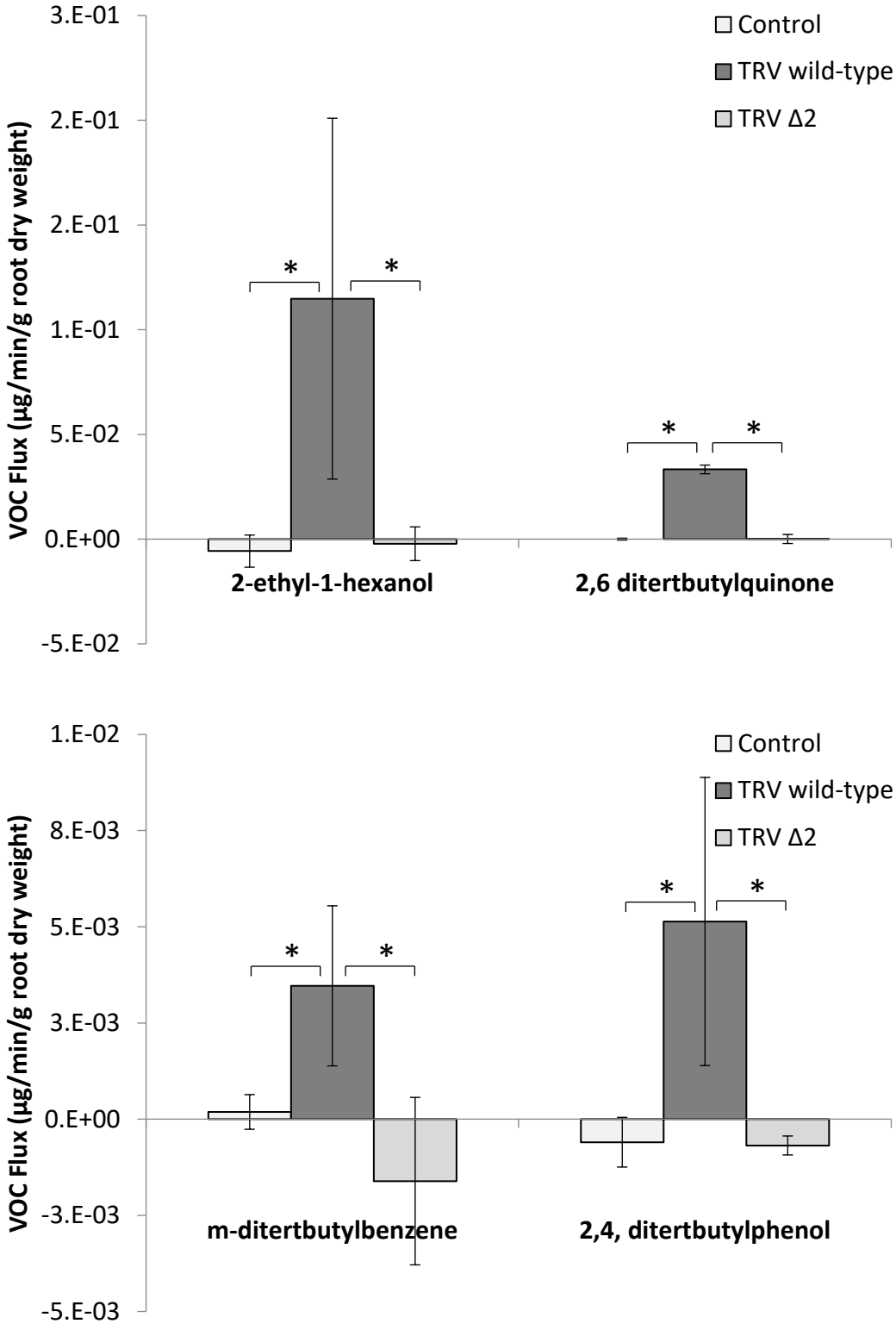

Figure 2.10 Average root VOC fluxes from roots of plants infected with TRV. Control plants were infected with Agrobacterium only, while both TRV treatments were infected with Agrobacterium transformed with TRV. TRV-2 (CP+2b+2c) are plants infected with wild-type TRV with an intact RNA-2, whereas TRV-2 (CP+GFP) were plants infected with mutant TRV missing two genes from RNA-2. The fluxes are shown at two scales due to variability in the size of fluxes and are µg min^{-1} g^{-1} root dry weight. Error bars are standard error, n=6 for all treatments. * $p<0.05$.

Compound IDs for all selected compounds were confirmed by comparison of the spectra and retention times with known standards (Sigma-Aldrich). There was large variation in 2-ethyl-1-hexanol production (± 1 SE) in wild-type infected plants, but this was not significantly explained by viral load (Figure 2.11). There was no significant difference in the variation in normalised expression of the TRV RNA-2 coat protein gene within treatments (Figure 2.11). Levels of infection were relatively similar across all TRV-infected plants with no significant difference between wild-type infected and mutant-infected plants. The levels of the two reference genes were different (Ct=27.4±0.5 for APR and Ct=24.3±0.4 for EF1α) but when normalised to the highest relative value for each gene, and then compared to each other, there was no significant difference in the mean normalised expression for each root sample depending on the reference gene used.

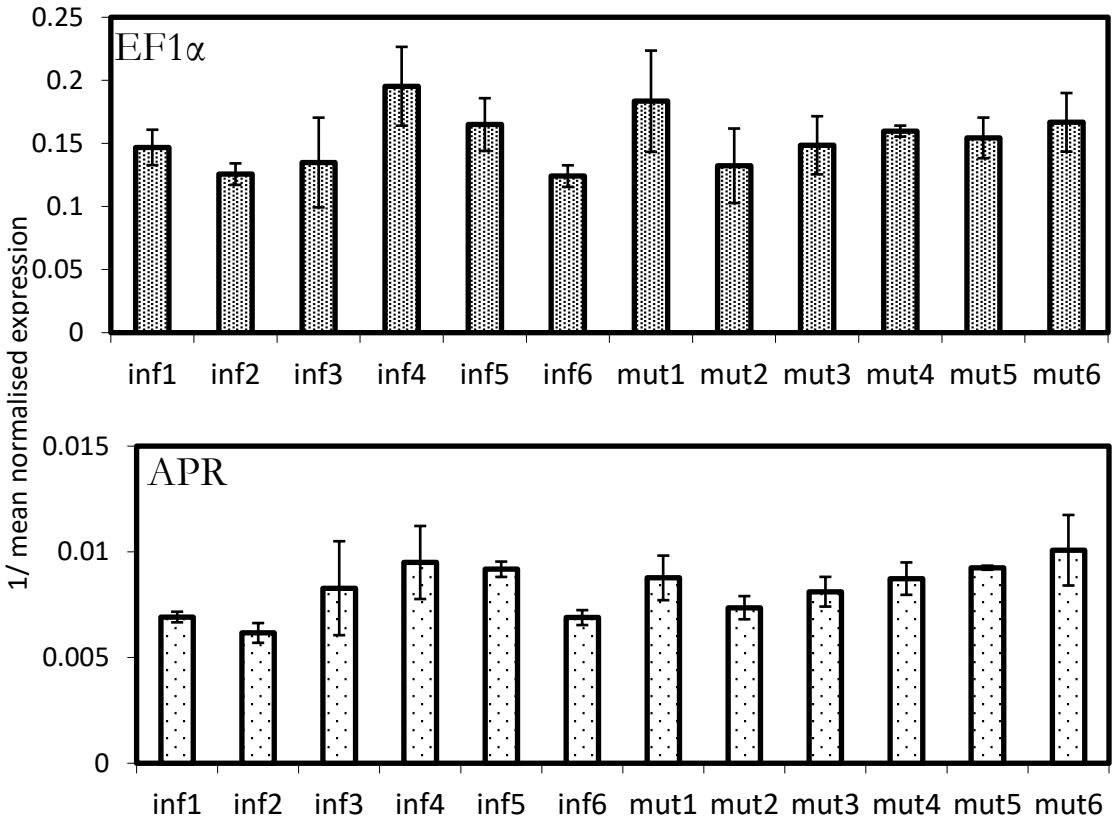

Figure 2.11 Normalised expression of RNA-2 coat protein gene to two different N. benthamiana reference genes (APR and EF1α) in cDNA extracted from plant roots for all wild-type and mutant TRV infected plants used in the root volatile experiment. Data shows means of the three technical replicates with error bars showing standard error. 1/normalised expression is given at different scales for the two reference genes due to the difference in expression of these two genes.

Dynamic solvent-based headspace sampling

These samples were analysed by comparing profiles of t=45 chromatograms to the lab air blanks (example given in Figure 2.12). Any peaks present in the sample or missing or reduced by a factor of 2x in the lab air blanks would be flagged as a compound of interest. Across all three time points, when comparing with lab air blanks, no compounds of interest were found in either infected or *Agrobacterium* control plant samples. The DCM blanks showed no discernible peaks. Infection testing with end point RT-PCR showed that all plants were successfully infected. RT-qPCR was not carried out on these plants, due to the lack of volatiles of interest present in these samples.

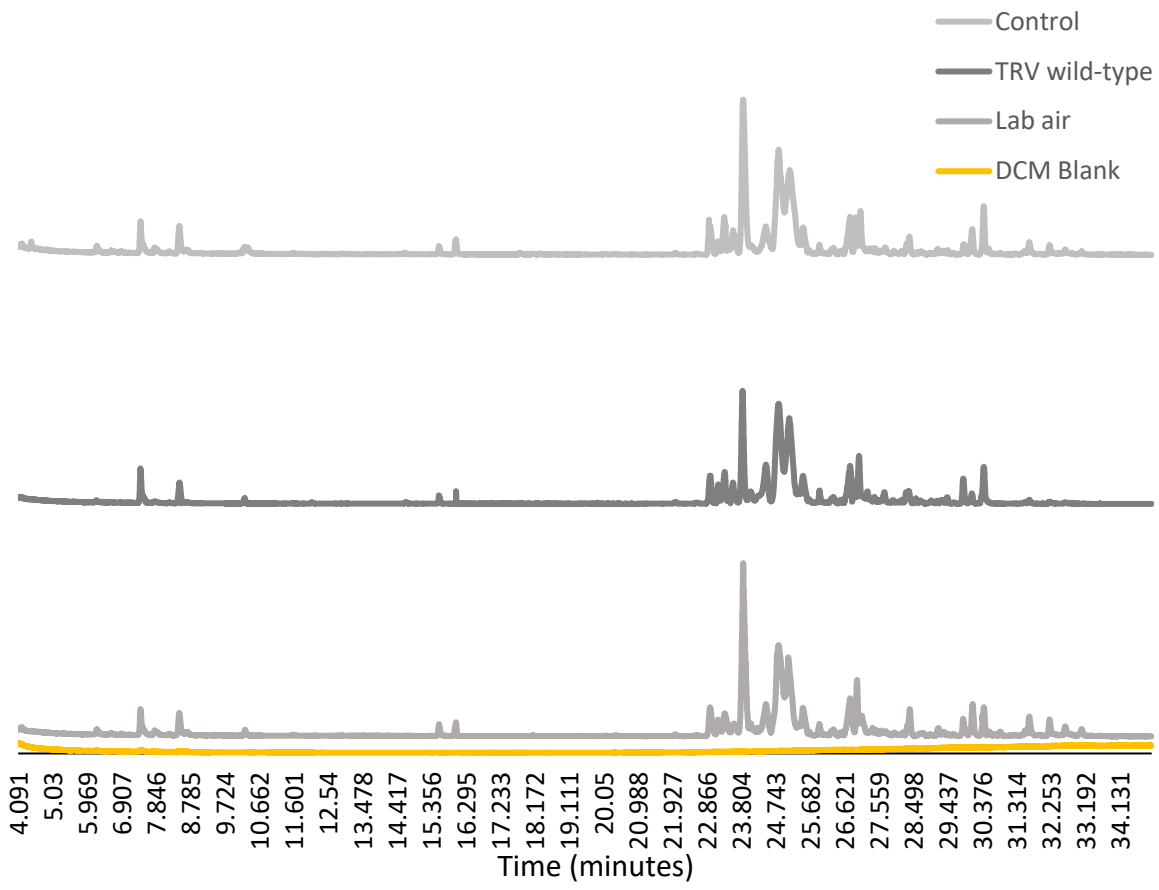

Figure 2.12 Example chromatograms of dynamic headspace solvent-based root VOC sampling. The chromatograms are staggered to show the similarities in traces between treatments with time post injection on the X axis and abundance on the Y axis with arbitrary units due to the stagger. There are no peaks present in the dichloromethane blank, showing no contamination of the solvent used, but no significant differences in peaks present in the lab air compared to *Agrobacterium* control infected plants and TRV infected plants. All peaks identified with this method were compounds present in the background air This pattern was repeated across all samples analysed. The samples used here for examples are all from 7 days post infection.

For the testing of standards, all compounds were detected, even at the lowest concentration of 1ng ml^{-1} (Figure 2.13), showing that these compounds were detectable by this chromatography method.

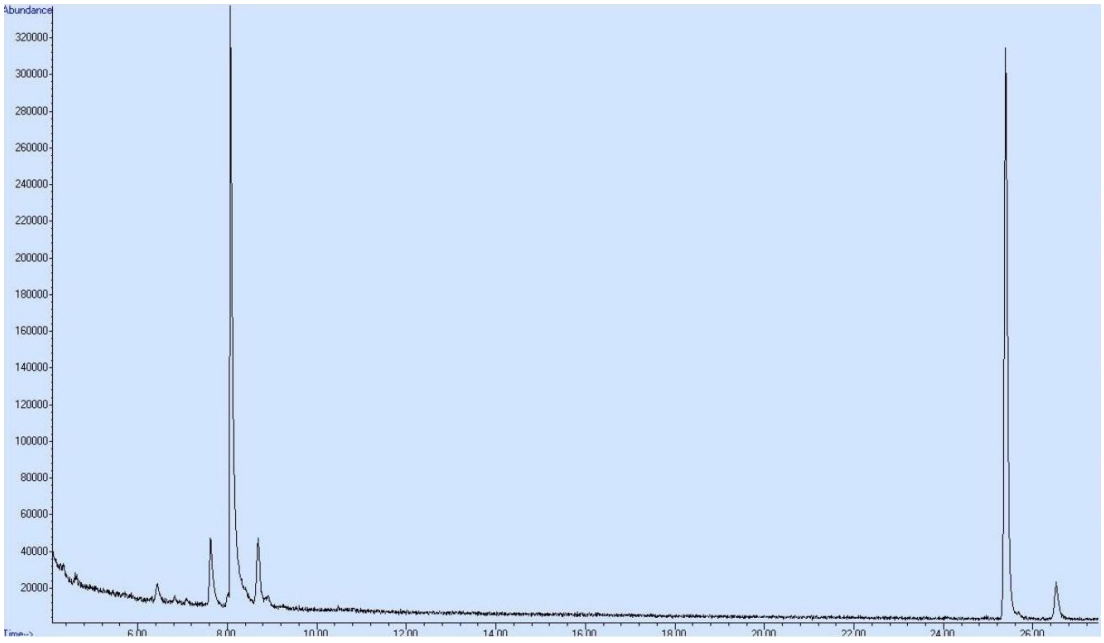

Figure 2.13 Raw chromatogram of 4,5 dimethyliazole and β caryophellene at concentrations of 10ng ml^{-1}. 4,5 dimethyliazole is the peak at 8.07 minutes while β caryophellene is the peak at 25.4 minutes.

Trichodorid assays

Pot treatment significantly affected trichodorid count numbers (GLM-p $\chi^2_{2, 30}$=88.8, p<0.001), with trichodorids preferentially moved through the tube towards the TRV-infected plant (Figure 2.14) when given the choice between an *Agrobacterium* control plant and a wild-type infected plant. Double the number of nematodes moving towards the infected plant (8±1 trichodorids) than the control plants (4±1). Of the 12 choice experiments run, one failed as the arms of the olfactometer were not sufficiently sealed into the central arena, and water that potentially contained trichodorids was released; this sample was discarded and the analysis was consequently carried out on 11 samples. Of the samples recorded, recapture rate was 58%±1.8, with 43% of those recaptured responding to one of the treatments.

RT-PCRs of root RNA from these plants confirmed infection in all the TRV-infected plants and confirmed absence of infection in control plants. Relative gene expression of the RNA-2 coat protein gene, normalised to the two reference genes, in the 11 TRV-

infected plants was compared to the number of nematodes moving towards those plants (Figure 2.14). Normalised viral load explained a little of the observed variance in trichodorid preference for wild-type infected plants (Figure 2.15, r^2=0.47, p<0.05 when normalised to *APR* and r^2=0.38, p<0.05 when normalised to *EF1a*).

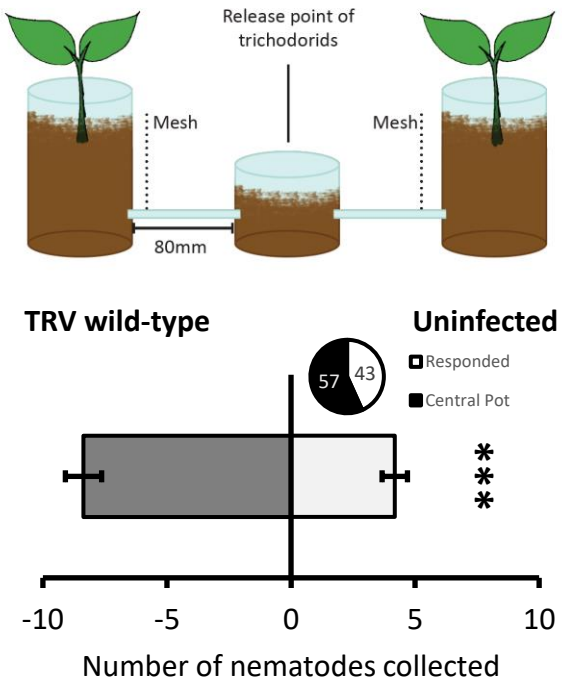

Figure 2.14 Response of trichodorids within olfactometers presented with uninfected and TRV infected plants Mean trichodorids collected from arms leading to either the *Agrobacterium* control or the TRV-infected plant. This data is from three pooled repeats of the olfactometer tests, n=11 as one sample failed. 43% of recaptured nematodes responded to one of the treatments, 57% remained in the central pot. Error bars are standard error. *** p<0.001.

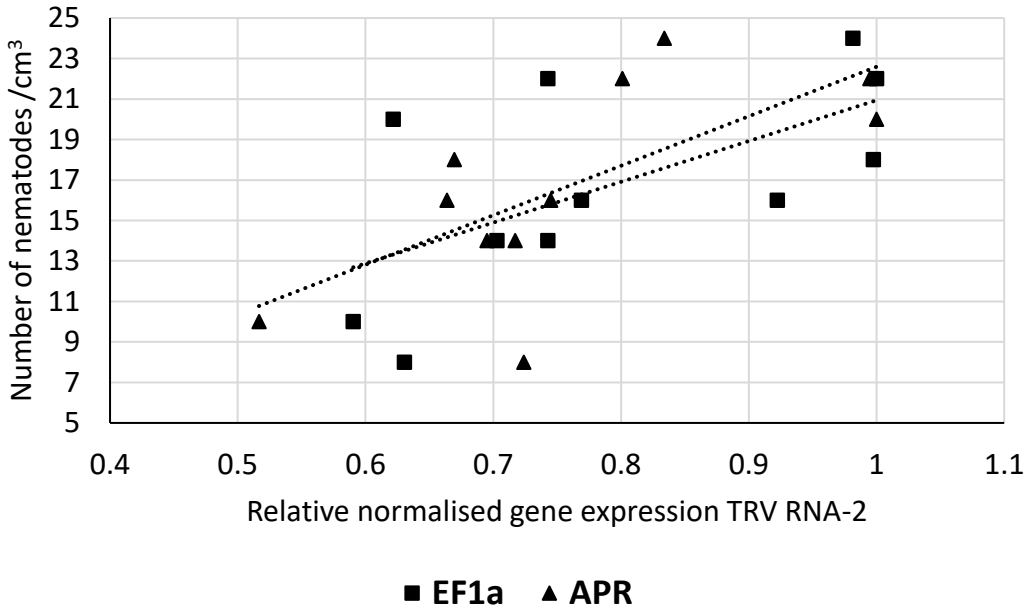

Figure 2.15 Comparison of nematode movement towards infected plants with two indicators for degree of infection. Both are relative abundances of TRV RNA-2 coat protein gene normalised to a different N. benthamiana reference gene.

Discussion

TRV infection had a deleterious effect on plant growth, allocating residual growth capacity to root systems and accelerating these impacts between 7 and 14 dpi. As predicted, there was a significant decline in total plant biomass with infection, inclusive of stunted root elongation which reduced the surface area to volume ratio of the roots. The number of root tips was also reduced in infected plants, showing a decline in root proliferation. At 7 days, infected plant root mass constituted a lower percentage of overall plant biomass than in controls, despite relatively equivalent aboveground biomass, showing that the reduction in biomass from infection through to day 7 was stronger in the root system in infected plants. There was a switch in the trends of root:shoot ratio between infected and uninfected plants between 7 and 14 dpi, with infected plants allocating a greater proportion of their total weight to roots, which may be an attempt for infected plants to redirect residual growth capacity to the roots, to try and provide more resources for growth.

