Olfactory responses of the leafhopper vector, *Mgenia fuscovaria* Stål (Hemiptera: Cicadellidae), to volatiles from aster yellows phytoplasma-infected and uninfected grapevine (*Vitis vinifera* L.)

By

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Declaration of authorship

I, the undersigned, hereby declare that the dissertation which I hereby submit for the degree *Magister Scientiae* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Signature Date

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Thesis summary

The leafhopper *Mgenia fuscovaria* Stål (Hemiptera: Cicadellidae) is a vector of aster yellows phytoplasma (AY), '*Candidatus* Phytoplasma asteris', in grapevine, *Vitis vinifera* L. (Vitaceae), in South Africa. In a previous study, *M. fuscovaria* was preferentially attracted to AY-infected compared to uninfected grapevine branches, although the mode of attraction was not determined. Phytoplasma infection may alter the volatile profiles of plants, rendering them more attractive to the insect vector. This may lead to an increase in the number of vectors transmitting the pathogen. The volatile compounds that attract or repel insect pests could be used in behavioural manipulation strategies to manage pests. The objective of this study was to determine the effect of AY-infection on the volatile composition of the grapevine cultivars Colombard and Chenin blanc in summer and autumn, and the associated behavioural and electrophysiological responses of *M. fuscovaria* towards these changes in volatile profiles.

Volatile analyses of AY-infected and uninfected grapevine branches revealed both qualitative and quantitative differences. In summer, methyl salicylate was produced in significantly higher amounts or only produced in AY-infected branches in cv. Chenin blanc and cv. Colombard, respectively. Similarly, ethyl salicylate was recorded only from AY-infected branches of both cultivars during summer. There was a significant increase in the total volatile emissions in AY-infected compared to uninfected grapevine cv. Colombard, including several green leaf volatiles. The compounds that differed significantly between AY-infected and uninfected branches in autumn were produced exclusively or in greater quantities in uninfected branches. (*E,E*)-α-farnesene was the most abundant compound recorded in all cases. Grapevine branches infected with AY often had a greater mass than uninfected branches with the same leaf area.

In behavioural studies, *M. fuscovaria* displayed no consistent preferences toward volatiles from AY-infected and uninfected grapevine branches cv. Colombard or cv. Chenin blanc in summer

or autumn. In summer, there were no significant differences in the choices made by leafhoppers for both cultivars. In autumn, leafhoppers preferred purified air over AY-infected cv. Colombard branches and AY-infected cv. Chenin blanc branches over purified air. There was no difference in the choices made between male and female leafhoppers.

In electrophysiological tests, *M. fuscovaria* antennae displayed weak responses to grapevine volatiles collected in summer. Consistent responses were identified to 1-octen-3-ol, phenol, (*E,E*)-α-farnesene, which is produced at elevated concentrations by AY-infected grapevine, and aromadendrene, which was only produced by AY-infected branches and not by uninfected branches cv. Colombard. For grapevine cv. Chenin blanc, insects responded to the co-eluting green leaf volatiles (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol and 1-hexanol as well as nonane from uninfected branches.

The results from this study suggest that *M. fuscovaria* is not preferentially attracted toward AY-infected grapevine branches based solely on olfactory cues. Based on the weak responses observed in electrophysiological and behavioural tests, as well as results obtained in studies on other leafhopper species, the observed attraction could be a result of visual cues rather than olfactory cues or a combination of both.

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Chapter 1

The role of plant volatiles in host plant selection and their use in integrated pest management strategies

1.1 General introduction

Phytophagous insects often transmit disease-causing pathogens such as bacteria, viruses and fungi, to their plant hosts. In economically important plants, this may cause devastating losses in crop yields and profits (Oerke, 2006). For pathogens that are vectored by insects or other arthropods, control is often aimed at the vector to reduce pathogen spread. Due to the numerous negative factors associated with the use of pesticides, integrated pest management has become a popular method of control. To develop such control strategies, thorough knowledge of the vector's biology and ecology is essential (Pickett et al., 1997; Cook et al., 2007).

The phloem-feeding leafhopper, *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae), was recently identified as a vector of aster yellows phytoplasma (AY) '*Candidatus* Phytoplasma asteris' (Krüger et al., 2011), a disease causing bacterium (Lee et al., 2004), in grapevine *Vitis vinifera* L. (Vitaceae) in South Africa. Current management methods include using pesticides to control leafhopper populations and removing AY-infected plants from vineyards. There is a need to develop management strategies to control insect pests in vineyards that are cost-effective, environmentally friendly and sustainable. In initial choice tests, *M. fuscovaria* adults were preferentially attracted to AY-infected grapevine branches compared to uninfected branches (Krüger et al., 2015), although the mode of attraction, either visual or olfactory, was not determined. In similar cases where insect vectors showed increased attraction to pathogen-infected plants, vectors were attracted to the altered volatiles produced by infected host plants (e.g. Eigenbrode et al., 2002; McLeod et al., 2005; Srinivasan et al., 2006; Mayer et al., 2008b; a; Mauck et al., 2010; Mann et al., 2012). If insect vectors

are preferentially attracted to pathogen-infected plants, this could lead to an increase in vectors settling on infected plants, increasing disease incidence (McElhany et al., 1995; Mayer et al., 2008b). However, the specific compounds or blend of compounds responsible for attracting insect vectors could also potentially be used to lure insect pests away from important crop plants towards a trap or trap crop as part of an integrated pest management strategy (Agelopoulos et al., 1999; Cook et al., 2007).

To understand the rationale behind the current study, this section provides a brief overview of insect pests in agriculture; the process of host plant selection by phytophagous insects; the importance of olfactory cues in this and other processes; and ultimately the use of these cues to manipulate insect pests as part of a sustainable integrated pest management strategy. The study pathogen, insect and plant are also introduced.

1.2 Insect pests

Many insect species are considered pests in various industries, and with almost 50% feeding on living plants (Schoonhoven et al., 2005), insect herbivory is one of the major threats in plant production industries such as agriculture, horticulture and forestry. Damage may be either direct through the consumption of plant material or indirect by transmission of disease-causing viruses, bacteria and other pathogens. Other biotic as well as abiotic stresses might further influence plant production and yield, consequently reducing crop performance and resulting in lower harvests than what is attainable (Oerke, 2006). In addition to the direct quantitative losses resulting from these stresses, qualitative losses are also eminent. Pests reduce the market quality of products by, for example, altering aesthetic features and storage characteristics, or by contaminating products with toxins from pests, such as mycotoxins (Oerke, 2006). Both the quantitative and qualitative losses have economic implications.

Oerke (2006) provided estimates of the crop losses occurred from 2001 to 2003 that can be attributed to different pests and the effectiveness of controlling these pests with current control measures. Crops included in the study were wheat, rice, maize, potatoes, soybeans and cotton. On a global scale, the total potential loss due to pests, including weeds, pathogens, insects and other animals, varied from approximately 50% in wheat to more than 80% in cotton. The success of pest control varied considerably, with efficacy reaching 75% for weeds, 32% for pathogens and 39% for animal pests. In reality, pathogens and animal pests cannot be separated as pathogens are often a direct consequence of animal feeding. According to Oerke (2006), the more efficient control of weeds can be attributed to the use of both mechanical and chemical control methods, whereas the management of other pests rely heavily on synthetic chemicals. Only pre-harvest losses were included in this survey, post-harvest losses may reduce yield even further.

Due to a global population growth rate of 3.57% (as measured from 2000 to 2010, Food and Agriculture Organization of the United Nations Statistics Division, 2010) and limited cultivable land, losses from pests in both plant production industries and animal husbandry need to be minimized to increase food production and security (Godfray et al., 2010). The conventional use of pesticides for pest control is undoubtedly effective. However, due to pest resistance, toxic residues, negative environmental impacts and associated costs, these methods are decreasing in popularity (Miller & Cowles, 1990). There is a demand for control strategies that are more environmentally friendly, cost effective and sustainable.

1.3 Integrated pest management

Before the introduction of powerful pesticides, crop protection specialists relied profoundly on pest biology and cultural practices to create multi-tactical strategies for controlling pests and protecting crops (Gaines, 1957). However, by the early 1940's organosynthetic insecticides were introduced and the research focus shifted to testing chemicals rather than to research further pest biology and non-insecticidal methods of control (Kogan, 1998). Even though these new chemical

pesticides were very effective and reduced agricultural losses through pests considerably, by the late 1950s concerns about the risks involved began to be expressed (Ripper, 1956). These included insecticide resistance, resurgence of primary pests, increase in secondary pests, reduction or even elimination of beneficial natural enemies and bee populations, alteration of decomposition rates in soil organic matter, and overall environmental contamination (Ripper, 1956; Pimentel & Andow, 1984; Kogan, 1998). In addition, pesticides can damage crops if, for example, dosages were applied improperly or under unfavourable environmental conditions or if pesticides drifted from treated crops to nearby susceptible crops (Pimentel & Andow, 1984). A more environmentally friendly, reliable and sustainable pest control system was needed – the concept of "integrated pest management" was born.

Integrated pest management (IPM), as defined by Kogan (1998), is "a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interests of and impact on producers, society and the environment". IPM tactics include genetically modified organisms, the sterile insect technique, biological-control, cultivation of mixed crops, trap crops, and the use of semiochemicals to manipulate pest species.

This study focuses on the use of semiochemicals for behavioural manipulation of pest species. In order to understand how this mechanism works, it is important to understand how insects select host plants and what role chemical cues play in this selection process.

1.4 Host plant selection by herbivorous insects

All phytophagous insects exhibit some degree of selectivity towards the plants they feed on, and consequently need to be able to locate these suitable hosts in an array of non-host plants (Thorsteinson, 1960; Dethier, 1982; Bruce et al., 2005). This is a complex process, which is maintained by an intricate relationship of both neural and metabolic responses, as well as genetics

unique to each species, population or even individuals (Dethier, 1982; Visser, 1988; Jaenike, 1990). Insects use a variety of cues in their environment, such as olfactory, visual, mechanical and gustatory cues to locate host plants. The importance of these cues depends on the species, life stage and proximity of the insect to the host plant. In general, olfactory and visual cues play an important role in the first stage in the search for a host plant (host finding), whereas mechanical and gustatory cues are more important after contact is made (host acceptance) (Ramaswamy, 1988; Eigenbrode et al., 2015).

1.4.1 Host finding

Attraction from a distance usually involves olfaction or vision, or both (Bernays & Chapman, 1994; Bruce et al., 2005). Depending on the species, insects will often first encounter olfactory cues during the search process, usually present in the form of odour pockets separated by periods without odour (or odours from other, non-host plants), travelling down-wind from a host plant (Bernays & Chapman, 1994). In the search process, an insect will perceive a series of bursts of odour separated by periods without odour. When an insect perceives this odour and responds to it, it is said to become aroused, after which the insect will orientate itself upwind, towards the odour source (odour-induced anemotaxis) (Nottingham, 1988; Visser, 1988; Bernays & Chapman, 1994). Insects usually recognise host plant odours through either species-specific compounds, or species-specific ratios of compounds emitted by the plant (Bruce et al., 2005). Host finding is a complex process as there are millions of different molecules in the atmosphere that the insect has to identify or distinguish between. Most plants have the ability to synthesize and emit a large variety of volatile organic compounds (VOCs) from different organs such as flowers, fruits and leaves (Pichersky & Gershenzon, 2002; Tholl et al., 2006). In addition, the VOCs emitted by plants may change due to numerous internal and external factors, further complicating volatile profiles in the atmosphere. Different VOCs may be emitted in response to light and temperature changes (Holzinger et al., 2000; Sharkey et al., 2001), abiotic stresses like flooding or drought (Ebel et al., 1995; Holzinger et al.,

2000; Kreuzwieser et al., 2000), herbivory (Paré & Tumlinson, 1996; Dicke, 1999; Kessler & Baldwin, 2001; Pichersky & Gershenzon, 2002), to attract pollinators (Pichersky & Gershenzon, 2002), for inter- and intraspecific communication (Holopainen, 2004; Mescher & De Moraes, 2014) or as a consequence of infection with pathogens such as bacteria and viruses (Mayer et al., 2008b; Mancini et al., 2010). Changes in volatile emission often result in alteration of the behaviour of surrounding organisms (De Moraes et al., 1998; Kessler & Baldwin, 2001; Halitschke et al., 2008; Signoretti et al., 2012; Mescher & De Moraes, 2014).

As an insect approaches a plant, visual cues such as plant colour and form, including shape and size, become increasingly important (Nottingham, 1988). However these cues are relatively unspecific (Yamamoto et al., 1969) and are considered to be of critical importance only when employed in conjunction with olfactory cues (Bernays & Chapman, 1994). Nonetheless, some studies have demonstrated the attraction of insects towards visual cues in the absence of olfactory cues (Prokopy et al., 1983; Stenberg & Ericson, 2007), with visual cues being especially important in the absence of wind (De Wilde, 1976). Even though the compound eyes of many insects are well adapted to perceive the form of plants, colour is probably the most important visual cue for most phytophagous insects, especially in the case of insects that feed or oviposit on flowers (Bernays & Chapman, 1994). Attraction of insects towards specific colours is usually dependent on both the wavelength and the intensity of the colour. The peaks of maximal sensitivity for many insects lie in the ultraviolet spectrum, the blue part of the spectrum (450 nm) and the green part of the spectrum (540 nm) (Bernays & Chapman, 1994).

1.4.2 Host acceptance

Once an insect has located a potential host plant, the insect needs to establish whether to accept the plant in question for feeding and/or oviposition, or reject it. Olfaction and vision may still be important in this process. However, contact chemoreception (taste or gustatory stimuli) and mechanoreception (tactile stimuli) also play an important role (Fenemore, 1988; Nottingham, 1988;

Bernays & Chapman, 1994). Insects sense these foliar stimuli by moving around on the plant, exploring, touching, tasting and smelling the plant (Harris et al., 1987; Backus, 1988). In the case of feeding, insects will often start nibbling or probing, ingesting small amounts of food (Backus, 1988), whereas in the case of oviposition, female insects may drag or insert their ovipositor over/into foliage or soil without depositing eggs (Harris et al., 1987; Harris & Miller, 1988; Hattori, 1988; Ramaswamy, 1988). If insects continue feeding or eventually deposit eggs, they have accepted the host plant. This process is usually affected by stimulants, deterrents and arrestants, as well as other physical characteristics of the plant such as leaf texture, the presence of trichomes, surface wax and leaf angles (Bernays & Chapman, 1994). Sometimes, however, insects choose plants as hosts even though they are not optimal. This may be due to a combination of several factors such as the energy required for further searching (Mayhew, 1997), the presence of enemies/predators on these plants (Price et al., 1980), associative learning of the insect (Chapman, 1988; Bernays & Chapman, 1994; Wäckers & Lewis, 1994), or if a plant is more suitable for the insect's offspring than for the adult or *vice versa* (Cunningham et al., 2001; Mayhew, 2001).

1.5 Insect olfaction

Of all the cues used by insects, olfactory cues have a high specificity and can often be perceived over long distances. Olfactory cues are important, not just to find host plants, but also to locate prey in the case of carnivorous insects, mates and other conspecifics especially in the case of social insects, or to avoid dangerous situations such as predators. Olfactory cues are recognised and distinguished by insects with external sense organs called sensilla, located in most cases, on the insect antennae (Schneider, 1969; Bernays & Chapman, 1994). However, chemical receptors may also be present on the maxillary and labial palps, tarsi or ovipositors of some insects (Chapman, 1982; Bernays & Chapman, 1994). A typical olfactory sensillum consists of one-to-several sensory neurons and three accessory cells (Schneider, 1969; Bernays & Chapman, 1994). The sensory neurons relays olfactory information via their axon to the first olfactory centre in the brain, the

antennal lobe (Schneider, 1969; Vosshall et al., 2000). The antennal lobe consists of a series of distinct neuropile regions called glomeruli where integration of the olfactory information occurs (Bernays & Chapman, 1994). These glomeruli act as centres where inputs from sensory neurons with similar response characteristics converge, amplifying the signal and improving the signal-to-noise-ratio (Bernays & Chapman, 1994; Vosshall et al., 2000; Marin et al., 2002).

Not all insects, however, are equally dependent on olfactory cues and even within the same species, males and females or larvae and adults, may differ substantially in this regard (Carey & Carlson, 2011). In most cases, the length of an insect's antenna is generally proportional to the number of olfactory receptors it bears (Bernays & Chapman, 1994). Therefore, insects with longer antennae are assumed to be more dependent on olfactory cues than insects with shorter antennae. Antennae may also have quite varied shapes ranging from long and threadlike as in the case of cockroaches, to the leaf-shaped, feathery antennae of moths (Schneider, 1969). The antennae of some insects may also, in addition to olfactory receptors, house receptors for taste, temperature, humidity and mechanical stimulation (Schneider, 1969).

1.6 Chemical ecology in pest management

Chemical ecology entails the study of the origin, function and importance of natural chemicals that mediate interactions between organisms (Pickett et al., 1997). These chemicals are referred to as semiochemicals and encompass pheromones that mediate interactions within the same species, and allelochemicals which mediate interactions between species (Brown et al., 1970; Law & Regnier, 1971). Allelochemicals can further be divided into allomones, kairomones and synomones. Allomones are chemical signals advantageous to the emitter such as defensive secretions, whereas kairomones are advantageous to the receiver, including volatile compounds that can be detected by a predator or parasite to locate prey or hosts (Brown, 1968; Brown et al., 1970; Dicke et al., 1990). Synomones are advantageous to both the emitter and receiver (Dicke et al., 1990). For insects, this chemical information is essential at all stages of development e.g. to locate food, hibernation and

oviposition sites, to find mating partners and other conspecifics, and to avoid unsuitable hosts and habitats (Agelopoulos et al., 1999). Semiochemicals that have the ability to attract or repel insects have been incorporated into pest control strategies (Agelopoulos et al., 1999) as a sustainable and economical alternative to broad spectrum insecticides (Pickett et al., 1997). One such approach is the push-pull strategy first conceived by Pyke et al. (1987).

Push-pull strategies use semiochemicals to manipulate insect pests by using stimuli that render the protected resource less attractive to the pests (the push component) while a different stimuli simultaneously lures the insect pest towards a more attractive resource (the pull component) where the pests are subsequently removed (Pyke et al., 1987; Miller & Cowles, 1990; Cook et al., 2007; Eigenbrode et al., 2015). The push component may include visual distractions, non-host volatiles, anti-aggregation pheromones, alarm pheromones, oviposition deterrents or antifeedants (chemicals that prevent or interrupt feeding activity by contact chemoreception or by post-gustatory effects) (Cook et al., 2007; Zhang et al., 2013; Eigenbrode et al., 2015). The pull component may include visual stimulants, host volatiles, aggregation pheromones, sex pheromones, oviposition stimulants or gustatory stimulants (Cook et al., 2007; Mu et al., 2012; Eigenbrode et al., 2015). Chemical stimuli are preferred over visual stimuli due to the lack of specificity of visual cues and the general impracticality of changing visual stimuli to affect behaviour. However, more often these stimuli are combined for optimal results (Foster & Harris, 1997; Zhang et al., 2013).

There are a number of attributes that need to be considered when choosing a stimulus for behavioural manipulation, including accessibility (the stimulus must be suitable for presentation in a form that the insect can perceive), definability and reproducibility (the stimulus must be well-defined to reproduce it artificially), controllability (greater control in terms of behavioural manipulation can be achieved with the ability to control various parameters of a stimulus, such as longevity and intensity), specificity (the more specific a stimulus is to a particular behaviour of a pest, the more likely it can be used to manipulate that behaviour) and practicability (cost of

protecting a resource must be within practical limits) (Foster & Harris, 1997). Consequently, such control strategies, as is the case with most IPM strategies, require in depth research and a thorough knowledge on the pest's biology, behaviour, chemical ecology and interactions with hosts, conspecifics and natural enemies (Pickett et al., 1997; Cook et al., 2007; Zhang et al., 2013). The research may entail extensive funding and take decades before implementation. In addition, monitoring and decision systems for integrated approaches currently require higher operational costs than the sole use of insecticides, and semiochemical registration is often expensive due to the small and specialized market (Cook et al., 2007). However, there are also numerous advantages to using semiochemicals for behavioural manipulation as part of IPM strategies.

Semiochemicals used in pest management strategies are usually non-toxic and may reduce pesticide input considerably, especially if pest populations can be monitored to allow accurate timing of treatments or if insects are concentrated in predetermined areas, usually traps or trap crops (Martel et al., 2005b). This will ultimately result in a reduction of the negative environmental impacts as well as reduce costs associated with pesticide use (Pickett et al., 1997). In addition, the behaviour-modifying stimuli are usually species specific and only affect the pest species considerably, with no or little impact on other faunal species. Stimuli are, however, not highly effective when employed alone and therefore do not select strongly for resistance, as is the case with most insecticides, making this approach more sustainable (Pickett et al., 1997; Cook et al., 2007). In addition, by combining push and pull components, the push-pull strategy has increased efficiency compared to the individual components (Cook et al., 2007).

Since the concept of the push-pull strategy was first proposed by Pyke et al. (1987), numerous studies have investigated the potential of using semiochemicals for pest control in agriculture, horticulture, forestry, stored products and the veterinary and medical industries. One such strategy, which is probably the most successful to date, was designed to reduce stem borers, mainly *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae) and *Busseola fusca* Fuller (Lepidoptera: Noctuidae), as

well as striga (*Striga hermonthica* Benth; Scrophulariaceae) attack on maize plants (*Zea mays* L., Poaceae), and has already been implemented in several countries in sub-Saharan Africa (Khan et al., 2000; Khan et al., 2001; Hassanali et al., 2008; Midega et al., 2015). The strategy uses Napier (*Pennisetum purpureum* Schumach, Poaceae) and Sudan grass (*Sorghum vulgare sudanense* Stapf., Poaceae) as trap crops to attract stemborers, and molasses grass (*Melinis minutiflora* Beav., Poaceae) and *Desmodium* spp. (Fabaceae) to repel the ovipositing stemborers from the maize crops (Figure 1.1) (Khan et al., 2000; Khan et al., 2006). Parasitoids, *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae), were also found to be more attracted to the molasses grass (Khan et al., 2000; Khan et al., 2001; Midega et al., 2015), further decreasing stemborer attack. Wherever possible, harvestable intercrops or trap crops should be used rather than sacrificial crops (Gurr et al., 2004). This strategy was followed in the push-pull system to control stemborers and striga in maize, where the trap crop and intercrop plants provide valuable forage for cattle (Khan et al., 2000).

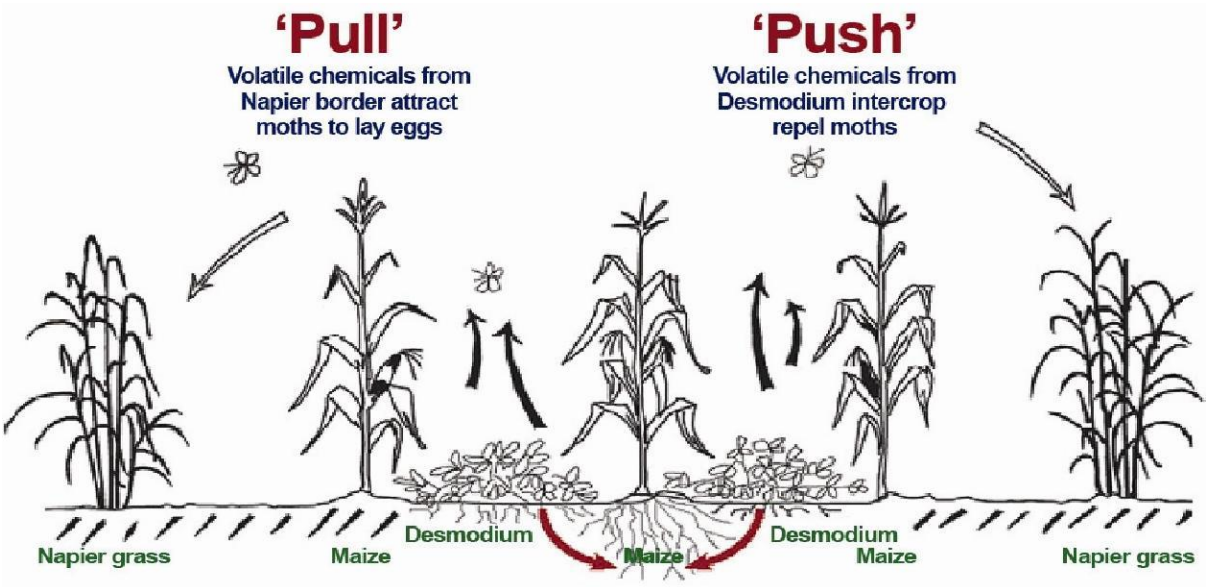

Figure 1.1: One of the most successful push-pull strategies to date uses Napier (*Pennisetum purpureum*) and Sudan (*Sorghum vulgare sudanense*) grasses as attractive trap crops, while molasses (*Melinis minutiflora*) and *Desmodium* spp. are used to repel ovipositing stemborer moths, mainly *Chilo partellus* and *Busseola fusca,* from maize (*Zea mays*). Damage to maize by the parasitic weed *Striga hermonthica* was also significantly reduced (source: Parrott, 2005).

