A University of Sussex DPhil thesis

Available online via Sussex Research Online:

http://sro.sussex.ac.uk/

This thesis is protected by copyright which belongs to the author.

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Please visit Sussex Research Online for more information and further details

Raspberry viruses manipulate plant–aphid interactions

Lindsay Sara McMenemy

Doctor of Philosophy

University of Sussex
November 2011

This thesis is dedicated to my parents,

Morag

&

Bernard

for always being there with love and support

thank you both

ABSTRACT

Plants come under attack by a variety of organisms, including insects and pathogenic micro-organisms such as viruses. Plant viruses can interact indirectly with their vectors by inducing changes to plant chemistry which may alter its attractiveness as a host for herbivore vectors. Using red raspberry as a study system, this study aimed to investigate the host plant mediated interactions occurring between the large raspberry aphid, *Amphorophora idaei*, and two of the viruses that it transmits, Black raspberry necrosis virus (BRNV) and Raspberry leaf mottle virus (RLMV).

In whole plant bioassays, BRNV and RLMV-infected plants were shown to be initially more attractive to *A. idaei* and aphids remained on the initially selected host plant for a period of approximately 30 minutes. In addition, *A. idaei* took three days longer to reach reproductive maturity compared with those feeding on non-infected plants, suggesting a virally-induced manipulation of aphid behaviour whereby a deceptive attraction of the vector to a host plant found to be nutritionally poor, presumably acts to promote virus transmission.

Investigations of the underlying plant chemistry revealed that raspberry viruses may be capable of facilitating aphid feeding by reducing leaf phenolic concentration when aphids are feeding and that infection with BRNV and RLMV resulted in significantly elevated levels of carbon and free amino acids in the leaves. While increased concentrations of amino acids might be expected to promote aphid performance, the amino acid composition was dominated by glutamate (77% of total content of infected plants), a previously suggested indicator of reduced host-plant suitability for aphids. Volatile entrainments from virus-infected plants showed elevated levels of the green leaf volatile (Z)-3-hexenyl acetate. Bioassays subsequently revealed that this compound acted as an aphid attractant at a concentration of 50 ng ml^{-1} but that aphid behaviour was unaffected by lower concentrations.

The combined utilisation of PCR diagnostics developed from newly sequenced viral genomes and the implementation of a non-invasive, targeted method of sampling plant headspace volatiles enabled this study to provide novel insights into the nature of host plant mediated interactions between aphids and the viral pathogens that they transmit.

CONTENTS

CHAPTER TWO The biology of the European large raspberry aphid (*Amphorophora idaei*): its role in virus transmission and resistance breakdown in red raspberry 34

CHAPTER THREE — The effect of Black raspberry necrosis virus and Raspberry leaf mottle virus on the recruitment and performance of *Amphorophora idaei* 62

CHAPTER FIVE Raspberry volatiles attract *Amphorophora idaei* to virus-infected raspberry plants 122

TABLES, FIGURES AND PLATES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors, Scott Johnson, Stuart MacFarlane (both SCRI) and Sue Hartley (University of Sussex) for giving me the opportunity to take up this project. Their continual enthusiasm and support of new ideas made the project a very enjoyable and productive experience and for that I am truly thankful. As my principle supervisor, Scott had to endure most of the trauma and the tears but never ceased to encourage me to pick myself up and try again. Stuart trained me in the lab with great patience and was always on hand with suggestions when the PCR reactions didn't go according to plan. Despite the distance between institutions, Sue was a constant source of support, providing advice and opinions on all aspects of the project and provided a very warm welcome on my various visits to Sussex. Also at Sussex, thanks are due to Liz Hill and her PhD student Lynne Robinson for teaching me the art of GC-MS extractions. I don't think I've ever worked so hard in all my life as I did during the week I spent in their lab!

A great number of people at SCRI helped me throughout the project. Many an aphid and raspberry plant owe their life to Carolyn Mitchell, Katy Clark, Sheena Lamond and Alison Dobson who would step in to take over their care when I was away from SCRI. I thank Wendy McGavin for her invaluable help with the virus testing and PCR; Alison Karley, Gill Banks and Fiona Falconer for their help with leaf chemical analyses, the genie of the GC - Tom Shepherd for all of his help with the volatile entrainments, Craig Simpson & my fellow Arab, Brian Fenton for acting as my internal assessors and also Jim McNicoll & Katrin MacKenzie at BIOSS for statistical advice. This project could not have been completed without their assistance and I am grateful to them all. I am also extremely grateful to my examiners, Jeremy Field at the University of Sussex and Glen Powell at Imperial College, London, for taking the time to read and comment on this thesis and for making the viva a very positive experience.

Finally, I'd like to say thank you to my family. My parents, Bernard and Morag – to whom I dedicate this thesis, Maria, Patrick and my brothers, David and Andrew, thank you for you love and support and for encouraging me to keep studying. And last, but not least, to Alex, thank you for all of your love and encouragement over the past years and in particular for helping me endure those dark months when it seemed we would never reach this point – I don't think I could have done it without you and our little rat family - I love you very much!

Chapter One

Introduction

1.1 Introduction

Like all other plants, raspberry is frequently attacked by a variety of damaging organisms, including insect herbivores and pathogenic microorganisms such as viruses and fungi. As plants are sessile and cannot physically move away from such attackers, they have developed a vast array of defence strategies to respond to grazing herbivores and invading pathogens and, ultimately, defend themselves against further attack (Baldwin & Preston, 1999; Dicke, 2009). Plant pathogens may trigger a defensive response from the plant which results in alterations to host plant physiology which then has effects on insect herbivores. This type of interaction is termed 'indirect' as the two attacking organisms need not interact directly in order for there to be profound changes to insect behaviour. Such indirect interactions, which are mediated by the host plant, received limited attention until about 20 years ago (Hatcher *et al.*, 2004) when their importance as drivers of arthropod and pathogen population dynamics began to be realised (see Khan & Saxena, 1985; Blua & Perring, 1992; Bacher *et al.*, 2002; Biere *et al.*, 2002). Such studies are especially complex for insects which act as vectors for pathogenic microorganisms, such as plant viruses, as in these cases there is scope for both direct interactions (e.g. circulation of virions in the insect haemolymph) and indirect interactions through virus-induced changes to the host plant (Stout *et al.*, 2006). Aphids, along with whiteflies and leafhoppers, are responsible for the transmission of around 80% of all insect transmitted plant viruses (Fereres & Moreno, 2009) and although many studies recognise that indirect interactions can have profound effects on aphid behaviour, few attempt to link these behaviours to changes in plant physiology which is central to both host selection and performance of the insect in response to infection of a host plant with viral pathogens. Several studies have addressed the role of the large raspberry aphid,

Amphorophra idaei, as a vector of several viral pathogens of red raspberry, *Rubus idaeus,* in the United Kingdom (see Chapter Two of this thesis) yet studies of the indirect interactions occurring between these debilitating viruses and their vector which are mediated by the host plant are non-existent. Such interactions may play a significant role in shaping the populations of many organisms, including insects, for which raspberry plants are an important resource. Investigations of the indirect interactions occurring between viral pathogens of raspberry and *A. idaei* are of key importance for long-term control of the aphid (and therefore spread of viral diseases) in the face of the large scale breakdown of plant resistance mechanisms that is known to be occurring in the UK and wider Europe as the aphid adapts and overcomes plant defence mechanisms (see Chapter Two).

1.2 Interactions between pathogenic micro-organisms and insect herbivores on a shared host plant

1.2.1 Plant defence against insects and pathogens

Pathogenic microbes that pose a threat to plants include bacterial pathogens, plant viruses and fungi. Their presence has been demonstrated to have a variety of effects on insect herbivores and both positive and negative interactions mediated by the host plant have been found. The first line of defence for a plant is provided by epicuticular waxes and trichomes which provide a physical barrier to an invading pathogen (Dangl & Jones, 2001). However, these constitutive defences are not always a successful deterrent and once the leaf surface is breached, a process of signal transduction may then be initiated in the plant to produce defensive chemicals, or secondary metabolites, which may affect the

susceptibility of the host plant to subsequent attack (Chen, 2008). Three main signalling pathways are known to be involved in the defence response to pathogens and insects; jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). The extent to which each pathway is activated appears to be attacker specific (Reymond & Farmer, 1998) which may result in antagonistic or synergistic interactions between plant attackers (Koornneef & Pieterse, 2008). For example, plant pathogens are usually associated with an SA-mediated response which may act to suppress the JA-mediated response to insect herbivores (Stout *et al.*, 1999). These types of interaction between signalling pathways are often referred to as 'cross-talk' (Kunkel & Brooks, 2002). In addition, plants have also been found to possess receptors which are capable of recognising the type of organism which is attacking e.g. mitogen-activated protein kinase (MAPK) proteins (Jonak *et al.*, 2002; Nakagami *et al.*, 2005) which activate a signalling cascade which regulates cellular activities such as gene expression. Plants therefore exhibit a complex array of biochemical responses and the result of this 'induced resistance' ultimately leads to alterations in the underlying plant chemistry which can subsequently exert a range of effects on a secondary attacker, such as an insect herbivore (Karban & Baldwin, 1997).

1.2.2 Effect of plant pathogens on non-vector insects

The biochemical processes mentioned in the previous section, and their products can indirectly alter host plant suitability for insect herbivores that play no direct role in the further transmission of the pathogen. For example, Kreuss (2002) showed that infection of creeping thistle with the pathogenic fungus, *Phoma destructiva*, was detrimental to larval development of the beetle *Cassida rubiginosa* and contrastingly, Johnson *et al.* (2003)

demonstrated a positive association between birch leaves that were infected by a fungal pathogen and natural populations of the birch aphid, *Euceraphis betulae*. Neither of these insects are vectors of the fungal pathogens and similar effects on non-vector herbivores have been found in response to plants infected with viral pathogens. For example, a recent study by Belliure *et al.* (2010) demonstrated the beneficial effect of Tomato spotted wilt virus (TSWV) infection on the two-spotted spider mite, *Tetranychus urticae* through promoted juvenile survival on TSWV-infected pepper plants and there is an extensive literature arising which investigates the effects of plant virus infection on other insect herbivores. However, here the story becomes increasingly complex as many insects actually act as vectors for plant viral pathogens and are therefore responsible for transmitting them to new host plants. In cases such as these, there may be further selection pressures acting on the pathogen to manipulate the behaviour of a vector which is ultimately responsible for its continued survival in the environment.

1.2.3 Effects of plant pathogens on insect vectors

Insects typically locate host plants using optical cues, such as foliage colouration (Prokopy & Owens, 1983), chemical cues such as detection of plant volatiles (Bruce *et al.*, 2005) and gustatory cues detected upon landing on the leaf surface (Powell *et al.*, 2006). A growing body of evidence suggests that plant attack by pathogenic micro-organisms alters their attractiveness as potential host plants for insects that vector the particular pathogen. Specifically, pathogenic microorganisms tend to make plants more alluring for the insect, presumably to increase the likelihood of transmission to a new host. For example, in choice tests involving the Mexican bean beetle vector of Southern bean mosaic virus

(SBMV) and Bean pod mottle virus (BPMV), *Epilachna varivestis*, adult beetles were preferentially attracted to leaf discs of bean plants that had been mechanically inoculated with the viruses over those from healthy plants (Musser *et al.*, 2003). The mechanisms responsible are often attributed to changes in foliage colouration (i.e. pathogen induced leaf senescence; Fereres *et al.*, 1999) which make the leaf more readily detected by the insect at distance. Increasingly experiments are demonstrating that the pathogen can also induce changes to plant volatile emissions that alter the attractiveness of the plant as a host for the insect. The study of McLeod *et al.,* (2005) demonstrated beyond doubt the role of elm tree semiochemicals in attracting the elm bark beetle vector, *Hylurgopines rufipes*, to trees infested with the fungal pathogen, *Ophiostoma novo-ulmi*, through first sampling and identifying sesquiterpenes from the tree bark and subsequently testing them in olfactometer bioassays in the laboratory and in more natural conditions in a plantation of American elm. The advantage of such a system to a pathogen that is dependent on a vector for dispersal is clear. What remains less clear is the benefit, if any, to the insect vector.

The performance of the insect vectors of plant pathogens can be affected by pathogen-induced changes to host plant metabolism and some studies have attempted to link insect performance on plants challenged by pathogens by quantifying alterations to nitrogen, sugars, proteins and amino acids (Blua *et al.*, 1994; Tinney *et al.*, 1998), all of which are important components of the insect diet. Intuitively, natural selection should favour a system by which the insect gains from feeding on pathogen-infected plant tissue and indeed several studies demonstrate this (Castle & Berger, 1993; Maris *et al.*, 2004; Belliure *et al.*, 2005). There is also evidence that pathogens can promote vector survival

by negatively impacting populations of natural enemies (Belliure *et al.*, 2008). In contrast however, there are also examples where plant pathogens exert negative effects on their vector. For example, Khan & Saxena (1985) demonstrated the negative effect of Tungro virus of rice through prolonged development of the leafhopper vector, *Nephotettix virescens* when feeding on tungro-infected rice plants. Tinney *et al*, (1998) showed that larvae of the moth, *Tyria jacobaeae*, were smaller and had longer relative growth rates when feeding on coltsfoot plants infested with the rust, *Coleosporum tussilaginis*, compared with larvae reared on healthy plants and the authors demonstrated that plants infected with *C. tussilaginis* had a lower leaf nitrogen content than healthy plants. Prolonged development time of insects often leaves them more susceptible to attack by natural enemies and/or predators (Clancy & Price, 1987) and therefore, in these cases, it is not in the best interests of the vector to feed on infected plant tissue.

1.3 Interactions between aphids and plant viruses

Aphids are specialised to feed on phloem sap and this makes them highly effective virus vectors as their specialised mouthparts facilitate feeding with a minimum amount of damage to the host plant while their characteristically high reproductive output and occasionally polyphagous nature ensures efficient virus transmission (Ng & Perry, 2004). This has provided the impetus for studies of indirect interactions between aphids and plant viruses as any alterations to the suitability of a plant as a host in response to virus infection may have a significant effect on aphid population dynamics and thus the spread of viral-disease (Ferriss & Berger, 1993; McElhany *et al.*, 1995; Jeger *et al.*, 2004).

1.3.1 Aphids as virus vectors

A direct interaction inevitably occurs between aphids and plant viral pathogens during virus transmission although there is currently no evidence to suggest that these interactions affect aphid behaviour (Medina-Ortega *et al.*, 2009). It is during this process that virus particles are transferred to the vector and also transmitted to new hosts. Plant viruses transmission by aphids occurs in three distinct steps: acquisition, retention and inoculation (Powell, 1991) and viruses can be generally classified in the following ways:

1.3.1.1 Nonpersistent and semiperistent viruses

Until recently, it was assumed that nonpersistent viruses were purely stylet-borne and semipersistent viruses were fore-gut borne. However, this view was revised after the work of Uzest *et al.* (2007) which demonstrated that the semipersistently transmitted Cauliflower mosaic virus (CaMV) is retained in the common food/salivary duct of the principal hemipteran vector, *Brevicoryne brassicae,* and not in the fore-gut as was previously assumed. The virus particles (virions) of nonpersistently transmitted viruses are likely to be subsequently released during intracellular salivation (Powell, 2005). Of all the true bugs which act as virus vectors, only aphids are known to transmit plant viruses in a nonpersistent manner (Ng & Falk, 2006). This is probably due to the intricate structure of aphid mouthparts which allows efficient uptake of virions during short exploratory probes of the plant tissue using the stylets. Acquisition of these viruses occurs only after cell membranes are punctured by the stylets (Powell, 1991) and the time taken to for the aphid to acquire the virus is very short (usually within minutes) as the stylets need only to pierce epidermal cells (Figure 1.1a). The virions can then be easily transmitted to a new host plant when the aphid migrates to a new feeding site. At this stage, it is believed that

transmission is facilitated by either of two mechanisms, the 'helper strategy' where virus-encoded proteins interact with the virion to aid binding to the mouthparts (Ng & Falk, 2006; Figure 1.1a), or 'the capsid strategy', where specific components of the viruses' protein coat (or capsid protein) aid adsorption and successful retention of the virions to the retention sites in the aphid mouthparts (Ng & Falk, 2006; Figure 1.1b). The time for which the virus is retained by the aphid is also very short and the virions are rapidly released when it begins to probe healthy plant tissue, usually within minutes. This type of transmission is termed 'nonpersistent' and most plant viruses are transmitted by this method. Semipersistent viruses differ slightly in that the virions are transmitted to and from the phloem. Virions may reach as far as the foregut, but as described above, may also be retained in the food/salivary canal of the stylets. The factors which may govern the subsequent release of virions from the vector to a new host plant (inoculation), which normally occurs within minutes of feeding on a new host, remain unexplored for nonpersistent and semipersistent viruses but are likely to involve interactions between certain virus-encoded proteins, vector proteins and other molecules as has been shown for certain persistently transmitted plant viruses (Ng & Falk, 2006) (see *1.3.1.2*)

1.3.1.2 Persistent viruses

A virus is termed 'persistent' when it actually enters the circulatory system of the aphid and virions are found in the aphid haemolymph and, in contrast to nonpersistent and semipersistent viruses, those that are persistently transmitted require a much longer acquisition period – often hours to days. As a consequence, the aphid can remain viruliferous (capable of transmitting the virus) for many days. Depending on whether the virus replicates within the aphid, persistent viruses can be further classed into circulative,

nonpropagative (viruses that do not replicate in the vector) or circulative, propagative (viruses which have a replicative phase in the vector). Circulative nonpropagative transmitted viruses remain in the aphid for periods of weeks and also through moulting. The virus is ingested from infected phloem tissue and passes from to the gut epithelium by endocytosis and exits into the hemocoel by exocytosis (Gray & Gildow, 2003).. After circulating in the hemocoel the virions are actively taken up by the accessory salivary gland cells where they can then be passed to plant tissue when the insect is feeding. The work of Gildow & Gray (1993) and later work reviewed by Gray & Gildow (2003) showed that the ability of Luteoviruses to cross barriers in the accessory salivary glands is virus-specific and in order to be successfully transmitted, virions must be capable of crossing both the basal lamina and basal plasmalemma of this gland. Movement through the aphid salivary glands is therefore highly specific and successful transmission of the same virus can vary not only between aphid species but also between virus isolates (Gray & Gildow, 2003). Aphid transmitted circulative, propagative plant viruses, which have only so far been found to occur in one family of viruses, the Rhabdoviridae (Hogenhout *et al.*, 2008) pass through the aphid gut and circulate in the haemolymph. Rhabdoviruses have to overcome barriers in the gut epithelium such as gut cell receptors which recognise certain viral proteins and, like Luteoviruses, must also be able to penetrate the accessory salivary gland in order for successful transmission to occur (Hogenhout *et al.*, 2003). Once they have reached the salivary glands, virions bud off from cellular membranes and can be transported to plant cells during salivation. Unlike circulative, nonpropagative viruses which only remain in the aphid for weeks after uptake, successful acquisition of a Rhabdovirus renders the aphid viruliferous for the rest of its life.

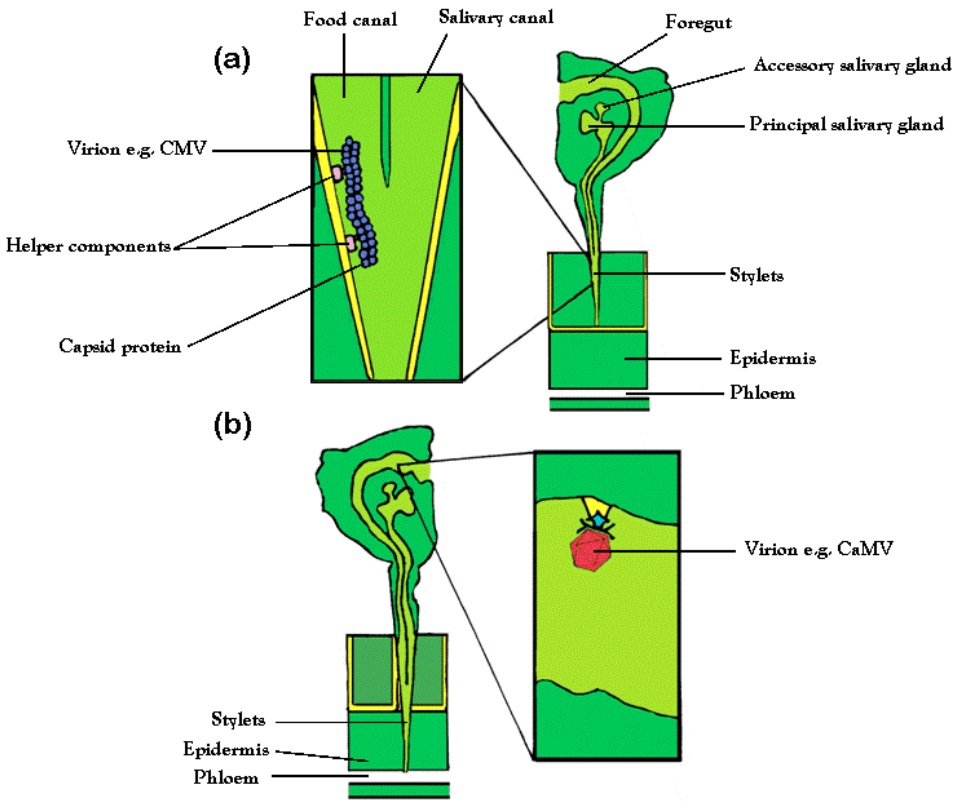


Figure 1.1. Schematic diagram of potential virus binding sites for nonpersistent viruses e.g. Cucumber mosaic virus (CMV) and semipersistent viruses e.g. Cauliflower mosaic virus (CaMV). (a) Helper strategy, showing helper component interacting with the capsid protein of the virion. (b) Capsid strategy showing the virion interacting with the retention site. Diagram based on that of Ng & Falk (2006).

1.3.2 Virus transmission and aphid preference-performance

Transmission of most plant viruses are thought to be the result of co-evolutionary processes between the virus and the vector (Ng & Perry, 2004) and evidence suggests aphids are often attracted to host plants already infected with the virus. For example, Eigenbrode *et al.*, (2002) demonstrated that the principle vector of Potato leaf roll virus, *Myzus persicae*, was preferentially attracted to potato (*Solanum tuberosum*) plants infected with the virus rather than to healthy plants. *M. persicae* is a generalist and will feed on a wide range of plants (Blackman & Eastop, 2000) yet studies of the same virus and vector conducted by Srininvasan *et al.* (2006) reported a similar result with less emigration of aphids from PLRV-infected hairy nightshade (*Solanum sarrachoides*). Eigenbrode *et al.* (2002) was the first study to report changes in aphid host preference in response to volatile cues from virus-infected plants and this work led to a number of subsequent studies using this particular study system which attempted to categorise the behaviour of *M. persicae* more specifically. For example, Alvarez *et al.* (2007) showed that the aphid's ability to differentiate between volatiles emitted from healthy and PLRV-infected potato plants was dependent on the age of the plant and Werner *et al.* (2009) demonstrated differences in aphid behavioural responses to PLRV-infection at different time intervals following host plant inoculation with the virus and found that fewer aphids migrated from infected plant leaflets at 4 and 6 weeks after virus-inoculation compared with 2, 8 and 10 weeks. The seminal study of Eigenbrode *et al.* (2002) has also led to investigations of the role of plant volatile attractants in other plant systems, and Medina-Ortega *et al.* (2009) demonstrated that another generalist aphid, *Rhopalosiphum padi*, was attracted to synthetic volatile blends made to mimic those produced by wheat plants that were infected with Barley yellow dwarf virus (BYDV).

The consequences for aphids of host plant virus infection can be variable, and while many studies report that virus-infected plants make superior hosts for aphids (Castle & Berger, 1993; Blua *et al.*, 1994; Jimenez-Martinez *et al.*, 2004; Srininvasan *et al.*, 2006) some report neutral (Hodge & Powell, 2008) or negative effects (Fiebig *et al.*, 2004; Donaldson & Gratton, 2007). However, to date, few studies have linked the consequences of aphid host plant choice in response to plant viruses to their reproductive success on the plant, nor have they related any changes in preference or performance to changes in host plant quality, so the causal mechanism of the observed effects remains unclear. For example, although the studies of Eigenbrode *et al.* (2002), Srinivason *et al.* (2006), Alvarez *et al.* (2007) and Werner *et al.* (2009) clearly demonstrated that *Myzus persicae* will preferentially settle and feed on host plants infected with PLRV, they focussed exclusively on aphid behaviour. No attempt was made to link aphid arrestment with the causal nutritional mechanism through investigations of the underlying leaf chemistry - the ultimate driver of the indirect interaction. A few studies have attempted to make these causal links but gaps remain. For example, the studies of Fiebig *et al.* (2003) demonstrated a reduction in the intrinsic rate of population increase of the cereal aphid, *Sitobion avanae*, on wheat plants infected with Barley yellow dwarf virus (BYDV) and linked this decrease in performance to a reduction in free amino acids and sugars in the phloem sap and the study of Blua *et al.* (1994) measured a higher rate of population increase as the concentration of free amino acids increased in squash plants infected with ZYMC, but both studies failed to assess the preference of the aphid or the potential role of volatile compounds in the interaction.

Given the differences that have been observed in insect performance on host plants infected with viral pathogens, methods could be employed to link vector behaviour and performance not only with the causal mechanism e.g. plant nutritional compounds important for aphid growth and development, but also with the mode of virus transmission. As viral pathogens can be categorized into those that are non-persistent, semipersistent and persistent (for examples see Table 1.1), their successful transmission depends critically on not only whether a competent vector remains on the plant long enough to acquire the virus, but also if the vector then feeds or probes for the necessary time period required for the virus to be transferred. For example, nonpersistent viruses require only brief probes for the virions to be transferred to the vector mouthparts and can be transmitted within minutes of access to a non-infected plant whereas persistent viruses require much longer acquisition and inoculation periods (Ng & Perry, 2004). Does it therefore follow that the virus can manipulate plant chemistry in such a manner that will promote optimal uptake and transmission by the vector?

A recent study by Mauck *et al.* (2010) demonstrated that although the cotton aphid, *Aphis gossypii,* was preferentially attracted to *Cucurbita pepo* plants infected with Cucumber mosaic virus (CMV), plants that were experimentally infected with the virus supported lower aphid populations than did uninfected plants. The attraction of the aphid and the lower number of aphids found on virus-infected plants therefore indicates a higher dispersal of *A. gossypii* from CMV-infected plants which may be indicative of a nutritionally poor host. As CMV is transmitted nonpersistently, the rapid dispersal of aphids from infected plants presumably results in virus spread. Conversely, a number of studies have suggested that a prolonged period of feeding by aphids in response to plant

infection with viruses that require a longer time period to be successfully acquired is facilitated by an attraction of the vector to the host plant (Alvarez *et al.*, 2007) and virus-induced improvements in host plant quality which encourage colonisation. In cases such as these, enhanced quality of the plant promotes rapid population growth and, with crowding, the production of alates (winged aphids) which disperse and transmit the virus to new plants (Gildow, 1980, 1983). For example, studies of Potato leafroll virus (PLRV), a persistently transmitted virus, have shown that the principal PLRV-vector *Myzus persicae* is preferentially attracted to virus-infected plants (Eigenbrode *et al.*, 2002) and that the insect actually performs better on these plants (Castle & Berger, 1993). This enhanced performance makes it more likely that the aphid will remain on the plant for prolonged feeding, a behaviour which is likely to increase the likelihood of successful acquisition, after which the aphid will remain viruliferous for an extended period (Ng & Perry, 2004).

These examples of virus-induced changes to host plant quality seem to support the hypothesis that aphid performance on virus-infected plants can be predicted from the mode in which the particular virus is transmitted by the aphid. Although comparative studies of aphid behaviour and performance on host plants infected with viruses with different modes of transmission are few, Castle & Berger (1993) showed that *M. persicae* was consistently more attracted to Potato virus X (PVX-), Potato virus Y (PVY-) and PLRV-infected potato plants over non-infected ones. Furthermore, this study showed that PVX-infected plants were the least attractive to the aphid vector and PLRV-infected plants attracted the most aphids. These findings are of significance as PVX is not transmitted by aphids, while PLRV is persistently transmitted by *M. persicae* and must replicate in the aphid. However, a similar study by Hodge and Powell (2008), which investigated the

effects of several viruses of legumes, which are transmitted nonpersistently and persistently by the aphid, *Acyrthosiphon pisum*, found that although there were differences in aphid performance, no relationship between virus transmission strategy and the induction of winged morphs could be found. For example, the persistently transmitted virus, Pea enation mosaic virus (PEMV) was found to exert little effect on aphid survival, growth and reproductive output despite the requirement for prolonged aphid feeding for the virus to be successfully acquired. A more recent study by the same authors which investigated the interaction between *A. pisum* and PEMV in further detail, found the same preference for PEMV-infected plants by *A. pisum* but noted that the performance of the aphid, measured by mean daily growth rate (MDGR), was only enhanced on older PEMV-infected plants with well developed virus symptoms (Hodge & Powell, 2010. The varying findings of these experiments, utilizing different plant systems, highlight the intimate associations between viral pathogens and their aphid vectors and suggest that these interactions are likely to be highly host specific.

The two viruses which are investigated in this thesis, Black raspberry necrosis virus and Raspberry leaf mottle virus, fall between the two categories of viruses studied in the above example. As semipersistent viruses, aphid ingestion of phloem is required for virions to be successfully acquired by the aphid. If a link between aphid performance and virus transmission strategy is to hold true, then their aphid vector, *Amphorophoro idaei,* should not only be preferentially attracted to infected raspberry plants, but should feed on the plant for periods of in excess of 30 min to successfully acquire the virus (Stace-Smith, 1955a) before migration to new, potentially uninfected host plants.

Mode of transmission	Acquisition period	Retention time	Family	Genus	Example
Nonpersistent	Seconds	Minutes Lost after molting	*Bromoviridae* *Bromoviridae* *Comoviridae* *Potyviridae* *Potyviridae*	*Alfamovirus* *Cucumovirus* *Fababirus* *Macluravirus* *Potyvirus*	Alfalfa mosaic virus Cucumber mosaic virus Broad bean wilt virus Maclura mosaic virus Potato virus Y
Semipersistent	Minutes	Hours Lost after molting	*Caulimoviridae* *Closteroviridae* *Sequiviridae* *Seqiviridae* Unassigned	*Caulimovirus* *Closterovirus* *Sequivirus* *Waikavirus* *Sadwavirus*†	Cauliflower mosaic virus **Raspberry leaf mottle virus** Parsnip yellow fleck virus Arthriscus yellows virus **Black raspberry necrosis virus**
Circulative, Nonpropagative	Hours, days	Days, weeks	*Luteoviridae* *Luteoviridae* *Luteoviridae*	*Luteovirus* *Polerovirus* *Umbravirus*	Barley yellow dwarf virus Potato leaf roll virus Carrot mottle virus
Circulative, Propagative	Hours, days	Lifespan of insect	*Rhabdoviridae* *Rhabdoviridae*	*Cytorhabdovirus* *Nucleorhabdovirus*	Lettuce necrotic yellows virus Sonchus yellow net virus

Table 1.1. Examples of aphid transmitted plant viruses. † Virus not yet assigned to genus or family by ICTV although phylogenetic analysis suggests placement in the genus *Sadwavirus* (Halgren *et al.*, 2007; see Chapter Two). Data after Ng & Perry (2004) and Hogenhout *et al.*, (2008).

1.3.3 Summary

Plant viruses can alter the behaviour of their aphid vectors through changes in host-plant attractiveness. Studies of aphid preference for healthy or diseased host plants have revealed the potential role of plant volatile compounds as attractants to virus-infected plants, yet the consequences of the host plant choice for aphid performance are variable and negative, neutral and positive effects of virus-infected host plants have been recorded. Relatively few studies have addressed the underlying plant nutritional chemistry that may be responsible for the differences in aphid performance but, plant nitrogen, particularly amino acids seems likely to play an important role.

1.4 Aims

The aim of this study was to characterise the behaviour and performance of the European large raspberry aphid, *Amphorophora idaei*, in response to host plant infection with two common viral pathogens of red raspberry in the United Kingdom, Black raspberry necrosis virus (BRNV) and Raspberry leaf mottle virus (RLMV). Specifically, this study aimed to:

1. establish whether *A. idaei* show a preference for raspberry plants infected with BRNV and RLMV and how virus infection affects aphid movement
2. investigate the effect of virus-infection of the performance of *A. idaei*

3. identify and where possible, quantify, changes to particular aspects of nutritional quality of the host plant which may be responsible for any differences observed in (1) and (2).
4. investigate the potential role of plant volatile compounds in aphid attraction to virus-infected raspberry plants.

1.5 Thesis structure

The following chapter (CHAPTER TWO), "The biology of the European large raspberry aphid (*Amphorophora idaei*), its role in virus transmission and resistance breakdown in red raspberry" provides a comprehensive review of the raspberry study system used for the experiments detailed later in this thesis. Raspberry is a small, but high value crop in the U.K. and this study aimed to synthesise existing knowledge of *A. idaei* as studies were previously fragmented across several disciplines. The review focuses on the role of *A, idaei* as a vector of raspberry pathogens, its interactions with other insects and the problems arising from breakdown of plant resistance to the aphid.

CHAPTER THREE, "The effect of Black raspberry necrosis virus and Raspberry leaf mottle virus on the recruitment and performance of, *Amphorophora idaei*", describes the results of several aphid bioassays which were conducted in order to ascertain if *A. idaei* exhibited a preference for virus-infected host plants as has been demonstrated by studies of other aphid species. Furthermore, this chapter explores aphid performance on healthy and virus-infected raspberry plants and movement subsequent to the initial host plant choice; with the hypothesis being that promoted aphid performance in response to virus infection would lead to prolonged aphid feeding on the plant, while reduced

performance may trigger aphid migration. The role of visual and olfactory cues in aphid host location is also explored.

CHAPTER FOUR, "Aphids and viral pathogens induce chances in *Rubus idaeus* leaf chemistry", describes the results of leaf chemical analyses conducted to explore the causal mechanisms for the depressed performance of *A. idaei* on virus-infected plants which was demonstrated by the experiments of Chapter Three. Specifically, this chapter aimed to test the hypothesis that increased leaf phenolics and decreased nitrogen and amino acids may account for the prolonged development time of *A. idaei* on virus-infected host plants.

CHAPTER FIVE, "Raspberry volatiles attract *Amphorophora idaei* to virus-infected raspberry plants", aimed to investigate alterations to plant volatile emissions in response to infection with raspberry viruses. In particular, this study aimed to characterise gross changes in volatile composition and test candidate attractants in bioassay with *A. idaei.*

CHAPTER SIX, "Discussion", reviews the key findings of the research and suggests future directions for the study of indirect interactions between pathogens and their vectors.

Chapter Two

The biology of the European large raspberry aphid (*Amphorophora idaei*): its role in virus transmission and resistance breakdown in red raspberry[†]

[†] *Agricultural and Forest Entomology* (2009) DOI: 10.1111/j.1461-9563.2008.00409.x

Abstract

The European large raspberry aphid, *Amphorophora idaei*, is the most important vector of viral diseases afflicting commercially grown red raspberry (*Rubus idaeus* L.) in the United Kingdom and wider Europe, with European raspberry production amounting to 416,000 tonnes per annum. *Amphorophora idaei* is the principle vector of three known viruses: Black raspberry necrosis virus, Raspberry leaf mottle virus and Rubus yellow net virus, with *A. idaei* taking as little as two minutes to transmit some viruses. Existing control strategies, including resistant cultivars, insecticides and eradication of disease from parent plants and associated problems are described in this chapter. For example, strong selection pressures have resulted in *A. idaei* overcoming genetic resistance in many raspberry cultivars and most insecticides are now ineffective at controlling the *A. idaei* populations and consequently the spread of viral diseases.

Information about trophic interactions with other insect herbivores and natural enemies is scarce and existing knowledge is also reviewed in this chapter. Another major pest of raspberry, the vine weevil (*Otiorhynchus sulcatus*) has been found to compromise aphid resistance in some raspberry cultivars, increasing *A. idaei* abundance by 80%. Parasitoids show mixed success in parasitizing *A. idaei*, although *Aphidius ervi* attack rates more than doubled when *A. idaei* fed on a partially susceptible raspberry cultivar, compared to a resistant variety.

Future directions for the sustained control of *A. idaei* are suggested, taking into consideration the possible effects of climate change and also changes in agronomic practices in UK agriculture.

2.1 Introduction

The European large raspberry aphid, *Amphorophora idaei*, is the most economically important aphid pest of commercially grown red raspberry (*Rubus idaeus*) cultivars in the UK and Northern Europe. While large populations of *A. idaei* can reduce plant vigour (Gordon *et al.*, 1997), it is a more serious pest of raspberry because of its role as a highly mobile and effective vector of at least three viral pathogens, all of which can cause severe damage and loss in fruit crop (Alford, 2007). The three viruses known to be transmitted by *A. idaei* all belong to the Raspberry mosaic disease (RMD) complex (Converse, 1987) and they are Black raspberry necrosis virus (BRNV), Raspberry leaf mottle virus (RLMV) and Rubus yellow net virus (RYNV). BRNV is often the first to infect raspberry (Jones, 1976) with the plant then rapidly becoming infected with other viruses such as RLMV.

Raspberry (*Rubus* spp.) is a high value and economically important crop in many parts of the UK, Europe and North America, with global production estimated at 500,000 tonnes per year (FAOSTAT, 2008). There is increasing emphasis on the importance of fruit and vegetables for human health, particularly those products, such as *Rubus*, which are rich sources of dietary antioxidants (Deighton et al., 2000; McDougall et al., 2005). Thus demand for *Rubus* is increasing, with European production increasing by 42% between 1994 and 2004 alone (FAOSTAT, 2008). One of the main threats to increasing yields are viral diseases transmitted by *A. idaei*, so it is not surprising that there have been extensive efforts to control *A. idaei* over the last 40-50 years, largely by breeding aphid resistant raspberry varieties (Sargent et al., 2007). However, the strong selection pressure exerted on the aphid by resistant varieties, together with the short generation time of *A. idaei*, has

led to the evolution of aphid biotypes capable of overcoming plant resistance (Birch et al., 2002). Aphid biotypes (also referred to as strains) are groups of multiple genotypes that share a phenotypic trait (in the case of *A. idaei*, the ability to colonise specific plant genotypes (Puterka & Burton, 1991). In particular, the spread of resistance-breaking genes between populations of *A. idaei* is believed to occur through migrations of parthenogenetic asexual females in the summer months as a result of overcrowding on host plants and subsequent migrations of sexually reproducing males and females in the autumn months which ensure extensive exchanges of genetic material during the *A. ideai* life cycle (Birch et al., 2002). In addition to concerns about resistance-breaking *A. idaei* biotypes, the few remaining certified insecticides are now largely ineffective at eradicating aphids in time to prevent viral transmission (which takes as little as two min - see Section 2.3). Insecticides have also proved problematic, as peak aphid populations occur at the optimum time for fruit harvesting (Gordon et al., 1997), when insecticide residues may pose a threat to human health due to toxicity.

The increasing problem of *A. idaei* in commercial raspberry production therefore requires novel approaches to control aphid populations and virus transmission, which could include combinations of genetically resistant plant varieties and novel strategies such as biocontrol (Ode, 2006) and semiochemical technologies (Agelopoulos et al., 1999). Developing such approaches requires a more detailed understanding of the biology of *A. idaei* and how it interacts with other organisms, yet this information remains fragmented across several disciplines. The literature reviewed in this chapter synthesises existing knowledge of *A. idaei* across these disciplines and describes the aphid interactions with other organisms, including its host plant and the viral pathogens it transmits.

2.2 General biology

2.2.1 Taxonomy and morphology

Classification of the large raspberry aphid is somewhat confused in early literature as it was originally assumed that all *Amphorophora* occurring on *Rubus* belonged to a single species, *Amphorophora rubi (*Kaltenbach). In 1939, after observations of both morphology and host-plant transfers, Börner concluded that there were in fact two distinct species of these aphids which he assigned to the genus *Nectarosiphon*, one occurring solely on red raspberry that was termed *Nectarosiphon idaei* (now *Amphorophora idaei*), and another on blackberry, *Nectarosiphon rubi* (now *Amphorophora rubi*) (Börner, 1939). This revision of aphid taxonomy was subsequently supported by cytological studies which confirmed that the chromosome complement of *Amphorophora* occurring on red raspberry in Europe differed from that of *Amphorophora* sampled from blackberry (2n = 18 and 20 respectively) (Blackman et al., 1977). Hill (1956) suggested that the North American and European vectors were also discrete species, as the raspberry cultivar Lloyd George was reported to be resistant to *A. idaei* in North America, even though it was known to be susceptible in the UK. Despite being almost identical in appearance, the taxonomy of these species differs, with the North American aphid vector named *Amphorophora agathonica* and the European vector named *Amphorophora idaei* (Alford, 2007).

The body length of apterous (wingless) *A. idaei* adults ranges from 2.5 - 4.1 mm (Blackman & Eastop, 2000). Adults are usually pale green to yellowish green in colour with long antennae and siphunculi (Figure 2.1a). Plate 2.1a shows a single apterous female and nymphs on the stem. The alatae differ slightly in that they possess a brown

head and thoracic region but are generally of a similar size, ranging from 2.5 - 4.0 mm in length.