These infection effects may be transient (Fernández -Calvino *et al.* 2014, Valentine *et al.* 2004), reducing in intensity and significance as time passes after infection (Shaw *et al.* 2014). This may explain why no published papers remark on deleterious effects of TRV infection on root systems, and only record tuber yield when looking at potatoes (Dale *et al.* 2000, 2004). Historically, the negative effects seen on growth, particularly in roots, in the trichodorid–TRV relationship in plants have been assigned to the trichodorids rather than the virus, such that trichodorids are referred to as "stubby root nematodes", ostensibly because of the reduced root mass due to their feeding on plant roots (e.g. Ruehle 1969, Standifer and Perry 1960, Whitehead *et al.* 1970, Yokoo 1964). These data suggest that viral infection may contribute to the damage and reduction in growth attributed solely to trichodorid infestations. It is clear that TRV infection does cause significant differences in both above and belowground biomass, at least until day 14 post infection. There was no significant difference in average root diameter, so whether the effects on roots are due to a change in root architecture or just reduced growth is less clear; however, due to the significant reduction in root length in infected plants at 14 dpi even while belowground biomass starts to recover, it suggests that the effect is less likely to be merely due to overall decline in growth with infection.

Using the direct headspace sampling method, four volatile compounds were identified as being overproduced in wild-type TRV-infected plants compared to plants

infected with either 2b- and 2c-missing TRV mutants or *Agrobacterium* controls. These were 2-ethyl-1-hexanol, 1,4-di-tertbutylbenzene, 2,6 ditertbutylquinone and 2,4 diterbutylphenol. 2-ethyl-1-hexanol is produced by many different plant species, including wheat (Birkett *et al.* 2004, Cruz *et al.* 2012), tobacco (Wei *et al.* 2004), lychee (Mahattanatawee *et al.* 2007, Wu *et al.* 2009) and olives (Ribeiro *et al.* 2008). It has reported bactericidal (Nakamura and Hatanaka 2002) and antifungal effects (Cruz *et all.* 2012, Fernando *et al.* 2005) as well as attractant effects on arthropod pests (Birkett *et al.* 2004). Insertion of the *Aspergillus niger* β-glucosidase gene, *BGL1*, by Cauliflower mosaic virus into *Nicotiana tabacum* leaves also increases production of 2-ethyl-1-hexanol compared to control plants (Wei *et al.* 2004). This would make it an ideal candidate for upregulated production through viral infection as it has been found to be commonly produced in multiple plant species. It has also been shown to be the result of an array of biotic attacks and stresses, such as beetle herbivory (Heil and Bueno 2007), fungal parasitism (Castelyn *et al.* 2015, de Lacy Costello *et al.* 2001) and pathogenic bacteria (Fernando *et al.* 2005, Yi *et al.* 2009). However, production of 2-ethyl-1-hexanol has not yet been reported as a result of viral infection. There are some concerns that 2-ethyl-1-hexanol can be a contaminant from industrial plasticisers in volatile sampling (Yi *et al.* 2009) but this does not explain the large difference found between treatments, although it may mean that the baseline levels of 2-ethyl-1-hexanol are affected and so slightly higher. Even with a potentially elevated and noisier baseline that could have affected starting concentrations, this investigation still identified significant differences in 2-ethyl-1-hexanol fluxes.

1, 4-di-tertbutylbenzene is produced by beech roots (Voglar *et al.* 2019), rhizobia associated with plant roots (Sang *et al.* 2011), the biocontrol fungus *Trichoderma asperellum* (Srinivasa *et al.* 2017) and the flowers of orchids (Baek *et al.* 2019). In this context, it is unlikely that these fluxes are the result of microbially-derived compounds as the plants were grown hydroponically and the roots rinsed prior to analysis. While there may still be bacteria associated with the roots, this is less likely. Production has not been reported to correspond to biotic or abiotic stress in plant roots (Voglar *et al.* 2019), nor does it have any reported antibiotic properties when tested (Sang *et al.* 2011). The likelihood of this compound being produced by root-associated microbes in this investigation is low, as the plants were grown hydroponically and rinsed before sampling.

Neither of the two remaining compounds have been identified in the literature as being produced by plants or microbes. It may be that they are produced as a result of contamination, and this study is repeated (Chapter 3) as a preliminary assay, with limited replicates, to identify and confirm the presence of virally mediated root volatiles and to confirm both the identity and role of these reported compounds in viral transmission.

The mutant infection treatment missing 2b and 2c showed volatile production that was statistically similar to the controls. This suggests that the observed differences in trace gas production were caused by products or interactions of one or both genes on infection. Alternatively, it is possible that the mutant infection was less deleterious to the plant and so did not elicit such a strong response, which is supported by the less severe effect on shoot and root biomass caused by the mutant infection than wild-type infection. In Chapter 3, two additional mutant treatments are tested to ascertain the effects of losing the functional effects of 2b and 2c in isolation. This way, any effects of a loss of function of one gene can be tested without confounding effects of the other.

Attempts to collect volatile and semi-volatile samples using the volatile collection traps failed to find any compounds released by *N. benthamiana* plant roots, irrespective of infection treatment. As the gas chromatography method used was the same as that for the analysis of the canister samples, and because all tested standards were detected using this method, even at very low concentration, it is unlikely that the lack of peaks can be attributed to this. As peaks were present in the lab air samples that were not present in the DCM blanks, the volatile traps had captured some volatiles, so it is also unlikely to be the fault of the polymers. Porapak Q as an adsorbent has a high affinity for lipophilic to medium polarity organic compounds of intermediate molecular weight. It is suitable for a wide range of VOCs including oxygenated compounds (Tholl *et al.* 2006) and should have captured some of the compounds seen in the canister samples, including 2-ethyl-1-hexanol. It is probable that there was a fault in the setup of the sampling equipment, and that the air being pulled over the traps was not solely from the sampling chamber. It is also likely that the plants were not left long enough in the sampling chamber for enough volatiles to have accumulated in the traps to give visible peaks. Other studies using adsorbent matrices to sample compounds from plants typically leave the plant material in the sampling apparatus for at least one and sometimes up to 10 hours (e.g. Bera *et al.* 2018, Mozūraitis *et al.* 2020, Steen *et al.* 2019). This is not a possibility for these investigations, as to leave roots exposed for that length

of time would cause drought stress on the plant, with a possible associated effect on volatile emissions (Salerno *et al.* 2017). Considering these factors, future volatile analysis in this thesis was carried out using the canisters and static headspace collection methods.

The olfactometer tests strongly indicate that trichodorids are responding positively to signals coming from TRV-infected plants that are not present in *Agrobacterium*-infected controls. This effect was significant in all three repeats of this trial and the RT-qPCR data shows that the strength of the movement correlates positively with increasing levels of infection. This is the first documented proof of trichodorids responding to TRV-infected plants and of potential chemotaxis for these nematodes, but it fits in with the understanding of how nematodes in general orient themselves in their soil environment and find food sources (Kergunteuil *et al.* 2019, Mondal *et al.* 2019, Oota *et al.* 2019, Rasmann *et al.* 2012, Schratzberger *et al.* 2019). With further work, the four compounds identified in the root volatile portion of this investigation should be tested on trichodorids in order to attempt to pinpoint the cause for this response. 2-ethyl-1-hexanol was selected for testing in Chapter 4, as it was produced in much larger quantities than the other identified compounds and has been documented in a wide variety of plant–pest interactions (p56-7). The Δ2b mutant mentioned above is tested in olfactometer assays against wild-type TRV-infected plants and control plants (Chapter 3) to see if there is variation in the strength of the attraction of the mutant treatments compared to wild-type.

This work has demonstrated that TRV infection has wide-ranging effects on plants and on plant–nematode interactions that were previously unknown. Infection does cause trichodorids to alter their behaviour and move towards infected plants which would help increase uptake of the virus by trichodorids and so theoretically increase transmission rates. This may be a driver in the huge variation in in-field transmission efficiencies of TRV (Ploeg *et al.* 1989). The volatile study has suggested a group of potential compounds that are overexpressed by wild-type infected plants compared to control plants. These compounds, either alone or in a blend of chemicals or even in concert with other factors, may explain the attractant effect seen in the olfactometer assays.

Chapter 3

Introduction

Work in the previous chapter showed clear effects of TRV infection on *N. benthamiana* plants; from impacting above and belowground growth and biomass allocation to the compounds released by living plant roots. There was also a significant difference in the preference of trichodorid nematodes when presented with plants infected with wild-type TRV and control plants. For effective viral control it is critical to establish which part of the virus is most responsible for the observed plant behaviour. Plant biomass data from Chapter 2 (Figure 2.8) demonstrated that plants infected with a TRV mutant that was missing two genes from RNA-2 responded in a similar way to uninfected plants in some respects (volatile fluxes, Figure 2.9). In terms of plant biomass and allocation, mutant-infected plants showed a reduced impact of infection (hydroponic plant weights, Figure 2.8) and showed an intermediate response between uninfected and wild-type infected plants.

While the previous work answers some questions about the direct effects of infection, it does generate others. Strong effects of infection were seen but the permanence of these effects was not rigorously tested. Apart from spraing symptoms in potato, the initial reduction in root to total plant biomass and dramatic reversal at 14 days post infection (dpi) with TRV infection have not been reported in the literature, while aboveground effects are well documented across different hosts (Otulak *et al.* 2012). This contradiction between our previous results and the published literature could be due to the temporary nature of TRV infection, consistent with previous reports of recovery from TRV symptoms in *N. benthamiana* from 18 dpi (Cadman and Harrison 1959, Ratcliff *et al.* 1999, 2001, Shaw *et al.* 2014). If this is the case, this has ramifications for agricultural systems since the time of infection may alter the severity of crop symptoms, e.g. potato seedlings that are virally infected may not be infected when tubers are set and spraing symptoms may not appear unless re-transmission occurs (Harrison and Robinson 1986, MacFarlane 1999). In order to address this, it is important to extend the timeline of infection further to see if and when recovery occurs. Linking this timeline to growth parameters such as those introduced in the last chapter (e.g. root length, root:total plant biomass allocation, root surface area) would also show if the infection effects mirror infection levels completely, or whether there is a lag period. It has been documented that TRV takes up to 4 days from leaf inoculation

to travel to the roots of *N. benthamaiana* plants and that infection peaks between 7 and 8 dpi (Valentine *et al.* 2004). In addition to this, it may be that the most deleterious effects do not occur at peak infection levels and may be noticed even after the expression of TRV genes is reduced; therefore, the full infection course should be sampled.

This requires further information on how TRV infection is driving these observable effects, including what part of the virus is responsible. Plant biomass data from Chapter 2 (Figure 2.8) showed that plants infected with a TRV mutant that was missing two genes from RNA-2 showed a reduced response to infection than plants infected with wild-type TRV.

RNA-2 is the second component of TRV's bipartite RNA genome (Lindner *et al.* 2018). It encodes the coat protein gene, which is responsible for production of the protein capsid around the viral genome and one or more non-structural genes associated with nematode transmission (Adams *et al.* 2012, Koenig *et al.* 2016, Lindner *et al.* 2018, MacFarlane 1999, MacFarlane *et al.* 1995, Ploeg *et al.* 1993b, Sahi *et al.* 2016), depending on the TRV isolate (Figure 1.5). The serotype used in these investigations, TRV-PpK20, has two genes in addition to the gene encoding for the coat protein, which are identified as 2b and 2c (Figure 3.1). TRV-PpK20 is one of the most well-reported serotypes and is known to be transmitted by a single species of trichodorid, *Paratrichodorus pachydermus* (Hernández *et al.* 1995, Ploeg *et al.* 1993a). It was first isolated in Kinshaldy, Scotland (Ploeg *et al.* 1992).

In TRV-PpK20, 2b has been shown to be essential for virus transmission by nematodes (Hernández *et al.* 1996, MacFarlane *et al.* 1996, Visser and Bol 1999), and this may be due to the formation of link bridges between the 2b protein and the nematode oesophagus (MacFarlane 1999). 2b genes from different TRV isolates and closely-related viruses (Pea early browning virus (PEBV) and Pepper ringspot virus (PepRSV)) have limited similarity (Figure 1.5, Tavares-Esashika *et al.* 2020) and it has been suggested that variations in 2b may determine vector species specificity (MacFarlane 1999). 2c proteins have much less similarity across isolates than 2b and their effect is not well understood. In an isolate of PEBV (TpA56), deletion or frame-shift mutation of 2c had a negative effect on nematode transmission (MacFarlane *et al.* 1996, Schmitt *et al.* 1998), but this effect was not seen for 2c genes in TRV-PpK20 (Hernández *et al.* 1996) or PaY4 (Vassilikos *et al.* 2001), whereas 2b remained essential. This isolate was chosen for this investigation since it is i) an isolate common to the UK and ii) well reported with a well-

characterised genome and there are confirmed differences in 2b and 2c function on nematode transmission.

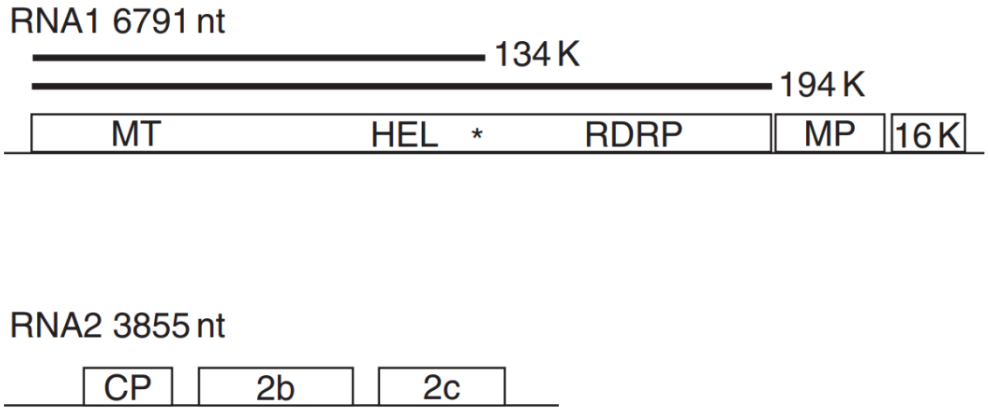

Figure 3.1 Genome diagram of TRV isolate PpK20. (from MacFarlane 2008) The open boxes represent virus genes while the solid lines above RNA-1 show the location of two overlapping helicase genes containing methyltransferase (MT), helicase (HEL) and RNA-dependent RNA polymerase (RDRP) motifs. The movement protein is also known as the 1a gene while the cysteine-rich 16k gene is also known as the 1b gene. In PpK20, RNA-2 contains two non-structural genes, 2b and 2c, in addition to the coat protein gene (CP).

This investigation aims to build on the previous work in Chapter 2 to i) quantify the effects of TRV on root volatiles, growth and architecture across a longer timeline of infection and ii) extend root sampling time to confirm the enhanced volatile production in infected plants and determine if further compounds of interest become apparent that were not detected during the shorter analytical procedure of Chapter 2. These trials will also expand on the work in Chapter 2 since they will examine plants infected with two TRV mutants (Δ2b, Δ2c) in addition to wild-type TRV and *Agrobacterium* controls. These assays will determine if the infection responses are reduced when one or both RNA-2 genes are inactive. As 2b is vital for nematode transmission (Adams *et al.* 2012, MacFarlane 1999, Ploeg *et al.* 1993b), plants infected with TRV-Δ2b mutants will be tested against wild-type TRV-infected plants and *Agrobacterium*-infected control plants in trichodorid preference trials.

It is expected that levels of viral load in the roots of *N. benthamiana* plants will significantly increase between 4 and 7 dpi, due to the delay in TRV reaching the roots, and then decline over the rest of the sampling timeline as the plant recovers. Observed effects to biomass and root architecture will follow a similar pattern but may lag infection, with the largest differences between treatments between 7 and 14 dpi. Based on the plant biomass data from Chapter 2, where a mutant missing both 2b and 2c

genes was tested, I hypothesise that the two mutant treatments will show intermediate effects between control plants and wild-type infected plants.

Root VOC data is expected to show a significant difference in production of the four compounds previously identified between wild-type infected plants and control plants. Plants infected with mutant versions of TRV are hypothesised to show an intermediate response. Roots that are sampled over longer times will show increased production of a range of compounds when compared to shorter sampling timeframes.

Based on my previous work I expect that trichodorids will preferentially move towards wild-type infected plants over TRV-Δ2b infected plants and towards TRV-Δ2b infected plants over control plants. I further postulate that there will be a positive relationship between level of infection and i) attractiveness to nematodes and ii) emission of VOCs elevated under wild-type infection.

Methods

TRV mutant design

To test for the importance of these genes in plant infection and nematode attractiveness, TRV RNA-2 mutants, which are identical to wild-type TRV except containing non-functional versions of those genes, were needed to be compared with wild-type infection and control plants.

In order to create the 2b mutant (TRV-Δ2b), the BstEII site 5'-GGTTACC-3' is cut with BstEII and in-filled with Klenow polymerase to produce 5'-GGTTACGTTACC-3', an insertion of 5 nucleotides. This causes a change in the reading frame of the gene, and this changes the predicted amino acid sequence of the protein and introduces a premature translation termination codon into the mutated gene sequence which destroys the BstEII site. Similarly, for the 2c frameshift mutant (TRV-Δ2c), this is at the NgoMIV site (5'-GCCGGC-3'), which after Klenow in-filling leads to 5'-GCCGGCCGGC-3', an insertion of 4 nucleotides and thus a frameshift during translation which destroys the site and creates a new Eag1 site. In each case, the frameshift causes a loss of function of that gene.

Plant growth and infection

Sterilised (see Chapter 2 Methods) *N. benthamiana* seeds were germinated on moist filter paper in a Petri dish at 20°C then transferred to trays of the 90:10 mix of sand:John Innes no2 compost and watered with ½ strength Hoagland's nutrient solution until two true leaves had expanded, then potted on to different media, depending on the experiment. Plants for root architecture and volatile collection assays were planted in 1L pots of sterilised sand and plants for trichodorid preference tests were directly planted into the olfactometer pots, as in Chapter 2 methods.

Plants were infected with one of four different treatments as soon as six true leaves were present. Seven days prior to testing, control plants were infected with untransformed *Agrobacterium* and "wild-type infected" plants with *Agrobacterium* transformed with TRV RNA-1 isolate Pp085 and TRV RNA-2 isolate PpK20 as in the previous experiments. The two new additional mutant treatments used *Agrobacterium* transformed with RNA-1Pp085 as with the wild-type infections but used altered PpK20 RNA-2 for altered 2b and 2c genes (see Chapter 3 Methods: TRV mutant design). In total, 5 different cultures were produced: untransformed *Agrobacterium*, *Agrobacterium*

transformed with TRV RNA-1 and *Agrobacterium* transformed with the three TRV RNA-2 variants (wild-type, Δ2b and Δ2c).

Infection solutions were prepared by making overnight stocks of each culture from frozen glycerol stocks and preparing infiltration cultures of mixed RNA-1/RNA-2 or untransformed *Agrobacterium* as described previously. All the TRV treatments used the same RNA-1 culture, so triple volumes were produced compared to the RNA-2 variants. Once mixed, 0.5ml of the combined infiltration medium were inoculated onto the apoplast of each leaf using a needleless syringe (Senthil-Kumar and Mysore 2014) and four leaves were inoculated per plant as in previous experiments (Chapter 2).

Successful infection was tested for with end-point RT-PCRs on leaf and root tissue from infected plants to check for expression of virus genes using primers from the 2004 paper by Boutsika and colleagues (Table 2.1). Quantification of infection was carried out for all investigation, normalised with the same *N. benthamiana* reference genes for internal control as previous work (Liu *et al.* 2012, see Table 2.2). In all investigations, shoot and root fresh weight was recorded and small samples of both leaf and root tissue were flash frozen in liquid nitrogen for RNA extraction and quantification of infection. The remaining plant material was dried at 70ºC for 3 days before dry weights were recorded.

Growth and root morphology

For this experiment 100 *N. benthamiana* plants that were potted onto 1L sand pots were used. Growth conditions were the same as the first root morphology experiment; plants were fed with 250ml ½ strength Hoaglands twice weekly and watered with dH_2O daily. Previously plants were harvested at 4-, 7- and 14-days post infection (dpi) and quantification of infection suggested there was a peak in viral load at 7 dpi and a slight, non-significant decrease between 7 and 14 dpi. To quantify the full infection time course, plants were also sampled at 21 and 28 dpi (Fernández -Calvino *et al.* 2014, Otulak *et al.* 2012, Valentine *et al.* 2004). Five plants per treatment were harvested at each time point, weighed, and growth parameters recorded as in the first experiment: root and shoot fresh weight, total root length, total root surface area, total root volume, number of tips and degree of root branching (tip number divided by total root length using WINRhizo software (Regent Instruments, Canada) on scanned imaged of the roots).