Other successful and potential push-pull strategies include using the aggregation pheromone 4-methyl-3,5-heptanedione as an attractant for the pea and bean weevil, *Sitona lineatus* L. (Coleoptera: Curculionidae), while using neem antifeedant as a repellent (Schmutterer, 1990; Smart et al., 1994). This strategy was tested in field trials with field beans (*Vicia faba* L. cv. Alfred, Fabaceae) as host crops and, especially in the case where the antifeedant was used, treatments reduced weevil damage significantly (Smart et al., 1994). In an attempt to control the onion fly, *Delia antiqua* (Meigen) (Diptera: Anthomyiidae), a pest on commercial onion, *Allium cepa* L. (Amaryllidaceae), chemical deterrents (cinnamaldehyde) have been used to repel ovipositing females away from the main crop towards attractive onion culls (Cowles et al., 1990; Miller & Cowles, 1990; Cowles & Miller, 1992) resulting in a significant decrease in oviposition (Miller & Cowles, 1990; Cowles & Miller, 1992). A faeces-contaminated surface as attractant and methyl neodecanamide-treated surfaces as repellent, manipulated the German cockroach, *Blattella germanica* (L.) (Blattodea: Blattellidae), sufficiently to ensure a reduction in insecticide use and show promise for implementation outside of a laboratory setting (Sakuma & Fukami, 1990; Sakuma et al., 1997a; b; Nalyanya et al., 2000). Push-pull strategies have also been investigated for the control of *Helicoverpa* spp. (Lepidoptera: Noctuidae) in cotton (*Gossypium hirsutum* L., Malvaceae) (Mensah, 1996; Cunningham et al., 1999; Guoqing et al., 2001; Duraimurugan & Regupathy, 2005; Liu et al., 2005; Liu & Liu, 2006; Xu et al., 2006; Mensah & Moore, 2011; Mensah et al., 2013), Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), on potato (*Solanum tuberosum* L., Solanaceae) (Visser & Avé, 1978; Landolt et al., 1999; Dickens, 2000; 2002; Dickens et al., 2002; Martel et al., 2005a; b; Dickens, 2006; Kuhar et al., 2006; Martel et al., 2007), beetle pests (Coleoptera) on oilseed rape (*Brassica napus* L., Brassicaceae) (Cook et al., 2004; Barari et al., 2005; Mauchline et al., 2005; Cook et al., 2006), flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), on chrysanthemums (Asteraceae) (Teerling et al., 1993; Bennison et al., 2002; MacDonald et al., 2002; Hamilton et al., 2005; Egger & Koschier, 2014) bark beetles (*Tomicus* spp., Coleoptera: Scolytidae) on conifers (Lindgren & Borden, 1993; Guerrero et al., 1997; Zhang &

Schlyter, 2004) and muscid flies (Diptera: Muscidae) (Birkett et al., 2004; Jensen et al., 2004), mosquitoes (Diptera: Culicidae) and midges (Diptera: Ceratopogonidae) (Blackwell et al., 1994; Bhasin et al., 2001; Costantini et al., 2001; Blackwell et al., 2004) on human and animal hosts.

Overall, semiochemicals are promising as a management method but are generally not sufficient when implemented alone and should preferably be combined with other approaches in integrated management strategies (Pickett et al., 1997). Also, the use of renewable sources, especially plants, for the production of semiochemicals is possible (Kos et al., 2013) and encouraged, even in the case of insect-produced pheromones (Bruce et al., 2015). Bruce et al. (2015) reported the first crop plant (a hexaploid variety of wheat, *Triticum aestivum* cv. Cadenza, Poaceae) genetically engineered to release an insect pheromone (*E*-β-farnesene – an aphid alarm pheromone) for defence. The aphids *Sitobion avenae* F., *Metopolophium dirhodum* Walker and *Rhopalosiphum padi* L. (Hemiptera: Aphididae) were successfully repelled and the parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) attracted by the volatiles from these plants in laboratory behavioural assays. However, field trials did not result in a reduction in aphids or an increase in parasitism. Nevertheless, this study contributes to the advancement of such strategies for eventual implementation in the field on a commercial scale.

1.7 Phytoplasmas

Some of the most important plant pathogens in agriculture are phytoplasmas, formerly known as mycoplasma-like organisms. These organisms are minute, gram-positive, bacterial plant pathogens that belong to the class Mollicutes, and are closely related to the spiroplasmas and mycoplasmas (Bai et al., 2006). These pathogens cause diseases in more than 200 economically important plant species world-wide (Bai et al., 2006) and are responsible for devastating losses in crop yields (Lee et al., 2000; Christensen et al., 2005). At present, there is no method to cure plants once infected, and control is mostly aimed at preventing further spread of the disease. Current

methods include eradication of infected plants, clearing infected areas of weeds that may act as alternative hosts, and chemical control of insect vectors (Angelini et al., 2007).

Phytoplasmas have complex life cycles requiring two hosts for successful dispersal and reproduction in nature, including a plant host and an insect host, which also acts as a vector for the disease (Figure 1.2) (Christensen et al., 2005). Insect hosts of phytoplasmas are phloem-feeding leafhoppers (Cicadellidae), planthoppers (Fulgoromorpha) or psyllids (Psyllidae) of the order Hemiptera (Weintraub & Beanland, 2006). Plant hosts include both monocot and dicot species in numerous different plant families world-wide (Lee et al., 2000; Hogenhout et al., 2008) including ornamentals (e.g. chrysanthemums), vegetables (e.g. tomato, lettuce, potato, canola and onion), fruit trees (e.g. apple, pears, papaya and stone fruits), cereals (e.g. oats and barley), sugarcane and grapevine (e.g. Lee et al., 2000; Guthrie et al., 2001; Cordova et al., 2003; Wei et al., 2004; Arocha et al., 2005; Bisognin et al., 2008; Hogenhout et al., 2008). In plants, phytoplasmas are restricted to the phloem tissue, especially the mature sieve tubes. In insects, phytoplasmas invade the haemolymph, reproduce in the mid-gut and other tissues of the insect and migrate to the salivary glands when the insect becomes infectious (Webb et al., 1999; Hogenhout et al., 2008). The bacteria are transferred between hosts during insect feeding – the insect becomes infected whilst feeding on an infected plant and *vice versa* (Christensen et al., 2005). To a lesser extent, plants may become infected with phytoplasmas through vegetative propagation or grafting with infected plants or vascular connections between host plants and parasitic plants if either are infected (Dale & Kim, 1969). Even though phytoplasma deoxyribonucleic acid (DNA) have been recorded from progeny embryos from infected plants, currently there is scarce evidence to suggest that phytoplasmas can cause disease in plants through seed transmission (Cordova et al., 2003; Nipah et al., 2007; Nečas et al., 2008; Hartung et al., 2010; Bertolini et al., 2015).

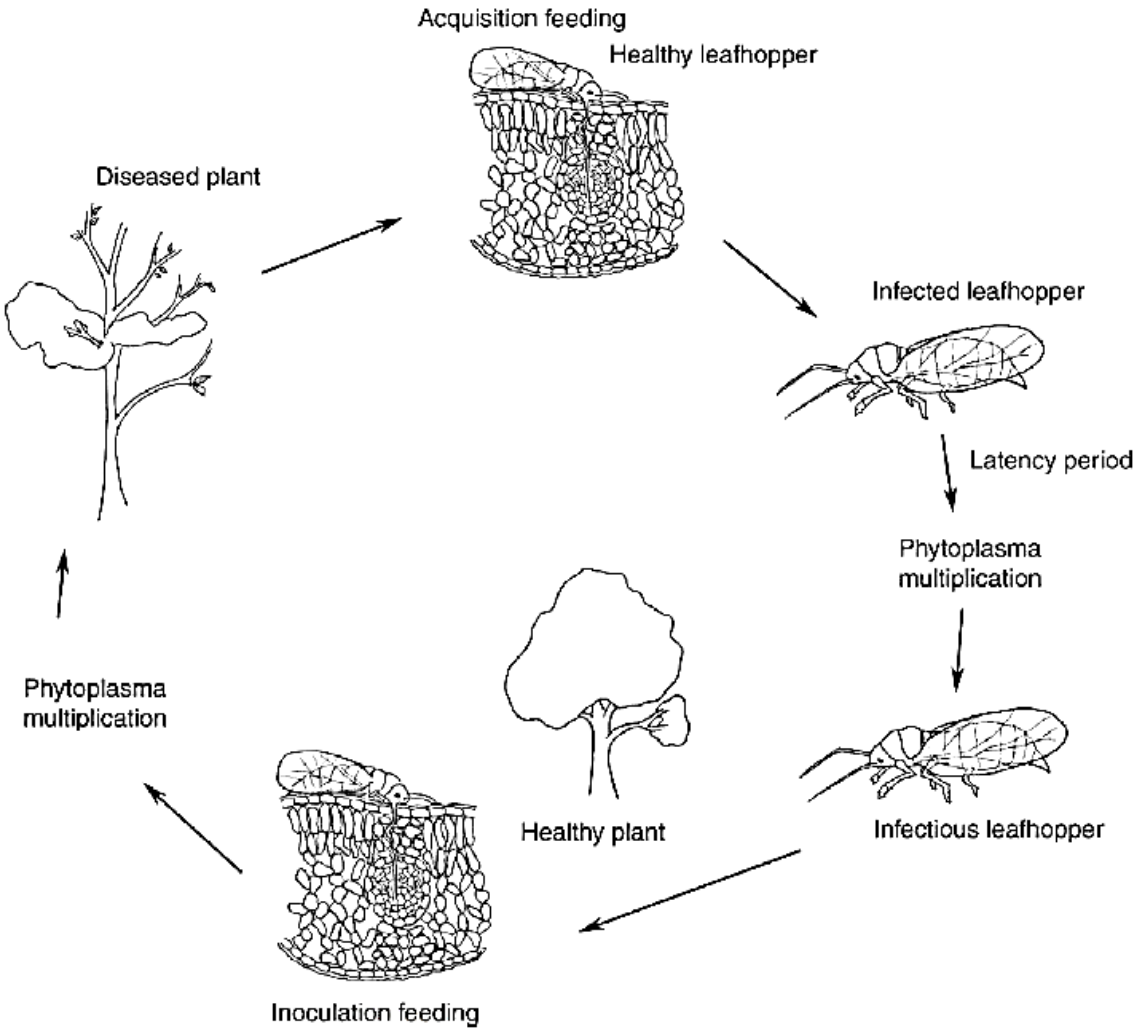

Figure 1.2: Host cycle of phytoplasmas. A leafhopper becomes infected whilst feeding on an infected plant (acquisition feeding). After a latent period during which the phytoplasma multiplies within the insect, the leafhopper becomes infectious and transmits the phytoplasma to uninfected plants during feeding (inoculation feeding) (source: Christensen et al., 2005).

Aster yellows phytoplasma (AY), '*Candidatus* Phytoplasma asteris', which belongs to the subgroup 16SrI-B, is the causative agent of grapevine yellows in South Africa and was recorded from grapevine, *Vitis vinifera* L. (Vitaceae), in the Western Cape in 2006 (Engelbrecht et al., 2010). The 16SrI group to which this phytoplasma belongs, is the most diverse and widespread, occurring in amongst others, North- and South America, Europe, Cuba, China, Saudi Arabia and Israel and is associated with more than 100 economically important diseases (Lee et al., 2004). Plant hosts are

usually negatively affected by infection. Depending on the phytoplasma strain, symptoms may include yellowing, stunting, virescence (greening of floral petals), phyllody (retrograde metamorphosis of floral parts into leaf like structures), witches broom and even plant death (Lee et al., 2004). After the introduction and discovery of AY in South Africa, Winetech (Wine Industry Network for Expertise and Technology) initiated a multi-disciplinary research programme with the aim to rapidly identify the vectors of AY to control AY in the country (Krüger et al., 2011). Results of transmission experiments, as well as field trials to determine leaf- and planthopper abundance in vineyards at the time of transmission, resulted in the identification of the leafhopper *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae) as a vector of AY in South Africa (Krüger et al., 2011).

In contrast to the plant hosts of phytoplasmas, studies on the insect vectors of these pathogens suggest that infection with phytoplasmas may increase survival and fecundity of some insect host species (Murral et al., 1996; Beanland et al., 2000). Moreover, infection may influence the plant host preference of these vectors (Christensen et al., 2005; Hogenhout et al., 2008) by, for example, altering host plant volatiles. Mayer et al. (2008a) showed that newly-emerged adults of *Cacopsylla picta* Foerster (Hemiptera: Psyllidae), a phloem-feeding psyllid and the main vector of '*Candidatus* Phytoplasma mali' (Seemüller & Schneider) which causes apple proliferation disease, is attracted to increased β-caryophyllene. This volatile compound is produced in greater quantities by infected apple (*Malus domestica* Borkh., Rosaceae) plants than by their uninfected counterparts (Mayer et al., 2008a; b). The attraction of insect vectors to infected plants has previously also been demonstrated for other insect vectors and diseases (Eigenbrode et al., 2002; McLeod et al., 2005; Srinivasan et al., 2006; Mauck et al., 2010). Mann et al. (2012) demonstrated that the psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is more attracted to '*Candidatus* Liberibacter asiaticus'-infected citrus plants (*Citrus* spp., Rutaceae) than to uninfected plants. This attraction of vectors to infected plants may lead to an increase in infected vectors (McElhany et al., 1995; Mayer et al., 2008b) and consequently faster spread of the disease. However, this attraction may also hold potential for the creation of a semiochemical mediated control strategy to control insect vectors.

1.8 Study insect

Leafhoppers, together with planthoppers, treehoppers, froghoppers, spittlebugs and cicadas, belong to the possibly paraphyletic suborder Auchenorrhyncha (Cryan & Urban, 2011) of the order Hemiptera. Approximately half of all hemipteran species are herbivorous (Backus, 1988). Due to their sap-feeding behaviour, they are responsible for the transmission of numerous plant diseases, and several leafhopper species are of economic importance (Mitchell, 2004). Leafhoppers follow a hemi-metabolic life-cycle with three distinct life stages: the egg, nymph, and adult stages. Two big compound eyes and long filiform antennae are present and haustellate mouthparts are used to feed on phloem sap.

Mgenia fuscovaria (Stål) (Hemiptera: Cicadellidae) (Figure 1.3), a leafhopper responsible for AY transmission to grapevine in South Africa, was first described in 1855 by Carl Stål. All species in the genus *Mgenia* feed on trees and shrubs and also lay their eggs on these plants, with only three species occurring in South Africa (M. Stiller, ARC-PPRI, Biosystematics Division, South Africa). *Mgenia angusta* occurs mostly in the Highveld but has also been recorded from the Western Cape, *M. capeneri* is restricted to the Limpopo province and *M. fuscovaria* has been mainly recorded in the southern and eastern parts of the country (M. Stiller, ARC-PPRI, Biosystematics Division, South Africa). *Mgenia fuscovaria* adults are approximately 6 mm in size and brown in colour whereas juveniles, morphologically similar to the adults, are somewhat smaller, green/yellow in colour and wingless. This species is commonly found in vineyards in the Western Cape feeding on grapevine plants. Currently, very little is known about this insect's ecology and biology due to the relatively recent realization of its economic importance as a vector of AY in South Africa.

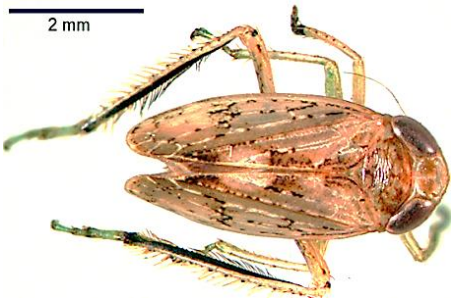

Figure 1.3: *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae) is a vector of aster yellows phytoplasma (‘*Candidatus* Phytoplasma asteris’) on grapevine (*Vitis vinifera*) in South Africa (photograph by M. Stiller).

1.9 Grapevine

Grapevine *Vitis vinifera* L. (Vitaceae) (Figure 1.4) is an important crop plant in South Africa and the world and used for the production of, amongst others, table grapes, non-alcoholic grape juice, raisins and wine. Numerous different cultivars with different qualities, dictated by the end-product the grapes are used for, exist.

Grapevine is a woody perennial, native to the temperate zones of the northern hemisphere. Plants are vigorous climbers and produce forked tendrils to gain structural support from other plants and objects in the vicinity. Leaves are long-stalked, palmately lobed and coarsely toothed, and are lost during autumn and winter months (deciduous). Flowers are small and green in colour, arranged in inflorescences, and fruits (grapes) are berries. Grapes are usually classified based on the skin colour of their fruits as either black/red or white.

The annual growth cycle of grapes commences in spring when buds can be observed between the stems and petioles of the plant. In the southern hemisphere this is around September. Flowering on the tips of new shoots in the form of inflorescences can be observed after bud break, and subsequently clusters of grapes will begin to form around the resulting seeds if flowers were fertilized. Ripe grapes are harvested at the end of summer and vines enter a period of dormancy at

the onset of winter. The timing and duration of events are subject to variations due to the grape variety, local climate, and seasonal weather (Goldammer, 2013).

Viticulture, including wine production, has taken place for thousands of years, possibly from as early as 2400 BC (Encyclopedia Britannica, 2016). Viticulture has since spread to numerous parts of the world and has become a very important commodity with almost 80 million tonnes of grapes produced globally in 2013, of which South Africa produced about 2.4% (Food and Agriculture Organization of the United Nations Statistics Division, 2013). Even though numerous different grape varieties are planted in South Africa, the majority are wine grapes, especially the cultivars Chenin blanc, Colombard and Pinotage (South African Wine Industry Information & Systems, 2013). Consequently, South Africa is regarded as one of the top 10 wine producers in the world (World Statistics, 2013).

In 2013, a total of 915.5 million litres of wine, excluding wine for brandy, distilling wine, grape juice concentrate and grape juice, were produced in South Africa (South African Wine Industry Information & Systems, 2013). This is 4% of the total wine production across the globe, generating an income of more than R3 826 million. More than half of the wine produced in the country is exported (525 586 145 litres in 2013), mostly to European countries, but also to North America, Asia, Australia and other African countries (South African Wine Industry Information & Systems, 2013).

Due to the economic importance of grapevine and associated industries in South Africa and around the world, threats such as insect pests, viruses, bacteria, fungi, other pathogens and abiotic factors that reduce product quantity and/or quality, threaten food and economic security. In the mid 1800's the European wine industry was almost devastated by grape phylloxera, *Daktulospaira vitifoliae* (Fitch) (Hemiptera: Phyttloxeridae), an insect that causes galls on the roots and leaves of the host plant. More than 1 million ha of vineyards were destroyed (Ordish, 1972). Fortunately it was found that grafting grapevine on resistant American rootstocks made them less susceptible to

attack (Granett et al., 2001). Hence, most susceptible commercial grapevines in affected areas to this day, are grafted on these resistant cultivars for protection from this pest (Granett et al., 2001).

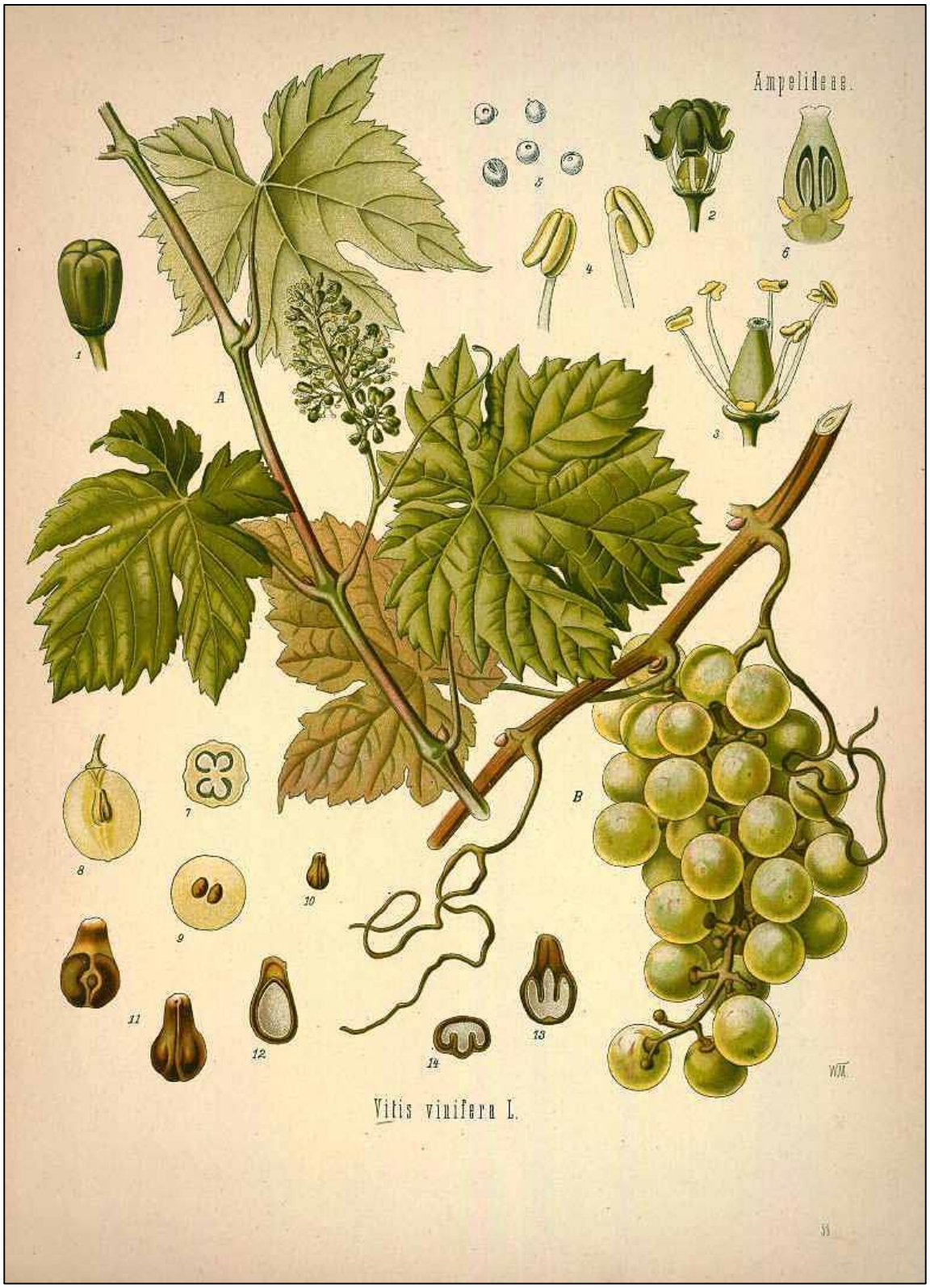

Figure 1.4: Botanical illustration of *Vitis vinifera* L. (Vitaceae) (Köhler & Pabst, 1883).

1.10 Conclusion

Grapevine is an important crop in South Africa and around the world, and losses to pests and diseases can have severe economic consequences. AY, which is responsible for numerous plant diseases resulting in yield losses world-wide, has recently been recorded in the country. Pesticides are not a reliable, long-term method of control and management strategies that are sustainable, environmentally friendly and economical are needed.

The work done by Mayer et al. (2008a; b) has demonstrated that phytoplasmas have the ability to alter the volatile composition of their host plants in such a way that insect vectors are more attracted to phytoplasma-infected plants compared to uninfected plants. This attraction of insect vectors to infected plants pose serious consequences for disease epidemiology and may lead to an increase in infected vectors and potentially faster spread of the disease (McElhany et al., 1995; Mayer et al., 2008b). However, due to the induced plant volatile changes that may attract insect vectors, there is also potential to develop a semiochemical-mediated control strategy to manipulate insect vectors away from important crop plants and towards a trap or trap crop.

1.11 Aims

The specific aims of this study were:

- To determine the headspace volatile profiles of AY-infected and uninfected grapevine plants of two cultivars, Colombard and Chenin blanc in summer and autumn, and identify changes in volatile profiles that can be attributed to AY infection.
- To examine the olfactory mediated responses of *M. fuscovaria* toward the volatiles emitted from AY-infected and uninfected grapevine plants and to determine any preferences displayed by the vector.