2.2.2 Life cycle

Amphorophora idaei undergoes an obligate holocyclic lifecycle (incorporating asexual and sexual phases) on red raspberry. Parthenogenetic (asexual) reproduction takes place throughout the spring and summer months until the emergence of males later in the year when the aphids then begin to reproduce sexually. The seasonal life cycle for a typical population in the UK is illustrated in Figure 2.1b, although it should be noted that the timing of events can be variable between seasons. In the UK, the fertilized eggs, deposited by the oviparae (the egg laying female), hatch in early March. The eggs are yellow-green when first laid and change to shining black prior to hatching (Dicker, 1940). The emergent nymphs feed at the leaf tip before moving to the underside of the leaf. These fundatrices (colony founders) are distinguishable from later generations by the presence of two rows of dark spots and bristles on the dorsal surface. These spots fade in the third and fourth instars (Dicker, 1940). Elsewhere in Europe, female *A. idaei* hatch from the eggs between late March and early April in Poland (Borowiak-Sobkowiak, 2006) and somewhat later (late April to early May) in Finland (Rautapää, 1967). Newly hatched nymphs do not tend to occur in large numbers, thus total population sizes are small at this time of year. The apterous nymphs and adults are very mobile and drop from the plant when they are disturbed (Converse, 1987). After several generations of parthenogenetic apterae, female alatae begin to appear, usually between the months of June and July (Alford, 2007). These alatae begin to migrate to new canes on the host plant or colonise new plants.

After migration, the female alatae again produce parthenogenetic, wingless daughters and oviparae begin to appear in October alongside sexual male alates. After mating, fertilised eggs are deposited near the base of the canes of the host plant from October right through until December where they remain until the following March. In the UK, peak populations of the large raspberry aphid occur in mid summer on most red raspberry genotypes (Jones, 1976). The number of generations produced over the summer period is dependent on prevailing environmental conditions. Borowiak-Sobkowiak (2006) recorded eight to ten generations in Poland, whereas Dicker (1940) recorded ten in Finland.

2.2.3 Seasonal occurrence of *A. idaei* on different raspberry cultivars in Scotland

The population dynamics of *A. idaei* can be highly variable on different cultivars of raspberry, depending on levels of resistance in the plant and the aphid biotypes in the area (see Section 2.4.3). In Scotland, surveys of aphid abundance on insecticide-free experimental plantations at SCRI (56°447'N, 3°012'W) showed that peak populations were observed between June and August (Mitchell, 2007). These surveys were carried out at weekly intervals during 2004 on plantations comprising two susceptible (Malling Jewel and Glen Ample) and one partially resistant cultivar (Glen Clova). Four independent plots of each cultivar were chosen, using four plants at random for each plot (16 in total for each cultivar). On each plant, 12 leaves were sampled at random from the top, middle and bottom stems (using both primocane and floricanes). See Mitchell (2007) for full details. Populations of *A. idaei* generally peaked in late July, but it was apparent that numbers were higher on susceptible varieties compared to the partially resistant variety (Figure2.2).

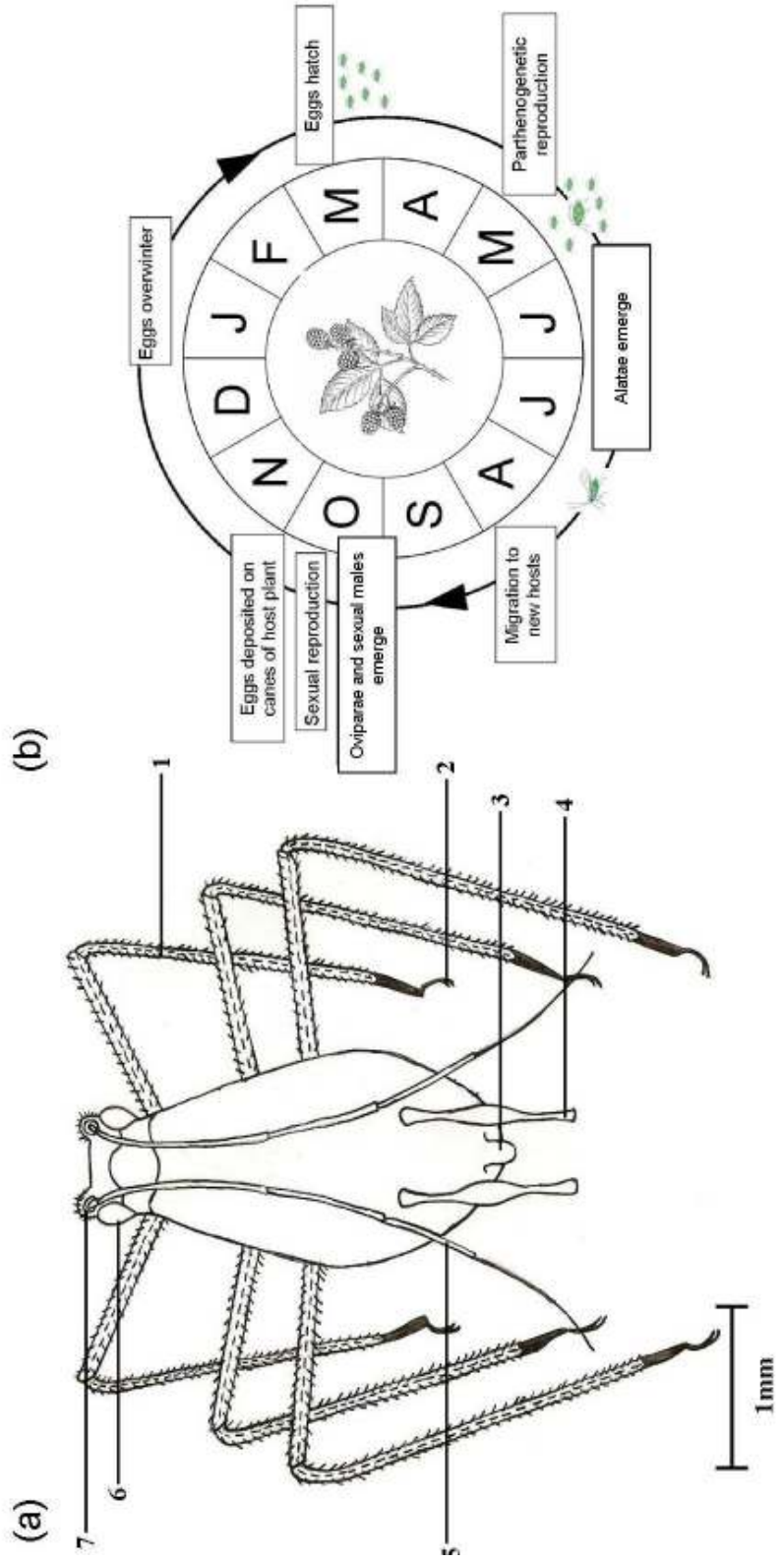


Figure 2.1 (a) General morphology of apterous female *A. idaei*, dorsal view showing: (1) tibia, fore tibia darkened; (2) tarsus, darkened; (3) cauda, longer than basal width; (4) siphunculus, swollen at distal end; (5) antenna, long with dark pigmentation; (6) compound eye (7) antennal tubercle, well developed;. (b) Seasonal life cycle of *A. idaei* as might typically occur on commercial raspberries in the UK

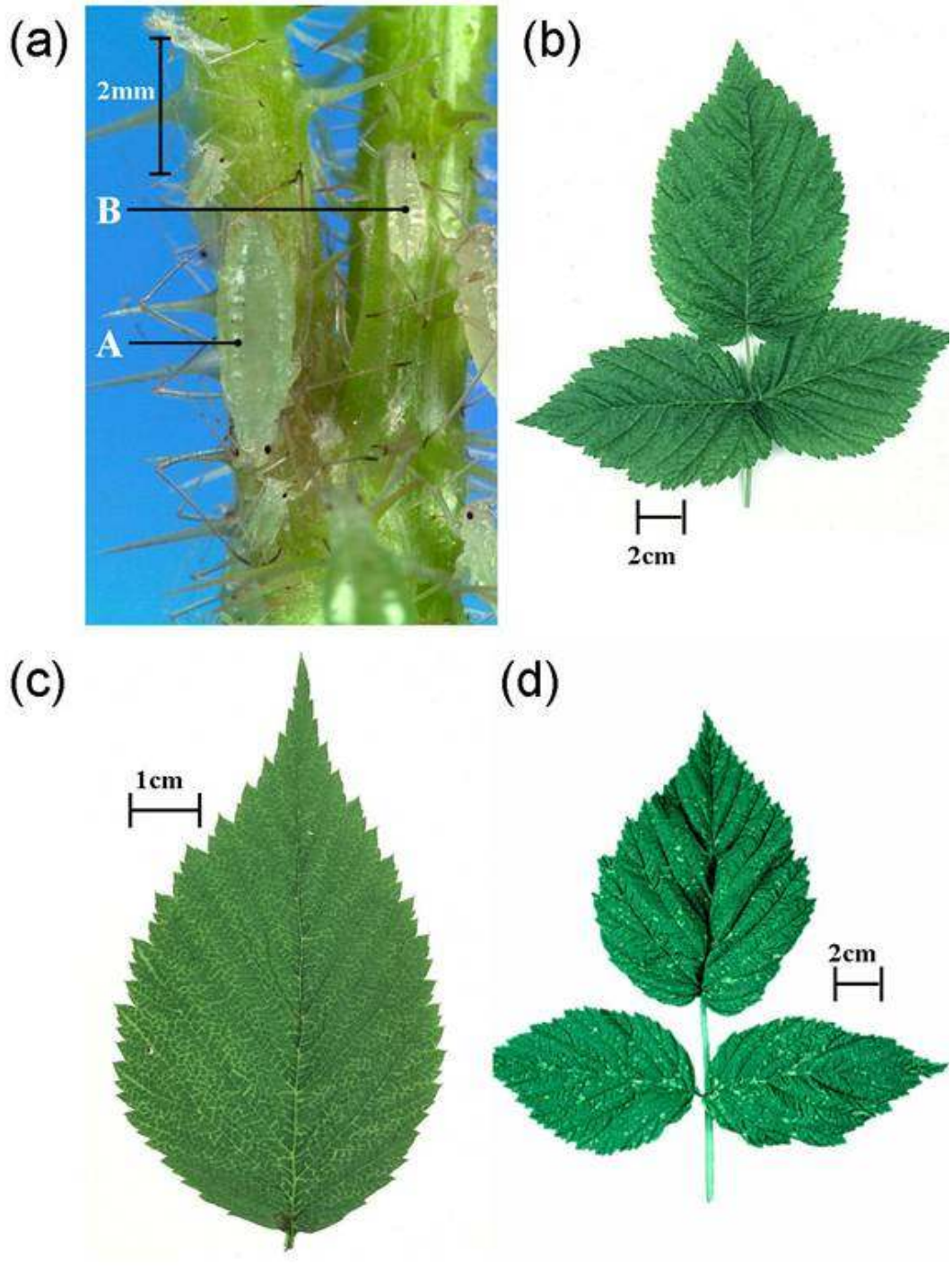


Plate 2.1. (a) Adult female *A. idaei* (labelled A) and nymphs (example labelled B) on stem of red raspberry. (b - d) Symptoms of viral pathogens known to be transmitted by *A. idaei* (b) Black raspberry necrosis virus (BRNV), (c) Rubus yellow net virus (RYNV) and (d) Raspberry leaf mottle virus (RLMV).

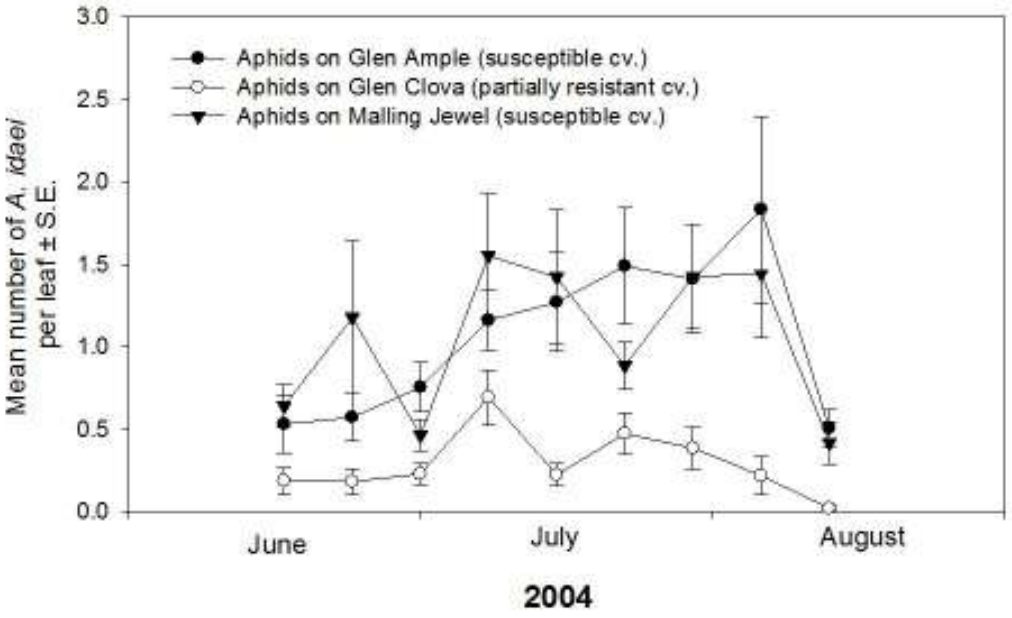


Figure 2.2. Seasonal occurrence of *A. idaei* on *Rubus idaeus* cultivars with varying degrees of aphid resistance: Malling Jewel (no resistance); Glen Clova (multigenic minor gene resistance) and Glen Ample (major gene A_1). Figure from Mitchell (2007).

2.2.4 Trophic interactions

Interactions with other organisms in the system have largely focussed on trophic interactions with other insects (reviewed below), although there is evidence that *A. idaei* becomes infected with fungal diseases. Dickson (1979) reported the occurrence of three fungal pathogens in *A. idaei* collected from the field. Of the three fungal pathogens identified, *Entomophthora aphidis*, *E. planchoniana* and *E. thaxteriana*, the latter was the most lethal for *A. idaei*, with mortality rates of 62% in laboratory experiments (Dickson, 1979).

2.2.5 Interactions with other herbivores

Other than the large raspberry aphid, notable pests of red raspberry in the UK include the small raspberry aphid (*Aphis idaei*), the raspberry beetle (*Byturus tomentosus*), the clay coloured weevil (*Otiorhynchus singularis*), the vine weevil (*Otiorhynchus sulcatus*), the raspberry cane midge (*Resseliella theobaldi*), the raspberry moth (*Lampronia rubiella*) and the two-spotted spider mite (*Tetranychus urticae*). A comprehensive review of the arthropod pests occurring on *Rubus* spp. is provided by Gordon et al., (1997). In spite of the potential diversity of arthropod species occurring on raspberry, studies of the interactions between the large raspberry aphid and other insect herbivores are remarkably scarce.

Despite this dearth of studies, the scope for interactions with other herbivores seems increasingly likely as several insect pests become more prevalent because of the removal of organochlorine pesticides due to environmental concerns (Gordon et al., 1997). For example, the removal of aldrin has led to sharp increases in populations of vine weevil (*O.*

sulcatus) in commercial raspberry production (Moorhouse et al., 1992). Root-feeding *O. sulcatus* larvae are the most damaging life stage, and it has recently been shown that they can impact on the population of *A. idaei* feeding aboveground (Johnson et al., 2008). In particular, feeding by two vine weevil larvae increased populations of *A. idaei* by 80% on raspberry plants with partial aphid resistance (cv. Glen Clova) (Figure 2.3a), whereas cultivars with more robust levels of aphid resistance (cv. Glen Rosa) showed no significant difference in aphid abundance (6-20 aphids per plant). The mechanism underpinning this interaction may be linked with nutritional changes in the phloem (Johnson et al., 2008).

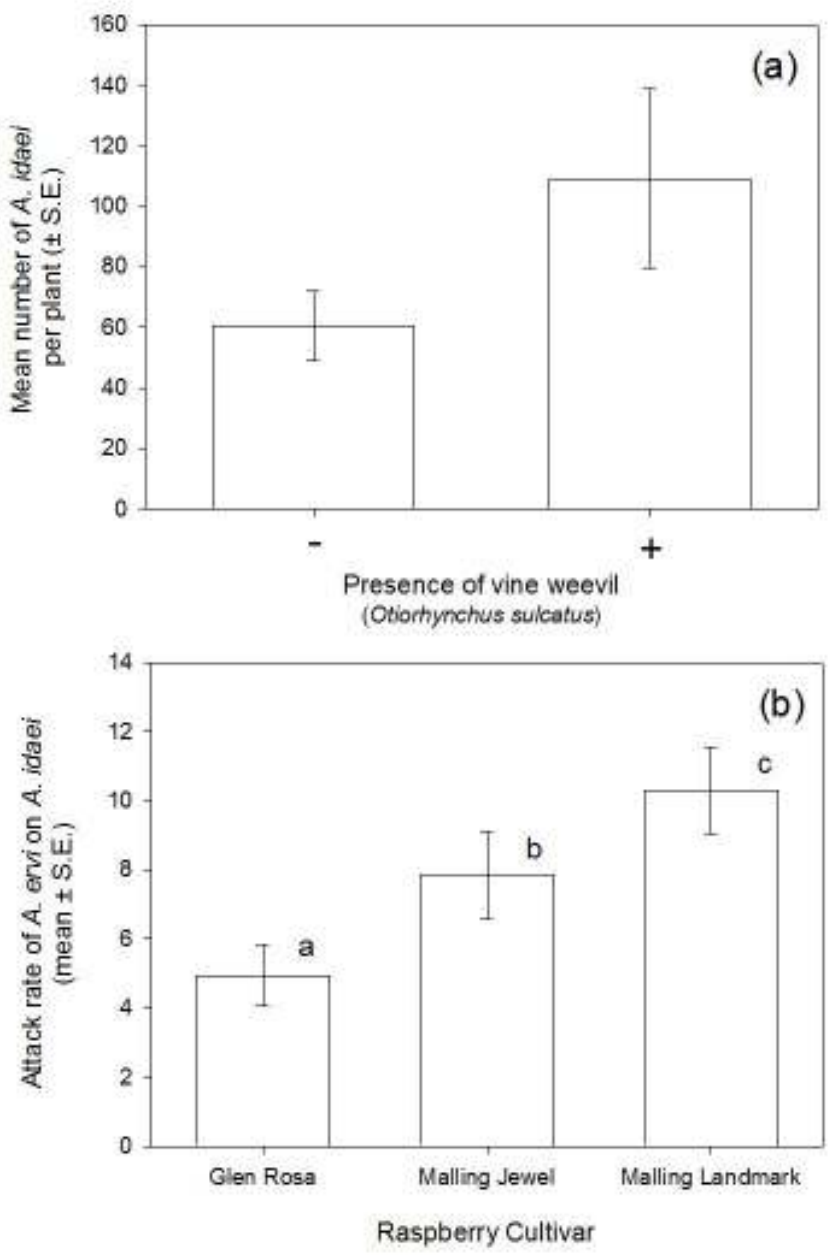


Figure 2.3. Trophic interactions between *A. idaei* and (a) the vine weevil, *Otiorhynchus sulcatis*, showing how the presence of vine weevils promoted *A. idaei* abundance on Glen Clova (analysis of variance: $F_{1,18}$= 5.31, P = 0.033), adapted from Johnson *et al.* (2008) and (b) the parasitic wasp *Aphidius ervi* oviposition rates on three cultivars of raspberry with increasing degrees of aphid resistance (Malling Jewel < Malling Landmark < Glen Rosa); ANOVA: $F_{2,56}$ = 27.11, P < 0.001 (aphid density fitted as covariate), adapted from Mitchell (2007). Lowercase superscripts indicate significant differences between cultivars. Data from Johnson *et al.*, (2008).

2.2.6 Potential biocontrol

The introduction of parasitoids or natural enemies, or both, to managed ecosystems has been suggested as a strategy for controlling insect pests (Price et al., 1980), so it is not surprising that this has been the primary motivation for investigating interactions between *A. idaei* and such organisms (Birch et al., 1996; Mitchell, 2007). There are currently very few studies of the interactions between the large raspberry aphid and natural enemies (see Birch et al., 1996). However, there is evidence that parasitoids frequently attack *A. idaei*. For example (Dickson, 1979) identified a selection of parasitoids that had emerged from mummified *A. idaei* collected from the field. Emerging parasitoids belonged to four families (Cynipoidae, Chalcididae, Encyrtinae and Braconidae) and one superfamily (Proctotrupoidae). The aphid mummies recovered from the field were predominantly at fourth instar (62%) and adult (30%) stages, with the remainder being third instar nymphs (Dickson, 1979).

Experiments investigating the interactions between *A. idaei* and parasitic wasps have recently been conducted by Mitchell (2007). In particular, parasitism of *A. idaei* by three common aphid parasitoids; *Aphidius colemani*, *Aphelinus abdominalis* and *Aphidius ervi* was examined, with only the latter successfully parasitizing the aphid. Parasitism levels by *A. ervi* were low, but there was evidence of an interaction between the resistance gene present in the raspberry cultivar (see Section 2.4) and the parasitoid's ability to successfully attack an aphid host. Although the mechanisms of the interaction remain uncertain, this study showed that attack rates by *A. ervi* were significantly higher on *A. idaei* feeding on the moderately susceptible cultivar, Malling Landmark, compared with those feeding on the highly susceptible cultivar, Malling Jewel and moderately resistant

cultivar, Glen Rosa (Figure 2.3b). Further studies of these interactions may provide a future strategy of combining aphid resistance with biological control, although a significant concern about biological control in raspberry is contamination of ripe fruit by parasitized aphid mummies, which can result in significant economic losses through rejection on aesthetic grounds (Hall *et al.*, 2009).

2.3 *Amphorophora idaei* vector biology

The three viruses known to be vectored by *A. idaei* belong to the Raspberry mosaic disease (RMD) complex and are widespread in Europe. Both the aphid's capability to transmit the viruses for several days after initial acquisition and recent sequencing and taxonomic placement (see McGavin & MacFarlane, 2010) suggests that their transmission is likely to be semi-persistent. This section describes each virus including their taxonomy (which has recently been revised) and the symptoms they commonly cause.

2.3.1 Black raspberry necrosis virus (BRNV)

BRNV has not as yet been assigned to a family or genus by the International Committee on Taxonomy of Viruses (ICTV), but phylogenetic analysis allows a tentative placement within the genus Sadwavirus (Halgren et al., 2007). Originally termed 52V virus in Europe (Jones & Murant, 1972), BRNV, so-called due to the effect it induces at the tips of black raspberry seedlings (Stace-Smith, 1955a), is often the first aphid-borne virus to arrive in new red raspberry plantations (Converse, 1987). After only one growing season, plantations of susceptible cultivars such as Malling Jewel can become 75% infected

(Jones, 1979). In the UK, BRNV is often found associated with a complex of viruses which only then results in the production of detectable physical symptoms. For example, in Europe the host plant develops symptoms of vein banding mosaic disease (Jones, 1991) when found in complex with RYNV and RLMV, which as the name suggests, produces visible chlorotic bands on the leaves. Few red raspberry cultivars show outward symptoms after infection with BRNV alone, but the disease is sometimes distinguishable by the presence of small patches of leaf discolouration (Plate 2.1b). The presence of the disease ultimately results in a decrease in plant vigour and fruit yield. However, the lack of outward symptoms in most commonly grown cultivars makes early diagnosis difficult unless plant tissue with a suspected infection is either grafted to a sensitive indicator (usually black raspberry, *Rubus occidentalis*) where the infection induces the characteristic necrosis at the tip of the seedling or PCR diagnostics are utilised to confirm infection.

2.3.2 Rubus yellow net virus (RYNV)

Nucleotide sequencing of RYNV has identified it as a new species (Jones et al., 2002) and it is now recognised by the ICTV as belonging to the *Badnavirus* genus in the family Caulimoviridae. It is a disease that is now found in almost every major raspberry growing area in the world, although the use of resistant red raspberry cultivars has had the effect of decreasing the incidence of the disease in Europe and North America. When the virus is present as a single infection (i.e. in the absence of other components of RMD), it produces a characteristic net-like chlorosis along the leaf veins of the host plant (Plate 2.1c). This chlorosis discolours the plant, making it appear pale green. If the infection is chronic, it can result in downward cupping of the leaves (Converse, 1987). More commonly, RYNV is found in a virus complex, which produces varying symptoms

depending on the components present. Cadman (1952) found that in some UK cultivars, when RYNV was present together with BRNV the infected plant showed symptoms of veinbanding mosaic disease. Stace-Smith (1956) found that the same combination of viruses in North American plants induced raspberry mosaic, but the work of Jones (1991) suggests that it is more likely to be the combination of RYNV and RLMV that causes veinbanding mosaic disease in UK cultivars, with the presence of BRNV simply resulting in more intense symptoms.

2.3.3 Raspberry leaf mottle virus (RLMV)

Recent sequencing and phylogenetic analysis has shown that a UK isolate of RLMV, previously termed Raspberry leaf spot virus (RLSV), and isolates of a similar Closterovirus found in North America, previously termed Raspberry mottle virus (RMoV), are actually all isolates of the same virus and should now be referred to under the collective name Raspberry leaf mottle virus (RLMV), the first of these viruses to be described (McGavin & MacFarlane, 2010). This section includes information from older literature where these viruses are still referred to using the old naming system.

RLMV is found commonly in cultivated red raspberry (Jones & Murant, 1972), with symptoms reported in plants from many European countries outside the UK. Single infection with RLMV is usually latent and symptomless, but in sensitive cultivars such as Malling Jewel, symptoms can be extreme, resulting in plant death (Jones & Jennings, 1980). The work of Jones (1980) showed that RLMV infection in the cultivar Glen Prosen resulted in an overall decrease in fruit yield. In such sensitive cultivars it is common for chlorotic yellow spots to develop on the plant leaves (Plate 2.1d). These spots

are often randomly distributed and measure 1-2 mm in diameter. In extreme cases, spotting of leaves can lead to the formation of large chlorotic patches as overlap occurs (Converse, 1987). Additionally, leaves on fruiting canes can become deformed and plant growth is stunted. Infection in sensitive cultivars ultimately leads to plant death within 2-3 years (Converse, 1987).

Currently, RLMV has not been assigned to a family or genus by the ICTV although recent nucleotide sequencing of the UK strain suggests placement within the family Closteroviridae (McGavin & MacFarlane, 2010). This sequence data is identical to that obtained by Tzanetakis et al., (2007) for a North American isolate of the virus, confirming that the two viruses are, in fact, the same.

2.3.4 Aphid transmission of viruses

All three of the viruses vectored by *A. idaei* are believed to be naturally transmitted during feeding on infected plant tissue, with the virus being picked up by the aphid's stylets. The virus is spread when the aphid migrates to a healthy host plant and subsequently inserts the stylets to feed. The spread of viruses in raspberry plantations usually occurs along plant rows (Rankin, 1931) and in addition to natural aphid migration, the viruses are also spread by passive aphid movements due to wind and rainfall (Converse, 1987). Studies conducted by (Stace-Smith, 1955a) revealed that BRNV could be transmitted by the aphid after an acquisition access period of between 15 and 30 minutes. Successful transmission required a subsequent inoculation period of two minutes on an uninfected host. These acquisition and inoculation times are slightly different in the case of RYNV. In this instance, the aphid must undertake an acquisition access period of in excess of an

hour in order to become virulent, with inoculation periods of four hours or more greatly increasing the incidence of successful transmission (Stace-Smith, 1955b). The ability of the aphid to transmit RYNV is lost after 4 hours of access to healthy plant tissue (Stace-Smith, 1955b) whereas aphids exposed to BRNV inoculated tissue only retain the ability to transmit the virus for c.3 hours (Stace-Smith, 1955a). The success of some aphid resistant raspberry cultivars has meant that research into the transmission of RLMV in raspberry has not been a high priority, although is likely to become more of an issue with resistance breakdown. Research by Cadman (1954) using the closely related *Rubus occidentalis* indicated that an initial acquisition access period of less than half an hour was required in order for the vector to acquire RLMV, with a subsequent inoculation period of less than an hour sufficient for the virus to be successfully transferred to a new host plant. Again, the frequency of successful transmission was greatly enhanced by increasing both the acquisition access and inoculation periods.

2.4 Resistant raspberry cultivars and resistance-breaking aphid biotypes

2.4.1 Resistant cultivars and plant resistance genes

By far the most effective method of aphid control is the planting of genetically resistant red raspberry cultivars. Many of these have been introduced over the last 40-50 years by commercial breeding programmes and have, until recently, been largely successful at controlling the spread of the viruses transmitted by *A. idaei* through resistance to the aphid vector. Plant resistance to *A. idaei* (and other pest species) is comprehensively reviewed by Hall et al., (2009), with a particular emphasis on plant breeding initiatives.

Cultivars introduced for commercial use over the last 40 years possess either single major gene resistance (either A_1 or A_{10}) or have multiple (multigenic) minor gene resistance (Jones, 1986) (Table 2.1). The resistance gene A_1 (Knight et al., 1959) was first incorporated into red raspberry cultivars in the 1980s (Birch et al., 2004) and the strongest gene, A_{10}, was isolated from black raspberry, *Rubus occidentalis* (Keep & Knight, 1967) and subsequently incorporated into red raspberry breeding. Birch & Jones (1988) reported that plants with either the A_{10} or A_1 gene conferred maximum resistance to *A. idaei*, while plants with multigenic resistance conferred slightly less resistance. This resistance is thought to be expressed in a number of ways, including reduced settling and feeding by aphids (i.e. antixenosis) (Birch & Jones, 1988) and decreases in fecundity and rate of larval development (i.e. antibiosis) (Mitchell, 2007).

A little over ten years ago, about 90% of raspberry plantations in the UK made use of plants containing resistance genes, with approximately 40% of these possessing the A_{10} resistance gene and 30% possessing the A_1 resistance gene (Birch et al., 1996). This particular method was highly successful in limiting both aphid population size and virus transmission until the increased selection pressure on the aphid inevitably led to the emergence of new resistance-breaking biotypes.

Birch & Jones (1988) noted that large numbers of *A. idaei* were found on the cultivars Glen Prosen and Glen Moy in the field, but that there were relatively small populations found on the cultivars Delight and Malling Landmark growing in close proximity. This observation was anomalous as these four cultivars were all assumed to possess the same resistance gene to the aphid, major gene A_1. Jones et al. (2000) suggested that either these

cultivars did not in fact all possess resistance gene A_1, or other genes (termed 'modifying genes') were in operation, leading to a variation in gene effectiveness against the aphid. Subsequent glasshouse experiments designed to test these hypotheses revealed that the genetic basis of resistance to *A. idaei* was much more complex than was previously believed, with major gene resistance apparently being altered by other minor genes, depending on the genetic background of the cultivar. In particular, it was noted that plants that had been shaded with black netting exhibited reduced resistance to *A. idaei*, indicating that the effectiveness of single major genes can be altered by varying environmental conditions during growth as well as genetic factors (Jones et al., 2000). Furthermore, the observation that root herbivory by vine weevils compromised multigenic resistance in Glen Clova and allowed aphid populations to successfully colonise the plant (described earlier - see Figure 2.3a) suggests that the presence of other herbivores feeding on the host may also alter plant resistance. Thus far, no gene has been identified in *Rubus* germplasm that can convey resistance to the virus itself (Jones & Jennings, 1980), so management continues to depend on control of the aphid vector.

2.4.2 Potential resistance mechanisms against *Amphorophora idaei*

The epicuticular wax layer found on most plants has long been understood to play a role in plant defence against desiccation and attack from herbivores and pathogens (Schoonhoven et al., 2005). There is also evidence that the epicuticular wax of *Rubus* leaves protects the plant from *A. idaei* attack (Robertson et al., 1991; Shepherd et al., 1999a, b). Attempts to identify an individual component that could underpin resistance to *A. idaei* have so far been unsuccessful although comparisons of the composition of leaf surface wax collected from the largely *A. idaei* resistant cultivar Autumn Bliss (A_{10}

resistance) and that of the entirely susceptible cultivar Malling Jewel have revealed 13 compounds common to both cultivars. These compounds were identified as belonging to four major classes: straight chain hydrocarbons; acetic acid esters of long chain alcohols; tocopherols and triterpenoid compounds and it is believed that their relative concentrations could influence resistance. Robertson et al. (1991) were able to relate the chemical composition of the leaf surface to aphid susceptibility using linear discriminant analysis. Using chromatographic data and results of bioassays, 24 out of 26 plants were classified correctly in terms of the level of aphid resistance possessed. Furthermore, small amounts of triacylglycerols detected by gas chromatography-mass spectrometry (GC-MS) in leaf wax collected from the susceptible cultivar Malling Jewel are believed to be to be a result of the incorporation of aphid exuviae and cornicle fluid into the leaf wax (Shepherd et al., 1999b). These compounds were detected in wax taken from the entirely aphid susceptible cultivar, Malling Jewel but not in wax from the resistant Autumn Bliss. These findings appear to indicate that the measurement of these leaf wax components could provide a future method of screening plants for aphid resistance (Shepherd et al., 2000).

Cultivar	Resistance gene	Viral pathogen				*A. idaei* biotype					
		BRNV	RLMV	RLSV	RYNV	1	2	3	4	X	A_{10}-breaking
Malling Jewel‡	None	✓S	✓NS	✓NS	✓S	✓	✓	✓	✓	✓	✓
Glen Ample	A_1	–	–	–	–	×	✓	×	–	✓	×
Glen Clova	Multigenic	–	✓NS	✓S	✓S	*	*	–	–	✓	ü
Glen Lyon	A_1	–	✓NS	✓ NS	–	×	✓	×	–	✓	×
Glen Magna	A_1	–	–	–	✓S	×	✓	×	–	✓	×
Glen Moy	A_1	–	–	✓S	✓S	×	✓	×	✓	✓†	×
Glen Prosen	A_1	✓NS	✓NS	✓ NS	✓S	×	✓	×	✓	✓†	×
Glen Rosa	A_{10}	–	–	✓ NS	✓S	×	×	×	×	×	✓
Glen Shee	A_1	–	–	✓NS	–	×	✓	×	-	✓	×
Glen Doll	A_{10}	–	–	–	–	×	×	×	×	×	✓

Table 2.1. Comparison of SCRI developed 'Glen' series of raspberry cultivars possessing differing genetic resistance with the entirely susceptible cultivar, Malling Jewel. Table shows the extent to which viral symptoms are visible when cultivars are infected, and the susceptibility of cultivars to different biotypes of *A. idaei*. Cultivars/biotypes not tested indicated by '-', S indicates recorded visible symptoms and NS indicates infection without visible symptoms. ✓ indicates biotypes capable of colonising cultivar, × indicates biotype is incapable of colonizing cultivar, * cultivar partially resistant. (Data from Birch & Jones, 1988; Jones & McGavin, 1998; Jones *et al.*, 2000).

2.4.3 Resistance-breaking aphid biotypes

The introduction of aphid resistant cultivars has led to strong selection pressure on *A. idaei* to overcome this resistance and has resulted in the emergence of several resistance-breaking biotypes (listed in Table 2.1). Of the five fully described *A. idaei* biotypes, two (biotypes two and X) are capable of overcoming A_1 resistance, found in cultivars such as Glen Moy and Glen Prosen (Birch et al., 1992). Biotype two is now found commonly on these cultivars. For example, in 1990-91, 77% of *A. idaei* collected from 22 field sites in the UK were identified as belonging to biotype two or X (Birch et al., 1994) compared with just 3% between 1958 and 1961 (Briggs, 1965). In an effort to combat this rising problem, cultivars possessing major gene A_{10} began to be used more extensively. This has led to the emergence of at least one further biotype with the genetic capacity to colonize these plants, currently referred to as 'A_{10}-breaking' in Table 2.1. More recently released cultivars expressing this gene, such as Glen Doll, are therefore no longer completely resistant to *A. idaei* attack. Biotype one is believed to now only to occur on wild raspberry, while there is a suggestion that biotypes three and four have either become, or are close to becoming, extinct (Sargent et al., 2007).

2.6 Alternative control strategies

Using resistant cultivars is by far the most widespread technique for controlling *A. idaei* populations, but other strategies have also been deployed. Insecticides have been used in the field against aphids already carrying viruses, whereas heat therapy and meristem culture can be exploited to eradicate the disease in parent plants used for propagation,

thereby limiting virus transmission back into *A. idaei* populations. However, both heat treatment and meristem culture do not protect the plant from subsequent attack by viruliferous *A. idaei*, so such remediation can be short lived.

2.6.1 Insecticides

The use of organophosphorous based (OP) insecticides to control raspberry aphid was relatively rare, and has declined still further with the introduction of aphid resistant red raspberry cultivars. Gordon et al., (1997), using survey data from agricultural agencies in the UK, reported that raspberry accounted for less than 5% of total OP insecticide usage on UK crops since the introduction of resistant cultivars. The short inoculation time required by the virulent aphid means that pesticides are largely unsuccessful at preventing virus spread in raspberry plantations, regardless of effectiveness at controlling aphid population size, as they simply do not kill the aphid quickly enough to prevent transmission (Taylor & Chambers, 1969).

2.6.2 Heat therapy

Heat therapy involves the application of either hot water or air at temperatures usually ranging between 35 and 54°C to eliminate virus particles from plant material. Wet heat is generally more effective than dry heat (Matthews, 1991), although the use of hot air on growing plants increases their survival rate. Research published by Chambers (1961) demonstrated that certain cultivars could be freed from some components of the raspberry mosaic complex by heat treatment of 35°C for periods of 2-3 weeks. RYNV is a heat stable virus (Stace-Smith, 1960) and as such, attempted eradication through whole plant heat treatment has proved unsuccessful although the heat-labile virus, BRNV, can

be eradicated from infected host plants by heat treatment at 32-37°C for a time period of 10 days (Stace-Smith & Mellor, 1957). Tip cuttings excised from the host plant and rooted during this heat treatment are found to be free from virus (Bolton & Turner, 1962). Plants that have been infected with other heat-stable raspberry viruses, such as RLMV, can be freed from infection by heat treatment at 32-37°C for time periods of between 10 and 20 days (Baumann, 1982).

2.6.3 Meristem culture

Plant viruses vary in their distribution within plant cells and, although difficult if the virus occurs in the apical meristem (Toussaint et al., 1984), it is possible to produce virus-free propagated plants by culturing of meristematic tissue. This is achieved by aseptically culturing the apical tips of the infected plants and the first pair of leaf primordia (Hollings, 1965) in a nutrient medium containing growth factors. It is also possible to use a combination of both heat therapy and meristem culture to produce virus-free plant material (Baumann, 1982).

2.7 Conclusions

The reported breakdown of the most effective aphid resistance in red raspberry has made the European large raspberry aphid a much more serious problem than has hitherto been the case. Insecticides are not a viable alternative in the long term, so new control strategies will need to be developed, which will inevitably rely on a better understanding of the biology and vectoring behaviour of *A. idaei*. Moreover, issues such as the effects of

global climate change on *A. idaei* have not yet been explored, but most predictions suggest that aphids are likely to become more of a problem due to increased overwintering survival (Zhou et al., 1995). Mitchell (2007) suggested that *A. idaei* is less well suited to higher temperatures, but the extent to which it could adapt to warmer climates (as it has done to resistant plant cultivars) remains unclear. Even if mid-season temperatures were disadvantageous to *A. idaei*, the overall increase in temperature would most likely extend the seasonal life cycle of *A. idaei* (see Figure 2.1) which in itself would be problematic in terms of viral transmission occurring earlier in the season. New strategies that incorporate several approaches in an integrated pest and disease management (IPDM) framework will undoubtedly be needed for effective control of *A. idaei*. For example, understanding the chemical ecology of host plant location, and whether viral infection plays a role in this through changes to plant chemistry, could be useful for disrupting *A. idaei* behaviour. Semiochemical traps, based on naturally occurring plant volatiles, have been used to control other insect pests of red raspberry (e.g. Robertson et al., 1995) so this is distinctly feasible.

Recent developments in raspberry management and research into host plant resistance provide two unique advantages, which may make biocontrol strategies more viable. Firstly, protected coverings (i.e. polytunnels) have become commonplace for growing raspberries since being introduced to the UK in 1993, and now account for 80% of soft fruit on sale in UK supermarkets (Anon, 2005). It is probable that parasitoids and/or predators would operate more effectively within the confines of such tunnels, having smaller areas to search for aphid prey and reduced ability to disperse from the tunnel. Secondly, the development of molecular and genetic tools, including molecular markers

such as Amplified Fragment Length Polymorphism (AFLP) and microsatellite markers such as Simple Sequence Repeats (SSR), has recently led to much better understanding of resistance mechanisms in *Rubus* (e.g. Graham et al., 2002; Graham et al., 2004; Stafne et al., 2005; Sargent et al., 2007) and may lead to more durable resistance against *A. idaei*. If the problem of fruit contamination with aphid mummies can be overcome, these twin approaches of bottom-up (host plant resistance) and top-down control (biocontrol agents) might therefore lead to more effective and sustainable control of *A. idaei* in the future.

Chapter Three

The effect of Black raspberry necrosis virus and Raspberry leaf mottle virus on the recruitment and performance of *Amphorophora idaei*

Abstract

Black raspberry necrosis virus (BRNV) and Raspberry leaf mottle virus (RLMV) are debilitating viruses of *Rubus idaeus* and are vectored by the large raspberry aphid, *Amphorophora idaei*. Plants infected with these viral pathogens were found to be initially more attractive to the aphid vector in light conditions as more adult aphids chose to move to these plants when offered a dual choice between a healthy plant and one infected with both viruses. In experiments conducted over different timescales aphid preference for virus infected plants was variable. Specifically, a significantly higher proportion of aphids were found on virus infected leaves over a short experiment of 30 minute whereas no difference in aphid preference was found over a longer experiment observed over a seven day period. Differences in leaf colouration measured from absorbance and reflectance of light from plant leaves were found, suggesting that there may be a visual cue to aphids which is responsible for their initial preference to virus infected plants.

Comparison of aphid performance on virus–infected plants and healthy plants revealed that feeding in the presence of BRNV and RLMV was detrimental to the aphid. *Amphorophora idaei* took longer to reach adulthood and begin to reproduce on plants that were infected with BRNV + RLMV when compared to healthy plants although the number of offspring produced in a seven day period was unaffected by plant infection with these viruses. These results are indicative of a deceptive attraction of *A. idaei* to a nutritionally poor host plant which may promote further transmission of the viruses as the aphid is attracted to the host plant over a period of time conducive to successful acquisition.

3.1 Introduction

3.1.1 Rationale

As was discussed in the previous chapter, *Amphorophora idaei* is prevalent in the United Kingdom and is a serious pest of red raspberry through its role as a vector of several viral pathogens (Converse, 1987). Understanding the mechanism(s) by which these pathogens are transmitted and how the viruses may influence aphid behaviour, is of fundamental importance to developing strategies to limit the spread of viral diseases in raspberry plantations as the selection pressure acting on aphid populations has led to large scale breakdown of resistance in cultivated *R. idaeus* (see Chapter Two). Novel control strategies based on manipulation of natural aphid behaviours could therefore provide solutions to this problem by exploiting plant derived chemicals which may act as aphid attractants or repellents. Knowledge of how the insect interacts with the host plant and how the plant mediates the interactions with viral pathogens is therefore of key importance to this research.

3.1.2 Plant viruses alter vector behaviour and reproduction

Research investigating the interactions between insect vectors, and the viruses they transmit, commonly demonstrate that insects preferentially settle on plants that are infected with viral pathogens when presented with a choice between a healthy and a diseased plant. Examples of this preference have been found for many different species of plant–feeding insects on a diverse range of host plants including the leafhopper vector of Tungro virus feeding on rice (Khan & Saxena, 1985) and the thrips vector of Tomato spotted wilt virus feeding on pepper (Maris *et al.*, 2004). These studies use a common

experimental design that involves releasing insects equidistantly between a healthy plant and one infected with the virus of interest. Interestingly, although these studies show that the insect is initially attracted to virus–infected plants it does not always follow that the insect will choose to feed there for a prolonged period of time. For example, the study of Khan and Saxena (1985) showed that the leafhopper, *Nephotettix virescens*, is initially attracted to diseased rice plants but after only 24 h, it disperses and settles on healthy plants. Plant nutritional quality is suggested as the trigger for such rapid movement as the presence of the viral pathogens in the host plant can actually be detrimental to vector development and reproduction (Khan & Saxena, 1985). Alterations to plant chemical composition in response to virus infection are discussed in detail in Chapter Four of this thesis.

It may at first seem counter–intuitive that a plant virus should induce changes in the host plant that may discourage the vector from prolonged feeding. However, as was discussed in Chapter One, most insect–transmitted plant viruses are not persistently transmitted (Ng & Perry, 2004), requiring the vector to probe the plant only for a very short time period (15-30 min in the case of BRNV and RLMV; see Chapter Two, Section2.3.4) before successful transmission occurs (within hours for BRNV and RLMV). The suitability of the plant as a host for the vector is therefore detected within a very short space of time as it probes the plant (Chapman, 2003). These brief probes are enough for virions to be successfully adsorbed to the mouthparts of the vector and they are then transported to a new host plant when the insect disperses. It must be stressed that the response of the vectors to feeding on plant tissue infected with viral pathogens varies between systems. Many studies report positive effects of plant pathogens on vector survival and reproduction. For example, Maris *et al.*, (2004) showed that offspring of the

thrips vector of Tomato spotted wilt virus (TSWV), *Frankliniella occidentalis*, reach pupation faster on TSWV–infected pepper plants, indicative of shortened development time. In studies using the same insect, Belluire *et al.*, (2008) showed that the shortened development time on TSWV–infected plants acts to reduce the susceptibility of the insect to predation by two species of mite as they are incapable of capturing larger thrips larvae, thus, the presence of the virus indirectly mediates a beneficial interaction for the vector through alterations in host nutritional quality.

Perhaps one of the most well studied interactions between a plant virus and its vector comes from another agricultural crop system and involves the interactions occurring between Potato leaf roll virus (PLRV) and the aphid vector *Myzus persicae* on potato plants. PLRV is a persistently transmitted virus (see Chapter 1, section 1.3.1.2) and requires the aphid vector to feed for prolonged periods (hours to days) in order to successfully transmit the virus to a new host plant. The comparative study of Castle & Berger (1993) demonstrated that *M. persicae* was preferentially attracted to virus-infected potato plants and the strongest attraction was for plants infected with PLRV which must replicate in the aphid before it can be transmitted to new host plants. In contrast, *M. persicae* were least attracted to plants infected with Potato virus X, a non-aphid transmitted plant virus. However, other studies report a neutral effect of plant viruses on their insect vectors. Hodge & Powell, (2008) showed that tic bean infection with Pea enation mosaic virus (PEMV) has no effect on survival, growth or reproduction of one of the aphid vectors, *Acyrthosiphon pisum*, despite the aphid's preference for settling on the virus–infected plants. This study suggests that the attraction of the vector to diseased

plants is caused by the yellowing of foliage and, unlike studies of potato viruses, found no evidence that vector preference for diseased host plants is related to the dependence of the virus on the vector for transmission.

In evolutionary terms, as the virus is dependent on the insect for survival, it would not be beneficial for it to cause damage of such severity as to risk the death of the host plant before acquisition has taken place. Therefore, natural selection should favour a system by which the virus has little or no negative effect on the aphid vector. At best, the virus should alter plant chemical composition in such a way as to actually benefit the insect and this scenario is indeed found to occur in the PLRV–potato system. The earlier work of Castle & Berger (1993) demonstrated that *M. persicae* benefits from feeding on infected potato tissue, reflected in shorter development times and increased number of progeny. The studies of Eigenbrode *et al.* (2002) and Alvarez *et al.* (2007) build on these findings by showing that increased volatile emissions from PLRV-infected plants are responsible for the attraction of the aphid to virus-infected plants.

The above examples demonstrate the variable effects that plant viruses can exert on their vectors. The interactions appear to be host and/or virus specific and in particular, are often related to the mode of virus transmission by the insect vector. This highlights the need for further studies of these interactions, particularly with regard to high value agricultural crop systems where novel strategies must be developed to address the problems arising through decreased pesticide application due to environmental concerns.

The apparent specificity of virus–vector interactions also increases the scope for investigations in other plant systems important for the UK economy, such as raspberry, which remain relatively poorly studied with regard to pathogen–induced changes to plant chemistry which may mediate interactions with insect herbivores.

3.1.3 Study system

Full details of the biology of *A. idaei* and current understanding of the role the aphid plays in the transmission of viral pathogens of raspberry are provided in Chapter Two of this thesis. In summary, in the UK, Black raspberry necrosis virus (BRNV) and Raspberry leaf mottle virus (RLMV) are components of Raspberry Mosaic Disease (RMD) and are therefore commonly found to infect raspberry host plants in combination. Glen Ample, an SCRI developed cultivar of red raspberry, is currently widely grown in commercial plantations in the UK as it is spine–free, vigorous and produces large, fleshy, bright red berries. This variety was bred to possess major gene resistance, A1, which previously conferred resistance to most biotypes of *A. idaei* (see Chapter Two, section 2.4.1). Glen Ample is now susceptible to biotypes 2 and X, prevalent in Scotland due to the selection pressure that the cultivar has exerted on aphid populations since its commercial release.

3.1.3 Aims and hypotheses

The aim of this study was to establish whether *A. idaei* preferred to feed on healthy host plants or those infected with BRNV and RLMV and determine how feeding on either host plant affected performance. As both BRNV and RLMV are transmitted

semipersistently and require short acquisition periods for the aphid to become viruliferous, aphid bioassays were set up using different timescales. The aim of the different designs was primarily to assess the initial host plant preference of the aphid for a particular plant (healthy or virus-infected) and further to this, the study specifically aimed to characterise whether:

1. Aphids remained on the plant they had initially selected
2. Aphid performance (juvenile development time, pre–reproductive period and number of nymphs laid) differed when feeding on healthy and infected plants.
3. Timescales for aphid migration to new host plants varied:
    a. Within 30 min (time required for successful virus acquisition)
    b. Over a seven day period (investigating migratory behaviour)

In addition to answering these questions, the experimental design allowed for investigation of potential cues to the aphid during host plant location, i.e. investigation of visual and olfactory attractants. These experiments were designed to test three main hypotheses:

1. Plants infected with viral pathogens would make more attractive hosts for *A. idaei* which would be reflected in an increased number of aphids choosing to move to infected plants.
2. Aphid performance would be affected negatively by the presence of BRNV + RLMV as semipersistent viruses require only brief virus acquisition periods.
3. Negatively affected performance of *A. idaei* would induce the aphid to move to a new host plant.

3.2 Materials and methods

3.2.1 Plant propagation

Plants were grown from vernalised root of the raspberry cultivar Glen Ample. Parent plant material was derived from virus–infected reference plants or virus–tested 'healthy' plants held at SCRI. Parent plants were originally produced by bottle grafting of scions from BRNV and RLMV–infected reference plants, which had been PCR–verified, onto healthy Glen Ample plants. Root balls from the infected plants were cold stored at –20 °C for at least six months prior to propagation. Root was sown in hotbox propagators at 20 ± 1 °C within an air conditioned, aphid–free glasshouse (20 ± 1 °C, 16:8; L: D photoperiod) in small trays using Bulrush bedding compost (Bulrush Horticulture Ltd., Londonderry, U.K.). From four weeks of growth, new seedlings were carefully transplanted to individual pots (diameter 12 cm) and allowed to grow for a further four weeks in the glasshouse (conditions as above) before being used in experiments.

3.2.2 Virus testing

All plants used in experiments were first verified for the presence or absence of virus using RT–PCR on total RNA extracted from plant leaves. Leaf samples for RNA extraction were taken from plant seedlings that were approximately six weeks old. A small leaf was excised from the growing tip of the plant, immediately placed in a 2 ml tube and snap frozen in liquid nitrogen. The sample was then ground to a homogenous powder in liquid nitrogen using a cooled, sterile mortar and pestle. 50 mg of the frozen plant powder was transferred into a 1.5 ml tube and 450 µl buffer RLT (Qiagen RNeasy plant mini kit), 45 µl plant RNA isolation aid (Ambion) and 4.5 µl β–mercaptoethanol

was added. The sample was vortexed vigorously and centrifuged for 5 min at top speed. The aqueous sample was transferred to a QiaShredder spin column and the extraction was continued according to the Qiagen RNeasy manufacturer's instructions. Ready–to–go™ RT–PCR beads (GE Healthcare) were used to set up 50 µl PCR reactions using total RNA extracts according to the manufacturers instructions using primers specific to virus RNA sequence (Table 3.1).

Target	Primer	Sequence (5' – 3')	Product size (nt)
BRNV	1153f 1154r	gcgcaatgaacccaagttta caacatcgaatccctcaagc	502
RLMV	1291f 1291r	gtccgacttagtgatgacgtatcg cctcggatggagtaagcccactg	373

Table 3.1. Primer sequences and product sizes for BRNV and RLMV RT-PCR reactions

The RT–PCR cycling conditions for BRNV amplification were: one cycle at 42 °C for 1 hour (reverse transcription); one cycle at 94 °C for 5 min, followed by 40 cycles at 94 °C for 1 min, 66.5 °C for one min and 72 °C for one min. The reaction then underwent a final extension at 72 °C for 20 min. The PCR conditions for RLMV primer pairs were as above but with an annealing temperature of 58 °C. Positive and negative control reactions were included in the PCR using RNA extracted from a known healthy plant and one which carried a reference isolate of RLMV or BRNV. A 10 µl aliquot of cDNA product was analysed by running on a 1% agarose gel stained with ethidium bromide solution and visualised under UV light.

3.2.3 Insects

A clone of *Amphorophora idaei* (biotype 2) were maintained in Perspex cages within a controlled environment laboratory at 19 ± 1 °C, 16:8, L:D photoperiod, the optimum for aphid development. The insects were reared on Malling Landmark raspberry plants to ensure that any behaviours observed were not a result of previous experience feeding on the Glen Ample test plant.

3.2.4 Aphid recruitment – initial preference

To investigate if *A. idaei* shows a preference for raspberry plants infected with viral pathogens, two choice tests were carried out. These choice tests were set up in a controlled environment at 19 ± 1°C with overhead lighting (16:8 L:D photoperiod). Two plants (one healthy and one infected with BRNV and RLMV) were positioned 30 cm apart and connected using a wooden bridge (30 cm x 3 cm) with a 15ml universal tube sunk into the centre. Bridge positions were checked using a spirit level. Three apterous *A. idaei* adults were transferred to the central tube and left for 30 minutes prior to the start of each experimental replicate in order to minimise the effects of stress on the insects. Apterae were used for ease of experimental manipulation as the 'open' nature of the experimental set-up did not allow for use of winged aphids. The screw cap of the tube was removed and aphids were allowed to ascend the inside of the tube and cross the wooden bridge to contact a plant, when they were assumed to have made an initial choice. Twenty replicates of the experiment were obtained in light conditions using different aphids and plants on each occasion. The experiment was repeated in the dark to ascertain whether preferences were driven by visual cues such as leaf senescence

induced by the raspberry viruses. The laboratory was darkened by blocking out all external light with black paper. Ten replicates were conducted using different aphids and plants. Aphid positions were checked by illuminating the experiment with a dim red light bulb for a few seconds at a time.

3.2.5 Aphid performance

The performance experiment was designed to test aphid development rate and reproduction on RT–PCR–verified healthy and virus–infected raspberry host plants. Plants were positioned on a raised platform within a water–filled plastic tray which served as a 'moat' barrier to aphid movement between plants within a controlled environment at 19 ± 1°C with 16:8 L:D photoperiod. Eight week old plants (cv. Glen Ample) were inoculated with one apterous adult aphid which was then monitored daily for production of offspring. At the onset of reproduction, all but one first instar aphid were gently brushed from the plant using a fine paintbrush. The remaining nymph was monitored daily and the date of adulthood (i.e. development time) and the time to onset of reproduction (i.e. pre–reproductive period) recorded. The aphid was allowed to remain on the plant for seven days from the onset of reproduction and the total number of offspring produced in this time was recorded at the conclusion of the experiment. Ten experimental replicates were obtained in this way using different aphids and plants. At the conclusion of the experiment, all aphids were brushed from the plant whereupon the leaves were excised, snap frozen in liquid N_2 and stored at –80 °C for chemical analyses (see Chapter Four). Ten control plants of each plant type (healthy and virus–infected)

that were not exposed to aphid treatment were also harvested at this time (see Chapter Four).

3.2.6 Aphid movement between plants

3.2.6.1 Over 30 min

One plant leaflet from each of two plants (healthy or virus-infected) was placed into a square Petri dish (23.5 cm × 23.5 cm) through a small hole cut in each side and the lid of the dish was placed on top to prevent aphids escaping. The leaves remained attached to the rest of the plant throughout the experiment and any potential strain on the stem was avoided by suspending the Petri dish on clamp stands so that it sat parallel to the bench (position checked with spirit level). A mirror was positioned beneath the arena to aid aphid observation if the insect walked underneath the leaf. An adult aphid was released from a central hole in the Petri dish after it had been starved for an hour and its position in the arena recorded every min for 30 min. The aphid was scored as being in a 'neutral' area if it was anywhere other than on one of the plant leaflets. A total of 43 experimental replicates were obtained in this way using different aphids and plant pairs (i.e. 43 healthy plants paired with 43 infected plants and 43 different aphids).

3.2.6.2 Over seven days

To further investigate aphid movement after the initial host plant choice was made, a migration experiment was conducted in order to look more closely at the patterns of aphid movement between host plants. Two plants, one healthy and one infected with

BRNV and RLMV, were positioned within a wire–mesh insect cage with a plywood base (54 cm × 50.5 cm) similar to those described by Clark *et al.* (2010). Plant pots were positioned through circular holes cut into the base (diameter 10 cm) which left the aboveground mass of the plant entirely enclosed within the cage and meant that the plants could be watered at the base of the plant pot without the need to open the cage. All cages were positioned in a controlled environment (conditions as above). Five apterous *A. idaei* adults were released from a dish in the centre of the cage base between the two plants and were then allowed to move freely within the cage. Aphid positions were recorded after 12 h and at 24 h then at 24 h intervals thereafter for a seven day period. A total of 28 replicates were obtained using different aphids and plants in each cage.

3.2.7 Foliage colouration

In order to ascertain if differences in foliage colouration may be a factor in aphid host plant location, measurements of both absorbance and reflectance were made using healthy and virus-infected plants. All measurements were taken using a UniSpec-SC spectral analysis system (PP Systems, Massachusetts, U.S.A). Absorbance and reflectance were measured from 20 plants (ten healthy and ten infected with BRNV and RLMV) which were grown in identical conditions to those used in aphid experiments and that had been verified for the presence of absence of the viruses using PCR tests (see section 3.2.2). The UniSpec system was calibrated in both light and dark to obtain a reference scan between 300 and 1100 nm. After calibration, the fibre-optic detector was clipped to a fully expanded leaf of the plant and absorbance and reflectance were measured between

400 and 800 nm at intervals of approximately 4 nm using a source white light intensity of 100%. All measurements were taken from leaves at a similar age and position on each plant. Absorbance and reflectance of each leaf was calculated by comparison with the reference scans as follows:

$$Absorbance = \log (I_0/I);$$

$$Reflectance = I/I_0;$$

where I is the raw sample data obtained from each leaf and I_0 is the reference data from the background scan.

3.2.8 Statistical analyses

Parametric statistical tests were applied where possible to all data confirmed to be normally distributed. Some aphid performance data (development time and pre–reproduction time) required $\log_{10}$ transformation in order to meet assumptions of normality. Aphid performance parameters were analysed using a one-way ANOVA and differences in foliage colouration (absorbance and reflectance of light) using a two-sample (unpaired) t-test in GenStat version 13.0 (VSN International).

The proportion of aphids initially choosing virus infected plants over healthy plants in the light and dark experiments were analysed using generalized linear models (GLM) with a binomial error structure and utilising a logit-link function in R version 2.12.1 (R Foundation for Statistical Computing). Model estimates of the proportion of aphids on virus infected plants (y-variable) were generated for both the experimental data and also for a null model which assumed no preference (proportion of aphids on infected plants

equal to 0.5). Significant deviations between these two models were therefore indicative of an aphid preference for virus infected plants and the results are reported as the χ^2 values generated from each comparison.

Due to the repeated measures on the same aphid individuals over time, the longer aphid migration experiments (over 30 min and 7 d) were analysed using a generalized linear mixed effects model (GLMM) assuming a binomial error structure and utilising a logit-link function. In each analysis, the proportion of aphids on infected plants was fitted as the y-variable and time was initially fitted as the x-variable. Cage or arena nested within time was initially fitted as the random term. Terms were subtracted from the model until any further removal led to significant increases in deviance and thus higher values of Akaike's Information Criterion (AIC; Akaike, 1974). All results and associated probabilities are reported based on the resulting minimum model for each experiment (for model summaries see Table 3.2). Aphids on the side of the arena or cage were assumed to be non-responsive and were excluded from the analyses. All mixed models were run using the lme4 package in R version 2.12.1 following the methods of Crawley (2007) to eliminate temporal pseudoreplication in the dataset.

3.3 Results

3.3.1 Aphid recruitment – initial preference

A higher number of *A. idaei* chose to move to plants that were infected with BRNV and RLMV when released from the universal tube under both light (Figure 3.1a) and dark (Figure 3.1b) conditions. All aphids tested moved to a plant within an hour of release with a total of 38 aphids out of 60 and 20 out of 30 choosing those infected with BRNV

+ RLMV under light and dark conditions respectively. The proportion of aphids choosing infected plants in light conditions was significantly different from 0.5 (χ^2_1 = 4.31, P = 0.038, Figure 3.1a). Although a higher proportion of aphids chose the infected plant in dark conditions, the result was not significantly different from 0.5 at the 95% confidence interval (χ^2_1 = 3.40, P = 0.065, Figure 3.1b).

3.3.2 Aphid performance

When reared on plants that were infected with BRNV and RLMV, the time taken for a newly laid first instar *A. idaei* to reach adulthood was significantly longer on BRNV + RLMV–infected plants compared with healthy plants ($F_{1, 18}$ = 4.75, P = 0.043; Figure 3.2a). The development time of *A. idaei* on healthy plants was only 12.3 (± 1.10) days, more than three days shorter than *A. idaei* feeding on infected plants (15.4 ± 0.90 days). Similarly, the time taken by the aphid to reach reproductive maturity and to begin to produce nymphs (pre–reproductive period) was significantly longer on virus–infected plants than on healthy plants ($F_{1, 18}$ = 6.15, P = 0.023; Figure 3.2b) at 16.8 (± 1.19) days compared to the 14.9 (± 1.14) days taken by aphids feeding on healthy host plants. Counts of the number of nymphs produced by an adult aphid over the seven days following the first reproduction on each plant revealed no effect of host plant infection with BRNV and RLMV (Figure 3.3).

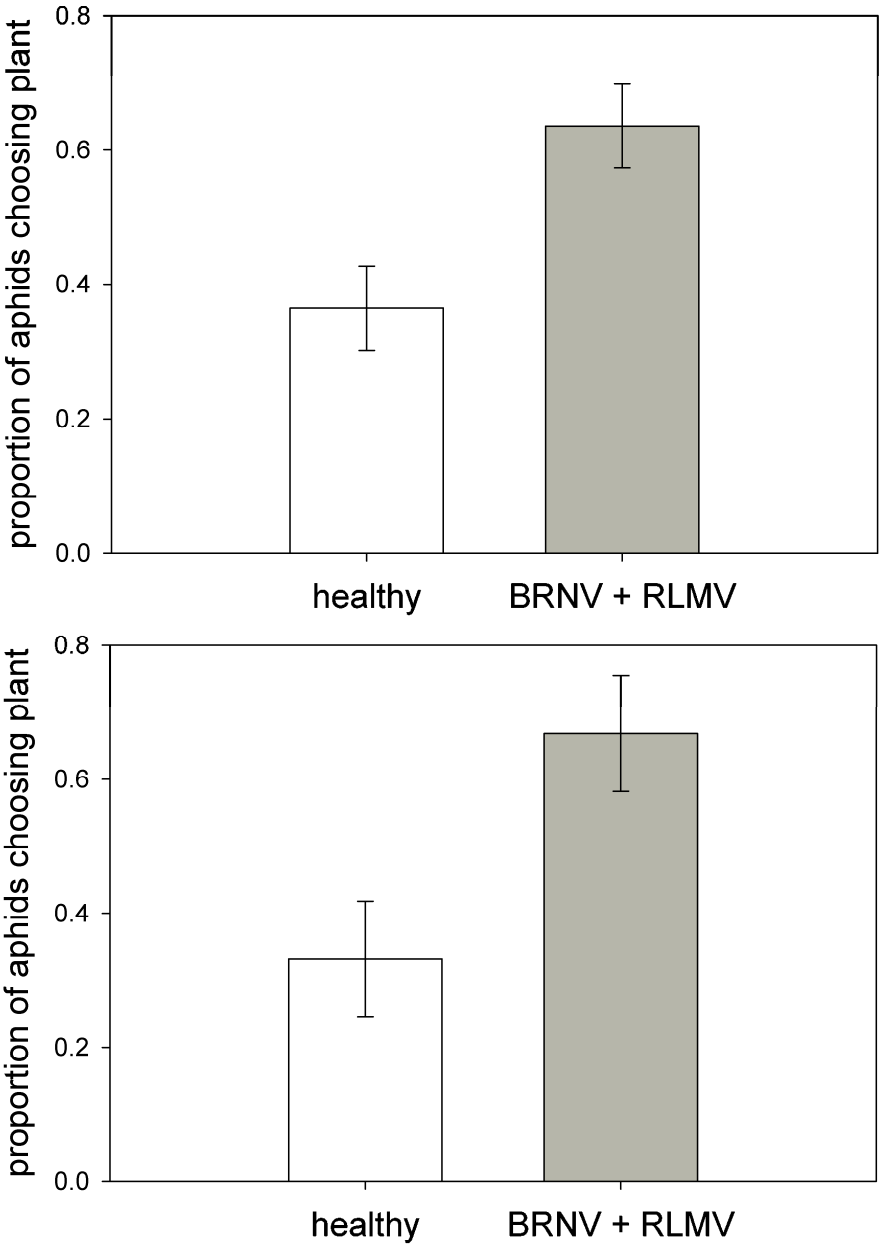


Figure 3.1. Proportion of aphids initially choosing healthy or BRNV + RLMV–infected plants in (a) light conditions, $\chi^2_1 = 4.31$, **P = 0.038** and (b) dark conditions, $\chi^2_1 = 3.40$, P = 0.065. Bars represent response from n = 20 and n = 10 respectfully.

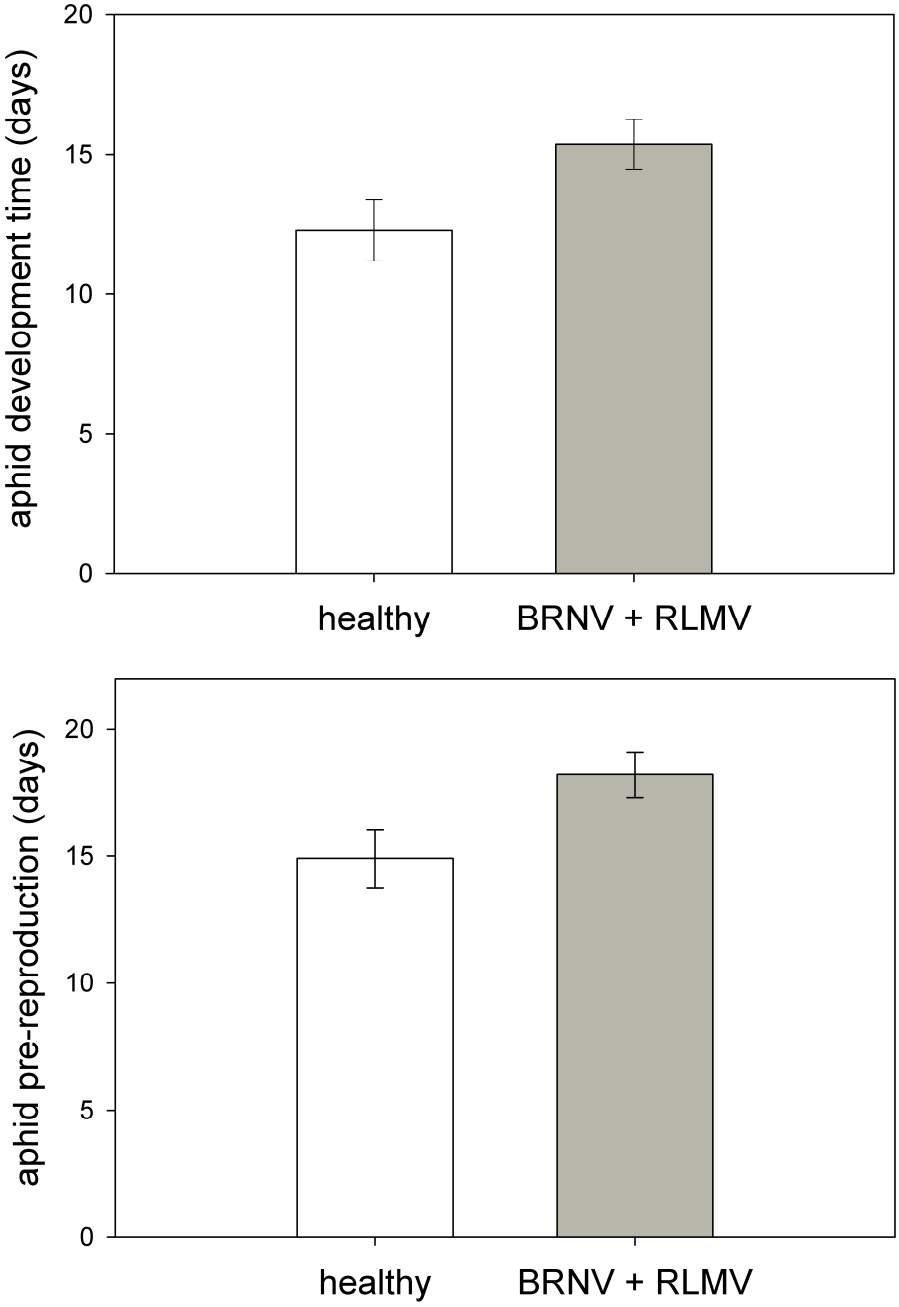


Figure 3.2. (a) *A. idaei* development time in days, $F_{1, 18} = 4.75$, **P = 0.043** and (b) *A. idaei* pre–reproductive period in days, $F_{1, 18} = 6.15$, **P = 0.023**. Mean values of n = 10 ± SEM are shown and back-transformed data are reported in both figures.

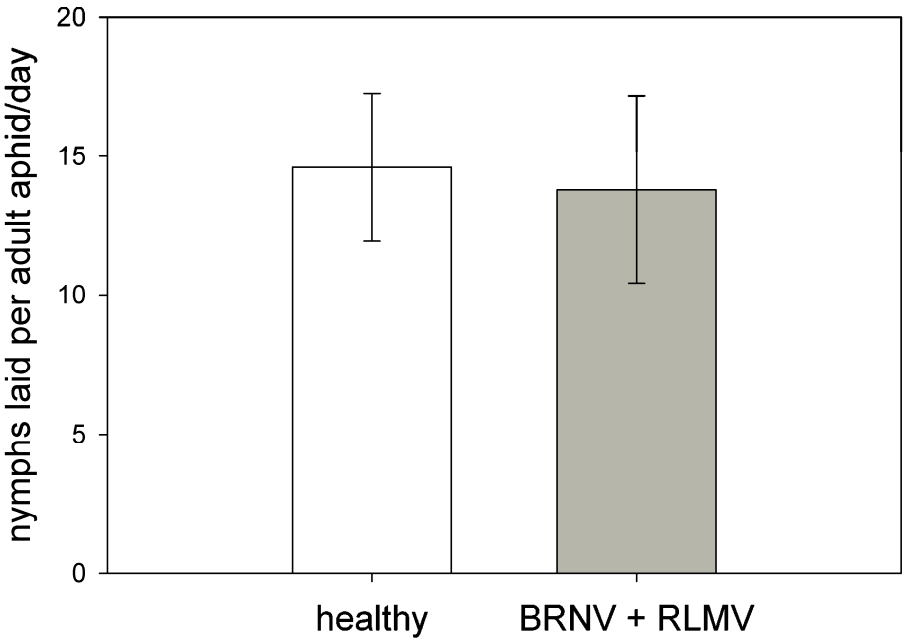


Figure 3.3. Number of nymphs laid by adult *A. idaei* per day, $F_{1, 18} = 0.035$, P = 0.854 Mean values of n = 10 ± SEM are shown.

3.3.3 Aphid movement between plants

3.3.3.1 Over 30 min

Observations of individual aphids released between a leaf of a non-infected and virus infected plant demonstrated that *A. idaei* were found in consistently higher proportions on infected leaves at all sample periods after 2 min of the start of the experiment (Figure 3.4a). The time for which the aphid was exposed to the leaves was found to exert a highly significant effect on aphid preference for virus infected leaves with a tendency for the proportion of aphids on infected leaves to increase significantly over time (Table 3.2).

3.3.3.2 Over seven days

Although a consistently higher proportion of aphids were present on virus-infected plants compared with healthy plants over the entirety of the seven day experiment (Figure 3.5), the proportion of aphids present on the infected plants was not found to differ significantly from 0.5 (see Table 3.2).

Experiment	AIC	Random Effects	Fixed Effects	Estimate	*z* value	P
30 mins	144.5	Arena	Intercept	1.70294	0.747	0.455
			Time	0.26868	4.086	**< 0.001**
7 days	158.1	Cage	Intercept	0.5222	1.064	0.287

Table 3.2. Summary of minimum adequate generalised linear mixed effects models (GLMM) for aphid behaviour assays showing the minimum AIC used for model selection, random and fixed effects specified in the model, model estimates and associated *z* values and probabilities.

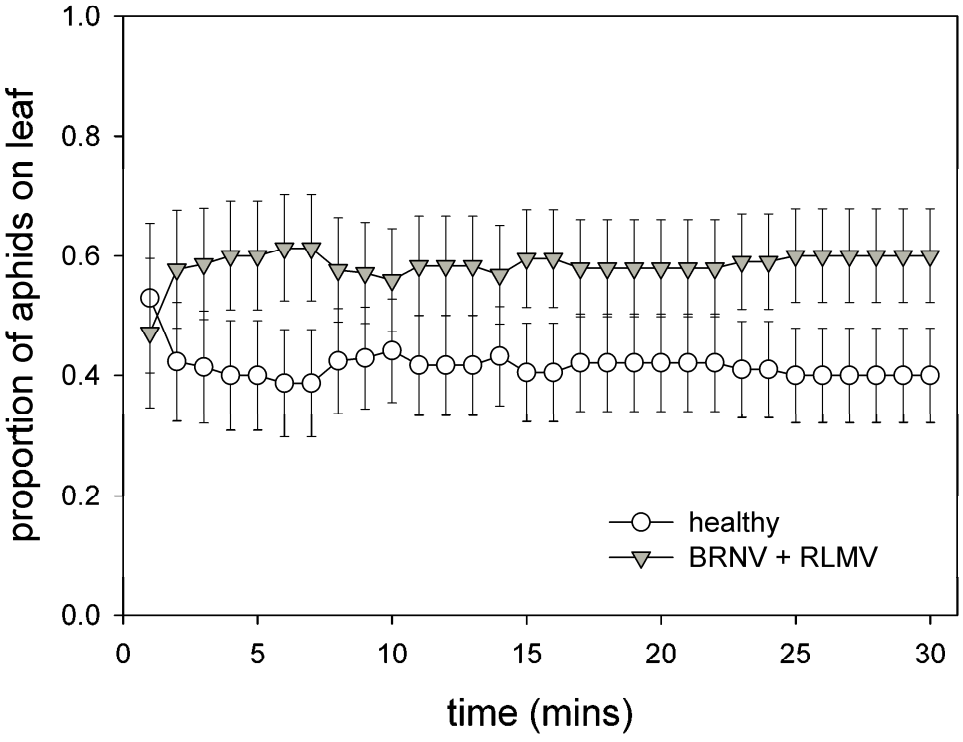


Figure 3.4. Proportion of aphids present on healthy or BRNV and RLMV-infected leaves over 30 minute time period, 1 adult aphid released per arena. Mean values ± SEM are shown. See Table 3.2 for details.

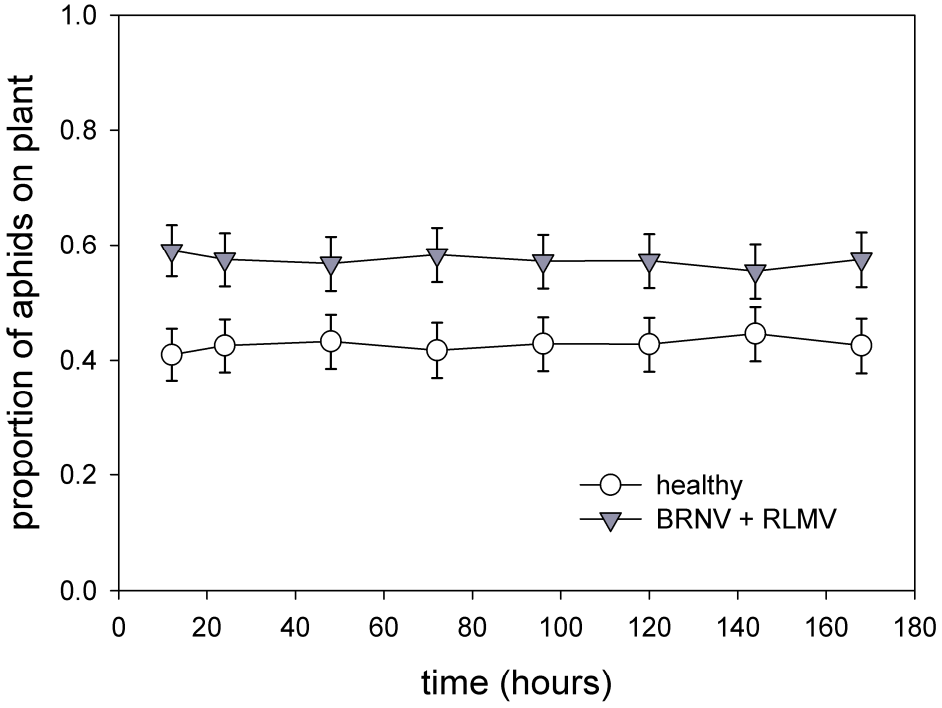


Figure 3.5. Proportion of aphids present on healthy or BRNV and RLMV-infected host plants over 7 d period. Mean values of n = 28 ± SEM are shown. See Table 3.2 for details.

3.3.4 Foliage colouration

Within the range of wavelengths at which absorbance and reflectance of white light was measured (400 – 800 nm), all healthy plants and infected plants exhibited a maximum absorbance in the blue and red regions of the spectrum of light (Figure 3.6a). There were no significant differences in the wavelength of light absorbed by healthy and virus-infected plant leaves between 412 and 712 nm but there were differences at wavelengths of 751.5, 771.3 and 791.2 nm; all values corresponding to the red region of the visible spectrum (Table 3.3). As expected, peak reflectance of both healthy and virus-infected leaves within the visible spectrum occurred at wavelengths corresponding to green light (Figure 3.6b). Analysis of reflectance revealed a significant difference in light reflected from healthy and virus-infected plants, particularly in regions of the spectrum corresponding to blue, green and red light (Table3.3).

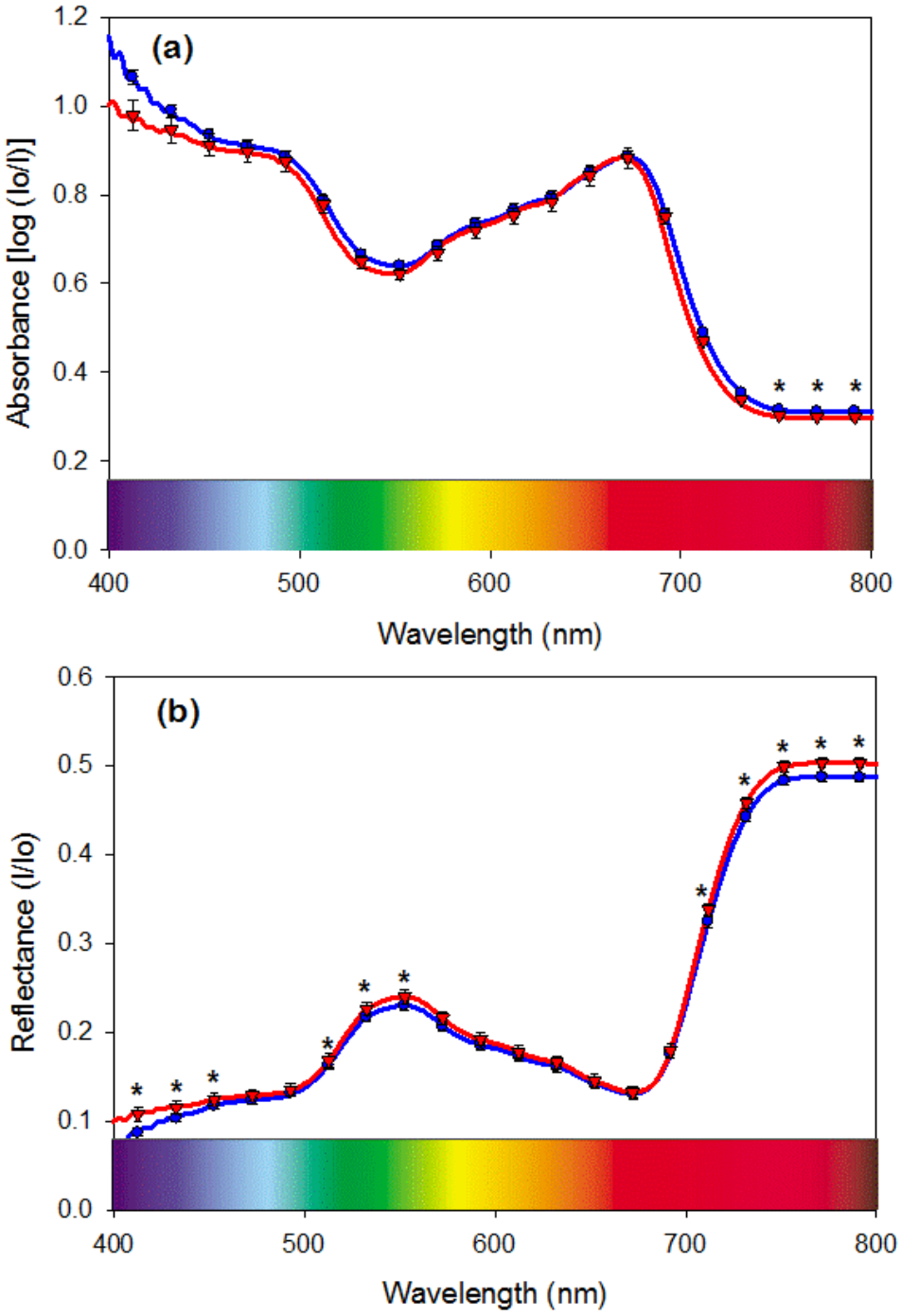


Figure 3.6 (a) Absorbance and **(b)** Reflectance of healthy and virus-infected plant leaves between 400 and 800 nm. Blue lines represent healthy plants and red lines represent virus-infected plants. Mean values of n = 10 ± SEM are shown. Asterisks denote significant differences (see Table 3.3 for details).

Wavelength (nm)	Absorbance					Reflectance				
	Healthy (± SE)	BRNV & RLMV (± SE)	t	d.f.	P	Healthy (± SE)	BRNV & RLMV (± SE)	t	d.f.	P
412.7	1.07 ± 0.017	0.98 ± 0.034	2.31	9	0.038	0.10 ± 0.003	0.11 ± 0.0086	-6.02	9	**<0.001**
432.8	0.99 ± 0.014	0.95 ± 0.028	1.39	9	0.187	0.10 ± 0.003	0.12 ± 0.007	-4.23	9	**0.001**
452.7	0.93 ± 0.013	0.91 ± 0.025	0.73	9	0.476	0.12 ± 0.003	0.12 ± 0.007	-2.95	9	**0.012**
472.7	0.91 ± 0.013	0.90 ± 0.024	0.52	9	0.607	0.12 ± 0.004	0.20 ± 0.007	-2.11	9	0.057
492.7	0.89 ± 0.013	0.86 ± 0.024	0.43	9	0.673	0.13 ± 0.004	0.14 ± 0.007	-1.56	9	0.144
512.7	0.79 ± 0.012	0.78 ± 0.020	0.47	9	0.643	0.16 ± 0.004	0.17 ± 0.007	-3.87	9	**0.001**
532.6	0.67 ± 0.011	0.65 ± 0.015	0.90	9	0.378	0.22 ± 0.005	0.23 ± 0.008	-5.54	9	**<0.001**
552.6	0.64 ± 0.011	0.62 ± 0.014	1.04	9	0.313	0.23 ± 0.006	0.24 ± 0.007	-4.02	9	**<0.001**
572.5	0.69 ± 0.013	0.67 ± 0.015	0.83	9	0.420	0.21 ± 0.006	0.22 ± 0.007	0.70	9	0.495
592.4	0.73 ± 0.014	0.72 ± 0.017	0.60	9	0.555	0.19 ± 0.006	0.19 ± 0.007	2.11	9	**0.049**
612.4	0.77 ± 0.015	0.75 ± 0.019	0.50	9	0.625	0.17 ± 0.006	0.18 ± 0.007	1.13	9	0.273
632.3	0.80 ± 0.015	0.78 ± 0.020	0.42	9	0.683	0.16 ± 0.006	0.17 ± 0.007	1.07	9	0.300
652.2	0.85 ± 0.015	0.84 ± 0.022	0.23	9	0.821	0.14 ± 0.005	0.15 ± 0.007	1.50	9	0.167
672.1	0.89 ± 0.013	0.88 ± 0.024	0.09	9	0.931	0.13 ± 0.004	0.13 ± 0.007	1.61	9	0.125
691.9	0.76 ± 0.012	0.75 ± 0.018	0.25	9	0.802	0.18 ± 0.005	0.18 ± 0.007	-3.83	9	**0.001**
711.8	0.49 ± 0.009	0.47 ± 0.009	1.44	9	0.166	0.33 ± 0.007	0.34 ± 0.007	-6.40	9	**<0.001**
731.7	0.35 ± 0.005	0.34 ± 0.006	2.05	9	0.055	0.44 ± 0.005	0.46 ± 0.006	-6.47	9	**<0.001**
751.5	0.32 ± 0.004	0.30 ± 0.005	2.14	9	**0.047**	0.48 ± 0.004	0.50 ± 0.006	-6.36	9	**<0.001**
771.3	0.31 ± 0.004	0.30 ± 0.005	2.17	9	**0.043**	0.49 ± 0.004	0.50 ± 0.006	-3.97	9	**<0.001**
791.2	0.31 ± 0.004	0.30 ± 0.005	2.15	9	**0.045**	0.49 ± 0.004	0.50 ± 0.006	-2.23	9	**0.039**

Table 3.3. Statistical analysis of absorbance and reflectance of healthy and virus-infected leaves using paired t-tests. Table shows mean values of n = 10 ± SE and accompanying critical values of t, degrees of freedom (d.f.) and probability (P) for each comparison.

3.4 Discussion

In aphid initial choice tests, *A. idaei* showed a significant preference for plants that were infected with BRNV and RLMV over those that were healthy. These findings are consistent with several other studies of aphid preference for diseased host plants (e.g. (Castle & Berger, 1993; Eigenbrode *et al.*, 2002; Mauck *et al.*, 2010). Aphids are known to exhibit a phototactic response to reflected light from host plants (Powell *et al.*, 2006) and many studies attribute aphid preference for virus–infected plants to the visual attraction of symptomatic yellow foliage (Fereres *et al.*, 1999; Hodge & Powell, 2008). The absorbance of light from healthy and virus-infected plants as measured by spectral analysis in this study showed that there were discrete differences in the wavelength of light absorbed by the leaf (Figure 3.6a) and the wavelength of light reflected (Figure 3.6b). As apterous *A. idaei* exhibited a preference for virus infected plants in light conditions, it is reasonable to assume that they may be attracted by a visual cue.

The eyes of herbivorous insects, including aphids, are known to possess three colour receptors which are sensitive to UV, blue or green light (see Chittka & Doring, 2007). In addition, the peach-potato aphid, *Myzus persicae* has been shown to possess a UV receptor with a peak sensitivity of 330 nm and a green receptor with a peak sensitivity of 530 nm (Kirchner *et al.*, 2005). Although photoreception of *A. idaei* has not yet been determined, if it is similar to that of *M. persicae* then the results of this study show that there are significant differences in the reflectance of light from healthy and virus-infected raspberry plants at the closest wavelength to that of peak sensitivity in *M. persicae* (wavelength = 532.6 nm, $t = -5.00$, $P < 0.001$; Table 3.2) and indeed throughout the green region of the

spectrum of light. Although, the influence of foliage colouration was negligible in this short-term laboratory experiment, it may be important for host plant location by *A. idaei* in field conditions and, as virus-infected plants measured in this study showed a greater reflectance of green light, it may be the case that these plants would be more apparent to the aphid at long distance. Additionally, as the virus infection progresses, visible symptoms develop on the leaves. For example, infection of red raspberry with BRNV and RLMV often results in the production of angular yellow patches on the leaves (see Chapter two; section 2.3.1) and yellow-green light has been shown to be the most attractive for other aphid species (Prokopy & Owens, 1983). Due to the high selection pressure acting on aphids, colour intensity may be used by the aphid as a method of discriminating between good quality hosts and poor ones. Significant differences between reflectance of light corresponding to the blue and red regions of the spectrum were also found in my study which may also influence *A. idaei* host selection at long distance however, until a red receptor is discovered in an herbivorous insect, the role of vivid red autumn colouration as a warning signal to herbivores (Hamilton & Brown, 2001) will remain unresolved.

In darkness, where the detection of reflected light by the aphid would have been impossible, there was a trend towards a preference for virus infected plants ($P = 0.065$). This particular experiment used only half the number of trials of that conducted in light conditions and it is therefore likely that an increased number of replicates would have resulted in a significant result. In conclusion, it is not possible to rule out the role of an olfactory cue mediated by changes in volatile compounds as in other plant pathogen –

aphid interactions (Eigenbrode *et al.*, 2002; Jimenez-Martinez *et al.*, 2004; Mauck *et al.*, 2010).

My studies showed that, after the initial attraction of the *A. idaei* to plants infected with BRNV and RLMV (Figure 3.4), they exhibited a significant preference for the infected plants over a 30 min period. Indeed, the proportion of aphids found on infected leaves was found to significantly increase over the 30 min the aphid was exposed to the two plants. However, the study which was conducted over a seven day period indicated no such significant preference for virus infected plants between 12 and 168 hours (Figure 3.5). This finding has several important implications as the attraction of the aphid to these plants appears to be deceptive and this is reflected in *A. idaei* performance parameters when forced to feed on virus–infected plant tissue. Newly laid instar I *A. idaei* took longer to develop to adulthood on plants infected with BRNV and RLMV than when compared to those which fed on healthy control plants. The time taken to reach reproductive maturity and to begin to produce offspring was subsequently longer and, although no difference was observed in the total number of offspring produced on each type of plant, the development times would undoubtedly have knock–on effects for total aphid population growth. It may actually be less costly to the aphid to invest energy in moving to a more suitable host.

Similar antagonistic effects of plant viruses on insect vectors have been found previously. For example, Donaldson & Gratton (2007) demonstrated that the population growth rate of *Aphis glycines* on soybean infected with Alfalfa mosaic, Soybean mosaic or Bean

pod mottle viruses, was reduced by an average of 20% and Mauck *et al.*, (2010) reported that squash plants infected with Cucumber mosaic virus supported lower populations of *Aphis gossypii* in the field. It may be argued that a pathogen–induced reduction in host suitability is actually of benefit to BRNV and RLMV in this instance as the semi–persistent nature of their transmission means that *A. idaei* need only feed on the virus-infected plant for approximately 30 min in order to become viruliferous. However, the mechanism of subsequent transmission remains unresolved. It is likely that the vector migrates between the 30 min and 12 h time periods recorded in these experiments and that this relocation was missed by the first sampling period of 12 h in the seven day experiment. A more frequent period of experimental observation (e.g. counts every hour from 0 to 12 h) may therefore be beneficial for the purposes of future studies.

Whatever the mechanism, be it physical or chemical, it is clearly detrimental for *A. idaei* to feed on virus–infected tissue when healthy raspberry plants are available and although this study did not measure longevity and fecundity of *A. idaei* for the entirety of the aphid's lifespan, the prolonged development time is likely to not only have an impact on overall population growth but could also have indirect implications for vector survival through an increased period of susceptibility to natural enemies and predators. The work of Alliaume *et al.* (2010) showed that the larval developmental stages of the seven–spot ladybird, *Coccinnella septempunctata*, exhibited a preference for smaller development stages of *A. idaei* in choice tests conducted in the laboratory. Although the behavioral response of the beetles requires further testing under field conditions, it is highly likely that a similar behaviour would be observed in plantations and further corroborate the slow-

growth, high-mortality hypothesis (Feeny, 1970; Clancy & Price, 1987; Williams, 1999). Similarly, juvenille developmental stages of *A. idaei* are less effective than adults at evading attack by the parasitic wasp, *Aphidius ervi* (Mitchell et al., 2010) and is therefore beneficial for the aphid to reach adulthood quickly. These insects are just two examples of a number of predators/natural enemies known to be naturally occurring in raspberry plantations and their presence would be therefore highly likely to impact aphid population size on both healthy and virus–infected plants. Thus, it is important to bear in mind that plant infection with viral pathogens not only has impacts for the insects that act as vectors, but the consequences of induced changes to host plant chemistry may be more far reaching and impact upon the population dynamics of higher trophic groups – particularly with changes in management practices which now tend to enclose raspberry plants under polytunnels and therefore also enclose insect populations (Mitchell et al., 2010).

The plant–mediated interactions between viral pathogens and aphids occurring in raspberry, and indeed other plant systems consist of several sequential stages. First, pathogen–induced changes to the host plant alter the visual and/or olfactory signals emitted by the host plant. This is probably in the form of increased volatile emissions providing a stronger or more easily detectible signal which attracts aphids preferentially to the diseased plant. Secondly, pathogen–induced changes to contact or gustatory cues detected by the aphid when it alights on the plant may operate through physical or chemical changes to the leaf surface which may facilitate probing and feeding. Finally, the pathogen may induce alterations to host plant nutritional chemistry which may induce the aphid to remain (arrest) on the plant and feed for prolonged periods

(Eigenbrode et al., 2002; Jimenez-Martinez et al., 2004; Sriniνasan et al., 2006) or might trigger aphid migration through the provision of a nutritionally poor host (Mauck et al., 2010). This study has addressed the first and last of these stages and identified the initial attraction of *A. idaei* to raspberry plants infected with BRNV and RLSV and characterised aphid behaviour subsequent to this host plant choice in the form of rapid migration away from the diseased plant and poorer development when forced to feed on virus–infected tissue. The next step is to further elucidate the mechanisms that may be responsible for these observations. In particular, the identification of altered volatile signals emitted from virus–infected raspberry plants is discussed in Chapter Five of this thesis while investigations of plant leaf chemistry are discussed in Chapter Four.

Chapter Four

Aphids and viral pathogens induce changes in *Rubus ideaus* leaf chemistry

Abstract

In order to investigate potential causal mechanisms for the poorer performance of *A. idaei* on raspberry plants infected with BRNV and RLMV compared with healthy plants, a series of chemical analyses were conducted on *R. idaeus* leaf tissue with the objective of exploring virus-induced changes to leaf chemical composition. These chemical analyses were conducted on freeze dried and milled leaf material excised from plants used in aphid choice and performance experiments. Leaf phenolic compounds were quantified to explore defensive responses of raspberry plants to aphid and virus attack and leaf carbon, nitrogen and amino acids were analysed to investigate potential differences in nutritional quality for the aphid.

Extraction of leaf phenolic compounds revealed that aphids feeding on healthy plants increased the level of leaf phenolic compounds, suggestive of a defensive response by the plant, but that aphids feeding on virus-infected plants failed to elicit a similar response. Aphid feeding actually led to a decrease in phenolic content in these plants. This decrease may be evidence of a virus-induced facilitation of aphid feeding.

Analysis of leaf carbon and nitrogen concentration by flash combustion showed that although the carbon concentration was significantly elevated in plants infected with BRNV and RLMV compared with healthy plants, leaf nitrogen did not differ. Although the subsequent C:N ratio calculated was higher in virus-infected plants, it did not differ significantly from that of healthy plants.

Quantification of free amino acids using HPLC demonstrated that their concentration in plants infected with BRNV and RLMV increased by over 200% from 8.45 μM g^{-1} in virus-free plants to 29.14 μM g^{-1}. The amino acid content of raspberry was dominated by the non-essential glutamate (65% of total in healthy plants) which further increased in response to plant infection with BRNV and RLMV (77% of total). High relative concentrations of glutamate have been previously suggested as being indicative of nutritionally poor host plants for aphids.

4.1 Introduction

4.1.1 Rationale

As was demonstrated in Chapter Three, the large raspberry aphid, *Amphorophora idaei*, is attracted to raspberry plants that are infected with BRNV and RLMV. This choice appears to have detrimental consequences for the aphid as when forced to feed on raspberry plants infected with these pathogens, *A. idaei* performs less well and takes longer to develop compared with *A. idaei* feeding on healthy raspberry. However, when given a free choice between host plants, despite an initial attraction to virus-infected host plants, the preference was short lived and the proportion of aphids on these plants was not significant after 12 h. The mechanism(s) that are responsible for these behaviours are likely to be related to pathogen induced changes in plant nutritional chemistry and the aim of this chapter was to identify specific changes to leaf chemistry which may explain the aphid behaviour and performance observed in the experiments that are detailed in Chapter Three.

4.1.2 Plants as resources for insects and pathogens

Aphids are phloem feeders and the phloem sap diet provides them with a concentrated solution of carbohydrate and a low concentration of amino acids (Dixon, 1998). Aphids require carbon, in the form of sugars, as an energy source and amino acids for protein metabolism (Rhodes *et al.*, 1996) but are understood to be limited by plant nitrogen (Dixon, 1998) and in particular, by the low availability of dietary amino acids (Douglas, 1993). Studies of aphid physiology using artificial diets have demonstrated the importance of these compounds for aphid growth (Douglas, 1998). Aphids, like all

animals, are unable to synthesise particular amino acids that are required for protein synthesis (essential amino acids) (Morris, 1991) and they therefore must acquire these in their diet or from symbionts. The ratio of essential to non–essential amino acids in the aphid phloem–sap diet is very low (Douglas, 1993) and in order for aphids to meet the high metabolic demand to fuel their characteristically high growth rate, they have formed symbioses with micro–organisms to provide them with additional essential amino acids (Buchner, 1965). Most aphids possess the symbiotic micro–organism *Buchnera aphidicola* and a recent study to quantify plant phloem amino acids revealed that this symbiosis is sufficient to meet the nutritional demand of the aphid when reared on artificial diets lacking individual component amino acids which would normally be available to them when feeding on their host plant (Gunduz & Douglas, 2009).

Plant pathogens are also reliant on the host plant metabolism to synthesise particular resources that they require in order to multiply and be transmitted to new hosts. For example, like aphids, plant viruses require amino acids which they use to synthesise new viral protein during replication and they are entirely dependent on the host plant metabolism to synthesise these components (Hull, 2002). Overlapping requirements for these resources inevitably leads to competition between insects and viruses for these resources (Fiebig *et al.*, 2004).

4.1.3 Plant mediated interactions between pathogens and insect herbivores

Plants are capable of mounting a wide array of defensive responses to pathogen infection and herbivory (Stout *et al.*, 2006). The induction of a defensive response to a first attacker may have implications for a secondary attacker through alterations to plant physiology or biochemical pathways which may alter the chemical composition of the leaf. For example, Kluth *et al.*, (2002) showed that although the aphids *Aphis fabae* and *Uroleucon cirsii* mechanically transmitted the rust fungus, *Puccinia punctiformis*, they interacted differently with the pathogen compared with the herbivorous beetle, *Cassida rubignosa* which is also a vector for the fungus. Aphids were shown to perform better on host plants infected with the pathogen, while development of juvenile beetles was impaired and adult biomass reduced. This study therefore demonstrates the variable effects of plant pathogen infection on insect vectors with one seemingly mutualistic interaction and one antagonistic interaction. Thus, plant–mediated effects on insects must be taken into account when attempting to study tri–lateral interactions such as those occurring between *A. idaei* and BRNV and RLMV on host raspberry plants. Many studies have successfully characterised insect behaviour on host plants that are infected with pathogens, but studies that actually investigate the underlying changes to plant physiology that may underpin the interaction are few in number. Knowledge of these chemical mechanisms is extremely important for furthering our understanding of host plant mediated interactions between pathogens and their vectors, particularly chemical mechanisms that may affect pathogen epidemiology through promoted or degraded insect performance which has implications for the spread of disease in natural systems.

4.1.4 Potential chemical mechanisms

As aphids are limited by dietary nitrogen and low concentrations of amino acids in the phloem sap diet, these nutrients may be important factors in determining the underlying plant chemistry which may act as the causal mechanism for changes in insect performance on host plants infected with pathogens. Fiebig *et al.* (2004) reported a significant reduction in total amino acids in response to wheat infection with Barley yellow dwarf virus (BYDV). This decrease had a knock–on effect for the cereal aphid, *Sitobion avenae*, which was shown to be less efficient at utilising the plant phloem sap and subsequently exhibited a lower intrinsic rate of increase on BYDV–infected host plants. Similarly, Johnson *et al.* (2003) showed that infection of birch leaves with a fungal pathogen, *Marssonina betulae,* led to a higher concentration of free amino acids in symptomatic leaves where performance of the birch aphid, *Euceraphis betulae*, was enhanced. In addition to pathogen–induced changes in amino acid composition, all higher plants produce allelochemicals such as polyphenols and therefore all herbivorous insects encounter these chemicals when feeding (Schoonhoven *et al.*, 2005). Many polyphenolics, e.g. catechin, are strong deterrents to insect feeding or act as insect toxicants. The studies of Abouzaid *et al.* (1993) demonstrated the detrimental effect of catechin on the performance of the European corn borer, *Ostrinia nubilalis*. Specifically, this antioxidant flavonol was shown to significantly impede larval development of the insect. The presence of such feeding deterrents in raspberry tissue is an important consideration for investigation of *A. idaei* performance as enzymes in aphid saliva have been found to be closely related to plant defence against aphids (Miles, 1999). For example, probing by aphids can cause localised accumulation of polyphenols in the leaf which may disrupt aphid digestion, but these are usually counteracted by enzymes in the

aphid's saliva which detoxify harmful plant defence compounds (Miles, 1999; Ma *et al.*, 2010). *Rubus* species are known to be rich in phenolic antioxidants (Deighton *et al.*, 2000) but most studies focus on the properties of berries due to their benefits for human health. Few studies have addressed the content of these compounds in leaves and their effects on herbivores that feed on raspberry, such as *A. idaei*.

4.1.5 Aims and hypotheses

The aim of this study was to investigate potential chemical mechanisms that could be responsible for the poorer performance of *A. idaei* on raspberry plant infected with BRNV + RLMV. In order to achieve this aim, four different chemical analyses were performed on raspberry leaf tissue. The specific aim was to quantify changes in levels of leaf phenolics, carbon, nitrogen and amino acids which may affect the response of *A. idaei* on when feeding on plants infected with BRNV and RLMV. Chemical analyses were as follows:

1. quantification of leaf polyphenols
2. quantification of leaf carbon and nitrogen content
3. identification and quantification of leaf free amino acids

These analyses were conducted to test the following hypotheses which were based on the reduced performance of *A. idaei* on virus infected plants:

1. production of polyphenolic compounds would be enhanced in response to infection with BRNV and RLMV and/or aphid feeding (defensive response)

2. leaf nitrogen would be decreased in plants infected with BRNV + RLMV, resulting in an increased C:N ratio

3. plants infected with BRNV and RLMV would have a lower concentration of amino acids than healthy plants.

4.2 Materials and methods

Leaves from plants used in the aphid performance experiments and individual aphid choice tests (over 30 min) were freeze dried and milled to a fine powder for all chemical analyses detailed below (see Chapter three). These plants included controls that were not exposed to aphids and a subset of six plants that had been exposed to aphids for approximately three weeks. In these cases, the aphid population never exceeded 35 aphids (adult plus offspring). Analysis of leaf C and N content was conducted on the leaves from aphid choice tests while analysis of leaf phenolics and amino acids was carried out on control plants from the aphid performance experiment (Chapter Three, Section 3.2.5) which consisted of 10 healthy plants and 10 plants infected with BRNV + RLMV that had been exposed to identical growth conditions as plants that had been inoculated with aphids during the experiment. Phenolic and amino acid analyses were also performed on a subset of six plants that had been exposed to aphids. All plants were harvested for analysis at the same time after the completion of the experiment.

4.2.1 Leaf phenolics

Analysis was carried out using the enzymatic method described by Stevanato *et al.*, (2004), which has the advantage of not being affected by interfering substances such as ascorbate, citrate and sulfite (Stevanato *et al.*, 2004). Extractions were conducted in a 10:1 ratio (leaf material to methanol) from 50 mg freeze dried material in 50% methanol at 80°C for 2.5 hr. The aqueous phase was removed and cleared by centrifugation. An enzymatic reaction was set up using 50 µl of supernatant mixed with 740 µl potassium phosphate buffer (pH 8.0), 100 µl 4–aminophenazone, 100 µl hydrogen peroxide and 10 µl horse radish peroxidase. The reaction was incubated at room temperature for 15 min and absorbance read at a wavelength of 500 nm. Absorbance data were converted to catechin equivalents using a standard curve produced by serial dilution (0 – 0.10 mg ml^{-1} catechin). All chemicals were obtained from Sigma–Aldrich (Dorset, UK).

4.2.2 Carbon and nitrogen

Carbon and nitrogen concentrations were analysed using flash combustion and chromatographic separation of approximately 2 mg of ground and homogenised plant material using an Exeter Analytical CE440 Elemental Analyzer (EAI, Coventry, UK). Combustion of the weighed sample occurred in pure oxygen using helium to carry the combustion products through the analytical system. The C and N concentrations of samples were calculated using standards (Acetanilide) with known C and N concentrations.

4.2.3 Quantification of leaf free amino acids

Free amino acids were extracted from 50 mg freeze–dried and milled leaf material in 80% HPLC grade methanol (Fisher Scientific). After addition of 1 ml of 80% methanol, samples were vortexed vigorously and placed on a shaking plate for 1 h to provide continuous agitation. The mixture was then centrifuged for 15 min at 10,000 rpm at 4 ^{o}C and the supernatant stored in a fresh tube. A second 1 ml aliquot of 80% methanol was then added to the plant pellet and a second extraction carried as described above. The supernatants were then pooled and mixed thoroughly by vortexing. A 1 ml aliquot of the extraction was transferred to a new tube and dried under vacuum until just dry. The resulting pellet was eluted in 1 ml Milli–Q water and stored temporarily at –20 °C.

Amino acids were separated using reverse phase HPLC after derivitisation using *o*–phthaldialdehyde (Jones, *et al.* 1981) using a Hewlett Packard autosampling LC system with Zorbax XDB-C_{18} column and fluorescence detection. Amino acids were quantified by comparison with AA–S–A18 (Sigma) reference mixture supplemented with asparagine, glutamine and tryptophan. All protein amino acids, with the exception of proline and cysteine, could be quantified using this method.

4.2.4 Statistical analyses

Parametric statistical tests were used where possible on data determined to be normally distributed (Shapiro–Wilk test). In order to investigate potential interactions occurring between plant viruses and aphids, leaf phenolics and amino acids were analysed using a two–way ANOVA with plant type (healthy or infected) and aphid treatment as factors.

Some amino acid data (asparagine, serine, glycine, arginine, histidine, lysine, methionine, tryptophan and valine) required $\log_{10}$ transformation in order to meet assumptions of normality (Shapiro-Wilk test). Carbon, nitrogen and C:N were analysed using a t-test or non-parametric equivalent, Mann-Whitney U as indicated. All figures show back transformed means and standard errors.

4.3 Results

4.3.1 Phenolics

Extraction of leaf phenolic compounds and comparison of absorbance with a serial dilution of catechin showed that although the concentration of phenolics was slightly elevated in control plants infected with BRNV + RLMV, the difference was not significant when compared with healthy control plants. However, the phenolic content of healthy plants that had been inoculated with adult *A. idaei* showed a significant increase compared with both healthy and virus–infected control plants that received no aphid treatment. Furthermore, plants infected with BRNV + RLMV which were inoculated with adult *A. idaei* showed a significant reduction when compared with healthy plants also inoculated with aphids but no difference was observed between either of the controls. There was a significant interaction between the presence of virus in the leaf tissue and aphid feeding (Figure 4.1).

4.3.2 Carbon and nitrogen

The carbon content of healthy leaves was 359.19 (± 10.650) mg g^{-1} of leaf dry weight. This measurement was lower than that found in leaves infected with BRNV + RLMV

which was calculated to be 420.14 (± 3.058) mg g^{-1} of leaf dry weight. This difference was determined to be highly statistically significant (Mann-Whitney U = 0.00, T = 55.0, P < 0.001, Figure 4.2a). A similar relationship was not found for leaf nitrogen. Although healthy plant leaves were found to have a slightly lower nitrogen concentration per gram of leaf dry weight compared with leaves infected with BRNV + RLMV (41.47 ± 2.224 mg g^{-1} c.f. 44.42 ± 2.409 mg g^{-1}), the difference was not significant at the 95% confidence interval (t = -0.898, d.f = 18, P = 0.381, Figure 4.2b). Similarly, the C:N ratios subsequently calculated showed that although healthy leaves had a slightly lower C:N ratio, calculated as 8.80 ± 0.377, it did not differ significantly from the ratio of 9.68 ± 0.467 of virus-infected leaves (t = -1.467, d.f = 18, P = 0.160, Figure 4.2c)

4.3.3 Quantification of leaf free amino acids

The total concentration of free amino acids in healthy plant leaves was 8.45 ± 1.574 µM g^{-1} which was lower than in plants infected with BRNV and RLMV where the total amino acid concentration was calculated to be 29.14 ± 5.173 µM g^{-1}; an increase of approximately 240%. There was no effect of aphids on the concentration of free amino acids ($F_{1, 28}$ = 0.246. P = 0.624) (Figure 4.3a). The essential amino acids had a total concentration of 0.91 ± 0.106 µM g^{-1} (14%) in healthy plants and 1.58 ± 0.179 µM g^{-1} (8%) in plants infected with BRNV + RLMV. Again, the presence of aphids feeding on leaves did not result in a significant effect on the content of leaf essential amino acids

($F_{1, 28}$ = 0.047, P = 0.428) (Figure 4.3b).

The dominant amino acid found in raspberry leaf tissue was the non–essential glutamine at a concentration of 6.19 ± 1.349 μM g^{-1} (65% of total) in healthy plants, and 24.90 ± 4.846 μM g^{-1} (77% of total) in plants infected with BRNV + RLMV. Another non–essential amino acid, aspartate, was the second most abundant and was found at a concentration of 0.64 ± 0.089 μM g^{-1} in healthy plants and 1.33 ± 0.165 μM g^{-1} in BRNV and RLMV infected plants.

Infection of raspberry plants with BRNV and RLMV had a significant effect on the concentrations of five of the eight non-essential amino acids quantified; aspartate (4.4a), aparagine (4.4b), tyrosine (4.4g), alanine (4.4h), and glutamate (4.4i) and one of ten essential amino acids, isoleucine (4.4k), were significantly elevated in response to virus infection and aphid feeding was found to significantly increase the concentration of tyrosine in otherwise healthy plants (4.4g). In addition, there was an interactive effect of virus infection and aphid feeding on the concentration of the essential amino acids arginine (4.4e) and methionine (4.4n). No significant effect of virus infection and/or aphid feeding was found for serine (4.4c), glutamine (4.4b), glycine (4.4f), histidine (4.4j), leucine (4.4l), lysine (4.4m), phenylalanine (4.4o), threonine (4.4p), tryptophan (4.4q) or valine (4.4r).

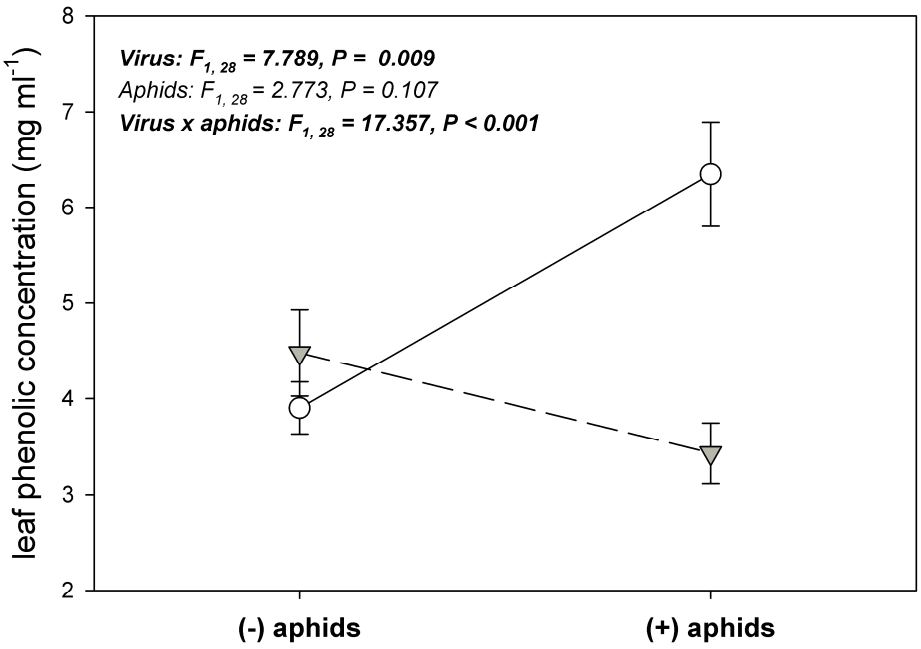


Figure 4.1. Leaf phenolic concentrations of virus–infected and healthy plants, with or without aphids feeding. White circles represent healthy plants and grey triangles represent virus-infected plants. Mean values of n = 6–10 ± SEM are shown. Significant effects ($P < 0.05$) are highlighted in bold.

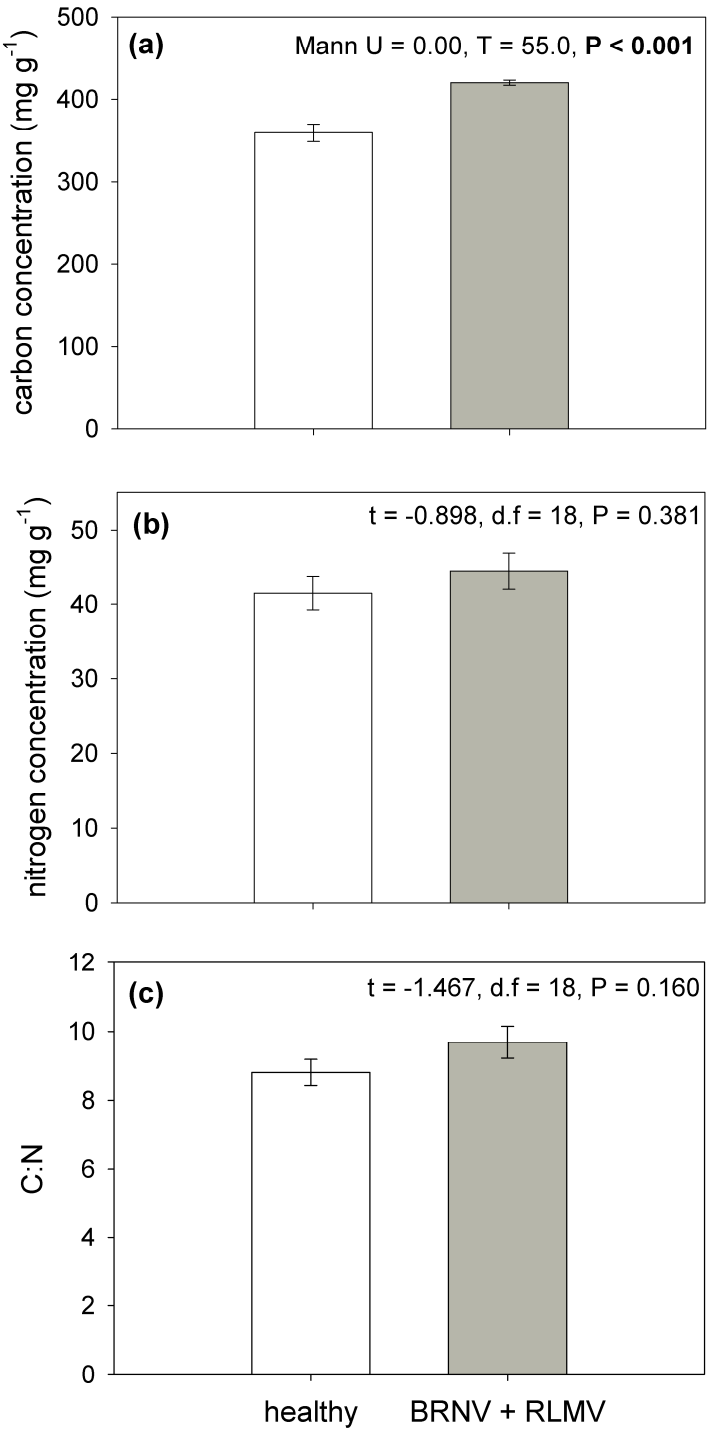


Figure 4.2 **(a)** carbon concentration, **(b)** nitrogen concentration and **(c)** C:N ratio of healthy and virus infected raspberry leaves. All figures show mean values of n = 10 ± SEM.

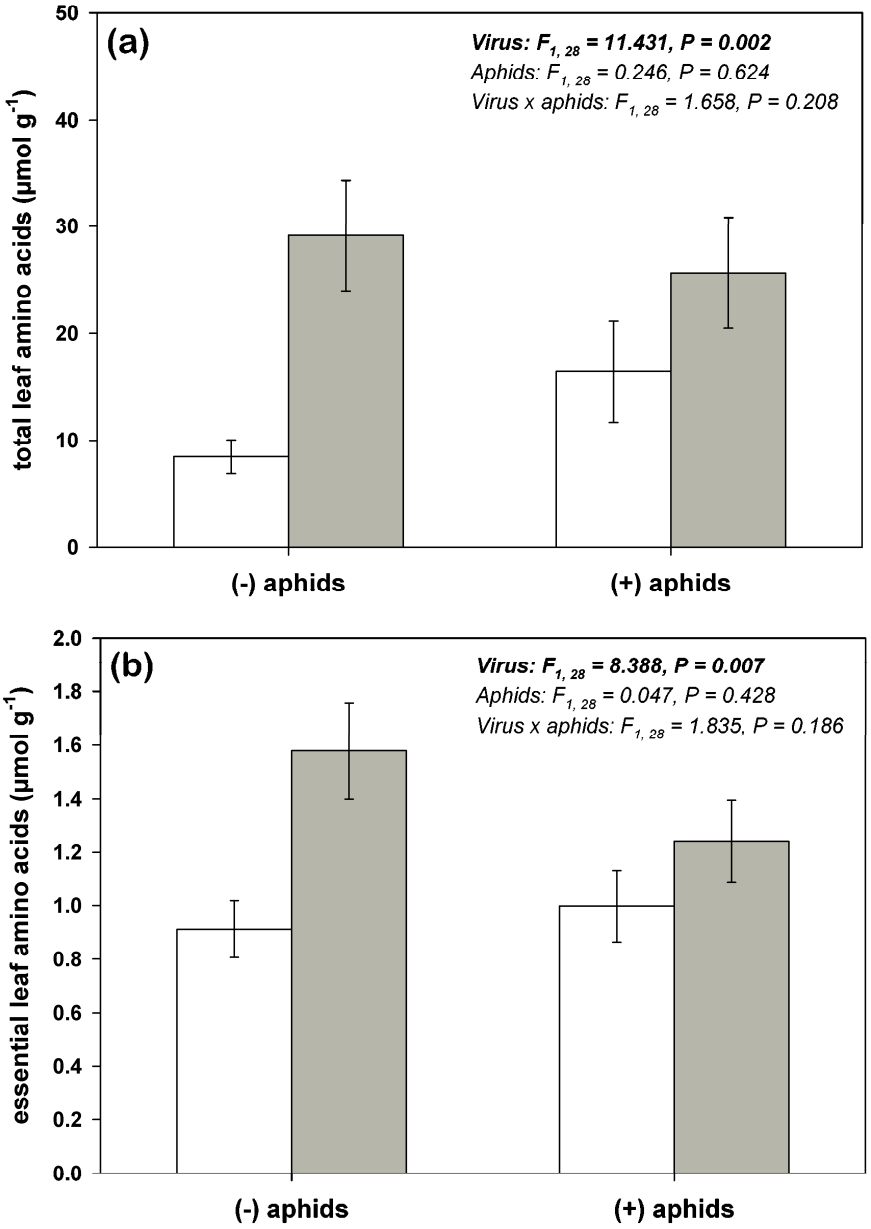
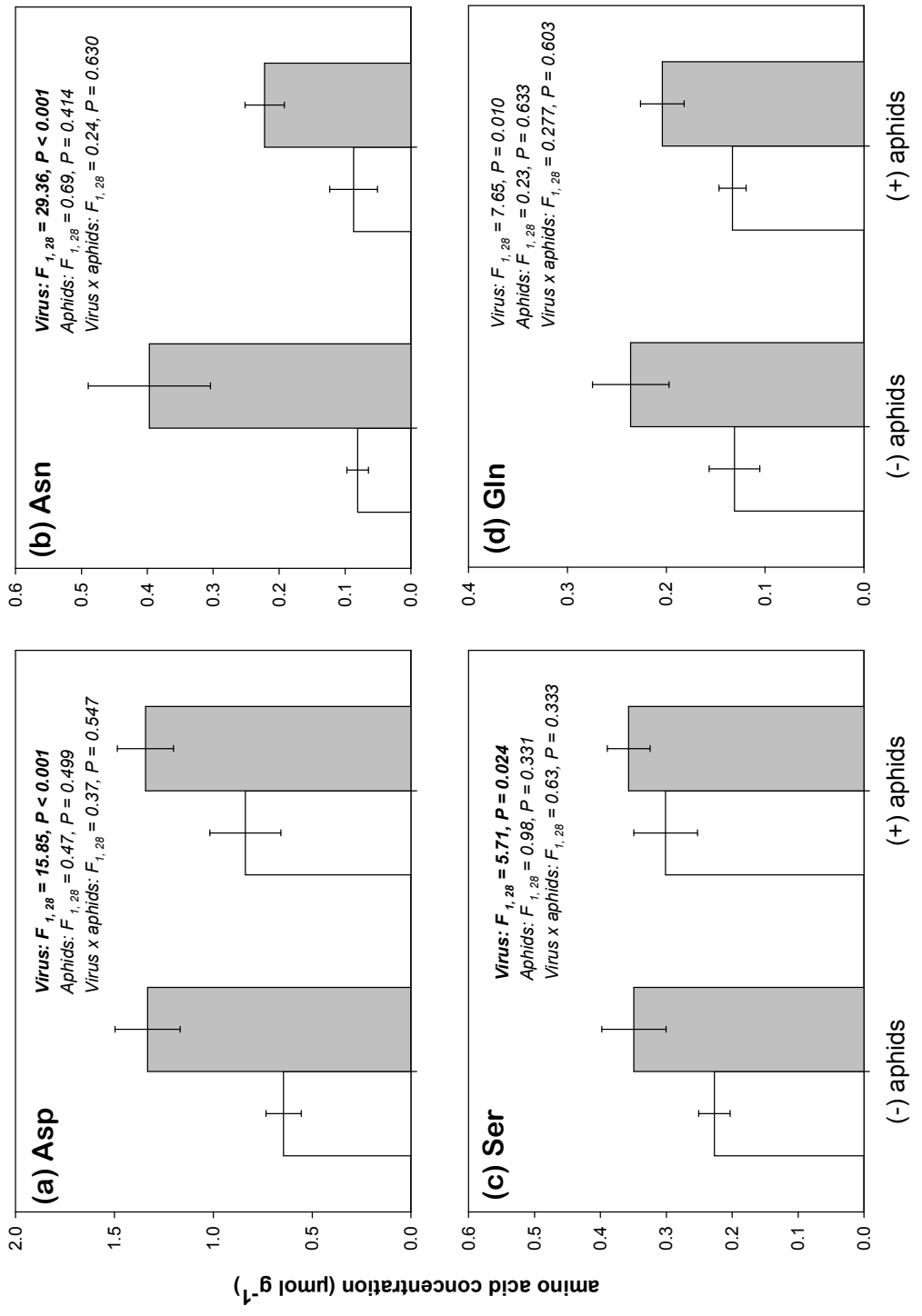
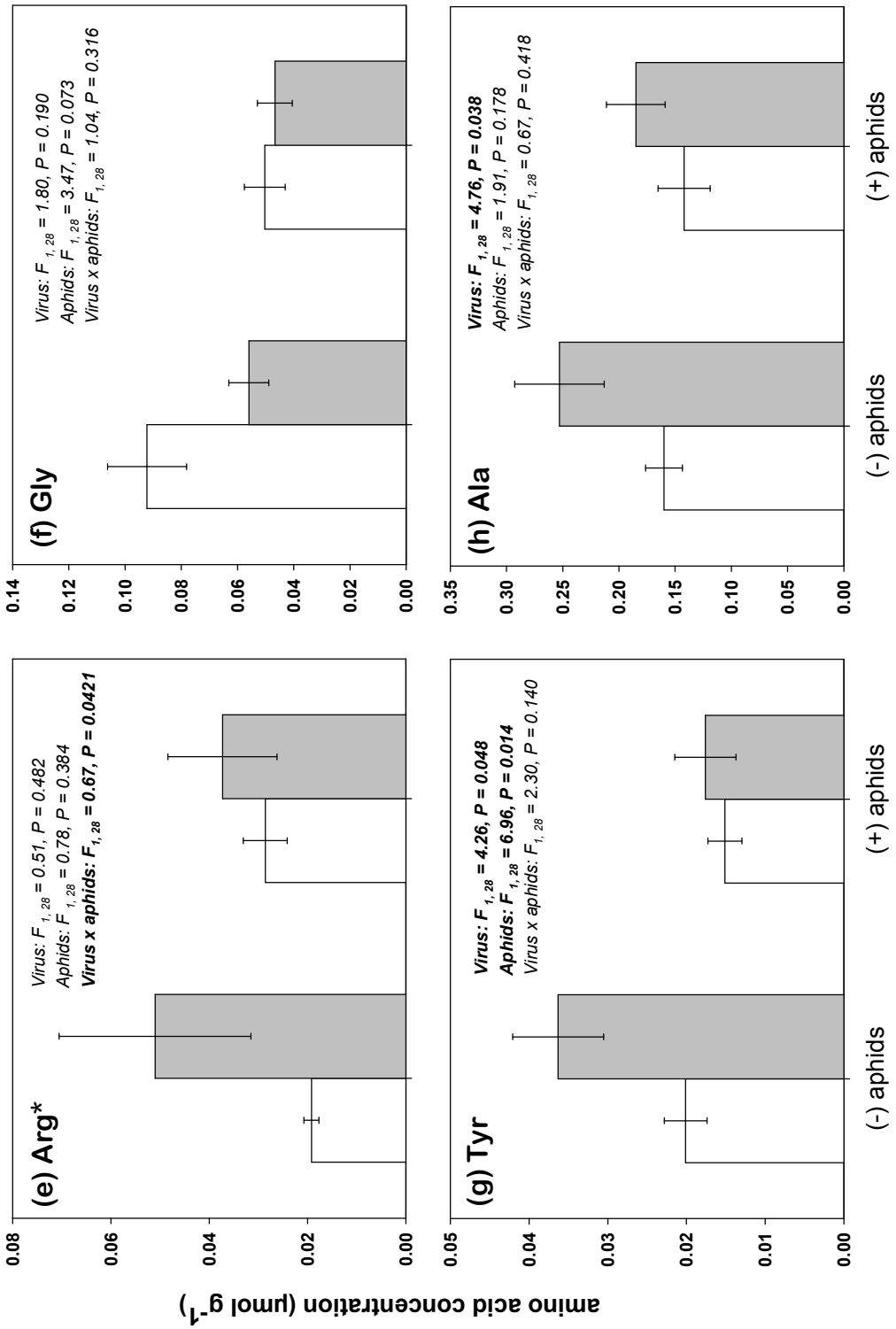
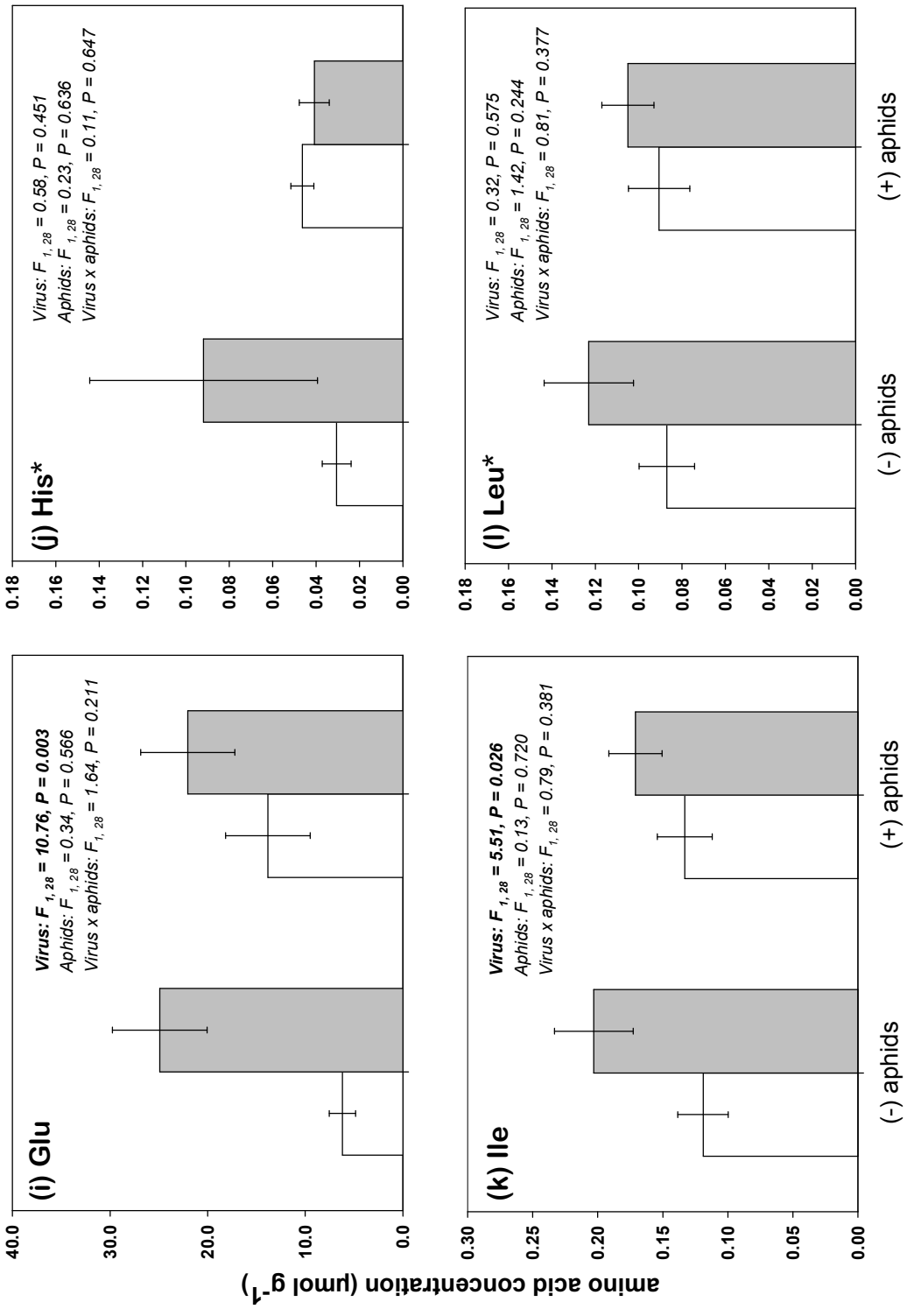
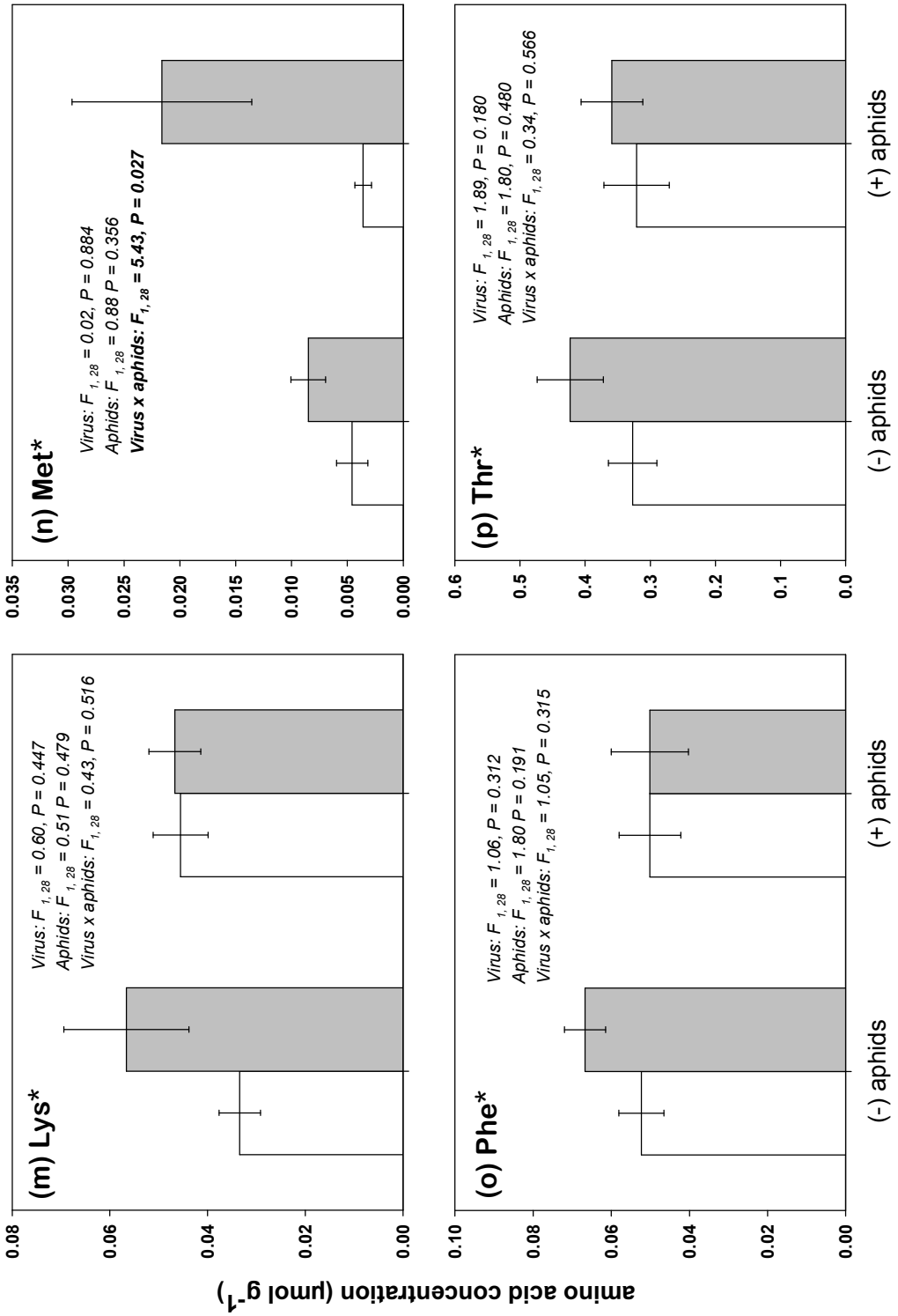


Figure 4.3 Concentration of **(a)** total free amino acids and **(b)** essential amino acids in healthy raspberry leaves (white bars) and leaves infected with BRNV + RLMV (grey bars) without aphids present (–) and with aphids feeding on leaves (+). Mean values of n = 6–10 ± SEM are shown. Significant effects ($P < 0.05$) are highlighted in bold.


(a) Asp
Virus: F 1, 28 = 15.85, P < 0.001
Aphids: F 1, 28 = 0.47, P = 0.499
Virus x aphids: F 1, 28 = 0.37, P = 0.547
(b) Asn
Virus: F 1, 28 = 29.36, P < 0.001
Aphids: F 1, 28 = 0.69, P = 0.414
Virus x aphids: F 1, 28 = 0.24, P = 0.630
(c) Ser
Virus: F 1, 28 = 5.71, P = 0.024
Aphids: F 1, 28 = 0.98, P = 0.331
Virus x aphids: F 1, 28 = 0.63, P = 0.333
(d) Gln
Virus: F 1, 28 = 7.65, P = 0.010
Aphids: F 1, 28 = 0.23, P = 0.633
Virus x aphids: F 1, 28 = 0.277, P = 0.603
(-) aphids
(+) aphids
amino acid concentration (μmol g-1)


(e) Arg*
Virus: $F_{1, 28}$ = 0.51, P = 0.482
Aphids: $F_{1, 28}$ = 0.78, P = 0.384
Virus x aphids: $F_{1, 28}$ = 0.67, P = 0.0421
(f) Gly
Virus: $F_{1, 28}$ = 1.80, P = 0.190
Aphids: $F_{1, 28}$ = 3.47, P = 0.073
Virus x aphids: $F_{1, 28}$ = 1.04, P = 0.316
(g) Tyr
Virus: $F_{1, 28}$ = 4.26, P = 0.048
Aphids: $F_{1, 28}$ = 6.96, P = 0.014
Virus x aphids: $F_{1, 28}$ = 2.30, P = 0.140
(h) Ala
Virus: $F_{1, 28}$ = 4.76, P = 0.038
Aphids: $F_{1, 28}$ = 1.91, P = 0.178
Virus x aphids: $F_{1, 28}$ = 0.67, P = 0.418
(-) aphids
(+) aphids
amino acid concentration (μmol g^{-1})


(i) Glu
Virus: F 1, 28 = 10.76, P = 0.003
Aphids: F 1, 28 = 0.34, P = 0.566
Virus x aphids: F 1, 28 = 1.64, P = 0.211
(j) His*
Virus: F 1, 28 = 0.58, P = 0.451
Aphids: F 1, 28 = 0.23, P = 0.636
Virus x aphids: F 1, 28 = 0.11, P = 0.647
(k) Ile
Virus: F 1, 28 = 5.51, P = 0.026
Aphids: F 1, 28 = 0.13, P = 0.720
Virus x aphids: F 1, 28 = 0.79, P = 0.381
(l) Leu*
Virus: F 1, 28 = 0.32, P = 0.575
Aphids: F 1, 28 = 1.42, P = 0.244
Virus x aphids: F 1, 28 = 0.81, P = 0.377
amino acid concentration (µmol g-1)
(-) aphids
(+) aphids


(m) Lys*
Virus: F 1, 28 = 0.60, P = 0.447
Aphids: F 1, 28 = 0.51 P = 0.479
Virus x aphids: F 1, 28 = 0.43, P = 0.516
(n) Met*
Virus: F 1, 28 = 0.02, P = 0.884
Aphids: F 1, 28 = 0.88 P = 0.356
Virus x aphids: F 1, 28 = 5.43, P = 0.027
(o) Phe*
Virus: F 1, 28 = 1.06, P = 0.312
Aphids: F 1, 28 = 1.80 P = 0.191
Virus x aphids: F 1, 28 = 1.05, P = 0.315
(p) Thr*
Virus: F 1, 28 = 1.89, P = 0.180
Aphids: F 1, 28 = 1.80, P = 0.480
Virus x aphids: F 1, 28 = 0.34, P = 0.566
(-) aphids
(+) aphids
amino acid concentration (µmol g-1)

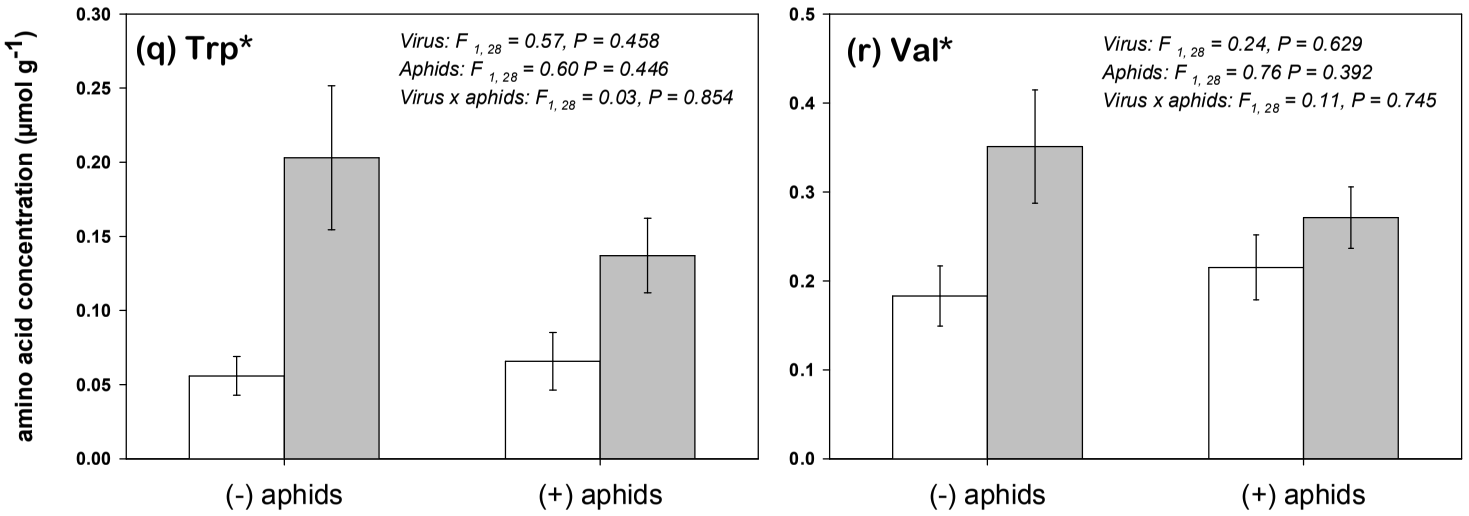


Figure 4.4. Concentration of individual free amino acids in healthy (white bars) and BRNV + RLMV– infected (grey bars) raspberry leaf with and without aphids feeding. Mean values of n = 6–10 ± SEM are shown. Asterisk denotes essential amino acid. **(a)** Asp – aspartate, **(b)** Asn – asparagine, **(c)** Ser – serine. **(d)** Gln – glutamine, **(e)** Arg – arginine, **(f)** Gly – glycine, **(g)** Tyr – tyrosine, **(h)** Ala – alanine, **(i)** Glu – glutamate, **(j)** His – histidine, **(k)** Ile – isoleucine, **(l)** Leu – leucine, **(m)** Lys – lysine, **(n)** Met – methionine, **(o)** Phe – phenylalanine, **(p)** Thr – threonine, **(q)** Trp – tryptophan and **(r)** Val – valine. Significant effects ($P < 0.05$) are highlighted in bold

4.4 Discussion

The results of this study demonstrate that there is an interactive effect of raspberry viruses and aphids on the phenolic content of raspberry leaves (Figure 4.1). In particular, leaf phenolic levels exhibited an upward trend in response to aphid feeding on otherwise healthy plants but showed a small decrease in response to aphid feeding on plants infected with BRNV and RLMV. Therefore, the two raspberry viruses studied seemingly induced the evasion of a plant defensive response to aphids as no major difference in phenolic content was observed between healthy plants and those infected with BRNV and RLMV. Elevated leaf phenolic content of plants in response to herbivore attack has been observed previously (Hartley & Firn, 1989; Felton *et al.*, 1994; Bi *et al.*, 1997) and polyphenolic compounds, such as polyphenol oxidase (PPO), have been previously implicated as markers for aphid host plant preference and selection and indicators of aphid resistant and aphid susceptible host plants (Jordens-Rottger, 1979; Dreyer & Jones, 1981; Peng & Miles, 1988). Montgomery & Arn (1974) showed that a simple phenolic substance, phlorizin, acted as a feeding and ingestion deterrent to the North American vector of BRNV and RLMV, *Amphorophora agathonica* and Han *et al.* (2009) showed that constitutive levels of PPO were higher in aphid resistant cultivars of wheat compared with susceptible varieties. The same study demonstrated that the application of aphid saliva induced an increase in PPO concentration in both resistant and susceptible varieties, although the increase was greater in susceptible plants (Han *et al.* 2009). The major signalling pathway associated with plant defence against insects is the jasmonic acid (JA) pathway. Methyl jasmonate (MeJA), together with jasmonic acid (JA), are key inducers of proteinase inhibitors which can protect plants from insect attack but are more often associated with responses to chewing herbivores such as Lepidopteran larvae rather than

phloem feeders such as aphids (Farmer & Ryan, 1990; Thaler *et al.*, 1996; Koo & Howe, 2009). Anti-aphid defence, similar to plant defence against pathogens, is more commonly shown to be associated with salicylic acid (SA) signalling (Bostock, 1999; Bostock *et al.*, 2001) and a weaker jasmonate response (Moran & Thompson, 2001; Rodriguez-Saona *et al.*, 2005; Thaler *et al.*, 2010). It may therefore be expected that the *R. idaeus* plants would mount a similar response to attack by *A. idaei* and by the two viral pathogens, BRNV and RLMV, as viruses stimulate an SA-mediated plant defence response. However, this was obviously not the case for the production of plant phenols and this could indicate that that "cross-talk" is occurring between other pathways i.e. a virus-induced SA response could diminish the activity of JA-responsive genes encoding anti-herbivore activity. Clearly, further studies are required to confirm this.

Although little is known about the induction of phenolic compounds in response to plant pathogens, the results of the studies which are available report variable results. For example, Arpita & Ghosh (2008) recorded lower levels of phenolics in *Hibiscus* plants infected with the viral pathogen causing Yellow vein mosaic disease, while Johnson *et al.* (2003) reported that birch leaves infected with the fungal pathogen, *Marssonina betulae,* produced higher levels of phenolics than asymptomatic leaves. As aphids performed less well on plants that were infected with BRNV and RLMV, where phenolic levels were reduced compared to healthy plants (Chapter Three), it is unlikely that that the levels of phenolic compounds present were responsible for the depressed development time of *Amphorophora idaei*. This observation would appear to be beneficial to the viral pathogens as the failure of the plant to mount an anti-aphid defensive response when infected with

BRNV and RLMV may facilitate the short probes the aphid is required to make in order to successfully acquire the viruses.

Leaf C:N increased, although not significantly so, in response to infection with BRNV and RLMV as a result of an increase in carbon content of the leaves. High C:N ratios are generally associated with decreases in performance of herbivorous insects, and, in particular high concentrations of carbon based compounds such as carbohydrate and lipids have also been associated with decreased insect performance as they tend to dilute other important nutrients (Awmack & Leather, 2002). The concentration of carbon in raspberry leaves was found to be significantly elevated in response to infection with BRNV and RLMV and further analysis of carbon composition, particularly sugars important for aphids such as sucrose, may reveal compositional changes to help explain the poorer performance of *A. idaei* on virus-infected plants. For example, Mittler, (1970) showed that plant sucrose content could be predicted by measuring honeydew carbohydrate content and the more recent study of Hale *et al.* (2003) demonstrated that a very high sucrose concentration can lead to reduced ingestion of phloem sap as the aphid restricts osmoregulation which becomes costly at high osmotic pressures (Hale *et al.*, 2003). In general, the ability of an insect to convert plant material into body mass increases as plant nitrogen increases (Schoonhoven *et al.*, 2005) and the effect of elevated nitrogen on aphids has been demonstrated previously (Cisneros & Godfrey, 2001). Based on performance parameters measured for *A. idaei* in Chapter Three, as leaf nitrogen, which is generally considered to be limiting to aphids, did not show any alteration in concentration in response to virus infection it seems unlikely to be the cause of the depressed performance of *A. idaei*. However, the lack of difference observed may

actually have been due to the presence of amino-nitrogen derived from BRNV and RLMV nucleic acids in the leaf tissue or the presence of plant derived N–containing compounds which are actually unavailable to the aphid but which may have masked any differences in N concentration. Furthermore, the free amino acid content of raspberry leaf tissue was shown to be elevated in response to infection with BRNV and RLMV and both the total amino acid content and the relative proportion of essential amino acids were found to be highly elevated in response to BRNV and RLMV infection in raspberry leaf tissue. As aphids are generally limited by amino acid availability (Douglas, 1993, 2006) it appears that virus-induced changes to amino acid content cannot explain the poorer performance of *A. idaei* on BRNV and RLMV-infected plants. However, in almost all of the individual amino acids quantified, the increase in response to virus infection was less pronounced in plants where aphids were feeding (Figure 4.4a-r). This could be the result of direct removal of amino acids from the leaf pool by aphids during phloem feeding but may also be another example of cross-talk occurring between plant signalling pathways as generally stress to plants causes reductions in protein synthesis and a corresponding increase in free amino acids (Brodbeck & Strong, 1987). In addition, plant viruses have a requirement for amino acids produced by metabolism of the host plant in order to synthesise new viral protein (Hull, 2002). If the amino acid requirements of BRNV and RLMV overlap with that of *A. idaei* then the reduced performance of the aphid on virus-infected raspberry plants may be due to direct competition for dietary amino acids between the herbivore and the pathogen. However, the exception to the general trend observed in *R. idaeus* is the essential amino acid methionine which, although unaffected by virus infection alone, exhibited an interaction with aphid feeding ($F_{1,28}$ = 4.53, P = 0.027). Methionine has previously been suggested as

a feeding stimulant for several aphid species (Mittler, 1967, 1970; Harrewijn & Noordink, 1971) and it could be hypothesised that the elevated levels in BRNV and RLMV–infected tissue is, like the leaf phenolics, a pathogen-induced mechanism to facilitate aphid acquisition of virions, which may be overridden by the natural plant defence response to aphid attack, although further studies are required in order to confirm this.

The dominant amino acid in both healthy and virus-infected raspberry leaf tissue was glutamate which accounted for 64% of the total amino acid content in healthy plants. This proportion was elevated in response to infection with BRNV and RLMV (77.0%) and when aphids fed on virus-infected leaves (82%). Glutamate has, in the past, been implicated in a reduction in host plant suitability when present in high relative concentration (Douglas, 1993; Karley *et al.*, 2002), as it is in raspberry leaf. Furthermore, high relative levels of glutamate have been shown to be present in aphid resistant plant cultivars and have been suggested as contributing to poorer aphid performance on these plants. For example, Weibull (1988) recorded higher levels of glutamate in barley and oat cultivars resistant to the bird cherry oat aphid, *Rhopalosiphum padi* while Chen *et al.* (1997) found the same relationship in melon plants resistant to the cotton aphid, *Aphis gossypii*. It seems that glutamate may act similarly as an indicator amino acid of host plant suitability for *A. idaei* in raspberry.

In conclusion, the leaf chemical analyses detailed in this chapter may in part begin to explain the poorer performance of *A. idaei* on raspberry plants infected with BRNV and

RLMV, particularly with respect to individual amino acids (see Table 4.1) such as glutamate which may act as indicators of a nutritionally poor host, although further studies are required in order to confirm specific relationships. Further investigation is required to confirm their role in *A. idaei* nutrition, particularly the essential amino acids such as methionine. Artificial diets are commonly utilised in investigation such as these and additional analyses of products of aphid digestion through examination of honeydew may also prove useful. All chemical analyses in this study were conducted on total leaf material, which has proved a reliable indicator of phloem composition for barley (Winter *et al.*, 1992; Johnson *et al.*, 2009) but in this system, further extraction and analysis of phloem using techniques such as EDTA exudation (King & Zeevaart, 1974; Douglas, 1993) or aphid stylectomy may provide further insights into the nutritional quality for *A. idaei* of raspberry plant infected with BRNV and RLMV.

	+ aphids	+BRNV + RLMV	+ BRNV +RLMV + aphids
Leaf phenolics	↑	↑	–
Carbon	**n/a**	↑	**n/a**
Nitrogen	**n/a**	_	**n/a**
His	_	_	_
Ile	_	↑	_
Leu	_	_	_
Lys	_	_	_
Met	_	_	↑
Phe	_	_	_
Thr	_	_	_
Trp	_	_	_
Val	_	_	_
Asp	_	↑	_
Asn	_	↑	_
Ser	_	_	_
Gln	_	_	_
Arg	_	_	↑
Gly	_	_	_
Tyr	↑	↑	_
Ala	_	↑	_
Glu	_	↑	_
Total		↑	

Table 4.1. Summary of results of leaf chemical analyses. Results reported as difference from healthy control plants. ↑, increase, _, no change and n/a, not tested. Essential amino acids are shaded.

Chapter Five

Raspberry volatiles attract *Amphorophora idaei* to virus-infected raspberry plants

Abstract

Aphid behavioural assays detailed in Chapter Three showed that *Amphorophara idaei* were more attracted to virus infected raspberry plants compared with healthy plants. Although there was evidence that there may be a visual cue to the aphid through changes in leaf colouration, the role of a volatile signal was not ruled out. In order to investigate potential alterations in the volatile composition of raspberry plants infected with BRNV and RLMV, entrainments of leaf headspace were taken simultaneously from healthy and virus infected plants using a novel solid phase microextraction (SPME) technique.

Analysis of the volatile entrainments using TOF-GC-MS successfully identified 27 individual volatile components in the headspace of raspberry plants and revealed an elevated level of overall volatile emissions from virus-infected plants compared with healthy controls in line with the results of previous studies of the effect of virus infection. Furthermore, comparison of the GC-MS signals from healthy and virus infected plants showed that 2 of the 27 plant-derived compounds, 2-hexenal and (Z)-3-hexenyl acetate, were elevated in response to virus-infection based on non-overlapping standard errors, making them candidate aphid attractants.

Results of aphid bioassays with (Z)-3-hexenyl-acetate showed that *A. idaei* was attracted to the compound at a concentration of 50 ng ml^{-1} but unaffected by lower and higher concentrations of this volatile.

5.1 Introduction

5.1.1 Rationale

Aphids have been shown to exhibit a phototactic response to reflected light from plants (Hardie, 1989) and in particular, an attraction to yellow colouration (Moericke, 1952; Prokopy & Owens, 1983). The results of Chapter Three indicated that there may be a visual cue operating to attract *A. idaei* to virus infected plants but the experiments did not conclusively rule out a role of volatile signals in host plant location. It may therefore be the case that aphids are attracted to plants infected with BRNV and RLMV in the presence and absence of visual stimuli. This chapter is concerned with the identification of potential volatile attractants and more consideration is given to the role of leaf colouration in Chapter Six.

5.1.2 Plant volatiles as host cues to aphids

All plants emit a range of volatile hydrocarbons which generally consist of C_6-aldehydes, C_6-alcohols and their acetates (Shiojiri *et al.*, 2006) which vaporise on exposure to air, most especially when damage has occurred to the leaf (Schoonhoven *et al.*, 2005). These so called green leaf volatiles (GLVs) are those which are responsible for the characteristic 'cut grass' smell from a damaged plant and consist mainly of saturated or monosaturated alcohols and adehydes which may occur as different isomers. It was first believed that phytophagous insects possessed the ability to discriminate between host plants based on perception of volatile compounds which were characteristic of a particular host plant (Fraenkel, 1959), however, it has since been shown that most insects, including aphids, are capable of detecting plant volatiles that are ubiquitous to higher plants (Bruce *et al.*,

2005) (Table 5.1) Plant chemical cues are detected by olfactory receptor neurons (ORNs) which are housed on the antennae of the insect. In aphids, the ORNs are found on sensilla known as rhinaria located on the antennae of the insect. These include the proximal and distal primary rhinaria and the secondary rhinaria. The antennal structure of alate and apterous aphid morphs differ in that alate aphids possess more secondary rhinaria, suggesting that these organs are involved in host location (Pickett *et al.*, 1992). On perception of a plant odour, the ORNs act to convert the chemical signal received from the plant to an electrical signal which acts as an input to the central nervous system of the insect (Hansson, 2002) and elicits the appropriate behavioural response. Whether the plant is subsequently deemed acceptable depends on a variety of cues which are detected after landing, such as antennal detection of odours at the leaf surface (Storer *et al.*, 1996) or repellent cues detected by probing the leaf with the stylets (Powell *et al.*, 2006).

Examples of aphid host perception using volatile signals come from a variety of different species feeding on different host plants. For example Visser *et al.* (1996) compared the electroantennogram (EAG) responses of four aphid species with an overlapping host plant range (*Megoura viciae*, *Aphis fabae*, *Myzus persicae* and *Brevicoryne brassicae*) to 35 plant volatiles and found that aphids were sensitive to general green leaf volatiles e.g. *(E)*-2-hexenal, benzaldehydes (e.g. 4-methoxybenzaldehyde), carvones (e.g. (-)-(*R*)-carvone, monoterpene aldehydes (e.g. citronellal), nitriles (e.g. hexanonitrile) and isothiocyanates (e.g. butyl isothiocyanate) (Table 5.1). Moreover, the polyphagous aphid, *M. persicae* showed increased sensitivity to 2-heptanone, 3-methoxy- and 4-methoxybenzaldehyde and

hexano- and heptanonitrile compared with the other aphid species studied and the non host-alternating *M. viciae* showed reduced sensitivity to some of the general green leaf volatiles which may aid in olfactory discrimination between host and non-host plants.

In addition, studies of olfactory responses of the black bean aphid, *Aphis fabae* demonstrated that this aphid species preferentially orientated towards the odour of undamaged host faba bean, *Vicia faba*, plants in olfactometer studies (Nottingham *et al.* 1991) and identification of the volatiles responsible revealed 15 different compounds that were electrophysiologically active with *A. fabae* (Webster *et al.*, 2008). A synthetic blend of these volatiles made to mimic *V. faba* compositionally was attractive to *A. fabae* and furthermore, the aphid showed no preference for the natural plant odour over the synthetic blend (Webster *et al.*, 2008).

It has been further hypothesized that not only are aphids tuned to detect ubiquitous plant volatile compounds (Bruce *et al.*, 2005) but they also use plant specific ratios to recognize their host plants. For example, Ngumbi *et al.* (2007) demonstrated that *Myzus persicae* was more attracted to synthetic blends made to mimic the headspace of potato plants compared with the individual component volatiles and a recent study by Webster *et al.* (2010) showed that a synthetic blend made to mimic faba bean elicited a positive response from the black bean aphid, *A. fabae*. Indeed, in the study by Webster *et al.*, the component volatiles tested individually surprisingly elicited a negative response from the aphid and the authors postulated that these responses are indicative of the aphids' ability to discriminate between odour sources qualitatively and that the response is dependent

on the blend properties of the particular host plant. Such responses would clearly be advantageous to an aphid for successful host plant location, but there is no indication that all aphid species would behave in a similar way.

	Aphid species					
	Aphis fabae	*Brevicoryne brassicae*	*Megoura viciae*	*Metopolophium dirhodum*	*Myzus persicae*	*Sitobion avenae*
Plant volatile						
Fatty acid derivatives						
Hexanol	+	+	+	+	+	+
Hexanal	+	+	+	+	+	+
(*E*)-2-Hexenal	+	+	+	+	+	+
(Z)-3-Hexenol	+	+	+	+	+	+
(*E*)-2-Hexenol	+	+	+	+	+	+
(Z)-3-Hexenyl acetate	+	+	+	+	+	+
2-Heptanone	+	+	+	+	+	+
1-Octen-3-ol	+	+	+	+	+	+
Phenyl-propanoids						
Benzaldehyde	+	+	+	+	+	+
Isoprenoids						
Limonene	-	-	-	+	-	+
Caryophyllene	+	+	+	+	+	+
α-Pinene	+	+	+	+	+	+
Linalool	+	+	+	+	+	+
Geraniol	+	+	+	+	+	+
Myrcene	-	-	-	+	-	+
3-Carene	-	-	-	+	-	+
β-Pinene	+	+	+	+	+	+
Reference	Visser *et al.*, (1996)	Visser *et al.*, (1996)	Visser *et al.*, (1996)	Visser & Yan (1995)	Visser *et al.*, (1996)	Visser & Yan (1995)

Table 5.1. Electrophysiological responses of aphids to common plant volatiles. + = compound responded to, – = compound not tested. Table redrawn from Bruce *et al.* (2005).

5.1.3 Plant viruses alter the attractiveness of host plants to aphids

As has been discussed previously, plants infected with viral pathogens are often shown to be more attractive to the aphids that vector the pathogen. By eliminating visual cues to the insect (i.e. conducting bioassays in darkness), the role of plant volatiles in the attraction can be demonstrated. Using this type of experimental set up, the seminal study of Eigenbrode *et al.* (2002) revealed that *Myzus persicae* was preferentially attracted to headspace volatiles of potato plants infected with Potato leaf roll virus (PLRV) over those present in the headspace of non-infected potato plants. The attraction of *M. persicae* was tested in response to a natural odour source (i.e. plant leaflets) and also in response to filter paper discs treated with volatiles eluted from Super-Q volatile traps onto filter paper. In both cases, volatiles from PLRV-infected plants preferentially attracted the aphid and analysis of the volatiles collected showed that total emissions from virus-infected plants were almost double those emitted from a non-infected control. Furthermore, analysis of the individual components revealed that 14 out the 21 individual component detected were elevated by 1.6 fold (β-sesquiphellandrene) to 5 fold (nonane). The results of behavioural bioassays coupled with the headspace volatile analysis make it likely that these increases are responsible for the attraction of *M. persicae* and indeed, studies conducted subsequently using the same system revealed that one of the constituent volatiles of PLRV-infected potato, β-pinene, acted as an aphid arrestant (Ngumbi *et al.*, 2007).

In experiments of a similar style, Jimenez-Martinez *et al.* (2004) demonstrated that the bird cherry–oat aphid, *Rhopalosiphum padi*, was more attracted to the headspace of wheat

plants infected with Barley yellow dwarf virus (BYDV) compared to the headspace of uninfected control plants. In addition, wheat plants infected with BYDV were found to emit a higher concentration of volatiles than uninfected control plants which could potentially explain the preference exhibited by the aphid for these plants. In more recent studies of the same system, Medina-Ortega *et al.* (2009) studied the response of *Rhopalosiphum padi* to both synthetic blends of volatiles made to mimic the headspace of BYDV-infected wheat plants and non-infected plants and the individual components of the blend. *R. padi* were found to be attracted by the synthetic blend made to mimic BYDV-infected plants over a blend made to mimic non-infected wheat and also to the individual components nonanal, (Z)-3-hexenyl acetate, decanal, caryophyllene and undecane when tested against a paraffin oil control. This study therefore succeeded in identifying several compounds from wheat which act as aphid attractants. The study of Mauck *et al.* (2010) employed a slightly different experimental approach and in addition to sampling volatiles of *Cucurbita pepo* plant in the laboratory, extended their study to include sampling from field grown plants. The outcome was the same. Plants infected with Cucumber mosaic virus (CMV) released an increased quantity of volatiles and were more attractive to the aphids vectors *M. persicae* and *Aphis gossypii* compared with non-infected plants (Mauck *et al.*, 2010).

5.1.4 Aims and hypotheses

The aim of the experiments described in this chapter was primarily to detect overall differences between headspace volatiles from healthy raspberry plants and those infected with BRNV and RLMV using a non-invasive headspace sampling technique and qualitative analysis. This is the first study, to my knowledge, that has attempted to identify difference in raspberry volatile emissions in response to plant infection with viral pathogens. Candidate aphid attractants from this analysis would then be tested to determine whether they attracted *A. idaei*. The key hypotheses for this study were:

1. Raspberry plants infected with BRNV and RLMV would produce elevated levels of volatile compounds in line with previous studies, showing the effect of viral infection on volatile emissions where aphids were preferentially attracted to virus-infected plants (Eigenbrode *et al.*, 2002; Srinivasan *et al.*, 2006)
2. If certain components of the blend of raspberry headspace volatiles were present in BRNV and RLMV- infected plants and absent from non-infected plants, or were found to be present in both, but elevated in response to viral infection, these components would be attractive to *A. idaei*.

5.2 Materials and methods

5.2.1 Volatile sampling using SPME-GC-MS

All volatile entrainments were taken from plants which were grown in identical conditions to those used in aphid experiments (see Chapter Three). Volatile entrainments were taken after at least 8 weeks of growth to mimic the age of plants used in aphid experiments. Volatiles were sampled using solid-phase micro-extraction (SPME) fibres (Supelco Ltd., Pennsylvania, USA). SPME fibres are advantageous for sampling the headspace of plants as they eliminate the need for solvents during sampling of the plant headspace. This is due to the fibre being coated with either a solid extraction phase which directly adsorbs organic compounds to its surface, or an absorbent compound which can be used for liquid phase analytes. In both cases, their sensitivity means that limits of detection can be as low as parts per trillion (ppt). SPME fibre design consists of a syringe-like mechanism which is used to expose the fibre during sampling and to retract it into the sheath prior to analysis (Figure 5.1). After the entrainment has been taken, the fibre can then be easily injected directly into a GC-MS system for desorption and final analysis.

Carboxen-PDMS SPME fibres were used to sample raspberry headspace volatiles in this study. These fibres are coated with carbon and polydimethylsiloxane which is an adsorbent polymer for gaseous entrainments. This particular fibre is reusable after thermal conditioning at 300 °C. Entrainments were taken simultaneously from one healthy plant and one verified to be infected with BRNV and RLMV using PCR tests (see Chapter Three). Each plant was positioned on a custom built metal rig which was used

to hold leaf cages and fibres in place on the plant (Plate 5.1a). One leaf of each plant was fitted with a copper wire cage (Plate 5.1b) which had been solvent washed in isohexane and methanol prior to use. The cage was held in place on the sampling rig with lengths of copper wire to remove any strain on the leaf and stem of the plant. Once in position, the leaf and cage were sealed in a transparent plastic cooking bag using plastic seals (Platee 5.1c). Cooking bags were used primarily to shield the SPME fibre from any contaminating air currents and as their principal use is in food preparation, they are unlikely to give off many chemical signals which could contaminate the volatile signal released from the plant (Tom Shepherd, personal communication). The leaf and bag were flushed with filtered air by passing an air flow through a molecular sieve and activated carbon which cleaned the air and removed any traces of water. Clean air was passed into the bag at a flow rate of 250 ml min^{-1} for 30 min prior to the volatile entrainment being taken by inserting a small copper pipe fitted to the filters and a vacuum pump into a small hole made in the bag. The bag was then resealed and the SPME fibre and holder was positioned approximately 50 mm from the leaf surface by piercing a small hole through the plastic with a syringe. The fibre was exposed above the leaf surface (Plate 5.1d) and the entrainment was taken for 90 min. The fibre was then retracted into its sheath and immediately placed into the GC-MS autosampler for desorption and analysis. The SPME fibre was injected into a ThermoFinnigan Tempus time of flight (TOF) GC-MS (Thermo Scientific) with DB 1701 column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, West Lothian, UK) using helium as a carrier gas. The column was held at 40 °C for 2 min and then heated to 250 °C at 10 °C min^{-1} and held for 10 min. Data was acquired using the Excalibur software package (Thermo Scientific) and peaks were identified by comparison with the integrated NIST/EPA/NIH electron

ionization (EI) library using 3 pairs of plants from which entrainments had been simultaneously obtained.

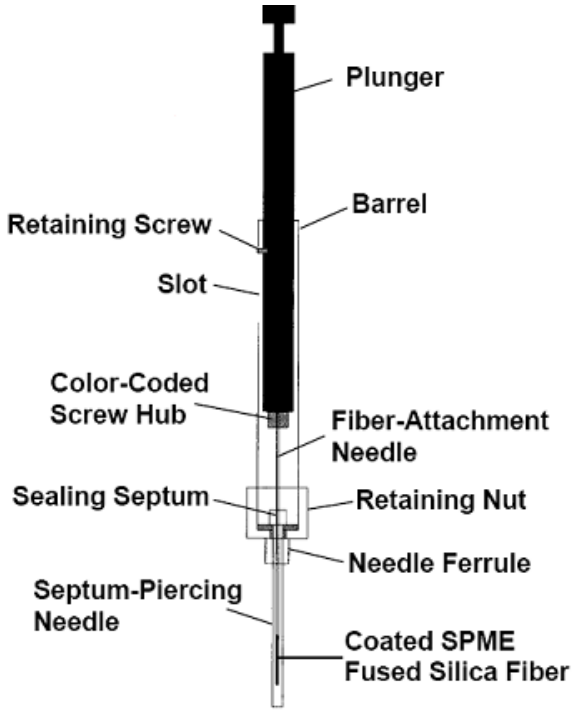


Figure 5.1. SPME fibre assembly for injection into a GC-MS system, reproduced from SPME-fibre user guide (Supelco Ltd., Pennsylvania, USA).

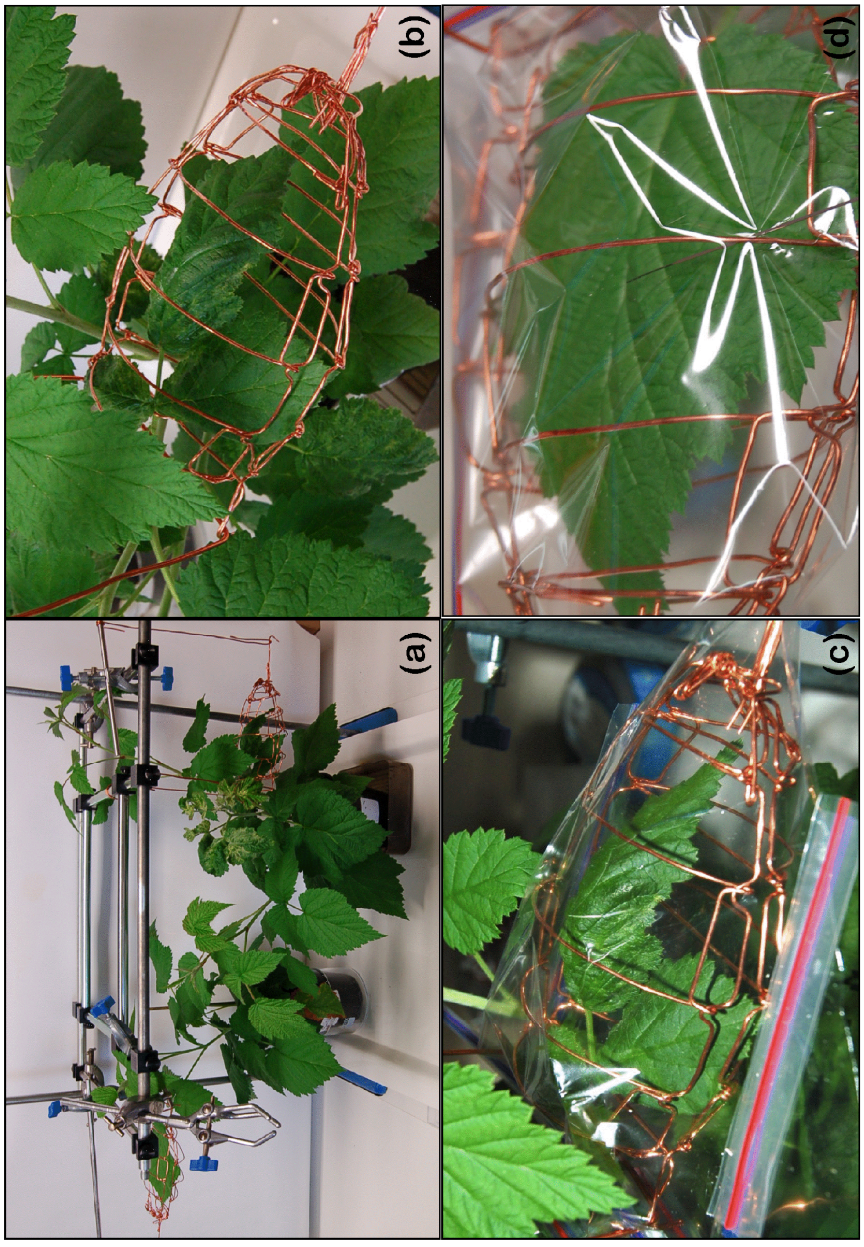


Plate 5.1 (**a**) SPME sampling rig for simultaneous sampling from healthy plant (left) and virus-infected plant (right) showing copper wire cages suspended on plants; (**b**) wire cage positioned around a virus-infected leaf; (**c**) leaf and cage sealed in transparent cooking bag and (**d**) SPME fibre exposed over leaf surface within cage

5.2.2 Aphid responses to (*Z*)-3-hexenyl acetate

As (Z)-3-hexenyl acetate was found to be highly elevated in raspberry plants infected with BRNV and RLMV, this chemical was used to conduct bioassays with adult *A. idaei*. The general protocol for individual choice tests was similar to that of Medina-Ortega *et al.* (2009) and involved the release of aphids in an arena containing two paper discs, one which was treated with (Z)-3-hexenyl acetate (Sigma-Aldrich, Dorset, UK) and another to act as a control. To prevent rapid evaporation of the volatile, it was first dissolved in pure (> 99%) paraffin oil (Sigma-Aldrich, Dorset, UK) which acted as a slow-release agent. Appropriate concentrations were obtained by serial dilution. Two paper discs (filter paper Whatman grade 1) measuring 5.5 cm in diameter were positioned in opposing sides of a rectangular glass dish (21.5 × 16.5 cm) with glass lid. One of the paper models was treated with 100 µl of paraffin oil and the other was treated with 100 µl of volatile solution. The models were positioned equidistantly from the centre of the glass floor and covered with a glass dish. An apterous adult *A. idaei* which had been starved for 1 hour was then released into the centre of the arena and its position recorded every 1 min for a total of 20 min. The bioassays were conducted in a darkened laboratory at room temperature. Arenas were positioned on a ventilated bench top and were lit from above by a single white LED bulb producing a light intensity in the glass arena of 350 ± 1 LUX. LEDs generally produce very little heat but to ensure temperatures in the bioassay arena were uniform, iButtons™ (Maxim Integrated Systems, CA) were used to record the temperature in each 'zone' of the glass dish prior to the experiment. Different aphids and paper leaf models were used to obtain each replicate and the arena was rotated through 180° after each replicate to avoid positional effects. Arenas were solvent washed in

methanol and baked in an oven prior to use. Aphid behaviour was investigated at four different concentrations (10 ng ml^{-1}, 50 ng ml^{-1}, 100 ng ml^{-1} & 250 ng ml^{-1}) and different aphids and paper discs were used to obtain 20 – 24 replicates per concentration.

5.2.3 Statistical analyses

Raspberry volatiles

The total volatile signals acquired from healthy and virus-infected plants were compared using a t-test. Individual volatile compounds were not subject to statistical analysis but standard errors were calculated. Non-overlapping standard errors were considered evidence of a change in abundance between samples (Eigenbrode *et al.*, 2002).

Aphid choice tests

Due to the repeated measures on the same aphid individuals over time, aphid choice tests were analysed using a generalized linear mixed effects model (GLMM) assuming a binomial error structure and utilising a logit-link function. In each analysis, the proportion of aphids on the volatile treated paper disc was fittted as the y-variable and time was initially fitted as the x-variable. Arena nested within time was initially fitted as the random term. Terms were subtracted from the model until any further removal led to significant increases in deviance and thus higher AIC, providing a minimum adequate model for each analysis. All results and associated probabilities are reported based on the resulting minimum model for each experiment (for model summaries see Table 5.3). Aphids on the side of the glass arena were assumed to be non-responsive and were excluded from the analyses. All mixed models were run using the lme4 package in R

version 2.12.1 following the methods of Crawley (2007) to eliminate temporal pseudoreplication in the dataset.

5.3 Results

5.3.1 GC-MS analysis of raspberry volatiles

Identification of raspberry volatiles emitted from fully expanded raspberry leaves using GC-MS successfully identified a total of 27 component compounds which comprised mainly aldehydes, monoterpenes and sesquiterpenes (Table 5.2). The total volatile emissions from plants infected with BRNV and RLMV was found to be elevated by approximately 25%, but this difference was determined not to be statistically significant ($t = -0.513$. $P = 0.635$). Entrainments from both healthy and virus-infected plants were dominated by acetic acid which accounted for between 40 and 50% of the total volatile signal detected. The next most abundant compound in terms of signal level detected was the monoterpene α-pinene which accounted for 18.58% of the signal from healthy plants and 19.82% of the signal from virus-infected plants. Although the same component volatiles were present in the entrainments from both healthy and virus-infected plants, several components showed differences in relative compositions between plant treatments based on non-overlapping standard errors. Of the 27 components which were found to make up the raspberry volatile profile (Figure 5.2), 17 were found to be elevated in response to infection with BRNV and RLMV while 11 showed a reduction or no change in signal level. Of these, 2-hexenal and (Z)-3-hexenyl acetate, phellandrene, 5-ethyl-25H-furanone and one of a family of five sesquiterpenes (termed unknown sesquiterpene 4 in Table 5.2) were considered as significantly altered based on non-overlapping standard errors. While 2-hexenal and (Z)-3-hexenyl acetate were elevated in virus-

infected plants, phellandrene, 5-ethyl-25H-furanone and the unknown sesquiterpene were markedly reduced.

Peak #	component	component expressed as % of total plant volatile signal: RT (min)	uninfected control	BRNV + RLMV-infected
1	*acetic acid*	3.93	49.88 ± 3.920	45.88 ± 8.910
2	*hexanal*	5.75	1.52 ± 0.414	2.02 ± 0.256
3	*α-pinene*	6.90	18.58 ± 2.842	19.82 ± 7.919
4	*2-hexenal*	7.11	**0.06 ± 0.008**	**0.19 ± 0.112**
5	*camphene*	7.27	0.65 ± 0.182	0.67 ± 0.196
6	*heptanal*	7.53	0.38 ± 0.099	0.43 ± 0.116
7	*linalyl acetate*	7.80	1.73 ± 0.466	1.64 ± 0.561
8	*ocimene*	8.30	4.00 ± 1.077	6.14 ± 3.849
9	*limonene*	8.70	2.03 ± 0.476	1.93 ± 0.578
10	*phellandrene*	8.83	0.28 ± 0.052	0.23 ± 0.088
11	*eucalyptol*	9.02	2.90 ± 1.181	1.42 ± 0.025
12	*(Z)-3-hexenyl acetate*	9.07	**2.00 ± 1.155**	**3.87 ± 0.357**
13	*benzaldehyde*	9.15	3.68 ± 0.805	3.50 ± 0.482
14	*octanal*	9.23	0.75 ± 0.274	1.06 ± 0.330
15	*butyrolactone*	9.86	6.30 ± 2.739	4.62 ± 2.771
16	*5-ethyl-25H-furanone*	9.93	**0.34 ± 0.032**	**0.24 ± 0.050**
17	*linalool*	10.84	0.83 ± 0.313	1.03 ± 0.252
18	*nonanal*	10.84	0.79 ± 0.332	0.86 ± 0.230
19	*camphor*	11.89	0.28 ± 0.037	0.31 ± 0.124
20	*d-valerolactone*	12.25	0.24± 0.041	0.19 ± 0.039
21	*decanal*	12.32	0.39 ± 0.173	0.51 ± 0.213
22	*bornyl acetate*	13.46	0.96 ± 0.273	1.69 ± 1.206
23	*UK sesquiterpene 1*	13.76	0.01 ± 0.002	0.01 ± 0.001
24	*UK sesquiterpene 2*	13.87	0.050 ± 0.011	0.04 ± 0.005
25	*UK sesquiterpene 3*	14.44	0.16 ± 0.026	0.25 ± 0.085
–	*UK sesquiterpene 4*	14.70	**0.03 ± 0.010**	**0.16 ± 0.101**
–	*UK sesquiterpene 5*	15.61	0.01 ± 0.002	0.01 ± 0.001

Table 5.2. Components of headspace of healthy and virus-infected raspberry leaves in order of elution during gas chromatography. Mean values of n = 3 ± SEM are shown. Components found to be elevated with non-overlapping standard errors are double underscored and components found to be reduced with non-overlapping standard errors are single underscored. UK - unknown. Peak # corresponds to peaks labelled in Figure 5.2.

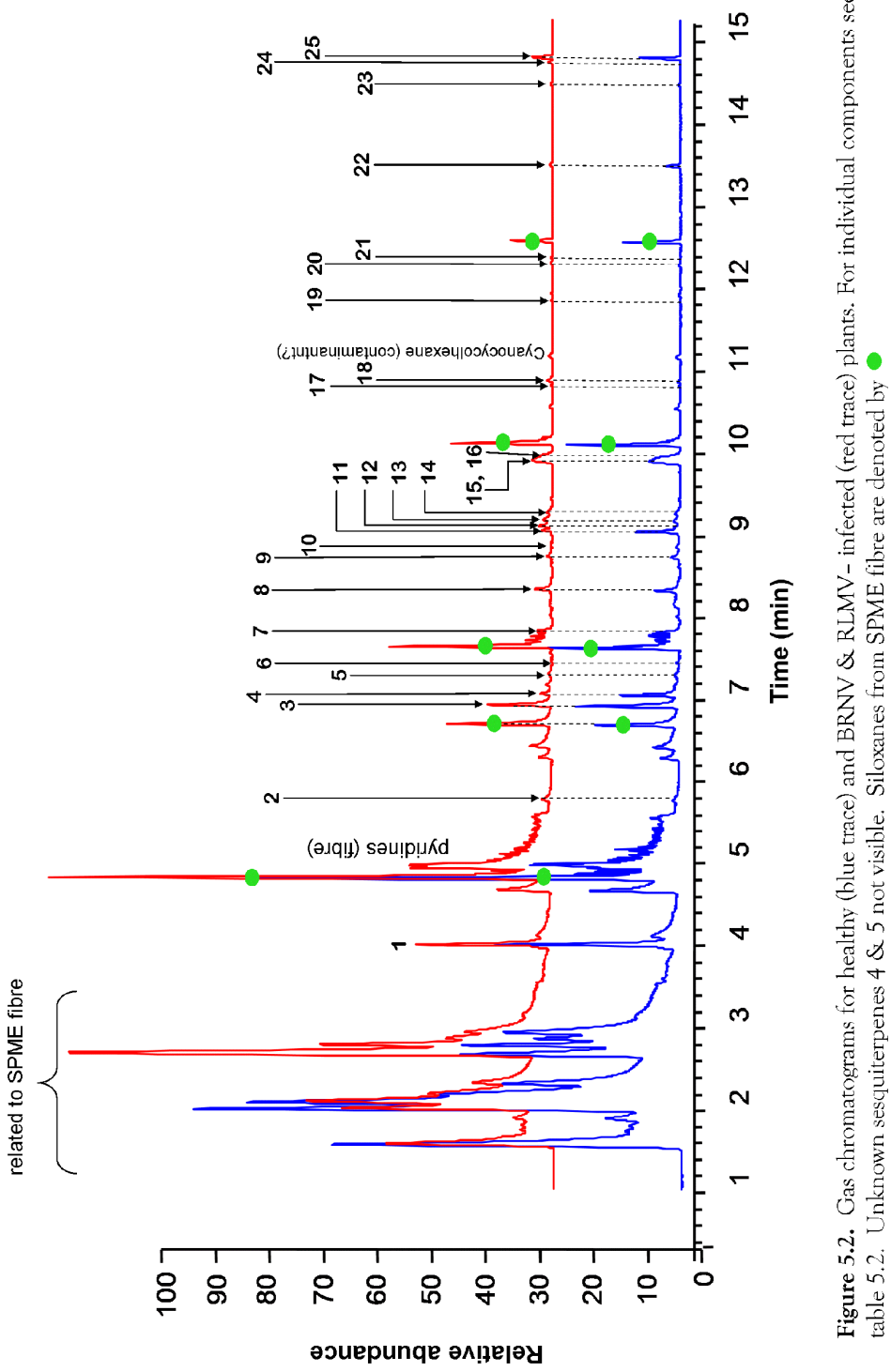


Figure 5.2. Gas chromatograms for healthy (blue trace) and BRNV & RLMV– infected (red trace) plants. For individual components see table 5.2. Unknown sesquiterpenes 4 & 5 not visible. Siloxanes from SPME fibre are denoted by ●

5.3.2 Aphid responses (*Z*)-3-hexenyl acetate

The response of *A. idaei* to concentrations of the green leaf volatile (Z)-3-hexenyl acetate (which is known to be naturally released by the host plant) was variable. At the lowest concentration tested (10 ng ml^{-1}) there was no effect of the volatile solution on aphid preference (see Table 5.3 and Figure 5.3). However, at the next concentration tested (50 ng ml^{-1}) the volatile exerted a significant effect on aphid preference for volatile treated discs (Table 5.3). Specifically, the proportion of aphids on the treated discs showed a significant tendency to increase over time (Figure 5.4). The higher concentrations of 100 and 250 ng ml^{-1} had no effect on the preference of *A. idaei* for volatile treated discs (Figure 5.5 and 5.6 respectively.

Assay	AIC	Random Effects	Fixed Effects	Estimate	z value	P
10 ng ml^{-1}	167.9	Time Arena	Intercept	167.85	-0.7685	0.193
50 ng ml^{-1}	70.73	Arena	Intercept Time	2.46940 0.21444	1.064 2.661	0.2873 **0.0078**
100 ng ml^{-1}	278.3	Arena	Intercept	0.5993	1.042	0.298
250 ng ml^{-1}	152.5	Arena	Intercept	0.3425	0.981	0.326

Table 5.3. Summary of minimum adequate generalised linear mixed effects models (GLMM) for aphid volatile assays showing the minimum AIC used for model selection, random and fixed effects specified in the model, model estimates and associated z values and probabilities.

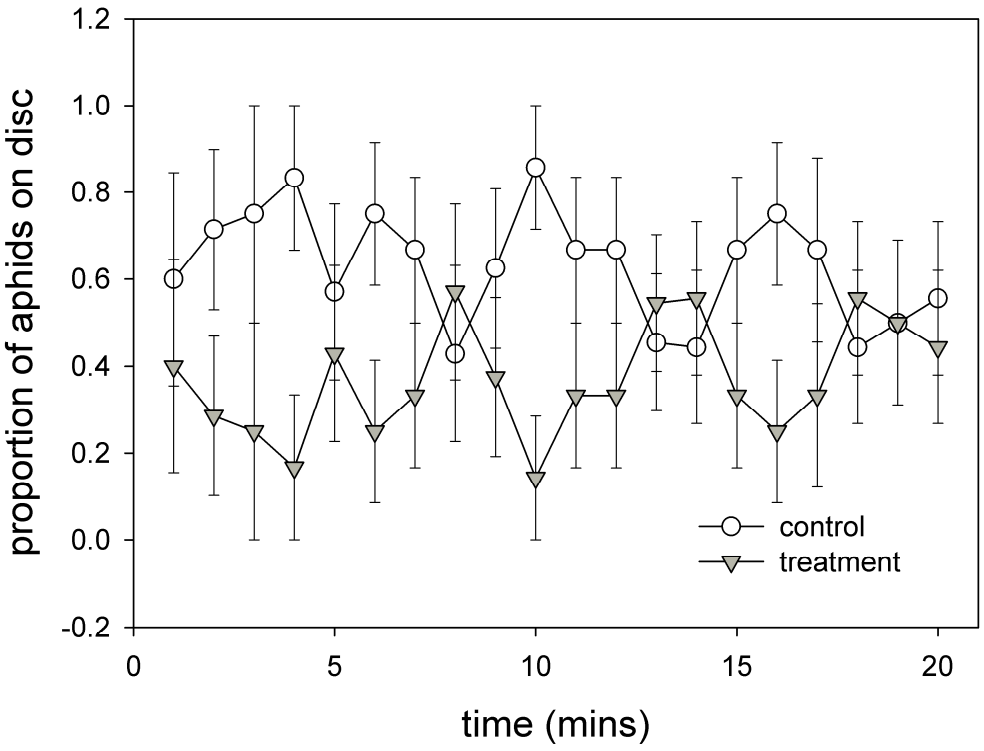


Figure 5.3 Aphid positions on paper leaf models treated with paraffin oil control or 10 ng (Z)-3-hexenyl acetate over 20 minute experimental period. Mean values of n = 20 ± SE are shown. See Table 5.3 for details.

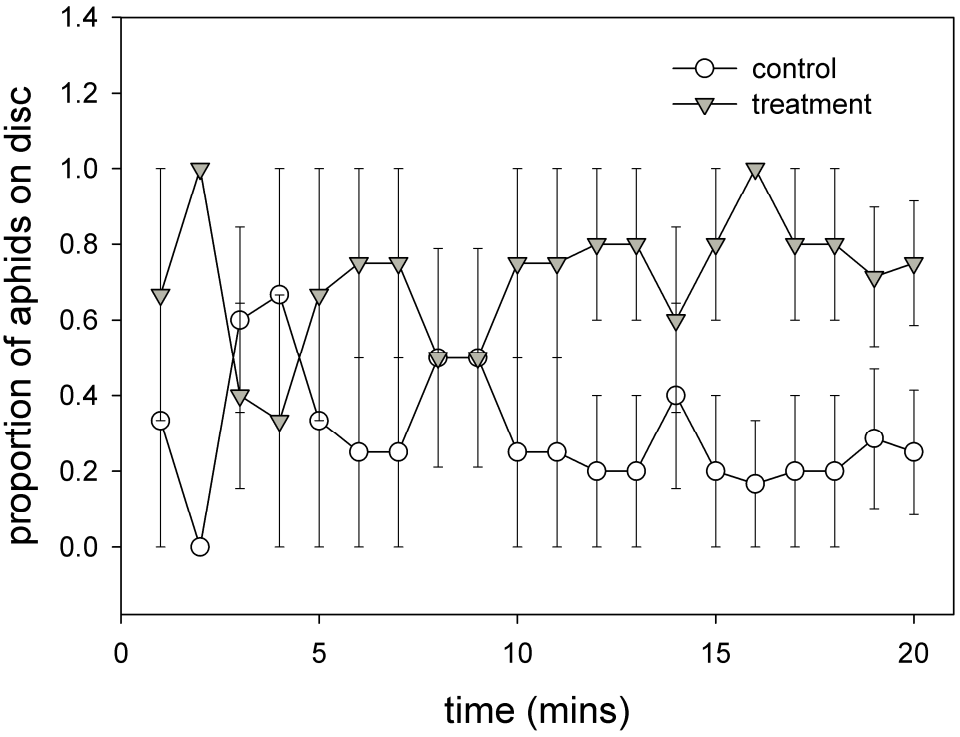


Figure 5.4 (a) Aphid positions on paper leaf models treated with paraffin oil control or 50 ng (Z)-3-hexenyl acetate over 20 minute experimental period. Mean values of n = 20 ± SE are shown. See Table 5.3 for details.

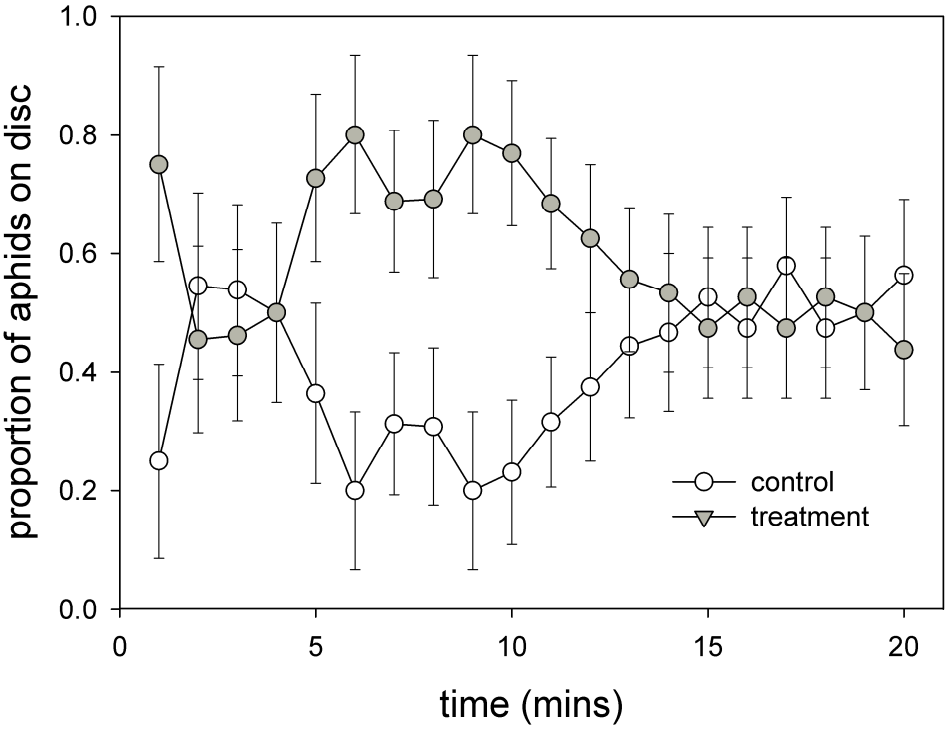


Figure 5.5 Aphid positions on paper leaf models treated with paraffin oil control or 100 ng (Z)-3-hexenyl acetate over 20 minute experimental period. Mean values of n = 24 ± SE are shown. See Table 5.3 for details.

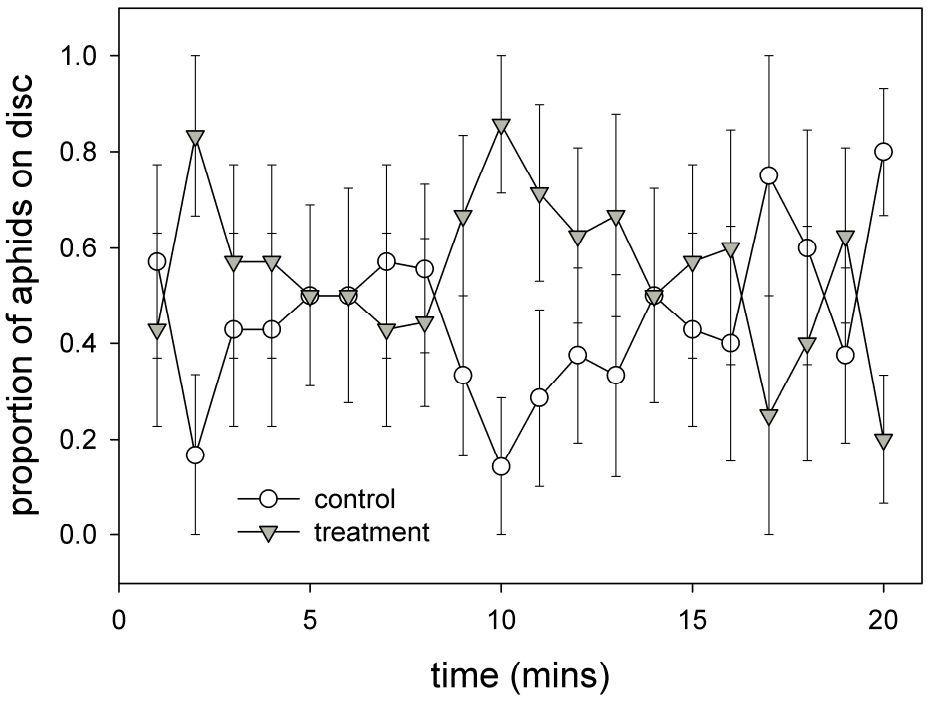


Figure 5.6 Aphid positions on paper leaf models treated with paraffin oil control or 250 ng (Z)-3-hexenyl acetate over 20 minute experimental period. Mean values of n = 24 ± SE are shown. See Table 5.3 for details.

5.4 Discussion

Comparison of the volatile profiles generated for healthy raspberry plants and those infected with BRNV and RLMV revealed that 17 of the 27 component volatile compounds were elevated in response to viral infection. Of these, 2-hexenal and (Z)-3-hexenyl acetate were determined to be considerably elevated on the basis of non-overlapping standard errors, making them candidate attractants for the virus vector, *Amphorophora idaei*. Both 2-hexenal and (Z)-3-hexenyl acetate are green leaf volatiles and have been previously shown to elicit electroantennogram responses from other aphid species, including the black bean aphid, *Aphis fabae*, and the peach–potato aphid, *Myzus persicae* (Visser *et al.*, 1996). A striking feature of headspace entrainments from both healthy and virus-infected raspberry plants was the presence of a family of at least five sesquiterpenes which had retention times of between 13 and 16 minutes in this study. Although they were not identified, these compounds seem to be a characteristic feature of *Rubus idaeus* plants and have been found previously in headspace entrainments taken from both canes and damaged leaves (Tom Shepherd, personal communication). However, the compounds which were found to be elevated in response to infection with BRNV and RLMV, 2-hexenal and (Z)-3-hexenyl acetate, have been previously identified and quantified from potato (Eigenbrode *et al.*, 2002), faba bean (Nottingham *et al.*, 1991), wheat (Jimenez-Martinez *et al.*, 2004) and squash (Mauck *et al.*, 2010). Green leaf volatiles such as these, are formed by the hydroperoxide lyase (HPL) pathway of oxylipin metabolism (Matsui, 2006; Shiojiri *et al.*, 2006) which is responsible for the oxidation of fatty acids to short chain aldehydes and their derivatives (Feussner & Wasternack, 2002). During this process, 2-hexenal, which is a six carbon adehyde, is metabolized by the

action of alcohol dehydrogenase to form its corresponding C_6 –alcohol, (*Z*)-3-hexen-1-ol or its isomer (*E*)-2-hexenal. This is then in turn involved in a reaction catalysed by acyltransferase to form (*Z*)-3-hexenyl acetate from the alcohol precursor and acetyl coenzyme A (acetyl CoA) (Matsui, 2006) (Figure 5.7).

The results of the volatile entrainments taken from raspberry plants indicated that the relative amount of (Z)-3-hexenyl acetate increased in response to host plant infection with BRNV and RLMV. This compound was selected for further study with *A. idaei* as it had been previously shown to be elevated in raspberry leaves subjected to mechanical damage, although to a lesser extent (Tom Shepherd, personal communication) and it is the end product of the metabolism of 2-hexenal, which was also shown to be elevated in this study. The range of concentrations tested was initially based on concentrations which had been previously shown to be naturally occurring in host plants of both *Myzus persicae* (Ngumbi *et al.*, 2007) and *Rhopalosiphum padi* (Medina-Ortega *et al.*, 2009). These bioassays demonstrated that individual *A. idaei* were attracted to (Z)-3-hexenyl acetate at a concentration of 50 ng per ml paraffin oil and were unaffected by the other concentrations tested. Although this study tested the role of only one compound from the raspberry headspace, the positive response of *A. idaei* to (Z)-3-hexenyl acetate at a concentration of 50 ng ml^{-1} suggests that that similar relationships may be found for other individual volatile components, particularly 2-hexenal which was also found to be highly elevated in response to infection with BRNV and RLMV. Various isometric forms of 2-hexenal have been previously shown to elicit electroantennogram responses from several aphid species (Table 5.1) and its elevation in virus-infected raspberry plants makes it another promising candidate as an attractant for *A. idaei.*

However, the fact that *A. idaei* responded to (Z)-3-hexenyl acetate at only one of the four concentrations tested suggests that further work is needed before firm conclusions can be drawn. In particular, future work should focus on elucidating the concentration of volatiles such as (Z)-3-hexenyl acetate which naturally occurs in the red raspberry headspace.

In contrast, infection of raspberry plants with BRNV and RLMV led to a considerable reduction in two volatile components, one identified as 5-ethyl-25H-furananone and the other one of the family of sesquiterpenes referred to previously. It may be that these components are unattractive to *A. idaei* and therefore their reduction in the headspace makes no difference to the behaviour of the aphid or perhaps their reduction enhances a compositional shift in volatile components in response to BRNV and RLMV-infection. This is, however, speculative, requiring further study of the effects of these components on *A. idaei* behaviour. However, similar studies of faba bean headspace volatiles have shown that although the intact blend acting as an aphid attractant, individual component volatiles can actually act as aphid deterrents when presented alone to the insecct (Webster et al., 2010).

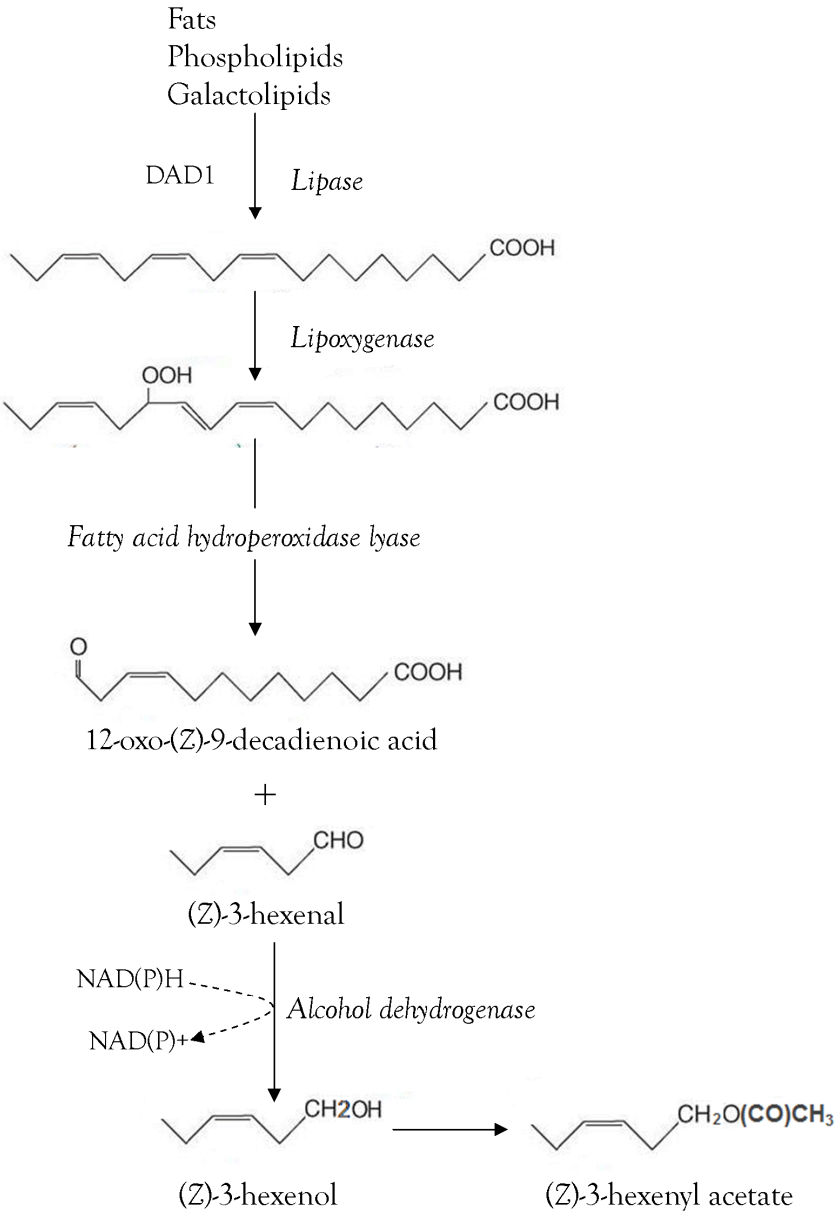


Figure 5.7. Part of the oxylipin biosynthetic pathway in plants showing reactions leading to the formation of (Z)-3-hexenyl acetate. For full pathway see Matsui (2006).

The findings of numerous investigations of plant volatile compounds have given rise to two general hypotheses in the ecological literature concerning insect host plant location. The first is that insects are able to recognize their host plants through detection of particular components of the plant headspace that are characteristic of a particular plant species exploited by the insect. The second hypothesis is that insects recognize specific ratios, or blends, of certain volatiles that are ubiquitous throughout the plant kingdom (Bruce *et al.*, 2005). The findings of this investigation appear to support the latter as both healthy and virus-infected plants contained the same suite of volatile components which were present in the headspace to a greater or lesser extent in response to virus infection, with the majority of components being compounds which have been characterized previously from different plant species. No one component was found to be unique to plants infected with BRNV and RLMV and therefore the preferential attraction of *A. idaei* to these plants detailed in Chapter Three is either the result of the elevation of individual components such as (Z)-3-hexenyl acetate and 2-hexenal, or the overall shift in the ratios of the volatile components which seems to result from host plant infection with the two viral pathogens studied here.

To my knowledge, this is the first study of alterations to raspberry headspace volatiles in response to virus infection. The results provide scope for further study of the exact mechanism by which the volatile components of the raspberry headspace act to preferentially attract *A. idaei* to virus infected plants. Although individual components have been tested and verified as aphid attractants in this study, many aspects remain to be investigated. For example, in order to translate these findings to a field situation, it would be interesting to not only test more component volatiles, such as 2-hexenal, but

also to investigate the distance over which they may remain physiologically active. This type of study would be crucial to the development of a chemical lure for *A. idaei* which may reduce the incidence of virus in a raspberry plantation by deceptive manipulation of the aphid's behaviour. Furthermore, behavioural testing of compositionally different synthetic blends based on the volatile ratios of both healthy and virus-infected raspberry plants would provide further insight into the mechanism of *A. ideai* attraction and may reveal which components are particularly important in the attraction of the aphid. This type of study requires a quantitative study of the raspberry headspace to be carried out, perhaps using volatile traps such as Super-Q™ which would allow known standards to be incorporated into the GC-MS analysis.

Chapter Six

Discussion

6.1 Summary

The primary aim of this thesis, as outlined in Chapter One, was to characterise the behaviour and performance of the European large raspberry aphid, *Amphorophora idaei*, in response to host plant infection with two viral pathogens of raspberry, Black raspberry necrosis virus and Raspberry leaf mottle virus. Towards this aim, experiments were designed to establish aphid preferences for healthy and virus-infected plants, investigate aphid performance in response to plant viral infection and identify and/or quantify changes in host plant chemistry which may be responsible for any differences that were observed. Chapter Three of this thesis showed that *A. idaei* was preferentially attracted to *Rubus idaeus* plants that were infected with Black raspberry necrosis virus and Raspberry leaf mottle virus when they were present in combination. This attraction appeared to be a viral manipulation of aphid behaviour as *A. idaei* performed poorly on these plants compared with those that were not infected. The aphid remained on the virus-infected host plant for a period of approximately 30 min.

Investigations of raspberry leaf chemistry detailed in Chapter Four showed that infection by these viral pathogens may facilitate aphid feeding by reducing the levels of phenolic compounds in the leaves which may otherwise act as a deterrent to the aphid and by increasing the levels of the amino acid methionine which has been implicated as an aphid feeding stimulant (Mittler, 1967, 1970; Harrewijn & Noordink, 1971). The amino acid composition of raspberry leaves was dominated by the non-essential glutamate, which further increased in response to infection with BRNV and RLMV. Glutamate may be an indicator of poor nutritional quality and may be linked to increased development time

observed for *A. idaei* when feeding on virus-infected plants. Finally, sampling of headspace volatiles from non-infected and infected raspberry plants (Chapter Five) revealed highly elevated levels of the green leaf volatile, (Z)-3-hexenyl acetate which was subsequently shown to act as an aphid attractant when presented to *A. idaei* at a concentration of 50 ng ml^{-1}. This study therefore makes a fundamental contribution to existing knowledge of indirect interactions which are mediated by a shared host plant and provides a basis for further work, either using the raspberry system or one of the many other plant systems which are simultaneously attacked by viral pathogens and their aphid vectors.

6.2 Aphids and plant chemistry

As was discussed in Chapter Four, aphids have a dietary requirement for energy rich sugars, and amino acids for protein metabolism (Rhodes *et al.*, 1996). In particular, aphids require their diet to be supplemented with essential amino acids either by acquiring them during feeding or through the metabolism of their symbionts. Aphids are limited by dietary nitrogen and low concentrations of amino acids in the phloem sap diet (Douglas, 1993; Dixon, 1998) therefore these nutrients may be important factors in determining the underlying plant chemistry which may act as the causal mechanism for changes in insect performance on host plants infected with pathogens. Furthermore, aphids must overcome plant defence mechanisms which may be mounted in response to herbivory and/or attack by viral pathogens. For example, all higher plants produce allelochemicals such as polyphenols and therefore all herbivorous insects encounter these toxic chemicals when feeding (Schoonhoven *et al.*, 2005). Aphids may have the capacity

to detoxify certain plant-derived feeding deterrents through enzymes present in their saliva (Miles, 1999) but many studies have shown that toxins, such as polyphenols, are deterrent to aphids (Jordens-Rottger, 1979; Dreyer & Jones, 1981; Peng & Miles, 1988; Chen *et al.*, 1997).

In addition to plant defensive responses to herbivory and infection with viral pathogens, this thesis investigated the indirect effects of virus infection on the aphid vector. Indeed, many studies have demonstrated that host plant infection with viruses can have significant effects on plant chemistry for aphids e.g. through reduced or elevated levels of amino acids in the phloem sap (Blua *et al.*, 1994; Fiebig *et al.*, 2004). The experiments described by this thesis show that both aphids and viruses can have profound effects on leaf chemistry, most notably compositional changes to carbon and free amino acids concentrations in the leaf tissue. For example, the overall leaf carbon concentration was found to be significantly elevated in response to host plant infection with BRNV and RLMV which may have the effect of diluting other important plant nutrients (Awmack & Leather, 2002). Furthermore, the concentration of certain amino acids was significantly elevated in response to plant viral infection. Elevated levels of amino acids have been previously shown to promote aphid performance so why wasn't this the case for *A. idaei*? The dominant amino acid quantified in raspberry was glutamate, a non-essential amino acid, which was found at a concentration of 24.9 ± 4.85 μM g^{-1} in virus-infected plants accounting for 77% of the total concentration of amino acids extracted from the leaf. High levels of glutamate have been implicated in reduced nutritional quality of phloem sap for aphids (Douglas, 1993). For example, a high relative concentration of glutamate was found in certain oat cultivars where *Rhopalosiphum padi* were found to perform poorly

in terms of development and reproduction (Weibull, 1988) and similar observations have been made of *Myzus persicae* and *Macrosiphum euphorbiae* feeding on potato where glutamate was most the most abundant amino acid in the phloem sap (Karley *et al.*, 2002). With studies of the mechanistic basis for these observations lacking, it is difficult to reach a definitive conclusion without further experimentation but investigations should focus on the potential of glutamate to disrupt efficient digestion of essential amino acids in the aphid gut, perhaps through competition between individual amino acids for receptor sites for readsorption.

Another possible explanation for the poorer performance of *A. idaei* on virus-infected plants could be competition between the insect and the pathogen for amino acids and other nutrients (Fiebig *et al.*, 2004) because viruses, like aphids, require amino acids for synthesis of new viral protein (Hull, 2002). The amino acids extracted from *Rubus* leaf tissue were *free* amino acids, meaning only those which may be directly available as nutrients for both organisms were quantified. This therefore allows further investigation of potential overlap in the amino acid requirements of both aphids and viral pathogens. The newly available full genomic sequence for BRNV and RLMV means that predictions of amino acid composition of viral proteins can be made (Appendix A). If these overlap with the dietary requirements of *A. idaei* then this may provide evidence for direct competition between the virus and the vector for these amino acids.

Analysis of amino acid composition of both BRNV and RLMV predict that serine, leucine and valine are the most abundant in terms of their relative concentration in total viral proteins of both BRNV and RLMV (Appendix A, Table A.2). It is well established

that aphids are limited by dietary nitrogen and amino acids in the phloem sap diet (Douglas, 1993; Dixon, 1998) which are considered to be a major determinant of aphid population increase (Brodbeck & Strong, 1987). The amino acid budget of the pea aphid, *Acyrthosiphum pisum*, calculated from the contribution of each amino acid to total aphid protein growth, revealed that *A. pisum* require aspartic acid and glutamic acid in the highest concentrations, followed by leucine and lysine (Gunduz & Douglas, 2009; Figure 6.1). Leucine is a major component of the viral protein of both BRNV and RLMV (see Appendix A). Extrapolation of this data for the dietary requirements of the large raspberry aphid would be inappropriate, especially with investigations of *A. idaei* symbionts lacking, but this data serves to highlight that the amino acid requirements of aphids may overlap with the requirements of viral pathogens. Similar dietary studies of *A. idaei* could not only confirm whether the dietary requirements of the aphid do indeed overlap with BRNV and RLMV but whether direct competition is likely to be occurring between these pest and pathogen species.

The findings of this thesis have therefore identified two potential mechanisms which may mediate the negative interaction between *A. idaei* and the two viruses that it vectors which require further investigation. The first is the role of non-essential amino acids, specifically glutamate, which may disrupt aphid digestion of phloem sap and the second is potential direct competition between aphid and virus for amino acids. The direct effects of viruses on their insect vectors are rarely quantified other than to investigate virus acquisition and inoculation times of the vector. Belliure *et al.* (2008) hypothesised that infection of vectors with plant viruses may increase their resistance to plant defence mechanisms. Through comparison of survival and development of the thrips vector of

Tomato spotted wilt virus (TSWV), *Frankliniella occidentalis,* that were virulent for or avirulent, their study showed that acquisition of virus did not consistently benefit the thrips vector when feeding. The authors concluded that the overall interaction between virus and vector was mainly indirect. However, if competition for amino acids is occurring between the vector and the virus in the raspberry system studied in this thesis, i.e. the replicating virus is removing amino acids from the phloem sap available to the aphid, then the overall negative effect of plant virus infection on *A. idaei* is likely to be a combination of both direct and indirect effects during aphid feeding.

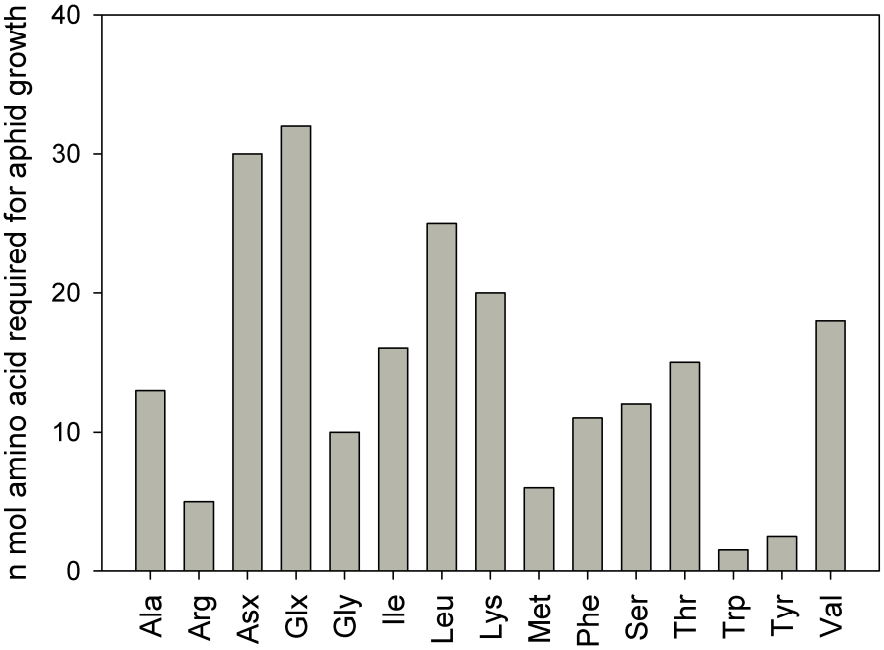


Figure 6.1. Concentration of individual amino acids required for aphid protein growth of the pea aphid, *Acyrthosiphon pisum*. ala – alanine, arg – arginine, asx – aspartic acid, glx – glutamic acid, gly – glycine, ile – isoleucine, leu – leucine, lys – lysine, met – methionine, phe – phenylalanine, ser – serine, thr – threonine, trp – tryptophan, tyr – tyrosine, val – valine. Figure redrawn from Gündüz & Douglas (2009).

6.3 From the lab to the field – the potential implications for aphid population dynamics

The reproductive capacity of *A. idaei,* and indeed all aphids, is such that small differences in aphid development time could have a massive impact on aphid population numbers. The prolonged development of *A. idaei* on virus-infected plants described in Chapter three is such that the so called 'telescoping of generations', whereby ovarian and embryonic development of the nymphs takes place inside the mother, would undoubtedly lead to a reduced intrinsic rate of increase (r_m) on plants infected with BRNV and RLMV where there is a delay in nymphs being laid by the mother onto the host plant. The results of a small field survey conducted in 2008 which aimed to explore seasonal aphid population dynamics on healthy and virus-infected plants produced some interesting results which may corroborate aphid behaviour in the laboratory (see Appendix B). PCR testing of 42 plants spread evenly across the plantation revealed that over 90% of the plants were infected with BRNV and RLMV in various combinations (i.e. single infections with either virus or dual infection) and of those infected, over 60% contained both viruses. Although the plants surveyed were of a different cultivar to that used in experiments detailed in this thesis, their parentage meant that at least some resistance to *A. idaei* should have been present and the prevalence of BRNV and RLMV in a field plot planted in 2002 highlights the need for ongoing studies of *A. idaei* with a view to controlling virus spread. Mathematical simulations of virus transmission suggest that virus transmission is optimal when aphids preferentially orientate to infected plants but remain there long enough to acquire the virus before migrating to a healthy host (Sisterson, 2008). The results of the laboratory studies show that this scenario occurs in

red raspberry and the results of the field survey demonstrate that BRNV and RLMV are capable of achieving high prevalence under certain conditions. This may prove energetically costly to *A. idaei* as the lack of nutrition provided by virus-infected plants reduces aphid performance and triggers migration. These costs may have negative impacts on aphid population numbers, reflected by the small population numbers found in this field study.

The observations from raspberry and other studies serve to highlight the importance of transferring laboratory results into field situations where many other biotic and abiotic factors may play a role in the interaction between host plants, viruses and the insects that vector them. In this case however, in order for laboratory and field studies to complement each other, further experiments must be carried out using alate *A. idaei* in order to ascertain host plant preference under field conditions and also, of particular importance to raspberry systems, the effects of enclosing plantations under polytunnels. Polytunnels provide a longer growing season for raspberry by increasing temperature but these variations in growing conditions are also likely to affect virus transmission by altering plant metabolism (Canto *et al.*, 2009). Specifically, fluctuations in plant nutrients are likely to alter aphid behaviour and physiology. For example, a recent study has demonstrated that raspberry plants grown under polytunnels, where temperatures were 7–10 °C higher than field plantations, had a lower foliar concentration of essential amino acids compared with field plots, associated with smaller aphid body size (Johnson *et al.*, 2010). Smaller *A. idaei* are likely to be more susceptibile to predation by ladybird predators (Alliuame *et al.*, 2010) and parasitism by wasps such as *Aphidius ervi* (Mitchell *et al.*, 2010). Therefore, it is important that studies continue to investigate, not only plant-

mediated effects on *A. idaei*, but also the knock-on effect on higher trophic groups such as predators and natural enemies.

6.4 Crosstalk

Three important signaling pathways are known to be involved in plant defence signaling against invading herbivores or pathogens, jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). The extent to which the pathways are activated depends on the type of organism that is attacking, thus enabling the plant to tailor its response to be attacker-specific (Reymond & Farmer, 1998). However, SA-mediated responses to pathogen attack have been shown to diminish the activity of JA-induced responses, normally associated with herbivory (Stout *et al.*, 1999). Such 'cross-talk' between the pathways may result in antagonistic or synergistic interactions between different attackers (Koornneef & Pieterse, 2008; van Dam, 2009). When *A. idaei* feeds on BRNV and RLMV-infected host plants the resulting interaction appears to be detrimental to the aphid, as demonstrated by the performance experiment where development time of the aphid was increased on virus-infected plants. In addition, the concentration of polyphenolics in the leaf tissue was found to be significantly elevated only when the plant was attacked by aphids and not in response to viral infection (see Chapter four, Figure 4.1). This surprising result suggests that aphids induce a plant defensive response which viruses are capable of evading. Previous studies have shown that aphids, like pathogens, tend to induce a response associated with SA signaling (Bostock, 1999; Bostock *et al.*, 2001) and a weaker JA response (Moran & Thompson, 2001; Rodriguez-Saona *et al.*, 2005; Thaler *et al.*, 2010). However, in the presence of viruses, *A. idaei* did not trigger the same elevated

levels of polyphenolics that it did in healthy plants. The benefit of this mechanism to the virus could be a non-deterrence of aphid feeding in order for it to acquire virions for subsequent transmission. Studies of electrical potential of aphids (EPG) on healthy and virus-infected plants would be required in order to confirm if phenolic compounds were indeed exerting an effect. In addition, further studies should focus on elucidating these complex interactions in order to ascertain which signaling pathways are involved in anti-aphid and anti-virus defence in raspberry plants (see section 6.5).

6.5 Future perspectives

The experiments described in this thesis provide a basis for a variety of future investigations of raspberry-virus-aphid interactions. Discussion in this chapter, and in those previous, have made suggestions for further whole plant experiments which may aid in disentangling the mechanisms which are in operation to deceptively attract *A. idaei* to what proves to be a poor host in terms of aphid development. However, conducting studies at a genomic level would also provide insight into specific plant signals and their timing in response to aphid and pathogen attack. For example, molecular studies of *Arabidopsis thaliana* have shown that plant attack by viral pathogens or aphids leads to a similar pattern of gene activation suggesting that the same signaling pathways are triggered (Bostock, 1999; Bostock *et al.*, 2001). However, as detailed above, feeding by *A. idaei* resulted in different responses to infection with BRNV and RLMV, indicating that the plant mounted a different mode of defence in response to each of these attackers. Insects and pathogens that attack plants induce signalling pathways that are responsible for the regulation of plant defence genes and metabolites involved in direct and indirect

defence against the attacker (Stout *et al.*, 2005). Analysis of attacker-specific gene expression profiles using microarray technology has proven valuable for untangling which plant signalling pathways are activated in response to different modes of attack (see DeVos *et al.*, 2005). To date, most studies of gene expression profiles have been generated by manipulation of the model plant *Arabidopsis thaliana* by subjecting plant leaves to attack by insects and pathogens utilising different feeding strategies for comparison of expression signatures. These include phloem feeding aphids (Mewis *et al.*, 2006; Couldridge *et al.*, 2007; Kusnierczyk *et al.*, 2008), leaf chewing caterpillars (Reymond *et al.*, 2004; de Vos *et al.*, 2005; Mewis *et al.*, 2006), microbial pathogens (leaf fungi or bacteria) (de Vos *et al.*, 2005) and simulated mechanical wounding (Reymond *et al.*, 2000).

Reymond *et al.* (2000) compared the effects on gene expression of both mechanical wounding of the plant and feeding by larvae of the cabbage butterfly, *Pieris rapae* (Lepidoptera: Pieridae), and found that each treatment generated very different transcript profiles. Removal of the plant's rosette leaves activated genes that are known to be associated with water-stress, such as PRODH (Kiyosue *et al.*, 1996) which was strongly down-regulated. Feeding by *P. rapae,* however, did not induce many of the genes that were inducible by mechanical wounding and instead induced other plant defence genes such as that encoding hevein-like protein (HEL). The overall transcript profile generated by *P. rapae* feeding damage suggests that the larvae can minimise the activation of host defence genes and actually generate an attacker-specific gene transcript profile. De Vos *et al.* (2005) reported similar findings with SA-, JA- and ET- responsive genes. These were found to vary greatly in both the timing of expression and in the quantity of transcript

present in response to insects with different feeding modes (e.g. leaf chewers, cell content feeders and phloem feeders), once again indicating attacker-specific gene transcript profiles. These studies have provided a detailed insight into the response of the whole plant genome to insect and pathogen attack with the advantage of the availability of full genomic sequence data for *Arabidopsis thaliana*. In contrast, sequencing of the *Rubus idaeus* genome is not yet completed and at present only limited sequence data are available from an in-house database held at SCRI. Crucially, full genomic sequence would allow for the development of a full cDNA microarray and subsequently, attacker-specific transcript patterns could begin to be identified. The studies from *Arabidopsis* have served to identify numerous candidate genes implicated in plant attack by insects and pathogens and the sequences for these genes are readily available from online databases such as The *Arabidopsis* Information Resource (TAIR). It is therefore highly likely that microarray technologies in conjunction with quantitative RT-PCR can be used as a molecular tool to identify attacker specific gene transcript profiles from raspberry in the very near future. Quantitative real-time PCR (qRT-PCR) is a powerful molecular technique that can be used to simultaneously amplify and quantify levels of a particular gene transcript present within a sample. Such investigations would further our understanding of complex plant signalling processes and identify differences and similarities between activation in response to pathogen and aphid attack.

6.6 Conclusions

While most studies to date have focussed on elucidating the mechanism by which aphids are attracted to virus-infected plants, they have either assessed the consequences of this choice in terms of subsequent aphid performance (Eigenbrode *et al.*, 2002; Srinivasan *et al.*, 2006; Alvarez *et al.*, 2007; Werner *et al.*, 2009), or have addressed aspects of plant nutrition which may affect aphids' host plant preference (Blua *et al.*, 1994; Fiebig *et al.*, 2004). This thesis aimed to incorporate all of these aspects and has tested not only aphid preference and performance in response to host plants infected with viral pathogens, but has also identified potential causal mechanisms for the behaviours observed in terms of attraction to the host plant and the underlying leaf chemistry which may be important for aphid development. Thus, this study provides novel insights into the nature of plant-aphid-virus interactions. Furthermore, the specialist aphid central to this investigation, *Amphorophora idaei*, has provided novel insights into the intimate associations occurring between the insect and its host plant, which cannot be achieved by studies of polyphagous aphids such as *Myzus persicae* and *Rhopalosiphum padi*. In conclusion, the findings of this thesis seem to suggest that infection of raspberry plants with BRNV and RLMV can lead to a virus-induced manipulation of aphid behaviour and performance which presumably acts to maximise transmission. Furthermore, the viruses appear capable of manipulating plant chemistry in such a way that facilitates aphids probing/feeding for a time period of 30 min, which is favourable for successful acquisition of virions but ultimately may induce migration to new feeding sites. Leaf chemical analyses and headspace volatile sampling have added crucial detail to the underlying plant physiological mechanisms which may mediate these interactions. This study therefore provides a platform for

further investigation at both genomic and field level to further our understanding of indirect interactions between aphids and the viruses they transmit, mediated by the host plant.

REFERENCES

Abouzaid, M.M., Beninger, C.W., Arnason, J.T. & Nozzolillo, C. (1993) The effect of one flavone, 2 catechins and 4 flavonols on mortality and growth of the European corn-borer (*Ostrinia nubilalis* Hubner). *Biochemical Systematics and Ecology*, **21**, 415-420.

Agelopoulos, N., Birkett, M.A., Hick, A.J., Hooper, A.M., Pickett, J.A., Pow, E.M., Smart, L.E., Smiley, D.W.M., Wadhams, L.J. & Woodcock, C.M. (1999) Exploiting semiochemicals in insect control. *Pesticide Science*, **55**, 225-235.

Akaike, H. (1974) A new look at the statistical model identification. *IEEE Transactions on Automatic Control* **19,** 716–723

Alford, D.V. (2007) *Pests of fruit crops - a colour handbook*. Manson Publishing, London.

Alliuame, A.A., McMenemy, L.S., Vasse, M., Artaux, A., Hubbard, S.F. & Johnson, S.N. (2010) Predatory behaviour of the seven-spot ladybird *Coccinella septempunctata* on the European large raspberry aphid, *Amphorophora idaei*. *Biocontrol*, ***submitted***.

Alvarez, A.E., Garzo, E., Verbeek, M., Vosman, B., Dicke, M. & Tjallingii, W.F. (2007) Infection of potato plants with potato leafroll virus changes attraction and feeding behaviour of *Myzus persicae*. *Entomologia Experimentalis et Applicata*, **125**, 135-144.

Anon. (2005) Why polytunnels are used. Fact sheet produced by British Summer Fruits. Available at: http://www.britishsummerfruits.co.uk (accessed 17 February 2008).

Arpita, C. & Ghosh, S.K. (2008) Alterations in biochemical components in mesta plants infected with yellow vein mosaic disease. *Brazilian Journal of Plant Physiology*, **20**, 267-275.

Awmack, C.S. & Leather, S.R. (2002) Host plant quality and fecundity in herbivorous insects. *Annual Review of Entomology*, **47**, 817-844.

Bacher, S., Friedli, J. & Schar, I. (2002) Developing in diseased host plants increases survival and fecundity in a stem-boring weevil. *Entomologia Experimentalis et Applicata*, **103**, 191-195.

Baldwin, I.T. & Preston, C.A. (1999) The eco-physiological complexity of plant responses to insect herbivores. *Planta*, **208**, 137-145.

Baumann, G. (1982) Elimination of a heat-stable raspberry virus by combining heat treatment and meristem culture. *Acta Horticulturae*, **129**, 11-12.

Belliure, B., Janssen, A., Maris, P.C., Peters, D. & Sabelis, M.W. (2005) Herbivore arthropods benefit from vectoring plant viruses. *Ecology Letters*, **8**, 70-79.

Belliure, B., Janssen, A. & Sabelis, M.W. (2008) Herbivore benefits from vectoring plant virus through reduction of period of vulnerability to predation. *Oecologia*, **156**, 797-806.

Belliure, B., Sabelis, M.W. & Janssen, A. (2010) Vector and virus induce plant responses that benefit a non-vector herbivore. *Basic and Applied Ecology*, **11**, 162-169.

Bi, J.L., Murphy, J.B. & Felton, G.W. (1997) Antinutritive and oxidative components as mechanisms of induced resistance in cotton to *Helicoverpa zea*. *Journal of Chemical Ecology*, **23**, 97-117.

Biere, A., Elzinga, J.A., Honders, S.C. & Harvey, J.A. (2002) A plant pathogen reduces the enemy-free space of an insect herbivore on a shared host plant. *Proceedings of the Royal Society B - Biological Sciences*, **269**, 2197-2204.

Birch, A.N.E. & Jones, A.T. (1988) Levels and components of resistance to *Amphorophora idaei* in raspberry cultivars containing different resistance genes. *Annals of Applied Biology*, **113**, 567-578.

Birch, A.N.E., Harrower, B., Malloch, G., Catley, M.A. & Jones, A.T. (1992) Potential use of RFLP analysis for rapidly distinguishing biotypes of the virus vector aphid, *Amphorophora idaei*. *Acta Horticulturae*, **308**, 95-97.

Birch, A.N.E., Fenton, B., Malloch, G., Jones, A.T., Phillips, M.S., Harrower, B.E., Woodford, J.A.T. & Catley, M.A. (1994) Ribosomal spacer length variability in the large raspberry aphid, *Amphorophora idaei* (Aphidinae: Macrosiphini). *Insect Molecular Biology*, **3**, 239-245.

Birch, A.N.E., Geoghegan, I.E., Majerus, M.E.N., Hackett, C. & Allen, J. (1996). Interactions between plant resistance genes, pest aphid populations and beneficial aphid predators. In *Scottish Crop Research Institute Annual Report 1996/7*. Scottish Crop Research Institute, Dundee.

Birch, A.N.E., Jones, A.T., Fenton, B., Malloch, G., Geoghegan, I., Gordon, S.C., Hillier, J. & Begg, G. (2002) Resistance-breaking raspberry aphid biotypes: constraints to sustainable control through plant breeding. *Acta Horticulturae*, **585**, 315-317.

Birch, A.N.E., Gordon, S.C., Fenton, B., Malloch, G., Mitchell, C., Jones, A.T., Griffiths, D.W., Brennan, R., Graham, J. & Woodford, J.A.T. (2004) Developing a sustainable IPM system for high value *Rubus* crops (raspberry, blackberry) for Europe. *Acta Horticulturae*, **649**, 289-292.

Blackman, R.L., Eastop, V.F. & Hills, M. (1977) Morphological and cytological separation of *Amphorophora* Buckton (Homoptera-Aphididae) feeding on European raspberry and blackberry (*Rubus* spp.). *Bulletin of Entomological Research*, **67**, 285-296.

Blackman, R.L. & Eastop, V.F. (2000) *Aphids on the world's crops: an identification and information guide*. John Wiley & Sons Ltd., Chichester.

Blua, M.J. & Perring, T.M. (1992) Alatae production and population increase of aphid vectors on virus-infected host plants. *Oecologia*, **92**, 65-70.

Blua, M.J., Perring, T.M. & Madore, M.A. (1994) Plant virus-induced changes in aphid population development and temporal fluctuations in plant nutrients. *Journal of Chemical Ecology*, **20**, 691-707.

Bolton, A.T. & Turner, L.H. (1962) Note on obtaining virus-free plants of red raspberry through the use of tip cuttings. *Canadian Journal of Plant Science*, **42**, 210-211.

Börner, C. (1939) Gattungen und arten der mitteleuroäischen Aphidenfauna. *Arbeiten uber physiologische und angewandte Entomologie aus Berlin-Dahlem* **6**, 75-83.

Borowiak-Sobkowiak, B. (2006) Bionomy and ecology of *Amphorophora idaei* (Börn.) on raspberry. *Journal of Plant Protection Research*, **46**, 169-180.

Bostock, R.M. (1999) Signal conflicts and synergies in induced resistance to multiple attackers. *Physiological and Molecular Plant Pathology*, **55**, 99-109.

Bostock, R.M., Karban, R., Thaler, J.S., Weyman, P.D. & Gilchrist, D. (2001) Signal interactions in induced resistance to pathogens and insect herbivores. *European Journal of Plant Pathology*, **107**, 103-111.

Briggs, J.B. (1965) The distribution, abundance and genetic relationships of four strains of *Rubus* aphid (*Amphorophora rubi* (Kalt.)) in relation to raspberry breeding. *Journal of Horticultural Science*, **40**, 109-117.

Brodbeck, B. & Strong, D. (1987). Amino acid nutrition of herbivorous insects and stress to host plants. In *Insect Outbreaks* (eds P. Barbosa & J.C. Schultz), pp. 347-364. Academic Press, San Diego.

Bruce, T.J.A., Wadhams, L.J. & Woodcock, C.M. (2005) Insect host location: a volatile situation. *Trends in Plant Science*, **10**, 269-274.

Buchner, P. (1965) *Endosymbioses of animals with plant microorganisms*. John Wiley, Chichester, UK.

Cadman, C.H. (1952) Studies in *Rubus* virus diseases. III. A veinbanding disease of raspberries. *Annals of Applied Biology*, **39**, 69-77.

Cadman, C.H. (1954) Studies in *Rubus* virus diseases. VI. Aphid transmission of raspberry leaf mottle virus. *Annals of Applied Biology*, **41**, 207-214.

Canto, T., Aranda, M.A. & Fereres, A. (2009) Climate change effects on physiology and population processes of hosts and vectors that influence the spread of hemipteran-borne plant viruses. *Global Change Biology*, **15**, 1884-1894.

Castle, S.J. & Berger, P.H. (1993) Rates of growth and increase of *Myzus persicae* on virus-infected potatoes according to type of virus-vector relationship. *Entomologia Experimentalis et Applicata*, **69**, 51-60.

Chambers, J. (1961) The production and maintenance of virus-free raspberry plants. *Journal of Horticultural Science*, **36**, 48-54.

Chapman, R.F. (2003) Contact chemoreception in feeding by phytophagous insects. *Annual Review of Entomology*, **48**, 455-484.

Chen, J.Q., Rahbe, Y., Delobel, B., Sauvion, N., Guillaud, J. & Febvay, G. (1997) Melon resistance to the aphid *Aphis gossypii*: behavioural analysis and chemical correlations with nitrogenous compounds. *Entomologia Experimentalis et Applicata*, **85**, 33-44.

Chen, M.S. (2008) Inducible direct plant defense against insect herbivores: A review. *Insect Science*, **15**, 101-114.

Chittka, L. & Döring, T.F. (2007) Are Autumn foliage colors red signals to aphids? *PLOS Biology*, **5,** 1640-1644.

Cisneros, J.J. & Godfrey, L.D. (2001) Midseason pest status of the cotton aphid (Homoptera : Aphididae) in California cotton: Is nitrogen a key factor? *Environmental Entomology*, **30**, 501-510.

Clancy, K.M. & Price, P.W. (1987) Rapid herbivore growth enhances enemy attack - sublethal plant defenses remain a paradox. *Ecology*, **68**, 733-737.

Clark, K.E., Hartley, S.E., Brennan, R.M., MacKenzie, K. & Johnson, S.N. (2010) Oviposition and feeding behaviour by the vine weevil (*Otiorhynchus sulcatus*) in relation to red raspberry (*Rubus idaeus*) cultivars and plant nutritional status. *Agricultural & Forest Entomology*, ***in press***.

Converse, R.H. (1987) *Virus diseases of small fruits: United States Department of Agriculture Handbook No. 631*. US Government Printing Office, Washington D.C.

Couldridge, C., Newbury, H.J., Ford-Lloyd, B., Bale, J. & Pritchard, J. (2007) Exploring plant responses to aphid feeding using a full *Arabidopsis* microarray reveals a small number of genes with significantly altered expression. *Bulletin of Entomological Research*, **97**, 523-532.

Crawley, M.J. (2007) The R Book. John Wiley & Sons Ltd., Chichester, England.

Dangl, J.L. & Jones, J.D.G. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826-833.

de Vos, M., Van Oosten, V.R., Van Poecke, R.M.P., Van Pelt, J.A., Pozo, M.J., Mueller, M.J., Buchala, A.J., Métraux, J.P., Van Loon, L.C., Dicke, M. & Pieterse, C.M.J. (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions*, **18**, 923-937.

Deighton, N., Brennan, R., Finn, C. & Davies, H.V. (2000) Antioxidant properties of domesticated and wild *Rubus* species. *Journal of the Science of Food and Agriculture*, **80**, 1307-1313.

Dicke, M. (2009) Behavioural and community ecology of plants that cry for help. *Plant, Cell and Environment*, **32**, 654-665.

Dicker, G.H.L. (1940) The biology of the *Rubus* aphids. *Journal of Pomology and Horticultural Science*, **18**, 1-33.

Dickson, A.T. (1979) The population dynamics of raspberry aphids in eastern Scotland. PhD Thesis, University of Dundee.

Dixon, A.F.G. (1998) *Aphid Ecology*. Chapman and Hall, London.

Donaldson, J.R. & Gratton, C. (2007) Antagonistic effects of soybean viruses on soybean aphid performance. *Environmental Entomology*, **36**, 918-925.

Douglas, A.E. (1993) The nutritional quality of phloem sap utilized by natural aphid populations. *Ecological Entomology*, **18**, 31-38.

Douglas, A.E. (1998) Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology*, **43**, 17-37.

Douglas, A.E. (2006) Phloem-sap feeding by animals: problems and solutions. *Journal of Experimental Botany*, **57**, 747-754.

Dreyer, D.L. & Jones, K.C. (1981) Feeding deterrency of flavonoids and related phenolics towards *Schizaphis graminum* and *Myzus persicae* - aphid feeding deterrents in wheat. *Phytochemistry*, **20**, 2489-2493.

Eigenbrode, S.D., Ding, H., Shiel, P. & Berger, P.H. (2002) Volatiles from potato plants infected with potato leafroll virus attract and arrest the virus vector, *Myzus persicae* (Homoptera : Aphididae) *Proceedings of the Royal Society of London B - Biological Sciences*, **269**, 2603-2603.

FAOSTAT (2008) Food and Agriculture Organization of the United Nations - Production Data Archives. Available at: http://faostat.fao.org/ (accessed 16 February 2008).

Farmer, E.E. & Ryan, C.A. (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proceedings of the National Academy of Sciences of the United States of America*, **87**. 7713-7716

Feeny, P. (1970) Seasonal changes in oak leaf tannins and nutrients as a cause of Spring feeding by winter moth caterpillars. *Ecology*, **51**, 565-581.

Felton, G.W., Summers, C.B. & Mueller, A.J. (1994) Oxidative responses in soybean foliage to herbivory by bean leaf beetle and three-cornered alfalfa hopper. *Journal of Chemical Ecology*, **20**, 639-650.

Fereres, A., Kampmeier, G.E. & Irwin, M.E. (1999) Aphid attraction and preference for soybean and pepper plants infected with potyviridae. *Annals of the Entomological Society of America*, **92**, 542-548.

Ferriss, R.S. & Berger, P.H. (1993) A stochastic simulation model of epidemics of arthropod vectored plant viruses. *Phytopathology*, **83**, 1269-1278.

Feussner, I. & Wasternack, C. (2002) The lipoxygenase pathway. *Annual Review of Plant Biology*, **53**, 275-297.

Fiebig, M., Poehling, H.M. & Borgemeister, C. (2004) Barley yellow dwarf virus, wheat, and *Sitobion avenae*: a case of trilateral interactions. *Entomologia Experimentalis et Applicata*, **110**, 11-21.

Fraenkel, G.S. (1959) Raison d'être of secondary plant substances. *Science*, **129**, 1466-1470.

Gildow, F.E. (1980) Increased production of alatae by aphids (Hemiptera, Aphididae) reared on oats infected with Barley yellow dwarf virus. *Annals of the Entomological Society of America*, **73**, 343-347.

Gildow, F.E. (1983) Influence of Barley yellow dwarf virus-infected oats and barley on morphology of aphid vectors. *Phytopathology*, **73**, 1196-1199.

Gildow, F.E. & Gray, S.M. (1993) The aphid salivary gland basal lamina as a selective barrier associated with vector-specific transmission of Barley yellow dwarf Luteoviruses. *Phytopathology*, **83**, 1293-1302.

Gordon, S.C., Woodford, J.A.T. & Birch, A.N.E. (1997) Arthropod pests of *Rubus* in Europe: pest status, current and future control strategies. *Journal of Horticultural Science*, **72**, 831-862.

Graham, J., Smith, K., Woodhead, M. & Russell, J. (2002) Development and use of simple sequence repeat (SSR) markers in *Rubus* species. *Molecular Ecology Notes*, **2**, 250-252.

Graham, J., Smith, K., MacKenzie, K., Jorgenson, L., Hackett, C. & Powell, W. (2004) The construction of a genetic linkage map of red raspberry (*Rubus idaeus* subsp. *idaeus*) based on AFLPs, genomic-SSR and EST-SSR markers. *Theoretical and Applied Genetics*, **109**, 740-749.

Gray, S. & Gildow, F.E. (2003) Luteovirus-aphid interactions. *Annual Review of Phytopathology*, **41**, 539-566.

Gunduz, E.A. & Douglas, A.E. (2009) Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proceedings of the Royal Society of London B - Biological Sciences*, **276**, 987-991.

Hale, B.K., Bale, J.S., Pritchard, J., Masters, G.J. & Brown, V.K. (2003) Effects of host plant drought stress on the performance of the bird cherry-oat aphid, *Rhopalosiphum padi* (L.): a mechanistic analysis. *Ecological Entomology*, **28**, 666-677.

Halgren, A., Tzanetakis, I.E. & Martin, R.R. (2007) Identification, characterization, and detection of Black raspberry necrosis virus. *Phytopathology*, **97**, 44-50.

Hall, H., Hummer, K., Jamieson, A., Jennings, S.N. & Weber, C.A. (2009) Raspberry breeding and genetics. *Plant Breeding Reviews*, **32** 39-353.

Hamilton, W.D. & Brown, S.P. (2001) Autumn tree colours as a handicap signal. *Proceedings of the Royal Society of London B - Biological Sciences*, **268**, 1489-1493.

Han, Y., Wang, Y., Bi, J.-L., Yang, X.-Q., Huang, Y., Zhao, X., Hu, Y. & Cai, Q.-N. (2009) Constitutive and induced activities of defense-related enzymes in aphid-resistant and aphid-susceptible cultivars of wheat. *Journal of Chemical Ecology*, **35**, 176-182.

Hansson, B.S. (2002) A bug's smell - research into insect olfaction. *Trends in Neurosciences*, **25**, 270-274.

Hardie, J. (1989) Spectral specificity for targeted flight in the black bean aphid, *Aphis fabae*. *Journal of Insect Physiology*, **35**, 619-626.

Harrewijn, P. & Noordink, J.P. (1971) Taste perception of *Myzus persicae* in relation to food uptake and developmental processes. *Entomologia Experimentalis et Applicata*, **14**, 413-419.

Hartley, S.E. & Firn, R.D. (1989) Phenolic biosynthesis, leaf damage and insect herbivory in birch (*Betula pendula*). *Journal of Chemical Ecology*, **15**, 275-283.

Hatcher, P.E., Moore, J., Taylor, J.E., Tinney, G.W. & Paul, N.D. (2004) Phytohormones and plant-herbivore-pathogen interactions: Integrating the molecular with the ecological. *Ecology*, **85**, 59-69.

Hill, A.R. (1956) Observations on the North American form of *Amphorophora rubi* Kalt. (Homoptera, Aphididae). *Canadian Entomologist*, **88**, 89-91.

Hodge, S. & Powell, G. (2008) Do plant viruses facilitate their aphid vectors by inducing symptoms that alter behavior and performance? *Environmental Entomology*, **37**, 1573-1581.

Hogenhout, S.A., Redinbaugh, M.G. & Ammar, E.D. (2003) Plant and animal rhabdovirus host range: a bug's view. *Trends in Microbiology*, **11**, 264-271.

Hogenhout, S.A., Ammar, E., Whitfield, A.E. & Redinbaugh, M.G. (2008) Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology*, **46**, 327-359.

Hollings, M. (1965) Disease control through virus-free stock. *Annual Review of Phytopathology*, **3**, 367-396.

Hull, R. (2002) *Matthews' Plant Virology*. Academic Press, London.

Jeger, M.J., Holt, J., Van den Bosch, F. & Madden, L.V. (2004) Epidemiology of insect-transmitted plant viruses: modelling disease dynamics and control interventions. *Physiological Entomology*, **29**, 291-304.

Jiménez-Martínez, E.S., Bosque-Pérez, N.A., Berger, P.H., Zemetra, R.S., Ding, H.J. & Eigenbrode, S.D. (2004) Volatile cues influence the response of *Rhopalosiphum padi* (Homoptera: Aphididae) to Barley yellow dwarf virus-infected transgenic and untransformed wheat. *Environmental Entomology*, **33**, 1207-1216.

Johnson, S.N., Douglas, A.E., Woodward, S. & Hartley, S.E. (2003) Microbial impacts on plant-herbivore interactions: the indirect effects of a birch pathogen on a birch aphid. *Oecologia*, **134**, 388-396.

Johnson, S.N., Karley, A.J., Hawes, C. & Mitchell, C. (2008). Aboveground-belowground trophic linkages. In *Scottish Crop Research Institute Annual Report 2007* (ed S. Stephens). Scottish Crop Research Institute, Dundee.

Johnson, S.N., Hawes, C. & Karley, A.J. (2009) Reappraising the role of plant nutrients as mediators of interactions between root- and foliar-feeding insects. *Functional Ecology*, **23**, 699-706.

Johnson, S.N., Karley, A.J. & Young, M.W. (2010) Effects of localised climate change on raspberry aphid populations and crop physiology. *Agriculture Ecosystems & Environment*, ***submitted***.

Jonak, C., Okresz, L., Bogre, L. & Hirt, H. (2002) Complexity, cross talk and integration of plant MAP kinase signalling. *Current Opinion in Plant Biology*, **5**, 415-424.

Jones, A.T. & Murant, A.F. (1972) Some properties of a mechanically transmissible virus widespread in raspberry (*Rubus idaeus*) in Scotland. *Plant Pathology*, **21**, 166-170.

Jones, A.T. (1976) Effect of resistance to *Amphorophora rubi* in raspberry (*Rubus idaeus*) on the spread of aphid-borne viruses. *Annals of Applied Biology*, **82**, 503-510.

Jones, A.T. (1979) Further studies on the effect of resistance to *Amphorophora idaei* in raspberry (*Rubus idaeus*) on the spread of aphid-borne viruses. *Annals of Applied Biology*, **92**, 119-123.

Jones, A.T. (1980) Some effects of latent virus infection in red raspberry. *Acta Horticulturae*, **95**, 63-70.

Jones, A.T. & Jennings, D.L. (1980) Genetic control of the reactions of raspberry to black raspberry necrosis, raspberry leaf mottle and raspberry leaf spot viruses. *Annals of Applied Biology*, **96**, 59-65.

Jones, B.N., Pääbo, S. & Stein, S. (1981) Amino acid analysis and enzymatic sequence determination of peptides by an improved o-pthaldialdehyde precolumn labeling procedure. *Journal of Liquid Chromatography*, **4**, 565-586.

Jones, A.T. (1986) Advances in the study, detection and control of viruses and virus diseases of *Rubus*, with particular reference to the United Kingdom. *Crop Research*, **26**, 127-171.

Jones, A.T. (1991) *Rubus* host ranges of rubus yellow net virus and its involvement with other aphid-borne latent viruses in inducing raspberry veinbanding mosaic disease. *Annals of Applied Biology*, **118**, 331-338.

Jones, A.T. & McGavin, W.J. (1998) Infectibility and sensitivity of UK raspberry, blackberry and hybrid berry cultivars to *Rubus* viruses. *Annals of Applied Biology*, **132**, 239-251.

Jones, A.T., McGavin, W.J. & Birch, A.N.E. (2000) Effectiveness of resistance genes to the large raspberry aphid, *Amphorophora idaei* Börner, in different raspberry (*Rubus idaeus* L.) genotypes and under different environmental conditions. *Annals of Applied Biology*, **136**, 107-113.

Jones, A.T., McGavin, W.J., Geering, A.D.W. & Lockhart, B.E.L. (2002) Identification of Rubus yellow net virus as a distinct badnavirus and its detection by PCR in *Rubus* species and in aphids. *Annals of Applied Biology*, **141**, 1-10.

Jordens-Rottger, D. (1979) Role of phenolic substances for host-selection behavior of the Black bean aphid, *Aphis fabae*. *Entomologia Experimentalis et Applicata*, **26**, 49-54.

Karban, R. & Baldwin, I.T. (1997) *Induced responses to herbivory*. University of Chicago Press Ltd., Chicago.

Karley, A.J., Douglas, A.E. & Parker, W.E. (2002) Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *Journal of Experimental Biology*, **205**, 3009-3018.

Keep, E. & Knight, R.L. (1967) A new gene from *Rubus occidentalis* L. for resistance to strains 1, 2 and 3 of the *Rubus* aphid *Amphorophora rubi* Kalt. *Euphytica*, **16**, 209-214.

Khan, Z.R. & Saxena, R.C. (1985) Behavior and biology of *Nephotettix virescens* (Homoptera, Cicadellidae) on Tungro virus-infected rice plants - epidemiology implications. *Environmental Entomology*, **14**, 297-304.

King, R.W. & Zeevaart, J.A. (1974) Enhancement of phloem exudation from cut petioles by chelating agents. *Plant Physiology*, **53**, 96-103

Kitrchner, W.H., Döring, T.F. & Saucke, H. (2005) Evidence for trichromacy in the green peach aphid, *Myzus persicae* (Sulz) (Hemiptera: Aphididae). *Journal of Insect Physiology*, **51**, 1255-1260.

Kiyosue, T., Yoshiba, Y., Yamaguchi-Shinozaki, K. & Shinozaki, K. (1996) A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in *Arabadopsis*. *Plant Cell*, **8**, 1323-1335.

Kluth, S., Kruess, A. & Tscharntke, T. (2002) Insects as vectors of plant pathogens: mutualistic and antagonistic interactions. *Oecologia*, **133**, 193-199.

Knight, R.L., Keep, E. & Briggs, J.B. (1959) Genetics of resistance to *Amphorophora rubi* (Kalt.) in raspberry. I. The gene A_1 from Baumforth A. *Journal of Genetics*, **56**, 261-280.

Koo, A.J.K. & Howe, G.A. (2009) The wound hormone jasmonate. *Phytochemistry*, **70**, 1571-1580.

Koornneef, A. & Pieterse, C.M.J. (2008) Cross talk in defense signalling. *Plant Physiology*, **146**, 839-844.

Kruess, A. (2002) Indirect interaction between a fungal plant pathogen and a herbivorous beetle of the weed *Cirsium arvense*. *Oecologia*, **130**, 563-569.

Kunkel, B.N. & Brooks, D.M. (2002) Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology*, **5**, 325-331.

Kusnierczyk, A., Winge, P., Jorstad, T.S., Troczynska, J., Rossiter, J.T. & Bones, A.M. (2008) Towards global understanding of plant defence against aphids - timing and dynamics of early *Arabidopsis* defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. *Plant, Cell and Environment*, **31**, 1097-1115.

Ma, R., Chen, J.L., Cheng, D.F. & Sun, J.R. (2010) Activation of defense mechanism in wheat by polyphenol oxidase from aphid saliva. *Journal of Agricultural and Food Chemistry*, **58**, 2410-2418.

Maris, P.C., Joosten, N.N., Goldbach, R.W. & Peters, D. (2004) Tomato spotted wilt virus infection improves host suitability for its vector *Frankliniella occidentalis*. *Phytopathology*, **94**, 706-711.

Matsui, K. (2006) Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. *Current Opinion in Plant Biology*, **9**, 274-280.

Matthews, R.E.F. (1991) *Plant Virology*. Academic Press, Inc., London.

Mauck, K.E., De Moraes, C.M. & Mescher, M.C. (2010) Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 3600-3605.

McDougall, G.J., Shpiro, F., Dobson, P., Smith, P., Blake, A. & Stewart, D. (2005) Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. *Journal of Agricultural and Food Chemistry*, **53**, 2760-2766.

McElhany, P., Real, L.A. & Power, A.G. (1995) Vector preference and disease dynamics - a study of Barley yellow dwarf virus. *Ecology*, **76**, 444-457.

McGavin, W.J. & MacFarlane, S.A. (2010) Sequence similarities between Raspberry leaf mottle virus, Raspberry leaf spot virus and the closterovirus Raspberry mottle virus. *Annals of Applied Biology*, **156**, 439-448.

McLeod, G., Gries, R., von Reuss, S.H., Rahe, J.E., McIntosh, R., Konig, W.A. & Gries, G. (2005) The pathogen causing Dutch elm disease makes host trees attract insect vectors. *Proceedings of the Royal Society of London B - Biological Sciences*, **272**, 2499-2503.

Medina-Ortega, K.J., Bosque-Perez, N.A., Ngumbi, E., Jimenez-Martinez, E.S. & Eigenbrode, S.D. (2009) *Rhopalosiphum padi* (Hemiptera: Aphididae) responses to volatile cues from Barley yellow dwarf virus-infected wheat. *Environmental Entomology*, **38**, 836-845.

Mewis, I., Tokuhisa, J.G., Schultz, J.C., Appel, H.M., Ulrichs, C. & Gershenzon, J. (2006) Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry*, **67**, 2450-2462.

Miles, P.W. (1999) Aphid saliva. *Biological Reviews*, **74**, 41-85.

Mitchell, C. (2007) Novel approaches to the development of integrated pest management in UK raspberry production. PhD thesis, University of Dundee, Dundee.

Mitchell, C., Johnson, S.N., Gordon, S.C., Birch, A.N.E. & Hubbard, S.F. (2010) Combining plant resistance and a natural enemy to control *Amphorophora idaei*. *Biocontrol*, **55**, 321-327.

Mittler, T.E. (1967) Effect on aphid feeding of dietary methionine. *Nature*, **214**, 386.

Mittler, T.E. (1970) Effects of dietary amino acids on feeding rate of aphid *Myzus persicae*. *Entomologia Experimentalis et Applicata*, **13**, 432.

Moericke, V. (1952) Farben als Landereize für geflügelte Blattlause (Aphidoidea). *Zeitschrift für Naturforschung* **7b**, 304-309.

Montgomery, M.E. & Arn, H. (1974) Feeding response of *Aphis pomi, Myzus persicae*, and *Amphorophora agathonica* to phlorizin. *Journal of Insect Physiology*, **20**, 413-421.

Moorhouse, E.R., Charnley, A.K. & Gillespie, A.T. (1992) A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera, Curculionidae). *Annals of Applied Biology*, **121**, 431-454.

Moran, P.J. & Thompson, G.A. (2001) Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways. *Plant Physiology*, **125**, 1074-1085.

Morris, J.G. (1991). Nutrition. In *Environmental and Metabolic Animal Physiology* (ed C.L. Prosser), pp. 231-276. John Wiley and Sons, New York.

Musser, R.O., Hum-Musser, S.M., Felton, G.W. & Gergerich, R.C. (2003) Increased larval growth and preference for virus-infected leaves by the Mexican bean beetle, *Epilachna varivestis* Mulsant, a plant virus vector. *Journal of Insect Behavior*, **16**, 247-256.

Nakagami, H., Pitzschke, A. & Hirt, H. (2005) Emerging MAP kinase pathways in plant stress signalling. *Trends in Plant Science*, **10**, 339-346.

Nault, L.R. (1997) Arthropod transmission of plant viruses: A new synthesis. *Annals of the Entomological Society of America*, **90**, 521-541.

Ng, J.C.K. & Perry, K.L. (2004) Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology*, **5**, 505-511.

Ng, J.C.K. & Falk, B.W. (2006) Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annual Review of Phytopathology*, **44**, 183-212.

Ngumbi, E., Eigenbrode, S.D., Bosque-Perez, N.A., Ding, H. & Rodriguez, A. (2007) *Myzus persicae* is arrested more by blends than by individual compounds elevated in headspace of PLRV-infected potato. *Journal of Chemical Ecology*, **33**, 1733-1747.

Nottingham, S.F., Hardie, J., Dawson, G.W., Hick, A.J., Pickett, J.A., Wadhams, L.J. & Woodcock, C.M. (1991) Behavioral and electrophysiological responses of aphids to host and nonhost plant volatiles. *Journal of Chemical Ecology*, **17**, 1231-1242.

Ode, P.J. (2006) Plant chemistry and natural enemy fitness: Effects on herbivore and natural enemy interactions. *Annual Review of Entomology*, **51**, 163-185.

Peng, Z.K. & Miles, P.W. (1988) Acceptability of catechin and its oxidative condensation products to the Rose aphid, *Macrosiphum rosae*. *Entomologia Experimentalis et Applicata*, **47**, 255-265.

Pickett, J.A., Wadhams, L.J. & Woodcock, C.M. (1992) The chemical ecology of aphids. *Annual Review of Entomology*, **37**, 67-90.

Powell, G. (1991) Cell membrane punctures during epidermal penetrations by aphids - consequences for the transmission of two potyvirus. *Annals of Applied Biology*, **119**, 313-321.

Powell, G. (2005) Intracellular salivation is the aphid activity associated with inoculation of non-persistently transmitted viruses. *Journal of General Virology*, **86**, 469-472.

Powell, G., Tosh, C.R. & Hardie, J. (2006) Host plant selection by aphids: Behavioral, evolutionary, and applied perspectives. *Annual Review of Entomology*, **51**, 309-330.

Price, P.W., Bouton, C.E., Gross, P., McPherson, B.A., Thompson, J.N. & Weiss, A.E. (1980) Interactions between three trophic levels: influence of plants on interactions between insect herbivores and natural enemies. *Annual Review of Ecology and Systematics*, **11**, 41-65.

Prokopy, R.J. & Owens, E.D. (1983) Visual detection of plants by herbivorous insects. *Annual Review of Entomology*, **28**, 337-364.

Puterka, G.J. & Burton, R.L. (1991). Aphid genetics in relation to host plant resistance. In *Proceedings of aphid-plant Interactions: population to molecules* (eds Peters, D.C., Webster, J.A. & Chlouber, C.S.), pp. 59-71. USDA Agricultural Research Service and Oklahoma State University, Stillwater, Oklahoma, USA.

Rankin, W.H. (1931). Virus diseases of black raspberries. In *Technical Bulletin 175*. New York State Agricultural Experiment Station, Geneva, N.Y.

Rautapää, J. (1967) The bionomics of the raspberry aphids *Aphis idaei* v.d. Goot and *Amphorophora rubi* (Kalt.) (Hom., Aphididae). *Annales Agriculturae Fenniae*, **6**, 127-144.

Reymond, P. & Farmer, E.E. (1998) Jasmonate and salicylate as global signals for defense gene expression. *Current Opinion in Plant Biology*, **1**, 404-411.

Reymond, P., Weber, H., Damond, M. & Farmer, E.E. (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell*, **12**, 707-719.

Reymond, P., Bodenhausen, N., Van Poecke, R.M.P., Krishnamurthy, V., Dicke, M. & Farmer, E.E. (2004) A conserved transcript pattern in response to a specialist and a generalist herbivore. *Plant Cell*, **16**, 3132-3147.

Rhodes, J.D., Croghan, P.C. & Dixon, A.F.G. (1996) Uptake, excretion and respiration of sucrose and amino acids by the pea aphid *Acyrthosiphon pisum*. *Journal of Experimental Biology*, **199**, 1269-1276.

Robertson, G.W., Griffiths, D.W., Birch, A.N.E., Jones, A.T., McNicol, J.W. & Hall, J.E. (1991) Further evidence that resistance in raspberry to the virus vector aphid, *Amphorophora idaei*, is related to the chemical composition of the leaf surface. *Annals of Applied Biology*, **119**, 443-449.

Robertson, G.W., Griffiths, D.W., Woodford, J.A.T. & Birch, A.N.E. (1995) Changes in the chemical-composition of volatiles released by the flowers and fruits of the red raspberry (*Rubus idaeus*) cultivar Glen Prosen. *Phytochemistry*, **38**, 1175-1179.

Rodriguez-Saona, C., Chalmers, J.A., Raj, S. & Thaler, J.S. (2005) Induced plant responses to multiple damagers: differential effects on an herbivore and its parasitoid. *Oecologia*, **143**, 566-577.

Sargent, D.J., Fernandez-Fernandez, F., Rys, A., Knight, V.H., Simpson, D.W. & Tobutt, K.R. (2007) Mapping of A(1) conferring resistance to the aphid *Amphorophora idaei* and dw (dwarfing habit) in red raspberry (*Rubus idaeus* L.) using AFLP and microsatellite markers. *BMC Plant Biology*, **7**, 1471-2229.

Schoonhoven, L.M., van Loon, J.J.A. & Dicke, M. (2005) *Insect-Plant Biology*. Oxford University Press, Oxford.

Shepherd, T., Robertson, G.W., Griffiths, D.W. & Birch, A.N.E. (1999a) Epicuticular wax ester and triacylglycerol composition in relation to aphid infestation and resistance in red raspberry (*Rubus idaeus* L.). *Phytochemistry*, **52**, 1255-1267.

Shepherd, T., Robertson, G.W., Griffiths, D.W. & Birch, A.N.E. (1999b) Epicuticular wax composition in relation to aphid infestation and resistance in red raspberry (*Rubus idaeus* L.). *Phytochemistry*, **52**, 1239-1254.

Shepherd, T., Robertson, G.W., Griffiths, D.W. & Birch, A.N.E. (2000). Plants and aphids: the chemical ecology of infestation. In *Scottish Crop Research Institute Annual Report 1999/2000*, pp. 122-124. Scottish Crop Research Institute, Dundee.

Shiojiri, K., Kishimoto, K., Ozawa, R., Kugimiya, S., Urashimo, S., Arimura, G., Horiuchi, J., Nishioka, T., Matsui, K. & Takabayashi, J. (2006) Changing green leaf volatile biosynthesis in plants: An approach for improving plant resistance against both herbivores and pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 16672-16676.

Sisterson, M.S. (2008) Effects of insect-vector preference for healthy or infected plants on pathogen spread: insights from a model. *Journal of Economic Entomology*, **101**, 1-8.

Srinivasan, R., Alvarez, J.M., Eigenbrode, S.D. & Bosque-Perez, N.A. (2006) Influence of hairy nightshade *Solanum sarrachoides* (Sendtner) and Potato leafroll virus (Luteoviridae : Polerovirus) on the host preference of *Myzus persicae* (Sulzer) (Homoptera : Aphididae). *Environmental Entomology*, **35**, 546-553.

Stace-Smith, R. (1955a) Studies on *Rubus* virus diseases in British Columbia. II. Black raspberry necrosis. *Canadian Journal of Botany*, **33**, 314-322.

Stace-Smith, R. (1955b) Studies on *Rubus* virus diseases in British Columbia. I. Rubus yellow net. *Canadian Journal of Botany*, **33**, 269-274.

Stace-Smith, R. (1956) Studies on *Rubus* virus diseases in British Columbia. III. Separation of the components of raspberry mosaic. *Canadian Journal of Botany*, **34**, 435-442.

Stace-Smith, R. & Mellor, F.C. (1957) Studies on virus diseases in British Columbia. IV. Transmission of raspberry viruses to *Fragaria vesca* L. *Canadian Journal of Botany*, **35**, 287-290.

Stace-Smith, R. (1960) Current status of bramble disease. *Canadian Plant Disease Survey*, **40**, 24-42.

Stafne, E.T., Clark, J.R., Weber, C.A., Graham, J. & Lewers, K.S. (2005) Simple sequence repeat (SSR) markers for genetic mapping of raspberry and blackberry. *Journal of the American Society for Horticultural Science*, **130**, 722-728.

Stevanato, R., Fabris, S. & Momo, F. (2004) Enzymatic method for the determination of total phenolic content in tea and wine. *Journal of Agricultural and Food Chemistry*, **52**, 6287-6293.

Storer, J.R., Powell, G. & Hardie, J. (1996) Settling responses of aphids in air permeated with non-host plant volatiles. *Entomologia Experimentalis et Applicata*, **80**, 76-78.

Stout, M.J., Fidantsef, A.L., Duffey, S.S. & Bostock, R.M. (1999) Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology*, **54**, 115-130.

Stout, M.J., Thaler, J.S. & Thomma, B. (2006) Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. *Annual Review of Entomology*, **51**, 663-689.

Taylor, C.E. & Chambers, J. (1969) Effects of insecticide treatments on aphid populations and on spread of latent viruses in raspberry cane nurseries. *Horticultural Research*, **9**, 37-43.

Thaler, J.S., Stout, M.J., Karban, R. & Duffey, S.S. (1996) Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *Journal of Chemical Ecology*, **22**, 1767-1781.

Thaler, J.S., Agrawal, A.A. & Halitschke, R. (2010) Salicylate-mediated interactions between pathogens and herbivores. *Ecology*, **91**, 1075-1082.

Tinney, G.W., Hatcher, P.E., Aqres, P.G., Paul, N.D. & Whittaker, J.B. (1998) Inter- and intra- species differences in plants as hosts to *Tyria jacobaeae*. *Entomologia Experimentalis et Applicata*, **88**, 137-145.

Toussaint, A., Dekegel, D. & Vanheule, G. (1984) Distribution of *Odontoglossum* ringspot virus in apical meristems of infected *Cymbidium* cultivars. *Physiological Plant Pathology*, **25**, 297-305.

Tzanetakis, I.E., Halgren, A., Mosier, N. & Martin, R.R. (2007) Identification and characterization of Raspberry mottle virus, a novel member of the Closteroviridae. *Virus Research*, **127**, 26-33.

Uzest, M., Gargani, D., Drucker, M., Hebrard, E., Garzo, E., Candresse, T., Fereres, A. & Blanc, S. (2007) A protein key to plant virus transmission at the tip of the insect vector stylet. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 17959-17964.

van Dam, N.M. (2009) How plants cope with biotic interactions. *Plant Biology*, **11**, 1-5.

Visser, J.H., Piron, P.G.M. & Hardie, J. (1996) The aphids' peripheral perception of plant volatiles. *Entomologia Experimentalis et Applicata*, **80**, 35-38.

Webster, B., Bruce, T., Dufour, S., Birkemeyer, C., Birkett, M., Hardie, J. & Pickett, J. (2008) Identification of volatile compounds used in host location by the black bean aphid, *Aphis fabae*. *Journal of Chemical Ecology*, **34**, 1153-1161.

Webster, B., Bruce, T., Pickett, J. & Hardie, J. (2010) Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. *Animal Behaviour*, **79**. 451-457.

Weibull, J.H.W. (1988) Free amino acids in the phloem sap from oats and barley resistant to *Rhopalosiphum padi*. *Phytochemistry*, **27**, 2069-2072.

Werner, B.J., Mowry, T.M., Bosque-Perez, N.A., Ding, H.J. & Eigenbrode, S.D. (2009) Changes in green peach aphid responses to Potato leafroll virus-induced volatiles emitted during disease progression. *Environmental Entomology*, **38**, 1429-1438.

Williams, I.S. (1999) Slow-growth, high-mortality - a general hypothesis, or is it? *Ecological Entomology*, **24**, 490-495.

Winter, H., Lohaus, G. & Heldt, H.W. (1992) Phloem transport of amino acids in relation to their cytosolic levels in barley leaves. *Plant Physiology*, **99**, 996-1004.

Zhou, X.L., Harrington, R., Woiwod, I.P., Perry, J.N., Bale, J.S. & Clark, S.J. (1995) Effects of temperature on aphid phenology. *Global Change Biology*, **1**, 303-313.

Appendix A

The complete sequence of a UK strain of Black raspberry necrosis virus & the amino acid composition of BRNV and RLMV

Archives of Virology (2010), doi: 10.1007/s00705-010-0807-9

Arch Virol
DOI 10.1007/s00705-010-0807-9

ANNOTATED SEQUENCE RECORD

The complete sequence of a UK strain of black raspberry necrosis virus

W. J. McGavin · L. S. McMenemy · S. A. MacFarlane

Received: 18 June 2010 / Accepted: 14 September 2010
© Springer-Verlag 2010

Abstract The complete nucleotide sequence of a UK strain of the sadwavirus Black raspberry necrosis virus (BRNV) was obtained by amplification and sequencing of virus RNA from infected plants grown in a raspberry plantation in Aylth, Scotland. The RNA1 was 7,572 nucleotides (nt) in size and RNA2 was 6,350 nt in size, each excluding the 3′ poly-A tail. The RNA1- and RNA2-encoded polyproteins are predicted to be processed into (RNA1) a protease cofactor, an RNA helicase, the VpG, a 3C-like protease, an RNA-dependent RNA polymerase and an AlkB protein, and (RNA2) a movement protein and two capsid proteins.

Introduction

Red raspberry (*Rubus idaeus*) and black raspberry (*R. occidentalis*) are high-value, perennial crops that are grown worldwide in temperate regions. Viral disease is a significant problem for raspberry growers, with more than 40 virus or virus-like agents being reported to infect these plants. In the UK, an aphid- and sap-transmissible virus code-named 52V was isolated from a large number of field-grown red raspberry plants and caused apical tip necrosis when transmitted by aphids to black raspberry [2]. These results were interpreted to suggest that 52V is an isolate of a previously described virus, black raspberry necrosis virus (BRNV) [7], and further work was done to characterize this virus following its mechanical transmission to herbaceous plants, including *Chenopodium quinoa* and spinach (*Spinacia oleracea*) [3]. Recently, the complete sequence was obtained from a virus causing leaf chlorosis, cane dieback and yield loss in black raspberry in Oregon, USA [1]. Comparison with partial (unpublished) sequences obtained in Scotland from 52V-infected spinach plants suggested that the American virus was an isolate of BRNV. Analysis of this sequence revealed that BRNV is a member of the genus *Sadwavirus* and has a genome of two polyadenylated, single-stranded, positive-sense RNAs, each of which is translated as a polyprotein, and encodes two coat proteins that assemble to form spherical virus particles. Interestingly, in contrast to the results obtained with 52V, the North American (NA) isolate of BRNV did not infect *Ch. quinoa* and *S. oleracea* and, unexpectedly, did not cause apical tip necrosis when infecting black raspberry.

W. J. McGavin · L. S. McMenemy · S. A. MacFarlane (✉)
Plant Pathology Programme, SCRI, Invergowrie,
Dundee DD2 5DA, UK
e-mail: s.macfarlane@scri.ac.uk

Experimental work

In an attempt to explain these differences in apparent behaviour of US and UK strains of BRNV, we decided to obtain the complete sequence of a Scottish strain of BRNV. The 52V virus in spinach cannot be inoculated back into raspberry, and so it was decided to identify a new source of BRNV from the field. Consequently, Glen Ample plants showing strong mosaic symptoms were identified in 2007 in a field at Aylth, near Dundee, and found by RT-PCR to contain BRNV. Symptomatic leaves were collected from the plants and stored at −80°C as a source of BRNV-infected material.

Virus sequences were obtained by a combination of approaches using both double-stranded (ds)RNA and total RNA extracted from the symptomatic leaves using methods described previously [4, 5]. Some virus sequence was derived by PCR amplification with DOP primers [6] of

Published online: 28 September 2010

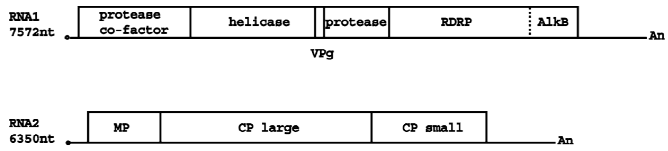


Fig. 1 Genome diagram of the Alyth strain of BRNV. Predicted locations of polyprotein cleavages are denoted by *solid vertical lines*. It is not known whether the AlkB protein is cleaved from the RDRP protein. An denotes poly-A tail at the 3′ terminus of the viral RNAs. *Small circle* denotes VPg attached to 5′ end of viral RNAs

random-hexamer-primed cDNA synthesised from dsRNA. Other sequence was obtained by PCR amplification of cDNA with BRNV-specific primers derived either from the US isolate of BRNV or from the UK virus sequence that was assembled as the project progressed. The 5′-terminal regions of the UK BRNV RNAs were cloned after PCR amplification using primer 995 (ATCAAAGCGCACTG AACCCAAG), derived from the 5′ terminus of the US isolate of BRNV, together with RNA1- or RNA2-specific primers complementary to UK BRNV. To obtain the 3′ terminal regions of the UK BRNV RNAs, cDNA was synthesised using primer 1,096 (GACTCGAGTCGACAT CGATTTTTTTTTTTTTTTTT), which annealed to the poly-A tail of the viral RNAs. The 3′ terminal regions were then amplified using RNA1- or RNA2-specific primers derived from UK BRNV and primer 1,097 (GACTCGAGT CGACATCGA), which matched part of the cDNA synthesis primer. The complete nucleotide sequences were deposited in EMBL-Bank with the accession numbers FN908128 (RNA1) and FN908129 (RNA2).

Sequence analysis

Analysis of the sequencing results show that the UK (Alyth) isolate of BRNV has an RNA1 of 7,572 nucleotides (nt) and an RNA2 of 6,350 nt (excluding the poly-A tail), which compares to 7,581 nt and 6,364 nt for the corresponding RNAs of US BRNV, and reveals a significant sequence diversity between the two isolates (overall nucleotide identity is only 79% for RNA1 and 82% for RNA2). The 5′ untranslated region (UTR) is 148 nt for RNA1 and 219 nt for RNA2. They are identical for 40 nt at the 5′ termini but are only 55% identical over the entire 5′UTR. The 3′UTRs of RNA1 and RNA2 are both 932 nt and are identical for 12 nt at the 3′ terminus, with 92% identity over the entire region.

Nucleotides 149–6,637 of RNA1 encode a single polyprotein of 2,163 amino acids (aa) that, by comparison with RNA1 of US BRNV, is predicted to be cleaved into a proteinase cofactor (coding sequence 149–1,693 nt), an RNA helicase (1,694–3,208 nt), the VPg (3,209–3,286 nt), 3C-like protease (3,287–3,988 nt) and RDRP (3,989–6,637 nt) (Fig. 1). As with US BRNV, the C-terminal part of the Alyth strain RDRP protein includes a domain of 192 aa with sequence similarity to AlkB proteins that are able to repair DNA and RNA nucleotides that have been modified by, for example, methylation.

Table 1 Sequence comparison of the proteins and their coding sequences of the UK (Alyth) and US (NA) strains of BRNV

RNA1	Identity (nt/aa)	RNA2	Identity (nt/aa)
Protease cofactor	82%/94%	MP	79%/85%
Helicase	76%/88%	CP L	84%/96%
VpG	79%/96%	CP S	74%/85%
Protease	77%/91%		
RDRP	75%/89%		
AlkB	69%/77%		

Nucleotides 220–5,418 of RNA2 encode a single polyprotein of 1,733 aa that is predicted to be cleaved into the movement protein (220–1,191 nt), the large coat protein (1,192–3,954 nt) and the small coat protein (3,955–5,418 nt).

Comparison of the amino acid sequences of the viral proteins from the UK and US BRNV strains (Table 1) shows that the greatest homology exists between the protease cofactor (94% identity) and the large coat protein (96% identity), with the least homology (77%) existing between the AlkB proteins of the viruses. In addition to the complete sequence of the NA strain of BRNV from northwestern America, a partial sequence of the RDRP-coding sequence of the GSMNP strain from the southern USA (Mississippi) has also been obtained. This sequence is only 76% identical to that of the NA strain, reinforcing the notion that significant sequence diversity exists for isolates of this virus. Using the Alyth strain sequence, we have been able to design PCR primers that allow detection of BRNV in raspberry plants collected from various locations in the UK. Further studies are needed to examine whether strains from elsewhere in Europe can also be detected using these primers.

A remaining issue to be addressed is whether or not BRNV strains from the UK cause tip necrosis when infecting black raspberry. This is important because tip necrosis is used as a diagnostic test for BRNV during pathogen testing of red raspberry plants in high health certification schemes. Recent investigation of raspberry plants held in the SCRI *Rubus* virus collection using a range of newly-available virus-specific PCR tests has shown that all the plants that were previously assessed as being infected only with BRNV actually are also infected with one or more additional viruses. It is possible that the black raspberry tip necrosis seen in earlier grafting experiments may have been caused, either in whole or in part, by these other viruses and not by BRNV.

Acknowledgments This work was funded by the Scottish Government Rural and Environment Research and Analysis Directorate (RERAD). LMS was supported by a PhD scholarship jointly funded by SCRI and Sussex University.

References

1. Halgren A, Tzanetakis IE, Martin RR (2007) Identification, characterization, and detection of Black raspberry necrosis virus. Phytopathology 97:44–50
2. Jones AT, Murant AF (1972) Some properties of a mechanically transmissible virus widespread in raspberry (*Rubus idaeus*) in Scotland. Plant Pathol 21:166–170
3. Jones AT, Roberts IM (1977) Rubus host range, symptomatology and ultrastructural effects of a British isolate of black raspberry necrosis virus. Ann Appl Biol 86:381–387
4. McGavin WJ, MacFarlane SA (2009) Rubus chlorotic mottle virus, a new sobemovirus infecting raspberry and bramble. Virus Res 139:10–13
5. McGavin WJ, MacFarlane SA (2010) Sequence similarities between *Raspberry leaf mottle virus*, *Raspberry leaf spot virus* and the closterovirus *Raspberry mottle virus*. Ann Appl Biol 156:439–448
6. Rott ME, Jelkmann W (2001) Characterization and detection of several filamentous viruses of cherry: adaptation of an alternative cloning method (DOP-PCR), and modification of an RNA extraction protocol. Eur J Plant Pathol 107:411–420
7. Stace-Smith R (1955) Studies on *Rubus* virus diseases in British Columbia. II. Black raspberry necrosis. Can J Bot 33:314–322

RLMV		BRNV		RLMV + BRNV	
Amino acid	(Mol %)	Amino acid	(Mol %)	Amino acid	(Mol %)
Ser	11.57	Leu*	8.61	Ser	9.76
Leu*	10.83	Ser	7.95	Leu*	9.72
Val*	7.76	Gly	7.26	Val*	7.37
Arg	7.74	Val*	6.97	Arg	6.51
Phe*	6.66	Glu	6.40	Gly	6.06
Ala	5.63	Ala	6.30	Ala	5.97
Thr*	5.63	Lys*	5.79	Phe*	5.74
Gly	4.86	Thr*	5.77	Thr*	5.70
Ile*	4.14	Ile*	5.64	Glu	4.99
Asp	4.02	Asp	5.52	Ile*	4.89
Lys*	3.69	Arg	5.28	Lys*	4.87
Glu	3.59	Phe*	4.82	Asp	4.77
Tyr	3.42	Asn	3.81	Tyr	3.52
Asn	2.95	Tyr	3.61	Asn	3.38
His*	2.49	Gln	3.02	Gln	2.55
Gln	2.08	His*	2.38	His*	2.43
Met*	1.15	Met*	2.36	Met*	1.76
Trp*	1.12	Trp*	1.48	Trp*	1.30

Table A.2. Amino acid compositions of RLMV, BRNV and both viruses combined. Table shows amino acids ranked in descending order of total molar percentage as determined from genomic RNA sequences of both viruses. * denotes essential amino acids. Predictions made using BioEdit vs 7.0 with GenBank accessions for RLMV (accession dq357281) and BRNV - (accessions dq344639 and dq344640)

Appendix B

Field survey of *Amphorophora idaei* and BRNV and RLMV prevalence

Background

The opportunity arose in 2008 to survey a raspberry field plantation at SCRI, Invergowrie, Dundee. The plantation contained plants which were progeny of crosses between the SCRI developed cultivar Glen Moy, which possesses the same A_1 aphid resistance gene as Glen Ample, and the North American cultivar Latham. These plants were crossed in attempt to confer resistance to raspberry root rot. The raspberry plot consisted of 18 rows of raspberry plants arranged into three replicates (six rows per replicate) each of which contained 241 individual plants. A total of 42 plants which were spread evenly across the site, were selected based on a historical record of virus symptoms recorded from 2006 to 2008, to investigate seasonal fluctuations in large raspberry aphid populations on both healthy and virus-infected plants.

Virus testing

Each of the 42 plants surveyed for the presence of large raspberry aphid was also tested for the presence or absence of BRNV and RLMV using the PCR diagnostic tests detailed in Chapter three of this thesis.

Aphid survey

A total of 15 leaves were labelled on each of the 42 plants, of which 5 were located at the top of the cane, 5 in the mid-section of the cane and 5 at the bottom of the cane. All leaves were from floricanes. Leaves were surveyed once every 2 weeks by gently turning over the leaf and recording the number of large raspberry aphid present.

Results

Virus testing

Of the 42 plants tested, 27 tested positive for both BRNV and RLMV, five tested positive for BRNV only, five tested positive for RLMV only and a further five were negative for both viruses and were therefore considered to be non-infected (Figure B.1).

Aphid survey

On the first survey date, conducted on the 22nd May 2008, the number of *A. idaei* was found to be highest on non-infected plants when compared with any of the combinations of virus (Figure B.2) with a mean of 2.80 aphids being found on non-infected compared with the next highest value of 0.25 on RLMV-infected plants. The number of *A. idaei* found on non-infected plants was much less at the second survey date on the 5th June (0.2), while the number on any of the combinations of virus-infected plants was slightly increased compared with the earliest survey date (max. of 0.44 on plants infected with BRNV and RLMV). By the third sampling date on 19th June, the number of *A. idaei* on RLMV-infected plants and those infected with both viruses was found to be higher than on non-infected plants with a mean of 1.48 aphids on RLMV-infected plants and 1.30 on those infected with BRNV and RLMV compared with 1.00 aphids found on non-infected plants. The number of *A. idaei* on BRNV-infected plants remained at the lowest level of 0.60 aphids. At the final sampling date on the 2nd July, the number of aphids on non-infected, BRNV-infected and RLMV-infected plants had declined to 0 with just 0.11 found on plants infected with BRNV and RLMV.

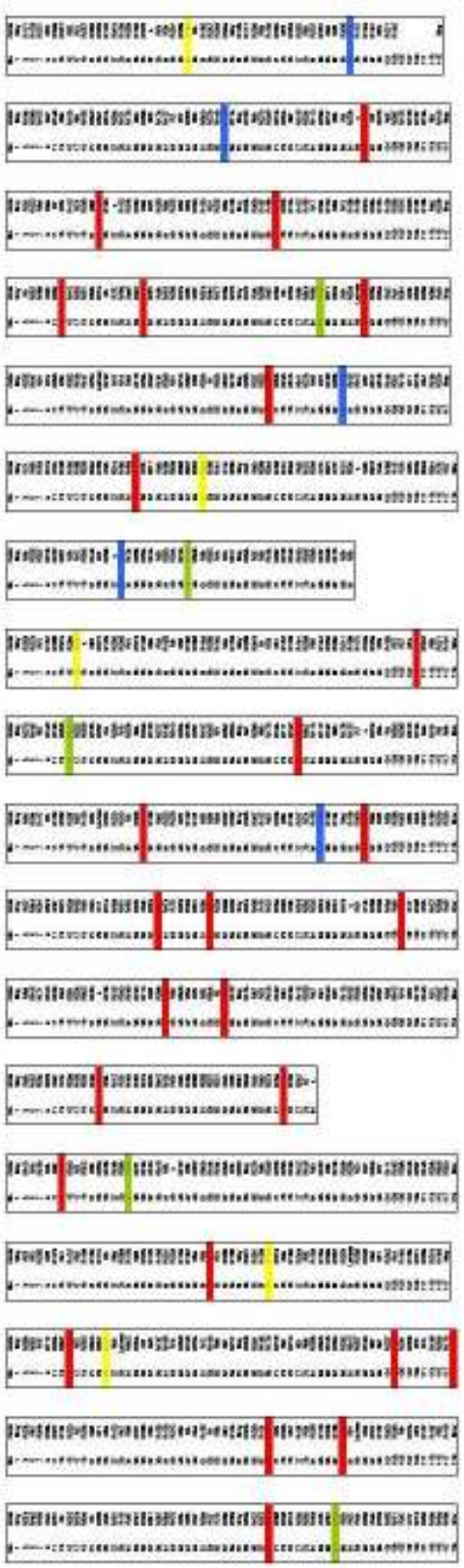


Figure B.1. Schematic diagram of field plot at SCRI. Coloured boxes show location of raspberry plants which were surveyed for large raspberry aphid. Blue boxes represent healthy plants, yellow boxes represent BRNV-infected plants, green boxes represent RLMV-infected plants and red boxes represent plant infected with both BRNV and RLMV as determined by PCR diagnostic tests.

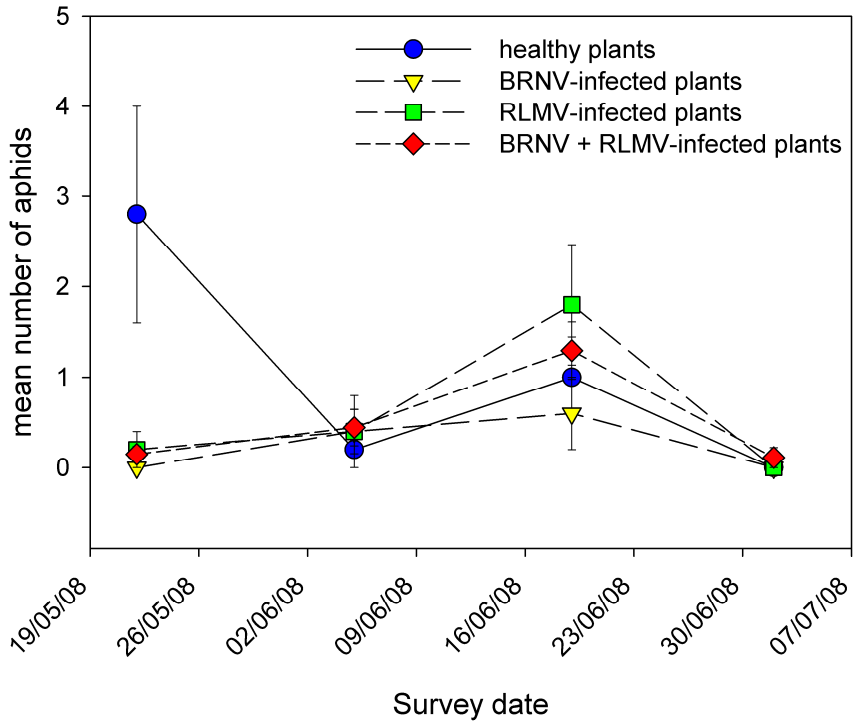


Figure B.2. Mean number of aphids on each plant type at each survey date. Mean number of 5 shown for healthy, BRNV-infected and RLMV-infected plants and mean number of 27 shown for BRNV and RLMV-infected plants. Error bars show SEM.

Concluding remarks

The aim of the field survey was to characterise seasonal fluctuations in aphid numbers on non-infected and virus-infected plants. However, on completion of virus testing it became apparent that the raspberry plot that was used was heavily infested with both BRNV and RLMV. Indeed, of 42 plants which were selected to represent an even spread of plants across the plantation, only 5 were found to be free from virus, representing just 11% of the plants investigated. In just 8 years, both BRNV and RLMV had quite clearly become prevalent in this plantation and the aphid population which was supported by these plants was low. The number of plants infected with both BRNV and RLMV represented 64% of the plants which were surveyed and corroborates the general observation that these viruses are commonly found to infect raspberry plants in combination. The high prevalence of the viruses in this plantation lend support to studies which are designed to find methods of controlling the spread of *A. idaei*, and through control of the vector, limit the spread of viruses.

This study was also aimed at investigating aphid populations, not only within one season, but over several. This was unfortunately impossible and the survey was abandoned after 10 weeks due to damage inflicted on the floricanes due to the weight of unharvested fruit which rendered further work impossible due the huge loss of leaves. Although the plants which were investigated were of a different cultivar to that used for the experiments detailed in the main body of this thesis, they were derived from Glen Moy parentage. This cultivar possesses the same resistance gene as Glen Ample and therefore there should have been some degree of aphid resistance in the field plants, making the high prevalence of the viruses all the more surprising.