Root VOC sampling

This analysis used 20 *N. benthamiana* plants grown in 1L pots of sand and watered with the same ½ strength Hoagland's solution regimen as the root morphology plants, contrary to the plants used in Chapter 2. As before, samples were taken 7 dpi using a sampling chamber (Figure 2.2) as described, but samples were taken from each plant at 0, 30 and 60 minutes after being placed in the box, rather than just 0 and 20 minutes (Chapter 2). The longer sampling period was used to enhance the concentration of affected volatiles in the sampling chamber, increasing the signals detected. Multiple timepoints can also provide greater confidence in production rates over time. All samples were analysed on a GC:MS system using a 30m DB-5 column (Agilent) with an initial oven temperature of 30°C, held for 5 minutes, first ramp of 4°C min^{-1} to 250°C (no hold) and a second ramp and bake-out of 40°C to 325°C with a 5 minute final hold (total runtime of 66 minutes). Peak areas were analysed on Agilent ChemStation Data Analysis software and converted to production over time by comparing values for 0, 30 and 60 minutes as described (Chapter 2 Methods: Root VOC sampling).

Trichodorid response

As the 2b gene on RNA-2 is known to be vital for nematode transmission (Hernández *et al.* 1997, MacFarlane 2003, Vassalakos *et al.* 2001, Vellios *et al.* 2002) and trichodorid numbers were limited, two sets of choice assays were carried out: TRV wild type vs TRV-Δ2b and control (untransformed *Agrobacterium*) vs TRV-Δ2b. The experiments were carried out as described in Chapter 2, except each set was only run twice instead of 3 times. Statistical analysis showed that significant differences in the original data were already apparent after the second repeat.

Statistical analysis

Nematode numbers were analysed using a GLM as described in Chapter 2, with the same steps used to check the model. Models without day had a better AIC and so only pot was included as independent variable in the final models. Analyses of deviance were performed on the models with Tukey HSD post hoc tests with p value adjustment to investigate significance.

Comparisons between nematode numbers and expression of viral RNA was carried out with Pearson's product moment correlation (Pearson 1985). All other data were analysed using analysis of variance. Analysis of variance model assumptions were checked with Shapiro-Wilk normality tests and Levene's test for homogeneity of

variance. All root architecture parameters were transformed using cube root transformation to fit the analysis of variance assumptions. All qPCR data was also transformed using cube root transformation. The root architecture data were analysed per timepoint, as well as across the entire dataset. The R packages car (Fox and Weisberg 2019) was used to carry out Levene's test for homogeneity of variance, emmeans (Lenth 2021) to carry out post hoc comparisons on the GLMs and AER (Kleiber and Zeileis 2008) to check overdispersion of the GLM data. The packages car, rcompanion (Mangiafico 2020) and MASS (Venables and Ripley 2002) were used for data transformation and visualisation.

Results

Growth and root morphology

There was a significant effect on infection on both shoot and root biomass 7 and 14 dpi (ANOVAs-Day 7: $F_{(3,16)}$=4.1, p<0.05 [shoot dry], $F_{(3,16)}$=15.51, p<0.001 [root dry], Day 14: $F_{(3,16)}$=4.88, p<0.05 [shoot dry], $F_{(3,16)}$=11.32, p<0.001 [root dry]), showing that TRV infection has a negative effect on plant growth and that this is more marked in roots than shoots (Figure 3.2). No significant effect of infection on root or shoot biomass was seen at 4 dpi, as in previous work (Chapter 2). The extended timepoints of 21 and 28 dpi show that any differences between treatments are no longer significant. Post-hoc tests show that *Agrobacterium*-infected control plants are the largest and differ significantly compared to wild-type TRV-infected plants for fresh and dry weights of root and shoot portions of the plants. The TRV mutant treatments show an intermediate response between control and wild-type infection. Plants infected with TRV with an altered 2c gene (TRV-Δ2c) only differed significantly from control plants in root dry weight at 14 dpi (0.03g±0.006 and 0.07g±0.011 respectively, p<0.05); for other measures, they were similar in size to control plants. For root biomass, they were significantly larger than wild-type infected plants at 7 dpi (fresh and dry weight p<0.05). Plants infected with 2b-altered TRV (TRV-Δ2b) never differed from wild-type plants, although Figure 3.2 suggests that they may have been slightly larger and had significantly smaller roots than control plants at 7 and 14 dpi (p<0.001, for root fresh and dry at both timepoints). These suggest slightly reduced costs of infection for these mutant infections, especially for TRV-Δ2c plants.

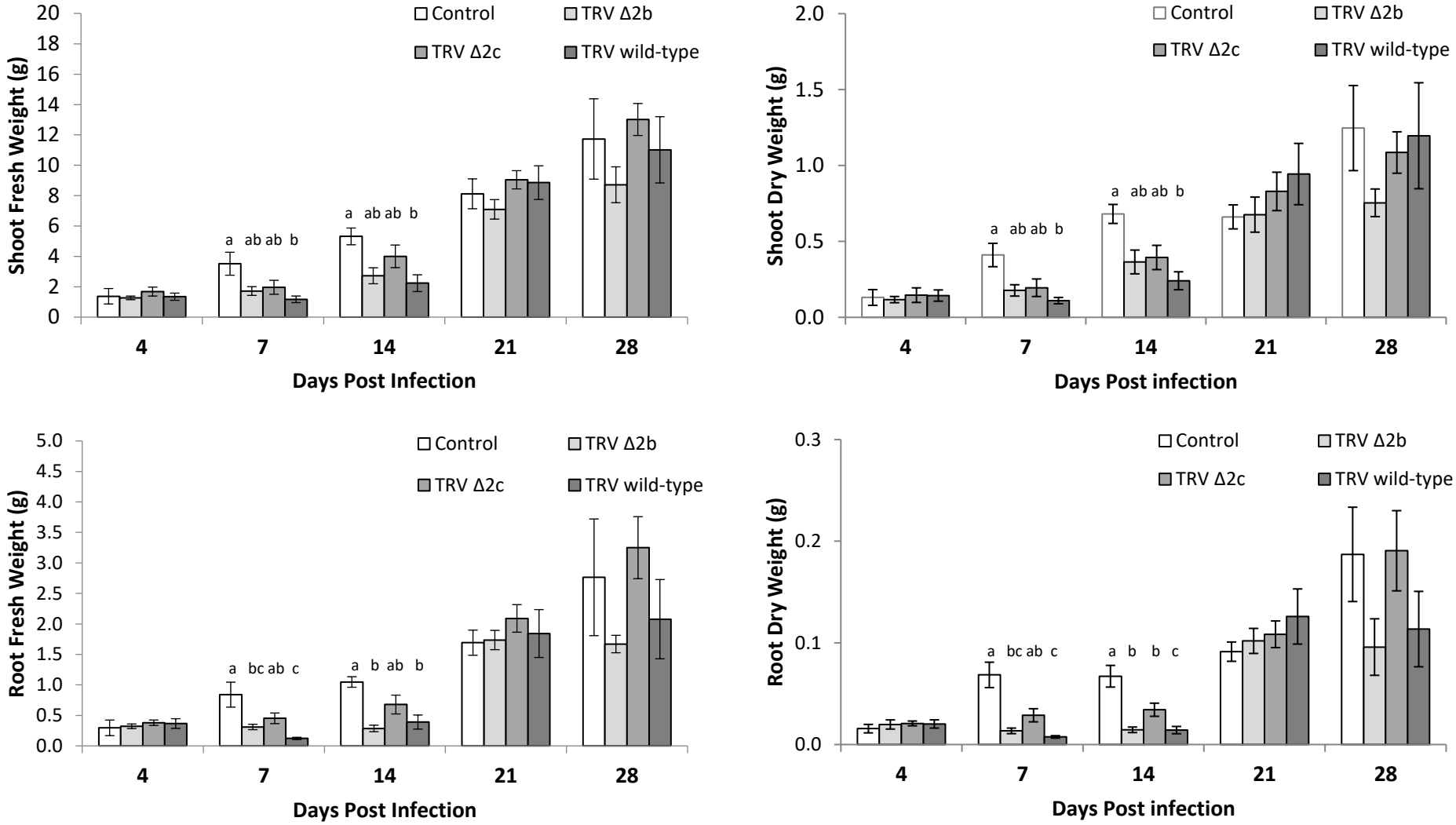

Figure 3.2 The effect of infection with wild-type TRV and frameshift mutants on root and shoot biomass across an infection timeline. These figures show comparisons between *N. benthamiana* plants that are infected with different TRV treatments. Significant differences between treatments were only seen at 7 and 14 days post infection. Letters represent statistically significant bars. Means and standard errors are shown and n=5 for all bars.

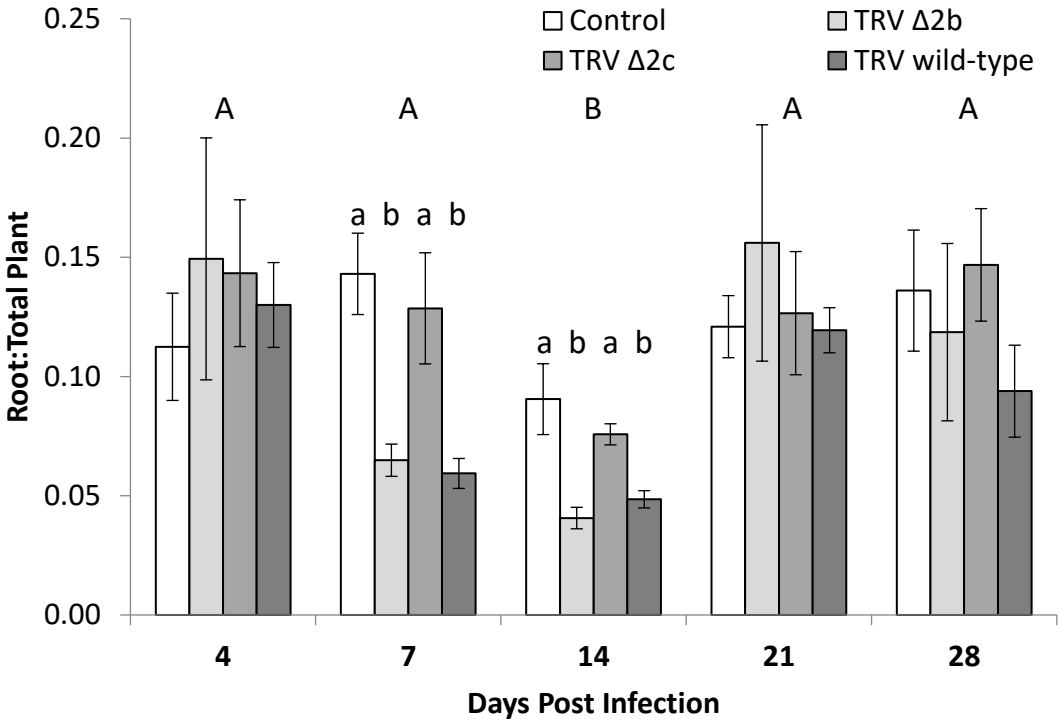

Figure 3.3 The effect of infection with wild-type TRV and frameshift mutants on root to total plant weight ratio across an infection timeline. This figure shows how biomass allocation to roots changes between *N. benthamiana* plants infected with different TRV treatments. Significant differences between treatments were only seen at 7 and 14 days post infection. Lowercase letters represent statistically significant differences within sampling days, while uppercase letters show differences across sampling timeline. Means and standard errors are shown and n=5 for all bars.

There was a significant difference in the biomass allocation to roots compared to the rest of the plant (Figure 3.3) between the different sampling days on the infection timeline ($F_{(4, 75)}$=8.00, p<0.001) and a significant interaction between infection type and sampling day ($F_{(12,75)}$=2.44, p<0.01), but no significant difference between infection treatments when looking at the dataset as a whole (p=0.61). Post hoc tests showed that there was a significant dip in root to total plant allocation at 14 dpi compared to all other days, with no other differences found to be significant. Figure 3.3 suggests that this difference is driven primarily by a drop in TRV-Δ2b and wild-type TRV-infected plants, and analysis of the day 7 and day 14 data supports this. At both timepoints there was a significant difference between treatments (Day 7: $F_{(3,16)}$=8.23, p<0.01, Day 14: $F_{(3,16)}$=5.60, p<0.01). Post hoc tests showed that TRV-Δ2b and wild-type TRV-infected plants had significantly less root allocation than control or TRV-Δ2c plants on both days (Day 7: p<0.01 between control and wild type and p<0.05 for all other interactions, Day 14 p<0.05 for all interactions). There was no significant difference between control

plants and TRV-Δ2c infected plants or between TRV wild-type and TRV-Δ2b infected plants. This suggests that in addition to making plants smaller, infection also temporarily alters the relative size of root systems to the whole plant.

Infection also affected other aspects of the *N. benthamiana* plant architecture (Figure 3.4). Infection reduced total root length (Day 7: $F_{(3,16)}$=8.23, p=0.01, Day 14: $F_{(3,16)}$=13.81, p=0.001) with significant differences between control and wild-type infected (p<0.001 both days) and control and TRV-Δ2b infected plants (p<0.001 both days). Plants infected with TRV-Δ2c had significantly longer roots than either TRV-Δ2b (p<0.05 at day 7, p<0.01 at day 14) or wild-type (p<0.01 at day 7, p<0.05 at day 14) infected plants across both days but were not significantly different to control plants.

Root surface area (Day 7: $F_{(3,16)}$=25.27, p<0.001, Day 14: $F_{(3,16)}$=8.38, p<0.01) and volume (Day 7: $F_{(3,16)}$=10.82, p<0.001, Day 14: $F_{(3,16)}$=7.19, p<0.01) also showed differences at these two timepoints (Figure 3.4). At 7 dpi, control plants had significantly greater surface area than TRV-Δ2b (p<0.001), TRV-Δ2c (p<0.01) and wild-type infected plants (p<0.001) and TRV-Δ2c infected plants had greater surface area than wild-type infected plants (p<0.01). This difference was slightly reduced at 14 dpi, with the only significant differences seen between control and TRV-Δ2b (p<0.01) and wild-type (p<0.01) infected plants, with TRV-Δ2c infected plants giving an intermediate, but not significantly different response (Figure 3.4). For root volume, the strongest effect was also seen 7 dpi. Control plants had significantly greater volume than TRV-Δ2b (p<0.01 both days) and wild-type (Day 7: p<0.001, Day 14 p<0.01) infected plants at both timepoints and greater than TRV-Δ2c (p<0.05) infected plants at 7 dpi. The data for root volume is not shown, as it mirrors that of root surface area.

For total root length, total root surface area and total root volume there were no significant differences between treatments at 21 or 28 dpi. Figure 3.4 suggests a significant increase for both metrics in plants infected with TRV-Δ2c (root length: 1056 cm ± 55, surface area: 213.3 ± 36.8) at 21 dpi compared to control and TRV-Δ2b plants, but the overall variability at that time point is too high for a significant result.

There was no overall significant effect on root length, root surface area or root volume across with infection treatment across the whole timeline. There were no significant differences found in number of root tips, average root diameter or surface area to volume ratio either across the whole timeline or at 7 and 14 dpi.

There was a significant difference in viral RNA load across days for both housekeeping genes used (EF1α $F_{(4,59)}$=1100.87 p<0.001, APR: $F_{(4,59)}$=1498.67 p<0.001) with a significant increase at 7 and 14 dpi (Figure 3.5) which then drops off again between 14 and 21 dpi. At 4 dpi, there are some detectable levels of infection for wild-type plants whereas there is none for either of the mutant infected treatments. No significant difference between treatments was found at any other timepoint, even when analysing each timepoint in isolation.

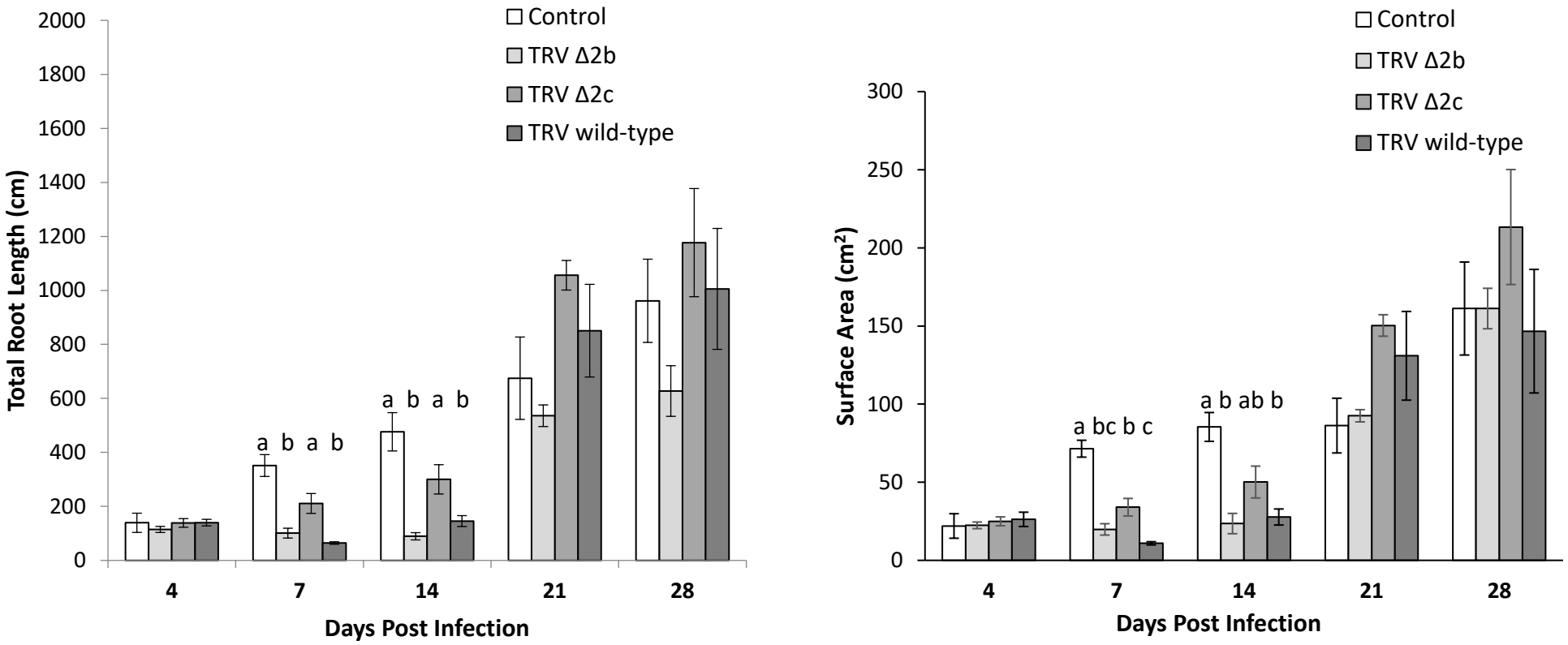

Figure 3.4 The effect of infection with wild-type TRV and frameshift mutants on total root length and root surface area across an infection timeline. This figure shows the differences of some root architecture parameters in *N. benthamiana* plants infected with different TRV treatments. Significant differences between treatments were only seen at 7 and 14 days post infection. Letters represent statistically significant bars. Means and standard errors are shown and n=5 for all bars.

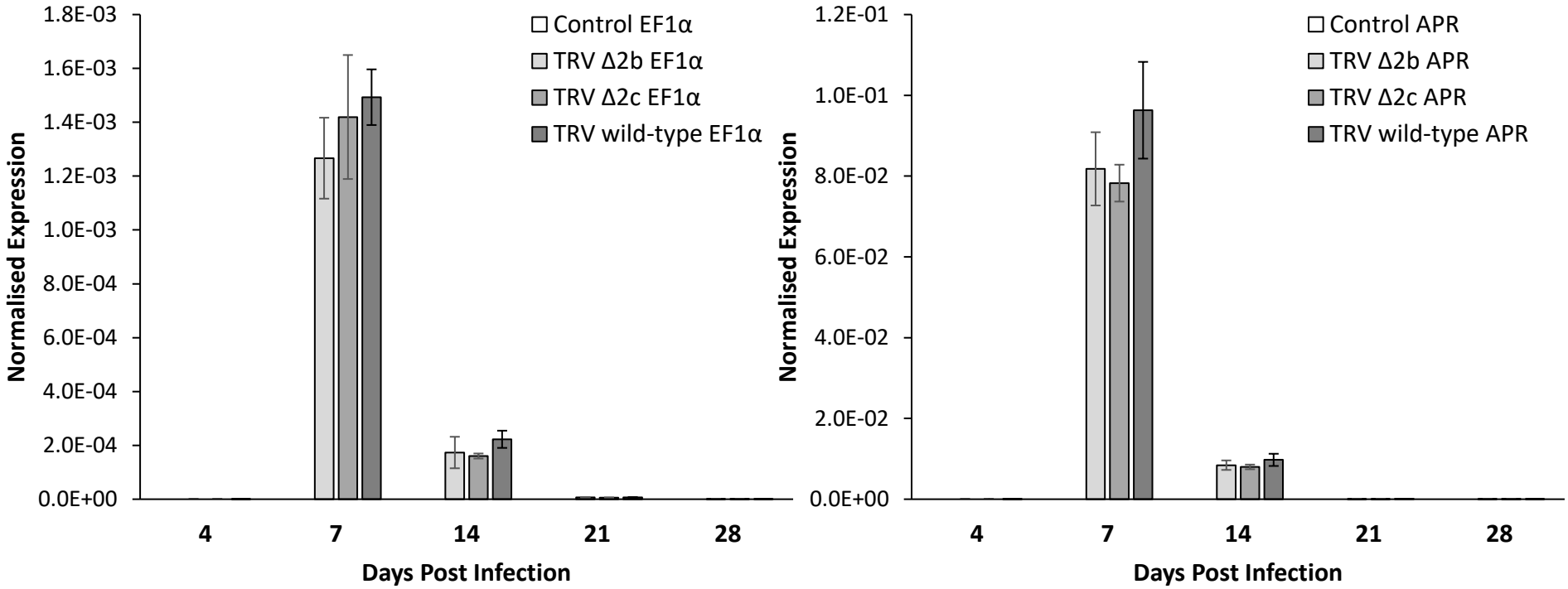

Figure 3.5 The normalised expression of TRV coat protein gene in *N. benthamiana* roots across a 28-day infection timeline. This figure shows the changes in expression of a TRV gene normalised to two different *N. benthamiana* housekeeping genes (*EF1a* and *APR*) over time. Means and standard errors are shown and n=5 for all bars.