- To determine which volatile compounds produced by AY-infected and uninfected grapevine plants can be detected by *M. fuscovaria* and could therefore play a role in host plant location for this species. These compounds could potentially be used for vector manipulation.

The results of this study should contribute towards the understanding of the role of plant volatiles in host plant location and selection by the phytoplasma vector *M. fuscovaria*, to ultimately determine whether or not there is potential to develop a push-pull strategy for the control of leafhoppers in vineyards infected with AY in South Africa.

1.12 Thesis outline

The chapters of this thesis are written in the form of research papers, therefore there is some overlap between chapters with regards to parts of the text. Chapter 2 examines the effect of AY-infection on the volatile composition of grapevine cultivars Colombard and Chenin blanc in summer and autumn. Chapter 3 reports on the behavioural responses of *M. fuscovaria* towards these grapevine volatiles. Chapter 4 identifies volatile compounds that induce electrophysiological responses in *M. fuscovaria* antennae and could potentially play a role in host plant selection by this species. Chapter 5 provides a general discussion and conclusion of the results.

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Chapter 2

Volatile composition of aster yellows phytoplasma-infected and uninfected grapevine (*Vitis vinifera* L.)

Abstract

Phytopathogens can alter the physiology of host plants resulting in changes in the composition of volatile compounds. To determine if aster yellows phytoplasma (AY) ('*Candidatus* Phytoplasma asteris') infection alters volatile emissions of grapevine *Vitis vinifera* L. (Vitaceae) hosts (cultivars Colombard and Chenin blanc), volatiles were identified and quantified from field-collected AY-infected and uninfected grapevine branches in summer and autumn. AY-infection induced both qualitative and quantitative differences in its grapevine host. In summer, methyl salicylate was emitted in higher amounts from AY-infected compared to uninfected branches of cv. Chenin blanc, whereas in cv. Colombard methyl salicylate was produced in AY-infected branches only. Similarly, ethyl salicylate was only produced in AY-infected branches for both cultivars and the total volatile emissions by AY-infected grapevine branches were higher compared to uninfected branches for cv. Colombard in summer. In autumn, the compounds that differed significantly between AY-infected and uninfected branches were produced in greater quantities or only produced in uninfected branches. (*E,E*)-α-farnesene was the most abundant compound recorded from both AY-infected and uninfected grapevine branches of both cultivars in summer and autumn. In general, AY-infected branches had a greater mass than their uninfected counterparts with the same leaf area. AY-infection alters the physiology of its grapevine host leading to changes in the volatile composition of infected plants which could potentially influence insect vector behaviour.

Keywords: *Vitis vinifera*, grapevine, Colombard, Chenin blanc, aster yellows phytoplasma, '*Candidatus* Phytoplasma asteris', plant volatiles.

2.1 Introduction

The phenomenon where insect vectors of pathogens show increased attraction toward pathogen-infected plant hosts compared to uninfected hosts has been demonstrated in several cases (Eigenbrode et al., 2002; Srinivasan et al., 2006), including for bacterial (Mayer et al., 2008a; b), viral (Mauck et al., 2010) and fungal (McLeod et al., 2005) pathogens. This attraction may lead to an increase in the number of vectors settling on infected plants and acquiring pathogens, and consequently an increase in disease incidence (McElhany et al., 1995; Mayer et al., 2008b). Recently, aster yellows phytoplasma (AY) ('*Candidatus* Phytoplasma asteris'), a pathogenic bacterium that causes disease in numerous economically important plants world-wide (Lee et al., 2004), has been recorded in grapevine *Vitis vinifera* L. (Vitaceae) in South Africa (Engelbrecht et al., 2010). In a previous study, the leafhopper *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae), a vector of the disease (Krüger et al., 2011), was shown to be preferentially attracted to AY-infected grapevine branches when given a choice between AY-infected and uninfected grapevine branches cv. Colombard in summer (Krüger et al., 2015). The mode of attraction, whether visual or olfactory, was not determined.

Phytopathogens can alter the physiology of their plant hosts in many ways. Infection often results in changes in the primary (Chisholm et al., 2006; Berger et al., 2007) and secondary (Huang et al., 2003) metabolism of host plants. In some cases, infection with pathogens results in a change in the composition of volatile compounds produced by host plants. For example, tobacco plants (*Nicotiana tabacum* L.; Solanaceae) inoculated with different strains of the bacterium *Pseudomonas syringae,* emitted volatile compounds that differed both qualitatively and quantitatively from uninfected tobacco plants (Huang et al., 2003). Similarly, peanut plants *Arachis hypogaea* L. (Fabaceae) infected with white mould *Sclerotium rolfsii* Sacc. (Cardoza et al., 2002) and bean *Phaseolus vulgaris* (L.) (Fabaceae) cv. Red Mexican leaves exposed to the bacterium *Pseudomonas syringae* pv. *phaseolicola* (Croft et al., 1993) had different volatile profiles than their uninfected

counterparts. Some insect vectors are attracted to pathogen-infected host plants as a result of these differentially produced volatiles (e.g. Eigenbrode et al., 2002; McLeod et al., 2005; Srinivasan et al., 2006; Mauck et al., 2010; Mann et al., 2012).

Apple plants *Malus domestica* Borkh (Rosaceae) infected with the bacterium '*Candidatus* Phytoplasma mali' (Seemüller & Schneider) produced higher amounts of the compound β-caryophyllene which attracted the psyllid vector *Cacopsylla picta* (Foerster) (Hemiptera: Psyllidae) (Mayer et al., 2008a; b). Mann et al. (2012) observed that '*Candidatus* Liberibacter asiaticus'-infected plants are more attractive to the psyllid vector *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) than uninfected plants. Infected plants produced significantly more methyl salicylate, less methyl anthranilate and D-limonene. Methyl salicylate was shown to attract psyllids in behavioural assays. Moreover, four semiochemicals produced by American elms, *Ulmus americana* L. (Ulmaceae), infected with Dutch elm disease (*Ophiostoma novo-ulmi*), synergistically attracted the elm bark beetle *Hylurgopinus rufipes* Eichhoff (Coleoptera: Scolytidae), to infected plants (McLeod et al., 2005).

Differences in the volatile profiles between AY-infected and uninfected grapevine branches may potentially influence vector behaviour and disease epidemiology. The aim of this study was to determine the qualitative and quantitative differences in the volatile organic compounds (VOCs) produced by AY-infected and uninfected grapevine branches of the cultivars Chenin blanc and Colombard in summer and autumn.

2.2 Materials and methods

Portable volatile entrainment unit

A portable volatile entrainment unit was assembled inside a metal case for easy transport. It consisted of two vacuum pumps (ACO-001, Resun, China), one push pump and one pull pump, a charcoal filter to purify the air as it entered the collection chambers, a flow-meter (Dwyer® RMA-26-

SSV, 0.5-5 l/min, USA) to regulate the air-flow into the collection chambers and three flow-meters (Dwyer® Instruments, model VFA-22-SSV, 0.1-1 l/min, USA) to regulate the airflow out of the collection chambers through glass tubes containing the adsorbent. Tubing and fittings used were polytetrafluoroethylene (PTFE, 2 mm inner diameter) and metal respectively, to prevent leakage of compounds and minimize contamination (Figure 2.1).

Preparation of equipment

All equipment used in experiments were cleaned by washing with detergent (Teepol soap), rinsing with ethanol (99.5%) or acetone and distilled water and baking for a minimum of 2 h at 160 °C. Baking bags (Pick 'n Pay Giant Roasting Bags, 415 x 550 mm, South Africa) were baked at 140 °C for a minimum of 2 h. Activated charcoal filters were prepared by baking for a minimum of 8 h at 160 °C with N_2 gas (Nitrogen Baseline 5.0, 99.999%, Afrox) flowing through the filters to prevent oxidation of breakdown products during baking. Foil used in experiments was also baked for 2 h at 160 °C before use.

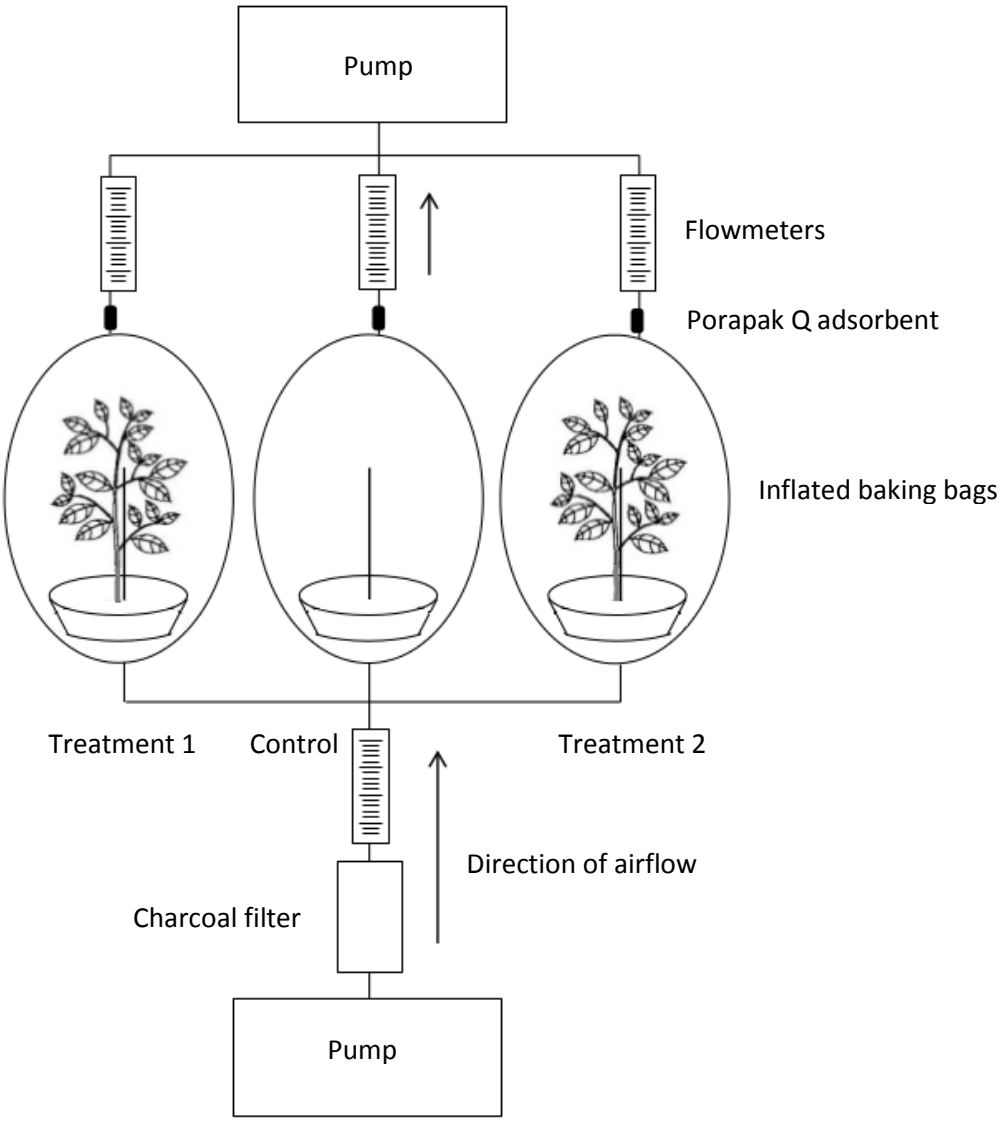

Figure 2.1: The set-up of the volatile entrainment unit. The different parts of the entrainment unit were connected with polytetrafluoroethylene (PTFE) tubing and metal Swagelok fittings.

Plant material

Grapevine branches of the white wine cv. Chenin blanc and cv. Colombard, two of the most common cultivars grown in South Africa (South African Wine Industry Information & Systems, 2013) were used for volatile entrainments. Grapevine cv. Colombard was collected from two vineyards, De Villa (31°40'11.49"S; 18°28'31.14"E) and Karoovlakte (31°40'11.49"S; 18°28'31.14"E) (Vredendal, Western Cape, South Africa) infected with AY. Grapevine cv. Chenin blanc was collected from

Karoovlakte. Due to the quarantine status of AY in Vredendal it was not possible to grow infected plants outside of the quarantine area. In addition, potted plants could not be used as young AY-infected plants die within a few weeks after infection and older potted plants were not available. Therefore, grapevine cuttings (*ca*. 40 cm) were made in the field and placed in water for transportation. For the experiments, shorter cuttings (*ca*. 10 - 15 cm) were made from the field-collected cuttings with secateurs while stems were submerged in water. Cuttings were kept in water at all times. Care was taken to ensure that the cuttings used in experiments were of comparable sizes. Branch mass was recorded for all plant samples with a balance (Ohaus® Scout Pro SPU602, China) immediately after removing the branches from the volatile collection bags. Thereafter, the leaf area was determined by attaching the leaves of the branches to paper with a ruler placed next to the leaves as reference while taking a photo. The photo was then prepared for leaf area analysis by converting the image to a TIF format in grayscale using the image manipulation program GIMP (GIMP V 2.6.6, 2001-2015). Measurements were taken after calibrating the software with the known distance provided by the ruler using the scientific image analysis program ImageJ 1.47v (Rasband, 2014).

Grapevine volatile collection

Each volatile entrainment set consisted of an AY-infected grapevine branch, an uninfected grapevine branch, and purified air as control (Figure 2.2). Each cutting was placed in a glass beaker (250 ml volume) containing distilled water. The top of the beaker was covered with aluminium foil, surrounding the stem of the cutting, to decrease water evaporating into the volatile collection chamber. Glass rods (30 cm) were placed inside the glass beakers in an upright position to prevent the volatile collection bags from collapsing on the branches and damaging them. After the branches were enclosed in the baking bags, the bags were sealed with rubber elastics around the PTFE tubing (2 mm inner diameter) through which charcoal-filtered air entered the bags at a flow-rate of 2 l/min. One flow-meter controlled the airflow for three bags with an approximate airflow of 0.66 l/min per

bag. The control was identical to the plant treatments and consisted of a glass beaker with a glass rod and aluminium foil but did not contain any plant material. Once the bags were fully inflated, the top corner of each of the bags were cut off and the push pump delivering purified air into the system was allowed to run for an hour before starting the experiment to expel un-purified air from the collection chambers. Thereafter, glass tubes containing Porapak Q adsorbent (60/80, 50 mg, Supelco, Bellefonte PA, USA) were inserted into each of the bags through the cut-off corners and the openings around the tubes were sealed using PTFE tape. Air was pumped from the collection chambers through the Porapak Q tubes at a flow rate of 0.4 l/min. Each bag was controlled by a separate flow-meter. The Porapak Q tubes were loosely covered with foil to protect light sensitive compounds during the experiment. Volatiles were entrained for 48 h after which the Porapak Q tubes were sealed in glass tubes under N_2 gas and sent to the Swedish University of Agricultural Sciences, Uppsala, Sweden for gas chromatography (GC) and combined GC-mass spectrometry (GC-MS) analysis.

Figure 2.2: Collection of the aboveground volatile compounds produced by aster yellows phytoplasma (AY)-infected and uninfected grapevine (*Vitis vinifera*) branches with portable entrainment units. Each entrainment set consisted of an AY-infected branch, an uninfected branch and a control, which was identical to the plant treatments without any plant material.

Chemical analysis of volatiles

Plant compounds were eluted from the adsorbent with 500 µl dichloromethane. After elution, an internal standard was added (1-nonene to give a concentration of 1 ng/µl), and the extracts concentrated to 50 µl under a gentle nitrogen flow. Volatiles were analysed on an Agilent 6890 GC (Agilent Technologies, Santa Clara CA, USA) equipped with a cold-on-column injector and flame ionisation detector, and fitted with an HP-1 column (100% dimethyl polysiloxane, 30 m, 0.25 mm inner diameter and 0.25 µm film thickness, J&W Scientific, USA). GC temperature program was: 1 min hold at 30 °C, 5 °C/min to 150 °C, 0.1 min hold, 10 °C/min to 250 °C, 15 min hold. The carrier gas was hydrogen with flow rate determined by constant pressure at 7.6 psi. A 1 µl aliquot of the entrainment sample was injected. Compounds were quantified by peak area in comparison with that of the internal standard. Compounds were tentatively identified by GC-MS on an Agilent 7890N GC (Agilent Technologies) coupled to an Agilent 5975C mass selective detector (electron impact 70 eV). The carrier was helium with a flow rate of 1 ml/min. Identifications were made by comparing mass spectra and retention indices against a commercial library (NIST 08) and authentic standards where available.

Molecular analysis

Grapevine branches were tested for AY-infection, after volatile collection experiments, with real-time polymerase chain reaction (PCR).

Nucleic acid extraction – Total nucleic acids were extracted from leaf veins with the NucleoSpin Plant II protocol (Macherey-Nagel, Germany). Veins were excised from the grapevine leaf (0.1 g) using sterile surgical blades and grinded using a Mini Mill Pulverisette 23 (Fritsch, Germany) and bearing balls. Each sample of grounded plant material was added to a separate 2 ml micro-tube tube. 10 µl of RNase A solution (5 ng/µl) and 300 µl of lysis buffer (PL2) was added to each tube and mixed using a Vortex mixer (FineVortex, FinePCR, Korea). Samples were incubated at 65 °C for 10

min (AccuBlock Digital Dry Bath, Labnet International Inc., USA). 75 µl of precipitation buffer (PL3) was added to each tube, mixed (Vortex) and incubated on ice for 5 min. 450 µl of binding buffer (PC) was added to the sample mixture which was then filtered by centrifuging for 2 min at 11 000 relative centrifugal force (RCF) through the NucleoSpin violet filter. The clear liquid containing the deoxyribonucleic acid (DNA) was collected and the filter with the plant material discarded. 700 µl of the sample was loaded onto the green NucleoSpin filter. The filter membrane binds the DNA during the wash phases and releases it when the elution buffer (PE) is added. The sample was placed in a centrifuge (PrismR, Labnet, USA) for 1 min at 11 000 RCF and the flow-through discarded. 400 µl of wash buffer (PW1) was loaded onto the filter membrane and centrifuged for 1 min (11 000 RCF) and the flow-through liquid was discarded. 700 µl concentrated wash buffer (PW2) was loaded onto the filter membrane and centrifuged for 1 min (11 000 RCF) and the flow-through liquid was discarded. 200 µl of concentrated wash buffer was loaded onto the filter membrane and centrifuged for 2 min at 11 000 RCF and the flow-through liquid was again discarded. The filter was placed over a new collection tube and 50 µl of elution buffer (70 °C) was loaded onto the membrane. The sample was incubated for 5 min at 70 °C and centrifuged for 1 min at 11 000 RCF. This step was repeated with a different collection tube and the elution buffer containing the nucleic acids were pooled into one collection tube. Samples were stored at -20 °C until used for real-time PCR analysis. Leaf veins from plants known to be infected with AY served as positive extraction controls.

Real-time PCR – Nucleic acid extractions from plants were tested for the presence of AY DNA with real-time PCR using the LightCycler® TaqMan® Master kit (Roche Applied Science, Germany), the LightCycler® 1.5 instrument (Roche Applied Science, Switzerland) and the protocol adapted from Angelini et al. (2007). The AY-specific primers and probes used were designed by Angelini et al. (2007) and consisted of a 19 base pair (bp) forward primer (5'-TTG GGT TAA GTC CCG CAA C-3'), a 22 bp reverse primer (5'-CCC ACC TTC CTC CAA TTT ATC A-3') and a 23 bp (5'- CCA GCA CGT AAT GGT GGG GAC TT-3') probe resulting in a 102 bp amplicon. The reaction mixture for each sample (2 µl) contained 13 µl of PCR grade H_2O, 1 µl of each of the forward primer, reverse primer and probe

mixtures, and 4 µl of the TaqMan® master mix. The mixture was loaded into a glass capillary and centrifuged for a few seconds at 140 RCF. The thermal protocol (45 cycles) included an incubation period of 10 min at 95 °C; amplification at 95 °C for 15 sec, 60 °C for 1 min and 72 °C for 1 sec; and a cooling period of 40 °C for 30 sec. For each PCR run, a positive PCR control containing DNA from a grapevine plant known to be infected with AY and a negative PCR control consisting of the reaction mixture only were included. Analyses were qualitative. Samples were regarded as positive for AY-infection based on the crossing point values of the samples, the height of the curves and the number of cycles after which the fluorescence increased.

Statistical analysis

Volatile analyses results are expressed as ng compound/g branch mass. Significant differences in the VOCs emitted between AY-infected and uninfected grapevine was determined for total volatile concentration, as well as for each compound. Analyses were carried out separately for each season and cultivar using Mann-Whitney U tests. Differences in the branch mass and leaf area of AY-infected and uninfected grapevine branches were also analysed for each cultivar and season with Mann-Whitney U tests (IBM SPSS Statistics 23, USA). The significance level was set at $P < 0.05$ for all analyses.

2.3 Results

Molecular analyses

All of the samples collected from grapevine branches from plants that were thought not to be infected with AY based on a lack of symptoms, tested negative for AY infection with real-time PCR. Some of the plant samples that showed AY-symptoms, especially samples collected during autumn 2014, did not test positive for infection and were consequently omitted from the analyses. All of the remaining samples that showed AY-symptoms tested positive for infection (Figure 2.3).

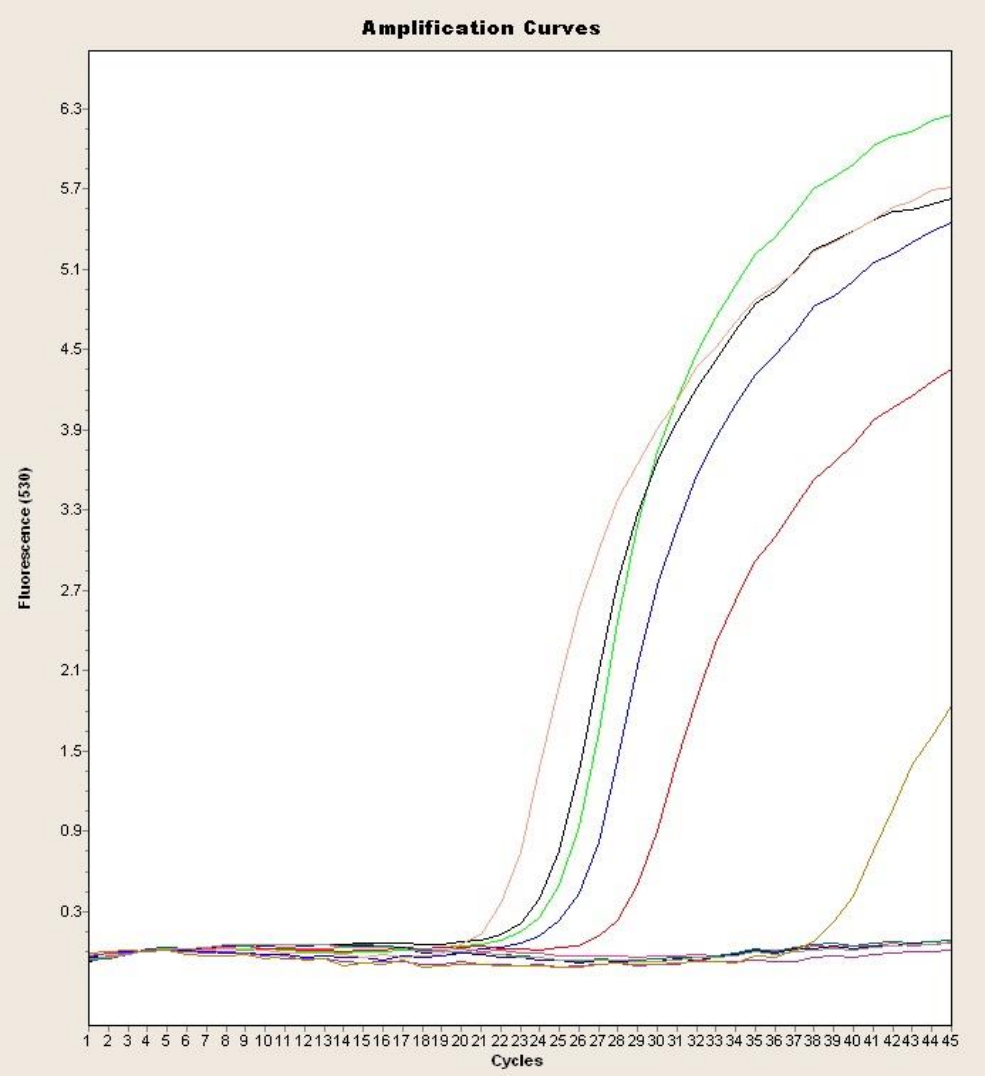

Figure 2.3: Real-time polymerase chain reaction (PCR) amplification of aster yellows phytoplasma (AY) deoxyribonucleic acid from grapevine leaves (*Vitis vinifera*). Samples were considered to be positive based on the crossing point values of the samples (CP value – point at which the fluorescence achieves a defined threshold), the height of the curves and the number of cycles after which the fluorescence increased. [AY-positive: AY1, AY2, AY3, AY4 and the AY+ control; AY-negative: U1, U2, U3, U4 and PCR- (reaction mixture)]. Even though the negative PCR control (reaction mixture) showed an increase in fluorescence, the sample is regarded as negative because fluorescence only increased after 38 cycles which can be attributed to the degradation of the quencher molecule due to the constant fluctuation in temperature during cycles (AY = aster yellows phytoplasma-infected; U = uninfected).