Root VOC sampling

Of the root volatiles found in the previous investigation (Chapter 2), only 2-ethyl-1-hexanol and 1,3-ditertubutylbenzene were detected in this experiment. Both 2-ethyl-1-hexanol and 1,3-ditertubutylbenzene showed similar trends to before, with significant differences (Figure 3.6) in volatile production between control, mutant TRV and wild-type TRV-infected plants ($F_{(3,17)}$=5.02, p<0.05 for 2-ethyl-1-hexanol and $F_{(3,17)}$=3.25, p<0.05 for 1,3-ditertubutylbenzene). Wild-type TRV-infected plants produced significantly more 2-ethyl-1-hexanol (p<0.01 compared to control and TRV-Δ2b, and p<0.05 compared to TRV-Δ2c) and 1,3-ditertubutylbenzene (p<0.05 for all, only at 60 mins timepoint) than other treatments (Figure 3.6). For all treatments, there seemed to be an increase in flux rate for both compounds between the 20 and 60 minutes timepoints (data not shown), but post hoc tests showed this was only significant for TRV wild-type 2-ethyl-1-hexanol (p<0.001) and 1,3-ditertubutylbenzene (p<0.05) and TRV-Δ2c 2-ethyl-1-hexanol (p<0.05).

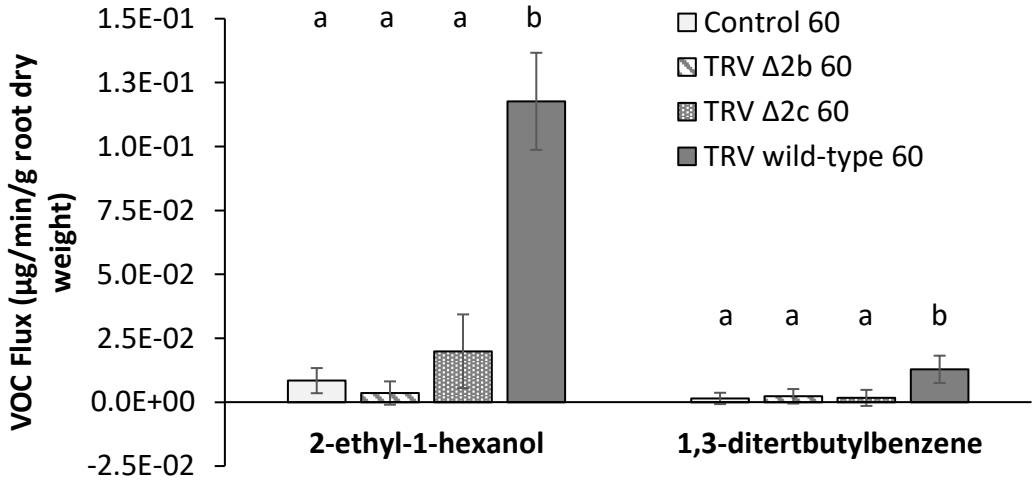

Figure 3.6 Average root VOC fluxes from roots of plants infected with different TRV treatments, showing the effects of 2b and 2c. Data is only shown from 60 minutes after plants were placed in the headspace sampler as the previous timepoint (20 minutes) showed little significant differences between treatments. The fluxes are shown at two scales and are µg min^{-1} g^{-1} root dry weight. In comparisons between treatments for the same compound, bars with different letters are statistically different from each other, error bars are standard error, n=5 for all treatments.

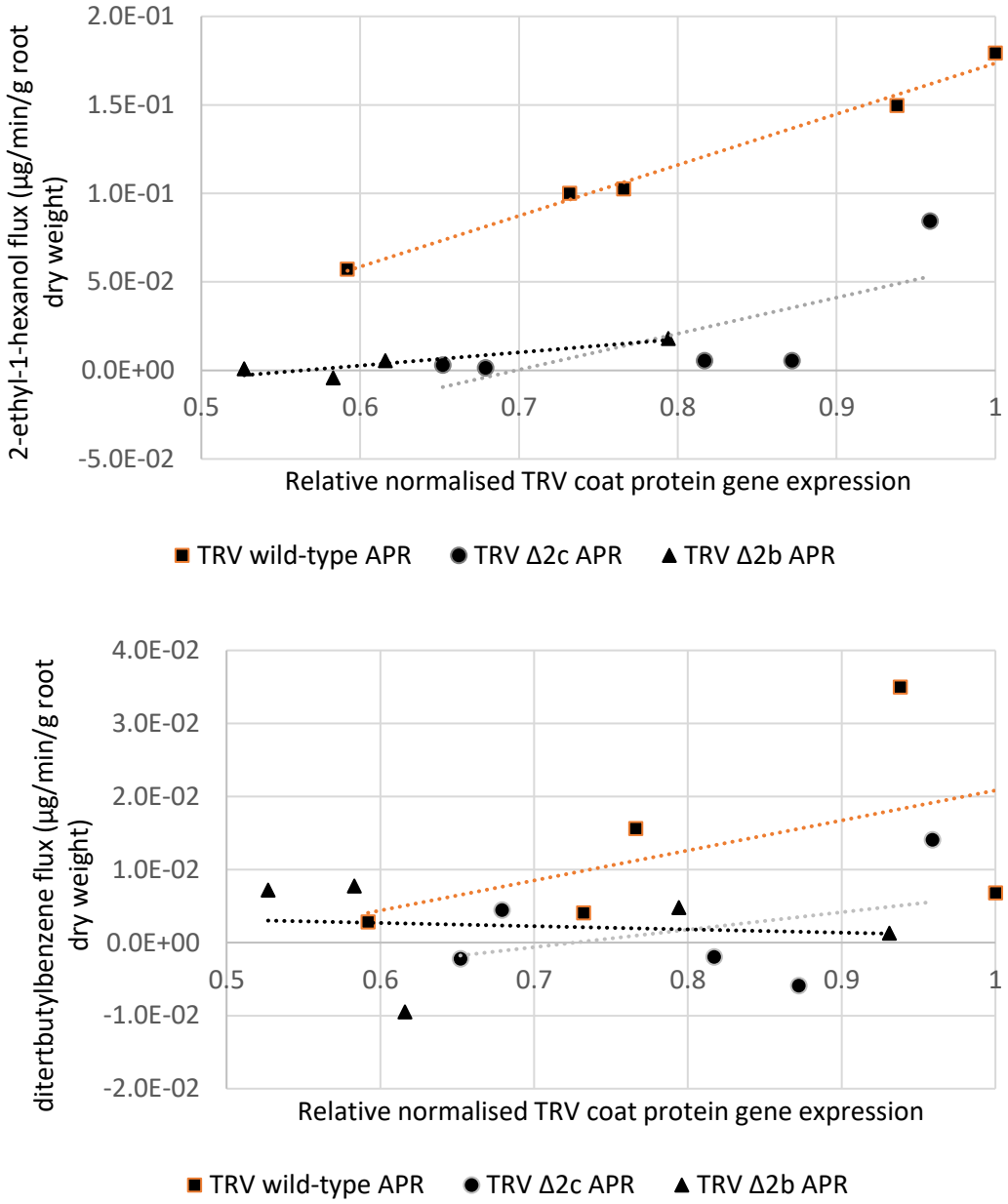

Figure 3.7 The relationship of 2-ethyl-1-hexanol (top) and 1,3-ditertbutylbenzene (bottom) fluxes with viral load. Comparison of root volatile emissions from plants infected with wild-type TRV and mutant TRV with altered 2c genes. With relative viral load normalised to two *N. benthamiana* housekeeping genes, APR and EF1α. Lines of best fit are for wild-type infected plants.

There was no significant difference in viral load across the three infection treatments. Normalised viral load explained nearly all of the observed variance in 2-ethyl-1-hexanol emissions for wild-type infected plants (Figure 3.7 top; r^2=0.99, p<0.001) and some of the variance for TRV-Δ2b infected plants (Figure 3.7top; r^2=0.94, p<0.01).

There was no significant relationship between viral load and 2-ethyl-1-hexanol production and in TRV-Δ2c infected plants (Figure 3.7 top; r^2=0.54, p=0.16) and no significant relationships between viral load and 1,3-ditertbutylbenzene production in any of the treatments (Figure 3.7 bottom: wild-type- r^2=0.26, p=0.39, Δ2b- r^2=0.01, p=0.86, Δ2c- r^2=0.15, p=0.51).

Trichodorid response

Building on the preference tests in Chapter 2, trichodorid preference was tested in a factorial design with wild-type TRV-infected plants, TRV-Δ2b infected plants and *Agrobacterium* control-infected plants (Figure 3.8). The control vs wild-type comparison is reprinted from Chapter 2 for visual comparison. Trichodorids preferred wild-type infected plants to plants infected with the Δ2b mutant (GLM-p $\chi^2_{2,21}$=55.89, p<0.001, post hoc p<0.05), but while the model showed a significant difference in the distribution of nematodes between the central pot and two arms, (GLM-p $\chi^2_{2,21}$=41.2, p<0.001), Tukey HSD post hoc tests showed there was no significant difference in preference between *Agrobacterium* control plants and Δ2b mutant-infected plants (p=0.36).

Across these assays, the average recapture rate was 61.75% ±2.53, with an average of 46% of recaptured nematodes responding to a treatment.

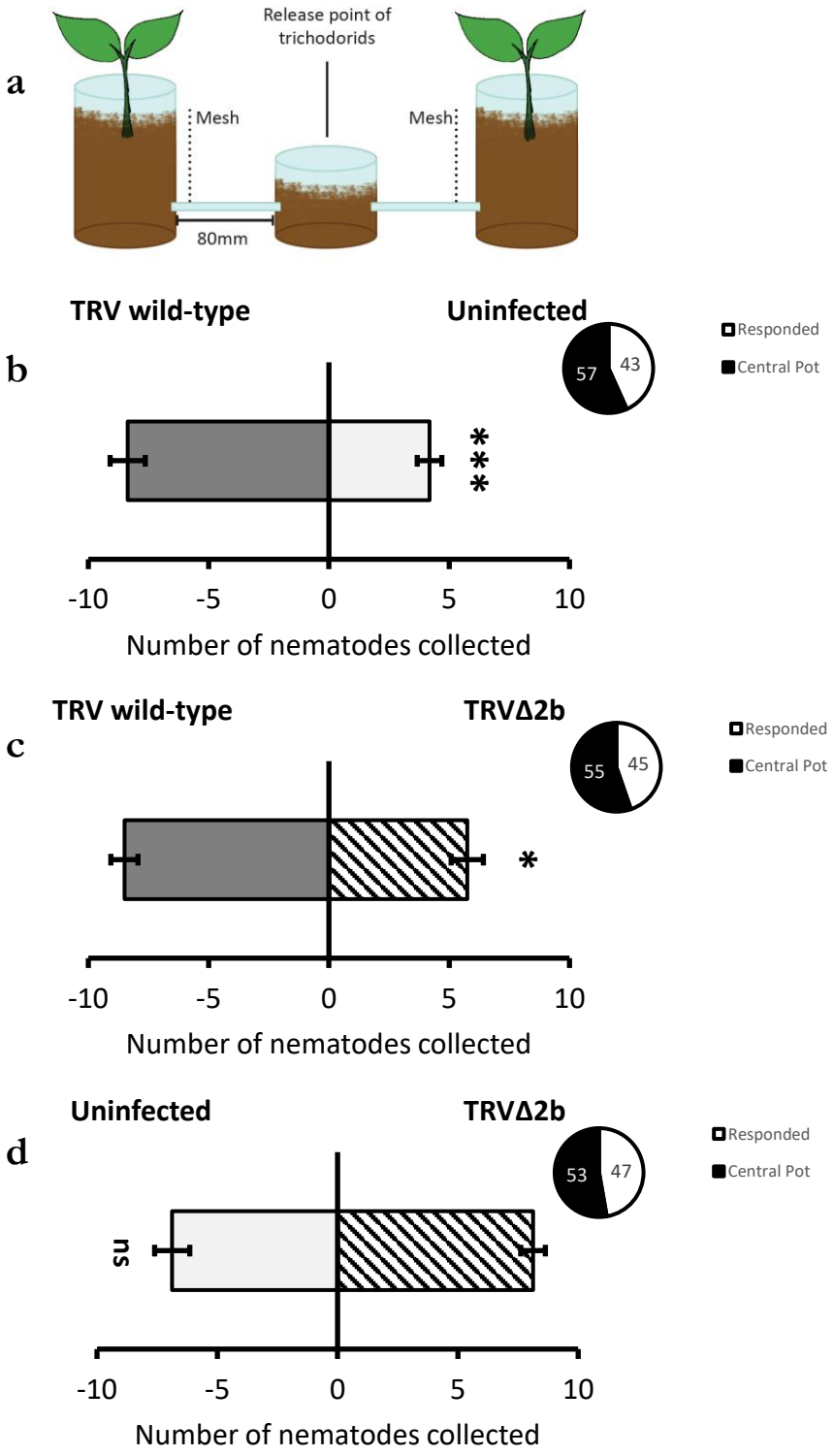

Figure 3.8 The preference of trichodorid nematodes given a choice between plants infected with different TRV treatments. This figure shows the number of trichodorids that moved down an olfactometer arm towards *N. benthamiana* plants infected with either wild type, TRV-Δ2b or control *Agrobacterium* (uninfected). 3.8a shows the olfactometer setup including central release of trichodorids. Pie charts show % of recaptured nematodes that responded to treatments. Means and standard errors are shown and n=11 for 3.8b, n=8 for 3.8c and d. 3.8b contains data presented in Chapter 2 (Figure 2.11). ns $p>0.05$ * $p<0.05$, *** $p<0.001$.

RT-qPCR data showed no significant difference in viral load for the TRV wild-type vs TRV-Δ2b comparisons. Normalised viral load explained some of the observed variance in trichodorid preference for both wild-type infected plants (r^2=0.79, p<0.01 when normalised to *APR* and r^2=0.71, p<0.01 when normalised to *EF1a*) and for TRV-Δ2b infected plants (r^2=0.78, p<0.01 when normalised to *APR* and r^2=0.78, p<0.01 when normalised to *EF1a*, Figure 3.9).

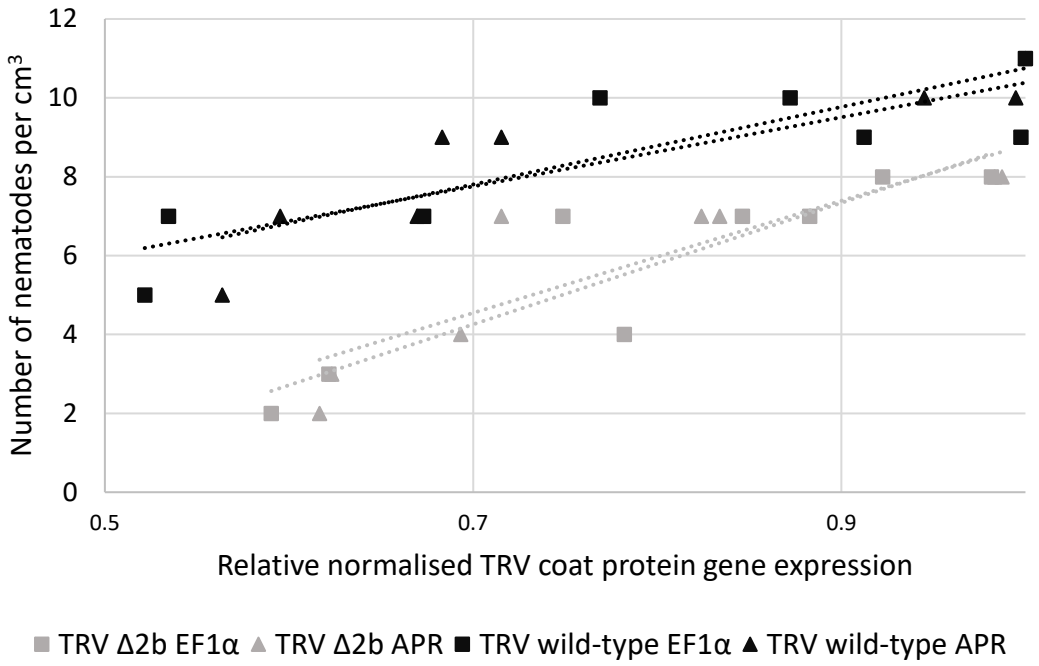

Figure 3.9 The relationship of nematode preference and viral load. Comparison of nematode movement towards plants infected with wild-type TRV and mutant TRV with altered 2 genes. With relative viral load normalised to two *N. benthamiana* housekeeping genes, APR and EF1α. Trendlines show the positive increase of plant attractiveness to trichodorids with increased levels of infection.

Discussion

For all infection treatments, where deleterious effects of infection were found for plant biomass and root growth, these were found at 7- and 14-days post infection. There was strong evidence of recovery in *N. benthamiana* roots and shoots after 21 dpi in plant biomass and the root architecture parameters studied. Viral activity in the roots peaked at 7 dpi, and the predicted lag between viral load and symptoms appears to be on the order of 7 days, since by 21 days post-viral load had declined to negligible levels.. This ties in to what has been established in the literature for TRV infection in *N. benthamiana,* with peak infection in the roots 6-8 dpi (Fernández-Calvino *et al.* 2014, Shaw *et al.* 2014, Valentine *et al.* 2004) and recovery from 18 dpi (Ratcliff *et al.* 1997, 1999, Shaw *et al.* 2014). In these cases, viral replication was still maintained and detectable, although at a much lower level than at peak infection. This difference may be due to these examples using leaf samples, compared to the root samples used in this investigation. The lag between peak infection and progression of symptoms in leaves is like that seen in roots in this investigation, both for TRV and other plant viruses, even if it occurs sooner (Bubici *et al.* 2015, Rimbaud *et al.* 2015, Valentine *et al.* 2004).

The reduction of viral accumulation over an infection timeline was initially linked to RNA silencing (Ma *et al.* 2015, Ratcliff *et al.* 1999), but it has been suggested that the nucleolus (Hiscox 2007, Greco 2009, Kalinina *et al.* 2018, Taliansky *et al.* 2010) and Cajal bodies (Love *et al.* 2017, Shaw *et al.* 2019) which are sub-nuclear structures contained in the nucleus have roles in responses to viral infection. Coilin, a protein produced by Cajal bodies, has been implicated for recovery from infection and suppressing host infection for TRV infections in *N. benthamiana* specifically (Shaw *et al.* 2014). *N. benthamiana* plants where coilin production had been knocked down (KD plants) did not recover from infection, and the leaves continued exhibiting severe systemic symptoms e.g. curling leaf malformation and necrosis (Shaw *et al.* 2014). Coilin seems to respond to TRV infection by recognising the protein products of TRV cysteine-rich 16K gene present on RNA-1 (Fernández-Calvino *et al.* 2016) and activating salicylic-dependant defence pathways (Shaw *et al.* 2019).

Differences were seen in the severity of infection symptoms with different mutant infection treatments. Plants infected with wild-type TRV and TRV-Δ2b always displayed the most severe stunting of root growth compared to shoot growth and reduction in root length and root surface area. Plants infected with TRV-Δ2c showed an

intermediate response and *Agrobacterium* control-infected plants were the least affected. This suggests that there is quite a large cost to the plant when 2c is present, since making it non-functional seemed to reduce the magnitude of several symptoms. Removing functional 2b (TRV-Δ2b) does not appear to have a substantial fitness cost from infection when compared to wild-type plants.

Comparing trends in root architecture between 4 and 14 dpi between the experiments described in Chapter 2 and Chapter 3 throws up some differences in certain root growth responses. Root dry weight in particular, which then affects the root to total plant root weight. This could be of concern, especially for the *Agrobacterium* controls, for the reproducibility of the investigations and conclusions that may be drawn on the effects of TRV infection on root parameters. However, the main driver of this difference was that root weight did not significantly increase for all treatments in this second experiment, presented in Chapter 3. This suggests that all plants in this investigation were impacted equally by this effect, which may be external in nature. A wide variety of external factors can impact root growth (Walter *et al.* 2009) including growth conditions. Unfortunately, between the two investigations, certain external parameters were changed. Firstly, the seeds used were from different batches, and the conditions and environment of the parent plants may have been different, which could have affected the growth rates of the seedlings (Elwell *et al.* 2011). Secondly, the growth room used was changed, and while the temperature the rooms were set to and the day/night cycle were unchanged, there may have been differences in humidity or the light the plants were exposed to, both in terms of wavelengths and intensity, which could also have impacted root growth rates (Johkan *et al.* 2010). With these extra factors, it is challenging to draw meaningful conclusions about differences between the two investigations. The key finding then, is that infected plants responded differently to control plants across an infection timeline in both, showing that TRV infection has an impact on root growth.