Chemical analysis of volatiles

The total amount of volatile compounds produced by AY-infected grapevine was significantly higher compared to uninfected grapevine for cv. Colombard in summer (P = 0.029). There were no significant differences in the amount of volatile compounds produced between AY-infected and uninfected grapevine for cv. Chenin blanc in summer or autumn, or cv. Colombard in autumn (P > 0.05) (Appendix A).

Summer

Grapevine cv. Colombard: 34 compounds were recorded from AY-infected branches and 18 compounds from uninfected branches (n = 4). (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 3-methyl-syn-butyl adoxime, 2-methyl-syn-butyl adoxime, (*E*)-2-hexen-1-ol, methyl benzoate, ethyl benzoate, methyl salicylate, ethyl salicylate, indole, β-cububene, aromadendrene, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), (*Z*)-3-hexenyl benzoate, methyl jasmonate and an unknown sesquiterpene were recorded from AY-infected branches only. Fourteen compounds, 6-methyl-5-hepten-2-one, benzyl alcohol, linalool oxide, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), α-cububene, cis-jasmone, α-copaene, β-bourbonene, germacrene D, (*Z,E*)-α-farnesene, cadinene, caryophyllene oxide and (*E,E*)-α-farnesene, were produced by both AY-infected and uninfected branches but were emitted in significantly higher amounts by AY-infected branches (Table 2.2).

Grapevine cv. Chenin blanc: 32 compounds were recorded from AY-infected branches and 31 compounds from uninfected branches (n = 8). Ethyl salicylate was the only compound that was not recorded from uninfected grapevine, only from AY-infected grapevine. Four compounds, benzyl alcohol, methyl benzoate, ethyl benzoate and methyl salicylate, occurred in significantly higher amounts in AY-infected than in uninfected grapevine branches, whereas linalool was recorded in significantly lower amounts in AY-infected than in uninfected grapevine branches.

Autumn

Grapevine cv. Colombard: 27 compounds were recorded from both AY-infected and uninfected branches (n = 4). Three compounds, (*Z*)-3-hexenyl acetate, indole and (*E*)-caryophyllene, occurred in significantly higher amounts in uninfected grapevine branches than in AY-infected branches.

Grapevine cv. Chenin blanc: 33 compounds were recorded from AY-infected branches and 34 compounds from uninfected branches (uninfected: n = 10; AY-infected: n = 6). β-caryophyllene was the only compound that was recorded from uninfected branches but not from AY-infected branches. Uninfected branches produced significantly more DMNT and (*Z*)-3-hexenyl acetate than AY-infected branches.

(*E,E*)-α-farnesene was the most abundant compound recorded from all treatments. In summer, (*E,E*)-α-farnesene was produced in significantly higher amounts in AY-infected grapevine cv. Colombard branches than in uninfected branches. The highest concentration of this compound (230.52 ng/g) was recorded from an uninfected grapevine cv. Colombard branch in autumn. In summer, methyl salicylate was produced in significantly higher amounts in AY-infected grapevine branches cv. Chenin blanc. For cv. Colombard, methyl salicylate was only produced in AY-infected branches and not in uninfected branches. Similarly, ethyl salicylate was only produced in AY-infected grapevine branches for both cultivars in summer (Appendix A).

Fewer replicates than anticipated could be obtained for grapevine cv. Colombard in both summer and autumn. Grapevine cuttings were field-collected based on visual AY symptoms and several of the plant samples that were thought to be infected with AY tested negative for infection with real-time PCR. This could be a consequence of herbicide damage which induces similar symptoms. Consequently, the samples were omitted from the analyses. In addition, irrigation in the vineyard initially used to collect grapevine cv. Colombard was unexpectedly discontinued as the

grower was planning to uproot the vineyard. Thus, no replicates for cv. Colombard were obtained during the autumn of 2014.

Leaf area and branch mass

The difference in leaf area and branch mass was compared between AY-infected and uninfected grapevine branches within cultivar and season. For grapevine cv. Colombard, leaf area and branch mass differed significantly between AY-infected and uninfected grapevine branches in autumn. AY-infected grapevine branches weighed more but had a smaller leaf area than uninfected branches. For grapevine cv. Chenin blanc, AY-infected branches had a greater mass than uninfected branches with the same leaf area in summer and autumn (Table 2.1).

Table 2.1: Leaf area (cm^2) and branch mass (g) (mean ± SEM) of aster yellows phytoplasma (AY)-infected and uninfected grapevine (*Vitis vinifera*) branches of the cultivars Colombard and Chenin blanc in summer and autumn (Mann-Whitney U test; $P < 0.05$).

			Uninfected	AY-infected	*Z*-value	*P*-value
Vitis vinifera cv. Colombard	Summer	Leaf area (cm^2)	445.68 ± 13.07	432.17 ± 28.06	-0.289	0.773
		Branch mass (g)	17.19 ± 1.50	17.70 ± 1.10	<0.001	1.000
	Autumn	Leaf area (cm^2)	518.20 ± 19.65	331.73 ± 24.59	-2.309	**0.021**
		Branch mass (g)	15.37 ± 0.71	25.98 ± 4.06	-2.309	**0.021**
Vitis vinifera cv. Chenin blanc	Summer	Leaf area (cm^2)	377.30 ± 11.75	473.44 ± 33.31	-1.785	0.074
		Branch mass (g)	12.93 ± 0.47	21.84 ± 1.84	-3.151	**0.002**
	Autumn	Leaf area (cm^2)	388.79 ± 26.04	413.46 ± 57.79	-0.245	0.806
		Branch mass (g)	10.71 ± 0.85	24.61 ± 2.71	-3.062	**0.002**

Table 2.2: Mean amount (ng compound/g branch mass ± SEM) of volatile compounds identified from aster yellows phytoplasma (AY)-infected and uninfected grapevine (*Vitis vinifera*) cv. Colombard and cv. Chenin blanc in summer and autumn. Means within a cultivar, season and row followed by different letters are significantly different (Mann-Whitney U test, $P < 0.05$).

	Vitis vinifera cv. Colombard				*Vitis vinifera* cv. Chenin blanc			
	Summer		Autumn		Summer		Autumn	
Compound	Uninfected	AY-infected	Uninfected	AY-infected	Uninfected	AY-infected	Uninfected	AY-infected
Total	3.024 ± 0.422[a]	43.211 ± 9.571[b]	88.090 ± 53.192	18.563 ± 7.386	32.572 ± 7.978	60.884 ± 19.725	64.261 ± 25.864	28.930 ± 10.037
Aldehydes								
(*E*)-2-hexenal		0.743 ± 0.382	0.084 ± 0.032	0.065 ± 0.058	0.308 ± 0.184	0.259 ± 0.126	0.122 ± 0.055	0.052 ± 0.022
Hexanal					0.254 ± 0.179	0.292 ± 0.129		
Ketones								
Cis-jasmone	0.016 ± 0.005[a]	0.100 ± 0.010[b]						
Geranyl acetone	0.054 ± 0.022	0.194 ± 0.080					0.036 ± 0.022	0.040 ± 0.040
6-methyl-5-hepten-2-one	0.064 ± 0.015[a]	0.482 ± 0.214[b]					0.834 ± 0.441	0.651 ± 0.640
Alcohols								
Benzyl alcohol	0.034 ± 0.006[a]	1.048 ± 0.269[b]	0.222 ± 0.085	0.179 ± 0.154	0.163 ± 0.030[a]	1.139 ± 0.533[b]	0.314 ± 0.093	0.374 ± 0.170
1-hexanol			0.111 ± 0.048	0.045 ± 0.034	0.088 ± 0.046	0.156 ± 0.085	0.051 ± 0.031	0.080 ± 0.055
1-octen-3-ol			0.027 ± 0.009	0.009 ± 0.002	0.031 ± 0.005	0.062 ± 0.017		
(*E*)-2-hexen-1-ol		0.084 ± 0.040	0.054 ± 0.007	0.038 ± 0.028	0.137 ± 0.098	0.088 ± 0.043	0.051 ± 0.028	0.044 ± 0.018
(*Z*)-3-hexen-1-ol		0.029 ± 0.015	0.028 ± 0.011	0.022 ± 0.012	0.101 ± 0.039	0.073 ± 0.035	0.156 ± 0.077	0.057 ± 0.036
Linalool	0.226 ± 0.081[a]	0.836 ± 0.199[b]	0.237 ± 0.129	0.044 ± 0.151	0.128 ± 0.049[a]	0.026 ± 0.004[b]	0.307 ± 0.082	0.223 ± 0.106
Linalool oxide	0.058 ± 0.016[a]	0.300 ± 0.053[b]	0.160 ± 0.043	0.504 ± 0.151	0.039 ± 0.010	0.101 ± 0.033	0.086 ± 0.014	0.285 ± 0.106
Esters								
(*Z*)-3-hexenyl acetate	0.138 ± 0.071	0.078 ± 0.048	0.359 ± 0.275[a]	0.021 ± 0.005[b]	1.587 ± 0.491	0.661 ± 0.344	2.787 ± 1.063[a]	0.709 ± 0.597[b]

	Vitis vinifera cv. Colombard				*Vitis vinifera* cv. Chenin blanc			
	Summer		Autumn		Summer		Autumn	
Compound	Uninfected	AY-infected	Uninfected	AY-infected	Uninfected	AY-infected	Uninfected	AY-infected
(*Z*)-3-hexenyl butyrate					0.044 ± 0.013	0.042 ± 0.010	0.025 ± 0.015	0.008 ± 0.008
(*Z*)-3-hexenyl benzoate		0.122 ± 0.033						
Ethyl benzoate		1.079 ± 0.402			0.021 ± 0.004[a]	1.131 ± 0.481[b]	0.034 ± 0.019	0.140 ± 0.107
Methyl benzoate		0.076 ± 0.022			0.014 ± 0.004[a]	0.033 ± 0.007[b]		
Methyl jasmonate		0.319 ± 0.108						
Methyl salicylate		2.261 ± 0.613	0.308 ± 0.110	0.884 ± 0.361	0.808 ± 0.242[a]	7.153 ± 1.716[b]	1.308 ± 0.434	2.529 ± 0.917
Ethyl salicylate		0.273 ± 0.077				0.305 ± 0.123	0.038 ± 0.024	0.066 ± 0.039
Terpenoids								
β-bourbonene	0.028 ± 0.007[a]	0.161 ± 0.064[b]	0.162 ± 0.039	0.179 ± 0.030				
α-caryophyllene					0.063 ± 0.019	0.039 ± 0.013		
β-caryophyllene	0.158 ± 0.035	0.469 ± 0.091					0.009 ± 0.006	
α-copaene	0.023 ± 0.006[a]	0.787 ± 0.194[b]	0.052 ± 0.013	0.028 ± 0.012			0.086 ± 0.028	0.050 ± 0.009
α-cubebene	0.057 ± 0.010[a]	0.784 ± 0.187[b]	0.083 ± 0.023	0.064 ± 0.019	0.197 ± 0.020	0.262 ± 0.050	0.024 ± 0.014	0.057 ± 0.025
β-cubebene		0.100 ± 0.030	0.016 ± 0.003	0.022 ± 0.006				
α-humulene	0.044 ± 0.015	0.118 ± 0.0230	0.105 ± 0.065	0.048 ± 0.024			0.029 ± 0.013	0.07 ± 0.032
β-farnesene							0.096 ± 0.051	0.020 ± 0.019
α-pinene							0.428 ± 0.308	0.582 ± 0.562
(*E*)-caryophyllene			0.540 ± 0.189[a]	0.056 ± 0.014[b]	0.236 ± 0.070	0.243 ± 0.088	0.091 ± 0.045	0.124 ± 0.053
(*E*)-ocimene					0.523 ± 0.200	0.567 ± 0.194	5.935 ± 4.087	0.438 ± 0.197
(*E,E*)-α-farnesene	1.638 ± 0.359[a]	22.135 ± 5.939[b]	78.985 ± 51.009	13.140 ± 6.561	23.746 ± 6.488	44.516 ± 17.617	43.972 ± 17.583	17.260 ± 7.217
(*Z,E*)-α-farnesene	0.071 ± 0.011[a]	1.036 ± 0.317[b]					0.465 ± 0.234	0.219 ± 0.210
(*E*)-β-farnesene			0.182 ± 0.110	0.052 ± 0.015	0.088 ± 0.014	0.095 ± 0.028	0.050 ± 0.0350	0.037 ± 0.017
Aromadendrene		0.150 ± 0.044						
Cadinene	0.027 ± 0.007[a]	0.791 ± 0.169[b]	0.129 ± 0.080	0.050 ± 0.018	0.049 ± 0.024	0.100 ± 0.024	0.064 ± 0.013	0.126 ± 0.070
Elemene			0.126 ± 0.042	0.102 ± 0.031				
Limonene							0.612 ± 0.425	0.537 ± 0.532
Longifolene					0.058 ± 0.009	0.403 ± 0.007		
Myrcene							0.007 ± 0.007	0.076 ± 0.076

	Vitis vinifera cv. Colombard				*Vitis vinifera* cv. Chenin blanc			
	Summer		Autumn		Summer		Autumn	
Compound	Uninfected	AY-infected	Uninfected	AY-infected	Uninfected	AY-infected	Uninfected	AY-infected
Ylangene			0.067 ± 0.022	0.052 ± 0.013				
Unknown sesquiterpene 1					0.108 ± 0.046	0.126 ± 0.034		
Unkown sesquiterpene 2					0.050 ± 0.010	0.055 ± 0.012		
Unknown sesquiterpene 3					0.102 ± 0.028	0.106 ± 0.037		
Unknown sesquiterpene 4		0.183 ± 0.044						
TMTT		0.128 ± 0.069	0.329 ± 0.275	0.046 ± 0.028	0.084 ± 0.018	0.122 ± 0.029	0.055 ± 0.033	0.059 ± 0.025
DMNT	0.175 ± 0.042[a]	4.873 ± 1.302[b]	2.244 ± 0.841	0.280 ± 0.093	0.755 ± 0.341	0.615 ± 0.161	3.892 ± 2.260[a]	0.308 ± 0.107[b]
Nerolidol							0.437 ± 0.229	0.047 ± 0.046
(*E*)-nerolidol			0.374 ± 0.243	0.051 ± 0.022	0.310 ± 0.105	0.248 ± 0.085	0.087 ± 0.040	0.083 ± 0.033
Caryophyllene oxide	0.052 ± 0.007[a]	0.668 ± 0.238[b]	0.259 ± 0.093	0.154 ± 0.065	0.081 ± 0.016	0.138 ± 0.045	0.586 ± 0.235	0.392 ± 0.257
Farnesene epoxide					0.104 ± 0.017	0.180 ± 0.056		
Germacrene D	0.161 ± 0.031[a]	1.761 ± 0.343[b]	2.802 ± 0.918	2.413 ± 0.622	2.299 ± 0.660	1.911 ± 0.735	1.190 ± 0.280	3.182 ± 2.041
Aromatic								
Indole		0.826 ± 0.338	0.046 ± 0.018[a]	0.013 ± 0.002[b]				
Amines								
2-methyl-syn-butyl adoxime		0.148 ± 0.067						
3-methyl-syn-butyl adoxime		0.069 ± 0.025						

DMNT: (*E*)-4,8-dimethyl-1,3,7-nonatriene; TMTT: (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

2.4 Discussion

As in the case of several other plant-pathogen relationships (e.g. Huang et al., 2003; Mauck et al., 2010; Bosque-Pérez & Eigenbrode, 2011), infection with AY altered the volatile composition of its grapevine host. In summer, cv. Chenin blanc branches produced increased amounts of methyl salicylate when infected with AY, and in cv. Colombard, methyl salicylate was only recorded from AY-infected branches and not from uninfected branches. Similarly, ethyl salicylate was only recorded from AY-infected branches and not from uninfected branches. For grapevine cv. Colombard, AY-infected branches also produced significantly more VOCs than uninfected branches, both qualitatively and quantitatively. Compounds induced by AY-infection include several green leaf volatiles, such as (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol. (*E,E*)-α-farnesene was the most abundant compound recorded from AY-infected as well as uninfected branches.

Methyl salicylate, which was produced in significantly higher amounts by AY-infected than uninfected branches in summer, is a known plant defence compound and the volatile form of salicylic acid. This endogenous plant signal associated with plant systemic acquired resistance (Ryals et al., 1996) has been demonstrated to have a plant-plant signalling function (Shulaev et al., 1997; Ozawa et al., 2000) and is a key compound in the induced resistance of tobacco and related plants against pathogens such as the tobacco mosaic virus (TMV) (Shulaev et al., 1997; Seskar et al., 1998; Zhu et al., 2014). Shulaev et al. (1997) showed that tobacco plants inoculated with TMV produced methyl salicylate after inoculation, while healthy, mock-inoculated or mechanically wounded plants did not. Healthy plants exposed to volatile compounds produced by TMV-inoculated plants as well as plants exposed to salicylic acid show induced expression of a molecular marker for acquired resistance (Yalpani et al., 1991). Shulaev et al. (1997) concluded that methyl salicylate is a likely candidate for an airborne signal which is detected by healthy tissues located close to the site of infection and could function as a long-distance transport of salicylic acid between plants if plants exposed to volatile methyl salicylate are able to incorporate this compound in their tissues as

salicylic acid. In addition, ethyl salicylate, an ester formed by the condensation of salicylic acid and ethanol, was only recorded from both cultivars from AY-infected branches in summer and not from their uninfected counterparts. The increased production of these compounds in AY-infected plants suggests that grapevine follow the salicylic acid-related signalling pathway of resistance (Durner et al., 1997) and that methyl salicylate might be produced as a consequence of AY-infection.

Green leaf volatiles (GLVs), which were induced by AY-infection in cv. Colombard in summer, are C_6 -aldehydes, C_6 -alcohols and their esters (Matsui 2006) emitted by most green plants. Intact plants produce these compounds in trace amounts, however the amounts rapidly increase when plants suffer wounding or other stresses (Hatanaka, 1993; Kishimoto et al., 2005; Dudareva et al., 2006; Holopainen & Gershenzon, 2010;). Several GLVs have been shown to possess antimicrobial activity (Hamilton-Kemp et al., 1992; Vaughn et al., 1993; Caccioni et al., 1995; Song et al., 1996; Archbold et al., 1997; Fallik et al., 1998; Archbold et al., 1999; Kubo & Fujita, 2001; Nakamura & Hatanaka, 2002; Frost et al., 2008; Scala et al., 2013) and the production of some of these compounds can be induced by pathogen infection (Croft et al., 1993; Shiojiri et al., 2006). In the current study, cut branches of field-collected grapevine were used for volatile collection. The cutting of these branches would have inevitably contributed to an increase in GLVs in both AY-infected and uninfected grapevine branches. However, as both AY-infected and uninfected grapevine branches were cuttings and treated equally, the increased GLVs recorded from AY-infected branches are likely due to AY-infection.

(*E,E*)-α-farnesene, the most abundant volatile compound present in all samples, was produced in significantly higher amounts in AY-infected grapevine than in uninfected grapevine cv. Colombard in summer. This compound has previously been shown to be emitted in response to pathogen infection (Vuorinen et al., 2007; Toome et al., 2010). Willow plants (*Salix burjatica* Nasarow x *S. dasyclados* Wimm., Salicaceae) inoculated with leaf rust produced (*E,E*)-α-farnesene, which was not recorded from healthy plants (Toome et al., 2010) and silver birch *Betula pendula* Roth (Betulaceae)

infected with the fungus *Marssonina betulae* produced increased amounts of this compound compared to their uninfected counterparts (Vuorinen et al., 2007). (*E,E*)-a-farnesene has also been shown to be induced by herbivory (Loughrin et al., 1994; Boevé et al., 1996; Röse et al., 1996; Scutareanu et al., 1997; Landolt et al., 2000).

Few studies have determined the volatile compounds produced by intact grapevine leaves. No published studies could be found on the volatile composition of grapevine cv. Colombard leaves; however a study on the volatile compounds produced by grapevine leaves cv. Chenin blanc was done by Wildenradt et al. (1975). The compounds recorded from grapevine cv. Chenin blanc in this study were similar to those recorded by Wildenradt et al. (1975). However, several compounds that were recorded in this study were not recorded by Wildenradt et al. (1975) and *vice versa.* This could be attributed to different environmental conditions or to different methods used.

In some cases, AY-infected plants had a greater mass than their uninfected counterparts with the same leaf area. This could possibly be attributed to lignification whereby plants accumulate lignin or lignin-like phenolic compounds as a response to pathogen infection as a mode of plant defence (Vance et al., 1980; Nicholson & Hammerschmidt, 1992). Results were standardised and expressed as ng compound/g branch mass to compensate for differences in branch mass.

In conclusion, AY-infection altered the VOCs produced by grapevine plants, in some cases qualitatively as well as quantitatively. AY-infected grapevine cv. Colombard branches produced more compounds than their uninfected counterparts in summer. The increased production of methyl salicylate and the induced production of ethyl salicylate in infected plants suggest that grapevine follow the salicylic acid-related pathway of resistance. Changes in volatile composition between AY-infected and uninfected grapevine could potentially influence vector behaviour.

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Chapter 3

Behavioural responses of *Mgenia fuscovaria* (Hemiptera: Cicadellidae) to volatiles from aster yellows phytoplasma-infected and uninfected grapevine (*Vitis vinifera* L.)

Abstract

Many phytopathogens that are responsible for disease in economically important plants are vectored by herbivorous insects. Some insect vectors are more attracted to pathogen-infected host plants compared to uninfected plants, which could result in an increase in disease incidence. In a previous study, the leafhopper *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae) was shown to be preferentially attracted to grapevine *Vitis vinifera* L. (Vitaceae) cv. Colombard infected with aster yellows phytoplasma (AY) ('*Candidatus* Phytoplasma asteris') compared to uninfected branches, in summer. The mode of attraction, either visual or olfactory, was not determined. Y-tube olfactometer bioassays were used to determine the role of olfaction for uninfected, inexperienced *M. fuscovaria* adults to volatiles from AY-infected and uninfected grapevine cv. Colombard and cv. Chenin blanc branches in summer and autumn. Leafhoppers did not show any preferences in summer for AY-infected or uninfected branches for either cultivar. In autumn, leafhoppers preferred purified air over AY-infected grapevine cv. Colombard branches and AY-infected cv. Chenin blanc branches over purified air. There were no significant differences in the choices made between male and female leafhoppers. The preference displayed by *M. fuscovaria* toward AY-infected grapevine when both visual and olfactory cues were presented, was not evident when only olfactory cues were employed. Olfactory cues could be supplementary to other, such as visual, cues.

Keywords: *Vitis vinifera*, grapevine, Colombard, Chenin blanc, *Mgenia fuscovaria*, leafhopper, vector, aster yellows phytoplasma, '*Candidatus* Phytoplasma asteris', olfaction.

3.1 Introduction

Aster yellows phytoplasma (AY) ('*Candidatus* Phytoplasma asteris'), a bacterial plant pathogen of the Class Mollicutes, was first recorded in grapevine (*Vitis vinifera* L.) (Vitaceae) in South Africa in 2006 (Engelbrecht et al., 2010). Thereafter, the leafhopper *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae) was identified as a vector of the disease in the Western Cape Province (Krüger et al., 2011). In dual choice tests in summer, adult *M. fuscovaria* were more attracted to AY-infected grapevine branches compared to uninfected grapevine branches cv. Colombard (Krüger et al., 2015). The mode of attraction, either visual or olfactory, was not determined.

In some cases, insect vectors of pathogens have been shown to be preferentially attracted to pathogen-infected host plants as a result of a change in olfactory cues induced by pathogen infection (Eigenbrode et al., 2002; Srinivasan et al., 2006; Mayer et al., 2008a; b; Mauck et al., 2010; Mann et al., 2012). This attraction to pathogen-infected plants due to induced volatile changes has also been observed for phytoplasmas, specifically apple plants (*Malus domestica* Borkh.; Rosaceae) infected with the phytoplasma '*Candidatus* Phytoplasma mali' (Seemüller & Schneider) (Mayer et al., 2008a; b). Infected plants released increased amounts of β-caryophyllene which attracted the insect vector of the disease, the psyllid *Cacopsylla picta* (Foerster) (Hemiptera: Psyllidae) (Mayer et al., 2008a; b). This was the case for both psyllids that were infected with the phytoplasma and psyllids that had no previous contact with the phytoplasma. Psyllids that developed on infected plants without becoming infected were, however, not attracted to infected plants, but preferred uninfected plants (Mayer et al., 2008b). This suggests that, in addition to the induced volatile changes in the plant host, the phytoplasma within the insect may also affect insect behaviour (Mayer et al., 2008b). If insect vectors are preferentially attracted to infected hosts, this could lead to an increase in the amount of vectors transmitting pathogens, increasing disease incidence (McElhany et al., 1995; Mayer et al., 2008b).

Olfactory cues are important host plant location cues for many insects as they allow them to locate host plants even though the plants are visually hidden among an array of other non-host plants (Bruce et al., 2005). Recognition of a host plant through olfactory cues is usually dependent on either species-specific compounds or specific ratios of compounds released into the atmosphere by host plants (Bruce et al., 2005). Some insects show relatively weak responses to odours and rely more on visual cues or a combination of both (Saxena & Saxena, 1974; Bullas-Appleton et al., 2004). Others, however, show great sensitivity and discriminatory powers towards odours. The aim of the current study was to investigate the olfactory response of uninfected, inexperienced *M. fuscovaria* to AY-infected and uninfected grapevine branches cv. Colombard and cv. Chenin blanc in summer and autumn, in the absence of visual cues.

3.2 Materials and methods

Olfactometer development and optimization

Despite the numerous Y-tube olfactometer designs available (e.g. Bullas-Appleton et al., 2004; Sharon et al., 2005; Mazzoni et al., 2009; Oluwafemi et al., 2011; Mu et al., 2012; Riolo et al., 2012), it was necessary to design and optimize an olfactometer set-up for *M. fuscovaria*. Based on a combination of different olfactometer designs by Oluwafemi et al. (2011), Sharon et al. (2005), and Wenninger et al. (2009), a Y-tube was designed for testing the behavioural response of *M. fuscovaria* (glass Y-tube, 1 cm inner diameter, 10 cm arms, 10 cm base, 60° angle between arms) (Figure 3.1a).

The common occurrence of turbulence where the arms of the olfactometer meet and the mixing of odours required that the air-flow of the olfactometer be tested to ensure an even airflow in both arms. Consequently, pH indicator strips (Whatman®, pH 1-14, EU and Merck, Germany) and volatile ammonia was used to visualise the airflow in the olfactometer. The pH indicator strips were placed inside the base and arms of the olfactometer, while cotton wool drenched in ammonia was placed into one of the odour containers. The other odour container contained purified air. Where

the ammonia came into contact with the pH indicator strips in the olfactometer, the yellow strips turned green. The airflow was visualised in this way while testing airflow rates ranging from 0.2 l/min to 1.0 l/min. Optimal airflow was determined as 0.6 l/min, the lowest airflow rate at which the ammonia did not enter the opposite arm (Figure 3.1b).

Based on initial tests and the work by Oluwafemi et al. (2011), the olfactometer was placed on a ramp with an incline of 15° from the surface as the leafhoppers preferred to walk in an upward direction. The ramp resulted in leafhoppers moving faster towards the odour sources and overall more active insects. Insects were considered to have made a choice at the point where the airflow test indicated that the odours did not mix (Figure 3.2).

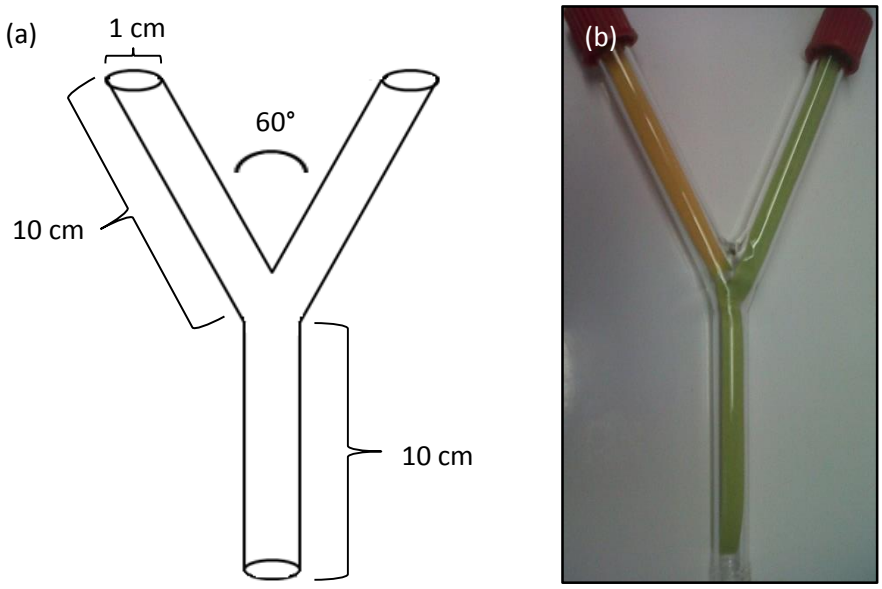

Figure 3.1: (a) Dimensions of the olfactometer design; (b) the airflow of the olfactometer was tested with pH indicator strips and ammonia – the yellow pH indicator strips turned green where it came into contact with the alkaline ammonia. The optimal airflow was determined as 0.6 l/min, the lowest airflow rate at which the ammonia did not enter the opposite arm of the olfactometer.

Figure 3.2: *Mgenia fuscovaria* adult choosing an arm during a test. The insect was considered to have made a choice if the whole body of the insect passed the point where the arms of the olfactometer split and where odours did not mix during the airflow test (as indicated by the line).

Insects

Adult *M. fuscovaria* were collected from an uninfected vineyard (31°43'3.66"S; 18°32'7.98"E) in Vredendal, Western Cape using sweep nets and manual aspirator tubes (Figure 3.3). Insects were kept in mesh cages on grapevine cuttings from the field in which they were collected. Insects were used in olfactometer tests on the same day as collection, as insects became less active after being held in captivity for long periods of time. Active insects were selected for trials from the walls and ceiling of the cage as these insects were more likely to make a decision in olfactometer tests.

Plant material

Field-collected grapevine cv. Chenin blanc and cv. Colombard branches were used in olfactometer tests. Cuttings were used in the experiments instead of intact plants. Branches were

collected from one of two vineyards infected with AY. Grapevine cv. Chenin blanc branches were collected from Karoovlakte (31°43'44.84"S; 18°32'7.98"E) and cv. Colombard branches were collected from De Villa (31°40'11.49"S; 18°28'31.14"E) and Karoovlakte.

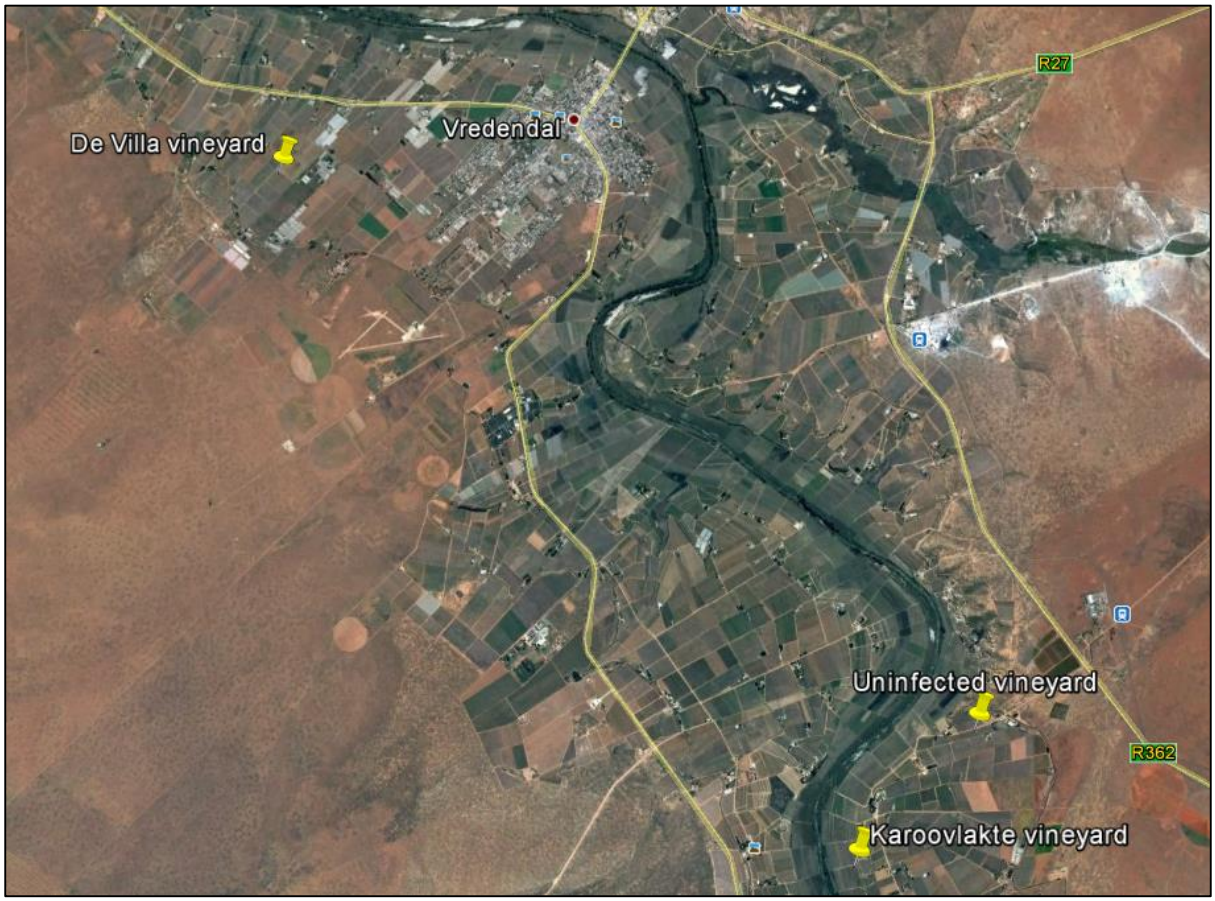

Figure 3.3: The study sites where grapevine plants (*Vitis vinifera*) and leafhoppers (*Mgenia fuscovaria*) were collected in Vredendal. De Villa and Karoovlakte were both infected with aster yellows phytoplasma (AY) during the study. Grapevine cv. Colombard was collected at De Villa and Karoovlakte; cv. Chenin blanc was collected at Karoovlakte. Uninfected insects were collected from a grapevine cv. Chenin blanc vineyard not infected with AY near Karoovlakte.

The following combination of treatments was tested in Y-tube olfactometer tests for both cultivars in summer and autumn:

- AY-infected grapevine vs. purified air

- Uninfected grapevine vs. purified air
- AY-infected grapevine vs. uninfected grapevine

AY-infected grapevine plants were identified and stem cuttings collected based on visible AY symptoms. Uninfected cuttings were obtained from grapevine plants that were symptom-free in the same vineyard. Long grapevine cuttings (*ca*. 30 - 40 cm) were made in the field and placed in water for transportation. For the experiments, shorter cuttings (*ca*. 10 - 15 cm) were made from the field-collected cuttings with secateurs while stems were submerged in water. Care was taken to ensure that the cuttings used in the experiments were of similar size to fit in the odour containers and had similar leaf coverage for comparable results. Cuttings used in olfactometer tests were used on the same day they were collected. For experiments, the stem cuttings were placed in glass vials containing distilled water and covered with sterile aluminium foil. The control was identical to the plant treatments but did not contain any plant material.

Preparation of equipment

All equipment used in experiments were cleaned with detergent (Teepol soap), rinsed with 99.5% ethanol (metal fittings and polytetrafluoroethylene tubing) or acetone (glassware) and distilled water, and baked for a minimum of 2 h at 160 °C. Activated charcoal filters were prepared by baking for a minimum of 8 h at 160 °C with N_2 gas (Nitrogen Baseline 5.0, Afrox, 99.999%) flowing through the filters to prevent oxidation of breakdown products during baking. Foil used in experiments was also baked for a minimum of 2 h at 160 °C before use.

Olfactometer bioassays

The custom-designed Y-tube olfactometer was used to investigate the response of adult *M. fuscovaria* to AY-infected and uninfected grapevine volatile compounds in the absence of visual cues. Teflon fittings with silicone stoppers sealed the ends of the olfactometer and the air containing the plant odours entered the arms of the olfactometer through glass inlet tubes with an inner diameter of 2 mm. The two arms of the olfactometer were connected to two 3 l glass jars containing the odour treatments (AY-infected grapevine, uninfected grapevine or a control). For each jar, tubing entered the jar through two holes in the side of the jar, sealed with silicone tubing. The hole through which purified air entered the jar was situated near the base of the jar and the exit hole was situated near the top of the jar. This ensured that the air flowed over the treatment plant before exiting the odour container, delivering the maximum amount of plant volatiles to the insect in the olfactometer. The top of the jar was sealed with sterile aluminium foil. For each jar, air entering the odour container was regulated at 0.6 l/min with a flowmeter (Dwyer® VFA-22-SSV, 0.1-1 l/min, USA). The air current that entered the system was provided by a vacuum pump (KNF Laboport, model N86KN.18, France) and an activated charcoal filter was used to purify the air as it entered the system. The different components of the olfactometer set-up were connected with 2 mm inner diameter polytetrafluoroethylene (PTFE) tubing and metal Swagelok fittings (Figure 3.4).

Because *M. fuscovaria* is diurnal, the behavioural bioassays were done during the day in a white box to avoid any directional bias towards external colours and shapes. A light (Opple compact fluorescent light, natural white, 12W, China) was positioned directly above the arms of the olfactometer. The pump was allowed to run for 5 minutes before commencing tests to ensure non-purified air was expelled from the system. An insect was placed into the base of the Y-tube and observed for 3 min, recording the movements of the insect using the computer program OLFA (Exeter Software, USA). A choice was considered to be made if the entire body of the insect passed the split of the Y-tube into one of the arms. Initially, insects were observed for a period of 10 min.

However it became obvious that insects that did not make a choice in the first 3 min, rarely made a choice at all. In addition, insects that made a choice usually remained in the first arm chosen and rarely moved from the one arm to the next (a total of 3 insects out of the 311 changed arms during tests). Consequently, insects were only observed for a period of 3 min and only the first choice was used in the analysis. If the insect did not choose an arm within the 3 min observation period, the insect was labelled as non-responsive and omitted from subsequent analyses. A new insect was used for each choice and thereafter preserved in ethanol (99.5%) to determine the sex and AY-infection status of the insects using real-time polymerase chain reaction (PCR). After each insect/replicate, the olfactometer was flipped 180°. After every set of 5 insects, the position of the odour containers was switched in order to avoid any directional bias and clean equipment and new plant treatments were used.

Molecular analysis

All plant material and insects used in the experiments were tested for infection with AY in the laboratory with real-time PCR. Nucleic acid extractions from insects was done using a non-destructive extraction protocol using TNES buffer (Sambrook & Russell, 2001). *Mgenia fuscovaria* adults stored in 96% ethanol were dried on paper towel and placed singly in 1.5 ml micro-tubes. 5 µl of proteinase K (5 ng/µl) and 190 µl of TNES buffer (double distilled H_20; 1M Tris-HCl; 0.5 M EDTA; 5 M NaCl; 10% SDS) was added to each tube. After mixing the content and pulse centrifuging (spinning the tubes in the centrifuge for a few seconds to ensure the entire contents of the tube mixes and accumulates at the bottom of the tube), tubes were incubated overnight at 55 °C (AccuBlock Digital Dry Bath, Labnet International Inc., USA). Proteins were removed by adding 5 M NaCl to each tube whereafter the content was mixed and centrifuged for 30 min at 3 700 revolutions per minute (rpm) at 4 °C. To precipitate the deoxyribonucleic acid (DNA), 200 µl of the supernatant of each tube was transferred, each to a new tube containing 400 µl of absolute ethanol (EtOH). Tubes were kept at -80 °C for 15 min and centrifuged for 30 min at 3 700 rpm at 4 °C. The alcohol in the tubes was drained

and 200 µl of 70% EtOH was added to each tube to rinse the DNA. The samples were centrifuged for 30 min at 3 700 rpm at 4 °C. Alcohol was eliminated as before and samples were dried by incubating the tubes with open lids at 37 °C in a dry heat-block for 30 min. The samples were rehydrated with 60 µl of double distilled water (ddH_20). Extracts were stored at -20 °C until analysed by means of real-time PCR. Plant DNA was extracted with the NucleoSpin Plant II protocol from Macherey-Nagel (Chapter 2). Samples were prepared for real-time PCR assays with a LightCycler® TaqMan® Master kit using primers and a probe specifically designed for AY detection by Angelini et al. (2007). Real-time PCR assays were done on a LightCycler® 1.5 instrument (Roche Applied Science, Switzerland) (Chapter 2).

Statistical analysis

The Fischer's exact test was used to determine any significant differences in the choices made by male and female leafhoppers. Differences in the proportion of *M. fuscovaria* individuals choosing a particular odour source were analysed using the first choice data and the two-tailed binomial test to determine if responses to two odour sources differed from a 50:50 distribution. Non-responsive insects were omitted from the analysis. The level of significance was set at $P < 0.05$. All tests were carried out with SPSS Statistics Software (IBM SPSS Statistics 23, USA).

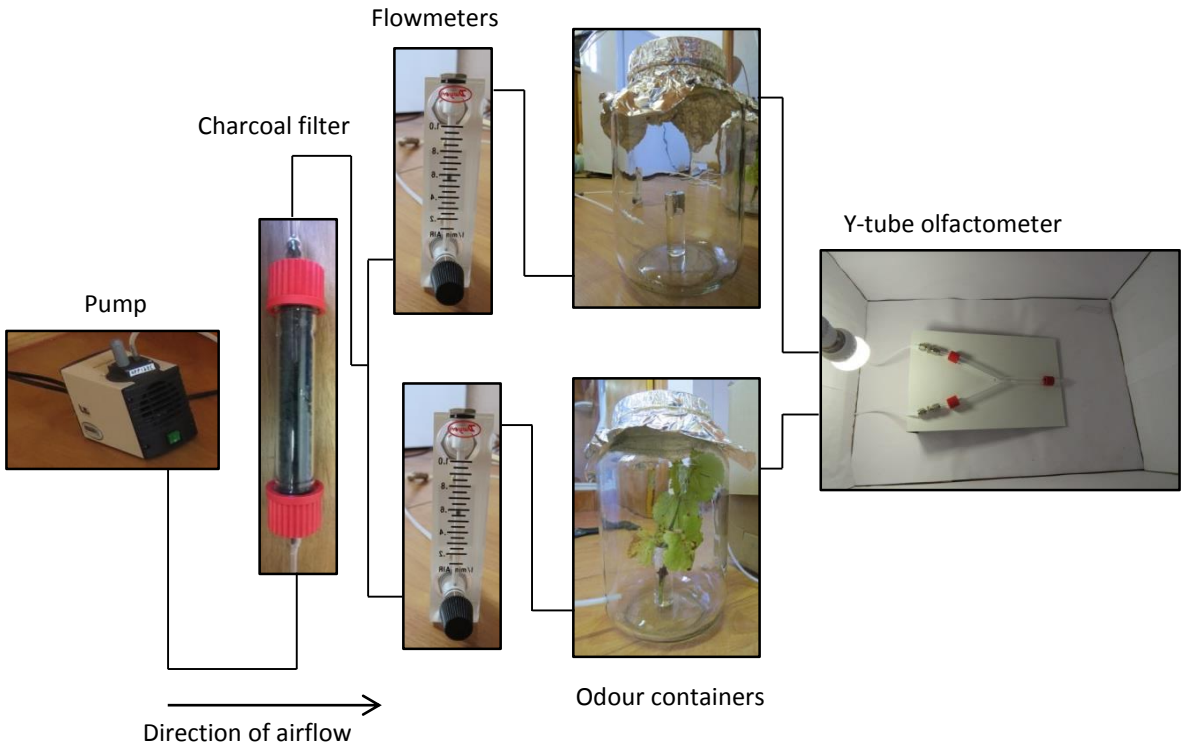

Figure 3.4: The olfactometer set-up. A vacuum pump produced an air current; the air was purified by an activated charcoal filter as it entered the system; the airflow for each arm of the olfactometer was regulated with a flowmeter (two flowmeters – one for each arm, each set at 0.6 l/min); the clean air flowed over the plant/s in the odour containers. The volatiles entered the arms of the olfactometer which was set-up in a white box with a white light situated directly above the arms of the olfactometer. The olfactometer was placed on a ramp that created a 15° angle from the ground surface.

3.3 Results

Molecular analysis

All insects used in olfactometer bioassays tested negative for AY infection. None of the uninfected plant samples tested positive for AY infection. Several of the plant samples that were initially thought to be infected with AY based on symptoms, including most of the samples collected during autumn 2014, however, did not test positive for infection in the laboratory, possibly due to

similar symptoms induced by herbicide damage. This resulted in some of the tests having fewer replicates than planned because the tests carried out with "false" positive AY-branches were omitted from the analyses.

Olfactometer bioassays

There were no significant differences in the choices made between male and female leafhoppers for any of the combinations tested (Fischer's exact test, $P > 0.05$). Hence, data for male and female leafhoppers were pooled for subsequent analyses. Of all insects tested, 35% were non-responsive and consequently not included in statistical analysis. There were no differences in the choices made by leafhoppers for any of the combinations tested in summer. In autumn, leafhoppers showed a preference for purified air over AY-infected grapevine branches cv. Colombard (Figure 3.5). In contrast, more leafhoppers chose AY-infected cv. Chenin blanc branches over purified air (Figure 3.6). There were no observable differences in the choices made by leafhoppers for any of the other combinations tested (Table 3.1).

Table 3.1: Data and test statistics for the differences in choices made by *Mgenia fuscovaria* in olfactometer bioassays (binomial test; $P < 0.05$). AY: aster yellows infected grapevine; U: uninfected grapevine; C: purified air.

			***Z*-value**	***P*-value**
Vitis vinifera L. Colombard	Summer	AY vs. U	1.7	0.143
		AY vs. C	1.41	0.211
		U vs. C	0.37	0.856
	Autumn	AY vs. U	1.40	0.230
		AY vs. C	2.40	**0.027**
		U vs. C	1.10	0.362
Vitis vinifera L. Chenin blanc	Summer	AY vs. U	0.00	1.000
		AY vs. C	1.00	0.424
		U vs. C	0.39	0.845
	Autumn	AY vs. U	0.21	1.000
		AY vs. C	2.83	**0.007**
		U vs. C	1.18	0.327

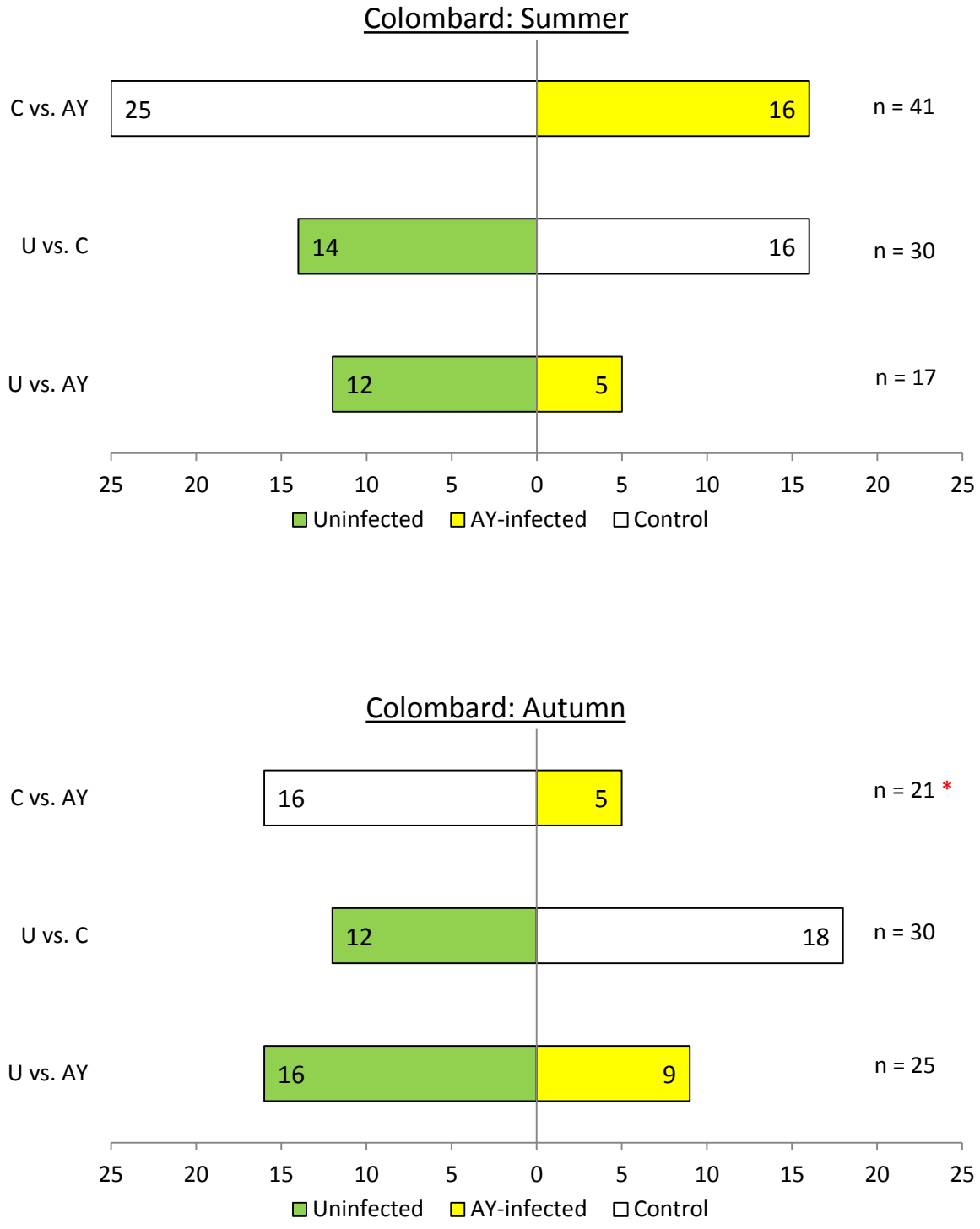

Figure 3.5: Responses of *Mgenia fuscovaria* adults to odours from grapevine (*Vitis vinifera*) cv. Colombard in summer and autumn. Asterisks indicate significant differences (binomial test, $P < 0.05$). AY: aster yellows-infected grapevine; U: uninfected grapevine; C: purified air.