TRV constructs with the 2b gene present have been found to move and infect leaves and root meristem more efficiently than those without (Valentine *et al.* 2004) and the 2b protein produced may antagonise host defences (MacFarlane 2010). It has been suggested that 2b protein interacts with the TRV coat protein (Holeva and MacFarlane 2006) in order to form a bridge between the virus particle and the nematode feeding apparatus (Velios *et al.* 2002), but this effect seems not to come at too high a cost to the

plant host. It is hard to pinpoint the role of 2c, as previously mentioned. Different RNA-2 isolates are very variable in the genes present and the 2c genes may have different functions in different isolates of TRV. In TRV-PpK20, 2c is not essential for nematode transmission (Hernández *et al.* 1996, MacFarlane *et al.* 1996, Schmitt *et al.* 1998, Vassilikos *et al.* 2001). When both 2b and 2c were removed from TRV-PpK20 and it was inoculated into two different potato cultivars, atypical extreme and hypersensitive-like resistance were observed (Ghazala and Varrelmann 2007).

Plants were sampled for root VOCs 30 and 60 minutes after being placed in the sampling chamber; the results from both timepoints showed similar trends, with differences more marked at 60 minutes. This may be because plants left in the chamber for an extended time suffered abiotic stress on the plant roots (Salerno *et al.* 2017, Timmusk *et al.* 2014, Vives-Peris *et al.* 2017), which led to an interaction between infection and stress, causing greater emissions of stress-related compounds. There were significant differences in 2-ethyl-1-hexanol production between plants infected with wild-type TRV and the two mutant treatments, with both types of mutant-infected plants having significantly lower production of 2-ethyl-1-hexanol than wild-type infected plants and no difference compared to control plants. This pattern was replicated for 1,3-ditertbutylbenzene, but not for the other two compounds previously identified; 2,6-ditertbutylquinone and 2,4-ditertbutylphenol.

The products of 2b and 2c are relatively simple proteins and the function of the 2b protein appears to be well understood (MacFarlane 2010). Combined with statistically similar reductions in production of both VOCs for both mutant treatments compared to wild-type infected plants, it is unlikely that the gene products of 2b and 2c are directly responsible for VOC production. It is currently not possible to confirm if this is the case, but they do influence VOC fluxes. It is more likely that either i) VOC emissions are elevated as a by-product of the plants being under infection stress, or ii) the gene products are modifying existing plant metabolism to generate compounds of interest, neither of which is contradicted by the increase in production seen with increase of viral activity. It is reasonable to suggest that as viral load increases, overall stress on the plants is increased, which can lead to overproduction of 2-ethyl-1-hexanol particularly. If it is a stress response, it may have been expected that production would mirror the differences between treatments seen in the root architecture and biomass investigations, with Δ2b mutant infections showing similar VOC production to wild-

type and Δ2c mutant infections staying like control plants. There was only one timepoint (7 dpi) for VOC analysis, so in order to confirm that this is the relationship that is seen across the infection timeline, it may be helpful to also sample plants at multiple points between 6 and 14 dpi. In this investigation, only one timepoint was selected due to constraints with equipment and the length of analysis of samples coupled with the 2-week shelf life of the samples in the canisters before degradation (Low *et al.* 2003).

Trichodorid preference trials built on the work in the previous chapter that demonstrated that wild-type TRV-infected plants were more attractive than *Agrobacterium* infected controls by showing that Δ2b infected plants are of an intermediate attractiveness compared to wild-type and control infected plants. This suggests that the root VOCs found may have an attractant effect on trichodorids, as they were elevated in wild-type infected plants, but that they cannot explain all of the differences between treatments, otherwise there would not have been any difference in attractiveness between Δ2b infected plants and controls. This may be due to some further unidentified compounds that are produced by plant roots; potentially some semi-volatiles that were not picked up with the sampling method. It is well documented that differences in volatile and semi-volatile sampling preparation methods and final determination method can affect what compounds are detected, and also the ratio of compounds (Agelopoulos and Pickett 1998, Met and Yeşilçubuk 2017, Sghaier *et al.* 2016, Soria *et al.* 2015), with certain compounds only detected in one sampling method (Met and Yeşilçubuk 2017). Between the root VOC investigations in the previous chapter and the ones described here, other methods of volatile and semi-volatile extraction were trialled for this system including frozen root extraction (Rasmann *et al.* 2005), adsorbent packed columns in a dynamic headspace (Trujillo-Rodríguez *et al.* 2017) and desorption of semi-volatiles from growth media using dichloromethane as a solvent. There were problems of sensitivity associated with all these techniques and they did not lead to the identification of any novel compounds.

It will be important to test 2-ethyl-1-hexanol and 1,3-ditertbutylbenzene on trichodorids to see if hypothesised attractant effects can be demonstrated. As the emission of 2-ethyl-1-hexanol from plant roots was nearly 10x greater than that of 1,3-ditertbutylbenzene, and numbers of trichodorids available for testing is limited due to difficulties of culturing trichodorids (Neilson pers. comms.), 2-ethyl-1-hexanol will be

tested in preference. The comparison of the attractiveness of Δ2c infected plants compared to Δ2b and controls on trichodorids would also be informative; as both Δ2b and Δ2c infection showed control-like emissions of the potential attractants but Δ2b-infected plants were still more attractive than controls, any differences in attractiveness with Δ2c may help to understand what this difference may be based on. It may also be interesting to compare attractiveness of infection of wild-type TRV of isolates where 2c is also non-essential for nematode transmission, but the structure of RNA-2 is very different (e.g. PaY4). If attractiveness of infected plants is due to by-products of infection stress making plants more visible to nematodes, rather than a coupled virus–vector relationship, then it may be that similar volatiles are produced by other potato viruses not transmitted by nematodes e.g. Potato virus X (no vector), or viruses with different soil borne vectors: Potato mop-top virus (cercozoan), Tobacco necrosis virus (fungi), or nematode-spread nepoviruses (Hewitt *et al.* 1958, Lister 1964, Smith 1946).

This work has further demonstrated the severe belowground effects of TRV infection on plants but has shown that these effects are transient in line with recovery times described in the literature and provided more evidence that two root VOCs, 2-ethyl-1-hexanol and 1,3-ditertbutylbenzene are produced as a result of wild-type infection and that production increases as viral load increases. It suggests that infection severity decreases when RNA-2 genes 2b (volatile emissions) and 2c (infection symptoms and volatile emissions) are not present, and that absence of 2b makes TRV-infected plants less attractive to trichodorid vectors. This is important information in trying to understand the relationship between TRV infection and transmission rates.

Chapter 4

Introduction

It has been demonstrated that *N. benthamiana* plants are more attractive to trichodorid nematodes with increasing viral activity, especially for wild-type infections (Chapter 2), but this positive relationship is also seen in plants infected with TRV-Δ2b mutants (Chapter 3). In TRV wild-type plants, increasing viral load also correlates with increased 2-ethyl-1-hexanol production from roots, which is not seen for control or TRV mutant-infected plants.

As discussed, 2-ethyl-1-hexanol is a widespread phytochemical and is produced in varied plant species and from different parts of those plants. It is commonly a green leaf volatile, from trees to grasses such as maize and wheat, chickpea, lima beans and aquatic ferns (Cruz *et al.* 2012, Khedive *et al.* 2017, Pereira *et al.* 2009, Solé *et al.* 2010, Yi *et al.* 2009). In some of these cases, it may be emitted as a response to biotic stress and attack from different sources. 2-ethyl-1-hexanol is also released from flowers of plants, where seems to have a role in attracting honeybee (Rering *et al.* 2018) and bumblebee (Schaeffer *et al.* 2019) pollinators, along with other volatile compounds, and production is elevated when nectar-inhabiting microorganisms are present, particularly yeasts (Rering *et al.* 2018, Schaeffer *et al.* 2019). It has been suggested that yeast species produce 2-ethyl-1-hexanol and other volatile compounds to inhibit growth of other microbes in the nectar (Sobhy *et al.* 2018).

Most relevant to this investigation, 2-ethyl-1-hexanol has been shown to be produced by plant roots in chickpea (Bazghaleh *et al.* 2016) and potato tubers (de Lacy Costello *et al.* 2001). In both of those cases, it was produced as a response to fungal attack.

Nematodes have been shown to be attracted to a wide range of different belowground compounds, as previously discussed in Chapter 1. Functionally different nematodes (plant-feeding, plant-associated, fungal hyphae-feeding, bacterial-feeding, unicellular eukaryote-feeding, animal-parasitic and omnivorous) can be attracted by the same phytochemical (reviewed in Rasmann *et al.* 2012), with CO_2 being the most common across different phylogenies and functional groups (Gaugler *et al.* 1980, Johnson and Nielsen 2012, McCallum and Dusenbery 1992, O'Halloran and Burnell 2003, Pline and Dusenbery 1987, Robinson 1995). Some compounds act to attract

multiple organisms with the same signal; for instance, sesquiterpenes released by *Citrus* spp. trees as a response to herbivore attack are attractive to both plant parasitic nematodes (*Tylenchus*) and entomopathogenic predators of the herbivore (Ali *et al.* 2011). Although nematode and plant interactions involving phytochemicals continue to be actively researched, the work primarily focuses on entomopathogenic nematodes (EPNs) (Andaló *et al.* 2017, Kergunteuil *et al.* 2018, 2019, 2020, Mondal *et al.* 2019) and endoparasitic plant nematodes (Borges 2018, Čepulytė *et al.* 2018, Khaki *et al.* 2017, Neupane *et al.* 2019, Oota *et al.* 2019, Yousuf *et al.* 2018). Trichodorids are free-living plant parasites and belong to an entirely different clade of nematodes: Enoplia. No current information exists on what their potential chemoattractants may be. It is likely that they would respond to CO_2 and some terpenes, as responses to these seem to be conserved across phylogenies, possibly as a result of convergent evolution (Rasmann *et al.* 2012).

2-ethyl-1-hexanol is a branched 8-carbon alcohol. Work on EPNs from different clades has shown positive responses to a variety of alcohols, including plant-derived alcohols (Hallem *et al.* 2011, O'Halloran and Burnell 2003) such as 1-hexanol. All plant-derived alcohols are fatty acid derivatives; one pathway includes those formed from linolenic acid, a C_{18} unsaturated fatty acid (Figure 4.1, Dudareva *et al.* 2013). These precursors are oxygenated to form two intermediates (Figure 4.1) (Feussner and Wasternack, 2002) and these intermediates are further reduced by alcohol dehydrogenases (Gigot *et al.* 2010). 2-ethyl-hexanol could be a modification of 1-hexanol synthesised through this pathway. Alcohols are often esterified (D'Auria *et al.* 2007), or further modified; for instance, methyl jasmonate is produced from one intermediate via the allene oxide synthase branch, separate to these reductions via alcohol dehydrogenase (Song *et al.* 2005). Many of these alcohols and modified compounds are classified as defensive green leaf volatiles and are released from plants as a response to wounding. These compounds form an important class of phytochemicals involved in multi-trophic interactions (e.g. Engelberth 2020, Joo *et al.* 2019, McMenemy *et al.* 2012, Michereff *et al.* 2019), and as cues for herbivores to find targets (e.g. Ahmed *et al.* 2019, Karmakar *et al.* 2018, McArthur *et al.* 2019).

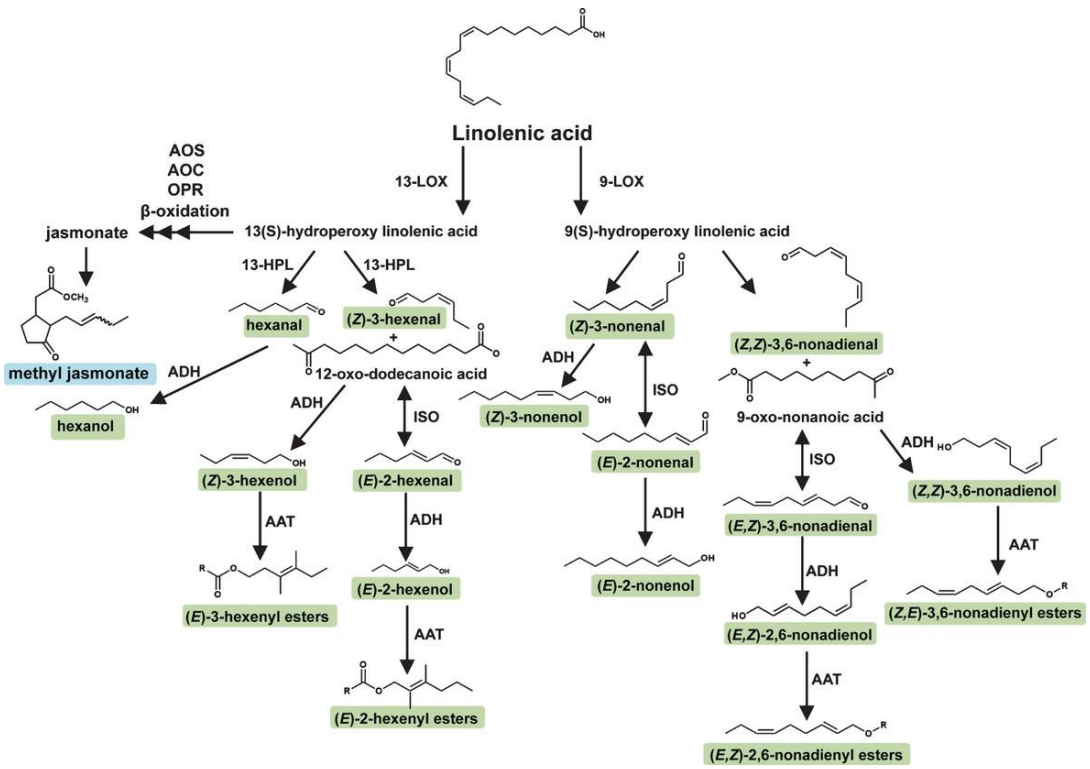

Figure 4.1 Synthesis of fatty-acid derived volatiles in plants. (From Dudareva *et al.* 2013) Showing the different paths that linoleic and linolenic acid precursors form methyl jasmonate via jasmonic acid (JA) and other volatiles via the "lipoxygenase (LOX) pathway" including hexanol, a probable precursor to 2-ethyl-1-hexanol.

Most nematodes studied respond to multiple different compounds including CO_2 (as reviewed in Rasmann *et al.* 2012). If 2-ethyl-1-hexanol is a chemoattractant to trichodorids, it is likely to be part of a panoply of chemical cues that the nematodes exploit in order to locate suitable plants, including 1,3-ditertbutylbenzene (Chapter 3).

The testing of potential chemoattractants using olfactometers is common within the context of plant-mediated multitrophic interactions, both on herbivores (Arif *et al.* 2020, Dada *et al.* 2020, Dardouri *et al.* 2019, Markheiser *et al.* 2020, Mitra *et al.* 2020) and natural enemies of those herbivores, either predators or parasitoids recruited by phytochemicals (Cai *et al.* 2020, Colazza *et al.* 2004, De Boer and Dicke 2004, De Boer *et al.* 2004). Nearly all studies using olfactometers have studied systems of aboveground herbivorous arthropods, such as aphids, caterpillars, mites, and beetles (Cai *et al.* 2020, Dardouri *et al.* 2019, De Boer *et al.* 2004, Mitra *et al.* 2020, Vuorinen *et al.* 2004). These studies generally used Y tube olfactometers, where individuals are placed at the bottom end of a branched olfactometer and given two options, either i) the compound of interest or sample of interest or ii) a relevant control. Attractiveness is quantified by the percentage of individuals moving towards the attractant and

compared to those moving towards the control (Vuorinen *et al.* 2004, Dicke *et al.* 1990). Y tube olfactometers limit the options to a binary choice, and for this reason, multiple-armed olfactometers have been developed in order to allow for fully factorial experimental designs (Turlings *et al.* 2004).

Studies carried out on belowground systems pose unique challenges. Due to the need for a soil or soil-like substrate, the organisms tested cannot be readily observed. In order to address this issue, the studied organisms are allowed to move towards the attractant but are stopped from reaching it by an impenetrable mesh (Ali *et al.* 2010, Hiltpold *et al.* 2011, Rasmann and Turlings 2008, Rasmann *et al.* 2005, Rivera *et al.* 2017, Willett *et al.* 2017) and are extracted from the substrate at that point. Inevitably, some organisms are not recovered and in larger multi-armed olfactometer studies it is not feasible to perform extraction on the entire substrate (Ali *et al.* 2010). For studies utilizing easily culturable organisms, such as EPNs, this is less of an issue, as thousands (Ali *et al.* 2010, Oliveira-Hofman *et al.* 2019, Rasmann *et al.* 2005, Rivera *et al.* 2017, Tourtois *et al.* 2017) can be released. However, for organisms that are unculturable and require specialist harvesting from natural field samples, it can be a limiting factor, as in this investigation. Additionally, trichodorid nematodes are much slower than EPNs, so the length of the arms used in these investigations (Figure 4.2) were adjusted from designs in the literature based on EPN movement rates (Ali *et al.* 2010, Rasmann *et al.* 2005. Willett *et al.* 2017).

These olfactometer experiments aim to explore the role of 2-ethyl-1-hexanol, as a primary chemical signal, on trichodorid behaviour. I hypothesise that it is an attractant to trichodorid nematodes, as is suggested by previous work presented in this thesis. It has been demonstrated that *N. benthamiana* plants infected with wild-type TRV produce significantly more 2-ethyl-1-hexanol than control plants or plants infected with TRV mutants with altered RNA-2 genes, and that trichodorids preferentially move towards wild-type infected plants over control or Δ2b mutant-infected plants. The two experiments presented here explore the mechanism of this preference through the analysis of nematode preference for 2-ethyl-1-hexanol in olfactometer tests and whether the addition of 2-ethyl-1-hexanol overcomes the reduced attraction previously seen in TRV-Δ2b mutant infections compared to wild-type infection. In the first olfactometer test, trichodorids will be given a choice between *Agrobacterium*-infected (non-viral) controls with and without the addition of 2-ethyl-1-hexanol. In the second,

plants infected with TRV-Δ2b mutants with added 2-ethyl-1-hexanol will be compared to plants infected with wild-type TRV.

- In the first test, the choice of *Agrobacterium* control plants + 2-ethyl-1-hexanol vs *Agrobacterium* control plants, it is expected that trichodorids will significantly move towards the plants with added 2-ethyl-1-hexanol, as it is expected that 2-ethyl-1-hexanol is acting as a foraging cue.

- In the second test it is hypothesised that there will be no significant difference in preference between wild-type infected plants and TRV-Δ2b infected plants with added 2-ethyl-1-hexanol, as the additional presence of the dominant foraging cue, 2-ethyl-1-hexanol, will raise the profile of the mutant infected plant to be as attractive as the wild-type infected plant that is naturally releasing 2-ethyl-1-hexanol.

- I further hypothesise that, as in previous investigations, there will be a positive relationship between TRV infection levels and nematode movement, where tested plants are infected with TRV.

Methods

Plant growth and infection

Plants were prepared as in previous olfactometer tests (see Chapters 2 and 3) and planted directly into the olfactometer pots once two true leaves had expanded. Plants for "control vs control + 2-ethyl-1-hexanol" trials were infected with untransformed *Agrobacterium*. Plants used for "TRV wild-type vs TRV-Δ2b + 2-ethyl-1-hexanol" were infected with *Agrobacterium* transformed with unmodified TRV ("wild type") and with unmodified TRV RNA-1 and altered TRV RNA-2 with non-functional 2b gene (TRV-Δ2b). Infection solutions were prepared as previously described (Chapters 2 and 3) and plants were inoculated 7 days after being planted into the olfactometers.

Shoot and root fresh weight was recorded and <100mg samples of root tissue were flash frozen for RNA extraction and quantification of infection through RT-qPCR (see Chapters 2 and 3). The remaining plant material was dried at 70ºC for 3 days before dry weights were recorded.

Trichodorid responses

In order to test the effects of 2-ethyl-1-hexanol on trichodorids, an amended olfactometer design was used (Figure 4.2). This allowed liquid 2-ethyl-1-hexanol to be added to the root system of one of the plants, close to the arm leading to the central pot. The added 2-ethyl-1-hexanol could then reach equilibrium with the vapour phase and diffuse via the gaseous phase of the sand matrix.