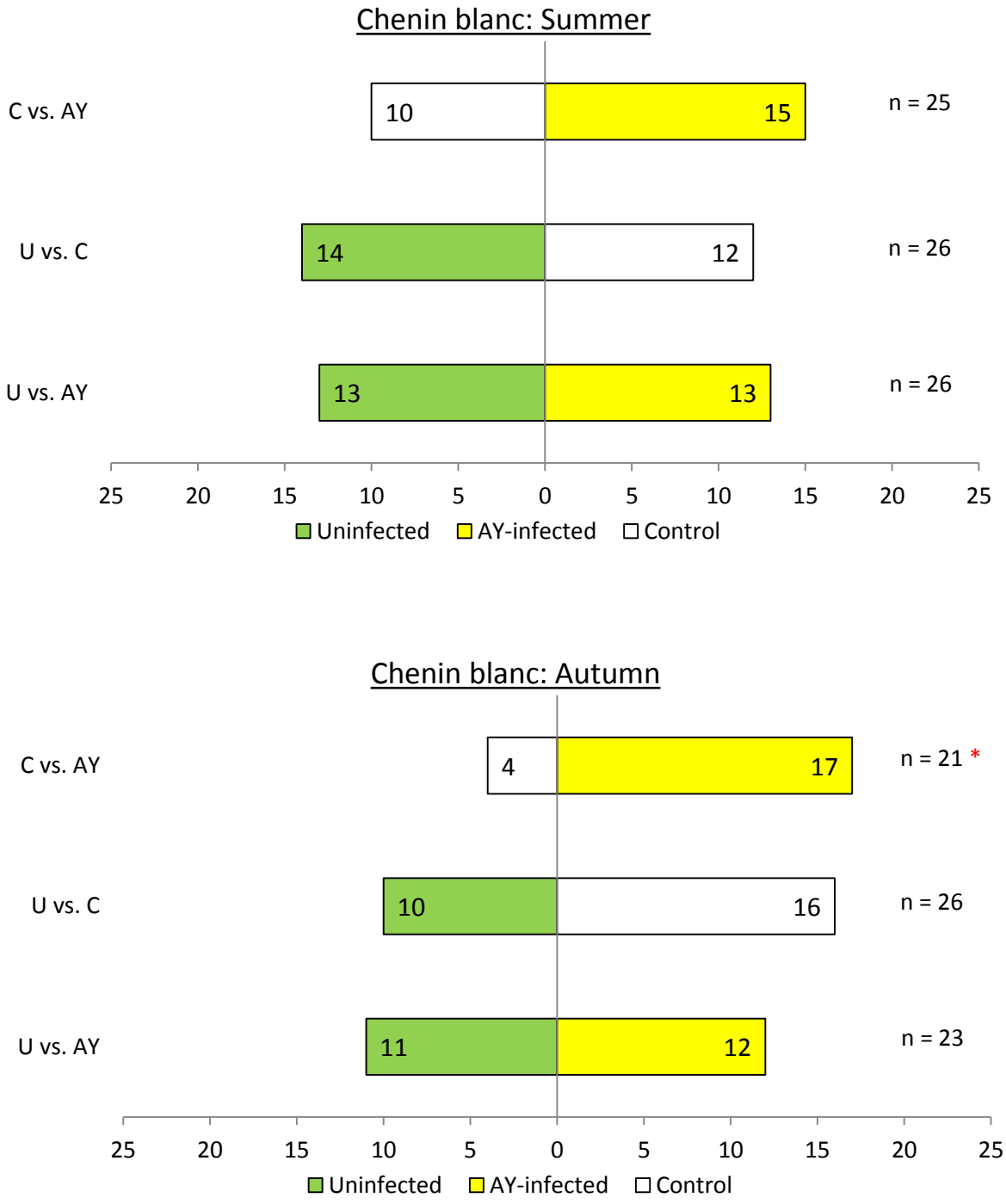

Figure 3.6: Responses of *Mgenia fuscovaria* adults to odours from grapevine (*Vitis vinifera*) cv. Chenin blanc in summer and autumn. Asterisks indicate significant differences (binomial test, $P < 0.05$). AY: aster yellows-infected grapevine; U: uninfected grapevine; C: purified air.

3.4 Discussion

There was no consistency in the choices made by *M. fuscovaria* between AY-infected and uninfected grapevine branches for both cultivars and seasons. In some cases insects seemed to be repelled by AY-infected grapevine branches, whereas in other cases insects were attracted. In autumn, leafhoppers preferred purified air over AY-infected grapevine cv. Colombard branches. In contrast, leafhoppers were attracted to AY-infected grapevine branches cv. Chenin blanc when tested against purified air. There were no observable differences in the choices made by leafhoppers for any of the other combinations tested or in the choices made by male and female leafhoppers.

The preference for AY-infected grapevine branches cv. Colombard displayed by *M. fuscovaria* observed by Krüger et al. (2015) included both visual and olfactory cues. When offered olfactory cues only, leafhoppers avoided AY-infected cv. Colombard branches in autumn when given a choice between odours from AY-infected branches and purified air. However, no difference in choices was observed when leafhoppers were presented with odours from AY-infected and uninfected branches. In autumn, AY-infected branches produced significantly less (*Z*)-3-hexenyl acetate, indole and (*E*)-caryophyllene than uninfected branches (Chapter 2). Conversely, leafhoppers showed attraction toward AY-infected grapevine cv. Chenin blanc branches in autumn compared to purified air. AY-infected branches produced significantly less (*Z*)-3-hexenyl acetate and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) than uninfected branches (Chapter 2). DMNT has been identified as a strong repellent for some herbivorous insects (Hatano et al., 2015) and therefore the reduction of this compound in AY-infected branches could contribute toward the attractiveness of AY-infected branches. However these compounds may not have influenced leafhopper choices as there was no difference in the choices made between AY-infected and uninfected grapevine branches.

Leafhoppers and other insects of the suborder Auchenorrhyncha (treehoppers, planthoppers, spittlebugs and cicadas) are in general less dependent on and responsive to volatile chemicals for intraspecific communication and host-finding compared to their relatives in the suborder

Sternorrhyncha (aphids, whiteflies, mealybugs and psyllids) (Todd et al., 1990b). Even though leafhoppers have been shown to respond to host volatile compounds, this response seems to be largely supplementary to other cues such as visual, gustatory and acoustic cues (Claridge, 1985; Todd et al., 1990b) and not the primary mode of host or mate location. Bullas-Appleton et al. (2004) found that the leafhopper *Empoasca fabae* (Harris) (Hemiptera: Cicadellidae) did not show any preference for different edible bean cultivars when only olfactory stimuli were presented in olfactometer tests. However, leafhoppers preferred one bean cultivar over two other cultivars when presented with a choice of leaflets of uniform shape and size from an aerial position in a Plexiglas chamber, suggesting that leaf colour played an important role in host plant selection. Similarly, Saxena and Saxena (1974) found that the leafhopper *Empoasca devastans* Distant (Hemiptera: Cicadellidae) showed attraction to cotton or castor leaves when volatile cues were excluded. Studies that have investigated the importance of visual cues in host plant selection by leafhoppers and other closely related species, reported that colour is an important cue with insects being mostly attracted to yellow and green (Meyerdirk & Oldfield, 1985; Todd et al., 1990a; Mu et al., 2012). *M. fuscovaria* is also attracted to yellow sticky colour cards in the field (Krüger et al., 2015) and since AY-infection causes grapevine leaves to turn yellow resulting in leaves with a peak reflectance higher than that of uninfected leaves (Krüger et al., 2015), this change in visual cues possibly contribute towards the attractiveness of AY-infected grapevine for this leafhopper species. In addition, there was no difference in the choices made between male and female leafhoppers for any of the combinations tested. Insects often show some level of sexual dimorphism in terms of olfactory responses due to their different requirements, e.g. males need to locate female mating partners whereas females need to locate suitable oviposition sites. Differences in choices between sexes are important to take into account as males and females may differ in their ability to acquire and transmit AY (Beanland et al., 1999) and thus influence disease epidemiology. The lack of sexual dimorphism in the choices made by leafhoppers could advocate that *M. fuscovaria* is to a lesser extent dependent on olfactory cues. However, in

some cases, males may also make use of host plant volatile cues to locate female conspecifics (Landolt & Phillips, 1997; Soroker et al., 2004).

Some leafhopper and related species do however respond to host odours. For the leafhopper *Dalbulus maidis,* a combination of green light and maize volatiles were almost twice as attractive when compared to green light and hexane (Todd et al., 1990b). Also, the American grapevine leafhopper, *Scaphoides titanus* Ball (Hemiptera: Cicadellidae), chose leaves or apical shoots over a control in an olfactometer tests and in electroantennogram tests showed significantly higher responses to a plant extract compared to a blank control (Mazzoni et al., 2009). Riolo et al. (2012) and Sharon et al. (2005) showed that the Palearctic planthopper, *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae), responds to olfactory cues in the absence of any visual cues when selecting a host plant. Other insects that occur on grapevine, such as the grapevine moth, *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae), have been shown to be attracted to other parts of the plant such as the flowers or berries (grapes) (Thiéry & Gabel, 2000; Tasin et al., 2005; Tasin et al., 2006a; Tasin et al., 2006b). In this study, these volatiles were excluded but could potentially also play a role in host plant selection by *M. fuscovaria*.

AY has only recently been recorded in South Africa (Engelbrecht et al., 2010) whereas *M. fuscovaria* is an indigenous leafhopper species. Generally it is believed that the longer an insect vector is associated with a pathogenic mollicute, the less virulent the mollicute infection will be on the insect host (Beanland et al., 2000). Some well adapted insect hosts may even benefit from the association (Purcell, 1988; Purcell & Nault, 1991; Beanland et al., 2000). Feral aster leafhoppers, *Macrosteles quadrilineatus* Forbes, exposed to AY lived longer and may lay more eggs than leafhoppers not exposed to this pathogen (Beanland et al., 2000). Also, the leafhopper *D. maidis* (DeLong & Wolcott) (Hemiptera: Cicadellidae) showed increased survival on AY-infected asters *Callistephus chinensis* L. Nees (Asteraceae), than on uninfected asters (Purcell, 1988). In these cases, insects were attracted to their pathogen-infected hosts due to a change in volatile cues produced by

infected plants. For insects to be attracted to infected plants, possibly due to such benefits, the pathogen and vector should have had time to co-evolve for these benefits to arise and for the insect vector to realise these benefits and respond accordingly. The lack of attraction of *M. fuscovaria* to AY-infected grapevine volatiles may be a consequence of the short evolutionary time these species have had to co-evolve. However, the lack of attraction could also be attributed to many other factors such as the insect's dependence on visual cues rather than, or in conjunction with, olfactory cues.

In conclusion, the attraction of *M. fuscovaria* to AY-infected grapevine branches may be a result of visual rather than olfactory cues, or a combination of both. Other volatile compounds produced by other plant parts which were not included in this study could also influence leafhopper behaviour, but remains to be tested. Currently, AY-phytoplasma and the leafhopper vector *M. fuscovaria* have had very little time to co-evolve and knowledge on the biology and ecology of this leafhopper species is still lacking. Due to several plant samples that were thought to be infected with AY but tested negative for infection in the laboratory, replicates were fewer than anticipated. The low number of replicates may disguise possible attractions/repulsions. Nonetheless, this study provides insight into the use of olfactory cues by leafhopper species in the process of host plant location to ultimately determine the feasibility of using such cues in pest management strategies.

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Chapter 4

Antennal responses of *Mgenia fuscovaria* (Hemiptera: Cicadellidae) to volatiles from aster yellows-infected and uninfected grapevine (*Vitis vinifera* L.)

Abstract

Mgenia fuscovaria (Stål) (Hemiptera: Cicadellidae) is a vector of aster yellows phytoplasma (AY) ('*Candidatus* Phytoplasma asteris') in grapevine *Vitis vinifera* L. (Vitaceae) in South Africa. In a previous study, this leafhopper species was shown to be preferentially attracted to AY-infected grapevine branches compared to uninfected branches. The mode of attraction, whether olfactory or visual, was not determined. Volatile analysis from AY-infected and uninfected grapevine branches revealed several qualitative and quantitative differences in the volatiles produced by AY-infected and uninfected grapevine branches. These induced volatile changes may influence leafhopper behaviour. Combined gas chromatography and electroantennography was used to investigate the responses of *M. fuscovaria* antennae toward volatiles produced by AY-infected and uninfected grapevine branches of the cultivars Colombard and Chenin blanc in summer. Four compounds with physiological activity were identified from AY-infected grapevine cv. Colombard, and five compounds from uninfected grapevine cv. Chenin blanc. (*E,E*)-α-farnesene, emitted at elevated amounts by AY-infected grapevine cv. Colombard, and aromadendrene, emitted by AY-infected branches only, induced responses from leafhopper antennae. If found to be behaviourally active, these compounds could influence disease epidemiology. Responses were also recorded for the co-eluting volatiles, 1-octen-3-ol and phenol from AY-infected grapevine cv. Colombard. For grapevine cv. Chenin blanc, insect antennae responded to the co-eluting green leaf volatiles, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-

2-hexen-1-ol and 1-hexanol, as well as nonane from uninfected branches. In general, electrophysiological responses were weak suggesting that *M. fuscovaria* uses olfactory cues in combination with other, such as visual cues, for host plant location.

Keywords: *Vitis vinifera*, grapevine, *Mgenia fuscovaria*, leafhopper, electroantennogram, olfaction.

4.1 Introduction

Phytopathogens can alter the volatile composition of host plants in such a way that insect vectors are more attracted to infected compared to uninfected plants (Eigenbrode et al., 2002; Srinivasan et al., 2006; Mayer et al., 2008a; b; Mauck et al., 2010; Mann et al., 2012). This phenomenon where insect vectors are preferentially attracted to pathogen-infected plants may potentially lead to an increase in disease incidence (Mayer et al., 2008b). However, this attraction may also hold potential for the development of a behavioural manipulation strategy, such as the push-pull strategy, where volatile cues that induce behavioural responses in insect vectors can be used to repel vectors away from important crop plants, attracting them to a trap or trap crop (Pyke et al., 1987; Miller & Cowles, 1990; Cook et al., 2007). The leafhopper *Mgenia fuscovaria* Stål (Hemiptera: Cicadellidae), a vector of aster yellows phytoplasma (AY) ('*Candidatus* Phytoplasma asteris') on grapevine (*Vitis vinifera* L.; Vitaceae) in South Africa (Krüger et al., 2011), was observed to be more attracted to AY-infected than uninfected grapevine branches (Krüger et al., 2015). The mode of attraction, whether visual or olfactory, was not determined. Analysis of the volatile compounds produced by AY-infected and uninfected grapevine branches revealed several qualitative and quantitative differences (Chapter 2), suggesting that olfactory cues may play a role in this selection process. In olfactometer bioassays, *M. fuscovaria* were both attracted and repelled by volatiles produced by AY-infected grapevine branches, however results were inconsistent (Chapter 3). If olfactory cues play a noteworthy role in the host plant selection process of *M. fuscovaria* and specific compounds can be identified from the headspace of AY-infected plants that influence leafhopper behaviour, these compounds could be used as part of a behavioural manipulation strategy to manage leafhopper populations in infected vineyards.

Few studies to date (Saxena et al., 1974; Saxena & Saxena, 1974; Khan & Saxena, 1985; Todd et al., 1990; Bullas-Appleton et al., 2004; Patt & Sétamou, 2007) have investigated the role of olfactory cues in host plant selection by leafhoppers and related species. In general, leafhoppers and

other insects of the suborder Auchenorrhyncha are considered to be less dependent on olfactory cues compared to their relatives in the suborder Sternorrhyncha (Todd et al., 1990). However, some leaf- and planthopper species respond to olfactory cues in behavioural and electrophysiological studies in the absence of other cues (Sharon et al., 2005; Mazzoni et al., 2009; Riolo et al., 2012). The leafhopper *Scaphoides titanus* Ball (Hemiptera: Cicadellidae) (Mazzoni et al., 2009) and the planthopper *Hyalesthes obsoletus* Signoret (Hemiptera: Cixxidae) (Sharon et al., 2005; Riolo et al., 2012) were both attracted by host plant volatiles in behavioural bioassays when presented with olfactory cues only. Both species also responded to host plant volatiles in electrophysiological tests. If leafhoppers are found to be dependent on olfactory cues to locate host plants to a significant extent, these volatile compounds could be used to manipulate pest species. Plant volatiles that influence insect behaviour have successfully been incorporated into pest management strategies as effective and sustainable alternatives to toxic pesticides (Hassanali et al., 2008).

The present study aimed to identify specific volatile compounds that may act as olfactory cues in the process of host plant selection by *M. fuscovaria* through identifying volatile compounds in the headspace of AY-infected and uninfected grapevine branches of the cultivars Colombard and Chenin blanc that induce an electrophysiological response in leafhopper antennae. Compounds eliciting a response which are produced by AY-infected branches but not by their uninfected counterparts may influence disease epidemiology and spread.

4.2 Materials and methods

Insects

M. fuscovaria adults were collected in an uninfected vineyard in Vredendal (31°43’3.66”S; 18°32’50.74”E), Western Cape, South Africa, with sweep nets and manual aspirator tubes. Insects were kept in mesh cages on grapevine and/or periwinkle plants (*Catharanthus roseus* L.;

Apocynaceae) for a maximum of 6 weeks, until used in electroantennogram (EAG) recordings. Insects were collected during late summer and early autumn from March to May.

Volatile entrainment samples

Volatiles were collected from the headspace of AY-infected and uninfected grapevine plants cv. Colombard and cv. Chenin blanc in summer. The adsorbent (Porapak Q, Supelco, Bellefonte PA, USA; 60/80, 50 mg) with collected volatile compounds were sealed in glass tubes under N_2 gas (Nitrogen Baseline 5.0, Afrox, 99.999%) and sent to the Swedish University of Agricultural Sciences for combined gas chromatography and mass spectrometry (GC-MS) analysis (Chapter 2). Plant volatile samples of the same cultivar and treatment (AY-infected vs. uninfected) were pooled to be used for combined gas chromatography and electroantennographic detection (GC-EAD) analysis.

Combined gas chromatography and electroantennographic detection (GC-EAD)

Insects used for GC-EAD were immobilized by cooling for a short period of time (± 3 min at -12 °C until insects were no longer active) before excising the head and thorax from the rest of the insect's body using micro-scissors. The thorax was removed together with the head to avoid rapid desiccation of the preparation, which was evident when only the head was excised. The front legs attached to the thorax were removed to avoid the movement of the legs interfering with recordings. Glass capillaries (Hirschmann®, 80 mm, 0.95 mm inner diameter, 1.35 mm outer diameter) containing Ag/AgCl electrodes immersed in Kaissling Ringer solution (0.175 g KCl, 3.75 g NaCl, 0.139 g $CaCl_2$ x $2H_2O$, 0.1 g $NaHCO_3$), to which polyvinylpyrrolidone was added in order to avoid rapid desiccation of the preparation, were used for the recordings. The reference electrode was inserted into the back of the insect's head with the tip situated close to the base of the antenna. After the head with attached thorax was mounted, the recording electrode was brought into contact with the distal cut end of the same antenna. The whole preparation was moved so that the antenna came into contact with the air delivered from a glass stimulus delivery tube. Charcoal-filtered, humidified

air flowed over the preparation at a constant airflow rate of 14 ml/min at room temperature. Eluted compounds, with helium as carrier gas, entered the airstream 90 mm upstream from the preparation. The GC-EAD system consisted of a GC (Agilent Technologies 6890N, Chemetrix, Midrand, South Africa) equipped with a cold-on-column injector and a HP5 column (Agilent Technologies, 30 m, 0.32 mm inner diameter, 0.25 film thickness). The GC column temperature was programmed as: injection at 40 °C for 1 min, whereafter the temperature increased by 20 °C min^{-1} to 300 °C, the temperature was held at 300 °C for 3 min. The transfer line (Syntech, The Netherlands) connecting the GC and EAD (Syntech, Hilversum, The Netherlands) was maintained at a constant temperature of 300 °C which was the maximum oven temperature used during runs. 2 µl of sample was injected for each recording. A one-to-one split ratio of the GC effluent was obtained by using a Y-shaped glass splitter to allow simultaneous detection and synchronization of the GC flame ionization detector (FID) and the EAG signals. During recordings, the preparation was covered with a Faraday cage to prevent excessive electrostatic and electromagnetic interference.

Insect sex determination and molecular analysis

The sex of the leafhoppers used in GC-EAD experiments was determined after completion of a recording, using images provided by M. Stiller (National Collection of Insects, ARC-Plant Protection Research Institute, South Africa). Thereafter, leafhoppers were tested for AY-infection with real-time polymerase chain reaction (PCR) analysis (Chapter 2). Nucleic acid extractions were done with a non-destructive extraction protocol using TNES buffer (Sambrook & Russell, 2001) (Chapter 3). A LightCycler® TaqMan® Master kit was used to prepare samples for real-time PCRs on a LightCycler® instrument (model 1.5, Roche Applied Science, Switzerland), using primers and a probe designed by Angelini et al. (2007) for AY detection.

Identifying electrophysiologically active compounds

Compounds were regarded as being electrophysiologically active if the responses observed from *M. fuscovaria* deviated sufficiently from the typically observed baseline drift, and coincided with the elution of a compound. To identify the compounds eliciting responses, chromatograms were both visually compared, and retention indices determined, to compare already identified peaks (Chapter 2).

The Kovats retention index (*KI*) (Kovats, 1958) for an unknown peak (*x*) was calculated by making use of the retention time (*tr*) of the peak in question, as well as the retention times of the n-alkanes/hydrocarbons that eluted on either sides of this peak – standard n-alkanes were run daily on the same GC, under the same conditions as the samples to compensate for day-to-day variation in the system (Figure 4.1). While retention times vary with the individual chromatograph and are dependent on several parameters (e.g. column length, film thickness, carrier gas properties), the derived retention indices are less dependent on these parameters and allow for comparable values that may be calculated under varying conditions.

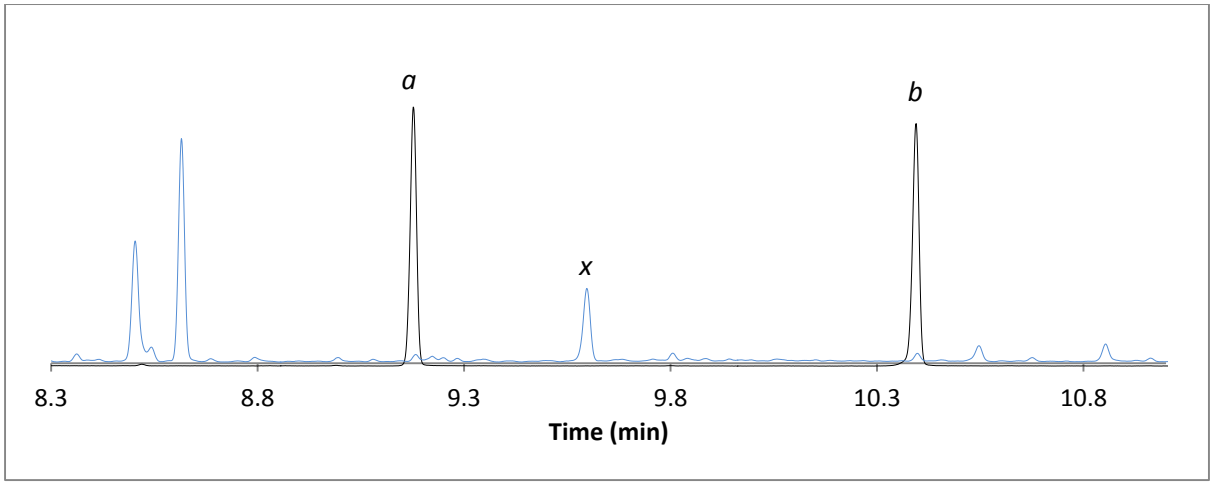

Figure 4.1: Chromatogram of an unknown peak *x* from a sample (blue chromatogram), between two consecutive hydrocarbon peaks (black chromatogram), *a* and *b*.

$$KI_x = (KI_b - KI_a)\left(\frac{tr_x - tr_a}{tr_b - tr_a}\right) + KI_a$$

The retention indices were calculated based on the retention times of the peak apex. It is also possible to use the peak start or end times, especially in the cases where peaks deviate from the normal Gaussian distribution.

4.3 Results

GC-EAD analysis commenced in the late summer months and continued into late autumn (March to June). Recordings became increasingly difficult as winter approached due to the dry weather conditions and consequent static electricity interference. The use of a humidifier during these times decreased the static interference somewhat. However, the noise levels in many of these recordings were severe enough that several had to be omitted. In addition, the transfer line was a significant source of electromagnetic interference, which could not always successfully be eliminated with the Faraday cage and these recordings also had to be omitted from the analysis (Figure 4.2). A total of 44 GC-EAD recordings were done, which included recordings with AY-infected and uninfected grapevine volatiles of both cultivars Colombard and Chenin blanc that were collected in summer, however results are only presented for AY-infected Colombard and uninfected Chenin blanc.

Responses were in the range of 0.1 – 0.6 mV. All of the presented recordings were done with female insects except for one male insect used in a recording with volatiles from uninfected grapevine, cv. Chenin blanc. All individuals tested negative for AY-infection with real-time PCR.

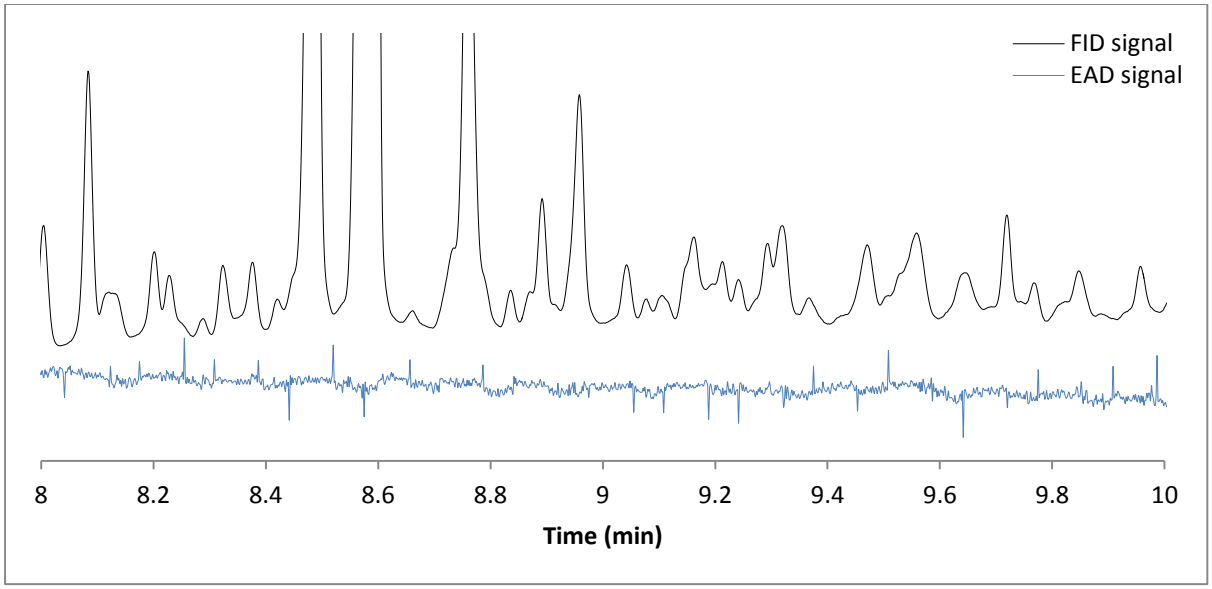

Figure 4.2: Part of a chromatogram (top) illustrating the effect of electromagnetic interference on the electroantennography detection signal (bottom), originating from the transfer line (sample illustrated – aster yellows infected grapevine cv. Colombard, summer).

In the case of AY-infected grapevine cv. Colombard (n = 2), three responses were identified. Compounds were identified as either 1-octen-3-ol or phenol (4.47 min, KI = 982), aromadendrene (8.34 min, KI = 1480) and (*E,E*)-α-farnesene (8.62 min, KI = 1520). 1-Octen-3-ol and phenol co-eluted and therefore the response could be attributed to either of these compounds (Figure 4.3). Three responses were identified for uninfected grapevine cv. Chenin blanc (n = 4). In two cases, more than one compound eluted simultaneously and therefore responses could be attributed to any of these co-eluting compounds. The first response was for (*E*)-2-hexenal (3.28 min, KI = 855) and/or (*Z*)-3-hexen-1-ol (3.30 min, KI = 858). The second response was for (*E*)-2-hexen-1-ol (3.40 min, KI = 869) and/or 1-hexanol (3.41 min, KI = 871). The third response was for nonane (3.67 min, KI = 900) (Figure 4.4). Phenol and nonane are both regarded as artefacts/contaminants and are likely breakdown products of the adsorbent. Due to the limited number of leafhoppers that responded, responses were not confirmed by EAG recordings using synthetic standards.

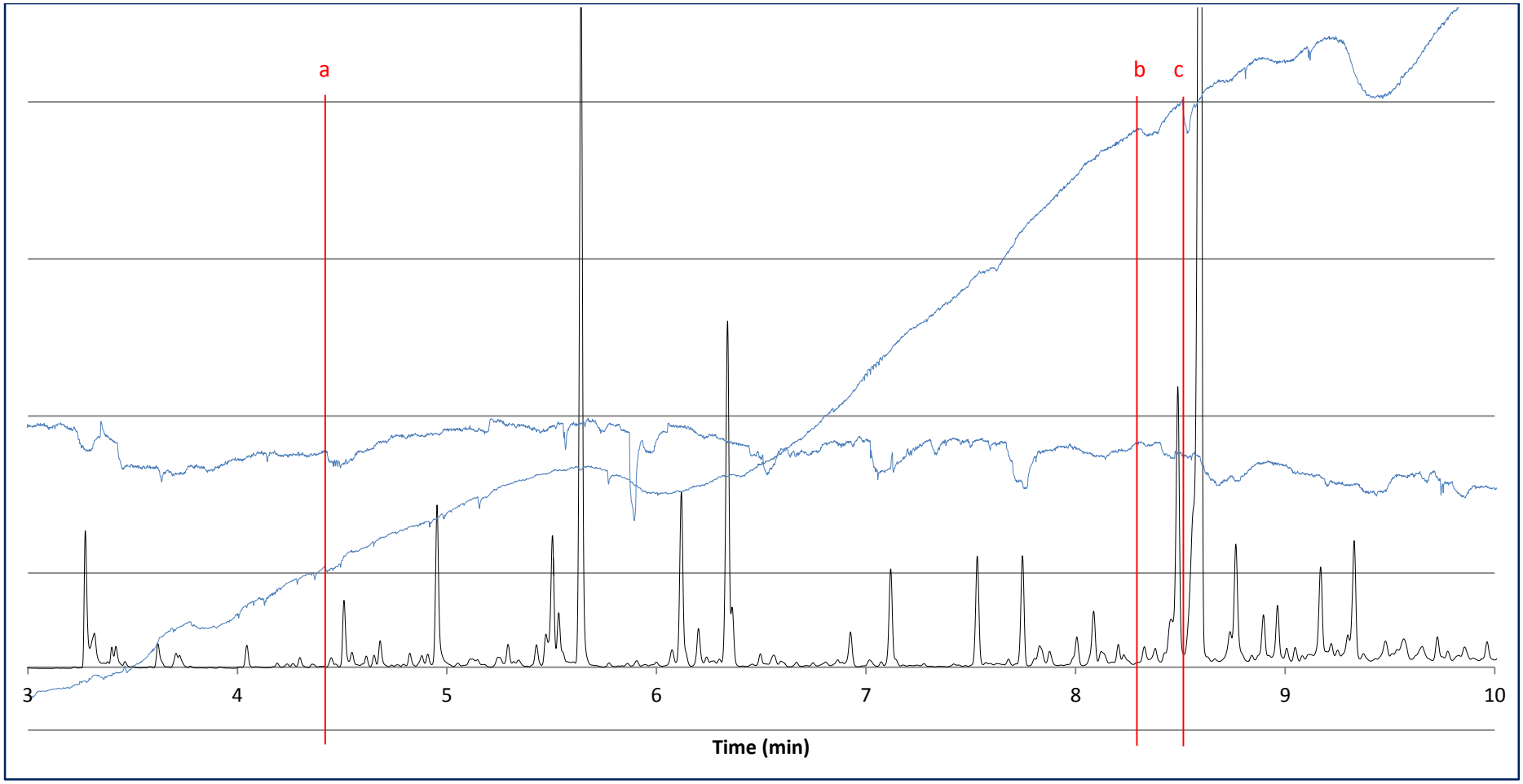

Figure 4.3: Part of a chromatogram (black) from aster yellows-infected grapevine (*Vitis vinifera*) cv. Colombard and combined electroantennogram responses (blue) from *Mgenia fuscovaria* (n = 2). Responses are indicated by the red lines at elution times 4.47 min, 8.34 min and 8.62 min. These compounds were identified as (a) either 1-octen-3-ol or phenol, (b) aromadendrene and (c) (*E,E*)-α-farnesene.

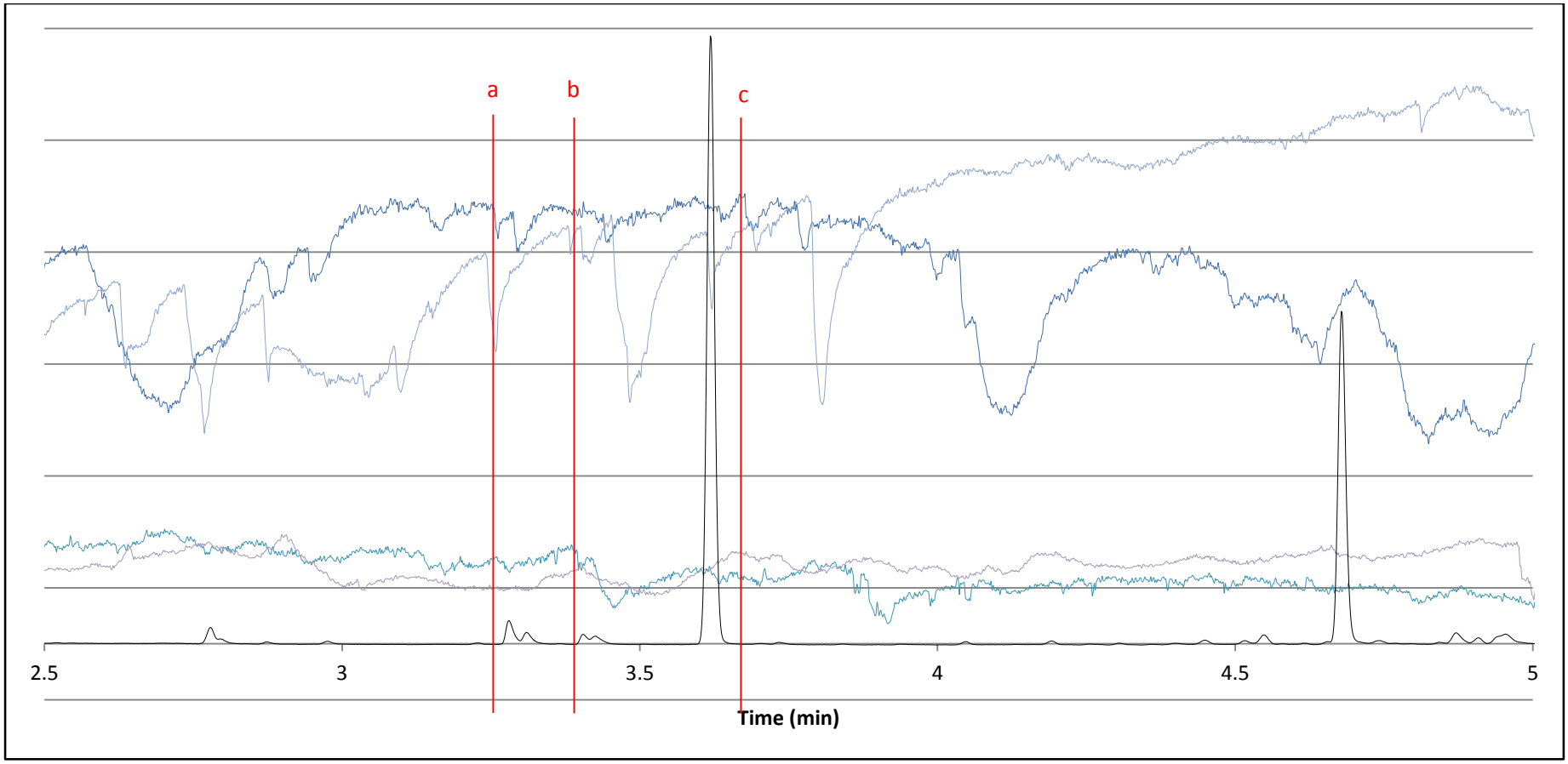

Figure 4.4: Part of a chromatogram (black) from uninfected grapevine (*Vitis vinifera*) cv. Chenin blanc and combined electroantennogram responses (blue) from *Mgenia fuscovaria* (n = 4). Responses are indicated by the red lines at elution times 3.28/3.30 min; 3.40/3.41 min and 3.67 min. Responses a and b occurred where more than one compound co-eluted. Because responses were not confirmed by compound standards, these responses can be attributed to either of the co-eluting compounds (a) (*E*)-2-hexenal / (*Z*)-3-hexen-1-ol; (b) (*E*)-2-hexen-1-ol/1-hexanol; (c) nonane.

4.4 Discussion

In GC-EAG tests with *M. fuscovaria*, four compounds with physiological activity were identified from AY-infected grapevine cv. Colombard, and five compounds from uninfected grapevine cv. Chenin blanc. (*E,E*)-α-farnesene, aromadendrene, 1-octen-3-ol and phenol from AY-infected grapevine cv. Colombard, and (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, 1-hexanol and nonane from uninfected grapevine cv. Chenin blanc, induced antennal responses from this leafhopper. In general however, responses were weak.

(*E,E*)-α-farnesene, which leafhoppers responded to in the headspace of AY-infected grapevine cv. Colombard, although present in uninfected branches, was recorded in significantly higher amounts in AY-infected than uninfected grapevine branches (Chapter 2). *M. fuscovaria* did not respond to (*E,E*)-α-farnesene from cv. Chenin blanc although uninfected samples contained similar amounts of this compound as AY-infected grapevine cv. Colombard. Aromadendrene, which also elicited a response from *M. fuscovaria*, was only present in AY-infected grapevine cv. Colombard headspace and not in the headspace of uninfected branches (Chapter 2). The increased production of (*E,E*)-α-farnesene and the production of aromadendrene were both induced by AY-infection and therefore, if found to be behaviourally active, may influence disease epidemiology and spread, but may therefore also hold potential for the use in a behavioural manipulation strategy. This will require future work as an electrophysiological response does not indicate the behavioural valence of a given compound. These compounds are, however, good candidates for further investigation in behavioural studies (Ramachandran & Norris, 1991; Weissbecker et al., 2000). For other insect species, (*E,E*)-α-farnesene have been demonstrated to act as an attractant and oviposition stimulator for some lepidopterans (Sutherland & Hutchins, 1972; Wearing & Hutchins, 1973; Hern & Dorn, 1999; Landolt et al., 2000; Bengtsson et al., 2001; Tasin et al., 2006). It has also been reported as an alarm pheromone component for the termite *Prorhinotermes canalifrons* (Isoptera: Rhinotermitidae) (Šobotník et al., 2008) and the aphid *Myzus persicae* Sulz. (Hemiptera: Aphididae)

(Pickett & Griffiths, 1980). Aromadendrene is a potential insect attractant (Oates et al., 2015) and were shown to attract Mediterranean fruit fly females, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) in field trials (Casaña-Giner et al., 2001) and induce electrophysiological responses in the Eucalyptus brown looper *Thyrinteina arnobia* Stoll (Lepidoptera: Geometridae) (Batista-Pereira et al., 2006).

Leafhoppers also responded to the co-eluting volatile compounds, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol and 1-hexanol from uninfected grapevine cv. Chenin blanc. These compounds are regarded as common green leaf volatiles, produced by most green plants and attractive to several insect species (e.g. Visser & Avé, 1978; Campbell et al., 1993; Ruther et al., 2002; Mu et al., 2012). In the case of the planthopper *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), Youn (2002) reported that hexanal and (*E*)-2-hexenal gave the largest responses in EAG recordings, with planthoppers also responding to several other green leaf alcohols.

Electrophysiological responses of leafhoppers towards plant volatiles have been reported for the American grapevine leafhopper *Scaphoides titanus* Ball (Hemiptera: Cicadellidae) towards headspace volatiles of grapevine rootstock (*Vitis riparia* X *rupestris* 101/14; Vitaceae) (Mazzoni et al., 2009). This leafhopper, which showed attraction towards grapevine volatiles in olfactometer tests, displayed significantly higher EAG responses towards plant extracts compared to a blank control. However, the overall EAG response was still rather low. Scanning electron microscopy (SEM) observations revealed few antennal sensilla potentially associated with olfaction in both nymphs and adults of both sexes (Mazzoni et al., 2009). Kristoffersen et al. (2006), who observed a similar sparse sensillar setup in the carrot psyllid *Trioza apicalis* Förster (Hemiptera: Triozidae), hypothesized that this could be an adaptation of a specialized species to a high stimulus level of host odours. This could also be the case for *M. fuscovaria* as grapevine is abundant in the landscape where insects were collected. The antennal responses of the planthopper *H. obsoletus* (Sharon et al., 2005), were stronger when compared to the leafhopper responses reported by Mazzoni et al. (2009) for volatiles

from *Vitex agnus-castus* L. (Verbenaceae), but not from grapevine cv. Cabernet Sauvignon, which elicited the same response as the blank control. Because the size of EAG responses are considered to be proportional to the number of antennal sensilla being stimulated (Stürckow, 1965; Mayer et al., 1984; Bernays & Chapman, 1994), the low responses observed from *M. fuscovaria* could be a consequence of few antennal sensilla associated with olfaction, as is the case with *S. titanus* (Mazzoni et al., 2009), however this remains to be investigated.

In conclusion, *M. fuscovaria* does not elicit strong electrophysiological responses toward host plant volatiles, at least in the case of the grapevine volatiles tested. It is possible that this leafhopper, like its relative *S. titanus*, has few antennal sensilla associated with olfaction (Mazzoni et al., 2009) and is not predominantly dependent on olfactory cues for host plant location. However, as olfactory responses were still evident in both behavioural (Chapter 3) and electrophysiological tests, it is likely that olfactory cues are important. Compounds that elicited electrophysiological responses from *M. fuscovaria* include several green leaf volatiles, (*E,E*)-α-farnesene and aromadendrene. The increased production of (*E,E*)-α-farnesene and production of aromadendrene was both induced by AY-infection. Compounds that elicit a response in electrophysiological tests are good candidates to investigate as attractants or repellents for the use in a semiochemical-based management strategy.

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Chapter 5

General discussion, conclusions and recommendations

This study examined the role of olfactory stimuli during host plant selection by the leafhopper *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae), a vector of aster yellows phytoplasma (AY) ('*Candidatus* Phytoplasma asteris') on grapevine (*Vitis vinifera* L.; Vitaceae) in South Africa (Krüger et al., 2011). In a previous study, *M. fuscovaria* was more attracted to AY-infected grapevine branches when compared to their uninfected counterparts (Krüger et al., 2015). The mode of attraction, however, was not determined. Attraction of insect vectors to pathogen-infected plants has been demonstrated previously (e.g. Eigenbrode et al., 2002; McLeod et al., 2005; Srinivasan et al., 2006; Mayer et al., 2008b; a; Mauck et al., 2010; Mann et al., 2012). For those studies, the mode of attraction was attributed to a change in olfactory cues induced by pathogen infection. This phenomenon, where insect vectors are more attracted to pathogen-infected plants may lead to an increase in disease incidence (Mayer et al., 2008b). However, the change in olfactory cues and vector behaviour may also hold potential for the development of a behavioural manipulation strategy where volatile chemicals are used to repel insects away from important crop plants towards traps or trap crops to control insect pests (Pyke et al., 1987). The aim of the current study was to determine the role of olfactory cues in the attraction of *M. fuscovaria* to AY-infected grapevine in the absence of visual cues. The objectives were (1) to identify the volatile profiles of AY-infected and uninfected grapevine branches in order to determine if there are any differences in the volatile profiles that might influence the behaviour of this leafhopper species, (2) to evaluate the behavioural response of *M. fuscovaria* to AY-infected and uninfected branches in olfactometer tests, and (3) to determine if any of the compounds produced by AY-infected and uninfected grapevine branches induce an electrophysiological response from *M. fuscovaria* antenna, and therefore potentially act as attractants or repellents for this species.

Grapevine plants infected with AY and uninfected grapevine differed significantly in the headspace volatiles produced, both qualitatively and quantitatively, except for cv. Colombard in autumn, in which compounds differed only quantitatively (Chapter 2). There was an increase in the amount and number of compounds recorded from AY-infected grapevine branches compared to uninfected branches in summer. The total volatile concentration was also significantly higher for AY-infected grapevine cv. Colombard branches compared to uninfected branches. In behavioural bioassays however, adult leafhoppers were not consistently attracted or repelled by AY-infected grapevine branches in summer or autumn (Chapter 3). Even though differences in the volatile composition of AY-infected and uninfected branches were more pronounced in summer, differences in choices were only observed in autumn when leafhoppers were presented with AY-infected branches and purified air. Leafhoppers preferred AY-infected grapevine cv. Colombard branches over purified air and purified air over AY-infected grapevine cv. Chenin blanc branches. Of the compounds identified as induced by AY-infection, methyl salicylate, a known plant defence compound associated with systemic acquired resistance, has been shown to attract predatory arthropods (Dicke et al., 1990; Drukker et al., 2000; Ozawa et al., 2000) and repel herbivorous ones (Hardie et al., 1994; Pettersson et al., 1994; Lösel et al., 1996; Ninkovic et al., 2003). This compound is also induced in tobacco plants inoculated with tobacco mosaic virus (Shulaev et al., 1997). However, methyl salicylate did not elicit any responses from *M. fuscovaria* antennae in electrophysiological tests – this could be a consequence of the low number of replicates. (*E,E*)-α-farnesene, the most abundant compound recorded from both AY-infected and uninfected grapevine, was produced in significantly greater quantities in AY-infected grapevine in cv. Colombard in summer. This compound has been shown to be emitted in response to pathogen infection (Vuorinen et al., 2007; Toome et al., 2010). In electrophysiological studies, *M. fuscovaria* responded to these elevated concentrations of (*E,E*)-α-farnesene in AY-infected plants, but no responses were recorded from uninfected plants (Chapter 4). Aromadendrene, which was found specifically in AY-infected grapevine, also induced an electrophysiological response from *M. fuscovaria*. In general,

electrophysiological responses from *M. fuscovaria* were, however, low. Mazzoni et al. (2009) also observed low electroantennogram responses toward host plant volatile compounds for the grapevine leafhopper *Scaphoides titanus* Ball (Hemiptera: Cicadellidae), and scanning electron microscopy revealed few antennal sensilla potentially associated with olfaction. While some leafhoppers and related species use olfactory cues for host plant location (Todd et al., 1990b; Sharon et al., 2005; Mazzoni et al., 2009; Riolo et al., 2012), others do not respond to olfactory cues alone (Saxena & Saxena, 1974; Bullas-Appleton et al., 2004) but may be more dependent on visual cues. In general, insects of the suborder Auchenorrhyncha, which includes leafhoppers, appear to be less dependent on olfactory cues than their relatives in the suborder Sternorrhyncha (Todd et al., 1990b). The results from behavioural tests as well as the low electrophysiological responses suggest that the preference for AY-infected grapevine observed by Krüger et al. (2015) could be a result of visual cues rather than olfactory cues, or a combination of both.