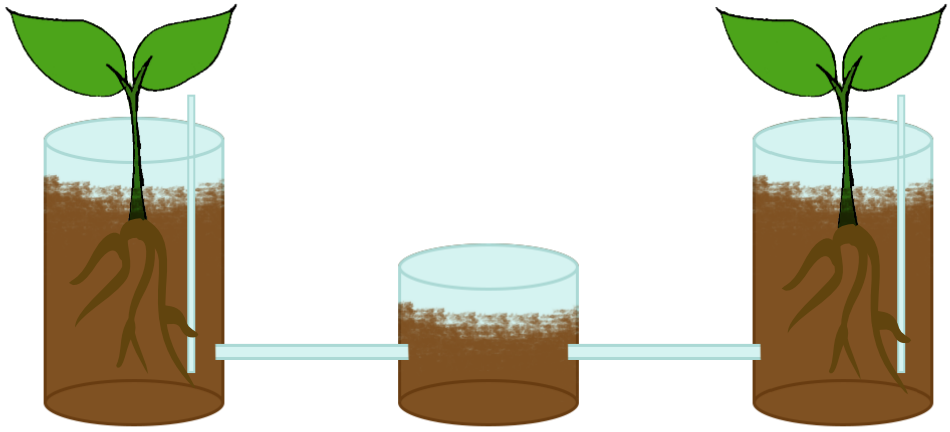

Figure 4.2 Amended olfactometer design for 2-ethyl-1-hexanol trials. Glass capillary tubes were inserted to the rooting depth with the *N. benthamiana* seedlings as they were transplanted. The tubes were capped with parafilm to prevent volatile compounds escaping the test environment through the capillary. Capillaries were added to both sides to mitigate any effect the presence of the capillaries may have had on volatile emissions. Olfactometer pots were constructed as previously described.

The trial was run for 48 hours after 50 nematodes were added to the central pot. Estimates of 2-ethyl-hexanol released from *N. benthamiana* plants at 7 dpi were calculated as being 31-67 μg per plant over 48 hours. This was based on an average flux of 0.18 μg min^{-1} g root dry $weight^{-1}$ (Figure 3.6) for wild-type TRV-infected plants, and root dry weights ranging from 0.06-0.13g for plants used in VOC analysis (Chapter 3, data not shown). Based on the density of liquid 2-ethyl-1-hexanol standard (Sigma-Aldrich, 833 kg m^{-3}), 37-81μl of standard would be added for similar production to wild-type infected plants.

Doses of 2-ethyl-1-hexanol were calculated so that over the run, double the upper bound of this estimate was added to improve the likelihood of detection, in line with other work (Rasmann *et al.* 2005), and 160μl were added over the run. 16μl of standard were added to one pot via the capillary at 0, 4, 24 and 28 hrs and 48μl were added at 8 and 32hrs after the start of the trial to allow for sufficient overnight soil concentrations. The nematodes from the central pot and the two arms leading to the planted pots were extracted from the sand by Baermann funnel, collected after 48 hours in the funnels (see Chapters 2 and 3) and counted under a dissecting microscope.

There were two sets of trials: a comparison between the attractiveness of control plants with added 2-ethyl-1-hexanol to other control plants and a second that compared plants infected with TRV-Δ2b with added 2-ethyl-1-hexanol to plants infected with wild-type TRV. As in Chapter 3, each trial was run twice, with 4 replicates per trial. Trichodorid count data were analysed using a generalised linear model with Poisson distribution as previously explained in Chapter 2 and 3 methods. For both models, AIC was better with only pot as an independent factor, so sampling run was removed from the model

Results

Agrobacterium control + vs *Agrobacterium* control

There was a significant difference in trichodorid distribution in the arms leading to the outer pots (GLIM-p $\chi^2_{(2,21)}=12.4$, $p<0.001$) with more nematodes moving towards the plants with added 2-ethyl-1-hexanol (11 nematodes ± 1) than those without (8 nematodes cm^{-3}±1, Figure 4.3, $p<0.05$). Average recapture of the nematodes was 70.5%±1.5, with 55% of those recaptured responding to one of the treatments.

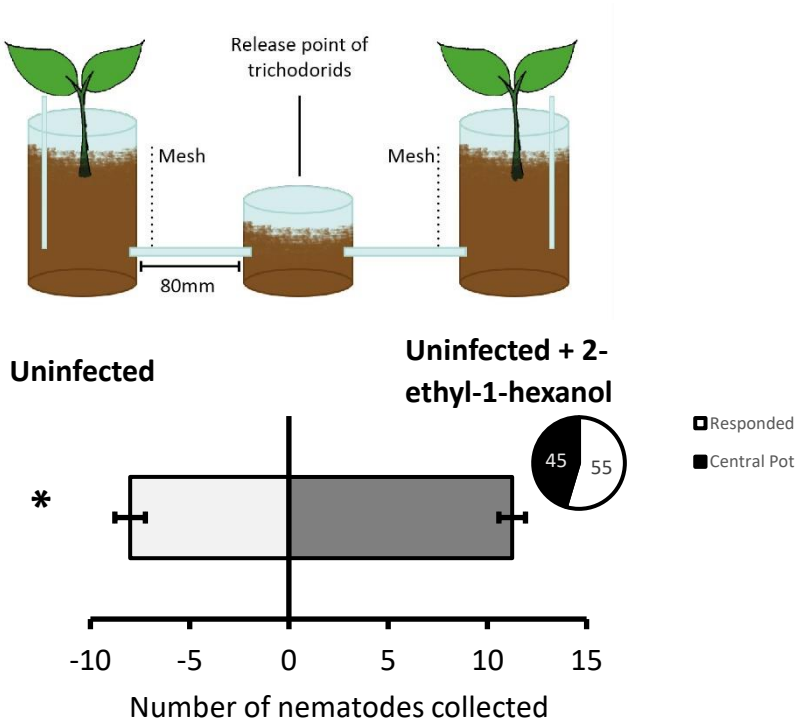

Figure 4.3 The preference of trichodorid nematodes given a choice between *Agrobacterium*-infected plants ("uninfected" with TRV) with or without the addition of 2-ethyl-1-hexanol. This figure shows the number of trichodorids that moved down an olfactometer arm towards *N. benthamiana* plants in the presence or absence of added 2-ethyl-1-hexanol. Diagram shows side view of olfactometers with trichodorids added to central pot. The pie chart shows the percentage of trichodorids that moved towards one of the treatments compared to those that remained in the release pot. Means and standard errors are shown and n=8 for all. * $p<0.05$.

TRV-Δ2b infection + vs TRV wild-type infection

The model showed that there were significant differences in trichodorid counts between the different pots (GLIM-p $\chi^2_{(2,21)}=55.9$, $p<0.001$) with a significant preference for wild-type infected plants (11 nematodes ±1) to plants infected with the Δ2b mutants with 2-ethyl-1-hexanol (8 nematodes ±1, Figure 4.4, $p<0.05$). Average recapture rate was 66.5% ±2.7, with 55% of those recaptured responding to a treatment.

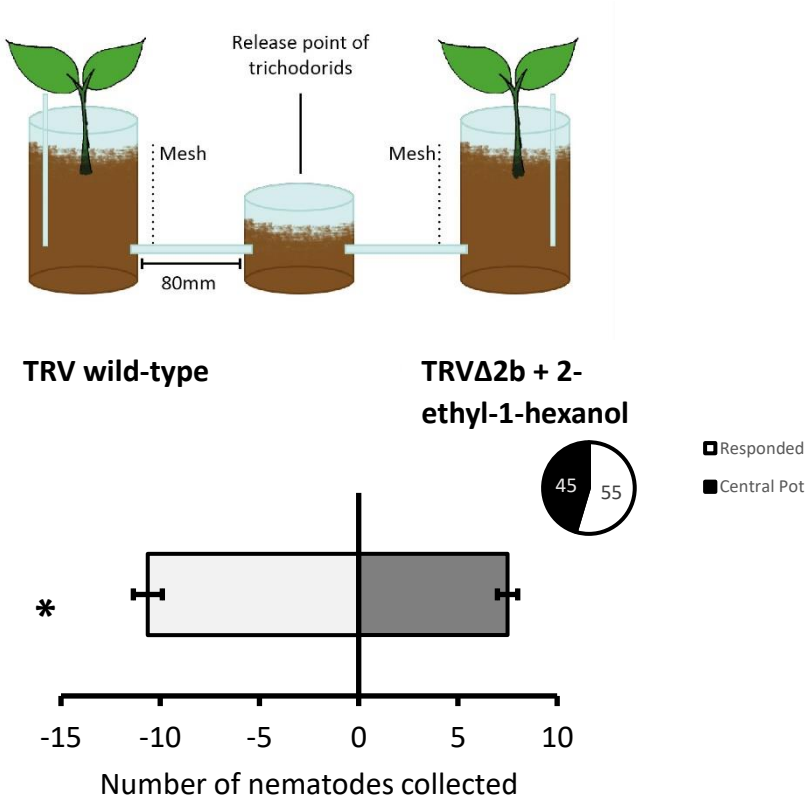

Figure 4.4 The preference of trichodorid nematodes given a choice between TRV wild-type infected plants and TRV-Δ2b infected plants with added 2-ethyl-1-hexanol. This figure shows the number of trichodorids that moved down an olfactometer arm towards *N. benthamiana* plants in the presence or absence of added 2-ethyl-1-hexanol. Diagram shows side view of olfactometers with trichodorids added to central pot. The pie chart shows the percentage of trichodorids that moved towards one of the treatments compared to those that remained in the release pot. Means and standard errors are shown and n=8 for all. * p<0.05.

There was no significant difference in viral load between infection treatments in these trials. Regression analysis of relative normalised expression to both housekeeping genes to nematode movement (Figure 4.5) and significant simple linear regression equations were found for both wild-type TRV infections (APR: $F_{(1,6)}=48.05$, $p<0.001$,

r^2=0.889 and EF1α: $F_{(1,6)}$=158.9, p<0.001, r^2=0.964) and TRV-Δ2b mutant infections (APR: $F_{(1,6)}$=23.84, p<0.01, r^2=0.799 and EF1α: $F_{(1,6)}$=32.54, p<0.01, r^2=0.844).

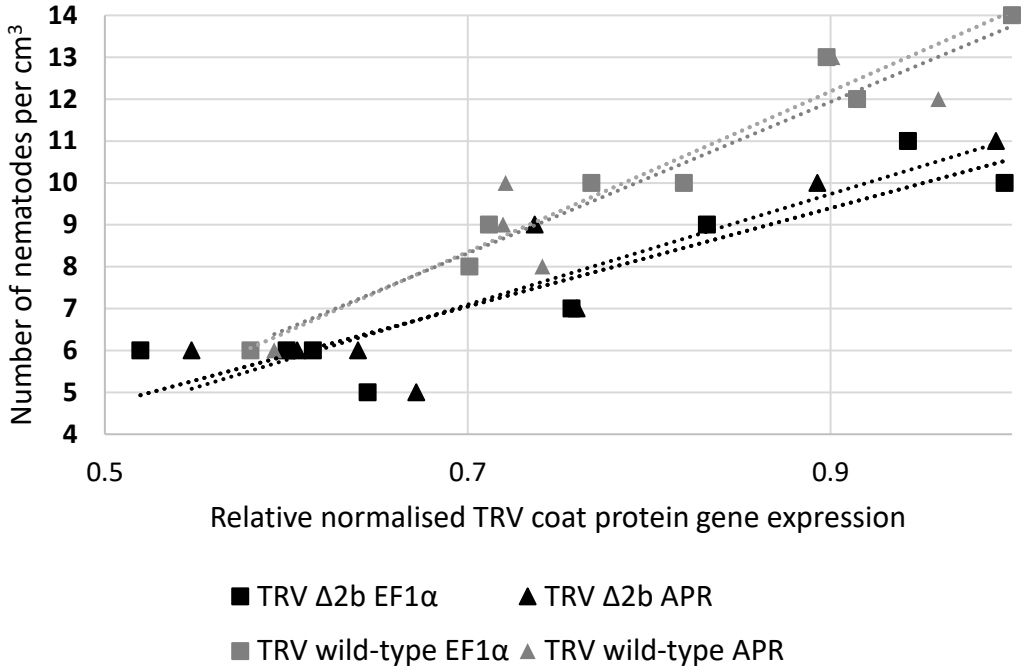

Figure 4.5 The relationship of nematode preference and viral load. Comparison of nematode movement towards plants infected with wild-type TRV and TRV-Δ2b with added 2-ethyl-1-hexanol. Relative viral load is normalised to two *N. benthamiana* housekeeping genes, APR and EF1α. Trendlines show the positive increase of plant attractiveness to trichodorids with increased levels of infection for each infection treatment compared to each housekeeping gene.

Discussion

There was a significant difference in attractiveness between *Agrobacterium*-infected control plants with and without the addition of 2-ethyl-1-hexanol, with the presence of the compound increasing nematode preference. This strongly suggests that trichodorids can both detect this compound and that it may constitute an important component of the chemical cues that they use to orient themselves in the soil (Rasmann *et al.* 2012). As 2-ethyl-1-hexanol can be produced in plants as a result of biotic stress (Castelyn *et al.* 2015, de Lacy Costello *et al.* 2001, Fernando *et al.* 2005, Heil and Bueno 2007, Yi *et al.* 2009), it may form a reliable signal for foraging trichodorids searching for a food source. While the observed difference in nematode movement preference is significant, there was still substantial movement towards the control plant with no added 2-ethyl-1-hexanol. As previous work has shown (Chapters 2 and 3), uninfected *N. benthamiana* plants do not release detectable amounts of 2-ethyl-1-hexanol from their roots, so the observed nematode attraction cannot be explained by background production of this individual compound. This suggests that while 2-ethyl-1-hexanol is acting as an important foraging cue for trichodorids, it may not constitute the entire signal. Nematodes, in general, respond to basic environmental cues like CO_2 gradients (reviewed in Rasmann *et al.* 2012), and while this has not been specifically tested for trichodorids, these responses are seen across phylogenetic and functional groups (Rasmann *et al.* 2012). It seems likely that trichodorids would also respond to several of these biological cues.

When plants infected with wild-type TRV were compared to plants infected with TRV-Δ2b with added 2-ethyl-hexanol, there was also a significant difference in preference, with trichodorids moving more towards wild-type infected plants. This ran contrary to the hypothesis that the addition of 2-ethyl-1-hexanol would "repair" the reduction in attractiveness between wild-type TRV and TRV-Δ2b seen in previous work (Chapter 3). This hypothesis was based on the evidence that 2-ethyl-1-hexanol made plants more attractive (first comparison) and that TRV-Δ2b plants were not significantly different from wild-type TRV infected plants in any of the root parameters analysed previously (total root length, root surface area, root fresh and dry weight or root to total plant weight) apart from volatile production. This previous work (Chapters 2 and 3) suggested that the only detectable differences in infection were root VOC production, and the addition of root VOC that was demonstrably attractive to trichodorids would

remove the difference in attractiveness between treatments. Even with addition of 2-ethyl-1-hexanol to TRV-Δ2b infected plants, trichodorids again preferred plants with increasing viral loads (Figure 4.5), which matches the previous findings of these investigations.

This supports the conclusion that 2-ethyl-1-hexanol is not the sole driver of plant-nematode interaction but may be part of a more complex cocktail of chemical cues produced by TRV-infected plants. Other work on exudates of plants infested by biotic agents, including arthropods (Krips *et al.* 2001, Kuhns *et al.* 2014, Penaflor *et al.* 2011) and nematodes (Hallem *et al.* 2011, Zhao *et al.* 2007), found that a variety of compounds were released by infected roots. When root exudates are tested in choice experiments on other nematodes, the full mixture of root exudates has the greatest effect on behaviour (Beeman *et al.* 2019, Wang *et al.* 2020). This work has already shown that this is the case for this interaction, with *in vivo* releases from infected plants attracting trichodorids (Figures 2.11 and 3.8). The purpose of the work in this chapter was to highlight and define a chemical compound that is involved in these interactions that is modified by viral infection.

We were unable to test 1,3-ditertbutylbenzene, and it is possible we were unable to collect and detect other attractants that were being released by plant roots. An early concern was that no terpenes were detected from samples, since these are a well-established and important group of attractant phytochemicals (Ali *et al.* 2011, Avellaneda *et al.* 2019, Krips *et al.* 2001, Kuhns *et al.* 2014, Penaflor *et al.* 2011, Zhao *et al.* 2007). Standards for well-documented attractants β-caryophellene (Hammack 2001, Rasmann *et al.* 2005, Sadeh *et al.* 2017), α-pinene (Hallem *et al.* 2011, Zaka *et al.* 2015, Zhao *et al.* 2007), limonene (Sadeh *et al.* 2017) and linalool (Hallem *et al.* 2011, Hammack 2001) were obtained. These were run using the gas chromatography method described in Chapters 2 and 3 and were detected with similar response factors to other compounds quantified in this research. Chromatograms, mass spectra and retention times of a range of isoprenoid standards were compared to the chromatograms of samples collected and no evidence was found to suggest they were present.

Isoprenoids are not the only possibility for further important chemical cues. The analytical methods that we chose allowed us to capture and analyse a broad range of compounds but is biased towards high volatility compounds (Redeker *et al.* 2018). Alternative methods using volatile adsorptive packing collection traps, which allow for

the concentration of lower volatility compounds (Phillips 1997, Poli *et al.* 2005, Ras *et al.* 2009, Xiu *et al.* 2019), were tested, but no novel compounds were detected.

It is unlikely that there was insufficient 2-ethyl-1-hexanol added for the trichodorids to respond to. Based on the two averages (data from Chapters 2 and 3) for 2-ethyl-1-hexanol emissions from wild-type infected plants, we estimate biological production to be 0.15-0.18μg min^{-1} g dry root $weight^{-1}$, which is 25.92-67.39μg/plant over 48 hours, using the range of plant root measures from Chapters 2 and 3. Converting into ml for use with liquid standard gives estimates of 31.1-80.9 μl of 2-ethyl-1-hexanol. The amount added in these experiments was double the largest estimate and is in line with previously published work (Ali *et al.* 2011, Rasmann *et al.* 2005).

A shortage of trichodorids meant that we could not test the attractiveness of ditertbutylbenzene, the second VOC of interest, which has also been reported to be produced by tree roots (Voglar *et al.* 2019). As this compound is produced in much lower quantities than 2-ethyl-1-hexanol, the priority was to test the latter as it is more likely to be biologically relevant. Further trichodorid testing, in addition to exploring the effects of ditertbutylbenzene, could involve manipulating CO_2 gradients to confirm whether they respond to CO_2 in similar ways to other nematode species. As roots create a CO_2 gradient (Johnson *et al.* 2006, Reinecke *et al.* 2008), this would seem a logical foraging cue for trichodorids to orient themselves in the soil. Tests that start to mimic field conditions more closely, and using potatoes rather than a model plant species, may give even more valuable information that is more specific to the agricultural reality.

These experiments have shown that 2-ethyl-1-hexanol is an important chemoattractant phytochemical for this interaction, and that it forms a significant part of the total signal that trichodorids respond to. In absence of viral infection, it seems to drive trichodorid attraction towards plants, but when compared to a wild-type TRV infection, trichodorids do not respond to 2-ethyl-1-hexanol addition. This finding is valuable both for our understanding of nematode chemotaxis, giving novel information on a previously unstudied compound and nematode group, as well as being valuable information for understanding the attraction behind the transmission of TRV in arable systems.

Chapter 5 : General Discussion

Overview

In the context of Tobacco rattle virus, exploring the impacts of infection on the structure of root systems and the effects of chemical signals from infected roots on trichodorid behaviour has ecological and agricultural implications in understanding and potentially managing this important pest. Sub-surface plant viruses transmitted by nematodes, including trichodorids, face similar challenges to attracting vectors as aboveground vector-borne viruses while operating in different environments (Shelef *et al.* 2019). Understanding rhizosphere interactions aids overall understanding of above- and belowground biota (Wardle *et al.* 2004). This thesis aims to further current understanding of TRV–trichodorid relationships by using an *N. benthamiana* model plant system to show the physiological effects of infection, including identifying chemical cues for attraction and the effects of those chemicals on the movement of vector nematodes. It also explores the potential impacts of two TRV genes on the strength of these physiological responses.

Through the experiments described in Chapters 2, 3 and 4, I explored infection effects within the *N. benthamiana* system, discovering impacts on growth and structure of host root systems, differences in root VOCs emitted and influences on trichodorid attraction. Focusing on potential causes behind these observations, I investigated how the absence of two genes on the smaller virus particle, RNA-2, of TRV altered these effects. A reduction in the strength of effects became evident as these genes were absent, including reduction in vector attractiveness. Finally, I looked at how one candidate VOC for chemoattraction affected the movement of trichodorids towards plants, showing that it increased trichodorid preference and is likely to be part of a more complex signal. In this final chapter, I bring together the results from these investigations to draw conclusions about these interactions and explore the context of these findings and possible implications for future work.

Synthesis of findings

Table 5.1 Summary of hypotheses relating to changes in biomass and root architecture as a result of infection. The chapter that each hypothesis relates to is given, along with the outcome. Further details are given in the relevant section of this chapter.

Chapter	Hypothesis	Accepted/Rejected
2	Wild-type TRV infection causes a significant reduction in aboveground biomass vs uninfected plants	**?**- difference only seen 14 dpi
2	Wild-type TRV infection causes a significant reduction in belowground biomass vs uninfected plants	✔ - differences seen 7 and 14 dpi
2	Wild-type TRV infection causes a significant reduction in root:total plant allocation vs uninfected plants	**X**- reduction seen at 7pi, increase at 14 dpi
2	Wild-type TRV infection causes a significant reduction in root surface area: volume vs uninfected plants	✔ - differences seen 7 and 14 dpi
2	Wild-type TRV infection causes a significant reduction in root length vs uninfected plants	✔ - differences seen 7 and 14 dpi
3	Significantly elevated viral loads 7 days post infection in infected plants vs other timepoints (4,14- and 21-days post infection)	✔
3	Most significant reduction in aboveground biomass between wild-type TRV infected and uninfected plants at 7- or 14-days post infection.	✔
3	Most significant reduction in belowground biomass between wild-type TRV infected and uninfected plants at 7- or 14-days post infection.	✔
3	Most significant reduction in root:total plant allocation between wild-type TRV infected and uninfected plants at 7- or 14-days post infection.	✔
3	Most significant reduction in root surface area:volume between wild-type TRV infected and uninfected plants at 7- or 14-days post infection.	✔
3	Most significant reduction in root length between wild-type TRV infected and uninfected plants at 7- or 14-days post infection.	✔

Table 5.2 Summary of hypotheses relating to presence of root volatile emissions as a result of viral infection. The chapter that each hypothesis relates to is given, along with the outcome. Further details are given in the relevant section of this chapter.

Chapter	Hypothesis	Accepted/Rejected
2	Significant difference in rVOC emissions from infected plants than uninfected plants	✔ - differences seen for canister sampling method, not volatile collection traps
3	Significantly greater 2-ethyl-1-hexanol, 1,3-ditertbutylbenze, 2,6-diterbutylquinone and 2,4-ditertbutylphenol emissions from roots of infected plants	**?**-only 2-ethyl-1-hexanol and 1,3-ditertbutylbenze identified
3	Increased emissions from plants sampled for longer (canister method: 60 mins vs 20 mins)	✔
2 &3	Viral load will explain a significant amount of the observed variance in root VOC emissions from infected plants	**?**- not in Chapter 2, only 2-ethyl-1-hexanol in Chapter 3

Table 5.3 Summary of hypotheses relating to the removal of RNA-2 genes reducing effects of infection. The chapter that each hypothesis relates to is given, along with the outcome.

Chapter	Hypothesis	Accepted/Rejected
3	Δ2b and Δ2c mutant treatments will show intermediate responses between wild-type TRV infected plants and uninfected plants for aboveground biomass.	✔
3	Δ2b and Δ2c mutant treatments will show intermediate responses between wild-type TRV infected plants and uninfected plants for belowground biomass.	✔
3	Δ2b and Δ2c mutant treatments will show intermediate responses between wild-type TRV infected plants and uninfected plants for root:total plant allocation.	X
3	Δ2b and Δ2c mutant treatments will show intermediate responses between wild-type TRV infected plants and uninfected plants for root surface area: volume.	✔
3	Δ2b and Δ2c mutant treatments will show intermediate responses between wild-type TRV infected plants and uninfected plants for root length.	✔
3	Δ2b and Δ2c mutant treatments will show intermediate responses between wild-type TRV infected plants and uninfected plants for rVOC emissions.	X

Table 5.4 Summary of hypotheses relating to trichodorid attraction to plants infected with different treatments. The chapter that each hypothesis relates to is given, along with the outcome.

Chapter	Hypothesis	Accepted/Rejected
2	Given a choice between wild-type TRV infected and uninfected plants, trichodorids will preferentially move towards wild-type TRV infected plants.	✔
2&3	Viral load will explain a significant portion of the observed variance in trichodorid preference towards wild-type TRV infected plants.	?
3	Given a choice between TRV-Δ2b infected plants and wild-type infected plants, trichodorids will preferentially move towards wild-type infected plants.	✔
3	Given a choice between TRV-Δ2b infected plants and uninfected plants, trichodorids will preferentially move towards TRV-Δ2b infected plants.	✔
3	Viral load will explain a significant portion of the observed variance in trichodorid preference towards TRV-Δ2b infected plants.	✔

Table 5.5 Summary of hypotheses relating to effects of 2-ethyl-1-hexanol on trichodorid attraction. The chapter that each hypothesis relates to is given, along with the outcome.

Chapter	Hypothesis	Accepted/Rejected
4	Given a choice between uninfected plants and uninfected plants with 2-ethyl-1-hexanol added to the roots, trichodorids will preferentially move towards plants with added 2-ethyl-1-hexanol.	✔
4	Given a choice between wild-type TRV infected and TRV-Δ2b infected plants with added 2-ethyl-1-hexanol, there will be no significant difference in attractiveness between the two options.	X
4	Viral load will explain a significant portion of the observed variance in trichodorid preference towards TRV infected plants.	✔

Changes in biomass and root architecture as a result of infection

Infection with TRV results in a variety of symptoms, depending on the host plant, both in above and belowground tissues, and these have been well characterised for crop and ornamental plant hosts (Taylor and Brown 1997). Although response to infection varies across species, within species and across plant tissues (Harrison 1968), there has been little research focus on retardation of belowground growth, nor whether the structure of roots change with infection. My preliminary research that tested model plants for TRV and trichodorid work (*Nicotiana benthamiana, Arabidopsis thaliana, Lactuca sativa* and *Petunia × hybrida*) for successful infection and plant growth responses (data not in thesis) suggested that infected *N. benthamiana* plants had reduced root systems with roots appearing shorter in length, as well as overall reduction in plant size compared to uninfected plants.

The feeding action of trichodorid nematodes has long been associated with "stubby root" symptoms as well as galling and swelling of roots (Christie and Perry 1951, Pitcher 1967), and the intensity of the plant response increases with both density of trichodorids present and the type of plant species (Winfield and Cooke 1975). My preliminary work suggested that TRV infection may play a role in these reported effects. Based on this, we hypothesised that there would be a decrease in above and belowground biomass as a result of infection, but that these effects would be more severe in roots, leading to an overall reduction in plant biomass and root:shoot ratios in infected plants. The root parameters analysed included total root length, average root diameter, root surface area and root volume.

This work was presented in Chapter 2, and most of these hypotheses were accepted (Table 5.1); wild-type TRV infection did significantly reduce aboveground and belowground biomass, although a significant reduction in the former was only evident two weeks after infection, whereas differences were seen in the latter at 7 and 14 days post infection. Infected plants also had reduced root systems compared to their total plant biomass. These root systems had a reduced total root length and surface area to volume ratio. This drives a stronger reduction in root systems relative to aboveground biomass when compared to healthy plants. Therefore, in addition to deviation from the normal growth of plants, such as reduction in total size, retarded growth and delayed senescence (Dale *et al.* 2004), I propose that TRV infection may play a role in causing

the observed and well characterised "stubby root" effects of trichodorid feeding (Coiro and Sasanelli 1994, Otulak *et al.* 2012, Whitehead and Hooper 1970). The hypothesis that infected plants had reduced root systems relative to the total plant mass was rejected as while this was seen 7 days post infection, the opposite was seen 14 days post infection. However, this increase in root mass still supports the idea that TRV infection could be at least partially responsible for the "stubby root" effects seen, as root lengths were still reduced compared to infected plants.

Trichodorid feeding can result in long-term root symptoms that may not become apparent until harvest (Brown and Sykes 1971). The TRV infection itself, however, appears to be transient (Cadman and Harrison 1959, Shaw *et al.* 2014), with viral load dropping to low levels from 18 days post-infection when plants enter the recovery phase (Ratcliff *et al.* 1997, 1999). However, the virus is often present at low levels even if the infected plant no longer demonstrates symptoms (Ma *et al.* 2015, Xenophontos *et al.* 1998) and developed resistance to subsequent reinfection with TRV is observed (Ratcliff *et al.* 1999, Shaw *et al.* 2019). The time course of infection and the appearance of symptoms vary with species of plant host (Cadman and Harrison 1959, Schmelzer 1957) and mechanism of infection. For example, potato and tobacco plants planted in *Trichodorus primitivus*-infested soil still showed symptoms of infection 4 weeks after exposure to viruliferous nematodes (Otulak *et al.* 2012), whereas *N. benthamiana* plants mechanically infected with the virus showed recovery from symptoms 21 days after infection (Shaw *et al.* 2019). The work in Chapter 3 tested the hypotheses that that viral load in the *N. benthamiana* variety used peaked around 7 days post infection, and the symptoms of infection as identified in the previous chapter would be strongest at the same time or lag slightly behind peak viral load and be strongest 14 days post infection. The infection timeline used follows a similar course to that seen in the work by Shaw and colleagues and was carried on to 28 days, to see if differences in architecture parameters between wild-type infected and control uninfected plants started to disappear from 21 days post infection, due to recovery (Shaw *et al.* 2014, 2019).

The data supported these hypotheses (Chapter 3 hypotheses, Table 5.1), although there was disagreement in the data from Chapters 2 and 3 on the root to total plant weight ratio; work in Chapter 2 suggested increased allocation to the roots between 14 days post infection, whereas Chapter 3 showed the opposite. These differences are likely due to the growth stage of plant treatments, as plants in Chapter 3 were infected when

smaller to ensure that plants at 28 days post-infection would be small enough to sample properly in the root scanner. It may be that larger plants recover more quickly, and this recovery begins with increasing resource allocation to root systems. While the infected plants recovered biomass over time, inoculated and systemically infected leaves retained the visual symptoms of infection (mottling and necrotic zones), even as new leaves showed few symptoms.

These findings suggest that biomass and root/shoot effects are transient, with the time frame of infection-dependent outcomes in line with previous work on *N. benthamiana*. TRV infection may exacerbate trichodorid feeding symptoms in certain conditions, rather than being an underlying cause. Not all trichodorid populations that damage plants are viruliferous; estimates range from approximately 10% in the Pacific Northwest (Mojtahedi *et al.* 2003) to 12-42% of viruliferous populations in Flanders, Belgium (reviewed in Ploeg and Decraemer 1997). Cooper (1971) estimated that 12% of Scottish potatoes are grown in soil containing TRV, whereas 15-20% are grown in trichodorid-infected soils, and while TRV has a wide host range (Taylor and Brown 1997), it does not infect all plants affected by trichodorids such as apple (Pitcher 1967) or sweetcorn (Christie and Perry 1951).

These findings suggest that TRV infection may have some additional physical effects on belowground root systems that have not previously been reported (Conti and Masenga 1977, Cremer and Schenk 1967, Dale 2009, Harrison and Robinson 1978) and provide novel information on how TRV infection affects belowground systems. The observed root physiology and biomass effects are transient, which may explain why they have not been previously reported, especially as research on field-grown TRV-infected plants tends to focus on final outcomes (Dale *et al.* 2000, 2004). If these responses are replicated in other species, it may have implications for nematode feeding and viral transmission. Trichodorids feed primarily on epidermal cells just behind the elongation zone at the root tip, feeding for no more than 6 minutes on individual cells (Wyss 1977, 1982) then moving to other sites (Taylor and Brown 1997). They are also readily disturbed by other nematodes, causing them to abandon feeding on that cell and to move to another (Karanastasi *et al.* 2003). When enough nematodes converge on an area of a root system, this leads to a decline in the roots and the movement of the nematodes to other zones (Pitcher 1967). Root tips of high viral load plants, 7-14 days post infection, are closer together due to reduced overall root length, which may cause a

combination of i) increased disturbance by other nematodes due to reduced space between feeding zones and ii) quicker depletion of high quality feeding areas due to smaller root systems. These effects may cause trichodorids that have fed successfully, and may therefore be carrying TRV, to move on to new, uninfected hosts.

Presence of root volatile emissions as a result of viral infection

Plant roots release a plethora of compounds into the rhizosphere and low molecular weight secondary metabolites such as amino acids, organic acids, sugars and phenolics are important constituents (Bais *et al.* 2006). Volatile organic compounds (VOCs) form about 1% of all root-released exudates (Venturi and Keel, 2016). Volatiles are particularly interesting as chemoattractants, because they can easily diffuse through gas- and water-filled pores, depending on their solubility, and so have a wider range in soils than water-soluble, non-gaseous exudates (Schulz-Bohm *et al.* 2018).

VOCs are an important group of molecules within the rhizosphere, used as intra- and interspecies signals, mediating interactions between different groups of organisms (Venturi and Keel 2016) and most commonly produced by plant roots and microbes (Peñuelas *et al.* 2014). VOCs are thought to be important in plant–microbe interactions; volatiles from plant growth promoting rhizobacteria (PGPR) directly improve plant growth (Delaplace *et al.* 2015, Fincheira *et al.* 2016), alter root architecture (Bavaresco *et al.* 2020) and improve resistance to stress and diseases (Naznin *et al.* 2014). Root VOCs can act as a carbon source for bacteria (Macey *et al.* 2020) and are used to attract bacteria to roots (Schulz-Bohm *et al.* 2018) and communicate with other plants, both in mutually beneficial and antagonistic interactions (Ens *et al.* 2009, Huang *et al.* 2019, Jassbi *et al.* 2010). Root herbivory is a common stimulus for the release of VOCs and different VOC groups have been detected in response to herbivory: fatty acid derivates, such as aldehydes (Robert *et al.* 2012); ketones and alcohols (Lawo *et al.* 2011, Palma *et al.* 2012); acids (Dudareva *et al.* 2006); terpenes (Ali *et al.* 2011, Jassbi *et al.* 2010, Rasmann *et al.* 2011, Rasmann and Turlings 2008); thiocyanates, isothiocyanates and other sulphur-containing compounds (Crespo *et al.* 2012). Some of these volatiles attract natural enemies of the feeding herbivores, including some species of nematode (Ali *et. al.* 2010, Degenhardt *et al.* 2009, Rasmann *et al.* 2005). These herbivory-response VOCs may also attract plant-parasitic nematodes (Ali *et al.* 2010).

In the initial work presented in Chapter 2, it was hypothesised that TRV-infected plants would show different root VOC profiles to uninfected plants, as many plant viruses have been documented as driving differential release of plant volatiles relative to uninfected plants, attracting vectors to enable spread to new hosts (e.g. Claudel *et al.* 2018, McMenemy *et al.* 2012), and nematodes are known to respond to root VOCs. The exact volatiles that were expected were unknown as no work had been carried out on volatiles emitted from roots of TRV infected plants. Significant differences were detected in volatiles emitted between wild-type infected plants and uninfected plants (Chapter 2, Table 5.2) sampled with the canister method.

It appears to be rare for novel products to be released by infected plants; infection either increases the total concentration of emitted volatiles or alters the concentration of a few compounds within the blend emitted (Eigenbrode *et al.* 2018). The findings of this research were consistent with these previous observations. The four compounds that showed significant differences between infected and uninfected treatments were also present in the chromatograms of samples from uninfected plants, just at much lower concentrations. When this experiment was repeated (Chapter 3), it was hypothesised that these same four compounds would be present again and with significantly greater emissions from the roots of infected plants than uninfected plants. Only two of the four, 2-ethyl-1-hexanol and 1,3-ditertbutylbenzene, were identified in the traces. It is possible that the other two compounds were produced by an alternate source, potentially microbial.

2-ethyl-1-hexanol can be produced by *Bacillus* spp. as well as plants (Almenar *et al.* 2007, Suryadi *et al.* 2019) and has been suggested to have anti-fungal properties (Almenar *et al.* 2007, Cruz *et al.* 2012). If 2-ethyl-1-hexanol is being produced by rhizosphere-associated microbes such as *Bacillus*, then this may help boost the signal for passing nematodes and increase attraction. However, many soil organisms are able to use 2-ethyl-1-hexanol as a carbon source, and oxidise it to ethylhexanoic acid, which is far less volatile (Nalli *et al.* 2006). This may reduce its diffusion distance, depending on the microbial community within the rhizobiome.

Aboveground, 2-ethyl-1-hexanol has been shown to be an important component of *Prunus persica* floral scents, attracting pollinators to flower heads in a variety of growing conditions (Du *et al.* 2018). As part of a six-component blend, it was found to be attractive to female orange wheat blossom midges on wheat (Birkett *et al.*

2004). Sun and colleagues (2019) found that belowground feeding of *Bikasha collaris* larvae on plants stimulated the release of 2-ethyl-hexanol from leaves, attracting adult conspecifics to the aboveground portion of the plant to feed. The action of the adults feeding aboveground increased root nutrient allocation and improved larval performance. Not only does 2-ethyl-1-hexanol mediate belowground and aboveground signalling, but it is also shaping dynamics where the rhizosphere and phyllosphere may interact.

The biosynthesis of 2-ethyl-1-hexanol in plants is likely to be ethylation of hexanol produced in the fatty acid pathway (see Dudareva *et al.* 2013). Other end products of this pathway include 3-hexenyl acetate, 2-hexenal, 1-hexanol, octanal, 3-hexen-1-ol, decanal and undecanal. These have been well studied in terms of volatile signalling including host location (Engelberth 2020, Hu et al. 2018 Joo et al. 2019, McMenemy et al. 2012, Michereff et al. 2019, Webster *et al.* 2008), and by comparison 2-ethyl-1-hexanol is poorly represented. It is rare that it is identified as a major player in signals, showing little chemoattraction when tested, and is often a background component to volatile blends (Almenar *et al.* 2007, Birkett *et al.* 2004, Dias *et al.* 2016). It may be that it has a larger role in these interactions than previously thought, or that its impact is greater in the rhizosphere, which is less well studied. It is also possible that in some studies it has simply not been detected, as methodologies in collection and sampling of volatiles can affect the stability and detection of volatiles and cause losses from the sample (Kim and Kim 2012, Ullah *et al.* 2014).

It was hypothesised that viral load would explain a significant portion of the variation in root VOC emissions. This hypothesis was not met in the data from Chapter 2, and in Chapter 3, only 2-ethyl-1-hexanol emissions were significantly explained by viral load, with an incredibly high percentage of the variation explained by viral load in roots. This could suggest, as above, that not all of the emissions seen in Chapter 2 were directly attributed to TRV infection, or that the growth conditions also influenced and possibly exacerbated signals linked to infection. It may be that these compounds are not directly induced by infection as an overproduction of existing plant metabolism, but are instead a stress response (Holopainen and Gershenzon 2010, Sharkey *et al.* 2008) as a by-product of infection, but this has not been tested in this investigation.

This work has demonstrated, for the first time, that root VOC emissions are altered by infection with a nematode-transmitted virus and suggests that TRV utilises similar

VOC-dependent mechanisms to attract transmission vectors as aboveground viruses (Mauck *et al.* 2012, 2014). It is important to note that this has been carried out on intact and living roots and that these compounds would be released in normal conditions, unlike work carried out on destructively processed tissues (Joo *et al.* 2019, Rasmann *et al.* 2005).

Removal of RNA-2 genes reduces effects of infection

The genome of TRV is split into two particles of rod-shaped RNA, and the smaller RNA-2 contains genes necessary for nematode transmission of TRV (Hernández *et al.* 1995, MacFarlane *et al.* 1996). The size and organisation of this particle is extremely variable between different TRV isolates; usually the coat protein (CP) gene is located near the 5' end (Goulden *et al.* 1990) and is followed by one or more non-structural protein-encoding genes (2b and 2c). However, some strains only contain the CP gene, such as TRV-PSG (Cornelissen *et al.* 1986) and are not transmitted by nematodes.

In some tobravirus isolates, such as PEBV-TpA56, both 2b and 2c are involved in nematode transmission (Hernández *et al.* 1995, MacFarlane *et al.* 1996, MacFarlane 2003, Ploeg *et al.* 1993a,b) while in others, such as TRV-PpK20 used in these investigations, only 2b is essential (Hernández *et al.* 1995) and the function of 2c is unknown. As the 2b protein is vital for nematode transmission, and has also been suggested to have antagonistic effects on plant defences (Valentine *et al.* 2004), I hypothesised that plants infected with TRV RNA-2 mutants missing 2b (TRV-Δ2b) would lead to a less severe infection than wild-type, and also that infection with the TRV-Δ2b mutant would show intermediate responses in plant growth and root architecture measures compared to wild-type TRV infected plants and uninfected plants. While the function of 2c is unknown for TRV-PpK20, it is likely to play some role in infection, and TRV-Δ2c infected plants were also hypothesised to show less severe effects of infection. Responses varied in significance for effects on root architecture parameters and biomass, but plants infected with TRV-Δ2b showed responses broadly similar to plants infected with wild-type TRV, whereas plants infected with TRV-Δ2c showed intermediate responses. Notably, TRV-Δ2c mutants showed similar ratios to uninfected plants at 7 and 14 days post infection for root to total plant biomass. This suggests that removal of 2c from TRV-PpK20 stops the changes to root systems with infection. I further hypothesised that plants infected with both mutants would show intermediate

changes to root VOC profiles compared to wild-type infected plants and uninfected plants, but all differences in root volatile fluxes from mutant-infected plants compared to uninfected plants were non-significant.

While isolates completely lacking in 2b and 2c exist and can still cause spraing and stem-mottle symptoms in potato (Garbaczewska *et al.* 2012), Ghazala and Varrelmann (2007) found that removing 2b and 2c from TRV-PpK20 leads to extreme and hypersensitive-like resistance in some susceptible varieties of potato. Extreme resistance to plant viruses is typified as strong and durable resistance, with reduced or no visible symptoms of infection (Flis *et al.* 2005), while hypersensitive resistance results in rapid necrotic reactions, either locally or systemically (Valkonen *et al.* 1998) as a programmed cell death response to contain viruses at the infection site (de Ronde *et al.* 2014). The results of these experiments suggest than in *N. benthamiana*, infections with mutants missing either one of the 2b or 2c genes reduces severity of infection, although necrotic lesions and deleterious effects on plant growth were still observed. Just as vector specificity in TRV may occur as a result of interactions of 2b and CP (MacFarlane 2010, Vassilakos *et al.* 2001), the severity of symptoms, including changes in emitted volatiles, could be due to interactions between 2b and 2c.

However, the response of TRV-Δ2c mutant-infected plants that bear similarities to uninfected plants—particularly for root to total biomass and, to a lesser degree, for total root length—may have consequences for feeding behaviour and transmission if, as suggested above, the reduced root systems make disturbance and dispersal of feeding trichodorids more likely. This may be a contributory factor to the differential transmission rates seen for different TRV serotypes (Ploeg *et al.* 1989,1992). TRV RNA-2 is not conserved across different TRV serotypes (Figure 1.5) and 2c is not always present (MacFarlane 1999). When it is, 2c often changes in size and known function across serotypes (MacFarlane *et al.* 1996), which may have an impact on root architecture of plants infected by different TRV serotypes.

As well as being essential for nematode transmission and having a role in vector specificity (MacFarlane 2010), the TRV RNA-2b gene product (40kDa) has been reported to have a role in colonisation of TRV through the host plant from the inoculation zone, and also as a possible antagonist to host defences (Valentine *et al.* 2004). In Cucumber mosaic virus, the unrelated 2b gene encodes for a small (12kDa) protein that inhibits host defences (Ziebell *et al.* 2011). It also seems to have a role in

altering host VOC emissions in *Nicotiana tabacum* (Tungadi *et al.* 2017), which suggests that these smaller non-structural virus proteins may be multifunctional in their roles, including altering volatile products.

Trichodorids are attracted to TRV-infected plants

Just as arthropod vectors that transmit viruses aboveground are preferentially attracted to viruliferous plants (Bosque-Perez and Eigenbrode 2011, Fereres *et al.* 2016, Mwando *et al.* 2018), I hypothesised that trichodorids would preferentially move towards plants infected with TRV over uninfected plants, which was demonstrated, with greater attraction with increasing viral load. While trichodorids have been observed orienting themselves towards host plants and aggregating at the elongation zone of growing roots (Taylor and Brown 1997), and so must be able to respond to chemical cues from host plants in order to find appropriate feeding locations, this is the first time trichodorids have been shown to be recruited by a chemical signal from TRV infected plants.

The TRV-2b gene is essential for nematode transmission, so TRV-Δ2b infected plants were hypothesised to be less attractive than wild-type infected plants, and these plants showed intermediate attraction; more attractive than uninfected plants but significantly less attractive to trichodorids than wild-type infected plants. This could be due to a difference in volatile signals between these infection treatments. However, the preference of mutant-infected plants over uninfected plants in the absence of a detected difference in root VOC profile suggests that more chemical cues are present and not yet identified.

In other plant–virus–vector systems, viruliferous and non-viruliferous vectors behave differently to volatiles released from infected plants, both in terms of initial attraction and feeding behaviours (Dáder *et al.* 2017, Fereres *et al.* 2016, Rajabaskar *et al.* 2014). Therefore, a potential further research project should test viruliferous versus non-viruliferous trichodorid populations to see if they respond differently to uninfected/infected plants; or it may use unseparated nematodes, such as in this work, but test collected nematodes for presence of TRV (Boutsika *et al.* 2004, Holeva *et al.* 2006) to see if viruliferous and non-viruliferous individuals within a total population behave differently.

2-ethyl-1-hexanol as major causative agent of TRV-induced plant signals

As the compound most strongly affected by TRV infection, I hypothesised that 2-ethyl-1-hexanol, a fatty acid-derived plant alcohol, would form part of trichodorid foraging cues and be attractive to trichodorids. Fatty acid-derived alcohols and esters form an important class of phytochemicals, derived from linoleic or linolenic acids (Dudareva *et al.* 2013), and are released in volatile blends from roots under herbivory, such as grapevine attacked by phylloxera (Lawo *et al.* 2011) and red clover by root borer (Palma *et al.* 2012). They are involved in many multitrophic interactions above ground, including virus vector attraction (Jiménez-Martínez *et al.* 2004, Raguso and Roy 1998, Shapiro *et al.* 2012). 2-ethyl-1-hexanol has been shown to be part of a volatile blend released from carrot and potato roots damaged by wireworms and cockchafer larvae (Weissteiner 2010, Weissteiner and Schulz 2006). This may be an attractant for entomopathogenic nematodes, although it appears to act inconsistently between species (Laznik and Trdan 2016).

The rhizosphere has been defined as the 1-3mm zone around roots shaped by the root system of plants and is directly influenced by root exudates and associated soil microorganisms (Sasse *et al.* 2018, Venturi and Keel 2016). Since volatiles and other gases drive signalling and other biogeochemical processes around plant roots, it has been proposed that this wider, volatile-influenced volume should be taken into consideration as part of the rhizosphere (de la Porte *et al.* 2020). For example, O_2 is depleted around the roots as a result of root and microbial respiration, and gradients of O_2 from the root surface extended out 1-2cm (Uteau *et al.* 2015). Likewise, volatiles released by *Carex* plants can recruit soil bacteria from over 10cm away (Schulz-Bohm *et al.* 2018) and entomopathogenic nematodes are able to sense the release of (E)-β-caryophellene from maize roots over 50cm away (Turlings *et al.* 2012). In this study, 2-ethyl-1-hexanol helped recruit trichodorid nematodes from over 8cm away, and similar fatty acid-derived compounds have been found to maintain concentrations in the soil up to 12cm away from their source (Schulz-Bohm *et al.* 2018), suggesting that it may be an effective signalling molecule at longer distances than tested here.

I found that the addition of 2-ethyl-1-hexanol to the rhizosphere of uninfected *N. benthamiana* plants made them significantly more attractive to trichodorids than untreated control plants (Chapter 4). I then compared wild-type TRV-infected plants

against TRV-Δ2b infected plants (Chapter 4). The mutant infected plants had proven to be of intermediate attraction between wild-type infected and uninfected plants, with no elevated release (relative to uninfected controls) of 2-ethyl-1-hexanol in the volatile profiles. I hypothesised that 2-ethyl-1-hexanol would enhance the attractiveness of mutant-infected plants to the same level as wild-type plants and remove differences in attractiveness between those treatments. The results show that while addition of 2-ethyl-hexanol reduces the difference in attraction between untreated TRV-Δ2b infected plants and wild-type infected plants, nematodes still significantly prefer wild-type TRV-infected plants. This suggests that while 2-ethyl-1-hexanol is attractive to trichodorids, the observed differences in attraction between infections with and without 2b cannot be explained by the reduced 2-ethyl-1-hexanol emissions from mutant infected plants. Testing the motility of trichodorids directly in arenas (Liu *et al.* 2019, Maleita *et al.* 2017, Qi *et al.* 2015) would not only allow direct observation of 2-ethyl-1-hexanol attraction, but also enable testing of 1,3-ditertbutylbenzene and volatile combinations, as well as degassed, non-volatile root exudates and metabolites from infected plants (Čepulytė *et al.* 2018)

In other virus–vector systems, infection often affects more than one plant-produced VOC, and it is these blends of volatiles that have the largest effect on vectors (Eigenbrode *et al.* 2018). This is likely in this system with 2-ethyl-1-hexanol not solely responsible for influencing trichodorid attraction, as suggested by significant movement to plants infected with TRV-Δ2b over uninfected plants when there was no significant difference in 2-ethyl-1-hexanol emissions between those treatments. 2-ethyl-1-hexanol appears to be a strong attractant in the absence of other infection signals but when compared to the complete wild-type infection, other factors beyond 2-ethyl-1-hexanol appear to be required for comparable attraction. These could be other root VOCs, such as 1,3-ditertbutylbenzene, or further undetected compounds such as soluble, non-volatile root exudates (Peñuelas *et al.* 2014) released with infection that combine to give the full signal seen with full TRV infection. Chemical signalling belowground is incredibly important over long distances for many different organisms, including nematodes. Nematodes are able to detect multiple stimuli including photic, electrical, mechanical and thermal (Jones 2002), but chemical signals are among the most important cues for nematodes (Rasmann *et al.* 2012). The largest chemoreceptor organ for nematodes are amphids (Rasmann *et al.* 2012), located in the head, and contain a

group of neurons, whose cell bodies lie in a cuticle lined pit (Jones 1979) with a gap leading to the exterior.

This is the first time a chemoattractant has been demonstrated for trichodorid nematodes, but also for any plant-parasitic nematodes outside the order Tylenchida (Chin *et al.* 2018, Rasmann *et al.* 2012, Sikder and Vestergård 2019). Plant-parasitic nematodes are split into three orders: Triplonchida, Dorylaimida and Tylenchida. The first two contain all known nematode vectors of plant viruses—trichodorids (Triplonchida) and longidorids (Dorylaimida) (Decraemer and Robbins 2007, Subbotin *et al.* 2020, Taylor and Brown 1997)—which makes this the first demonstrated example of chemotaxis recorded for any nematode vectors of plant viruses. These findings have potential implications for plant diseases caused by longidorid-transmitted viruses, primarily nepoviruses (Taylor and Brown 1997), and are important for expanding knowledge of nematode chemotaxis, both in terms of novel chemoattractants and information about less-studied nematode groups (Rasmann *et al.* 2012).

Implications for agricultural management of TRV and other nematode-transmitted viruses

In agriculture, spraing disease is managed either through the growth of TRV-resistant cultivars or through control of trichodorid vectors. There are complications with the latter, as trichodorids are polyphagous and can overwinter on weed species (Davis 1975, Mojtahedi *et al.* 2003). Nematicides are increasingly controlled due to environmental and human health safety concerns (Ingham *et al.* 2000, Kerry 2003). In the UK, there are only two nematicides authorised for use on free-living nematodes (EU Directive 91/414/EC): Nemathorin (active ingredient fosthiazate) and Vydate (active ingredient oxamyl). Even using nematicides, control is difficult as populations must be severely reduced to reduce incidence of spraing disease, therefore often requiring multiple costly applications of nematicides (Ingham *et al.* 2007).

As a result, growing TRV-resistant cultivars is the preferred method of reducing crop losses due to spraing and plant viruses. It is more effective, economical and reduces environmental impact. The resistance to TRV in commercial potato varieties is tested by screening tubers grown in viruliferous nematode-infested soils, either in the glasshouse or in the field (Dale and Neilson 2006, Dale and Solomon 1988), then

scoring them for spraing symptoms in tubers. Especially in glasshouse trials, this does not take into consideration nematode preference for alternative food sources that may be available; if 2-ethyl-1-hexanol can be shown to be released from potato roots in addition to *N. benthamiana* roots, then screening potato varieties by 2-ethyl-1-hexanol emissions may provide another axis to cultivar selection. Growers could then pick varieties that are both more resistant to TRV and are less attractive to trichodorids in the case of infection. In addition, possible companion plants with high emissions of 2-ethyl-1-hexanol from uninfected roots could be planted alongside less attractive potato varieties to further reduce the likelihood of trichodorid feeding (Shelton and Badenes-Perez 2006). Commercial cultivars showing resistance to spraing have been divided into three classes, depending on the strength of resistance and severity of symptoms (Dale and Neilson 2006, Robinson and Dale 1994). Selecting resistant varieties to test from across the three classes would show if volatile emissions, particularly 2-ethyl-1-hexanol and 1,3-ditertbutylbenzene, change with increasing resistance. A complicating factor in cultivar selection is the symptomless expression of TRV in some cultivars (Xenophontos *et al.* 1998). These tubers produce systemically-infected plants with symptomless daughter tubers that themselves are a source of TRV for nematode transmission.

The withdrawal of around 20% of pesticide active ingredients by EU directive and the promotion of low pesticide-input faming in member states has created an increased drive for integrated pest management (Hillocks 2012). In the future, the UK will have to produce increasingly great amounts of food with less reliance on pesticides. This will impact control of all free-living plant parasitic nematodes, including trichodorids. Unlike Europe and North America, the UK was slow to start looking for new methods of integrated pest management for the purpose of controlling free-living nematodes (Neilson 2016). In addition to breeding for resistance and crop rotation influenced by the monitoring of free-living nematodes, direct management of free-living nematodes can be attempted with tillage regimes, biological control agents, soil amendments and cover crops (Neilsen 2016).

Cover cropping can be used for multiple purposes, including weed suppression during growth, and then incorporated into the soil as a green manure for nutrients (Campiglia *et al.* 2009) or as a biofumigant; especially cruciferous species of Brassicaceae (Doheny-Adams *et al.* 2018). The addition of a cover crop grown alongside a main crop

is called companion planting. Companion planting in a variety of push-pull systems is a well-established concept in sustainable and integrated pest management (Conboy *et al.* 2019, Pickett and Khan 2016, Sarkar *et al.* 2018) and examples exist of successful implementation in developing countries (reviewed in Hokkanen 1991). Trap plants can attract potential pests through release of volatile compounds or arresting visual cues that divert movement towards crop species, and planting multiple trap plants that combine multiple different cues can be more effective (Eigenbrode *et al.* 2016). For nematodes, *Tagetes* cultivars have been suggested for control of some plant-parasitic nematodes (Hook *et al.* 2010), particularly *Meloidogyne* species (El-Hamawi *et al.* 2004, Ploeg and Maris 1999, Pudasaini *et al.* 2006), but their effectiveness as companion plants in intercropping systems is variable and limited (Hook *et al.* 2010, Powers *et al.* 1993). They have also been implicated as having potential negative impacts on beneficial organisms that may control free-living nematodes (Owino 1992).

Plants that are damaged by pests can release stress-related signals, either aboveground (Pickett *et al.* 2012) or through the rhizosphere (Birkett *et al.* 2001), including via shared arbuscular mycorrhizal networks (Babikova *et al.* 2013a,b) that lead to indirect defence priming in undamaged plants nearby. A key challenge in these systems is the dispersal of the pests from the trap plants back to the crop; they must be retained or experience elevated mortality on the trap plants. This is particularly an issue for high-dispersal winged arthropods. Plant-parasitic nematodes rarely travel more than several metres annually through active dispersal (Taylor and Brown 1997), so trap plants cannot attract nematode pests from large distances. This would require close association of trap species with crop plants in order to be effective. This close association increases the risk of pest dispersal back to crop plants. Plant parasitic nematodes can also be passively transported in plant material or agricultural equipment, or by soil and wind erosion; primarily by surface runoff of water following rainfall events (reviewed in Baxter *et al.* 2013). Therefore, agricultural practice and field physical conditions must be taken into account when predicting potential movement of nematodes.

However, transience of symptoms of infection found in this investigation has implications for spraing disease in potato. Plants that were infected before tuber formation did not show any symptoms of spraing in the tubers at harvest and direct feeding by trichodorids on tubers, rather than plant roots, was required for disease development (van Hoof 1964). Potentially, trichodorids in an intercropping system

would only need to be attracted from crop plants during tuber formation, rather than the entire growing season. This may reduce the importance of back dispersal or finding nematicidal trap plants, although it would require precise knowledge of the start of tuber formation, which varies by cultivar and growing conditions.

The presence of intact TRV RNA-2 genes seemed to affect the volatile profiles in this investigation, which could have implications for other nematode transmissive isolates of TRV with different RNA-2 genes, such as TRV-TpO1 (MacFarlane 1999) which encodes a 2c protein half the size of that of TRV-PpK20 used in this investigation. Future work could focus on testing volatile profiles for this and other isolates of TRV and PEBV. A particularly interesting comparison would be to look at TRV-PaY4, which is usually transmitted by *Paratrichodorus anemones* (Vassilakos *et al.* 2001) but can also be transmitted by *P. pachydermus*. It is currently the only tobravirus isolate to not have strict vector specificity. RNA-2 isolates that are naturally missing 2b and 2c (MacFarlane 1999) should also be compared; it would be expected that they may behave similarly to the Δ2b and Δ2c mutants studied here. More broadly, the information learnt in this nematode–virus system could be applied to longidorid transmitted viruses, such as Grapevine fanleaf virus (Hewitt *et al.* 1958) which can cause economically important decline of harvest of up to 60% of fruit yield (Martelli 2014, Rudel 1985).

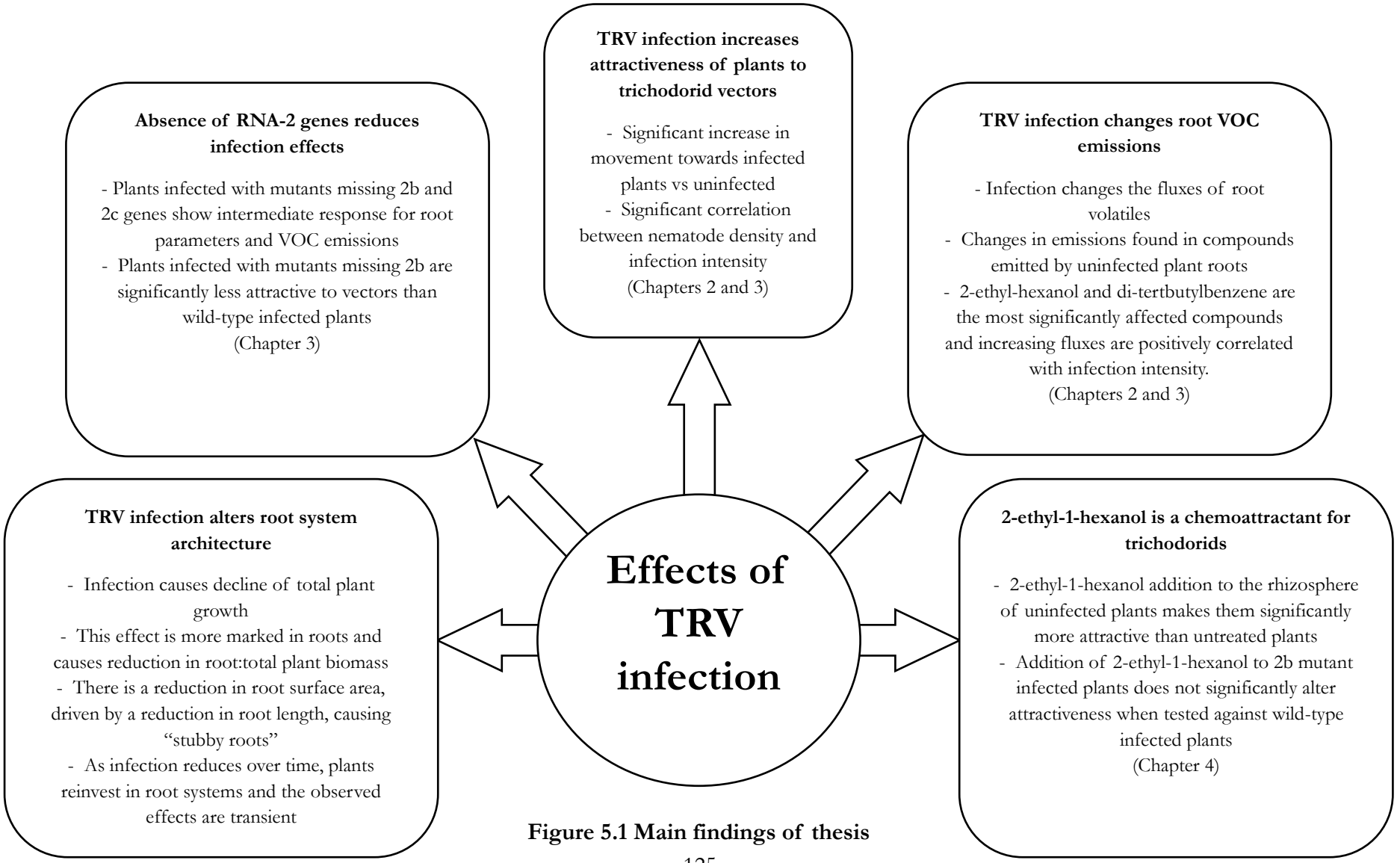

Figure 5.1 Main findings of thesis

Summary and conclusions

TRV–trichodorid interactions sit at the confluence of our understanding of plant virus–vector relationships, nematode chemotaxis and plant-mediated rhizosphere signalling, but while trichodorid feeding mechanisms have been well studied and characterised (see Tyler and Brown 1997) there is a paucity of background information to understand the wider picture of nematode foraging mechanisms. Work on plant virus–vector is dominated by studies on aboveground arthropod vectors, while nematode chemotaxis has focused on entomopathogenic and tylenchid plant-parasitic nematodes. Unlike aboveground interactions, nematode vectors can only rely on chemical and physical gradients to orient themselves towards plants, and chemical signals are readily consumed by the rhizobiome as carbon sources and broken down to other products, further complicating signals. Among this tumultuous backdrop, trichodorids must find a source, and TRV novel hosts, to allow it to spread.

The work in this thesis has shed light on this previously understudied interaction by using a model plant system. It has shown that TRV-infected plants have altered root systems and release root VOCs in response to infection, which attract vectors to infected plants and may encourage dispersal of viruliferous nematodes post feeding, as well as starting to suggest the molecular causes behind these effects (Figure 5.1). It has started to explore what compounds may be responsible for this signal and suggested a root VOC, 2-ethyl-1-hexanol, as being a driver of the nematode behaviours recorded.

Using this information as a starting point, there is potential to uncover more information on how trichodorid–virus interactions affect each other and are affected by the environment they are in; how viruliferous and non-viruliferous populations may differ and how these relationships may differ for other nematode–virus pairings; and how the rhizosphere fauna may influence chemical signals such as 2-ethyl-hexanol, with implications for pest management of viral disease in agriculture.

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