Measures to manage *M. fuscovaria* on grapevine in South Africa, other than chemical control, have not been documented before. To develop semiochemical-based control strategies, or any other integrated pest management strategy, a thorough knowledge on the insect's biology and ecology is essential. Due to the relatively recent discovery of *M. fuscovaria* as a vector of AY in grapevine, very little is known about this leafhopper species. The results of this study contribute towards the general knowledge on *M. fuscovaria* to potentially facilitate in identifying suitable control measures in the future. More work needs to be done to confirm the identified electrophysiological responses and possibly identify additional responses, in order to determine whether a control strategy which makes use of olfactory cues could be developed in the future. Identified compounds should also be tested in behavioural experiments as compounds that are perceived by the insect antenna and elicit an electrophysiological response, does not necessarily influence insect behaviour (Ramachandran & Norris, 1991). However, the results of this study suggest that *M. fuscovaria* does not display great sensitivity towards olfactory cues, at least in the case of grapevine cv. Colombard and cv. Chenin blanc and in the absence of visual cues which have been demonstrated to be important for other

leafhopper species (e.g. Todd et al., 1990a; Mu et al., 2012) and *M. fuscovaria* (Krüger et al., 2015). Research to develop a management strategy to control this leafhopper species by means of behavioural manipulation should focus on the use of visual cues in combination with olfactory cues rather than the sole use of olfactory cues.

5.1 References

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Appendix A

Volatile entrainment graphs and test statistics

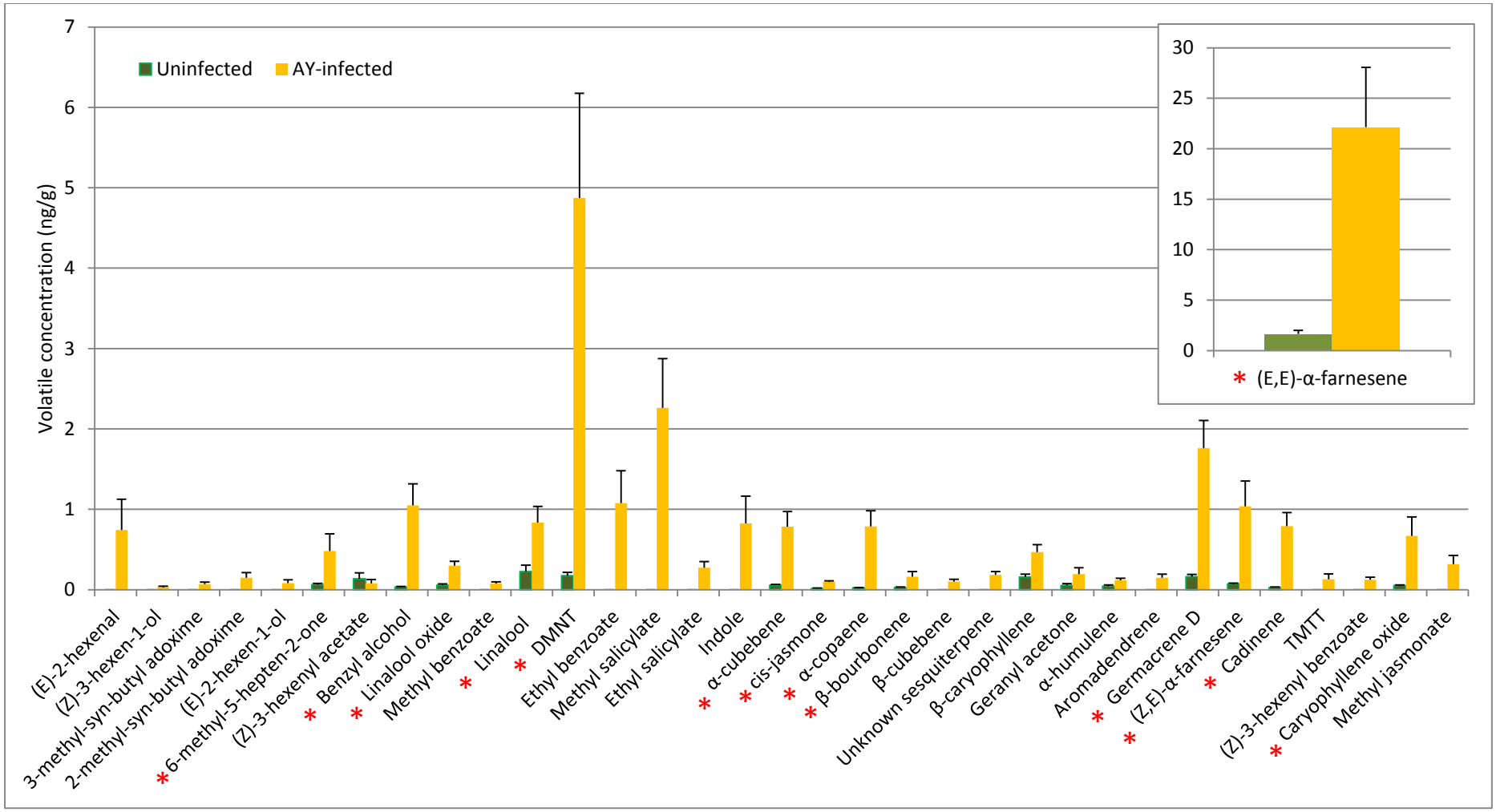

Figure A 1: Differences in volatile composition of aster yellows phytoplasma (AY)-infected and uninfected grapevine (*Vitis vinifera*) branches cv. Colombard in summer (n = 4; average ± SE). Significant differences between AY-infected and uninfected branches are indicated with an asterisk (Mann-Whitney U test, $P < 0.05$).

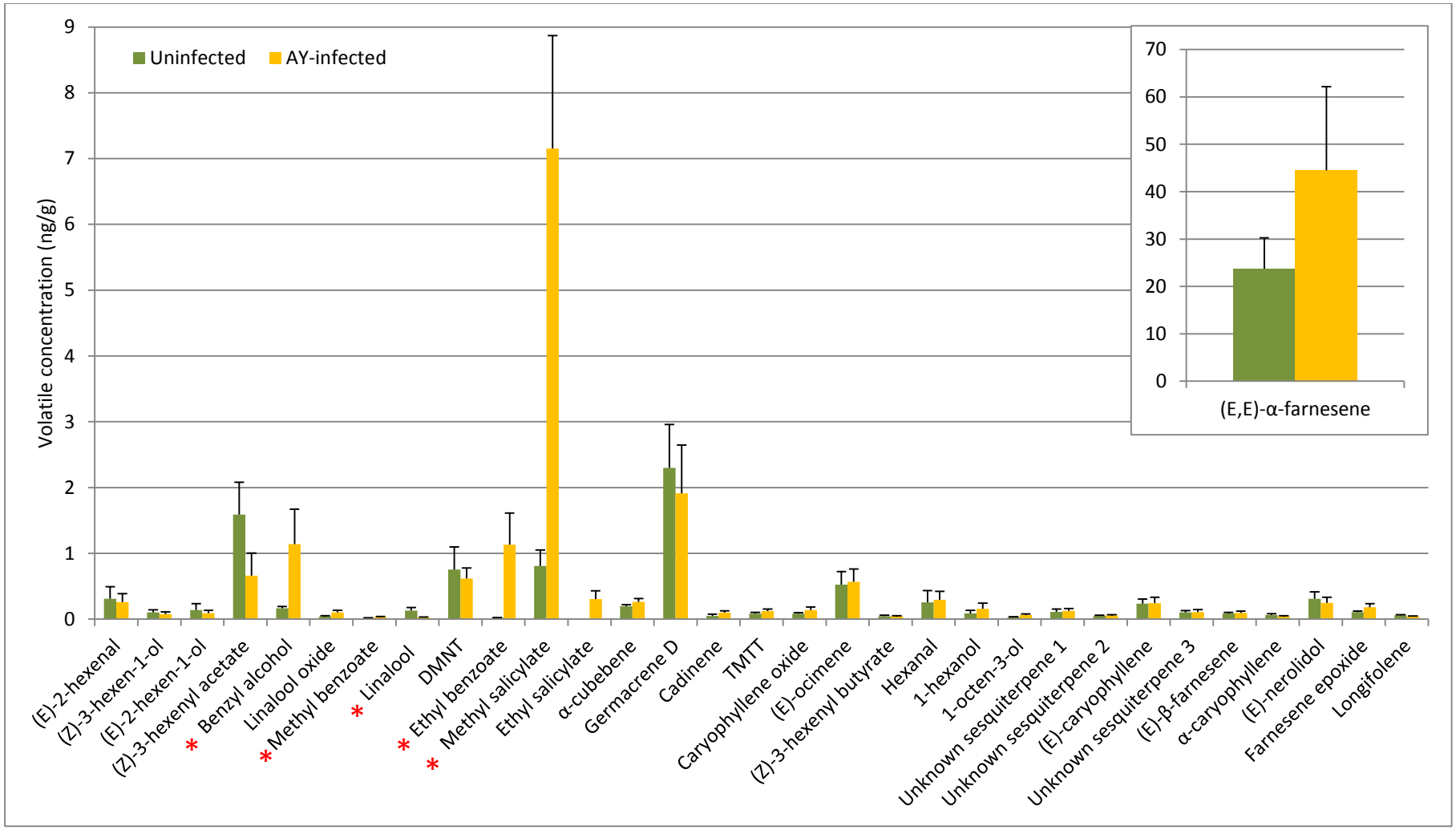

Figure A 2: Differences in volatile composition of aster yellows phytoplasma (AY)-infected and uninfected grapevine (*Vitis vinifera*) branches cv. Chenin blanc in summer (n = 8; average ± SE). Significant differences between AY-infected and uninfected branches are indicated with an asterisk (Mann-Whitney U test, $P < 0.05$).

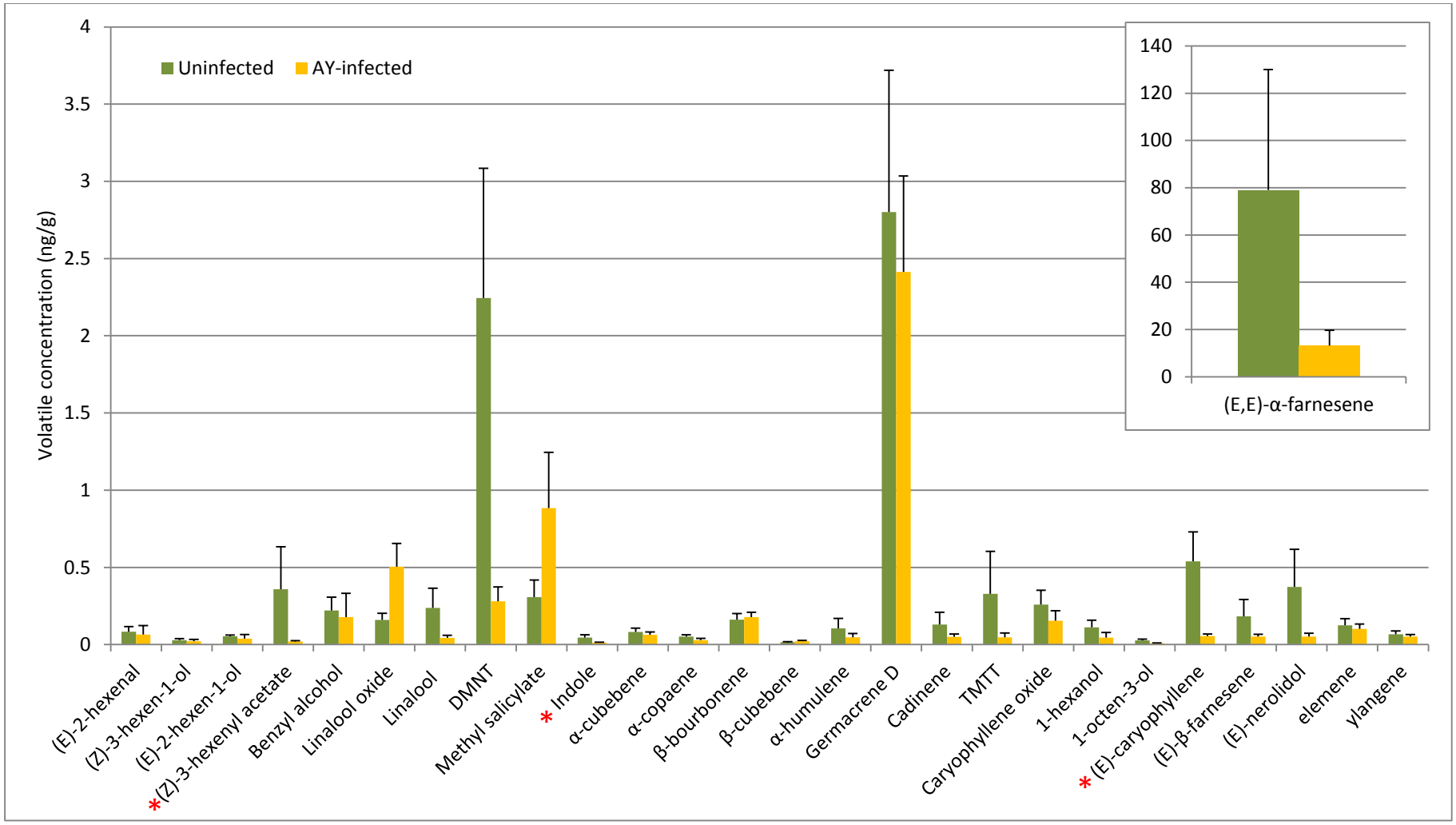

Figure A 3: Differences in volatile composition of aster yellows phytoplasma (AY)-infected and uninfected grapevine (*Vitis vinifera*) branches cv. Colombard in autumn (n = 4; average ± SE). Significant differences between AY-infected and uninfected branches are indicated with an asterisk (Mann-Whitney U test, $P < 0.05$).

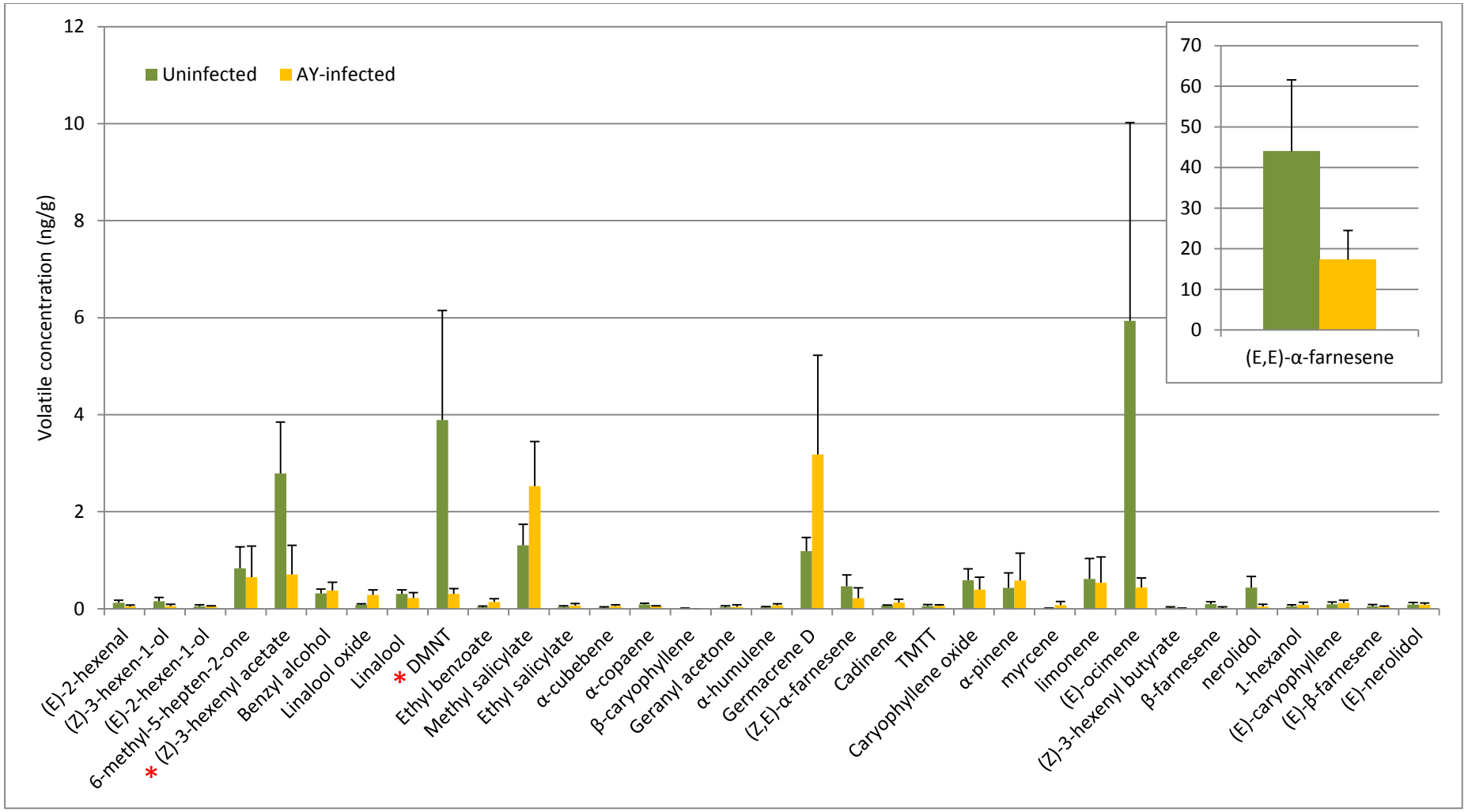

Figure A 4: Differences in volatile composition of aster yellows phytoplasma (AY)-infected and uninfected grapevine (*Vitis vinifera*) branches cv. Chenin blanc in autumn (uninfected n = 10; AY-infected n = 6; average ± SE). Significant differences between AY-infected and uninfected branches are indicated with an asterisk (Mann-Whitney U test, $P < 0.05$).

Table A 1: Test statistics for the differences in volatile organic compounds produced by aster yellows phytoplasma (AY)-infected and uninfected grapevine (*Vitis vinifera*) cultivars Colombard and Chenin blanc in summer and autumn (Mann-Whitney U test; $P < 0.05$).

	Vitis vinifera cv. Colombard				*Vitis vinifera* cv. Chenin blanc			
	Summer AY-infected vs. uninfected		Autumn AY-infected vs. uninfected		Summer AY-infected vs. uninfected		Autumn AY-infected vs. uninfected	
Compound	*Z*-value	*P*-value	*Z*-value	*P*-value	*Z*-value	*P*-value	*Z*-value	*P*-value
Total	-2.309	0.029	-1.732	0.114	-1.155	0.279	-1.085	0.313
Aldehydes								
(*E*)-2-hexenal	-2.460	0.029	-1.155	0.343	-0.735	0.505	-0.271	0.792
Hexanal					-0.840	0.442		
Ketones								
Cis-jasmone	-2.309	0.029						
Geranyl acetone	-1.443	0.200					-0.356	0.792
6-methyl-5-hepten-2-one	-2.309	0.029					-0.598	0.635
Alcohols								
Benzyl alcohol	-2.309	0.029	-1.155	0.343	-2.731	0.005	-0.542	0.635
1-hexanol			-1.732	0.114	-0.630	0.574	-0.811	0.492
1-octen-3-ol			-1.732	0.114	-0.840	0.442		
(*E*)-2-hexen-1-ol	-2.460	0.029	-1.155	0.343	-0.846	0.442	-0.724	0.492
(*Z*)-3-hexen-1-ol	-2.460	0.029	-1.443	0.200	-0.841	0.442	-1.197	0.263
Linalool	-2.309	0.029	-1.732	0.114	-2.310	0.021	-1.193	0.263
Linalool oxide	-2.309	0.029	-1.732	0.114	-1.050	0.328	-1.519	0.147
Esters								
(*Z*)-3-hexenyl acetate	-0.289	0.886	-2.309	0.029	-1.470	0.161	-2.169	0.031
(*Z*)-3-hexenyl butyrate					0.000	1.000	-0.784	0.562
(*Z*)-3-hexenyl	-2.460	0.029						

	Vitis vinifera cv. Colombard				*Vitis vinifera* cv. Chenin blanc			
	Summer AY-infected vs. uninfected		Autumn AY-infected vs. uninfected		Summer AY-infected vs. uninfected		Autumn AY-infected vs. uninfected	
Compound	*Z*-value	*P*-value	*Z*-value	*P*-value	*Z*-value	*P*-value	*Z*-value	*P*-value
benzoate								
Ethyl benzoate	-2.460	0.029	-3.361	0.000			-1.390	0.220
Methyl benzoate	-2.460	0.029			-2.207	0.028		
Methyl jasmonate	-2.460	0.029						
Methyl salicylate	-2.460	0.029	-1.155	0.343	-3.151	0.001	-1.193	0.263
Ethyl salicylate	-2.460	0.029			-3.590	0.000	-1.043	0.368
Terpenoids								
β-bourbonene	-2.309	0.029	-0.289	0.886				
α-caryophyllene					-0.841	0.442		
β-caryophyllene	-2.021	0.057					-1.131	0.562
α-copaene	-2.309	0.029	-1.155	0.343			-0.543	0.635
α-cubebene	-2.309	0.029	-0.866	0.486	-0.840	0.442	-1.159	0.313
β-cubebene	-2.460	0.029	-0.577	0.686				
α-humulene	-2.021	0.057	-0.289	0.886			-1.043	0.368
β-farnesene							-0.956	0.428
α-pinene							-0.695	0.562
(*E*)-caryophyllene			-2.309	0.029	-0.420	0.721	-0.811	0.492
(*E*)-ocimene					-0.210	0.878	-1.627	0.118
(*E,E*)-α-farnesene	-2.309	0.029	-1.732	0.114	-0.735	0.505	-0.976	0.368
(*Z,E*)-α-farnesene	-2.309	0.029					-0.927	0.428
(*E*)-β-farnesene			-1.443	0.200	-0.210	0.878	-0.811	0.492
Aromadendrene	-2.460	0.029						
Cadinene	-2.309	0.029	0.00	1.000	-1.904	0.065	-0.434	0.713
Elemene			-0.577	0.686				
Limonene							-1.043	0.368
Longifolene					-1.260	0.234		
Myrcene							-0.471	0.792
Ylangene			-0.289	0.886				
Unknown	-2.460	0.029						

	Vitis vinifera cv. Colombard				*Vitis vinifera* cv. Chenin blanc			
	Summer AY-infected vs. uninfected		Autumn AY-infected vs. uninfected		Summer AY-infected vs. uninfected		Autumn AY-infected vs. uninfected	
Compound	*Z*-value	*P*-value	*Z*-value	*P*-value	*Z*-value	*P*-value	*Z*-value	*P*-value
sesquiterpene 1								
Unkown sesquiterpene 2					-0.737	0.505		
Unknown sesquiterpene 3					-0.525	0.645		
Unknown sesquiterpene 4					-0.315	0.798		
TMTT	-2.460	0.029	-1.155	0.343	-0.945	0.382	-0.811	0.492
DMNT	-2.309	0.029	-1.732	0.114	-0.315	0.798	-2.495	0.011
Nerolidol							-1.274	0.263
(*E*)-nerolidol			-2.021	0.057	-0.105	0.959	-0.348	0.792
Caryophyllene oxide	-2.309	0.029	-0.866	0.486	-0.630	0.574	-1.193	0.263
Farnesene epoxide					-0.525	0.645		
Germacrene D	-2.309	0.029	-0.289	0.886	-0.315	0.798	-0.434	0.713
Aromatic								
Indole	-2.460	0.029	-2.309	0.029				
Amines								
2-methyl-syn-butyl adoxime	-2.460	0.029						
3-methyl-syn-butyl adoxime	-2.460	0.029						

DMNT: (*E*)-4,8-dimethyl-1,3,7-nonatriene; TMTT: (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene