Do mycorrhizal fungi facilitate root defence signalling in belowground predator-prey interactions?

A thesis submitted to the University of London for the degree of Doctor of Philosophy

By

James E. Hourston

School of Biological Sciences

Royal Holloway University of London

2015

Declaration of Authorship

I James Edward Hourston hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others or relied on others to carry out experimental work, this is always clearly stated.

Signed: _____

Date: _____

Acknowledgements

First I would like to thank my supervisors, Alan Gange, Alison Bennett and Scott Johnson, for their help and support throughout my PhD. I would also like to thank my advisor, Julia Koricheva for her helpful comments at some of the key stages of my PhD. I'd also like to thank Sergio Rasmann and Karita Saravesi for their help with my experimental work.

I would like to thank some of the very helpful people based at The James Hutton Institute such as Niki Jennings, Sandra Caul, Eleanor Barr, Sean Hackett, CJ Baird, Anna McRae, Tom Shepherd, William Deasy, Claudine, Susan Mitchell, and Susan Verrall as well as many more who helped all my experiments run smoothly and provided expert advice on a variety of topics.

At Royal Holloway I'd like to recognise Andrew Matthews, Gemma Baron, Neil Morley, Kai Graeber, Elli Amanatidou and Tina Steinbrecher for their help and support throughout my time there.

I would like to say a very big thank you to my friends and family who have been very supportive of me throughout the 3 and a half years I've been working on my PhD.

And finally, I would like to acknowledge my funding, from both the Natural Environment Research Council and The James Hutton Institute.

Royal Horticultural Society

Sharing the best in Gardening

MARSH CHRISTIAN TRUST

Marsh Horticultural Science Award

In association with

Marsh Christian Trust

and

Royal Horticultural Society

The 2014 award is presented to

Mr James Hourston

Alistair Griffiths
Head of Science
Royal Horticultural Society

Brian Marsh OBE
Chairman
Marsh Christian Trust

Abstract

The presence of arbuscular mycorrhizal fungi (AM fungi) in plant roots can have varied effects on insect herbivores. This thesis focused on the root feeding *Otiorhynchus sulcatus*, a pest of the perennial soft fruit crop *Rubus idaeus*, and subsequent interactions with two of its natural enemies, the entomopathogenic nematodes (EPNs) *Heterorhabditis megidis* and *Stienernema kraussei*. These interactions were thought to be mediated by plant signalling in the form of volatile organic compounds (VOCs) that are known to be modified by the presence of AM fungi.

A series of experiments were conducted to test the efficacy and taxis behaviour of EPNs when AM fungi were present or excluded from the root zone and to see if this was driven by plant VOC emissions. *Stienernema kraussei* was found to be the most effective EPN at controlling *O. sulcatus* under glasshouse conditions and in combination with resistant *R. idaeus* cultivars efficacy was even greater. When *H. megidis* taxis to *R. idaeus* plants was tested, the addition of AM fungi increased attraction of *H. megidis* regardless of *O. sulcatus* feeding pressures but this was not easily attributed to a difference in VOC production. Captured VOCs including the known semiochemicals, *α*-pinene and carene, were elevated under high *O. sulcatus* herbivory pressure indicating that EPNs in this system were indeed responding to herbivore induced VOCs. When EPN attraction was tested with a commercial inoculum however, similar effects were not seen, and EPNs were instead attracted by higher *R. idaeus* root biomass.

As this experimental system was developed to become more ecologically relevant, it was found that the effects seen under more controlled conditions were not reproduced. This indicates that while this system has promise, further study is required to unlock the potential of AM fungi to provide novel pest management options in agriculture.

Contents

List of Figures

List of Tables

1 General Introduction

The control of insect pests in horticulture, silviculture and agriculture is a constant battle for growers all over globe. Most conventional methods entail applications of chemical pesticides. However, the use of chemicals for this purpose is becoming increasingly controversial, with many being withdrawn from use due to safety and environmental concerns. Another issue that affects the chemical control of insects is that of resistance developing in pest populations. With the temporary ban of neonicotinoid pesticides across the EU in 2013, a number of pests traditionally controlled by these chemicals were left without an effective means to control them. New approaches to insect control which minimise or avoid pesticide use are consequently in high demand. In this thesis the application of utilising root defence signalling, in mycorrhizal plants, to enhance a biological control agent was explored.

1.1 The Black Vine Weevil *Otiorhynchus sulcatus*

1.1.1 *Otiorhynchus sulcatus* biology and life cycle

The black vine weevil, *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae) is a very successful polyphagous species with hundreds of known host plants (Moorhouse *et al.*, 1992). Adult *O. sulcatus* are between 9-13mm in length with a dark grey or black colouration with pale patches across the elytra and pronotum (Figure 1.1a). The elytra of *O. sulcatus* are fused, and all members of the species are flightless. All individuals are clonal triploid females and reproduce via mitotic parthenogenesis (Lundmark, 2010).

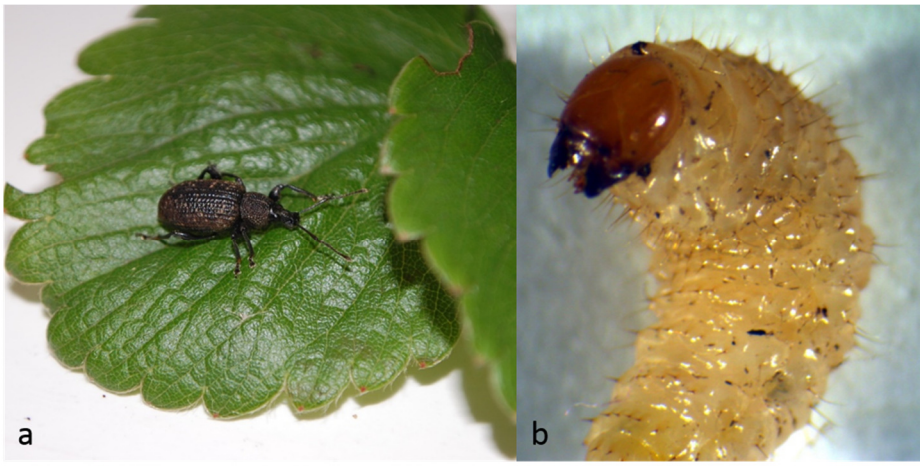


Figure 1.1a: An adult *O. sulcatus* feeding. 1.1b: An *O. sulcatus* larvae.

Eggs, white when first laid and later a caramel brown in colour, are laid by overwintering adults from March onwards, while overwintering larvae emerge as adults later in the season and lay eggs in July and August (Moorhouse *et al.*, 1992) as shown in Figure 1.2. The eggs are laid at the base of host plants in the leaf litter. Larvae, which are pale brown or white with a caramel brown coloured scleratised head, hatch after 1-2 weeks (Figure 1.1b) and travel down into the soil to feed on root material. This ensures that individuals of all life stages are present throughout the year.

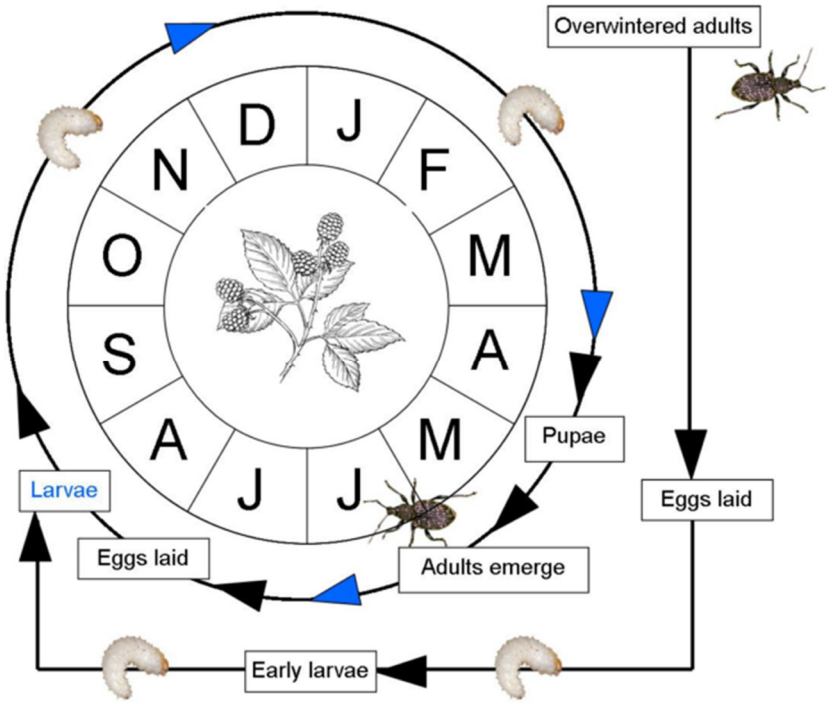


Figure 1.2: The life cycle of O. sulcatus living under field conditions in the Northern hemisphere (Clark, 2010). Based on data from Moorhouse, Charnley & Gillespie (1992)

The high number of eggs that *O. sulcatus* adults lay per day, 2- 4 eggs throughout their adult life stage (Clark *et al.*, 2011b), alongside their polyphagous nature make this species relatively easy to rear in the lab (section 2.1.8). The origin of *O. sulcatus* is in central Europe but through the shipment of plant material via trade routes it has spread to many temperate regions throughout the globe (Figure 1.3) (Moorhouse *et al.*, 1992).

Figure 1.3: Global distribution of *O. sulcatus*, indicated by light blue shading, after Lundmark (2010).

1.1.2 *Otiorhynchus sulcatus* as a pest species

Otiorhynchus sulcatus is a major pest of horticultural plants and soft fruit crops across the temperate zones of the world and can cause significant damage to a wide range of host plants. Adult *O. sulcatus* cause minor damage to the leaves of plants leaving characteristic notches on the leaf edge, while larvae feed on the root system of the plant and in high numbers can cause stunted growth, wilting and eventually death (Penman & Scott, 1976). As few as 1-3 larvae feeding on the roots of potted plants and can cause plant death in species such as *Cyclamen* and *Rhododendron* when kept under glasshouse conditions (Moorhouse *et al.*, 1992). The combination of *O. sulcatus* feeding belowground and further herbivores feeding aboveground can cause even greater damage to crops. In a study by McKenzie *et al.* (2013) an example of reciprocal feeding was observed in the red raspberry, *Rubus idaeus,* when *O. sulcatus* feeding belowground benefitted from carbon reallocation caused by aphids feeding aboveground. The adults when present in *R. idaeus* foliage can be a problem in crops that are harvested by mechanical means as they can contaminate the collected fruit (Kieffer *et al.*, 1983). Larval damage on soft fruit crops such as *R. idaeus* and strawberry, *Fragaria ananassa,* is of particular economic importance in the UK, with these crops worth in excess of £89.6m (2013) and £217.8m (2013) respectively (DEFRA, 2013). Both these crops are grown under polytunnels, which create conditions known to increase *O. sulcatus* performance (Johnson *et al.*, 2010). One way to improve pest resistance of *R. idaeus* is to use resistant cultivars (Hall *et al.*, 2008), this can influence oviposition by *O. sulcatus* when there is an alternative host present, but in the absence of a choice of host plants, egg laying is not influenced by host type (Clark *et al.*, 2011b). This could be an argument for the planting of trap crops adjacent to *R. idaeus* planting as part of an integrated pest management system.

1.1.3 *Otiorhynchus sulcatus* control.

Traditionally chemical control of *O. sulcatus* was achieved using Aldrin but this pesticide was withdrawn from use in 1990 and current methods typically entail a soil drench of chemicals such as the neonicotinoid; Imidacloprid, which has been withdrawn from use in the EU, under a temporary ban, from 2014, and the oganophosphate; Chlorpyrifos. The future use of these chemicals is uncertain and so alternative control measures must be sought (Moorhouse *et al.*, 1992; Cross & Burgess, 1997; Gill *et al.*, 2001).

The two main biological control agents used to manage *O. sulcatus* are an entomopathogenic fungus *Metarhizium anisopliae* and entomopathogenic nematodes (EPNs) but these methods are not currently as effective as pesticides. *Metarhizium anisopliae* treatments are usually most effective against *O. sulcatus* when applied in a pre-treated soil mix and are less effective when added to the soil surface, or through irrigation systems (Moorhouse *et al.*, 1993, 1994; Bruck & Donahue, 2007). This method is poorly suited to raspberry cultivation, as the plants are in the soil for around 5 years and cannot be replanted into pre-treated soil each time *O. sulcatus* is identified.

The two most commonly used genera of EPNs are the Steinernematidae and Heterorhabditidae. Deployed as biological controls they are most effective when added in water to the root zone of affected plants (Denno *et al.*, 2008). The nematodes are applied as motile infective juveniles. They swim through the thin water layer that covers soil particles and upon finding an insect host, via host odour cues such as kairomones and other various related odours (Dillman *et al.*, 2012), they enter through the spiracles whereupon they expel their bacterial endosymbionts which quickly break down and liquefy the host tissues finally killing the host via septicaemia. The nematodes then feed on this bacterially enriched soup and gather within the host body to reproduce. Several weeks after infection the host cadaver ruptures and thousands of the next generation of infective juveniles then emerge to seek out new hosts (Kakouli-Duarte *et al.*, 1997; Wilson *et al.*, 1999; Bruck *et al.*, 2005; Lola-Luz & Downes, 2007). EPNs have been shown to respond with taxis to the universal host cue of CO_2 as well as more host specific volatile organic compounds (VOCs) to find prey (Dillman *et al.*, 2012). It has been posed that the taxis behaviour of EPNs and the specific temperature tolerances of individual species, and strains of EPNs can be the biggest limiting factors in determining successful control of *O. sulcatus* (van Tol *et al.*, 1998). Through the use of EPNs active at low soil temperatures, typical of UK conditions, and the manipulation of VOC cues that can better attract, and maintain the interest of EPNs, better *O. sulcatus* control might be achieved. It is with this aim in mind that this thesis set out to investigate belowground predator prey interactions.

EPNs are well suited to *R. idaeus* cropping as they can take advantage of existing infrastructure for simple application. An aqueous delivery method recommended for EPNs (Bruck *et al.*, 2005) is ideal for a protected cropping system such as that used for raspberry production as it can be incorporated into an existing irrigation system. Improving the efficacy of EPNs could lead to a better suited and more cost effective biological control of *O. sulcatus*, eventually replacing existing conventional methods of control.

A possible way to improve the efficacy of existing biological controls may be a synergistic approach with resistant plants and plant symbionts, arbuscular mycorrhizal fungi are one such group of organisms that may provide a solution (section 1.5).

1.2 The Red Raspberry, *Rubus idaeus*

1.2.1 *Rubus idaeus* biology and cultivation history

The European or Red Raspberry *Rubus idaeus* (Rosales: Rosaceae) is a deciduous perennial shrub that performs best in slightly acidic soils. The perennial root stock of *R. idaeus* produces a biennial stem or 'cane' and usually produces fruit after 2 years of growth. Cultivated *R. idaeus* are usually planted in the winter, from November to March. Different cultivars produce fruit at different times in the summer and early autumn and are termed as either early, mid or late fruiters, with all cultivars bearing fruit in a window between June and October. Established *R. idaeus* rootstock and canes are typically maintained for around 5 years, after which, yield tends to tail off as pests and pathogens accumulate and become more of a problem. *Rubus idaeus* forms a fruit or raspberry that is in fact an aggregate of drupelets that retains a conical form after ripening, it is this fruit that gives the plant its common name. Despite global cultivation, *R. idaeus* has its native range in Europe through to Siberia. It is thought that *Rubus* ancestors likely originated from what is modern day China as this is the centre of *Rubus* diversity in the Northern hemisphere (Hall *et al.*, 2008). *R. idaeus* is a plant that readily forms associations with arbuscular mycorrhizal (AM) fungi, this affinity has even been applied with some success in a commercial setting with micropropagated *R. idaeus* inoculated with AM fungi to aid establishment (Varma & Schuepp, 1994).

In the UK, *R. idaeus* is a valuable crop with a farm gate value estimated at £89.6m in 2013 (DEFRA, 2013). This crop is grown in an area of 1,478 hectares, across the UK (DEFRA, 2013) and its cultivation and harvest provides a large amount of seasonal employment. Modern *R. idaeus* cropping in the UK and cultivation on similar latitudes is conducted almost entirely under protected cropping environments, most typically in polytunnels. Polytunnels are temporary structures consisting of long semi-cylindrical tunnels around 2.5m at their highest

point and formed of steel hoops and covered in translucent polythene sheeting. *Rubus idaeus* canes are planted in any number of rows inside the polytunnels and then a polythene ground covering is placed over a mulch at the base of the plants to exclude weeds and increase soil temperatures. Polytunnels can provide conditions that are between 4°C and 10°C greater than exterior temperatures and can result in a 50% increase in plant height and a 16% increase in leaf area across the growing season (Johnson *et al.*, 2010, 2012). *Rubus idaeus* that are grown in polytunnels are typically irrigated at the soil surface in order to have greater control of soil moisture throughout the seasons.

1.2.2 *Rubus idaeus* Pests and diseases

As with all cultivated species, *R. idaeus* has a number of pests and diseases that make its cultivation challenging for commercial and amateur growers alike. There is a wide range of fungal, bacterial and viral pathogens that infect *R. idaeus* (Hall *et al.*, 2008). The majority of these diseases are managed by the incorporation of resistant cultivars but the fungal pathogens often require regular fungicide applications to ensure they are controlled below an economic threshold (Hall *et al.*, 2008). The application of such fungicides can have a negative effect on the formation of AM fungi in *R. idaeus* and further reduce soil microbial diversity, which is already known to be low in arable fields (Daniell *et al.*, 2001). Another threat to *R. idaeus* yields are a variety of insect pests that also cause plant tissue damage (Alford, 2007). This thesis will focus on the insect pest *O. sulcatus*, as previously discussed (section 1.1.2), its pest status in *R. idaeus* cropping has recently become a more serious issue as cultivation practices move towards protected cropping. The implementation of polytunnels in *R. idaeus* cultivation has had the side effect of accelerating *O. sulcatus* development and increasing its impact as a pest. Johnson *et al.* (2010) demonstrated that *R. idaeus* carbon/nitrogen ratios were higher in polytunnels than in uncovered cropping. They showed that this decrease in nutritional quality led to significantly more plant material being consumed which, coupled with higher temperatures caused a 20 fold increase in eggs laid by the time adult *O. sulcatus* were 5 weeks old.

The increasing pest issue that *O. sulcatus* poses to *R. idaeus* cropping and the mycorrhizal affinity of the plant meant that it was a good choice for investigating the interactions of AM fungi on root defence signalling (discussed further in section 1.5).

1.3 Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi (AM fungi) are a group of obligate plant symbionts in the phylum Glomeromycota (Schüβler *et al.*, 2001) that form symbiotic relationships with around 70% of all vascular plants (Hodge, 2000; Smith & Read, 2008). AM fungi contribute to their symbionts by the provision of phosphorus, nitrogen and other nutrients and in return they receive carbon compounds produced in the plant by photosynthesis (Bever *et al.*, 2001). The presence of AM fungi in a plant root are not immediately obvious to the naked eye but can be discovered by the clearing and staining of root tissues and the application of microscopy (Figure 1.4). The AM fungal spores germinate in the soil when conditions are appropriate for plant seed germination and root growth. From the spore, hyphae form, which grow fairly slowly until they encounter sesquiterpene root exudates which induce extensive branching in hyphae and hence facilitate the location of host roots (Akiyama *et al.*, 2005). If the fungus encounters a root or root hair then an appresorium is formed and penetration occurs in the elongation zone of the root, in places where suberation has yet to occur. Hyphae then grow in or between the root cortical cells. Specialised hyphal tissues enter cortical cells to form arbuscules which are entirely contained in the cell plasma membrane. It is this site where nutrient exchange takes place (Smith & Read, 2008). The formation of arbuscules occurs in a cycle of between 4-15 days after which they break down and new ones are formed constantly. Vesicles, which are thought to be storage organs, form in cortex cells and are lipid filled sacs readily identified in stained root tissues (Figure 1.4).

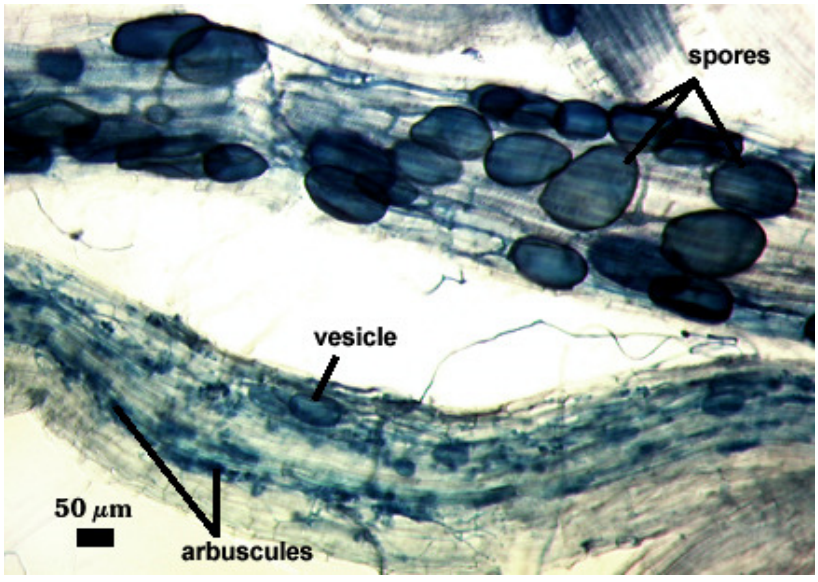


Figure 1.4: *Zea mays* root tissue stained with trypan blue to show colonisation by AM fungi and the different AM fungal structures. Image taken from (INVAM, 2014)**.**

As well as improved nutrient uptake, plants have shown a variety of other symptoms while in symbiosis with AM fungi. AM fungi have been shown to alter the interactions between plants and their pests and pathogens (Borowicz, 2001; Koricheva *et al.*, 2009). AM fungi have even been found to have an effect on the community of pollinators visiting the flowers of plants (Gange & Smith, 2005; Wolfe *et al.*, 2005; Cahill *et al.*, 2008), suggesting that their presence may produce a cascade of effects through a multi-trophic system.

A meta-analytical study by Koricheva, Gange, and Jones (2009) determined that most generalist insect herbivores were negatively affected by the presence of mycorrhizas while specialist insect feeders responded positively. This meta-analysis was however, mostly based on foliar feeding insects, due to the very small number of root feeding studies available, and although *O. sulcatus* could be considered a generalist feeder, previous studies have shown reduction in larval weights when in the presence of mycorrhizas (Gange *et al.*, 1994; Gange, 1996, 2001). In 2011 Currie, Murray, and Gange showed that this generalist/specialist trend in response to AM fungi may also be true in root feeding insects when they showed that the root clover weevil, *Sitona lepidus*, showed increased survival and no negative response to the presence of AM fungi. In addition to these trends in specialist/generalist responses to herbivory in the presence of AM fungi, a reduction in plant tolerance to herbivory has been observed as a consequence of AM fungi colonisation (Borowicz, 1997; Bennett & Bever, 2011). Observations by Gange & Ayres (1999) led to the proposal that there is a curvilinear relationship between AM fungal colonisation and plant benefit. Low levels of colonisation providing benefits to plants in the form of increased resource provisioning, whereas higher levels of colonisation produced more of a carbon drain than a nutrient benefit. This explanation fits with the observation of there being an AM fungi mutualism-parasitism continuum (Johnson *et al.*, 1997).

The process of AM fungal colonisation has the effect of priming plant defences that then give the plant a better chance of responding rapidly to pests or pathogens through the jasmonic acid and salicylic acid pathways (Van der Ent *et al.*, 2009; Jung *et al.*, 2012). The allocation of increased resources, provided by AM fungi can also contribute to constitutive plant defences, bolstering plant resistances against herbivores (Bennett *et al.*, 2006; Kempel *et al.*, 2010). In addition to this, AM fungi have been shown to change the secondary metabolite production of plants altering the proportion of monoterpenes and sesquiterpenes produced as volatiles (Rapparini *et al.*, 2008; Fontana *et al.*, 2009). An objective of this thesis was to measure the impact of AM fungi on root VOC production (section 1.5).

1.4 Herbivore Induced Natural Enemy Attraction

When a plant is damaged by a feeding herbivore a number of inducible defences are activated. This often includes an increase in the concentration of VOCs present in plant tissues which can act in many different ways, either as a direct toxin or a feeding deterrent (Bezemer & van Dam, 2005). These VOCs are exuded by the plant both above and belowground and in some cases this is used by additional herbivores to locate and identify an already damaged plant, but this can also act as an attractant for natural enemies that can come to the aid of the attacked plant. The notion of plants using insect predators and parasitoids as bodyguards is not new (Dicke & Sabelis, 1988) and there have been a number of discoveries of this behaviour in both insect and mite species (Dicke *et al.*, 1990; Turlings *et al.*, 1990; Elliot *et al.*, 2000; Gange *et al.*, 2003; Guerrieri *et al.*, 2004). Van Tol *et al.* (2001) showed that *Thuja occidentalis* roots under attack by *O. sulcatus* released chemicals attractive to the EPN; *Heterorhabditis megidis.* They failed however to detect noticeable changes in airborne volatile emissions and instead concluded that these chemicals were likely waterborne. The taxis of *H. megidis* was also found in a similar experiment by Boff *et al* (2001), using a Y shaped olfactometer choice experiment with a series of treatments comprising *Fragaria* roots and *O. sulcatus* larvae with the outcome that the combined treatment, with *O. sulcatus* larvae feeding on strawberry roots provided a strong attraction to *H. megidis*. Unfortunately no attempt was made to discover the mechanism behind this attraction but plant volatiles were again considered the likely attractant. Aratchige, Lesna, & Sabelis (2004) conducted a similar study on rust mites that feed belowground on tulips. Infestations by these mites affected the attraction of predatory mites. They found that predatory mites could discriminate between artificial wounding of tulip bulbs and a rust mite infestation. They did not test this system in a soil medium or measure the volatile compounds that may be responsible for such behaviour. The ability of natural enemies to discriminate between true herbivory and artificial wounding highlights the complexity of the herbivore induced VOCs to which natural enemies respond.

In 2005 Rasmann *et al.* conducted an experiment on western corn root worm, *Diabrotica virgifera,* induced volatile emissions in maize, *Zea mays,* plants and how these volatiles attracted *H. megidis*. This was carried out using a 6-arm root zone olfactometer to monitor the relative attraction of *H. megidis* to control, mechanical damage and *D. virgifera* infested *Z. mays* plants. Plants infested with *D. virgifera* were significantly more attractive to *H. megidis.* For the first time solid-phase micro-extraction samples were analysed by GC-MS to identify the

chemo-attractant responsible. They proposed the primary chemo-attractant was the sesquiterpene (E)-β-caryophyllene. In the field two cultivars were compared while under *D. virgifera* attack, one North American cultivar where (E)-β-caryophyllene emission doesn't occur and one European cultivar where (E)-β-caryophyllene emission is comparable to the wild *Z. mays* ancestor teosinte. As a consequence of elevated (E)-β-caryophyllene production the European cultivar showed a fivefold increase in nematode infection rate. This increase in nematode infection rate was also seen if the soil around the North American plants was spiked with (E)-β-caryophyllene. This research was then used in another study, by the same lab group, to restore the signal of (E)-β-caryophyllene in North American *Z. mays* plants (Degenhardt *et al.*, 2009). The method by which this discovery was achieved has provided a comprehensive framework for future research and application of that research into root defence (Rasmann & Agrawal, 2008). Rasmann & Turlings (2007) increased the scope of their olfactometry experiments to include both a below and above ground component with paired below and aboveground pests and natural enemies. They found that when both pests were present on the same plant that the relevant odour emissions were reduced and the attraction of both natural enemies were reduced. This may be the reason why reliance on volatile induced natural enemy attraction may always prove less effective in complex multi-trophic field environments. This reduced effectiveness of plant immune response is also seen in systemic plant defences in other plant species, where combinations of above and belowground pests feeding on a plant can cause a significant reduction in the production of host defence chemicals (Bezemer *et al.*, 2003, 2004).

Continuing in the rapidly expanding field of belowground herbivore-induced volatile emissions Ali, Alborn, & Stelinski (2010) investigated if there was a similar system that could be exploited with the root feeding weevil *Diaprepes abbreviatus* and citrus roots. Again a 6-arm root zone olfactometer was employed with control, mechanical damaging, non-feeding larvae and infested plant treatments. As may be expected it was found that plants infested with *D. abbreviatus* were more attractive to the EPN *Steinernema diaprepesi*. Four possible compounds were isolated as being the possible attractant in this system; geijerene (a break down product of pregeijerene), pregeijerene, α-santalene and α-Z-bergamotene, with pregeijerene thought to be the most likely attractant due to its relative abundance. Ali *et al.* (2012) then took this system to the field and showed that herbivore induced plant volatiles increased the activity of EPNs in the root zone of mature citrus roots with 4 species of nematodes responding to pregeijerene. Later tests showed that this volatile was a general signal as it had a similar effect on EPN activity when isolated and added to blueberry fields (Ali *et al.*, 2010).

There is a downside to olfactometry experiments which is worth considering when attempting to replicate results in the field. In a root-zone olfactometer the partitioning of soil or sand chambers with a barrier to insects and EPNs disrupts a potential foraging strategy which EPNs may employ to locate their host. This is through the following of host kairomone trails left behind as an insect moves through the soil, a method of host searching which is also common in many other organisms (Rogers & Potter, 2002; Inoue & Endo, 2008). This could mean that in a field setting the additional stimulus of host kairomone trails could make for a more complex network of attractant gradients leading to variation in nematode infection rates and consequently host mortality.

In a recent series of studies it has been shown that herbivore induced volatile emissions causing natural enemy recruitment have been shown to be enhanced by the presence of AM fungi. Schausberger *et al.* (2012) demonstrated that mycorrhizal plants infested with the red spider mite *Tetranychus urticae* were more attractive than non mycorrhizal plants to the spider mite predator, *Phytoseiulus persimilis*. Their studies suggest that predatory mites learn to recognise the altered plant VOCs of mycorrhizal plants (Patiño-Ruiz & Schausberger, 2014) which are more attractive due to their prey, *T. urticae*, being of higher nutritional quality (Hoffmann *et al.*, 2011c). This means that although there was an initial greater effect of *T. urticae* damage to plants this was more than compensated for by increased predation. Not only was *P. persimilis* more attracted to mycorrhizal plants but ovipostion was also greater leading to lasting *T. urticae* suppression (Hoffmann *et al.*, 2011b).

This presents an exciting development for any potential biological control programme on a belowground insect pest. As, although the presence of AM fungi has been shown to alter natural enemy attraction aboveground, very little is known about the impact it might have on belowground predator prey interactions. One of the objectives of this thesis was to see if the presence of mycorrhizas and feeding *O. sulcatus* change the root volatile emissions of *R. idaeus* and whether this influenced EPN attraction (see section 1.5).

1.5 Chapter outlines and objectives

The main aim of this thesis was to establish if mycorrhizal fungi influenced root defence signalling and if this, in turn, influenced belowground predator prey interactions. In particular, to discover if these effects were detectable in an AM fungal/*R. idaeus* system with the herbivore *O. sulcatus* and EPN predators. The root defence signals sampled were root VOC emissions.

1.5.1 Chapter 2

Chapter 2 details some of the specific methodologies employed in the experimental chapters (chapters 3-6).

1.5.2 Chapter 3

The main aim of chapter 3 was to see if the presence of AM fungi had an impact on *O. sulcatus* performance and to discover if *O. sulcatus* or AM fungi had an influence on plant VOC emissions. Key objectives in this chapter were:

- To investigate how different *R. idaeus* cultivars responded to *O. sulcatus* infestations of different densities.
- To test how *O. sulcatus* larvae performed under different population densities.
- To assess whether AM fungal colonisation was affected by *O. sulcatus* herbivory and/or *R. idaeus* cultivar.

1.5.3 Chapter 4

Chapter 4 aimed to determine how EPNs and AM fungal colonisation of *R. idaeus* affect root herbivore performance. To achieve the main aim of this study, a number of objectives were identified:

- To find out the effects of different EPN treatments on *O. sulcatus* mortality and larval mass.
- To ascertain the response of *R. idaeus* to *O. sulcatus* herbivory and EPN treatments through the collection of biomass data.
- To resolve the impact of AM fungal colonisation on *O. sulcatus* mortality and larval mass and to see if this was effected by the different EPN treatments.

1.5.4 Chapter 5

In chapter 5, two experiments are presented, both using belowground olfactometers to measure the taxis of EPNs towards different chemical cues.

1.5.4.1 Chapter 5.1

The main aim of this first olfactometry experiment was to see if the EPN *Heterorhabditis megidis* showed a preference for *R. idaeus* that were infested with *O. sulcatus* and inoculated with AM fungi and see if this was driven by plant VOC emissions. Further to this aim there were a number of objectives explored in this experiment:

- To see if AM fungi influenced the attraction of *H. megidis* and if this could be explained by different VOC emissions.

- To evaluate if the density of *O. sulcatus* populations on experimental plants had an effect on any observed herbivore induced natural enemy attraction effects and if this could be linked to VOC production.
- To determine if differences an *R. idaeus* cultivar could have an impact on *H. megidis* attraction and root emitted VOCs.
- To establish if levels of AM fungal colonisation recorded as percentage root length colonised (%RLC) by AM fungal structures (section 2.1.7) had an impact on *H. megidis* attraction, *R. idaeus* performance and *O. sulcatus* performance.

1.5.4.2 Chapter 5.2

The second of the two olfactometry experiments repeated the process applied in the first, but whereas the first olfactometry experiment used a field derived AM fungal inoculant this experiment used a commercial preparation alongside the EPN *Steinernema kraussei*. The aim of this experiment was to investigate if commercial AM fungal inoculant could increase the attraction of *S. kraussei* to *O. sulcatus* infested *R. idaeus*. Key objectives in this second experiment were:

- To see if the addition of a commercial AM fungal inoculant would influence *S. kraussei* distributions.
- To establish if *S. kraussei* were influenced by the presence of feeding *O. sulcatus* larvae, perhaps through herbivore induced VOCs.
- To investigate if the biomass and root to shoot ratio could have had an influence on the attraction of *S. kraussei*, and been influenced by *O. sulcatus* and AM fungal treatments.

1.5.5 Chapter 6

In chapter 6 the main aim was to determine if commercial AM fungi had an impact on *O. sulcatus* control when the EPN, *Steinernema kraussei* were added to *R. idaeus* and how this might compare to a field soil based inoculation. The objectives of this chapter were:

- To investigate if commercial inocula could enhance *S. kraussei* performance as effectively as a field derived spore inoculation.
- To assess the effects that different AM fungal treatments have directly on *R. idaeus* biomass
- To see if the %RLC by AM fungal structures can give an indication of the benefit derived by a host plant.

1.5.6 Chapter 7

In chapter 7 the findings of all chapters are summarised and then discussed.

2 General methods

2.1.1 Surface sterilisation of *Rubus idaeus* root stock at the James Hutton Institute

In order to prepare plants before inoculation with AM fungi a method of root surface sterilisation was used throughout this thesis. Rootstock from *R. idaeus* plants was washed over medium sieves (with 1mm and 0.5mm pores) with jets of cold water, so as to minimize loss of root material while ensuring that as much soil and other visible organic material was removed. The roots were then submerged in a bleach solution (4.5% Sodium Hyporchlorite) for 2 minutes. This was assessed to be sufficient time to dissolve as much of the finest root tissue, where AM fungal colonisation is concentrated, and fungal hyphae as possible while maintaining plant viability. The bleached root material was then thoroughly rinsed for 3 minutes in cold water and then allowed to soak in cold water for 2 minutes after a final 1 minute cold water rinse. The root material was then planted into seed trays with sterilised loam (Keith Singleton Nethertown, UK) and grown on top of under-heated benches in a controlled greenhouse environment (16:8 light:dark days at 18˚C). After 4 weeks the fresh growth from the rootstock was deemed sufficient for individual planting. The seed trays were carefully unearthed and the individual small *R. idaeus* plants were separated taking extreme care not to damage any of the roots of live plants, so as to minimise transplant shock, these small plants were then ready for experimental treatments.

2.1.2 Field extracted arbuscular mycorrhizal spores using sucrose centrifugation

Spores extracted from the field were extracted from soil taken from a field that has had *R. idaeus*, of multiple cultivars, grown on it for over 10 years. This allowed a soil community that was adapted to the presence of *R. idaeus* to be sampled and allowed for the extraction of a *R. idaeus* specific mixture of AM fungal species.

In a method adapted from Daniels and Skipper (1982) the AM fungal spores were extracted from soil using sucrose centrifugation. Initially large stones were removed from the soil using a Scheppach RS400 Electric Soil Sifter Sieve. Next, approximately 100g of sifted soil was added to 200ml of water and agitated in a BL305840 Blendforce Triplax blender (Tefal, Sarcelles, France) with 3 depressions of the pulse blend setting, before being poured into a stack of larger test sieves terminating in a 40µm test sieve. The soil was washed through the sieves with jets of cold water. The soil particles trapped on the 40µm sieve were then transferred into 50ml tubes containing 20ml of 60% sucrose solution and placed in a centrifuge (Sigma 4K15) for 3 minutes

at 2000rpm at a temperature of 22°C. The supernatant was then poured into a 40µm sieve and rinsed with water to remove sucrose from the spores. The collected spores were then re-suspended in water for short term storage and kept at 4°C. The spores were always used to inoculate soil within 4 days of extraction.

2.1.3 Preparation of arbuscular mycorrhizal fungal spore inoculations and a microbial wash

Excess water was removed from the spore suspension using a P10ml Gilson® (Luton, UK) Pipetman to create a microbial wash. This extracted volume was then put through a vacuum filter to remove any spores or detritus. The remaining spore suspension and the newly created microbial wash were then divided into equal volumes and half of each suspension was sterilised in an autoclave (Boxer laboratory equipment LTD, Wave 01920/468). This method was adapted from the methods outlined by Ames *et al* (1987) to control for microbial populations.

2.1.4 AM fungal trap cultures

Trap cultures were established using spore based inoculations taken from the field site mentioned in section 2.1.2 at the same time as the plants in section 3.2 were inoculated. Twelve 10L pots were filled with twice sterilised loam (Keith Singleton, Nethertown, UK) with each pot inoculated with 54±7.95SE spores and then sown with a grassland seed mixture. This seed mixture comprised 5% *Lotus corniculatus* 5% *Plantago lanceolata* 10% *Trifolium pratense* 80% *Agrostis capillaris* from The James Hutton seed stocks (The James Hutton Institute, Dundee,UK).

2.1.5 Staining protocol for arbuscular mycorrhizal fungi in roots using Quink Ink

One of the main methods by which *R. idaeus* roots were stained, in order to obtain information on colonisation, was an adapted version of the method proposed by Vierheilig *et al.* (1998) a method in which we used domestically available Parker (Newhaven, UK) Royal Blue Quink Ink. *R. idaeus* roots were prepared prior to staining by removing all the soil under running water. The roots were then cut into 1cm long pieces and placed into a labelled tissue Square mesh tissue embedding cassette made by Thermo Fisher scientific (Waltham, USA). The cassettes were then placed into a beaker containing 10% KOH (10% w/v: 10g KOH in 100ml aqueous solution) solution which has been preheated to 80˚C in a water bath. The samples were then left for 10 hours in the water bath, with the KOH solution being changed and refreshed every 2.5 hours. This process cleared the root cells of pigment and allows the mycorrhizal features within the root to become visible.

Following the clearing stage, the roots were then rinsed in water to remove the KOH and then blotted dry on tissue. This stage was essential as the stain will lose its colour if the liquid

attached to the roots is too basic. The samples were then added to a preheated beaker of staining solution (84.4:15:0.6, dH_2O:1%HCl: Royal Blue Quink), again in a water bath kept at 80˚C, for 15 minutes.

The stained root was then used to prepares slides ready for scoring using the treatment using the magnified intersections method (McGonigle *et al.*, 1990) see section 2.6.

2.1.6 Staining protocol for arbuscular mycorrhizal fungi in roots using trypan blue.

An alternative method for staining roots for assessing AM fungal colonisation is to use trypan blue, using a method adapted from Phillips & Hayman, (1970). Roots were prepared and placed into tissue cassettes exactly as described in section 2.4. An appropriate volume of 3% KOH (i.e. 3g/100ml or 30g/L) was boiled and then added to a beaker containing the tissue cassettes, these were then left for 30 minutes and then the solution was poured off and rinsed in water for 5 minutes. The samples were then placed into a 2% HCl solution and allowed to soak for 30 minutes and then drained, this time without a rinsing step. A Trypan Blue solution (1:1:1 lactic acid:water:glycerol with Trypan Blue at 0.05% of total volume) was then boiled and added to the cassettes and left for 20 minutes. Following this step the tissue cassettes were then thoroughly rinsed to remove excess Trypan Blue stain and the samples added to a de-stain solution (50:45:5 glycerol:water:1%HCl) and left to sit in the fridge for 2 days prior to being placed on slides ready for scoring, see section 2.1.7.

2.1.7 Preparing slides and scoring roots for arbuscular mycorrhizal colonisation

Labelled slides containing stained root tissue can be used to gain an indication of root length colonised by AM fungi using the magnified intersections method (McGonigle *et al.*, 1990). Glass slides were prepared with stained root tissue arranged length-ways along the slide, this was then mounted in de-staining solution (50:50 glycerol:water) with a glass coverslip sealed with clear nail varnish.

2.1.8 *Otiorhynchus sulcatus* cultured at the James Hutton Institute

The *O. sulcatus* culture maintained at the James Hutton Institute, Dundee consisted of gravid adult *O. sulcatus* that were originally captured as adults, at night in polytunnels containing a monoculture of *R. idaeus* on site. The adults were kept in culture maintained at 18˚C on a 16:8 Day/night cycle. They were kept in 9cm Petri dishes, with 5 individuals per Petri dish, lined with a moist tissue paper and fed twice weekly with fresh strawberry leaves of the 'Symphony' cultivar (Figure1.1a). Once a week the adults were transferred to fresh tissue lined Petri dishes and the old ones were stored at 4°C. The old dishes were never kept for more than 4 weeks, and it was from these that *O. sulcatus* eggs were sampled for experiments, taking care to use the freshest eggs available.

2.1.9 *Otiorhynchus sulcatus* cultured at Royal Holloway University of London

The *O. sulcatus* culture maintained at Royal Holloway, University of London consisted of gravid adult *O. sulcatus* that were captured as larvae feeding on strawberry plants onsite and then reared through to adults on 'Elsanta' strawberry (*Fragaria ananassa*) plants in a constant environment room kept at 22˚C on a 16:8 Day/night cycle. The adults were kept in culture maintained at 22˚C on a 16:8 Day/night cycle. They were contained in 9cm Petri dishes, with 5 individuals per Petri dish, lined with a moist tissue paper and fed twice weekly with fresh strawberry leaves of the "Elsanta" cultivar. Once a week the adults were transferred to fresh tissue lined Petri dishes and the old ones were stored at 4°C. The old Petri dishes were never kept for more than 4 weeks, and it was from these that *O. sulcatus* eggs were sampled for experiments, taking care to use the freshest eggs available.

2.1.10 Entomopathogenic nematode culturing

Entomopathogenic nematode cultures were maintained by *in vivo* production of infective juveniles under 18˚C on a 16:8 Day/night cycle conditions in 9cm Petri dishes. The wax moth *Galleria mellonella* (Livefood, Rooks Bridge,UK) was used as a surrogate host and infective juveniles were collected using white traps as devised by White (1927). Entomopathogenic nematodes were then stored in tap water at 5°C in 20ml glass vials.

2.1.11 Nematode extraction using Baermann funnels

Samples of sand or soil were placed into a Baermann funnel. Baermann funnels consist of a filter placed inside a funnel into which a sand or soil sample in placed (see Figure 2.1). The sample was placed on top of the tissue paper filter and then left for 48 hours. During this time the nematodes within the sample that were both living and motile, swam down through the filter and collected just above the pinch clamp, as indicated in Figure 2.1. After 48 hours the pinch clamp was then released and the first 20ml of water was run off into a 50ml centrifuge tube. This liquid sample was then transferred into a gridded 6cm petri dish and placed under a dissecting microscope into and the nematodes were counted.

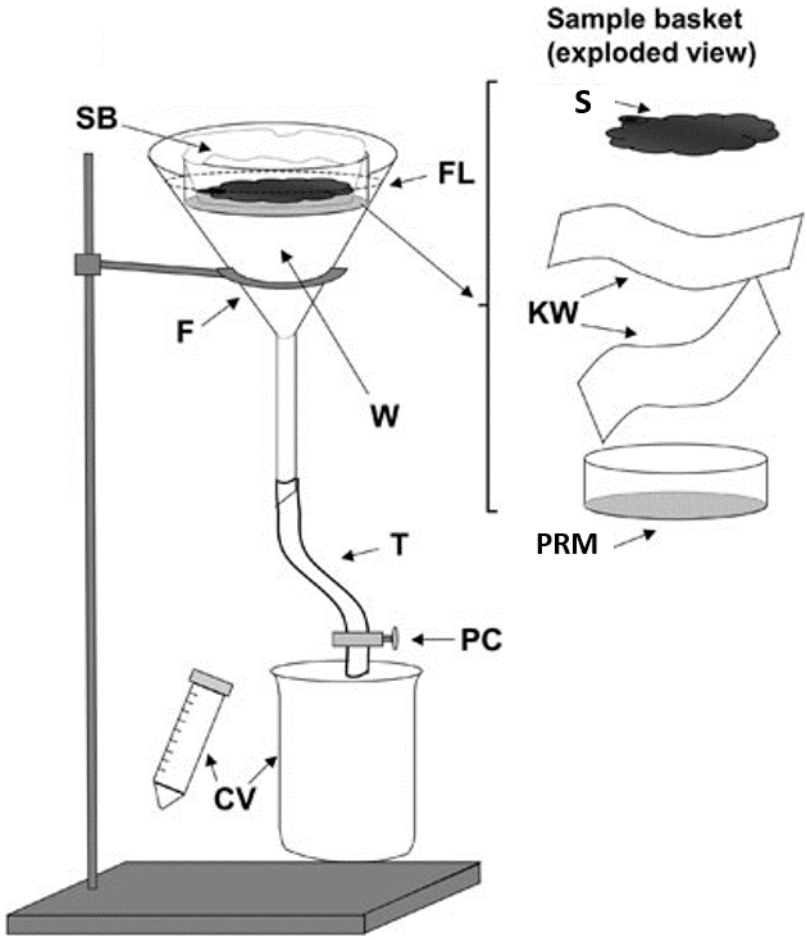


Figure 2.1: A Baermann Funnel. F, a glass funnel; W, tap water at 18°C; FL (dashed line) fluid level in funnel; SB, Sample basket. S, Sample of sand from olfactometer; KW, two layers of Kimwipes or other laboratory tissue; PRM Plastic ring with nylon mesh; T, rubber tubing; PC, pinch clamp; CV, catch vessels, 50ml centrifuge tube, or glass beaker. Modified diagram from (Lok, 2007)

2.1.12 Nematode extraction using wet sieving and sucrose centrifugation

To extract nematodes from sand or soil, samples were first placed into one of two 100ml beakers to which water was added. The suspended sand solution was then poured between the two 100ml beakers ten times in order to agitate the substrate and suspend the nematodes in the water. This was then left to settle for 15 seconds and then the supernatant was poured onto a 38µm sieve. The sieved sample was then transferred into a 50ml centrifuge tube along with 40ml of water. This was spun at 1700rpm (810g) for 5 minutes and then allowed to settle for 5 minutes. This process produced a pellet at the bottom of the tube, containing the nematodes. The supernatant was then pipetted out using a P10ml Gilson® (Luton, UK) Pipetman to approximately 1cm above the pellet. The 50ml centrifuge tubes were then topped up to 40ml volume with a 45.4% sucrose solution. This was then placed on a vortex at high speed for 10 seconds to ensure the pellet was completely dispersed. The 50ml centrifuge tubes were then loaded back into the centrifuge and brought up to 1000rpm (280g) over 30 seconds at which point the brake was applied. This re-suspends the supernatant with the last grains of sand and organic material collecting in a pellet at the bottom of the tube.

The supernatant was then poured back into a 38µm sieve and washed with tap water before being transferred into a gridded 6cm Petri dish that was sealed with parafilm®M made by Sigma-Aldrich® (ST. Louis, USA) ready for the population to be counted under a dissecting microscope.

2.1.13 Plant volatile sampling

The method for sampling volatiles in these experiments was the application of stainless steel tubes packed with 200mg of the sorbent powder, Tenax® TA. (2.6-diphenylene oxide polymer resin, 60–80 mesh, surface area 35 sq m/g; Markes International Ltd, Llantrisant, UK). Tenax® TA designed for the trapping of volatiles and semi-volatiles and has a very low affinity for water, it is therefore ideal for use in high moisture environments, such as soil. These tubes were conditioned prior to use in order to remove any residual components present within the tube either from previous use or exposure during storage. The tubes were conditioned in a conditioning oven at 240°C while maintaining a steady flow of 2kPa of the clean carrier gas Helium at 5N grade for 4 hours. The freshly conditioned tubes were then immediately capped with ¼ inch brass storage caps complete with ¼ inch PTFE ferrules. The caps were screwed on finger tight and then using the CapLok™ tool they were tightened a further quarter turn. The tubes then remained in a clean and dry environment and were not uncapped until immediately prior to sampling.

All experiments conducted used potted *R. idaeus.* To make the sampling using ATD tubes easier a short wooden dowel plug was inserted upright into the soil, at the time of potting the *R. idaeus*, with one end just breaking the soil surface. This dowel plug was 1mm greater in length and 5mm greater in diameter than the ATD tubes used. This dowel was removed just prior to sampling and the hole it left behind was the space in which the ATD tubes were placed (see Figure 2.2). This ensured that there was no unnecessary disruption of the root zone at the time of sampling volatiles. Immediately after sampling the caps were replaces by the same method and stored until desorption.

Figure 2.2: A *R. idaeus* cane with an automated thermal desorption tube in the soil for the passive collection of root volatiles.

2.1.14 ATD-GC-MS methodology

The volatile samples captured in ATD tubes were then analysed in a Unity™ automated thermal desorber (Markes International Ltd, Llantrisant, UK) with an ATD Ultra™ auto sampler (Markes International Ltd, Llantrisant, UK) coupled with an Agilent Technologies 6890N GC-MS system (Agilent Technologies 5975B). Sample tubes were alternated with a blank tube to ensure the independence of each chromatograph. Samples were desorbed over a period of 5 minutes per tube at a temperature of 240°C. The compounds eluted by this process then passed from the sample tube to a cryofocussing trap, containing Tenax® and kept at 10°C. The cryofocussing trap was then rapidly heated to 240°C whereupon, compounds were transferred along a transfer line heated to 150°C onto a DB1701 GC column (60.0m 0.25mm 1.00lm, J&W, Folsom, CA, USA). The helium carrier gas used in the column had a flow rate of approximately 0.5ml min^{-1}. The oven temperature was increased from an initial 40°C to 240°C at a rate of 5°C min^{-1} and maintained at 240°C for 20 minutes. Following a 2 minute solvent delay EI (70.0eV) mass spectra were acquired at 1.33 scans s^{-1} over a mass range of 20-300 a.m.u. sourced at 230°C. This data was read directly into MSD Chemstation software (G1710DA, Rev. D.03.00). This method was based entirely on the work of Cognat *et al.* (2012).

2.1.15 GC-MS based peak integration and identification

The data collected into Chemstation software through the process described in section 2.1.14 was then converted from the standard ".D" Agilent format to ".RAW" files for use in the mass

spectrometry software Xcalibur™ 2.0.7 (Thermo Scientific, USA). The peaks in the chromatographs were then manually integrated using the software and individual peaks were run through the NIST MS 2.0d Library (NIST, USA). Only library matches that returned matches in excess of 80% were considered as reliable identifications. Identification was further aided with reference to an above-ground *R. idaeus* volatile database provided by Dr Tom Shephard (The James Hutton Institute, Dundee, UK), which was created using the same sampling methods on the same machine setup. This database has been previously used in peer reviewed publications (McMenemy *et al.*, 2012). In order to account for the lack of an internal standard within samples, the peak area of each compound was divided by the total peak area of the entire sample. This created a value that represented a relative abundance of any given compound which could be used in further statistical analyses.

2.1.16 Graphs in this thesis

All graphs in this thesis that present error bars are shown with standard error, unless otherwise noted.

3 Is the black vine weevil, *Otiorhynchus sulcatus*, influenced by the presence of mycorrhizas when feeding upon *Rubus idaeus*?

3.1 Introduction

Otiorhynchus sulcatus causes significant damage to a range of silvicultural and horticultural crops throughout the world's temperate regions. Adult *O. sulcatus* feed on the foliage of a huge range of plants, inflicting relatively minor damage when compared to the root feeding larvae, which can reduce plant growth and if an infestation is severe, the death of a host plant (Penman & Scott, 1976). Conventional control of *O. sulcatus* is achieved using soil drench treatments of chemical pesticides. Until very recently the most commonly used treatment for an *O. sulcatus* infestation was the neonicotinoid; Imidacloprid, which has been temporarily withdrawn from use in the EU since 2014 due to non-target effects on bees. Future strategies to control *O. sulcatus* would be wise to therefore consider pesticide free alternatives as part of an integrated approach to pest management (Gill *et al.*, 2001).

One of the primary plant hosts of *O. sulcatus* which is of major economic importance is the red raspberry, *Rubus idaeus,* with over 13.8 thousand tonnes produced in 2013, worth £89.6 million to the UK economy (DEFRA, 2013). The production of *R. idaeus* is, in the UK, almost entirely under the protection of plastic tunnels which can raise temperatures by around 4°C compared to the surrounding field conditions and results in greatly increased growth (Johnson *et al.*, 2010). However these conditions are also very favourable for *O. sulcatus* performance with the insects consuming more *R. idaeus* biomass, completing their life cycles faster and with adults being more fecund (Johnson *et al.*, 2010). Two cultivars that have been studied previously with respect to their tolerance to *O. sulcatus* attack are Glen Ample and Glen Rosa (Clark *et al.*, 2012). Both these cultivars are autumn mid-season fruiters and typically produce fruit in their second year late in July through to the middle of August. Despite being sister cultivars they differ in their usage, with Glen Ample being a major commercial variety, due to fruit size and quality, and Glen Rosa being more popular on the amateur market due to its better tolerance to pests and diseases (Hall *et al.*, 2008; Clark *et al.*, 2011b). Like many

members of the Rosaceae, *R. idaeus* readily form mutualisms with arbuscular mycorrhizal fungi.

AM fungi form symbiotic relationships with the majority of plant taxa (Hodge, 2000), they contribute phosphorus and other plant limiting nutrients in return for plant sugars derived from photosynthesis (Whittingham & Read, 1982; Bever *et al.*, 2001; Smith & Read, 2008). The allocation of increased resources, provided by AM fungi can also contribute to plant resistance against herbivores (Bennett et al. 2006, Kempel et al. 2010). As well as improved nutrient uptake plants have shown a variety of other effects while in symbiosis with AM fungi. The process of AM fungal colonisation has the effect of priming plant defences that then give the plant a better chance of responding rapidly to pests or pathogens through the jasmonic acid and salicylic acid pathways (Van der Ent *et al.* 2009, Jung *et al.* 2012). Koricheva *et al.* (2009) conducted meta-analysis to compare the effects of AM fungi on different groups of insect herbivores. They concluded that while specialist insect herbivores often responded positively to AM fungal colonisation, generalists tended to be negatively affected. They postulated that this disparity due to AM fungi boosting plant defences and plant nutrition and that the effects of this would be more detrimental to generalists whose physiology is not specialised to counter the defences of any particular plant species and cannot therefore take advantage of increased nutrient availability, unlike a specialist herbivore. *O. sulcatus,* a generalist herbivore, have appear to fit this observed trend as studies on *Fragaria spp.* demonstrate a reduction in larval weights when a single species of AM fungi is present in plant roots (Gange *et al.*, 1994; Gange, 1996, 2001). This effect seems disappear however, when a mixed AM fungal inocula is added to plants with such treatments having no apparent effects on larval performance (Gange, 2001). Under field conditions plants are known to have associations with multiple AM fungal partners simultaneously, if this is at the cost of possible benefits of exclusive symbioses with one partner then this suggests that these relationships are more complex than at first glance (Gadhave *et al. unpublished*; Bakker *et al.*, 2013). This experiment was therefore designed to see if a mixed AM fungal inocula added to plants, that was indigenous to *R. idaeus* in the field, would show negative effects on *O. sulcatus* performance.

Plants can respond to herbivore attack with an array of inducible defences among these is the increased production of plant VOCs emitted by plant tissues; acting potentially as toxins or deterrents (Bezemer & van Dam, 2005). VOCs are released both above and below the soil surface and provide a chemical signature that is particular not only to the plant species but also the plants' status and can consequently be attractive to further herbivores but also natural enemies that can assist the beleaguered plant. This effect of plants attracting natural enemies has been observed in a number of plants species alongside both insects and mite

species (Dicke *et al.*, 1990; Turlings *et al.*, 1990; Elliot *et al.*, 2000; Gange *et al.*, 2003; Guerrieri *et al.*, 2004). Earlier studies investigating *O. sulcatus* larval feeding inducing increased attraction of the EPN; *Heterorhabditis megidis* unfortunately failed to detect any VOC emissions that could explain *H. megidis* distributions (Boff *et al.*, 2001; van Tol *et al.*, 2001). Later experiments investigating these effects in belowground herbivore induced VOCs attracting natural enemies have been more successful in capturing and identifying the VOC compounds responsible. These studies used techniques such as solid-phase microextraction fibres or thermal desorption sampling followed by gas chromatography and mass spectrometry techniques (Rasmann *et al.*, 2005, 2012b; Ali *et al.*, 2012). This study will employ similar methods to try and identify any changes in root volatile chemistry between *R. idaeus* treated with differing levels of *O. sulcatus* and AM fungi.

The main aim of this work was to see if the presence of AM fungi affected *O. sulcatus* performance on *R. idaeus*. With this as the main aim a number of further objectives were identified. It was investigated if the two *R. idaeus* cultivars responded differently to *O. sulcatus* infestations of different densities. The hypotheses were that high density treatments of *O. sulcatus* would have a greater negative effect on *R. idaeus* growth parameters and that Glen Ample would suffer greater levels of herbivore damage than Glen Rosa. To test how *O. sulcatus* larvae performed under different population densities the hypothesis that higher densities of *O. sulcatus* would result in lower *O. sulcatus* larval mass was tested. It is feasible that *O. sulcatus* herbivory on roots could result in lower AM fungal colonisation as the root tissue available for AM fungi to colonise would be diminished; differences between cultivar susceptibility would likely moderate this interaction. It was investigated whether levels of AM fungal colonisation was effected by *O. sulcatus* herbivory and/or *R. idaeus* cultivar. It was hypothesised that AM fungal colonisation would be influenced by *O. sulcatus* density and that there would be a difference between the AM fungal colonisation of Glen Rosa and Glen Ample. Root VOC emissions were captured and the data collected from this was analysed to see if there are changes in chemistry that reflect different AM fungal and herbivory treatments. It was hypothesised that root VOC emissions would be altered by AM fungal and *O. sulcatus* treatments

3.2 Materials and Methods

A 2 x 2 x 3 factorial experiment was conducted with two different *R. idaeus* cultivars (Glen Ample and Glen Rosa), two different mycorrhizal treatments (live or sterile spores) and three

different herbivore treatments using *Otiorhynchus sulcatus* (a control treatment, low 20 egg treatment and a high 40 egg treatment).

3.2.1 Experimental setup

Following surface sterilisation of their roots (see general methods 2.1.1) 108 individual plants, 54 of each cultivar, were separated and put into 1.8L size 6 pots containing 1.6L of a twice sterilised 1:1 loam (Keith Singleton sterilised loam) and sand mix. A 0.2L reduction in soil medium was incorporated into the design in order to reduce contamination of adjacent pots caused by splash-back during watering. A length of dowel (9mm diameter X 90mm) was placed vertically in each pot to create a column in the soil for the later insertion of automated thermal desorption (ATD) tubes, in a way that would not damage the roots.

Field extracted spores and a microbial wash was prepared following methods laid out in the general methods sections 2.1.2 and 2.1.3. All 108 plants were then inoculated with 2ml of spore solution, containing 54±7.95SE spores, and 2ml of microbial wash; with the live spores and a sterile microbial wash forming the "live" mycorrhizal treatment and the sterile spores and live microbial forming the "sterile" mycorrhizal treatment. Both the treatments were applied using a P10ml Gilson® (Luton, UK) Pipetman with spores injected 5mm below the soil surface at the base of the plant's main stem. A number of initial plant measurements were made at the time of inoculation (plant height and total number of leaves).

Seven weeks after the addition of mycorrhizal spores, *O. sulcatus* eggs were added to plants. One hundred and eight plants were divided equally, according to cultivar and AM fungal treatment, into three different groups. One group was kept as a control group with no eggs added; the second group had 20 eggs added, and the third group 40 eggs. The eggs were sourced from the JHI *O. sulcatus* culture (see general methods 2.1.8). Every two weeks after the addition of *O. sulcatus* eggs until the time of harvest, plant measurements of plant height and total number of leaves were taken. The length and width of the largest leaf were multiplied to give an estimation of leaf area.

Fourteen weeks after the addition of *O. sulcatus* eggs, preconditioned ATD tubes packed with Tenax TA (see general methods 2.1.13) were placed into the pots of 36 plants to sample root VOC emissions. The tubes were left in the soil for a 24 hour period before being sealed with brass long term storage end caps. The plants were then harvested with the *O. sulcatus* larvae recovered from the soil, counted and weighed, and the above and belowground portions of the plants were snap frozen in liquid nitrogen and then freeze dried to enable the recording of dry mass. Arbuscular mycorrhizal colonisation of roots was assessed using the gridline intersect method (McGonigle *et al.*, 1990) after being stained with Trypan blue (see general methods 2.1.7).

3.2.2 Data analysis

The data was then analysed with the statistical package R (version 3.1.2). The plant growth data, that was collected periodically, was analysed using a repeated measures analysis of covariance which compared the response variables; plant height and leaf number against the explanatory variables; mycorrhizal presence, number of *O. sulcatus* eggs added, experimental block and initial plant growth, as co-variants and time as an error function.

The data that were taken after the experimental harvest was analysed using an ANOVA to compare the response variables; biomass, *O. sulcatus* mortality and *O. sulcatus* mass against the explanatory variables; mycorrhizal presence, number of *O. sulcatus* eggs added, *R. idaeus* cultivar and the covariate, experimental block**.**

The VOC samples captured from the soil earlier in the experiment were then processed using the methods outlined in the general methods sections 2.1.14 and 2.1.15. The extracted data was then analysed using a principal component analyses and identified compounds of particular relevance were incorporated into generalised linear models (GLMs) with all the explanatory variables recorded in the experiment.

3.3 Results

3.3.1 Plant growth data

The major factor affecting the recorded size metrics of *R. idaeus* throughout the duration of the experiment was the cultivar. Glen Ample plants were taller but with fewer leaves, which were on average larger (Table 1) than those of Glen Rosa (Figures 1, 2 and 3). In each case the initial height, leaf number or leaf size recorded at the beginning of the experiment was included as a covariate in the model, so differences recorded were not just due to initial plant size. To further explore the variation within the two cultivars used in this experiment the data was split into two sub-sets, one for each cultivar. The significant interaction between the *O. sulcatus* treatment and the *R. idaeus* cultivar (Table 1) was due to Glen Ample plants being taller in the high density, 40 egg, *O. sulcatus* treatment (Figure 6). The estimated area of the largest leaf also showed this trend with increased size in the presence of *O. sulcatus* larvae in the larger density 40 egg treatment (Figure 7). A significant interaction between cultivar and AM fungal treatment when the number of leaves per plant were investigated was uncovered. This was explained by the number of leaves on Glen Rosa being influenced by the AM fungal treatment, with AM fungal control plants having more leaves (Table 1) at every time point in the experiment (Figure 8).

Table 3.1: Results of repeated measures ANOVAs on R. idaeus measurements taken throughout the experiment.

Parameter	Measurements taken over time					
	Plant height		Number of leaves		Estimated leaf area	
	F	*P*	*F*	*P*	*F*	*P*
O. sulcatus treatment	10.28	<0.001	3.033	0.04	3.05	0.05
AM fungal treatment	3	0.08	21.34	<0.001	14.18	<0.001
R. idaeus cultivar	331.684	<0.001	6.4	0.01	128.8	<0.001
*O. sulcatus**AM fungi	3.73	0.035	6.664	0.001	11	<0.001
*O. sulcatus**cultivar	8.282	<0.001	0.78	0.4	8.1	<0.001
AM fungi*Cultivar	0.896	0.344	5.13	0.02	0.13	0.9
Experimental block	17.38	<0.001	3.78	0.005	9.86	<0.001
Initial plant height	25.31	<0.001				
Initial leaf number			65.19	<0.001		

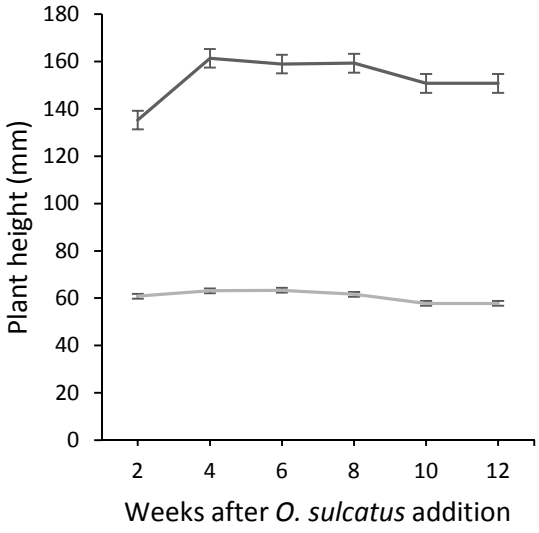


Figure 7: *R. idaeus* of the Glen Ample (dark grey) cultivar were taller throughout the experiment than Glen Rosa (light grey).

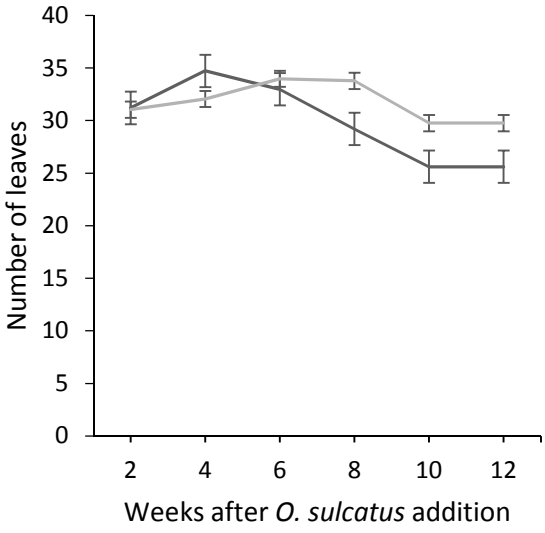


Figure 8: *R. idaeus* of the cultivar Glen Rosa (light grey) had more leaves over time than Glen Ample (dark grey).

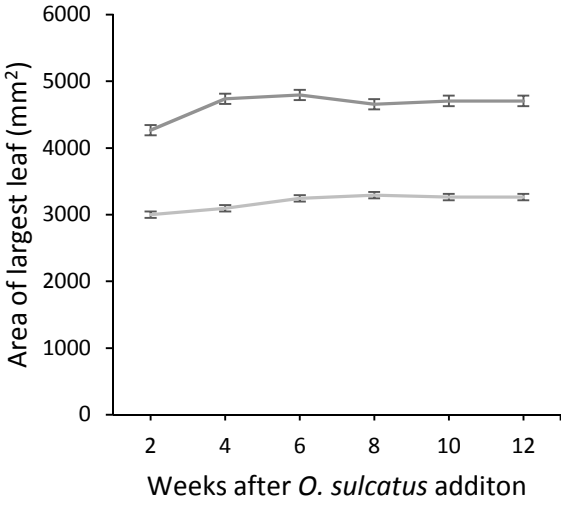


Figure 9: The area of the largest leaf on each plant was significantly different in the two cultivars tested; Glen Ample (dark grey) leaves being significantly larger than Glen Rosa (light grey).

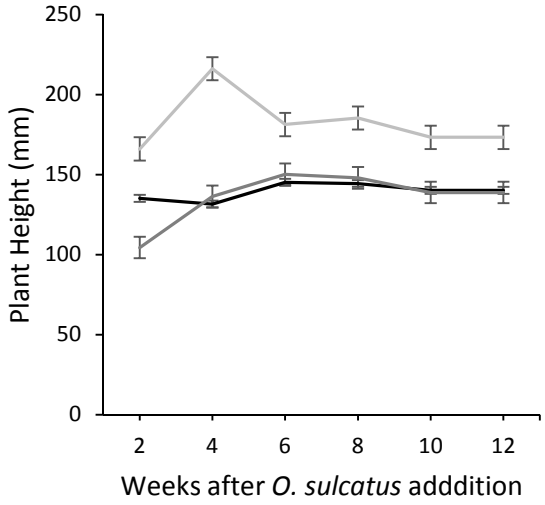


Figure 6: *O. sulcatus* egg density had a significant influence on the height of Glen Ample over time. Control treatment shown in black, 20 egg treatment in dark grey and 40 egg treatment in light grey.

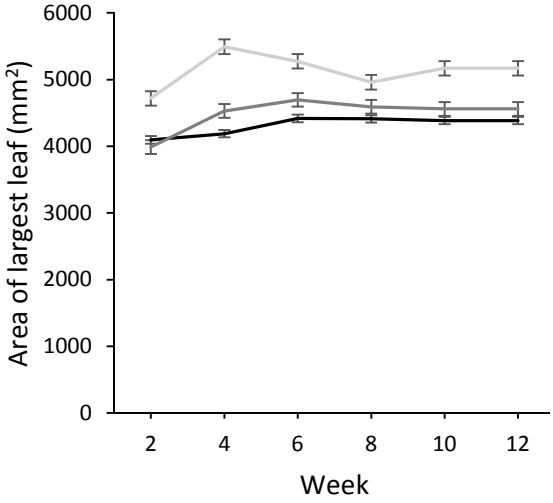


Figure 7: The *O. sulcatus* egg density added at the beginning of the experiment had a significant influence on the estimated area of the largest leaf on the Glen Rosa plants. Control treatment shown in black, 20 egg treatment in dark grey and 40 egg treatment in light grey.

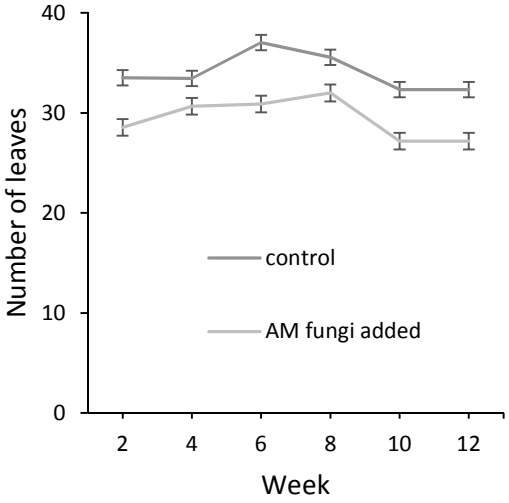


Figure 8: The AM fungal treatment (light grey) added to Glen Rosa plants caused a decrease in the number of leaves the plant grew when compared to the control (dark grey) treatment.

3.3.2 Herbivory and biomass data

After the experiment was harvested the biomass of the plant was recorded. The total biomass was not explained by either the mycorrhizal treatment, the number of *O. sulcatus* eggs added or the *R. idaeus* cultivar. However, when above and below ground biomass were tested separately using a root to shoot ratio as a response variable there was a clear effect of the *O. sulcatus* treatment. This root to shoot ratio was significantly different ($F_{2,98}$=7.78, P <0.001)

under the different *O. sulcatus* egg densities with a higher proportion of biomass distributed to the aboveground portion of the plant in the two treatments where *O. sulcatus* were added when compared to the insect free control treatment (Figure 13). The mortality of *O. sulcatus* larvae was found to be significantly higher ($F_{1,28}$=16.20, P<0.001) in the 40 egg treatment than in the 20 egg treatment (see Figure 10). The mortality of *O. sulcatus* larvae did not appear to be affected directly by the AM fungal treatment or by differences between cultivar biomass. The larval masses of *O. sulcatus* recorded was not found to be affected by the AM fungal treatment, *O. sulcatus* density or the *R. idaeus* cultivar.

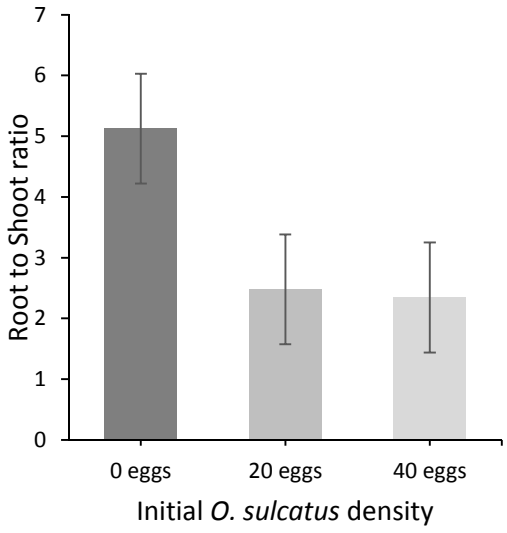


Figure 13: The Root to shoot ratio was different in the three *O. sulcatus* densities with the control treatment showing a higher distribution of biomass in the roots when compared to the two herbivory treatments.

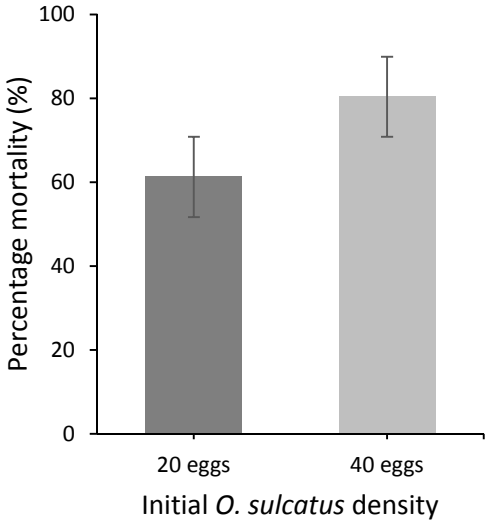


Figure 14: The percentage mortality of *O. sulcatus* larvae between the beginning and the end of the experiment was higher in the 40 egg density.

3.3.3 Mycorrhizal colonisation data

The percentage root length colonised by AM fungal structures indicated that the treatment in which AM fungi were added to plants exhibited much higher levels of colonisation (F=$_{1,20}$=20.81, P <0.001) than control treatments (Figure 15). When the individual structures were analysed separately it was found that hyphal colonisation and vesicle colonisation mirrored the overall trend ($F_{1,20}$=21.03, P <0.001 and $F_{1,27}$=5.03, P <0.05 respectively) but the presence of arbuscule formation was higher ($F_{1,27}$=10.04, P <0.001) in the Glen Ample cultivar when compared to Glen Rosa (Figure 16).

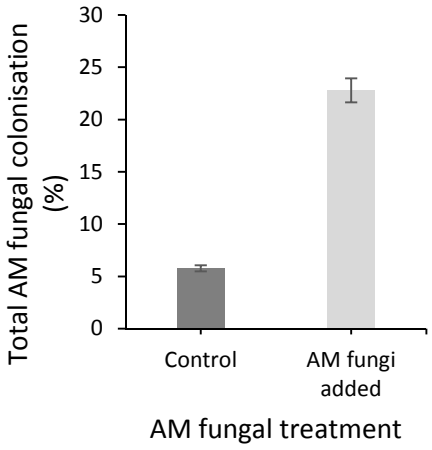


Figure 15: Total colonisation of roots was significantly greater in the treatment where AM fungi were added.

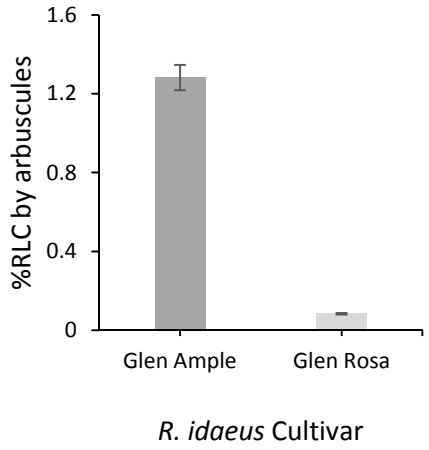


Figure 16: The formation of arbuscules was found to be higher in the Glen Ample cultivar than in Glen Rosa.

3.3.4 Volatile organic chemistry data

The compounds captured from the root zone in this experiment did not produce any useful results after principal component analysis and none of the identified compounds that were shown in the literature to elicit an attractant response in EPNs correlated with the AM fungal treatments. However, *α*-pinene and carene, both known semiochemicals, that have been previously shown to elicit such a response (Rasmann *et al.*, 2012a) were identified. The compound *α*-pinene was present in 58.82% of the samples, with large peaks, the areas of which comprised on average 30.94% ± 5.9SE of the total peak area of the chromatographs, making it the most abundant and ubiquitous compound within the range of detected and identified known semiochemicals. Secondly carene was detected in 52.94% of samples and comprised on average some 14.94% ± 2.6SE of the total peak area. It is worth mentioning that due to a large number of compounds falling outside the 80% threshold, for identification via the NIST library (details in 2.1.15), that duplicated compounds within a similar mass range may still exist in the data set. For the same reason it may be that there other un-identified VOCs that may be documented in the literature as being semiochemicals. The large peaks detected for some compounds, such as *α*-pinene represent very large outliers in the data set, the presence of which make the output of a PCA unreliable. The removal of such outliers for the purpose of re-analysis was not advisable as they represented a large proportion of the total peak area, and also known semiochemicals. The raw data used in this analysis can be found in digital appendix 1.

3.4 Discussion

In order to determine how different *R. idaeus* cultivars respond to *O. sulcatus* infestation plant metric data was collected throughout the experiment and then plant biomass was calculated at the end of the experiment. The main findings from the plant metrics collected over time was the strong differences in growth patterns observed between the two *R. idaeus* cultivars. Glen Ample was much taller with larger leaves than Glen Rosa which was a shorter, slower growing plant with more numerous but smaller leaves. This fits very well with the information available from the plant breeders about these two cultivars. Glen Ample has the higher yield and larger, sweeter fruit and is favoured commercially. Glen Rosa however is more tolerant to pests and diseases. It has the A_{10} resistance gene for protection against the large raspberry aphid, has smaller fruit and typically produces smaller yields when compared to Glen Ample (Hall *et al.*, 2008). A more vigorous growth is typically associated with Glen Ample leading to higher average biomass than in Glen Rosa (Hall *et al.*, 2008) which was not recorded directly in this experiment as both cultivars showed similar biomass but there is a pattern to indicate this seen in plant height and leaf size data.

It is when these two cultivars of *R. idaeus* are investigated separately that further details on their responses to AM fungi and herbivory treatments become apparent. In Glen Ample the higher density, 40 egg treatment resulted in significantly taller plants. This might suggest, at least when only looking at plant height, that these plants were exhibiting some form of over-compensatory growth in response to this high level of herbivory (McNaughton, 1983). A similar pattern was observed in Glen Rosa plants with the area of the largest leaf being significantly larger on plants exposed to the higher, 40 egg *O. sulcatus* treatment. Again, increased growth could be considered as an indication of a stressed plant that is exhibiting over-compensatory growth. However this is not borne out when the biomass data were taken into account. The biomass data collected reveals that there is no significant difference in biomass between herbivory treatments. Instead, it is the pattern of resource allocation that changes in the plant. The root to shoot ratio indicates that in the two *O. sulcatus* treatments a higher proportion of biomass is found in the aboveground portion of the plant when compared to control plants. This has been shown to occur as a response to root herbivory in *Zea mays*, and using radioactive $^{11}CO_2$ it was shown that this was an effect of carbon reallocation from roots to stem tissues (Robert *et al.*, 2014). A similar effect showing that plants allocate nitrogen away from root herbivores (Newingham *et al.*, 2007) has been shown in *Centaurea maculosa*. Although neither of these plants are closely related to *R. idaeus* these examples do provide explanations for how plants can respond to root herbivory which might explain the patterns observed in *R. idaeus* biomass.

Another observation made using plant metric data was that Glen Rosa plants had significantly fewer leaves in treatments where AM fungi was added. This was an unexpected result as commonly AM fungi are linked with an increase in biomass (Smith & Read, 2008). This could be another effect of AM symbiosis resulting in altered resource distribution, as this was not an effect that was found to be represented in overall biomass.

The initial density of *O. sulcatus* added to plants had a significant impact on the mortality of these populations. At higher densities, a significantly higher mortality was found. This is likely due to a greater competition for limited food resources. This is in accordance with the findings of La Lone and Clarke (1968) who found, while studying *O. sulcatus* larvae feeding on potted rhododendrons, that mortality of larvae increased with increasing density and that mortality was highest in early instars, findings mirrored by Gange (1996). The performance of *O. sulcatus* was not however affected by AM fungal treatments despite previous studies on *Fragaria ananassa* showing a decrease in larval performance (Gange *et al.*, 1994; Gange, 1996, 2001). While the majority of these studies used only single species of mycorrhizal inocula. Gange (2001) showed that when a mixture of two species was added then this effect on larval survival was lost. As this experiment used a mixed mycorrhizal inocula in order to mimic field conditions and a different plant species, it is perhaps unsurprising that these effects on larval performance were not observed.

The percentage root length colonised by AM fungi was higher in plants to which live AM fungal spores had been added and was very low or absent in untreated control plants. Overall this suggests that the surface sterilisation and subsequent inoculation of plants was a success. This low level of colonisation in control plants is likely to be due to residual AM fungal material in *R.idaeus* root material or it could have been material that survived soil autoclaving. Plants were separated by a minimum of 20cm so as to minimise the occurrence of soil splashing between pots of different treatments during watering but this is still a possibility. It should also be noted that a “microbe free plant” is neither attainable nor a necessarily useful baseline comparison as plants never exist in the absence of microbes (Partida-Martínez & Heil, 2011).

Another discovery when analysing the root colonisation data was the differing levels of arbuscule colonisation in the two *R. idaeus* cultivars tested. Arbuscule colonisation was on average low in both cultivars, which is not to say that this level of colonisation was not sufficient for the plant to receive a detectable benefit in plant performance (Gange & Ayres, 1999). While arbuscule colonisation was low, it was significantly lower in Glen Rosa plants than in Glen Ample plants. This is interesting as it shows that even closely related cultivars, can possess differing mycorrhizal affinity. This is a large area of research at the moment and breeding traits such as mycorrhizal affinity back into highly productive lines is of great interest

to plant breeders in order to move towards more sustainable agriculture with fewer inputs. This is of increasing need as the global availability of mineralised phosphorus continues to decline.

The VOCs collected via ATD-GC-MS were quantified and then identified, but those collected in the experiment did not correlate significantly with the experimental treatments. However a number of chemicals were identified that are frequently associated in entomopathogenic nematode ecology literature as semiochemicals governing chemotaxis towards insect hosts such as *O. sulcatus* (Rasmann *et al.*, 2012a). In the chromatographs produced by this experiment, the largest and most ubiquitous peak, was that of *α*-pinene. This compound has been previously identified as a subterranean herbivore-induced VOC when isolated from citrus roots (Ali *et al.*, 2010, 2011) as well as being shown to vary in emission levels with respect to AM colonisation (Rapparini *et al.*, 2008). It is worth noting that both *α*-pinene and *β*-pinene have been found previously in several other *R. idaeus* varieties (Aprea *et al.*, 2009) so its presence alone, both as a plant VOC emission and as a *R. idaeus* metabolite is well established. Due to the soil based sampling method used, it is not possible to determine if the emissions were detected from the plant via some kind of herbivore induced pathway or if these compounds were in fact also produced directly by *O. sulcatus* larvae feeding on *R. idaeus* roots. *Galleria mellonella,* an insect which is very susceptible to EPNs has been shown to produce both hexanal and alpha pinene and both these compounds stimulated a jumping response in the EPN *Steinernema carpocapsae* and to a lesser extent chemotaxis (Hallem *et al.*, 2011). It could be a peculiarity of *G. mellonella* which in turn could be the reason for its pronounced susceptibility to EPNs but we cannot rule out that this is a trait that *O. sulcatus* may have in common which may aid any biological control programme that aims to incorporate or enhance the effects of root herbivore induced VOCs that attract EPNs.

3.5 Conclusions

The main findings in this chapter were that both *R. idaeus* cultivars tested showed different growth patterns in response to AM fungal and *O. sulcatus* treatments. However both Glen Ample and Glen Rosa showed the same resource reallocation tolerance response to *O. sulcatus* herbivory. This study formed an experimental framework for the later experiments in this thesis as it provided evidence that the root surface sterilisation and subsequent inoculation with AM fungi protocol worked with *R. idaeus*. In addition to this it also provided information on how the two cultivars of *R. idaeus* responded to different densities of *O. sulcatus.*

The VOCs captured during the experiment did not provide any evidence of any VOCs that may be root herbivore of AM fungi induced, but perhaps with a greater level of compound identification this data set could provide evidence for these effects.

To build upon the information gathered in this experiment, new experiments were devised. An additional component was added to this tri-trophic system, in the form of EPNs. The interaction of these EPNs with the other components of the system were studied in two settings. The first was a 'no choice' experiment on *R. idaeus* to establish how effective different EPNs were at *O. sulcatus* control in the presence of AM fungi (Chapter 4). The second was an investigation into the preferences of EPNs when confronted with *R. idaeus* with different AM fungal and *O. sulcatus* treatments (Chapter 5).

4 Combining entomopathogenic nematodes and resistant cultivars to reduce *Otiorhynchus sulcatus* performance.

4.1 Introduction

Plants interact with a myriad of soil organisms, ranging from those that can be broadly described as mutualistic (e.g. mycorrhizal fungi and nitrogen fixing bacteria) to those that have detrimental effects on plant performance (e.g. herbivores and pathogens) (Gregory *et al.*, 2009). In addition to having a mutualistic relationship with soil microbes, more recent work suggests that plants have mutualistic relationships with invertebrate natural enemies of root herbivores, most notably EPNs (San-Blas, 2013). In particular, a number of studies have shown that plants under attack by root herbivores recruit EPNs, often by volatile cues released from the roots (Rasmann *et al.*, 2005; Ali *et al.*, 2010, 2012). EPNs infect root-feeding insects in their infective juvenile stage by penetrating the insect's cuticle or entering via orifices (Kaya & Gaugler, 1993).

Symbioses between AM fungi and vascular plants are extremely common with such associations occurring in around 70% of plant taxa (Hodge, 2000). Plants typically trade sugars

with AM fungi in exchange for phosphorus with this interaction varying in the degree of mutualism observed (Bever *et al.*, 2001; Smith & Read, 2008). As well as improved nutrient uptake, plants show a variety of other traits while in symbiosis with AM fungi. For example, there are numerous examples of AM fungi altering the performance of herbivorous insects feeding on shoots, either directly (Bennett, Alers-Garcia & Bever 2006; Koricheva, Gange & Jones 2009) or indirectly, via effects on the higher trophic levels, such as parasitoids (Gange, Brown & Aplin 2003). Despite sharing the same part of the plant (i.e. the roots), surprisingly few studies have addressed how AM fungi affect root herbivores, though there is ample scope for direct interaction and potentially an adaptive advantage to both fungi and plants in resisting root herbivory (Johnson & Rasmann, 2015). Indeed, of the eight studies reporting effects of AM fungi on root herbivores listed by Johnson and Rasmann (2015), all but one reported highly negative impacts of AM fungi on root herbivores.

Given that AM fungi and EPNs are both detrimental to root herbivores when studied individually, this raises the intriguing prospect that these two plant mutualists could work in concert to help host plants resist attack by root herbivores. To our knowledge, no previous studies have addressed whether AM fungi and EPNs synergistically affect the performance of root-feeding insect pests, despite the clear potential for combining these interactions to control a number of economic pests (Blackshaw & Kerry, 2008). While there are no studies investigating the interaction of AM fungi with EPNs, antagonistic interactions between AM fungi and plant parasitic nematodes have been reported (Elsen *et al.*, 2008). It could be hypothesised, however, that this antagonism would not occur with EPNs, given that they confer protection to the roots, and by implication AM fungi. AM fungi could facilitate EPN efficacy against root herbivores in a least two ways. Firstly, most AM fungi negatively affect root herbivore performance (Johnson & Rasmann 2015), which could render herbivores more vulnerable to EPN attack and penetration. Secondly, AM fungi have been shown to alter the volatile profile of plants (Rapparini *et al.*, 2008) which could also be true of volatiles released from the roots, including those attracting EPNs. Schausberger *et al.* (2011), for example, demonstrated that AM fungi changed the composition of herbivore-induced plant volatiles (caused by feeding by *Tetranychus utrticae*) emitted by the plant, which recruited the herbivore's natural enemy, *Phytoseiulus persimilis*. The use of mutualistic fungal associations with plants to supress shoot herbivores has been mooted (Vannette & Hunter, 2009; Pineda *et al.*, 2010), but their potential to synergistically interact with EPNs remains an, as yet, untapped management option against root herbivores.

O. sulcatus is a good model for testing the hypothesis that AM fungi and EPNs may work in tandem to supress root herbivore populations. There is evidence that plants form mutualistic

associations with both organisms individually to resist attack by *O. sulcatus*. AM fungi are known to reduce the performance of *O. sulcatus* (Gange, Brown & Sinclair 1994; Gange 1996; Gange 2001) and *Thuja occidentalis* roots attacked by *O. sulcatus* release attractant cues to the EPN, *Heterorhabditis megidis* Van Tol *et al.* (2001). *O. sulcatus* is a major pest of horticultural and nursery crops across temperate zones and causes significant damage to a wide range of host plants (Moorhouse, Charnley & Gillespie 1992). Adult *O. sulcatus* cause minor damage to the leaves of plants, while larvae feed on roots and cause significant reductions in plant vigour and yield (Penman & Scott, 1976). In a four year field study, Clark *et al*. (2012) reported that heavy infestations of *O. sulcatus* reduced yield by 39% and 66% in Glen Rosa and Glen Ample *Rubus idaeus* varieties, respectively. Traditionally, chemical control of *O. sulcatus* was achieved using Aldrin, but this pesticide was withdrawn from use in 1990. Current methods typically entail application of a neonicotinoid (Imidacloprid) or an oganophosphate (Chlorpyrifos), but the former has been suspended under recent EU legislation and both are damaging to non-target organisms, including many beneficial organisms (Gill *et al.*, 2001). In addition to the undesirable ecological impacts of these chemicals, many of the horticultural crops affected by *O. sulcatus* have moved to insecticide-free production because of consumer demand (Gordon *et al.*, 2006), so sustainable and environmentally sound control measures are urgently needed.

This study aimed to determine how root herbivore natural enemies (two EPN species) and AM fungal colonisation of the plant affect root herbivore performance. The study system used two cultivars of *R. idaeus*, known to be highly (Glen Ample) and moderately (Glen Rosa) susceptible to *O. sulcatus* (Clark *et al.*, 2012). *R. idaeus* is a small, but high value crop, known to be mycorrhizal (Taylor & Harrier, 2000) and widely attacked by *O. sulcatus* (Alford, 2007). The two EPN species incorporated into the experiment are both widely recommended and commercially available specifically for use against *O. sulcatus* (Haukeland & Lola-Luz, 2010). *Steinernema kraussei* Steiner is cold tolerant, active at <10°C, whereas *H. megidis* is active at >10°C, both are known to alter their dispersal and taxis depending on the substrate they are in and they possess different bacterial endosymbiont communities (Forst *et al.*, 1997; Kruitbos *et al.*, 2010; Ansari & Butt, 2011). These two species contrast well with one another and should provide an interesting comparison. *Otiorhynchus sulcatus* has a history, particularly in the horticultural sector, of being treated with a range of EPNs which creates a convenient multi-trophic system in which to study potential interactions. To assess how the different EPN treatments influenced *O. sulcatus* mortality and performance we proposed two hypotheses. First that EPN treatments would decrease *O. sulcatus* abundance and secondly that EPN treatments would lower *O. sulcatus* larval mass. Plant biomass was calculated at the end of the experiment to quantify the plant response to EPN treatments. We hypothesised that EPN treatments on plants infested with *O.sulcatus* would promote an increase in plant biomass.

Carbon allocation in *R. idaeus* was investigated as a response to herbivory. The root to shoot ratio of plant biomass was used as an index of changes in carbon allocation in response to herbivory. We predicted that the EPN treatments and subsequent abundance of *O. sulcatus* would influence the root to shoot ratio in *R. idaeus* cultivars.

To assess the influence of EPN and cultivar treatments on AM fungal colonisation a series of hypotheses were tested. We tested the assumption that different cultivars of *R. idaeus* would exhibit different levels of root length colonisation by mycorrhizal structures (arbuscules, vesicles or spores) based on evidence in other systems (Hetrick *et al.*, 1993; Zhu *et al.*, 2001). We then tested the assertion that the percentage of root length colonised (%RLC) by mycorrhizal structures (arbuscules, vesicles or spores) would increase EPN efficacy. Based on previous observations by other authors (Treseder, 2013) we hypothesised that the %RLC would be positively correlated with plant biomass. Finally based on work conducted on strawberry which indicated that AM fungi could impair the performance of *O. sulcatus* larvae (Gange, 1996) we predicted that the %RLC would be negatively related to *O. sulcatus* larval mass

4.2 Materials and Methods

4.2.1 Study system

Rootstock from existing *R. idaeus* plants of two cultivars; Glen Ample and Glen Rosa were prepared according to the methods outlined in section 2.1.1. Following this, 78 individual plants, 39 of each cultivar, were transplanted into 1.8L pots containing 1.6L of a twice sterilised 1:1 soil (Keith Singleton sterilised loam, Nethertown, Cumbria) and sand mix. The 39 plants of each cultivars were equally and randomly distributed between the 3 treatments giving a replication of 13 plants for each treatment. The plants were then incorporated into a randomised block design for the duration of the experiment. Two weeks after the plants were transplanted, and before any herbivore or EPN treatments were added, plant height was recorded in order to be used later as a covariate in statistical models to account for the initial variation in height between plants.

O. sulcatus eggs were taken from a pre-existing culture (cultured as described in chapter 2.1.8). The EPNs used in the experiment were purchased from commercial suppliers and were advertised as being a specific lines to control for *O. sulcatus. S. kraussei* (Becker and Underwood®, Littlehampton, UK) and *H. megidis* (Biobest®, Milton Bridge, UK). They were

both added to plants as separate treatments at their recommended dosages. This worked out as approximately 9000 *S. kraussei* added per pot and approximately 16000 *H. megidis* added to each pot.

4.2.2 Experimental setup

A 2 x 3 factorial experiment was conducted under controlled conditions (16:8 days at 18˚C), with two different *R. idaeus* cultivars (Glen Ample and Glen Rosa) and three different EPN treatments (a control treatment, and addition of *S. kraussei* or *H. megidis*).

Five weeks after the re-potting of *R. idaeus* cultivars, 40 *O. sulcatus* eggs were added into a 10mm indent in the soil surface, 20mm away from the stem of each plant. This egg density was selected to simulate arrival of a gravid adult feeding on plants for several weeks (Clark et al. 2012a). Four weeks after plants were infested with *O. sulcatus*, EPNs were added to plants, with control plants remaining untreated. Three weeks after nematodes were added, the plants were harvested and *O. sulcatus* larvae were retrieved, counted and fresh mass taken. Plants were then freeze dried to ascertain dry mass. In chapter 3.3 it was noted that although the *R. idaeus* rootstock was surface sterilised, even in control treatments using sterile soil, there was a low level of AM fungal colonisation in roots. For this reason, although no live AM fungal spores were added to plants, it was considered worthwhile assessing the colonisation of AM fungi at the end of the experiment. Roots were stained and mycorrhizal colonisation of roots was assessed using methods outlined in 2.1.62.1.5 and 2.1.72.1.7 but differed in that hyphal colonisation was not recorded separately when other features were identified. The root scoring carried out in this chapter was conducted by Alison E. Bennett (The James Hutton Institute, Dundee, UK). All results are reported at percentage root length colonised (%RLC).

4.2.3 Statistical Analyses

The mean mass and abundance of *O. sulcatus* larvae on each plant was analysed using generalised linear models (GLMs) incorporating Gaussian and Poisson errors respectively. These response variables were tested against the cultivar and EPN treatment and the interactions between the two. Experimental block and initial plant height were included as covariates. In order to simplify the models, the two cultivar treatments were then analysed separately. The biomass data taken from the dry mass of *R. idaeus* plants and the AM fungal colonisation data was then analysed using ANCOVAs using nematode treatment, *O. sulcatus* abundance and mean *O. sulcatus* mass as explanatory variables with experimental block incorporated as a covariate. All analysis was carried out using R3.1.2 ‘Pumpkin Helmet’ (R Core

Team, 2013) and models were simplified where appropriate with the best fitting minimal models reported.

4.3 Results

4.3.1 Insect herbivore performance

O. sulcatus abundance (Figure 4.1) and mass (Figure 4.2) was significantly lower on Glen Rosa than Glen Ample ($t_{1,65}$= -2.17, P <0.05, $t_{1,65}$=-2.39, P <0.05 respectively) and because of this the two cultivars were subsequently analysed separately to look at them both with a greater resolution. It should be noted that a very small number of dead *O. sulcatus* larvae were retrieved at the end of the experiment (two individuals), due to the small number retrieved, these larvae were not included in analyses. In Glen Rosa, the addition of *S. kraussei* caused *O. sulcatus* abundance to be much lower ($t_{2,35}$=-2.70, P <0.05) than in any other treatment (Figure 4.1).The larval mass of *O. sulcatus* on Glen Rosa Plants was not influenced by the nematode treatment (Figure 4.2).

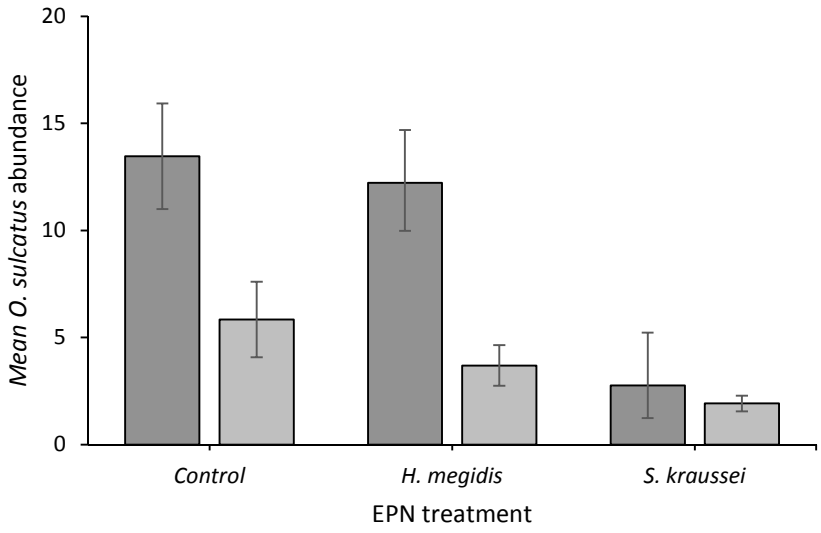


Figure 4.1: Mean *O. sulcatus* abundance, per plant, in different EPN treatments. Dark grey bars represent Glen Ample and light grey bars represent Glen Rosa plants.

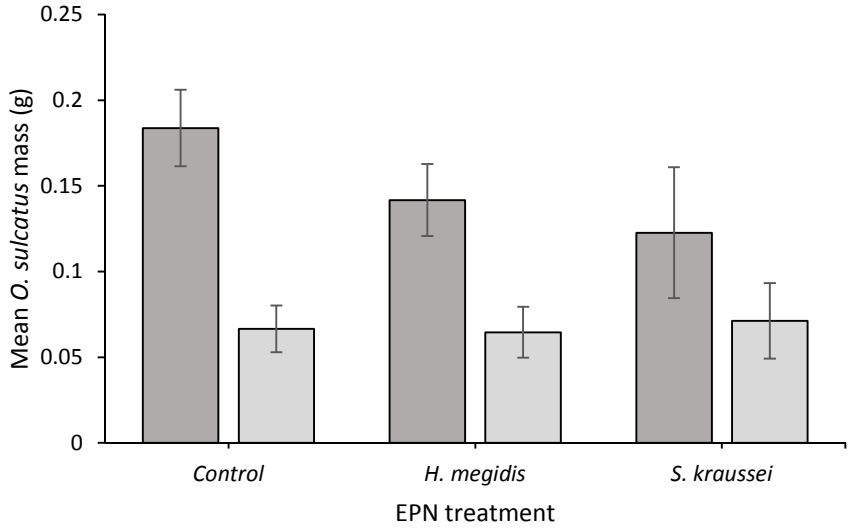


Figure 4.2: *O. sulcatus* larval mass on *R. idaeus*. Dark grey bars represent Glen Ample and light grey bars represent Glen Rosa plants.

In Glen Ample, the abundance and mass of *O. sulcatus* larvae were both reduced in treatments where *S. kraussei* ($t_{2,32}$=4.36, P <0.001) were added (Figure 4.3).

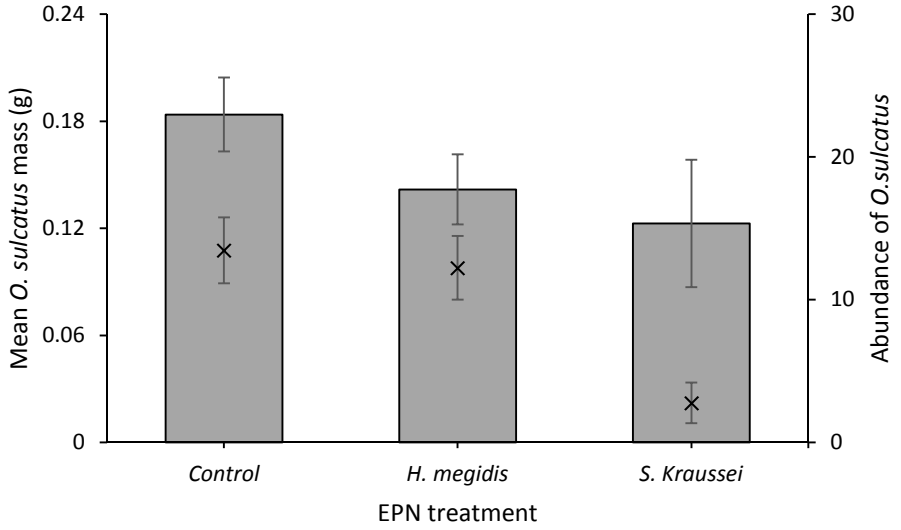


Figure 4.3: *O. sulcatus* larval mass and abundance, per plant, on Glen Ample. Grey bars represent mean *O. sulcatus* mass, and correspond to the left hand axis and the black points represent *O. sulcatus* abundance and corresponds to the right hand axis.

4.3.2 Plant biomass data

There were no significant differences between the biomass of the two *R. idaeus* cultivars tested. All the variation in whole plant biomass recorded at the harvest of the experiment was explained by the initial size of the plant at the beginning of the experiment ($t_{1,52}$= 6.13, P <0.001), with no significant effects of treatment conditions. To represent this, the non-

destructive measurement of initial plant height was added into the model as a covariate (Figure 4.4).

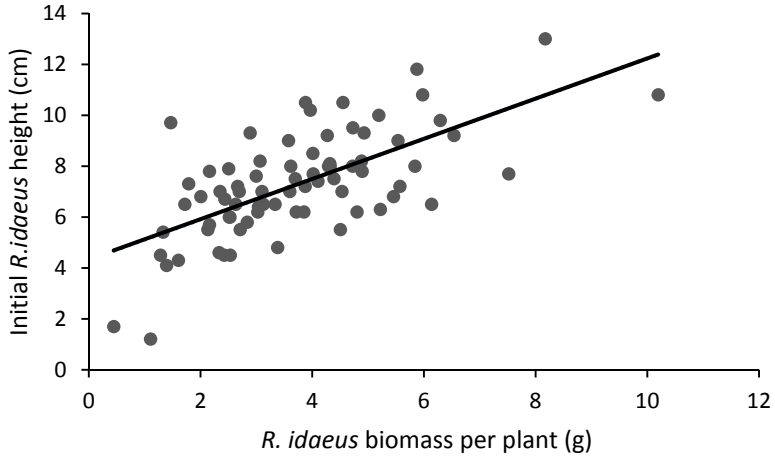


Figure 4.4: The initial plant biomass of *R. idaeus* at the beginning of the experiment plotted against the whole plant biomass recorded at the end of the experiment.

The root to shoot ratio was calculated using the biomass data from both Glen Rosa and Glen Ample. No patterns of above-belowground biomass were observed in Glen Rosa (Figure 4.5).

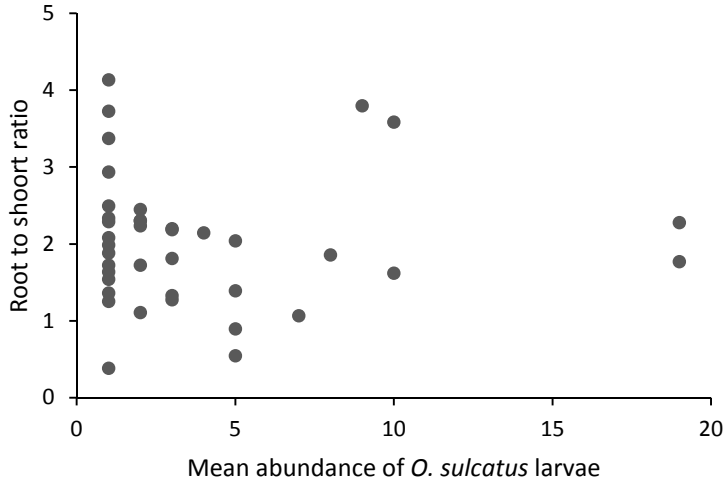


Figure 4.5: The relationship between root to shoot ratio of Glen Rosa and the mean abundance per plant of *O. sulcatus* larvae.

In Glen Ample a negative relationship was observed between the root to shoot ratio and the abundance of *O. sulcatus* larvae ($F_{1,32}$= 8.58, P <0.01). This showed that there was a higher proportion of above ground biomass, relative to belowground biomass when there were higher numbers of root feeding *O. sulcatus* larvae (Figure 4.6).

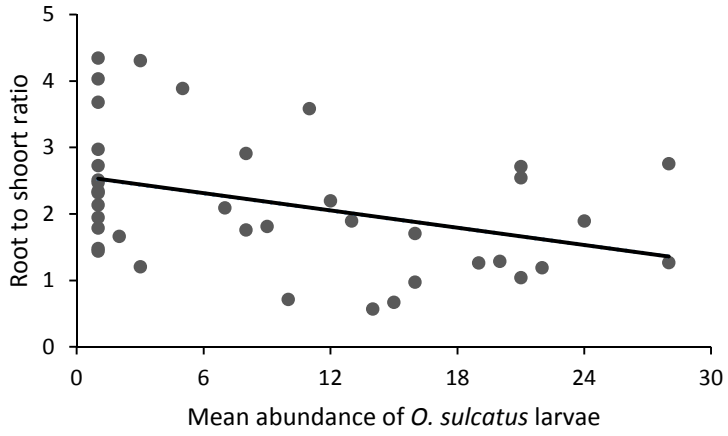


Figure 4.6: The relationship between root to shoot ratio of Glen Ample and the mean abundance per plant of *O. sulcatus* larvae.

4.3.3 Arbuscular mycorrhizal fungal colonisation data

When the %RLC by AM fungal features was analysed it was found that the distribution of these structures was quite variable across experimental treatments (Table 4.1)

Table 4.1: A summary of the percentage of root length colonised (%RLC) by different AM fungal structures.

Cultivar	Nematode treatment	Mean Arbuscular colonisation (%RLC with SE)	Mean Vesicle colonisation (%RLC with SE)	Mean Spore colonisation (%RLC with SE)
Glen Ample	control	38.0 ± 6	19.1 ± 5	27.3 ± 4
	H. megids	36.1 ± 7	22.3 ± 6	24.0 ± 6
	S. kraussei	57.0 ± 8	11.0 ± 4	35.0 ± 9
Glen Rosa	control	15.0 ± 6	4.0 ± 2	4.0 ± 4
	H. megids	14.0 ± 7	5.0 ± 4	5.4 ± 4
	S. kraussei	11.0 ± 7	4.0 ± 3	4.0 ± 4

It is clear from this table that there exists a big difference between the %RLC of root material of the two *R. idaeus* cultivars, with Glen Ample showing much higher levels of colonisation ($F_{1,30}$= 122.83, P < 0.001) than Glen Rosa (Figure 4.7).

Figure 4.7: The %RLC by all AM fungal features combined in two *R. idaeus* cultivars.

After it was discovered that the two cultivars tested had very different relationships with AM fungi the two cultivars data sets were then split and analysed separately. In Glen Rosa it was found that the %RLC by arbuscules had a positive relationship ($t_{1,31}$= 3.55, P <0.01) with the mean *O. sulcatus* larval mass recorded per plant (Figure 4.8). The other AM fungal structures showed no significant relationships with either the experimental treatments or the covariates included, such as *O. sulcatus* abundance, *O. sulcatus* larval mass and plant biomass.

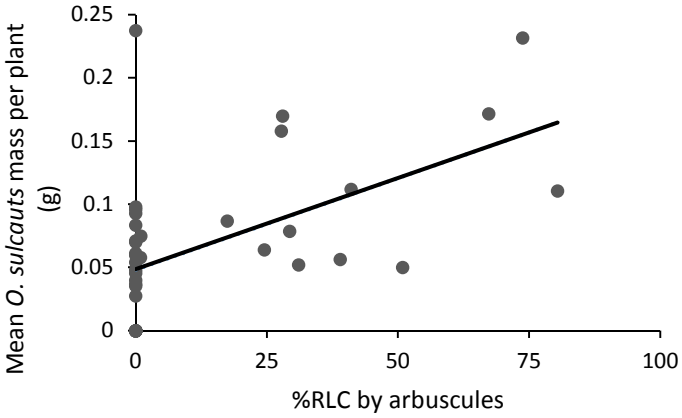


Figure 4.8: The relationship between the mean mass of *O. sulcatus* on each Glen Rosa plant and the %RLC colonised by arbuscules.

In Glen Ample it was found that the %RLC by arbuscules a positive relationship with the individual plant biomass and the different EPN treatments added to experimental plants (Figure 4.9). The amount of arbuscule colonisation and biomass were found to both be significantly higher in plants where *S. kraussei* were added ($t_{2,32}$= -2.14, P <0.05). Colonisation of other AM fungal structures showed no significant relationships with either the experimental treatments or the covariates included, such as *O. sulcatus* abundance, *O. sulcatus* larval mass and plant biomass.

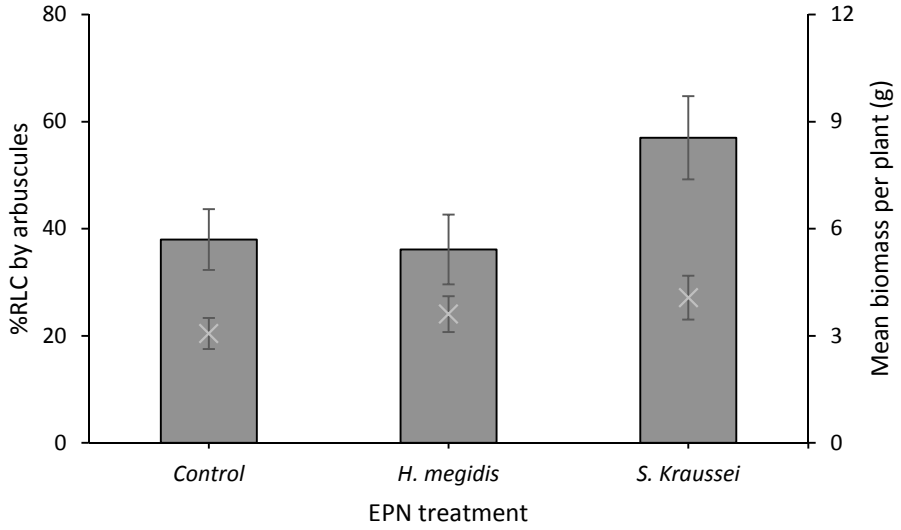


Figure 4.9: The relationship between the %RLC by arbuscules and the mean biomass Glen Ample plants. Dark Grey bars represent %RLC by arbuscules, and corresponds to the left hand axis and the light grey points represent *O. sulcatus* mass per plant and corresponds to the right hand axis.

4.4 Discussion

The comparison of two commercially available EPN species showed that *S. kraussei* was more effective at controlling *O. sulcatus* than *H. megidis* in both cultivars of *R. idaeus*, with the abundance and performance of *O. sulcatus* being substantially lower. Both these species are considered to be capable of cruise foraging, meaning they actively seek out hosts in the soil (Campbell *et al.*, 2003; Kruitbos *et al.*, 2010). The experiment was held at a constant 18°C meaning both species were operating within their optimal temperature range. Their contrasting performance could hence be due to other differences in behaviour and biology. There have been several studies that show soil media or substrate can have a significant effect on the dispersal behaviour of EPNs with different species showing greater taxis towards hosts in different media (Kruitbos *et al.*, 2010; Ansari & Butt, 2011). This could explain some of the variation between these species and consequently results may not be the same in the field. This said, *S. kraussei* has a lower cold tolerance (4°C) when compared to *H. megidis* (10°C) making it a better choice when treating plants at the beginning or end of a growing season (Haukeland & Lola-Luz, 2010). This is ideal for the protection of both Glen Ample and Glen Rosa, as these are both mid-season fruiting varieties, and the beginning of the season represents a period of critical growth, prior to flowering (Hall *et al.*, 2008).

O. sulcatus performed significantly better on Glen Ample plants than on Glen Rosa as shown in their larval mass, and this is supported by previous studies which found Glen Ample to be a

more susceptible cultivar when compared to other *R. idaeus* cultivars (Clark *et al.*, 2011a, 2012). This is likely due to the different traits bred into these two cultivars. Glen Ample is a more popular variety as it produces a higher yield of larger, sweeter fruit and is favoured commercially. Glen Rosa however is more tolerant to pests and diseases. It has been bred to have an A_{10} resistance gene which confers resistance to the large raspberry aphid but has smaller fruit and typically produces smaller yields when compared to Glen Ample (Hall *et al.*, 2008).

When *O. sulcatus* performance data was separated by cultivar an interesting effect was observed in Glen Ample plants that may hint at the reason for the difference in efficacy seen in the two EPN species. The interaction between *O. sulcatus* abundance and mass on Glen Ample plants showed that larval mass was reduced by a similar degree in both *H. megidis* and *S. kraussei* treatments. However it was only *in S. kraussei* treated plants where this fall in *O. sulcatus* mass was mirrored with a fall in abundance. This could be explained by *O. sulcatus* being infected by EPNs and this stress on their immune systems resulting in decreased performance, but not death. The level of tolerance to EPNs is known to vary greatly with some insect immune systems able to encapsulate and withstand up to 20 EPNs before the insect was killed (Thurston *et al.*, 1994). In *H. megidis* it would appear that this process of encapsulation is occurring, or perhaps the associated symbiotic bacteria, *Photorhabdus spp.*, is apparently not causing death by septicaemia normally associated with their infection of host tissues, unlike the *Xenorhabdus spp.* associated with *S. kraussei* (Dowds & Peters, 2002). This suggests that under the conditions of this experiment, *H. megidis* was behaving more like a parasite that a parasitoid. This is not a viable life strategy for an EPN as they must kill their hosts in order to complete their life cycle. It is possible that with more time, perhaps *H. megidis* may have proved more effective in killing *O. sulcatus* larvae but this would still make it a poor candidate for the biological control of a pest outbreak.

The plant height of *R. idaeus* taken just two weeks into the experiment was a reliable predictor for the biomass at the end of the experiment. Obviously the height of plants at this stage in the experiment could not have been affected by the subsequently added experimental treatments but it could have been affected by AM colonisation, which we assume at this point was already present in the transplanted rootstock. There was found to be no difference between the biomass of the two *R. idaeus* cultivars included in this study. This was unexpected, given the traits normally associated with these two *R. idaeus* lines. The more vigorous growth more typically associated with Glen Ample (Hall *et al.*, 2008) would usually lead to greater average biomass that in Glen Rosa which was not observed in this experiment as both cultivars showed similar biomass. It is feasible that Glen Ample was able to compensate for loss of biomass

despite a heavier *O. sulcatus* infestation as a consequence of its typically more vigorous growth but without an *O. sulcatus* free control we can only speculate on this.

In Glen Ample a negative relationship between root to shoot ratio and *O. sulcatus* abundance was observed. In short this means that when the population of *O. sulcatus* was higher, more carbon was being allocated away from *R. idaeus* roots and instead to the shoots of plants. This is the same pattern as was observed in chapter 3.4 and mirrored the effect seen in previous studies on plant response to root herbivory (Newingham *et al.*, 2007; Robert *et al.*, 2012). The fact that we have found evidence of this tolerance response in this cultivar again does suggest that this is a common response to root herbivory in Glen Ample.

Despite the *R. idaeus* rootstock being surface sterilised it was noted in chapter 3.3 that even in control treatments, using sterile soil, there was a low level of AM fungal colonisation in roots. For this reason, although no live AM fungal spores were added to plants, it was assumed that as it is impossible to sterilise a plant, that AM fungi would still be present in the root stock. It might be possible to avoid this source of contamination by growing all plants from seed but given the extra year that this would add to experiments, this was considered impractical, also *R. idaeus* is grown from rootstock commercially and so this allows us to better mimic field conditions. It is also worth noting that to create a totally sterile, microbe free plant, would create a highly contrived and artificial experimental model which would have no real-world parallels outside of other lab studies (Partida-Martínez & Heil, 2011). For this reason the possible presence of AM fungi was acknowledged and *R. idaeus* roots were stained and AM fungal colonisation recorded.

When these data were analysed it became clear that the two *R. idaeus* cultivars showed dramatically different levels of colonisation, with Glen Ample showing nearly 80% colonisation and Glen Rosa with only approximately 20% colonisation by AM fungi. The susceptible cultivar, Glen Ample, also showed a strong effect of increased carbon allocation away from roots and towards shoots in the root to shoot ratio analysis carried out. This resource allocation effect could be a possible explanation for the very high levels of %RLC recorded as stunted belowground growth leads to reduced elongation of roots due to growth, meaning that the density of mycorrhizal features was greater, the opposite of that often seen when a plant is growing rapidly (Titus & Lepš, 2000).

It has been shown in studies of other commercially important crops such as wheat that mycorrhizal responsiveness can vary greatly between cultivars and in wheat there has been a trend for modern cultivars to have very low mycorrhizal responsiveness compared to old (pre-1950s) cultivars (Hetrick *et al.*, 1993; Zhu *et al.*, 2001). Phosphorus uptake by plants in modern

wheat cultivars has become largely independent of AM fungi (Hetrick *et al.*, 1996), but from the perspective of moving towards sustainable agriculture, a strong argument could be made for re-introducing these traits into modern lines. With clear evidence of very high colonisation in Glen Ample, a modern and popular cultivar, it could be argued that what must be re-introduced in wheat, is still present in raspberries. However although increasing %RLC is nearly always linked with higher levels of plant growth and shoot phosphorus (Treseder, 2013), it has long been debated that there is a mutualism-parasitism continuum in AM fungal relationships (Penman & Scott, 1976; Smith & Smith, 2013). With very high levels of colonisation, no detected relationship with increase in growth and no clear idea of phosphorus levels in plant tissues it could be that the relationship between plant and AM fungi in Glen Ample plants has shifted towards commensalism or indeed parasitism.

The positive relationship observed in Glen Rosa plants between %RLC by arbuscules and the mean mass of *O. sulcatus* larvae could be due to the increased nutrients in plant tissues at higher levels of colonisation (Treseder, 2013). This increased access to a phosphorus rich source of food could explain this increased body size in *O. sulcatus* larvae as it is known that phosphorus limitation can impact on insect body size (Huberty & Denno, 2006). This pattern of increased larval weights in plants that are colonised with AM fungi is the opposite to that found in the meta-analysis by Koricheva *et al.* (2009) which showed that overall root feeders showed lower performance on mycorrhizal plants. This meta-analysis drew data from a number of studies that all looked at single AM fungal species inoculations compared with control treatments where AM fungi were totally absent or at very low levels which does not allow a direct comparison with this study.

In Glen Ample both the plant biomass and the %RLC of arbuscules was higher in *S. kraussei* treated plants. *S. kraussei* was the most effective EPN in controlling *O. sulcatus* and so this could be considered as the treatment in which herbivory was at its lowest levels. This may be significant as both AM fungi and *O. sulcatus* are actually competing over a similar resource, with *O. sulcatus* favouring the same un-lignified root tissue that AM fungi require in order to colonise (Smith & Read, 2008). It then follows that the combination of lower levels of herbivory, higher levels of biomass and higher levels of arbuscule colonisation would arise under these conditions.

There are of course limitations to staining to assess AM fungal colonisation as firstly it only provides a snapshot in time of what is a dynamic relationship and the stain itself cannot distinguish between dead and living material. This snapshot in time may also be misleading as the proportion of different AM fungal structures present in plant roots, changes significantly over time, as well as the species present (Šmilauer, 2001; Husband *et al.*, 2002). It has also

been shown in studies with control and herbivory treatments that herbivory can shift the proportions of mycorrhizal structures presence (Klironomos *et al.*, 2004) and that plants that are stressed, a higher proportion of vesicles and spores are present when %RLC is assessed (Duckmanton & Widden, 1994). This study doesn't have a herbivore free treatment, but it could be argued that vesicle and spore colonisation are quite high in proportion to arbuscles and the presence of root herbivores may well be the reason for this.

There was no indication of the suppressive effect of AM fungi known to occur in *O. sulcatus* (Gange, 1996) which we previously hypothesised could make *O. sulcatus* more susceptible to *S. kraussei.* Our thinking was that the bacterial endosymbiont carried by *S. kraussei, Xenorhabdus spp.* (Forst *et al.*, 1997), may more effectively overwhelm the insect immune response if the insect is already suffering from reduced performance. Not content that the level of colonisation alone would necessarily correlate with the function of the AM symbiosis we devised further experiments to manipulate AM fungal communities in the presence of EPNs.

4.5 Conclusions

The EPN, *S. kraussei,* provided superior levels of *O. sulcatus* control, when compared to *H. megidis* and control treatments. *Otiorhynchus sulcatus* had lower survival and performance on the *R. idaeus* cultivar, Glen Rosa, when compared to Glen Ample. Glen Ample, was however, far more susceptible to *O. sulcatus* herbivory. As Glen Ample is the commercial favourite, this finding underlines the need for improved protection against *O. sulcatus.* Further justification for the adoption by more resistant cultivars by growers was found when *O. sulcatus* infested Glen Rosa plants were treated with *S. kraussei*. This combined treatment of *S. kraussei* on Glen Rosa provided the highest levels of *O. sulcatus* suppression. Despite the relatively high populations of *O. sulcatus* on *R. idaeus*, even in the control treatments *O. sulcatus* larvae did not have a significant impact on overall plant biomass. They did however influence carbon allocation in the plants, with more being pushed to the shoots in Glen Ample as seen in chapter 3.

AM fungal colonisation was higher in Glen Ample than Glen Rosa but this was likely due to this susceptible cultivar investing less carbon resources belowground and a reduction in root elongation resulting in an artificially high %RLC. *Otiorhynchus sulcatus* performance actually increased in the presence of more arbuscules in Glen Ample plants. This could be caused by higher phosphorus concentrations in plant tissues as a consequence of higher levels of AM fungal colonisation. This contrasts to existing literature on AM fungi interacting with root feeding performance literature.

This experiment led to two new experiments. The taxis of EPNs in response to different AM fungi and *O. sulcatus* combinations was assessed (Chapter 5) and then a similar experiment to this study was devised in the more horticulturally relevant setting of a polytunnel in a design that incorporated different commercial AM fungal inocula..

5 Do AM fungi enhance the 'alarm signal' sent out by infested plants to natural enemies?

5.1 Introduction

The presence of arbuscular mycorrhizal fungi (AM fungi) in the roots of vascular plants is widespread (Hodge, 2000) and can improve a plant's nutrient uptake, especially of phosphorus. In addition to this, AM fungi can have effects that cascade through to higher trophic levels with varied effects on insect herbivores (Koricheva *et al.*, 2009) through to insect parasitoids and predators (Gange *et al.*, 2003; Hoffmann *et al.*, 2011c).

One of the insect herbivores shown to be affected by the presence of AM fungi is *Otiorhynchus sulcatus* a generalist herbivore in the Curculionidae. In three separate publications, Gange and colleagues showed that when *O. sulcatus* larvae were reared on plants inoculated with a single species of AM fungi, larvae suffered reduced growth when compared to untreated control plants (Gange *et al.*, 1994; Gange, 1996, 2001). In the most recent of these publications Gange (2001) demonstrated that while this effect reduced larval performance under single species inoculations of AM fungi, this was lost when multiple species were added. As plants almost never exist with only one AM fungal partner it means that the applications of such a system into a field trial are very unlikely to reproduce these results. If however there were combinations of AM fungal species and crop plants susceptible to *O. sulcatus* that did work to reduce their performance then such a system could be a very potent component of an integrated crop management system. This is of particular interest with regards to *O. sulcatus* as it is a pest that affects a wide range of plants of economic importance and is difficult to control. The conventional chemical approach to controlling *O. sulcatus* in crops is soil drench treatments of pesticides such as the temporarily banned (in the EU) neonicotinoid imidacloprid and various organophosphates. A Soil drench treatment of a pesticide uses far greater volumes than most foliar treatments and therefore pose an increased risk of both non-target effects and also environmental risk. Another method of control that is popular for the treatment of

plants that may be at risk of, or already under *O. sulcatus* attack, are entomopathogenic nematodes (EPNs). These have been shown in many studies to be effective in reducing both the performance and increasing mortality of *O. sulcatus* (Bruck *et al.*, 2005; Haukeland & Lola-Luz, 2010; Ansari & Butt, 2011). Previous studies have been conducted using an olfactometry based system and established that *O. sulcatus* feeding on various host plants caused a foraging preference in the EPN *Heterorhabditis megidis* over control or mechanically wounded treatments (Boff *et al.*, 2001; van Tol *et al.*, 2001). However neither of these studies were able to offer any evidence for the mechanisms behind this attraction beyond speculating that these are likely to be caused by herbivore induced plant volatiles. Neither of these studies were based on the same plant species, *R. idaeus*, as in the current study and nor did they consider how AM fungal colonisation may influence this relationship. There has been a recent surge in the number of publications covering the topic of herbivore induced plant volatiles and several of these have produced impressive results using belowground olfactometers to control root feeding insect pests with EPNs (Heil, 2014a). A study by Rasmann *et al.* (2005) identified the sesquiterpene (E)-β-caryophyllene as being the main herbivore induced volatiles caused by *Diabrotica virgifera* feeding on *Zea mays*. Infested plants were found to be much more attractive to *H. megidis* due to this VOC being present. Cultivars which produce higher levels of this VOC have been incorporated and the system has been shown to work in the field (Degenhardt *et al.*, 2009). In addition to plants being selected for the emissions of certain VOCs, EPNs can be cultured, using olfactometers and various VOC stimuli to enhance their attraction to root defence signals (Hiltpold *et al.*, 2010a,b).

In addition to the increase in literature investigating herbivore induced natural enemy attraction via VOCs there have been several papers that have shown that AM fungal colonisation can influence the production of the VOCs (Rapparini *et al.*, 2008; Fontana *et al.*, 2009; Hoffmann *et al.*, 2011c; Schausberger *et al.*, 2012; Henke *et al.*, 2015). However all these studies used single species of AM fungi and variation even between isolates of the same species can show very different effects (Wooley & Paine, 2007). In addition to this, these single species systems bear little resemblance to field conditions. In fact the application of AM fungi as commercial species mixtures in the field to improve agricultural sustainability has met with very little success. It is not enough to show that AM fungi even in species mixtures can have effects on VOCs that enhance pest control but that these effects can be replicated by growers with commercially available products.

To this end, two olfactometry experiments were carried out. The first of these two experiments used both the *R. idaeus* cultivars used and discussed in chapters 3 and 4, Glen Ample and Glen Rosa as host plants. The host plants were inoculated with a field derived

mixed species inoculum of AM fungal spores. The insect herbivores used were *O. sulcatus* to which the preference of *H. megidis* EPNs was tested. The main aim of this experiment was to see if *H. megidis* showed a preference for *R. idaeus* that were both infested with *O. sulcatus* and inoculated with AM fungi and to see if the mechanism by which this may occur was due to altered plant VOC emissions. Based on the work of previous authors mentioned earlier in this introduction it seemed likely that inoculation with AM fungi would cause plants to produce a different VOC cocktail that provided greater attraction to *H. megidis*. This led to the hypotheses that in the presence of AM fungi, VOC emissions would be modified and that *H. megidis* attraction would be greater. Different densities of *O. sulcatus* were used to create different levels of herbivory on *R. idaeus*. It was expected that these different densities would produce herbivore induced VOCs and consequently result in higher numbers of *H. megidis* attracted. The hypotheses were that high densities of *O. sulcatus* would lead to a change in VOC emissions and an increase in *H. megidis* taxis. An objective of this study was to see if there was a difference in VOC emissions and *H. megidis* attraction between the two cultivars. If this was the case then the reduced *O. sulcatus* survival seen on Glen Rosa alongside EPNs in chapter 4 could be explained by induced VOCs. It was hypothesised that Glen Rosa would have a distinct VOC profile from Glen Ample and that *H. megidis* would be more attracted Glen Rosa. The percentage of root length colonised (%RLC) by AM fungi was expected to be greatest in treatments where AM fungi was added, otherwise contamination may have occurred. Additionally it might be expected that the %RLC may be altered by either *O. sulcatus* herbivory, or the differences in root morphology between the two *R. idaeus* cultivars. It was hypothesised that %RLC would be different across *O. sulcatus*, AM fungal and cultivar treatments.

The second of these two olfactometry experiments repeated these tests, but in a way that would be more related to commercial practice. In particular, the AM fungal inoculant and the biological control agents (EPNs) used were commercially available. Also, the experimental methodology did not involve laboratory procedures that would not be achievable in the field (e.g. soil sterilisation). To represent this the commercial favourite Glen Ample cultivar of *R. idaeus* was used as the host plant system to which *O. sulcatus* were added. The AM fungal inoculant used was a commercially available preparation and the EPN was the readily available *S. kraussei* which proved most efficacious in *O. sulcatus* control in chapter 4. The aim of this experiment was to investigate if a commercial AM fungal inoculant could increase the attraction of *S. kraussei* to *O. sulcatus* infested *R. idaeus*. A key objective in this study was to see if the addition of a commercial AM fungal inoculant would influence *S. kraussei* distributions. It was hypothesised that the addition of an inoculant would increase *S. kraussei* attraction. Secondary to this objective was to establish if *S. kraussei* were influenced by the presence of feeding *O. sulcatus* larvae perhaps through herbivore induced VOCs. The

hypothesis was that *S. kraussei* would show a preference for plants that had an added *O. sulcatus* population. The biomass and root to shoot ratio recorded in this experiment could have also had an influence on the attraction of *S. kraussei*, and been influenced by *O. sulcatus* and AM fungal treatments. It was hypothesised that added AM fungi would increase plant biomass, and that conversely the addition of *O. sulcatus* would decrease plant biomass and alter root to shoot ratio, as in previous experiments (chapters 3 and 4). It was also hypothesised that the plant biomass or root to shoot ratio could have influenced *S. kraussei*.

5.2 Olfactometry experiment 1:

5.2.1 Materials and Methods

Under controlled conditions (16:8 light:dark days at 18˚C), a 2 x 2 x 3 factorial experiment was conducted with two different *R. idaeus* cultivars (Glen Ample and Glen Rosa), two different mycorrhizal treatments (live or sterile spores) and three different herbivore treatments using *O. sulcatus* (a control treatment, low 20 egg treatment and a high 40 egg treatment). A randomised block design was used to account for spatial variation within the climate controlled glasshouse.

Rubus idaeus was prepared using the methods outlined in 2.1.1. After 4 weeks 48 individual plants, 24 of each cultivar, were transplanted into 1.8L pots containing 1.6L of a twice sterilised 1:1 soil (Keith Singleton sterilised loam) and sand mix. A length of wooden dowel, 9mm diameter X 90mm, (B&Q, Eastleigh, UK) was placed vertically in each pot to displace soil for the later addition of automated thermal desorption (ATD) tubes.

AM spores were extracted from trap cultures (see section 2.1.4) containing spores taken from a field site that has had raspberry plants grown on it for over 10 years. The spores were extracted using the sucrose centrifugation method (2.1.2) and AM fungal inoculum was prepared using methods described in 2.1.3. All 48 plants were then inoculated with 1.5ml of spore solution, containing 17±4SE spores, and 1.5ml of microbial wash; with the live spores and a sterile microbial wash forming the 'live' mycorrhizal treatment and the sterile spores and live microbial wash forming the 'sterile' mycorrhizal treatment. Both the treatments were applied using a P10ml (Gilson®, Luton, UK) Pipetman and spores were injected 5mm below the soil surface at the base of the plant's main stem.

Three weeks after the addition of mycorrhizal spores *O. sulcatus* eggs were added to plants, the culture from which these eggs were taken is described in 2.1.8. The plants of each cultivar

were divided equally into three groups. One group was kept as a control group with no eggs added; the second group had 20 eggs added, and the third group 40 eggs. These levels of *O. sulcatus* treatment were selected to represent a low high herbivory pressure, respectively, relative to plant size (Clark *et al.*, 2012)

Three weeks after the addition of weevil eggs the plants were then placed into a belowground olfactometer. The olfactometer used in this experiment was a 6 arm choice chambers for assessing the preferences of EPNs (Figure 5.1). For full schematics of the olfactometers see appendix 1. The two cultivars were tested separately with two consecutive runs on the olfactometer.

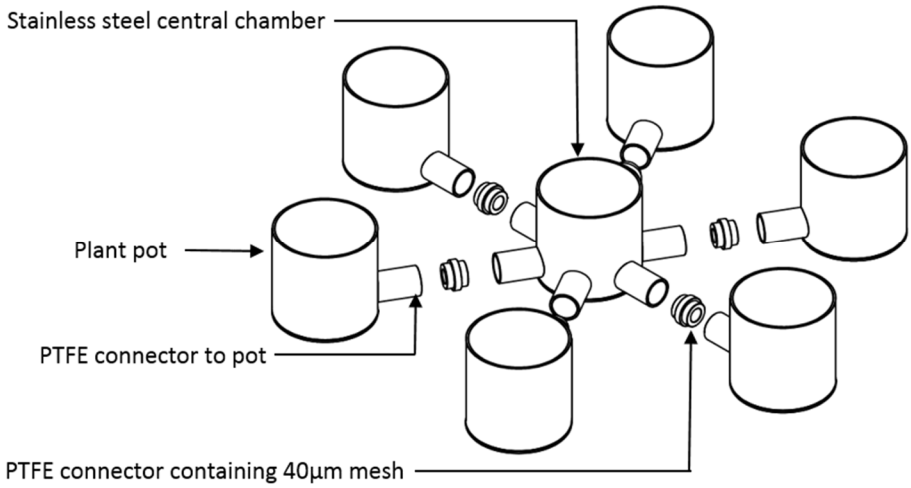


Figure 5.1: A 6 arm belowground olfactometer. After Rasmann *et al*. (2005).

Plants from a single cultivar were randomly selected within treatment and then randomly placed in a position around the olfactometer so that all treatments were represented at any one time but any "push" effects were mitigated. After the olfactometer was loaded with plants, silver sand was added to the central chamber and arms which was kept damp with 10% water by weight of sand. The EPNs used in this experiment were from a culture of *Heterorhabditus megidis* used by Rasmann *et al.*, (2005) and were maintained using the culturing methods shown in 2.1.10. This strain of *H. megidis* was known to respond to the plant VOC (E)-β-caryophyllene (Rasmann *et al.*, 2005). For each run of the olfactometers approximately 300 *H. megidis* were added to the central chamber. Over the course of 24h these EPNs then dispersed towards the plants at the ends of the arms. At the end of the arms the EPNs were trapped by a fine 40µm wire mesh and collected in a small detachable section of the arm (Figure 5.2). This sand sample was then placed in a Baermann funnel (see section

2.1.11) and the numbers of retrieved EPNs were assessed. This process repeated for each cultivar separately.

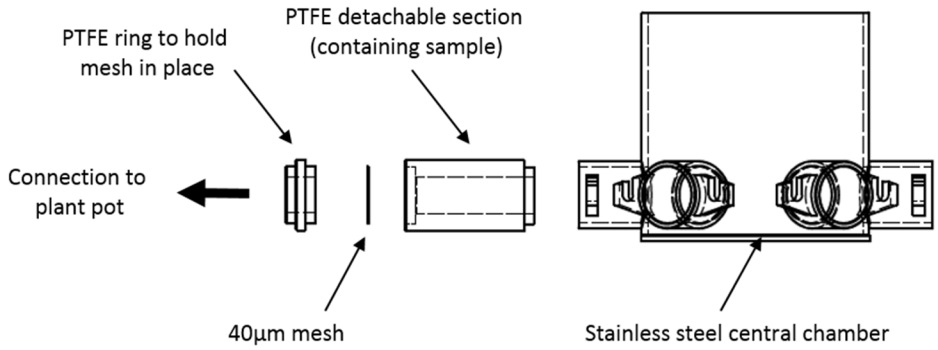


Figure 5.2: A detailed diagram of one of the olfactometer arms. The PTFE detachable section contained the sand sample from which EPNs were extracted.

Preconditioned ATD tubes (see section 2.1.13) were placed into the pots to sample root VOC emissions at the same time that the nematodes were added. The olfactometer was then left to run for 24h then it was harvested, sterilised and reset to obtain 8 replicates. The plants were then harvested with the weevils removed from the soil, counted and weighed, and the above and belowground portions of the plants were oven dried to enable the calculation of dry mass. Arbuscular mycorrhizal colonisation of roots was assessed using the gridline intersect method after being stained with Quink Ink Royal blue (2.1.5 and 2.1.7). ATD tubes retrieved from the soil and then the VOC samples they had captured were desorbed and run through an ATD-GC-MS set-up (see 2.1.14).

5.2.2 Data analysis

The number of *H. megidis* retrieved showed a positive skew, as is typical of count data, and was consequently incorporated into a generalised linear model (GLM) with Poisson errors as a response variable. This was tested against the explanatory variables of AM fungal treatment, *R. idaeus* cultivar and *R. idaeus* biomass. The experimental block and olfactometer run number were included as covariates in order to account for spatial and temporal variation in the variables recorded.

In order to ensure that different numbers of *O. sulcatus* eggs actually represented different sized populations of *O. sulcatus* larvae a GLM with Poisson errors was carried out. The number of retrieved larvae were tested against the explanatory variables of *O. sulcatus* treatment, AM fungal treatment and *R. idaeus* biomass and the covariates; experimental block and

olfactometer run. This test was then repeated but with *O. sulcatus* performance (total larval mass per plant) as the response variable.

Rubus idaeus biomass and root to shoot ratio were used in ANCOVAs with the explanatory variables; *O. sulcatus* treatment and AM fungal treatment with the experimental block and olfactometer run as covariates.

The total %RLC of all recorded AM fungal structures combined and the %RLC of individual structures (arbuscules, vesicles, hyphae and spores) were tested as response variables in a series of GLMs. They were tested with the explanatory variables; abundance, AM fungal treatment, *O. sulcatus* treatment and *R. idaeus* cultivar. The experimental block was included as a covariate.

VOC data was extracted from GCMS software and relative peak areas were calculated as detailed in 2.1.15. A principal component analysis (PCA) was carried out on the relative abundance of VOCs. This VOC relative abundance was used in order to help account for the PCA known sensitivity to outliers. The PCAs were carried out using the statistical package 'car' in R3.1.2 (R Core Team, 2013). However due to the presence of large outliers, the data set failed to meet the assumptions of a PCA, making the results of this test unreliable. Unfortunately these data points could not be omitted as they constituted a large proportion of the data set. Consequently a series of GLMs were used on specific VOCs of interest which had been mentioned previously in the literature (pinenes and carenes, both monoterpenes (Rasmann *et al.*, 2012a) to see if they correlated with *H. megidis*, AM fungal or *O. sulcatus* distributions. The total signal (the sum of all peak areas) and the total number of compounds isolated in each sample were also used as response variables and tested against the distribution of nematodes and the AM fungal, *R. idaeus* cultivar and *O. sulcatus* treatments. Although the total signal provides a less informative data set, with regards to the relative composition of VOCs present, it is also less sensitive to misidentified compounds than more targeted analyses.

All analysis was carried out using R3.1.2 'Pumpkin Helmet' (R Core Team, 2013) with the best fitting minimal models reported.

5.2.3 Results

The olfactometry experiment showed that *H. megidis* had a preference for plants which were inoculated with AM fungi ($t_{1,38}$=2.23, P <0.05). This preference was also affected by the *O. sulcatus* treatment that plants received as indicated by a significant interaction between these two variables ($t_{2,38}$= 2.38, P <0.05). Plants inoculated with AM fungal spores were significantly

more attractive to *H. megidis* when *O. sulcatus* were either absent or present at the lower, 20 egg treatment when compared to AM fungal control treatments. At the higher, 40 egg, level of *O. sulcatus* herbivory this relationship was not significant (Figure 5.3). The *R. idaeus* cultivar was found have no effect on *H. megidis* distributions.

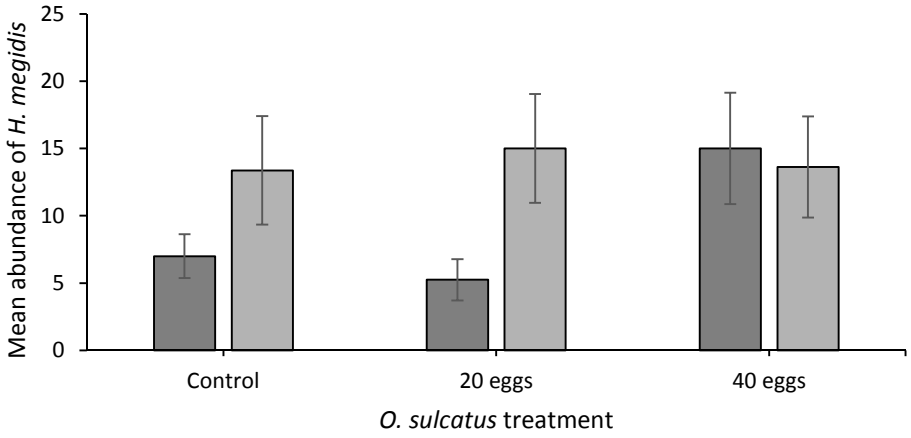


Figure 5.3: The mean abundance of *H. megidis* attracted to differently treated *R. idaeus.* Dark grey bars represent the AM fungal control treatment while light grey bars represent the treatment to which live AM fungal spores were added.

The two different *O. sulcatus* densities included in the experiment, created by the addition of either 20 or 40 *O. sulcatus* eggs, were found to reflect in a resulting larval abundance that was significantly higher ($Z_{2,44}$= 2.58, P<0.01) in the 40 egg treatment (Figure 5.4). This meant that egg addition proved a reliable predictor of *O. sulcatus* larval density and was therefore used to represent this in statistical models.

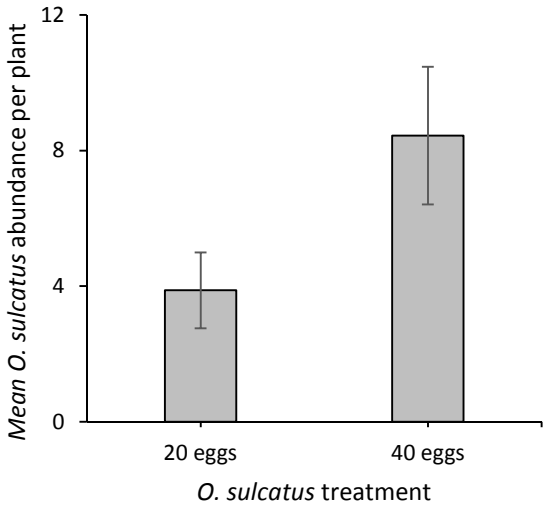


Figure 5.4: Initial *O. sulcatus* egg densities resulted in a comparable relative larval density with the addition of twice as many eggs, resulting in twice as many larvae.

The mean mass and larval performance of *O. sulcatus* was not found to vary significantly, either between different larval densities, between AM fungal treatments or between the two *R. idaeus* cultivars.

The *R. idaeus* biomass measured at the end of the experiment was not found to differ under the different *O. sulcatus*, AM fungal or *R idaeus* cultivar treatments. The same was also true for the root to shoot ratio of each plant, calculated from the *R. idaeus* biomass data.

The %RLC by different AM fungal structures varied across all treatments (Table 5.1). As only 3 spores were identified across all treatments, spore colonisation was omitted from analyses but was included in the total number of AM fungal structures.

Table 5.1: A summary table of the %RLC by different AM fungal structures. All means are presented with standard error.

AM fungal treatment	*R. idaeus* cultivar	*O. sulcatus* treatment	Total colonisation of AM fungal structures (%RLC)	Arbuscule colonisation (%RLC)	Vesicle colonisation (%RLC)	Hyphal colonisation (%RLC)
Sterile treatment	Glen Ample	Control	5.75 ± 1.93	3.40 ± 1.16	2.94 ± 0.69	2.35 ± 0.79
		20 eggs	4.67 ± 3.46	1.62 ± 1.27	0.00 ± 0.00	3.05 ± 2.20
		40 eggs	50.23 ± 13.19	14.10 ± 4.83	7.28 ± 1.00	35.66 ± 8.20
	Glen Rosa	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		20 eggs	0.31 ± 0.31	0.16 ± 0.16	0.00 ± 0.00	0.16 ± 0.16
		40 eggs	0.32 ± 0.21	0.21 ± 0.12	0.00 ± 0.00	0.11 ± 0.11
AM fungal spores added	Glen Ample	Control	56.48 ± 3.66	13.74 ± 5.08	3.51 ± 0.30	42.05 ± 5.46
		20 eggs	57.97 ± 2.38	19.21 ± 0.65	3.99 ± 1.07	37.98 ± 2.95
		40 eggs	40.34 ± 0.71	15.62 ± 0.65	18.35 ± 6.27	23.80 ± 1.52
	Glen Rosa	Control	58.07 ± 13.92	21.90 ± 4.84	1.86 ± 1.31	35.85 ± 9.07
		20 eggs	34.60 ± 19.21	11.15 ± 7.86	4.19 ± 0.41	23.28 ± 11.46
		40 eggs	39.22 ± 15.30	9.25 ± 3.57	6.50 ± 4.70	28.66 ± 11.39

All the different structures recorded showed that %RLC was higher in the AM fungal treatment where live AM fungal spores were added. This is perhaps best represented by the level of overall %RLC recorded for all AM fungal structures which showed a substantial difference ($F_{1,35}$= 51.77, P <0.001) in the %RLC between the two AM fungal treatments (Figure 5.5).

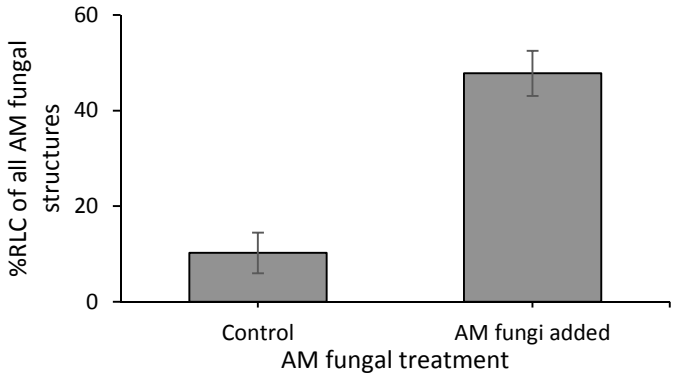


Figure 5.5: The %RLC by AM fungal structures was higher in the additive AM fungal treatment.

Although the %RLC of vesicles was mostly explained by the AM fungal treatment added ($F_{1,35}$=11.17, P <0.05), there was also a significant influence of the *R. idaeus* cultivar in determining the colonisation by vesicles. The %RLC by vesicles was significantly higher ($F_{1,35}$=4.48, P <0.01) in Glen Ample under high levels of *O. sulcatus* herbivory (Figure 5.6).

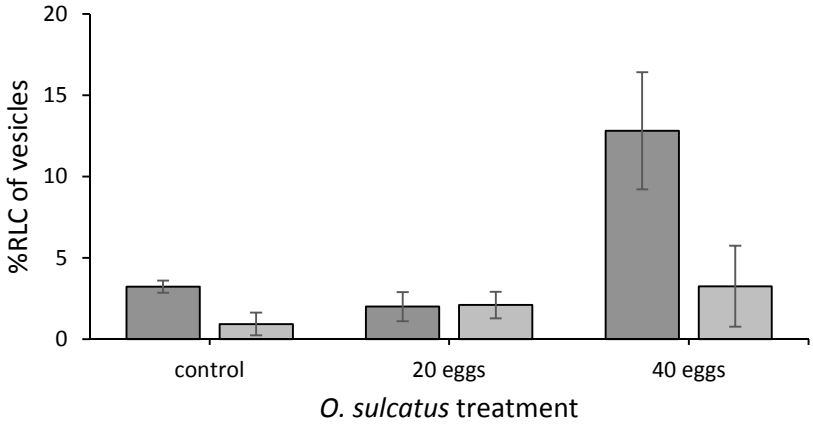


Figure 5.6: The %RLC of vesicles was found to be higher when there was high levels of *O. sulcatus* herbivory on Glen Ample. The dark grey bars represent the *R. idaeus* cultivar, Glen Ample and the light grey bars Glen Rosa.

The PCA carried out on VOCs failed to identify any significant components. The compounds identified as being possible herbivore induced VOCs (pinenes and carenes) were not found to be linked to either the *O. sulcatus* treatment or the *H. megidis* distributions. While these specific compounds were not found to be linked to herbivory or *H. megidis* distribution these two factors did appear to be linked to plant VOC emissions. The high *O. sulcatus* herbivory

treatment caused an increase in the total VOC signal recorded which was linked to a corresponding increase in the number of *H. megidis* attracted ($t_{1,36}$=-2.353, P <0.05) to heavily infested plants (Figure 5.7). The raw VOC data is included in digital appendix 2.

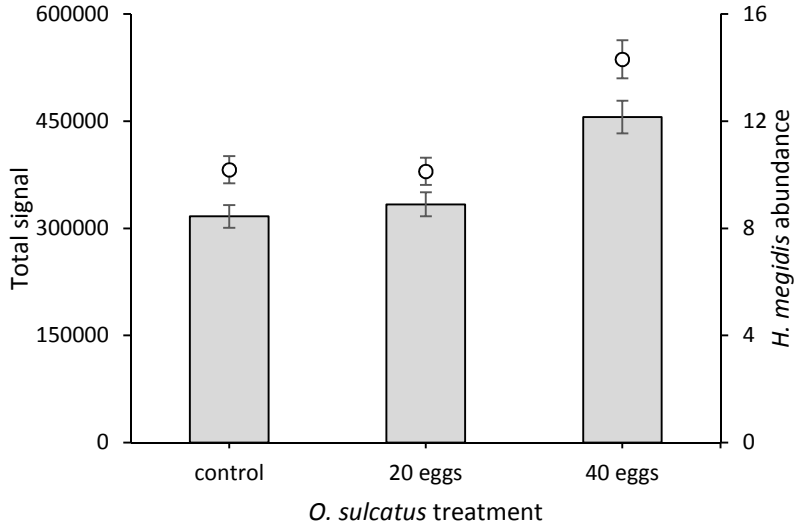


Figure 5.7: The total signal of plant VOCs was found to be higher in plants treated with a high *O. sulcatus* herbivory treatment, and in turn was more attractive to *H. megidis*. Grey bars represent the total signal of plant VOCs recorded and correspond to the left hand axis and the black points represent *H. megidis* abundance and correspond to the right hand axis.

5.2.4 Discussion

Olfactometers have proven to be a valuable tool to study the interactions of plants, herbivores and natural enemies, as well as the mechanisms behind these interactions. Olfactometers allow us to deconstruct the continuum of interacting organisms present in the field and restrict these to a finite and quantifiable level. This drastically reduces the complexity of a system and enables us to understand some of the core processes occurring in the natural world, in this case, a specific set of belowground predator prey interactions in a system comparable to a *R. idaeus* crop.

In this experimental set up, the presence of AM fungi appears to influence *H. megidis* attraction when *O. sulcatus* populations are low or absent. This effect appears to no longer be modified by AM fungi when *O. sulcatus* herbivory is at a higher level, with *H. megidis* attraction elevated regardless of AM fungal treatments. The recovery of *O. sulcatus* larvae at the end of the olfactometry experiment and the assessment or *R. idaeus* root tissue for AM fungal colonisation confirmed that both the herbivory and AM fungal treatments were successful manipulations of these two organisms.

The PCA carried out on the data failed to isolate any principal components of interest. It may be that even though the data was standardised the GCMS data set was still dominated by a few very large outliers, which makes the output of any PCA unreliable. Analysis using just these dominant compounds that were identified as likely being α-pinene and delta 3-carene could not explain the distributions of *H. megidis*, nor did they appear to be as a consequence of AM fungal and *O. sulcatus* treatments. Both these VOCs are known *R. idaeus* metabolites (Aprea *et al.*, 2009) as well as VOCs recognised as eliciting nematode taxis (Ali *et al.*, 2010; Rasmann *et al.*, 2012a). The total signal of VOCs recorded in the experiment was found to have a strong relationship with the levels of *H. megidis* attracted to different densities of *O. sulcatus*. The control and low *O. sulcatus* treatments did not result in a higher total signal but the high treatment did and this was matched with a marked increase in the level of *H. megidis* attraction. This supported the earlier *H. megidis* results that showed increased attraction to high *O. sulcatus* densities but did not explain the differences in the populations of *H. megidis* attracted to AM fungal and non AM fungal plants. The fact that total signal data did not support this is unsurprising as previous studies exploring secondary metabolite production of AM fungal and non AM fungal plants have shown that total signal did not vary but the proportion of monoterpenes and sesquiterpenes was altered (Rapparini *et al.*, 2008; Fontana *et al.*, 2009). So in future, if greater expertise was brought to bear on the identification of these compounds then perhaps the levels of these two groups of VOCs could be assessed and incorporated into analyses.

A phenomenon that adds to the complexity of herbivore induced VOC production and EPN taxis systems was demonstrated by Hallem *et al.* (2011). They showed that EPNs were responding directly to VOC emissions from insect larval hosts as opposed to herbivore induced plant VOCs. They discovered that hexanal and α-pinene released from *G. mellonella* larvae both simulated a jumping response in *S. carpocapsae* and to lesser extent chemotaxis. Discerning the true mechanism behind these EPN taxis effects may not be important for their reproducibility but may go some way towards explaining why mechanical damage treatments often elicit a slightly weaker response from EPNs than treatments containing actual insect herbivores (As reviewed by Dicke, 1999).

The presence of an AM fungal community in plant roots seems to mislead *H. megidis* into responding to treated plants as if there was outbreak of *O. sulcatus*. This high level of *O. sulcatus* herbivory elicits the same elevated *H. megidis* response in both mycorrhizal treatments, suggesting that this is the level of herbivory at which these two cultivars respond to herbivory regardless of AM fungal colonisation. The fact that these two cultivars appear to exhibit very similar reactions under these conditions is surprising as Leitner *et al.* (2010) found

that the presence of a single AM fungal species caused detectable changes in herbivore induced VOCs in *Medicago truncatula* but only on specific cultivars. However as Glen Rosa and Glen Ample are 'sister' cultivars it could be that this similarity in response to herbivory and AM fungal colonisation could be due to their very similar genetic backgrounds. These results also conflict with the findings of Henke *et al.* (2015) who found that plant VOCs changed when there was AM fungal colonisation, only when the plant was stressed, be it herbivory or an abiotic stress. This differs to the results found in this study as even in control treatments the difference in natural enemy attraction was apparently, purely due to the difference in AM fungal treatment and independent of herbivory. It could be that colonisation by AM fungi, primes *R. idaeus*, triggering the plant's natural resistance to pests regardless of whether they are present or not (Jung *et al.*, 2012). Alternatively it could be that the increased phosphorus (P) uptake linked with AM fungal colonisation (Smith & Read, 2008) increases the provisions a plant needs to produce VOC signals that are attractive to natural enemies. However, a recent study by Babikova *et al.* (2014), which showed that plant VOC emissions were independent of P availability, implies that this is perhaps an over simplistic hypothesis and still an area where a lot more work needs to be carried out in order to reveal the true mechanisms behind these effects. The fact that *R. idaeus* with an AM fungal community appears to attract *H. megidis* as if they are under heavy *O. sulcatus* attack, regardless of if these pests are present, is potentially of great use in an integrated crop management system. EPNs can be applied directly to plant roots via the use of irrigation systems such as T- Tape® (John Deere Ltd., Langar, UK) to plants inoculated to tailored AM fungal community. Such a system if easily applied to a cropping system could potentially ensure that EPNs would be attracted to plant roots regardless of insect presence and be able to perform as body guards in the event that *O. sulcatus* were to oviposit on a protected plant. The idea that such a system that works in individual pots could be easily scaled up into a field scenario where multiple plants share the same uninterrupted rhizosphere is rather unrealistic as this presents a very different environment. A major difference, beyond the obvious lack of compartmentalisation that isolates root feeders in a pot system, is the connectivity that common mycelial networks (Walder *et al.*, 2012) bring to such a system. These provide a communication network that can warn nearby plants (Babikova *et al.*, 2013) about pests and may therefore create VOC gradients that could draw EPNs away from un-infested plants.

There was one anomaly discovered in the %RLC data. In the high herbivory treatment there was a higher population of vesicles in Glen Ample. This was likely driven by two factors associated with the known susceptibility of Glen Ample to *O. sulcatus* (Clark *et al.*, 2011a). Firstly high vesicle colonisation is commonly associated with an AM fungal symbiotic relationship that is under stress (Duckmanton & Widden, 1994), in this case most likely from

O. sulcatus herbivory. Secondly a plant under this high level of herbivory will be unlikely to be growing rapidly, which would normally result in root elongation, and is associated with lower densities of AM fungal features (Titus & Lepš, 2000). It is therefore more likely in this scenario to have a higher density of AM fungal colonisation. This is further supported by the results in chapters 4.3 and 3.3 which both discuss how this effect could arise from the recorded carbon allocation patterns seen in Glen Ample under high herbivory treatments.

If it were possible to avoid the use of a tailored, *R. idaeus* specific, AM fungal inoculant then this would greatly increase the feasibility of applying such a system in a commercial setting. For this reason, in the second olfactometry experiment in section 5.3, a commercially available AM fungal inoculant was used to see if the same effects would be present.

Whereas *H. megidis* is commercially available and was purchased for experimental work in chapter 4 the strain used in this study was sourced from Prof. Sergio Rasmann (University of California, Irvine, USA) and was a strain specific to his work published in Rasmann *et al.* (2005). This strain is therefore not available commercially and may not compare well in terms of attraction to herbivore induced VOCs to those sourced from Biobest® (Milton Bridge, UK). In chapter 4, where the efficacy of both *H. megidis* and *S. kraussei* were compared it was clear that *S. kraussei* provided superior levels of *O. sulcatus* control. For this reason it made sense to use in subsequent experiments the commercially available and superior *S. kraussei*.

In order to further increase the validity to *R. idaeus* growers, the commercial favourite, Glen Ample was used in experiments from this point on. This is because, in this experiment, at least from the perspective of herbivore induced VOCs, and subsequent natural enemy attraction, the two cultivars were indistinguishable.

5.3 Olfactometry experiment 2: Is this effect commercially viable?

5.3.1 Materials and Methods

A 2 x 2 factorial experiment was carried out, in a constant temperature room (22˚C on a 16:8 Day:night cycle), using one cultivar of *R. idaeus* (Glen Ample) to test the effects of two

different mycorrhizal treatments (live or sterile commercial AM fungal inoculum) and two herbivore treatments of *O. sulcatus* (a control treatment and a 40 egg treatment). These experimental treatments were incorporated into a randomised block design.

R. idaeus was prepared using the methods outlined in 2.1.1. After 4 weeks 40 plants were transplanted into 2L size pots containing 1.8L of compost (John Innes No. 3, Levingtons, UK) and an AM fungal inoculum.

The commercially available AM fungal inoculant known as 'MycoForce Mycorrhizal Transplanter™' produced by Symbio® was used in this experiment to more closely mimic the crop management tools available to a commercial grower. The inoculant was added to plants at the commercially recommended dose of 5ml per 2L pot which contains approximately 1100 propagules. A control treatment of inoculant was created by heat sterilising the inoculum at 400°C twice for 20mins, with an hour to cool between heat treatments.

Five weeks after plants were potted into 2L pots the plants of each AM fungal treatment were split randomly into two equal groups of 20 plants. To half the plants, 20 *O. sulcatus* eggs (taken from a culture maintained on site, see 2.1.9) were added and to the other half, no eggs, in order to create an *O. sulcatus*-free control treatment.

After three weeks the potted *R. idaeus* were then connected to two belowground olfactometers. The olfactometers in this experiment were four arm choice chambers for analysing the dispersal of nematodes, and other than the different number of arms, their construction differed in no way to those discussed in 5.2.1. The 40 experimental plants were divided randomly into ten groups of four, with each plant in each group representing a different treatment group. These plants were then placed in a random order into a series of five 24hr olfactometry runs. As mentioned in 5.2.1, randomising the order of plants enables there to be a distinction between "push" and "pull" effects, both of which could be useful tools in such a system (Pickett *et al.*, 2014).

Once plants were incorporated into the olfactometer, moist silver sand (10% water by weight relative to sand) was added to the central chamber and four arms of the olfactometer. After the addition of moist sand, approximately 2,000 *S. kraussei* (Becker and Underwood®, Littlehampton, UK) suspended in 10ml of water were added to the centre of the central chamber using a P10ml (Gilson®, Luton, UK) Pipetman. The olfactometer was then allowed to run uninterrupted for 24hrs at which point it was dissembled and the removable sections depicted in Figure 5.2 were carefully set aside so that EPNs could be retrieved using wet sieving and sucrose centrifugation (see 2.1.11) enabling the numbers of nematodes attracted to each treatment to be assessed. The two olfactometers, used in this experiment, were then

sterilised and reset with fresh plants a total of 5 times, creating 10 replicates. After plants had been used in the olfactometer they were harvested, *O. sulcatus* larvae were retrieved from the compost, counted and weighed. The fresh above and belowground portions of the plants were weighed and then oven dried at 50°C to enable the calculation of dry mass. Prior to oven drying, 30mg sub samples of fresh root material were frozen in liquid nitrogen and the freeze dried. This was in preparation for DNA extraction Ion Torrent^{TM} (Life Technologies Ltd, Paisley, UK) next generation sequencing to identify the AM fungal community colonising the roots. This will be done in collaboration with Dr Karita Saravesi (University of Oulu, Finland).

The abundance of *S. kraussei* recaptured was analysed using an ANCOVA and tested against the explanatory variables of *O. sulcatus* treatment, *R. idaeus* biomass and AM fungal treatment. The experimental block in which plants were grown was included as a covariate in order to account for spatial variation within the constant temperature room as was the olfactometer run date, in order to account for any temporal effects.

The number of retrieved *O. sulcatus* larvae as well as their performance (total *O. sulcatus* weight per plant) were both used as response variables in GLMs using a Poisson error structure as both these data sets exhibited a negative skew consistent with the Poisson distribution. These two response variables were both tested against the explanatory variables of AM fungal treatment and *R. idaeus* biomass with experimental block and olfactometer run, as covariates.

The response variables of *R. idaeus* biomass and root to shoot ratio were used in ANCOVAs and tested against the *O. sulcatus* treatment and the AM fungal treatment with the experimental block and olfactometer run included as covariates. The change in mass between wet and dry *R. idaeus* aboveground biomass was used as a response variable in an ANCOVA to see if the explanatory variable of *O. sulcatus* treatment and AM fungal treatment influenced water concentration, again experimental block and olfactometer run were used as covariates.

All analysis was carried out using R3.1.2 'Pumpkin Helmet' (R Core Team, 2013) with the best fitting minimal models reported.

5.3.2 Results

The numbers of *S. kraussei* recovered from the different olfactometer arms were not explained by the different experimental treatments applied to plants, with neither the presence of *O. sulcatus* nor an added AM fungal inoculum making a detectable difference (data not shown).

The total biomass of *R. idaeus* did not have a relationship with either the AM fungal treatment or *O. sulcatus* presence. There was however a positive relationship ($F_{1,21}$ 4.46, P <0.05)

between the *R. idaeus* root to shoot ratio and the abundance of *S. kraussei* (Figure 5.8). This means that when root mass is larger relative to shoot mass, more *S. kraussei* were attracted.

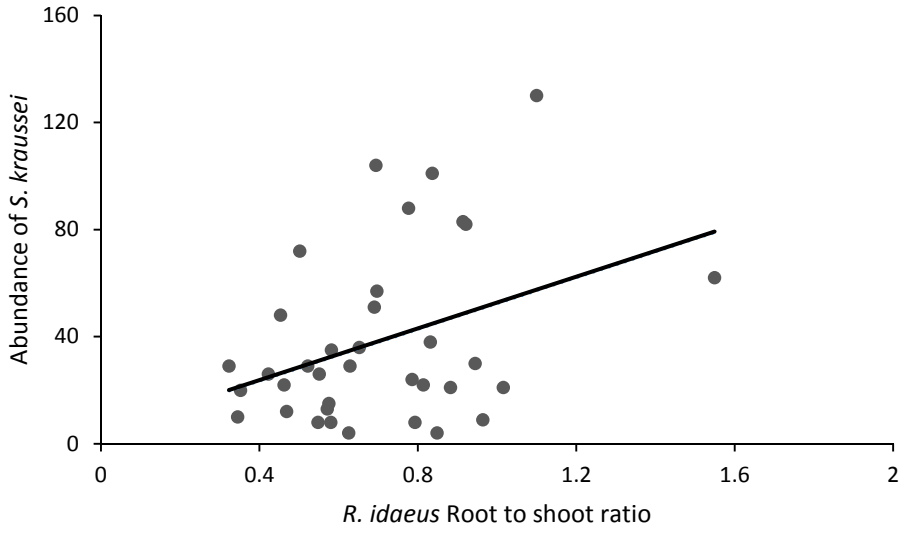


Figure 5.8: The abundance of *S. kraussei* had a positive relationship with *R. idaeus* root to shoot ratio.

The root to shoot ratio of *R. idaeus* was also lower ($F_{1,21}$=8.81, P <0.01) when *O. sulcatus* larvae were present when compared to control treatments (Figure 5.9)

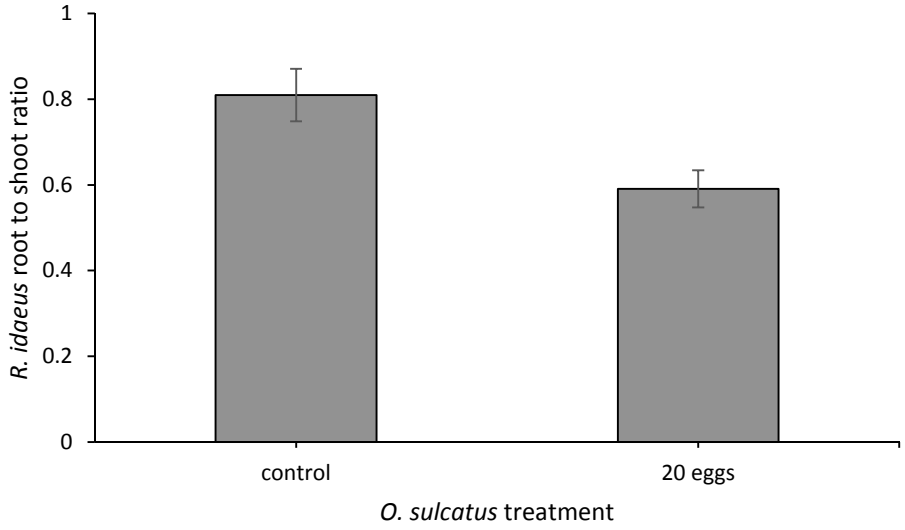


Figure 5.9: *R. idaeus* biomass was lower when *O. sulcatus* larvae were present.

In order to ascertain if the relationships exhibited by *S. kraussei* and *O. sulcatus* with root to shoot ratio were driven by root biomass, as opposed to fluctuations in aboveground biomass the analysis was repeated with the two sets of biomass data separately. *Steinernema kraussei* attraction appeared to only be affected by root biomass ($F_{1,21}$=89.281, P <0.01) with aboveground biomass having no effect on their distributions (Figure 5.10 and Figure 5.11)

Separating the shoot and root data in order to further explain the relationship between *O. sulcatus* and *R. idaeus* did not reveal any further relationships.

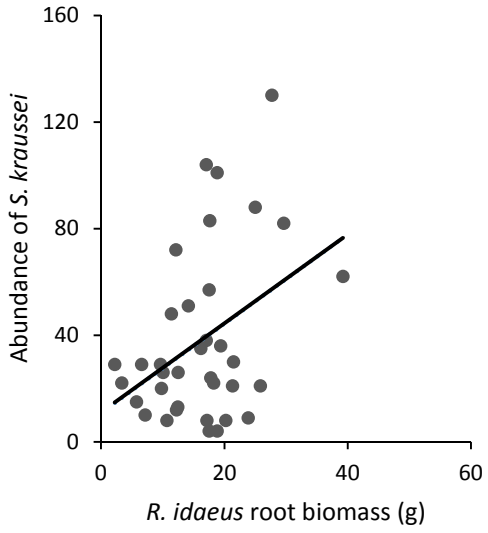


Figure 5.10: Root biomass is positively related to *S. kraussei* attraction.

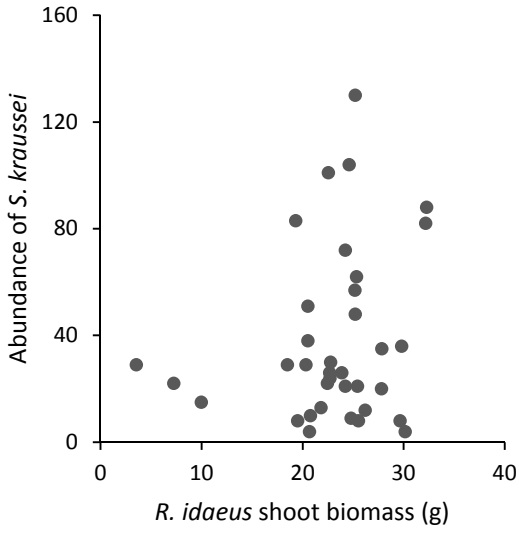


Figure 5.11: Shoot biomass has no effect on *S. kraussei* attraction.

The water content of *R. idaeus* shoots was not found to be influenced by either herbivory or AM fungal treatment. The abundance and performance of recovered *O. sulcatus* larvae was not found to vary significantly between AM fungal treatments.

5.3.3 Discussion

In this second olfactometry experiment, an attempt was made to test an experimental set up which was closer to what might be usefully applied in the field by a commercial *R. idaeus* grower for enhancing pest control. In the field, for example, it is not possible to sterilise soil and so any AM fungal treatments are in fact additive treatments to a pre-existing soil microbial community, which is what has been simulated in this experiment. Alongside this key difference, the more effective EPN species, *S. kraussei*, as shown in trials in chapter 4, was used in addition to the industry standard *R. idaeus* cultivar, Glen Ample.

The abundance of *S. kraussei* attracted to plants was not affected by the addition of an AM fungal inoculant in the way that *H. megidis* were shown to respond to a field derived inoculant in 5.2.3. If *S. kraussei* addition in combination with a commercial inoculant were to have followed the same pattern as in 5.2.3 then it would be expected that an increased population of *S. kraussei* would have been recorded in olfactometer arms that lead to both arms terminating in plants that had been inoculated with AM fungi, regardless of *O. sulcatus* treatment. The fact that this was not observed could be for one of two primary reasons. Firstly,

this could be simply due to the differences in these two EPN species, while both are termed as being 'cruise' foragers (Campbell *et al.*, 2003; Kruitbos *et al.*, 2010) meaning they are mainly observed actively seeking prey via chemotaxis, as opposed to the 'ambusher' tactics employed by some closely related species. They are however both from different families, the Steinernematidae and Heterorhabditidae, while most of the recorded differences in these two species are related their biology that is key to their pathogenicity (Boemare, 2002), once in contact with a host insect, there are fewer differences documented in their taxis behaviour. However, their distinct genetic backgrounds may predispose them to respond to host and plant chemical cues differently. Also they may not be equally capable of dispersal and efficient host seeking in the same substrate types as one another. EPNs have been shown to have very different levels of host finding performance and efficacy in different substrate types (Choo & Kaya, 1991; Kruitbos *et al.*, 2010). While *S. kraussei* may outperform *H. megidis* in a sandy loam (as used in chapter 4.2), it may be that *H. megidis* can outperform *S. kraussei* in terms of host location in a sand based system. If this is the case then a useful follow-up experiment to this one would be to test the same system but with different substrate types within the olfactometer central chamber and arms. Another confounding factor that may preclude the comparison of these two EPN species is that the *H. megidis* used in 5.2.1.2 were a strain that were used by Rasmann *et al.* (2005) and may have become optimised in terms of their response to herbivore induced VOCs in a sand based system, over several generations of culturing. It may therefore be interesting to compare the results obtained in section 5.2 with a new experiment which could investigate the performance, in an identical set-up, of a commercially available strain of *H. megidis*.

Secondly, in addition to the differences between the EPN species is the difference in the AM fungal treatment. With one experiment using a field derived, *R. idaeus* tailored, AM fungal inoculant and the other, an 'off the shelf' general purpose commercial preparation. Although there are isolated examples of when a commercially available inoculum has been applied in an agricultural setting with some success (Ceballos *et al.*, 2013), the literature is dominated by examples of how effects seen in laboratory tests with these products fail to have any detectable effects when used in the field (Herrmann & Lesueur, 2013). This could be due to the fact that the general purpose species mixtures of these inoculum tend not to be specific to the species of plant to which they are being added and generally just contain the most abundant species, or the species most often readily identified in the scientific literature. The literature is also dominated by experiments showing pronounced multi-trophic effects under the artificial scenarios in which only one AM fungal species is colonising a plant. These effects are often seen to break down when more than one species is added (Gange, 2001; Gange *et al.*, 2005; Vannette & Hunter, 2013). This could be caused by so called priority effects, whereby the

species already in association with a plant are more likely to outcompete an added species (Werner & Kiers, 2014) but it is also likely that plant species such as *R. idaeus* have a locally adapted community and added 'exotic' species are far less likely to establish. The addition of an inoculum that is tailored to the plant species in question could therefore interact in a more beneficial way with the population of AM fungi already native to the plant's roots. This may be an effect that might be hard to incorporate and develop into a commercial product, which usually contain many species, but evidence for this is certainly apparent when comparisons are made between experiments 5.2 and 5.3 and also apparent in chapter 6.

The presence or absence of *O. sulcatus* made no difference to the preference of *S. kraussei* in this olfactometry experiment. This was perhaps not unexpected as in the previous experiments it was only under a higher level of *O. sulcatus* herbivory that plants responded with chemical changes and recruited EPNs when AM fungal were absent, or with very low abundance. The lower density of 20 *O. sulcatus* eggs added to the plants in this case was used in order to look specifically at the differences between AM fungal treated and non-treated plants. If a higher density of *O. sulcatus* were used and effects observed were similar to those in 5.2 then the AM fungal effects would have been masked by a more general herbivore induced natural enemy attraction effect.

Although *S. kraussei* were not influenced in their foraging direction by AM fungi or *O. sulcatus*, they were influenced by the amount of *R. idaeus* root biomass. EPNs have been shown to be attracted to the roots of plants both in the presence (Kanagy & Kaya, 1993; Cutler & Webster, 2003) and absence of insect hosts (Bird & Bird, 1986). There are many reasons why this may be but in the absence of a detectable host EPNs are known to use chemotaxis to seek out plant roots (Kanagy & Kaya, 1993) and that the change in water gradients and soil structure around plant roots allows for improved host location and speed of dispersal (Bal *et al.*, 2014; Demarta *et al.*, 2014).

The change in root to shoot ratio that *S. kraussei* was responding to was, after further scrutiny almost entirely explained by the relationship directly with root mass and not by any aboveground patterns of biomass. This was not found to be the case with *O. sulcatus*' relationship with the root to shoot ratio of *R. idaeus*. The distribution of *R. idaeus* biomass was in fact found to be driven by the presence of *O. sulcatus* herbivory. This effect of changing above/belowground biomass ratios, in Glen Ample, has been observed and discussed multiple times already in this thesis (chapters 2.4 and 3.4). In this case it is perhaps serendipitous for the *O. sulcatus* larvae that their low population and herbivory on these Glen Ample plants is actually making them less appealing to *S. kraussei*. It should however be noted that even

plants with low root mass attracted between 20 and 30 *S. kraussei* and so it is likely that this set of circumstances may not have saved these *O. sulcatus* larvae from harm.

There wasn't any significant variation in the water concentration of aboveground *R. idaeus* tissues that was explained by experimental treatments. It was originally theorised that water uptake could have been impaired by root herbivory (Zvereva & Kozlov, 2012) or modified by AM fungal presence (Smith & Read, 2008) but no evidence was found for this. This theory could be more vigorously pursued in a future experiment with the additional information that could be inferred by having the wet weight of the *R. idaeus* root tissues. This was however reasoned to be impractical as the complete removal of soil particles from a root system invariably requires the application of a lot of time and fine jets of water both of which would lead to potential inaccuracies when calculating wet weights.

The abundance and performance of *O. sulcatus* larvae retrieved at the end of the olfactometry runs were not found to be affected by AM fungal treatment nor *S. kraussei* attraction. The absence of an AM fungal effect is perhaps to be expected, as previously discussed with reference to commercial inocula, and inocula of multiple AM fungal species. This lack of an effect of AM fungal treatment on *O. sulcatus* performance was also seen in a study by Gange, (2001) which showed that while single species of AM fungi reduced *O. sulcatus* performance, when multiple species of AM fungi were added, this effect was lost. The absence of an impact of *S. kraussei* populations on *O. sulcatus* abundance and performance is encouraging as it suggests that *S. kraussei* are effectively trapped by the 40µm mesh and are not reaching the experimental pots, which could have led to either the death of *O. sulcatus* larvae before retrieval or an underestimation in the populations of *S. kraussei*.

5.4 Conclusions

The two different olfactometry experiments discussed in this chapter have enabled a greater understanding of this complex multi-trophic system and also raised many more avenues of enquiry for later investigation. The first of these two experiments established if the observation of effects seen in other herbivore induced VOC attraction studies with EPNs could be repeated in a *R. idaeus*-AM fungi-*O.sulcatus* system. The second olfactometry experiment attempted to apply the findings of the first into a system more similar to that found in the field, to see what could be reasonably achieved in a *R. idaeus* cropping system with commercially available products.

The two *R. idaeus* cultivars tested in the first olfactometry experiment did not show any difference in their attractiveness to the EPN, *H. megidis*, nor were there any differences in VOC

production detected. For this reason, experiments subsequent to this focused on the more commercially relevant cultivar, Glen Ample.

When field derived spores were added to plants, those plants were found to be more attractive to *H. megidis* when *O. sulcatus* densities were low. This result was not found to be attainable when a more generalised population of AM fungi were added, in the form of a commercial inoculum. This commercial inoculant was used as an additive treatment to non-sterile soil. The second experiment carried out in this chapter is therefore much more reflective of conditions encountered in the field by *R. idaeus* growers. It suggests that the application of these inocula are not the answer to harnessing any AM fungal induced VOC effects on natural enemy attraction. Instead, if the compounds responsible for enhanced EPN attraction could be isolated and added as part of an integrated crop management regime with EPNs then this could provide a much more reliable pest management tool. This could be a way of improving EPN efficacy and bringing the level of *O. sulcatus* control that they provide in line with chemical pesticide treatments. It is unlikely that such a development would remove the need for pesticide applications but it could mean that such EPN treatments could reduce the frequency that pesticides would need to be applied. If enhanced EPNs were more effective at safeguarding plants when *O. sulcatus* populations were at low levels then they could prevent the establishment of *O. sulcatus* infestations in a *R. idaeus* crop. Allowing growers to reduce pesticide use, decreasing their reliance on such products, potentially reducing costs, but more importantly decreasing their impact on the environment and non-target organisms such as pollinators and natural enemies. Developing such a system would require further lab and field based trials to determine if such effects could be realised.

In a future olfactometry experiment it would be good to test a commercially available strain of *H. megidis* to see if they produced the same results in section 5.1. It would be interesting to also measure the relative success of the two EPN species taxis towards hosts where different substrate types, other than sand were used in the centre of the olfactometer.

The results of this chapter and of chapter 4 lead to a new study, presented in chapter 6, in a polytunnel environment comparing multiple commercially available AM fungal inocula and their effects of *O. sulcatus* performance and subsequent *S. kraussei* efficacy.

6 Investigating *Steinernema kraussei* efficacy in protecting raspberry plants with commercial AM fungal inoculants in a protected cropping environment

6.1 Introduction

Positive effects on plant nutrition and negative effects on insect herbivory have been reported over many years of controlled laboratory and field studies on AM fungal plants (Koide & Mosse, 2004). This body of literature provides some very encouraging results that imply, with the exceptions of some functional groups of insect herbivores, that AM fungi can provide improved resistance to plant pests (Koricheva *et al.*, 2009). These effects are often mediated by changes in tolerance to pest damage, reviewed in Vannette & Hunter (2009), either by direct effects that alter herbivore performance (Gange *et al.*, 1994; Gange, 2001) or indirect effects that attract natural enemies (Hoffmann *et al.*, 2011a; Schausberger *et al.*, 2012). Despite these reported positive effects, AM fungi have yet to be adopted into mainstream agriculture.

The lack of uptake by mainstream agriculture may be explained by a series of reasons. Firstly there is an absence of high quality and reliable, mass produced, and commercially available AM fungal inoculums available to growers (Herrmann & Lesueur, 2013). With some commercial preparations even containing many undeclared species of AM fungi, *Trichoderma spp.* and bacteria (Faye *et al.*, 2013). Part of the problem in terms of the production of inocula is a lack of scalability in the production of AM fungi. The continuous culturing of AM fungi in trap cultures creates selection pressures which may not favour performance in the field and reduce diversity (Trejo-aguilar *et al.*, 2013). The lack of plant and region specificity when it

comes to AM fungal formulations are also known to have an impact on their performance (Rowe *et al.*, 2007; Berruti *et al.*, 2013). The addition of less generic and more complementary AM fungal species may overcome this issue which could be caused by so called priority effects, by which established, native species, outcompete introduced species (Werner & Kiers, 2014). The effects observed in laboratory studies are rarely reproduced in the field with commercial inocula. A rare example of field success with field applications of AM fungi was reported by Ceballos *et al.* (2013), who demonstrated that Cassava yield in Colombia was increased after the addition of a single species inoculation of the AM fungi, *Rhizophagus irregularis*, but this may be a phenomenon that is not globally relevant. A recent study by Soudzilovskaia *et al.* (2015) identified distinct patterns of global AM fungal root colonisation driven by climate and soil chemistry that favour colonisation in regions with milder, more continental climates. The bulk of literature testing commercial inocula demonstrates very poor performance of inocula and frequently negative effects on plants after addition (Corkidi *et al.*, 2004; Rowe *et al.*, 2007; Berruti *et al.*, 2013; Faye *et al.*, 2013).

Another aspect that can be off-putting to growers is the limited shelf life of AM fungi and the variable effects of the sterile 'inert' carriers. The shelf life of AM fungal inocula is brief, and even with refrigeration they last for weeks to months rather than the years that mineral fertilisers and chemical pesticides can be stored. This also means that if inoculation of a crop is not soon after purchase then large scale refrigeration must be invested in to enable storage. The inert carriers used with AM fungal formulations have been shown to contain growth promoters and micronutrients that may have unexpected effects and in some cases be more effective at increasing plant yields than the AM fungal component (Corkidi *et al.*, 2004; Faye *et al.*, 2013). AM fungi, even if effective do not provide the immediate and dependable impact on crops of their chemical fertiliser and pesticide equivalents and applications are currently a more expensive option (Ceballos *et al.*, 2013). Another issue that may preclude the inclusion of AM fungi into an integrated crop management system is their sensitivity to applications of foliar systemic fungicides. Fungicides are vital for soft fruit production in order to combat rusts and powdery mildews but are detrimental to AM fungi (Kough *et al.*, 1987) and are even used for this reason to create control treatments in AM fungi studies (Gange & West, 1994). Not all fungicides have detectable effects on AM fungi after single applications (Sukarno *et al.*, 1993), but how they perform in the long term, after successive treatments throughout several growing seasons remains to be seen. This at the very least, restricts a grower to the products they can use in concert with AM fungi and may well provide a barrier to growers on the grounds of increased cost.

In an effort to test whether the effects seen in chapters 3 and 5.1, on *Otiorhynchus sulcatus* and *Steinernema kraussei* performance after adding an indigenous soil based spore inoculation could be reproduced with a commercial inoculum a polytunnel trial was carried out. The main aim was to determine if commercial AM fungi had an impact on *O. sulcatus* control when the EPN, *Steinernema kraussei* was added to *R. idaeus* and how this might compare to a soil based inoculation. The primary objective was to investigate if commercial inocula could enhance *S. kraussei* performance as effectively as a field derived spore (FDS) inoculation. It was hypothesised that *O. sulcatus* survival and larval mass would be lowest in plants treated with a FDS inoculation. Another objective was to assess the effects that different AM fungal treatments would have directly on *R. idaeus*. It was assumed that plant biomass and dormancy breaking would be increased when the most beneficial AM fungal community was present. Two hypotheses were tested to this effect. First that *R. idaeus* growth would be highest in the FDS treatment, secondly that dormancy breaking would be highest in the FDS treatment. The percentage root length colonised (%RLC) by AM fungal structures can sometimes give an indication of the benefit derived by a host plant. It was hypothesised that the different AM fungal communities added to *R. idaeus* would result in different levels of %RLC.

6.2 Materials and methods

An experiment was set up to investigate how two different commercial AM fungal inocula compared to the field derived AM fungi used in chapters 4 and 5.1, when added to *R. idaeus*. After inoculation with AM fungi all *R. idaeus* then had an *O. sulcatus* herbivory treatment added which was then controlled using the EPN, *S. kraussei.*

In preparation for the experiment, 160 *R. idaeus* canes of the industry favourite Glen Ample cultivar were purchased from Hargreaves Plants® (Kings Lynn, UK), a major commercial distributor of this cultivar. This meant that plants were purchased in the same life stage and condition as a commercial *R. idaeus* grower would receive them. These dormant *R. idaeus* canes were then weighed so that their initial biomass could be included as a covariate in analyses. Glen Ample canes were potted up into 2L pots with 1.8L of compost (John Innes No. 3, Levingtons, UK). Soil was not sterilised, as this is a facility that is not available to *R. idaeus* growers, and not possible in field planting and so all soil treatments are purely additive treatments. Plants were inoculated with one of four AM fungal treatments at the time of planting. Two treatments were commercially available AM fungal inoculants added at their

recommended dosages. The first of these four treatments received 5ml per pot, of 'MycoForce Mycorrhizal Transplanter™' (MF) produced by Symbio® with each 5ml dose containing approximately 1100 propagules. The second treatment received 15ml of 'rootgrow™ mycorrhizal fungi' (RG) produced by PlantWorks Ltd. (Sittingbourne, UK) containing approximately 2500 propagules per dose. The third treatment received a field derived spore (FDS) population of 41 ± 7.4 AM fungal spores taken from trap cultures (see section 2.1.4). This was prepared in exactly the same way as outlined in section 2.1.2 but instead of the creating of a microbial wash, as in other chapters, after the wet sieving stage, collected spores were surface sterilised with 9% Sodium Hyporchlorite solution to control for other soil microbes following methods used by Klironomos (2002). All of these three treatments contained sterilised (heat sterilised twice at 400°C for 20mins) material from the other two treatments so as to control for any additional nutrients contained in the inert clay carrier contained in the two commercial inocula and trace (equalling less than 1% by volume) additives such as chitin, alginates and humates. The fourth and final AM fungal treatment consisted of sterilised inocula from all three of the AM fungal inoculants and therefore represented a treatment where only the microbe community present in the compost and or already associated with the dormant *R. idaeus* canes was present.

The 160 potted, and inoculated dormant *R. idaeus* canes were arranged into a randomised block design consisting of 4 experimental blocks, within which all treatments were represented equally, inside a polytunnel (located at 51°25'37.8"N 0°34'01.2"W). Plants were put into the polytunnel in early September 2013. A five week period of uninterrupted growth enabled AM fungi and *R. idaeus* to establish. After this five week period a population of 40 *O. sulcatus* eggs (taken from culture as described in 2.1.8) was added to all plants from all treatments to simulate a high level of root herbivory (Clark *et al.*, 2012). After four weeks (as in section 4.2) approximately 9000 *S. kraussei* (Becker and Underwood®, Littlehampton, UK) were added to each *R. idaeus* pot to represent a grower responding to and treating an *O. sulcatus* infestation using the recommended dosage of this biological control agent. Three weeks after the application of *S. kraussei*, in early December, the plants were harvested. During this time the temperatures had been mild (Table 6.1) and in November a thermostatic heater was installed to keep the temperature in the polytunnel above 6°C. This ensured that neither *O. sulcatus* growth (Moorhouse *et al.*, 1992) nor *S. kraussei,* (Richardson *et al.*, 2002), activity was impaired by low temperatures.

Table 6.1: Climate data taken from the Met Office historical data set at their Heathrow weather station (51°28'34.9''N 0°29'39.3"W) in 2013, just 7.5km from the experimental site. *Sun hours were recorded using an automatic Kipp & Zonen sensor.

Month	Maximum recorded temperature (°C)	Minimum recorded temperature (°C)	Number of days with air frost	Sun hours*
September	19.7	11.1	0	118.9
October	17.0	10.6	0	89.6
November	10.4	4.7	1	80.4
December	10.2	3.5	3	51.3

When the plants in the experiment were harvested, an attempt was made to recover *O. sulcatus* larvae from the root system, then the above and belowground portions of *R. idaeus* were cleaned of soil and placed in a drying oven at 50°C. Subsequent to desiccation the plant tissues were weighed and their dry mass recorded. A 2g sub sample of root tissue was taken from each plant in order for AM fungal colonisation to be assessed using the methods outlined in 2.1.6 and 2.1.7.

No *O. sulcatus* larvae, live or dead, were retrieved from *R. idaeus* at the end of the experiment. This meant that no statistical analyses could be carried out on this data as in previous chapters. At the end of the experiment, *R. idaeus* had started to flower, but due to time and equipment constraints the exact numbers of flowers were not recorded but their presence was noted on approximately 80% of *R. idaeus*.

The *R. idaeus* total biomass data and the *R. idaeus* root to shoot ratio were both analysed separately in linear regression models against the AM fungal treatment with the initial dormant cane weight as a covariate. The dormancy breaking recorded in *R. idaeus* canes was analysed using a generalised linear model, with a quasi-binomial errors structure, against the AM fungal treatment applied.

The percentage root colonised (%RLC) by each of the three different AM fungal structures; arbuscules, vesicles and hyphae were analysed using linear regression models, with a quasi-gaussian errors, against the AM fungal treatment added and the initial *R. idaeus* cane mass. To further understand the distribution of %RLC in plants an analysis of plant benefit vs arbuscular colonisation was carried out to see if the data fitted with the model proposed by Gange & Ayres (1999). Plant benefit was calculated as the percentage change (positive or negative) in *R. idaeus* biomass and run in a linear model against %RLC of arbuscules.

All analysis was carried out using R3.1.2 'Pumpkin Helmet' (R Core Team, 2013) and models were simplified where appropriate with the best fitting minimal models reported.

6.3 Results

The biomass of *R. idaeus* was found to vary across AM fungal treatments with control and FDS treatments producing very similar biomass but RG and MF treatments resulted in lower biomass (Figure 6.1). The biomass in RG treated *R. idaeus* was found to be lower ($t_{1,29}$=-2.68, P <0.01) than the other treatments while the lower biomass seen in MF treated plants was found to be due to the initial differences in cane biomass which was included as a covariate.

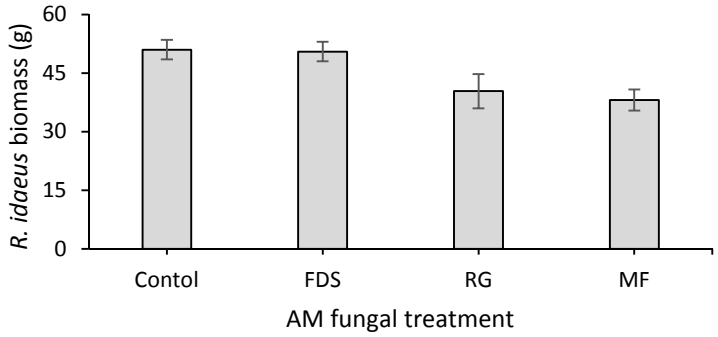


Figure 6.1: The mean biomass of *R. idaeus* was found to be lower in treatments where the RG AM fungal inoculant was added.

The root to shoot ratio calculated for *R. idaeus* plants was found not to be affected by the added AM fungal treatments and did not differ between treatments.

Some of the *R. idaeus* canes that were planted at the beginning of the experiment remained dormant, or died and failed to bud and produce fresh growth of any kind. It was found that fewer broke dormancy when treated with RG ($t_{1,159}$=2.87, P <0.01) than in any other treatment. There was also a similar trend in plants inoculated with MF (P >0.05). Plants in the control and FD AM fungal treatment groups fared equally well with both treatments producing fresh growth on canes in 31 of 40 *R. idaeus* planted (Figure 6.2).

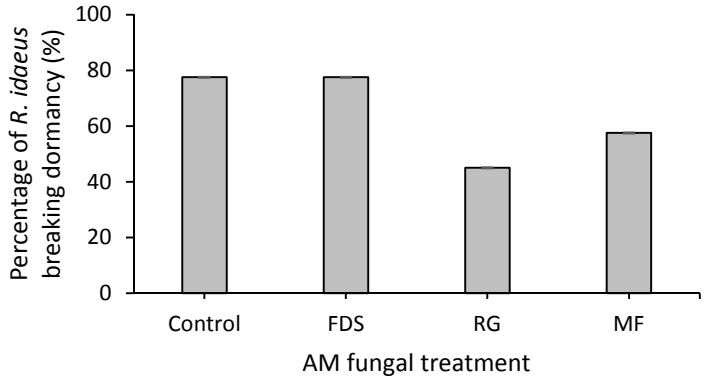


Figure 6.2: The percentage of *R. idaeus* canes that broke dormancy was found to be lower in the Rootgrow™ (RG) AM fungal treatment.

AM fungal colonisation, reported as %RLC of different AM fungal structures, was recorded at fairly low levels in all AM fungal treatments (Table 6.2).

Table 6.2: The %RLC of AM fungal structures across different AM fungal treatments.

AM fungal treatment	Mean Arbuscular colonisation (%RLC with SE)	Mean Vesicle colonisation (%RLC with SE)	Mean Hyphal colonisation (%RLC with SE)
Control	7.1 ± 3	3 ± 1	14 ± 7
FDS	3.0 ± 1	5.9 ± 3	9.0 ± 3
RG	8.1 ± 3	8.7 ± 3	16.3 ± 4
MF	4.8 ± 2	8.5 ± 4	12.9 ± 4

The mean %RLC by arbuscules was found to be lower in plants when the field derived AM fungal treatment was added ($t_{1,25}$=-2.28, P <0.05) when compared to other treatments (Figure 6.3).

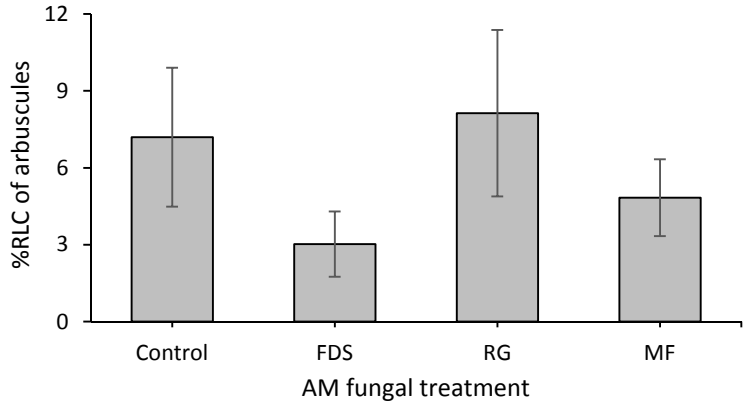


Figure 6.3: The %RLC of arbuscules was found to be lower in the field derived spores (FDS) AM fungal treatment than in other treatments.

In addition to the %RLC of arbuscules being lower in FDS inoculated plants, there was found to be an overall negative relationship between the %RLC of arbuscules and the *R. idaeus* cane mass ($t_{1,25}$= -2.39, P <0.05), taken at the beginning of the experiment (Figure 6.4). This relationship was found to be very similar to the negative relationship ($t_{1,35}$= -2.30, P <0.05) between %RLC and the plant benefit (percentage change in biomass relative to control treated plants) recorded in *R. idaeus* plants (Figure 6.5). There was not found to be any difference in the relationship between %RLC and plant benefit in the different AM fungal treatments.

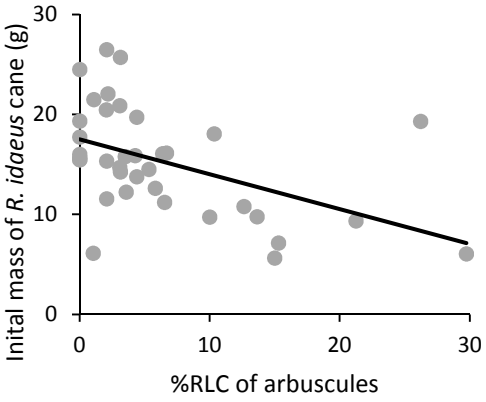


Figure 6.4: The *R. idaeus* cane mass recorded at the beginning of the experiment had a negative relationship with the %RLC of arbuscules.

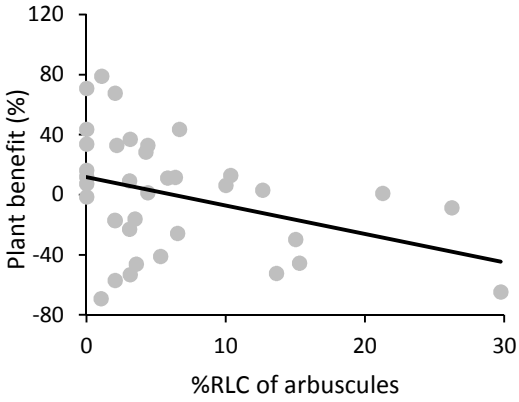


Figure 6.5: A negative relationship was observed between plant benefit and the %RLC of arbuscules in *R. idaeus*.

The %RLC of vesicles and hyphae was not found to differ across the four AM fungal treatments added to *R. idaeus* plants.

6.4 Discussion

This study investigated some of the challenges faced by *R. idaeus* growers when confronted with *O. sulcatus* infestation. It attempted to determine if AM fungal inoculants available to growers could make a difference to plant susceptibility to *O. sulcatus* and the efficacy of *S. kraussei* in its control. The major difference between this system to the conditions encountered in commercial *R. idaeus* cropping was that *R. idaeus* were grown in pots. This is because it is necessary to compartmentalise the rhizosphere when working with a subterranean root feeder such as *O. sulcatus*, but also removes plant to plant interactions via the rhizosphere.

Regrettably many of the planned objectives of this study had to be abandoned as no *O. sulcatus* were recovered from plants. The lack of *O. sulcatus* recovered from plants has proven quite anomalous when compared to other studies which used the same methods of *O. sulcatus* addition and EPN control (Chapter 4 and 5). There may have been a problem with the *O. sulcatus* larvae used in this experiment. *O. sulcatus* eggs were shipped from The James Hutton Institute (TJHI) in Dundee, and the details of the procedures used to maintain this culture are described in 2.1.8. As *O. sulcatus* eggs from this culture have been used in experiments in chapters 3, 4 and 5.1 it seems unlikely that the issue was with non-viable eggs from this culture. It had however been mentioned prior to receiving this shipment of eggs that the culture had been producing fewer eggs in recent months and fresh individual females had not been sourced (Dr Carolyn Mitchell, TJHI, personal communication, 30th September, 2013).

It could be that the viability of these eggs had started to decline along with the fecundity of the adults (Fisher & Bruck, 2004; Fisher, 2006). The eggs were transported in person over a 12h period and added to plants within one week and so no extreme temperatures which are known to affect egg viability occurred during this time (Fisher, 2006). The next possible explanation to explain *O. sulcatus* mortality from a chronological perspective was that *O. sulcatus* failed to survive on the established Glen Ample canes that they were added to. The eggs were added to plants in the poly tunnel with seasonal temperatures of between 10-17°C and this is not an extreme enough range to affect development (Fisher, 2006). The Glen Ample canes used in this experiment were approximately two years older than the plants used in previous chapters (3, 4 and 5) and age may feasibly have reduced their susceptibility to *O. sulcatus*. However the adult *O. sulcatus* that laid these eggs were captured from 2 -3 year old *R. idaeus* canes of various varieties and good *O. sulcatus* performance on mature Glen Ample is well documented (Clark *et al.*, 2012).

The *S. kraussei* treatment added to plants cannot fully account for *O. sulcatus* absence. Although *S. kraussei* were added to all treatments, the test plants, not included in this analysis which had *O. sulcatus* added for the purpose of determining the best time to add *S. kraussei*, also failed to produce any live *O. sulcatus* larvae. *S. kraussei*, given the right conditions could well produce 100% mortality in *O. sulcatus*, it is perhaps unfortunate that it was so successful in this case. The inclusion of two extra control treatments could have elucidated this particular point. A *S. kraussei* free treatment of 40 plants would have determined if *O. sulcatus* were indeed present and an *O. sulcatus* and *S. kraussei* free control could have established if *O. sulcatus* were having an effect on *R. idaeus* growth performance. The reason why these two additional treatments were not incorporated in the design was that *O. sulcatus* had been shown to have an effect on Glen Ample biomass in all previous experiments (chapters 3, 4 and 5) and *S. kraussei* had been shown in chapter 4 to provide around 84% control in a very similar system. In addition to this, including another 80 plants to this experiment would have made the scale of the experimental set up and harvest impractical for a single individual to conduct.

If the *O. sulcatus* and *S. kraussei* treatments, which were added to all plants across all AM fungal treatments are to be presumed to, on average have had, an equal effect on all plants then this does not diminish the potential of this data to enable speculation on the observed differences between AM fungal treatments.

The AM fungal treatment added to plants was found to have a number of different effects on both the plants and their colonisation by AM fungi. One of the key differences between the FDS and the two commercial inocula tested was the mode of application. The FDS treatment added only AM fungal spores to plants, the two other treatments consisted of infective

'propagules'. In this context, the number of propagules refers to the total number of spores, extraradical hyphae and infected root fragments present in any given volume of inoculum. This is unlikely to result in the same level of infectivity as a purely spore based inocula as not all AM fungal species have the same colonisation strategies. Klironomos & Hart (2002) assessed the levels of colonisation success of several AM fungal species when added as either spores, extraradical hyphae or infected root fragments. They demonstrated that while all species tested would readily colonise root tissue when added as spores, only a few genera could infect from hyphae and colonisation was very low when infected root fragments were applied. The two commercial inocula used in this experiment both contained a very high number of propagules, between 1100-2500, while the FDS treatment only contained 41 ± 7.4 spores. With no information on what proportion of propagules within the commercial products were spores it is hard to know how comparable these inoculants are.

One of the main reasons that the methods of controlling for microbial populations in the FDS treatment outlined in Klironomos (2002) was used as opposed to the microbial wash method devised by Ames *et al.* (1987) and used in chapters 3 and 5.1 was the perceived difficulty in applying this method to a commercial inoculum. Subsequent to carrying out this experiment it has been noted that West (1995) proposed a filtration based method for achieving a control for soil microbes. If this experiment was repeated then this method would be incorporated to provide a distinction between the different microbial communities that may be present in a commercial inocula. In fact it has even been shown that in some commercial inocula that several undeclared bacterial species and *Trichoderma spp.* were present (Faye *et al.*, 2013) and it would be good to try and control for any effects of these organisms if they are present.

The commercial product RG performed exceedingly poorly, with *R. idaeus* biomass greatly reduced as well as the numbers of *R. idaeus* canes breaking dormancy. An argument could be made that the addition of RG has introduced a population of AM fungi that are behaving parasitically under these conditions and imposing a fitness cost on *R. idaeus* (Smith & Smith, 1996; Johnson *et al.*, 1997). This is not the first time that negative effects have been recorded on plants when commercial inoculum is applied. Corkidi *et al.* (2004) evaluated the effects on plant growth and AM fungal infectivity of ten commercial inocula. A large range of between 0-50% colonisation was observed, which they concluded was due to a lack of viable propagules and differences in application rates. They determined that nearly all the effects of increased plant growth were due to the presence of growth promoters in the 'inert' carrier substrate as opposed to AM fungal colonisation. In addition to these findings, all but two of the ten inocula tested produced plants that were smaller than control plants, an effect that is far from dissimilar to that seen in this study. In fact, studies reporting success with commercial AM

fungal products appear to be the exception (Ceballos *et al.*, 2013) rather than the rule (Rowe *et al.*, 2007; Berruti *et al.*, 2013; Faye *et al.*, 2013). This is frequently due to the poor quality and consistency of commercial products that often only serve to discourage farmers from looking to improve crop sustainability with expensive biofertilisers (Herrmann & Lesueur, 2013).

As has already been discussed, the *R. idaeus* used in this experiment were older than those used in previous experiments (chapters 3, 4 and 5). Whereas this may not affect *O. sulcatus* directly it can most certainly affect the AM fungi-plant interactions and may in turn effect herbivores via secondary metabolites (Miller *et al.*, 2014).

The low levels of colonisation observed in *R. idaeus* at the end of the experiment could have been linked to the chemical changes in the plant due to flowering. The flowering of a plant has been shown to decrease the formation of new AM fungal structures (Johnson *et al.*, 1982). If this experiment was repeated, the numbers of flowers produced on each plant would provide an interesting covariate to incorporate into analyses. The %RLC of arbuscules recorded in *R. idaeus* was found to be significantly lower in FDS treated plants than in any other AM fungal treatment. This may be explained by the models for AM fungal colonisation versus plant benefit proposed by Gange & Ayres (1999). The dose-response effects of AM fungal colonisation were assessed with respect to plant benefit. The results presented may explain why there was significantly lower colonisation in the FDS treatment when compared to other treatments. These lower levels of arbuscule density on *R. idaeus* roots may produce maximum benefit to the plant, while higher levels of arbuscule colonisation density start to have a negative impact past approximately 5% colonisation on average. The effects in this experiment of %RLC versus plant benefit were found to have a linear relationship as opposed to the curvilinear predicted by Gange & Ayres (1999). This may be a consequence of low sample size, perhaps with a greater level of replication a curvilinear effect would emerge. This same effect may also explain the %RLC of arbuscules also having a negative relationship with the initial cane size of *R. idaeus*. Larger canes appeared to result in lower levels of arbuscule colonisation. These larger canes often had more developed root systems and as a consequence of this, they may have had a more established, pre-existing AM fungal community. Priority effects detected in AM fungal communities show that species that are already in association with roots, will outcompete added species (Werner & Kiers, 2014). Another possible explanation could be that large plants were less reliant on AM fungi for nutrition due to large reserves within tissues prior to dormancy.

Some of the limitations of staining as a method to determine AM fungal colonisation are that stained structures are not necessarily alive and active, and collected data is only reflective of

colonisation at the time of harvest. This means that changes in colonisation over time are not known, which limits the information that can be derived as such changes can be substantial (Šmilauer, 2001; Husband *et al.*, 2002). Plants under stresses such as herbivory can also present a different proportion of AM fungal structures in their roots than plants not enduring such stresses (Duckmanton & Widden, 1994; Klironomos *et al.*, 2004), a distinction that cannot be made in this study.

One of the original objectives for this experiment was to analyse the AM fungal community in association with the roots of the differently treated *R. idaeus* using molecular techniques. This would be useful information as it has been that there are species specific effects seen only when certain AM fungi are present (Gange, 2001). A collaborator was approached to help carry out this work but unfortunately the collaborator did not have a schedule that allowed for samples to be analysed quickly enough after the experimental harvest. This was a problem as long term storage in -80°C freezers were not available for this purpose at RHUL. A new collaborator was approached to analyse the roots of *R. idaeus* plants in chapter 5.2 to try and investigate how the AM fungal community changes after the addition of a commercial inoculum.

6.5 Conclusions

No *O. sulcatus* were retrieved from plants and so the focus of this study shifted from one of AM fungi/insect herbivore interactions to a comparative study on *R. idaeus* performance under different AM fungal treatments.

The infective propagules in commercial inocula consist of an unknown proportion of spores. As spores are the only reliable infective structure to produce colonisation in plant roots these high numbers of infective propagules reported may be misleading. The commercial inoculum known as Rootgrow™ appeared to have negative effects on *R. idaeus* performance, reducing biomass and dormancy breaking. This community was clearly very ill-suited to Glen Ample *R. idaeus*, behaving more like a parasite than a mutualist. The field derived spore inoculation performed in a very similar way to control plants with no detectable differences with regards to *R. idaeus* performance.

The differences in the percentage of root length colonised in the different AM fungal treatments was found to be explained by a dose response effect of AM fungal colonisation. High levels of colonisation produced negative effects on plant biomass, while low levels were either beneficial or benign.

7 Summary and General Discussion

7.1 Summary of results by chapter

7.1.1 Chapter 3

- A tritrophic system was set up to investigate the interactions between *Otiorhynchus sulcatus, Rubus idaeus* and arbuscular mycorrhizal (AM) fungi under glasshouse conditions.
- The Glen Ample and Glen Rosa *R. idaeus* cultivars tested showed different growth patterns in response to AM fungal and *O. sulcatus* treatments. Both Glen Ample and Glen Rosa showed the same resource reallocation tolerance response to *O. sulcatus* herbivory.
- Root emissions in the form of volatile organic compounds (VOCs) were captured during the experiment. Analysis did not identify that any VOCs were induced by herbivores or AM fungi but some known semiochemicals, *α*-pinene and carene, were identified.

7.1.2 Chapter 4

- In this chapter a multi-trophic system was set up to assess the relationships between *R. idaeus*, AM fungi, *O. sulcatus* and entomopathogenic nematodes (EPNs) under glasshouse conditions.
- The EPN, *Steinernema kraussei,* was found to provide superior levels of *O. sulcatus* control when compared to the EPN, *Heterorhabditis megidis,* and control treatments.
- *Otiorhynchus sulcatus* larvae had lower survival and performance on the *R. idaeus* cultivar Glen Rosa.
- The commercial favourite, Glen Ample, was far more susceptible to *O. sulcatus* damage, when compared to Glen Rosa.
- The application of *S. kraussei* on Glen Rosa provided the highest levels of *O. sulcatus* suppression.
- Surprisingly even in the control treatments the *O. sulcatus* larvae did not impact significantly on overall plant biomass but they did influence carbon allocation in the plants, with more being pushed to the shoots in Glen Ample, as seen in Chapter 3.
- AM fungal colonisation was a lot higher in Glen Ample than Glen Rosa but this was likely due to this susceptible cultivar investing less carbon resources belowground and

more aboveground. This reduced root growth can lead to artificially high percentage of root length colonised (%RLC).

- On Glen Ample plants, *O. sulcatus* performance increased in the presence of more arbuscules. This is possibly due to higher phosphorus concentrations in tissues. This contrasts to existing literature on AMF/root feeding performance.

7.1.3 Chapter 5

- Two olfactometry experiments were carried out in order to investigate the preferences of EPNs to differently treated *R. idaeus*. The EPNs were exposed to plants treated with different combinations of *O. sulcatus* larvae and AM fungi.
- The two *R. idaeus* cultivars, Glen Rosa and Glen Ample, which were tested in the first olfactometry experiment did not show any difference in their attractiveness to the EPN, *H. megidis*, nor were there any differences in VOC production detected.
- When field derived spores were added to plants, those plants were found to be more attractive to *H. megidis* even when *O. sulcatus* densities were low.
- High densities of *O. sulcatus* were found to produce greater VOC emissions which in turn made plants more attractive to *H. megidis*.
- In the second of these two olfactometer experiments commercial inocula were tested in combination with low populations of *O. sulcatus* to see if effects seen with field derived spores and *H. megidis* could be repeated.
- When commercial inocula was used, attraction of the more effective EPN, *S. kraussei*, was not affected. Instead, *S. kraussei* attraction was dictated largely by *R. idaeus* root biomass.

7.1.4 Chapter 6

- This chapter documented the interactions between *R. idaeus*, field derived and commercial AM fungi, *O. sulcatus* and *S. kraussei* in a protected cropping environment.
- No *O. sulcatus* were retrieved from plants under any of the different treatments added
- The commercial inoculum known as Rootgrow™ had negative effects on *R. idaeus*, reducing biomass and dormancy breaking. This AM fungal community behaved more like a parasite than a mutualist.
- Plants inoculated with field derived spores performed in a very similar way to control plants, with no detectable differences with regards to *R. idaeus* performance.
- Differences in the %RLC of arbuscules across the different AM fungal treatments were explained by a dose response effect of arbuscule colonisation. High arbuscule

colonisation resulted in negative effects on plant biomass, while low levels were either beneficial or benign.

7.2 General Discussion

This thesis set out with the aim of discovering if mycorrhizal fungi facilitated root defence signalling in belowground predator-prey interactions. This line of enquiry was posed as a consequence of work done by a number of different research groups, all with relevance to belowground interactions involving root feeding insects. Publications by Gange *et al*. (1993, 1996, 2001) demonstrated negative effects of AM fungi on the larval mass and survival of *O. sulcatus* providing evidence that this belowground herbivore may be influenced by the presence of AM fungi. In addition to this, work conducted by Dr Scott Johnson (University of Western Sydney, Australia) and colleagues (Clark *et al.*, 2011c,a, 2012; Johnson *et al.*, 2012) investigated how many aspects of *O. sulcatus* performance varied on different *R. idaeus* cultivars. *Rubus idaeus* is a good model plant for the investigation into the performance of this pest as it is highly mycorrhizal (Varma & Schuepp, 1994), a favoured host of *O. sulcatus* (Alford, 2007), and a valuable horticultural crop (DEFRA, 2013). An effective biological control agent for *O. sulcatus* is the application of EPNs (Haukeland & Lola-Luz, 2010) and recent studies have shown that plant cultivar (Degenhardt *et al.*, 2009), AM fungi (Schausberger *et al.*, 2012), and herbivory alone (Rasmann *et al.*, 2005) can have an impact on EPN attraction to plants. This third aspect of herbivore induced, natural enemy attraction draws together elements from both the research performed on *O. sulcatus* and AM fungi and that on *R. idaeus* cultivars. This area of research became the main focus of this study and its investigation led to the experimental chapters presented in this thesis.

7.2.1 Soil sterilisation

The sterilisation of soil via autoclaving was carried out in experiments reported in chapters 3, 4 and 5.1 prior to inoculation with AM fungal inoculum. A decision was made to discontinue this practice in the experiments reported in chapters 5.2 and 6. This decision was based on a few different factors. Firstly, if this research is to have particular relevance to *R. idaeus* growers, any effects observed need to be repeatable in the field. One of the key differences between the conditions of field and lab studies involving AM fungi is that field soils cannot be easily sterilised. This can produce a system that is very different from that of a lab based study. In a lab based study, experiments are straight forward 'Sterile' vs treatment comparisons. This kind of comparison is useful for advancing fundamental research but recently the biological

relevance of sterile, microbe free plants has been questioned, as it is a state that is almost never found in nature. This, as discussed by Partida-Martínez and Heil (2011) ignores the evolutionary history of plants and cannot be relied upon to produce normal ecological outcomes following interactions with experimental treatments. The ease with which AM fungi added to a system then colonise a plant is also understood to be quite different. Plants in the field that have an AM fungal community added in the form of an inoculum are likely to encounter a plant that has a pre-existing AM fungal community in residence in the root system. Werner and Kiers (2014) demonstrated that the order of arrival of AM fungal species matters, as indigenous AM fungi can outcompete invading species with no apparent reduction in fitness. This is an effect that is completely missed in studies where sterile soil has been applied and so reduces their ecological relevance.

The autoclaving of soil changes not just the indigenous AM fungal population but also has an effect on the free living microbial community. There are a large number of soil bacteria which are categorised functionally as mycorrhiza helper bacteria, the exclusion of these bacteria can inhibit AM fungal performance as they are known to enhance sporulation and mycelial growth (Frey-Klett *et al.*, 2007). It is also misleading to assume that the sterilisation of soil has the effect of ceasing all biological activity. In fact it often leaves only certain enzymes and microbes that create a very unnatural community when compared with untreated soil (Carter *et al.*, 2007). These considerations all contributed to the change in soil preparation protocols.

7.2.2 Soil/Sand mixtures in mycorrhizal studies

A number of studies that investigate AM fungal interactions have included in their methods a dilution of soil media with sand by around 50% by volume (Bennett & Strauss, 2013; Bennett *et al.*, 2013). These studies quote reasons such as improved drainage or an effort to reduce the chance that AM fungi will behave parasitically, but the creation of a phosphorus deficient medium ensures that plants are very dependent on their AM fungal symbionts and more likely to receive a positive benefit from colonisation (Hoeksema *et al.*, 2010). This provides more exaggerated effects than would otherwise be seen in a pure soil medium.

These high, >50%, sand systems bear little in common with the conditions faced by *R. idaeus* growers and are perhaps only directly relevant to the margins of agricultural land adjacent to sand dune systems. The behaviour and performance of soil organisms is impacted by soil texture and structure. Soils with high sand content are coarser and have greater porosity (Brady & Weil, 2007) and have been shown to have an impact on the growth and morphology of AM fungi (Drew *et al.*, 2003). Sandy soils are known to have a negative impact upon root herbivores (Brown & Gange, 1990) and in species with morphology similar to *O. sulcatus* larvae, which rely on existing cracks and fissures in the soil to aid movement, this may be

especially detrimental (Villani *et al.*, 1999). Soil texture also has an impact on EPN behaviour, with different species often more effective in different substrate types and foraging behaviours modified in sandy soils (Kruitbos *et al.*, 2010).

For these reasons, after the completion of experiments presented in chapters 3, 4 and 5.1, subsequent experiments were conducted in an undiluted soil media that had the same sand content as when purchased. This means that any effects seen are more likely to be replicated in a potted horticultural setting.

7.2.3 Relevance to *R. idaeus* cropping in the UK

Otiorhynchus sulcatus is a damaging pest on *R. idaeus*, a crop of major economic importance in the UK with the 'farm gate' value of production valued at £89.6 billion in 2013 (DEFRA, 2013). There are several reasons why *O. sulcatus* is a particular issue in *R. idaeus* cropping. One of the reasons for recent concern regarding this pests' impact is that over the last decade the majority of *R. idaeus* production has been converted to polytunnel based protected cropping systems. These polytunnels raise and moderate temperatures relative to ambient conditions and allow for greater control in water management. They also serve to extend growing seasons and produce higher quality fruit when compared to unprotected cropping (Demchak, 2009). These improved conditions for *R. idaeus* come at a cost. The same conditions that favour *R. idaeus* production also improve the performance of *O. sulcatus* (Johnson *et al.*, 2010). On top of this, the future for chemical insecticide based methods of this pest is by no means certain and so biological control alternatives are commonly employed as an alternative. One of the more popular choices for this is the application of EPNs (Haukeland & Lola-Luz, 2010). Some of the problems with the field application of EPNs is that they are often unreliable when compared to insecticides as they produce more variable results (Kakouli-Duarte *et al.*, 1997), hence any methods to improve their efficacy would be very welcome.

In this thesis an attempt was made to do just this, by investigating possible VOC attractants to EPNs and try to establish if mycorrhizal plants showed enhanced allure. Unfortunately there were no individual VOCs isolated from AM fungal plants that were shown to have a direct impact on EPN attraction. However, the presence of AM fungi was shown to increase attraction of EPNs when compared to control treatments. Further conclusions from herbivore induced VOCs are discussed in 7.2.5.

In chapter 4 AM fungal colonisation was found to reduce *O. sulcatus* larval mass, perhaps due to increased provisioning of nutrients, providing greater constitutive defence (Bennett *et al.*, 2006; Kempel *et al.*, 2010), or even a priming effect as a direct consequence of AM fungal colonisation (Jung *et al.*, 2012). *Rubus idaeus* with an AM fungal community were found to be

more attractive to EPNs (Chapter 5.1) again possibly due to extra provisioning or priming by AM fungi which could be leading to changes in the composition of VOC emissions (Rapparini *et al.*, 2008; Fontana *et al.*, 2009).

What was found which is of more immediate relevance to a *R. idaeus* grower is that when the EPN, *S. kraussei*, was applied to *R. idaeus* of the cultivar Glen Rosa then greatly improved control of *O. sulcatus* was achieved. If growers could be convinced to grow more pest resistant cultivars and apply EPNs throughout the growing season then high levels of *O. sulcatus* control could be maintained. Such applications could help to reduce chemical pesticide inputs which can avoid effects on non-target organisms such as pollinators and natural enemies.

The application of pest resistant, but not genetically modified, *R. idaeus* cultivars are compatible with farming with an organic certification (Soil Association Certification 2013). This can add significant market value to raspberries. Depending on the quality of fruit organic certified production can increase the end consumer price between 49% - 84% based on information from 3 of the UK's 5 biggest supermarkets (Table 7.1). While this data was taken from produce being sold out of season, in March, it does show that there is significant value added to raspberries when they are sold with an organic certification label.

Table 7.1: The retail prices of organic and non-organic raspberries at the UK's 5 largest supermarkets. Asda and Morrisons were not offering an organic alternative at the time this data was taken on 27/03/2015. Data taken was exclusive of promotional offers from the supermarket websites.

UK Supermarket	Price of Non-Organic Raspberries (£/kg)	Price of Organic Raspberries (£/kg)	Added value to Raspberries (%)
Tesco	£13.34	£20.00	49%
Sainsbury	£13.33	£22.00	65%
Waitrose	£15.16	£28.00	84%

If EPN efficacy against *O. sulcatus* can be further improved then this pest will become less of a barrier to *R. idaeus* growers interested in increasing their crop's value through organic production. Additionally, long term organic agriculture has been found to have a positive effect on soil microbial diversity (Birkhofer *et al.*, 2008) which could help to restore some of the benefits associated with AM fungal colonisation previously mentioned and those found by other authors (Smith & Read, 2008; Koricheva *et al.*, 2009).

7.2.4 The application of commercial AM fungi

The complex nature of the AM fungal communities associated with plant roots often leads to very variable results between studies. Crop plants are commonly associated with multiple species of AM fungi (Daniell *et al.*, 2001) and the AM fungal community can be specific to a

particular plant community (Johnson *et al.*, 2004). This can mean that generalised species mixtures of AM fungi compare poorly to plant specific communities (as discussed in chapter 6). Additions of commercial inocula were shown to result in negative or benign effects on plants when applied in chapters 5.2 and 6. This could be explained by priority effects whereby indigenous AM fungi outcompete all invader species (Werner & Kiers, 2014). Theories as to how these effects may be overcome are discussed in detail in Gadhave *et al.* (appendix 2). This thesis has added to the body of literature (Rowe *et al.*, 2007; Berruti *et al.*, 2013; Faye *et al.*, 2013) indicating that commercial producers of AM fungi are trying to run before they can walk, with bold claims of unspecified plant benefit. Until there is a greater understanding of the specificity and community ecology of soil microbes it seems very unlikely that a universal inoculum will provide anything approaching a reliable tool in a growers' arsenal of crop management techniques. Instead it is more likely that more context specific products, tailored to particular plant communities, may prove to be more effective products (Berruti *et al.*, 2013).

7.2.5 Herbivore induced volatiles and natural enemy attraction

There is definite evidence that herbivore induced volatile production can be modified by AM fungi (Rapparini *et al.*, 2008; Fontana *et al.*, 2009) and that this can result into enhanced natural enemy attraction (Schausberger *et al.*, 2012; Patiño-Ruiz & Schausberger, 2014). In this study however these effects were not identified. The production of VOCs by *R. idaeus* under different herbivore pressures was not found to produce a significant difference in the composition of VOCs (chapter 3.3). In chapter 5.1, it was found that there was elevated VOC production and EPN attraction when *R. idaeus* were under a high herbivore pressure. Despite these conditions and treatments being very similar to those in chapter 3, the VOC data set in chapter 3 did not show this effect of increased VOC production. There is however a trade off when trying to maximise ecological relevance as the populations of all four of the organisms in this system are genetically variable and phenotypically plastic and this can have an impact on the reproducibility of results (Heil, 2014b).

What was less clear was how the presence of AM fungi was influencing EPN attraction. Mycorrhizal plants were clearly more attractive to *H. megidis* in the olfactometry experiment but the distinction between mycorrhizal and non mycorrhizal plants was not evident in VOC data. This is probably because of the number of captured VOCs that have been identified was very low in the two data sets. Rapparini *et al.* (2008) demonstrated that it was the proportion of monoterpenes and sequesterpenes that was altered by AM fungal colonisation rather than the total signal of VOCs. With such a small number of potential monoterpenes and sequesterpenes identified it may be that the greater part of the story this data set has to tell is yet to be told.

As far as the application of AM fungi to augment herbivore induced defences in plants is concerned this thesis suggests that reproducibility even between lab studies is low, let alone application in the field. As has been previously discussed, commercial AM fungi produce unreliable and often unfavourable effects in plants. These products are not the answer to harnessing potential AM fungal induced VOC effects on natural enemy attraction. However, if the VOCs responsible for enhanced EPN attraction could be identified and then isolated then used as part of an integrated crop management system in conjuncture with EPNs then this could provide a reliable pest management tool. Such a development could reduce the frequency that chemical pesticides would need to be applied. If enhanced EPNs were more effective at safeguarding plants when *O. sulcatus* populations were at low levels then they could prevent the establishment of *O. sulcatus* on *R. idaeus* crops. If growers were able to reduce pesticide reliance this could potentially reduce costs. More importantly, a reduction in pesticide application frequency could decrease the environmental impact of pest control which would be better for non-target organisms such as pollinators and natural enemies.

Another way in which a very similar system has been implemented in the field with success has been to increase the production of VOC emissions, which recruit natural enemies, in plant tissues. The system developed by Rasmann *et al.* (2005), which identified a herbivore induced VOC attractive to EPNs, went on to restore this signal in a commercially viable cultivar which performed well in the field (Degenhardt *et al.*, 2009). The system described in this thesis has the added potential of being based around a crop that is commonly irrigated. This could be a definite advantage as both EPNs and VOCs can be delivered through such a system with no need to compromise on high yielding but pest susceptible cultivars. This would also avoid the need to use a genetically modified plant, which would avoid major regulatory issues in the EU.

7.3 Conclusions

The findings in this thesis have advanced the understanding of a specific multi-trophic study system, designed to apply knowledge of plant/AM fungi/insect interactions and herbivore induced natural enemy interaction to *R. idaeus* production.

When two cultivars of *R. idaeus* were investigated there was found to be a difference in resource allocation as a tolerance response in *R. idaeus* against *O. sulcatus*. Of the *R. idaeus* cultivars tested, Glen Rosa was found to be more resistant to *O. sulcatus* attack when compared to the commercially favoured Glen Ample cultivar. When two natural enemies in the form of two EPN species, *H. megidis* and *S. kraussei* were added to the system, differences in efficacy were recorded. The commercially available strain of *S. kraussei* was found to be the most effective EPN at controlling *O. sulcatus* under glasshouse conditions. The combination of

Glen Rosa and *S. kraussei* was found to provide exceptional levels of *O. sulcatus* control. When *H. megidis* taxis to *R. idaeus* plants was tested, plant cultivar was not found to influence either taxis or VOC production. Captured VOCs include known semiochemicals and were elevated under high *O. sulcatus* herbivory pressure. The addition of AM fungi increased attraction of *H. megidis* regardless of *O. sulcatus* feeding pressure but it was not possible to attribute this to a difference in VOC production. The production of VOCs and the attraction of *H. megidis* was increased under high levels of herbivory, independent of AM fungal treatment. When EPN attraction was tested with a commercial inoculum, these effects were not seen, and EPNs were instead attracted by higher *R. idaeus* root biomass. Further investigations into the applications of commercial inocula produced negative or an absence of effects on *R. idaeus* whereas a host specific AM fungal inocula produced more positive effects.

As the experimental system explored in this study was developed to become more ecologically relevant to field conditions, it was found that the effects seen under more contrived lab conditions were hard to reproduce. This implies that there are undiscovered interactions between the study organisms and their environment that added 'noise' to experimental data sets. As the field of multi-trophic microbe/plant/herbivore/predator expands and is better understood it is likely that this unexplained variation will be accounted for and these systems will come ever closer to having a direct impact upon how ecological knowledge is applied to cropping systems.

7.4 Suggestions for future work

This thesis has raised many new questions and there are many areas within this system that could benefit from further investigation. There is plenty of scope for further belowground olfactometry experiments as this system provides a very flexible platform with which to study ecological interactions. In future olfactometry experiments it would be good to test a commercially available strain of *H. megidis* to see if they produced the same results as those used in section 5.1, which were taken from a culture available only for research. It would be interesting to also measure the relative success of the two EPN species at taxis towards hosts where substrate types more similar to field soils were used instead of sand in the olfactometer arena. This would require some modifications to EPN extraction techniques but would be relatively simple to carry out, given the equipment and experience acquired in earlier experiments.

The VOC data collected could be expanded upon and added to if a collaboration with a chemical ecologist working on a similar system could be established. If further information

could be derived from the data sets then this could lead to a higher impact publication output subsequent to this PhD.

To further understand the AM fungal communities applied during the experiments reported in chapter 5.2, next generation sequencing to identify AM fungal community colonising the roots will be carried out. This will be done in collaboration with Dr Karita Saravesi (University of Oulu, Finland). It will establish what species were present before and after the addition of a commercial AM fungal inoculant and may enable the detection of priority effects or other AM fungal interactions.

The large scale application of AM fungi in the field to protect crops against insect herbivores may still be in its infancy due to the complexity of agroecosystems but this does not mean that this potential resource should be ignored. Some field trials of the experimental system investigated in this thesis could provide some very interesting results and help to advance our understanding of these complex systems.

References

Akiyama K, Matsuzaki K, Hayashi H. **2005**. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* **435**: 824–7.

Alford D V. **2007**. *Pests of Fruit Crops - A Colour Handbook*. Manson Publishing, UK.

Ali JG, Alborn HT, Campos-Herrera R, Kaplan F, Duncan LW, Rodriguez-Saona C, Koppenhöfer AM, Stelinski LL. **2012**. Subterranean, herbivore-induced plant volatile increases biological control activity of multiple beneficial nematode species in distinct habitats. *PloS one* **7**: e38146.

Ali JGJ, Alborn HHT, Stelinski LL. **2010**. Subterranean Herbivore-induced Volatiles Released by Citrus Roots upon Feeding by *Diaprepes abbreviatus* Recruit Entomopathogenic Nematodes. *Journal of chemical ecology* **36**: 361–368.

Ali JG, Alborn HT, Stelinski LL. **2011**. Constitutive and induced subterranean plant volatiles attract both entomopathogenic and plant parasitic nematodes. *Journal of Ecology* **99**: 26–35.

Ames RN, Mihara KL, Bethlenfalvay GJ. **1987**. The establishment of microorganisms in vesicular arbuscular mycorrhizal and control treatments. *Biology And Fertility Of Soils* **3**: 217–223.

Ansari M a., Butt TM. **2011**. Effect of potting media on the efficacy and dispersal of entomopathogenic nematodes for the control of black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Biological Control* **58**: 310–318.

Aprea E, Biasioli F, Carlin S, Endrizzi I, Gasperi F. **2009**. Investigation of Volatile Compounds in Two Raspberry Cultivars by Two Headspace Techniques : Solid-Phase Microextraction / Gas Chromatography-Mass Spectrometry Spectrometry (PTR-MS). *Journal of Agricultural and Food Chemistry* **57**: 4011–4018.

Aratchige NS, Lesna I, Sabelis MW. **2004**. Below-ground plant parts emit herbivore-induced volatiles: olfactory responses of a predatory mite to tulip bulbs infested by rust mites. *Experimental & applied acarology* **33**: 21–30.

Babikova Z, Gilbert L, Bruce TJA, Birkett M, Caulfield JC, Woodcock C, Pickett JA, Johnson D. **2013**. Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack. *Ecology Letters* **16**: 835–843.

Babikova Z, Gilbert L, Randall KC, Bruce TJA, Pickett JA, Johnson D. **2014**. Increasing phosphorus supply is not the mechanism by which arbuscular mycorrhiza increase attractiveness of bean (*Vicia faba*) to aphids. *Journal of experimental botany* **65**: 5231–41.

Bakker PAHM, Berendsen RL, Doornbos RF, Wintermans PCA, Pieterse CMJ. **2013**. The rhizosphere revisited: root microbiomics. *Frontiers in plant science* **4**: 165.

Bal HK, Taylor RAJ, Grewal PS. **2014**. Ambush Foraging Entomopathogenic Nematodes Employ 'Sprinters' for Long-Distance Dispersal in the Absence of Hosts. *Journal of Parasitology* **100**: 422–432.

Bennett AE, Alers-Garcia J, Bever JD. **2006**. Three-way interactions among mutualistic mycorrhizal fungi, plants, and plant enemies: hypotheses and synthesis. *The American naturalist* **167**: 141–52.

Bennett AE, Bever JD. **2011**. Mycorrhizal Species Differentially Alter Plant Growth and Response to Herbivory. *Ecology* **88**: 210–218.

Bennett AE, Macrae AM, Moore BD, Caul S, Johnson SN. **2013**. Early Root Herbivory Impairs Arbuscular Mycorrhizal Fungal Colonization and Shifts Defence Allocation in Establishing Plantago lanceolata. *PloS one* **8**: e66053.

Bennett AE, Strauss SY. **2013**. Response to soil biota in native, introduced non-pest, and pest grass species: Is responsiveness a mechanism for invasion? *Biological Invasions* **15**: 1343–1353.

Berruti A, Borriello R, Della Beffa MT, Scariot V, Bianciotto V. **2013**. Application of nonspecific commercial AMF inocula results in poor mycorrhization in *Camellia japonica* L. *Symbiosis* **61**: 63–76.

Bever JD, Schultz PA, Pringle A, Morton JB. **2001**. Arbuscular mycorrhizal fungi: more diverse than meets the eye, and the ecological tale of why. *BioScience* **51**: 923–931.

Bezemer TM, van Dam NM. **2005**. Linking aboveground and belowground interactions via induced plant defenses. *Trends in ecology & evolution* **20**: 617–24.

Bezemer TM, Wagenaar R, Van Dam NM, Van Der Putten WH, Wackers FL. **2004**. Above- and below-ground terpenoid aldehyde induction in cotton, Gossypium herbaceum, following root and leaf injury. *Journal of Chemical Ecology* **30**: 53–67.

Bezemer TM, Wagenaar R, Van Dam NM, Wackers FL. **2003**. Interactions between above- and belowground insect herbivores as mediated by the plant defense system. *Oikos* **101**: 555–562.

Bird AF, Bird J. **1986**. Observations on the use of insect parasitic nematodes as a means of biological control of root-knot nematodes. *International Journal for Parasitology* **16**: 511–516.

Birkhofer K, Bezemer TM, Bloem J, Bonkowski M, Christensen S, Dubois D, Ekelund F, Fließbach A, Gunst L, Hedlund K, *et al.* 2008. Long-term organic farming fosters below and aboveground biota: Implications for soil quality, biological control and productivity. *Soil Biology and Biochemistry* **40**: 2297–2308.

Blackshaw RP, Kerry BR. **2008**. Root herbivory in agricultural ecosystems. In: Johnson SN, Murray PJ, eds. Root Feeders - an Ecosystem Perspective. CABI, Wallingford, UK, 35–53.

Boemare N. **2002**. Biology, Taxonomy and Systematics of Photorhabdus and Xenorhabus. In: Gaugler R, ed. Entomopathogenic Nematology. Wallingford UK: CAB International, 35–57.

Boff MIC, Zoon FC, Smits PH. **2001**. Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer. *Entomologia Experimentalis et Applicata* **98**: 329–337.

Borowicz VA. **1997**. A fungal root symbiont modifies plant resistance to an insect herbivore. *Oecologia* **112**: 534–542.

Borowicz VA. **2001**. Do arbuscular mycorrhizal fungi alter plant-pathogen relations? *Ecology* **82**: 3057–3068.

Brady NC, Weil RR. **2007**. *The Nature and Properties of Soils.* Prentice Hall, Englewood Cliffs, NJ.

Brown VK, Gange AC. **1990**. Insect herbivry below ground. *Advances in Ecological Research* **20**: 1–58.

Bruck DJ, Donahue KM. **2007**. Persistence of *Metarhizium anisopliae* incorporated into soilless potting media for control of the black vine weevil, *Otiorhynchus sulcatus* in container-grown ornamentals. *Journal of invertebrate pathology* **95**: 146–50.

Bruck DJ, Shapiro-Ilan DI, Lewis EE. **2005**. Evaluation of application technologies of entomopathogenic nematodes for control of the black vine weevil. *Journal of economic entomology* **98**: 1884–9.

Cahill JF, Elle E, Smith GR, Shore BH. **2008**. Disruption of a belowground mutualism alters interactions between plants and their floral visitors. *Ecology* **89**: 1791–1801.

Campbell JF, Lewis EE, Stock SP, Nadler S, Kaya HK. **2003**. Evolution of host search strategies in entomopathogenic nematodes. *Journal of nematology* **35**: 142–5.

Carter DO, Yellowlees D, Tibbett M. **2007**. Autoclaving kills soil microbes yet soil enzymes remain active. *Pedobiologia* **51**: 295–299.

Ceballos I, Ruiz M, Fernández C, Peña R, Rodríguez A, Sanders IR. **2013**. The in vitro mass-produced model mycorrhizal fungus, *Rhizophagus irregularis*, significantly increases yields of the globally important food security crop cassava. *PloS one* **8**: e70633.

Choo HY, Kaya HK. **1991**. Influence of soil texture and presence of roots on host finding by *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology* **58**: 279–280.

Clark KE. **2010**. Linking aboveground and belowground insect herbivore interactions : a case study with the vine weevil (*Otiorhynchus sulcatus*). *PhD Thesis*.

Clark KE, Hartley SE, Brennan RM, Jennings NN, McMenemy LS, McNicol JW, Mitchell C, Johnson SN. **2012**. Effects of cultivar and egg density on a colonizing vine weevil (*Otiorhynchus sulcatus*) population and its impacts on red raspberry growth and yield. *Crop Protection* **32**: 76–82.

Clark KE, Hartley SE, Brennan RM, Mackenzie K, Johnson SN. **2011a**. Investigating preference-performance relationships in aboveground-belowground life cycles: a laboratory and field study with the vine weevil (*Otiorhynchus sulcatus*). *Bulletin of entomological research* **61**: 1–8.

Clark KE, Hartley SE, Brennan RM, MacKenzie K, Johnson SN. **2011b**. Oviposition and feeding behaviour by the vine weevil *Otiorhynchus sulcatus* on red raspberry: effects of cultivars and plant nutritional status. *Agricultural and Forest Entomology* **14**: 157–163.

Clark KE, Hartley SE, Johnson SN. **2011c**. Does mother know best? The preference-performance hypothesis and parent-offspring conflict in aboveground-belowground herbivore life cycles. *Ecological Entomology* **36**: 117–124.

Cognat C, Shepherd T, Verrall SR, Stewart D. **2012**. Comparison of two headspace sampling techniques for the analysis of off-flavour volatiles from oat based products. *Food Chemistry* **134**: 1592–1600.

Corkidi L, Allen EB, Merhaut D, Allen MF, Downer J, Bohn J, Evans M. **2004**. Assessing the Infectivity of Commercial Mycorrhizal Inoculants in Plant Nursery Conditions. *Journal of Environmental Horticulture* **22**: 149–154.

Cross J V, Burgess CM. **1997**. Localised insecticide treatment for the control of vine weevil larvae (*Otiorhynchus sulcatus*) on field-grown strawberry. *Crop Protection* **16**: 565–574.

Currie AF, Murray PJ, Gange AC. **2011**. Is a specialist root-feeding insect affected by arbuscular mycorrhizal fungi? *Applied Soil Ecology* **47**: 77–83.

Cutler GC, Webster J. **2003**. Host-finding ability of three entomopathogenic nematode isolates in the presence of plant roots. *Nematology* **5**: 601–608.

Daniell TJ, Husband R, Fitter a H, Young JPW. **2001**. Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. *FEMS microbiology ecology* **36**: 203–209.

Daniels BA, Skipper HD. **1982**. *Methods for the recovery and quantitative estimation of propogules from soil* (NC Schenck, Ed.). St. Paul, Minnesota: American Phytopathological Society.

DEFRA. **2013**. *Basic Horticultural Statistics 2013*.

Degenhardt J, Hiltpold I, Köllner TG, Frey M, Gierl A, Gershenzon J, Hibbard BE, Ellersieck MR, Turlings TCJ. **2009**. Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. *PNAS* **106**: 13213–13218.

Demarta L, Hibbard BE, Bohn MO, Hiltpold I. **2014**. The role of root architecture in foraging behavior of entomopathogenic nematodes. *Journal of invertebrate pathology*.

Demchak K. **2009**. Small fruit production in high tunnels. *Horttechnology* **19**: 44–49.

Denno RF, Gruner DS, Kaplan I. **2008**. Potential for entomopathogenic nematodes in biological control: a meta-analytical synthesis and insights from trophic cascade theory. *Journal of nematology* **40**: 61–72.

Dicke M. **1999**. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? *Entomologia Experimentalis et Applicata* **91**: 131–142.

Dicke M, Sabelis MW. **1988**. How plants obtain predatory mites as bodyguards. *Netherlands Journal of Zoology* **38**: 148–165.

Dicke M, Sabelis MW, Takabayashi J, Bruin J, Posthumus MA. **1990**. Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control. *Journal of Chemical Ecology* **16**: 3091–3118.

Dillman A, Guillermin M, Ha Lee J, Kim B, Sternberg P, Hallem E. **2012**. Olfaction shapes host–parasite interactions in parasitic nematodes. *PNAS* **109**: 1–10.

Dowds B, Peters A. **2002**. Virulence Mechanisms. In: Gaugler R, ed. Entomopathogenic Nematology. Wallingford UK: CAB International, 79–99.

Drew EA, Murray RS, Smith SE, Jakobsen I. **2003**. Beyond the rhizosphere: Growth and function of arbuscular mycorrhizal external hyphae in sands of varying pore sizes. *Plant and Soil* **251**: 105–114.

Duckmanton L, Widden P. **1994**. Effect of Ozone on the Development of Vesicular-Arbuscular Mycorrhizae in Sugar Maple Saplings. *Mycologia* **86**: 181–186.

Elliot SL, Maurice W, Janssen A, Leo PS, Geest V Der, Ellen AM. **2000**. Can plants use entomopathogens as bodyguards? *Ecology Letters* **3**: 228–235.

Elsen A, Gervacio D, Swennen R, De Waele D. **2008**. AMF-induced biocontrol against plant parasitic nematodes in *Musa sp*.: a systemic effect. *Mycorrhiza* **18**: 251–256.

Van der Ent S, Van Wees SCM, Pieterse CMJ. **2009**. Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. *Phytochemistry* **70**: 1581–1588.

Faye A, Dalpé Y, Ndung'u-Magiroi K, Jefwa J, Ndoye I, Diouf M, Lesueur D. **2013**. Evaluation of commercial arbuscular mycorrhizal inoculants. *Canadian Journal of Plant Science* **93**: 1201–1208.

Fisher JR. **2006**. Fecundity, longevity and establishment of *Otiorhynchus sulcatus* (Fabricius) and *Otiorhynchus ovatus* (Linnaeus) (Coleoptera: Curculionidae) from the Pacific North-west of the United States of America on selected host plants. *Agricultural and Forest Entomology* **8**: 281–287.

Fisher JR, Bruck DJ. **2004**. A technique for continuous mass rearing of the black vine weevil, *Otiorhynchus sulcatus*. *Entomologia Experimentalis et Applicata* **113**: 71–75.

Fontana A, Reichelt M, Hempel S, Gershenzon J, Unsicker SB. **2009**. The effects of arbuscular mycorrhizal fungi on direct and indirect defense metabolites of *Plantago lanceolata* L. *Journal of chemical ecology* **35**: 833–843.

Forst S, Dowds B, Boemare N, Stackebrandt E. **1997**. Xenorhabdus and Photorhabdus spp.: bugs that kill bugs. *Annual review of microbiology* **51**: 47–72.

Frey-Klett P, Garbaye J, Tarkka M. **2007**. The mycorrhiza helper bacteria revisited. *New Phytologist* **176**: 22–36.

Gadhave KR, Hourston JE, Gange AC. Developing soil microbial inoculants for pest management: Can one have too much of a good thing? *Journal of chemical ecology (invited for resubmission)*.

Gange AC. **1996**. Reduction in vine weevil larval growth by mycorrhizal fungi. *Biologische Bundesanstalt für Land- und Forstwirtschaft* **316**: 56–60.

Gange AC. **2001**. Species-specific responses of a root- and shoot-feeding insect to arbuscular mycorrhizal colonization of its host plant. *New Phytologist* **150**: 611–618.

Gange AC, Ayres RL. **1999**. On the relation between arbuscular mycorrhizal colonization and plant 'benefit'. *Oikos* **87**: 615–621.

Gange AC, Brown VK, Aplin DM. **2003**. Multitrophic links between arbuscular mycorrhizal fungi and insect parasitoids. *Ecology Letters* **6**: 1051–1055.

Gange AC, Brown VK, Aplin DM. **2005**. Ecological specificity of arbuscular mycorrhzae: Evidence from foliar- and seed-feeding insects. *Ecology* **86**: 603–611.

Gange AC, Brown VK, Sinclair GS. **1994**. Reduction of black vine weevil larval growth by vesicular-arbuscular mycorrhizal infection. *Entomologia Experimentalis et Applicata* **70**: 115–119.

Gange AC, M WH. **1994**. Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. *New Phytologist* **128**: 79–87.

Gange AC, Smith AK. **2005**. Arbuscular mycorrhizal fungi influence visitation rates of pollinating insects. *Ecological Entomology* **30**: 600–606.

Gill S, Lutz J, Shrewsbury P, Raupp M. **2001**. Evaluation of biological and chemical control methods for black vine weevil, *Otiorhynchus sulcatus* (Fabricius)(Coleoptera: Curculionidae), in container grown perennials. *Journal of Environmental Horticulture* **19**: 166–170.

Gordon SC, Williamson B, J G. **2006**. Current and future control for major arthropod and fungal diseases of red raspberry (*Rubus idaeus*) in Europe. In: Ramdane D, ed. Crops – growth, quality and biotechnology. WFL Publisher, Helsinki, Finland, .

Gregory PJ, Johnson SN, Newton AC, Ingram JSI. **2009**. Integrating pests and pathogens into the climate change/food security debate. *Journal of Experimental Botany* **60**: 2827–2838.

Guerrieri E, Lingua G, Digilio MC, Massa N, Berta G. **2004**. Do interactions between plant roots and the rhizosphere affect parasitoid behaviour? *Ecological Entomology* **29**: 753–756.

Hall H, Hummer K, Jamieson A, Jennings S., Weber CA. **2008**. Raspberry breeding and genetics. *Plant Breeding Reviews* **32**: 39–382.

Hallem EA, Dillman AR, Hong A V., Zhang Y, Yano JM, Demarco SF, Sternberg PW. **2011**. A sensory code for host seeking in parasitic nematodes. *Current Biology* **21**: 377–383.

Haukeland S, Lola-Luz T. **2010**. Efficacy of the entomopathogenic nematodes *Steinernema kraussei* and *Heterorhabditis megidis* against the black vine weevil *Otiorhynchus sulcatus* in open field-grown strawberry plants. *Agricultural and Forest Entomology* **12**: 363–369.

Heil M. **2014a**. Herbivore-induced plant volatiles : targets , perception and unanswered questions. *New Phytologist* **201**: 297–306.

Heil M. **2014b**. Relevance versus reproducibility-solving a common dilemma in chemical ecology. *Journal of chemical ecology* **40**: 315–316.

Henke C, Kunert M, Veit D, Kunert G, Krause K, Kothe E, Boland W. **2015**. Analysis of volatiles from Picea abies triggered by below-ground interactions. *Environmental and Experimental Botany* **110**: 56–61.

Herrmann L, Lesueur D. **2013**. Challenges of formulation and quality of biofertilizers for successful inoculation. *Applied microbiology and biotechnology* **97**: 8859–73.

Hetrick BAD, Wilson GWT, Cox TS. **1993**. Mycorrhizal dependence of modern wheat cultivars and ancestors: a synthesis. *Canadian Journal of Botany* **71**: 512–518.

Hetrick BAD, Wilson GWT, Todd TC. **1996**. Mycorrhizal response in wheat cultivars: relationship to phosphorus. *Canadian Journal of Botany* **74**: 19–25.

Hiltpold I, Baroni M, Toepfer S, Kuhlmann U, Turlings TCJ. **2010a**. Selective breeding of entomopathogenic nematodes for enhanced attraction to a root signal did not reduce their establishment or persistence after field release. *Plant signaling & behavior* **5**: 1450–1452.

Hiltpold I, Baroni M, Toepfer S, Kuhlmann U, Turlings TCJ. **2010b**. Selection of enomopathogenic nematodes for enhanced responsiveness to a volatile root signal helps to control a major root pest. *The Journal of Experimental Biology* **213**: 2417–2423.

Hodge A. **2000**. Microbial ecology of the arbuscular mycorrhiza. *FEMS Microbiology Ecology* **32**: 91–96.

Hoeksema JD, Chaudhary VB, Gehring CA, Johnson NC, Karst J, Koide RT, Pringle A, Zabinski C, Bever JD, Moore JC, *et al.* 2010. A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. *Ecology letters* **13**: 394–407.

Hoffmann D, Vierheilig H, Peneder S, Schausberger P. **2011a**. Mycorrhiza modulates aboveground tri-trophic interactions to the fitness benefit of its host plant. *Ecological Entomology* **36**: 574–581.

Hoffmann D, Vierheilig H, Schausberger P. **2011b**. Arbuscular mycorrhiza enhances preference of ovipositing predatory mites for direct prey-related cues. *Physiological Entomology* **36**: 90–95.

Hoffmann D, Vierheilig H, Schausberger P. **2011c**. Mycorrhiza-induced trophic cascade enhances fitness and population growth of an acarine predator. *Oecologia* **166**: 141–9.

Huberty AF, Denno RF. **2006**. Consequences of nitrogen and phosphorus limitation for the performance of two planthoppers with divergent life-history strategies. *Oecologia* **149**: 444–455.

Husband R, Herre EA, Turner SL, Gallery R, Young JPW. **2002**. Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. *Molecular Ecology* **11**: 2669–2678.

Inoue M, Endo T. **2008**. Below-ground host location by *Campsomeriella annulata* (Hymenoptera : Scoliidae), a parasitoid of scarabaeid grubs. *Journal of Ethology* **26**: 43–50.

INVAM. **2014**. International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi.

Johnson BY, Graham H, Leonard RT, Menge A. **1982**. Effect of Flower Bud Development in Chrysanthemum on Formation. *New Phytologist*: 671–675.

Johnson NC, Graham JH, Smith FA. **1997**. Functioning of mycorrhizal associations along the mutualism- parasitism continuum. *New Phytologist* **135**: 575–586.

Johnson SN, Petitjean S, Clark KE, Mitchell C. **2010**. Protected raspberry production accelerates onset of oviposition by vine weevils (*Otiorhynchus sulcatus*). *Agricultural and Forest Entomology*: 277–283.

Johnson SN, Rasmann S. **2015**. Root-Feeding Insects and Their Interactions with Organisms in the Rhizosphere. *Annual Review of Entomology* **60**: 517–535.

Johnson D, Vandenkoornhuyse PJ, Leake JR, Gilbert L, Booth RE, Grime JP, Young JPW, Read DJ. **2004**. Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. *New Phytologist* **161**: 503–515.

Johnson SN, Young MW, Karley AJ. **2012**. Protected raspberry production alters aphid-plant interactions but not aphid population size. *Agricultural and Forest Entomology* **14**: 217–224.

Jung SC, Martinez-Medina A, Lopez-Raez JA, Pozo MJ. **2012**. Mycorrhiza-Induced Resistance and Priming of Plant Defenses. *Journal of Chemical Ecology* **38**: 651–664.

Kakouli-Duarte T, Labuschagne L, Hague NGM. **1997**. Biological control of the black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with entomopathogenic nematodes (Nematoda: Rhabditida). *Annals of Applied Biology* **131**: 11–27.

Kanagy JMN, Kaya HK. **1993**. The possible role of marigold roots and ALPHA- terthienyl in mediating host-finding by Steinernematid nematodes. *Nematologica* **42**: 220–231.

Kaya HK, Gaugler R. **1993**. Entomopathogenic Nematodes. *Annual Review of Entomology* **38**: 181–206.

Kempel A, Schmidt AK, Brandl R, Schadler M. **2010**. Support from the underground: Induced plant resistance depends on arbuscular mycorrhizal fungi. *Functional Ecology* **24**: 293–300.

Kieffer J, Shanks C, Turner W. **1983**. Populations and Control of Insects and Spiders Contaminating Mechanically Harvested Red Raspberries in Washington and Oregon. *Journal of Economic Entomology* **76**: 649–653.

Klironomos JN. **2002**. Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature* **417**: 67–70.

Klironomos JN, Hart MM. **2002**. Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. *Mycorrhiza* **12**: 181–184.

Klironomos JN, McCune J, Moutoglis P. **2004**. Species of arbuscular mycorrhizal fungi affect mycorrhizal responses to simulated herbivory. *Applied Soil Ecology* **26**: 133–141.

Koide RT, Mosse B. **2004**. A history of research on arbuscular mycorrhiza. *Mycorrhiza* **14**: 145–63.

Koricheva J, Gange AC, Jones T. **2009**. Effects of mycorrhizal fungi on insect herbivores: a meta-analysis. *Ecology* **90**: 2088–97.

Kough JL, Gianinazzipearson V, Gianinazzi S. **1987**. Depressed Metabolic-Activity of Vesicular-Arbuscular Mycorrhizal Fungi after Fungicide Applications. *New Phytologist* **106**: 707–715.

Kruitbos LM, Heritage S, Hapca S, Wilson MJ. **2010**. The influence of habitat quality on the foraging strategies of the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis megidis*. *Parasitology* **137**: 303–9.

Leitner M, Kaiser R, Hause B, Boland W, Mithöfer A, Mithofer A. **2010**. Does mycorrhization influence herbivore-induced volatile emission in *Medicago truncatula*? *Mycorrhiza* **20**: 89–101.

Lok JB. **2007**. *Strongyloides stercoralis*: a model for translational research on parasitic nematode biology. *The C. elegans Research Community, WormBook*.

Lola-Luz T, Downes M. **2007**. Biological control of black vine weevil *Otiorhynchus sulcatus* in Ireland using *Heterorhabditis megidis*. *Biological Control* **40**: 314–319.

La Lone R, Clarke R. **1968**. Larval Development of *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) and Effects of Larval Density on Larval Mortality and Injury to Rhododendron. *Environmental Entomology* **6**: 397–418.

Lundmark M. **2010**. *Otiorhynchus sulcatus*, an autopolyploid general-purpose genotype species? *Hereditas* **147**: 278–82.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA. **1990**. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **155**: 495–501.

McKenzie SW, Vanbergen AJ, Hails RS, Jones TH, Johnson SN. **2013**. Reciprocal feeding facilitation between above- and below-ground herbivores. *Biology Letters* **9** .

McMenemy LS, Hartley SE, MacFarlane SA, Karley AJ, Shepherd T, Johnson SN. **2012**. Raspberry viruses manipulate the behaviour of their insect vectors. *Entomologia Experimentalis et Applicata* **144**: 56–68.

McNaughton SJ. **1983**. Compensatory Plant Growth as a Response to Herbivory. *Oikos* **40**: 329–336.

Miller RE, Gleadow RM, Cavagnaro TR. **2014**. Age versus stage: Does ontogeny modify the effect of phosphorus and arbuscular mycorrhizas on above- and below-ground defence in forage sorghum? *Plant, Cell and Environment* **37**: 929–942.

Moorhouse ER, Charnley AK, Gillespie AT. **1992**. A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Annals of Applied Biology* **121**: 431–454.

Moorhouse ER, Gillespie AT, Charnley AK. **1993**. Application of *Metarhizium anisopliae* (Metsch.) Sor. conidia to control *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) larvae on glasshouse pot plants. **122**: 623–636.

Moorhouse ER, Gillespie AT, Charnley AK. **1994**. The influence of temperature on the susceptibility of vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleptera:Curculionidae), larvae to *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Annals of Applied Biology* **124**: 185–193.

Newingham BA, Callaway RM, BassiriRad H. **2007**. Allocating nitrogen away from a herbivore: A novel compensatory response to root herbivory. *Oecologia* **153**: 913–920.

Partida-Martínez LP, Heil M. **2011**. The Microbe-Free Plant: Fact or Artifact? *Frontiers in Plant Science* **2**: 1–16.

Patiño-Ruiz JD, Schausberger P. **2014**. Spider mites adaptively learn recognizing mycorrhiza-induced changes in host plant volatiles. *Experimental and Applied Acarology* **64**: 455–463.

Penman DR, Scott RR. **1976**. Adult emergence and egg production of the black vine weevil in Canterbury. *New Zealand Journal of Experimental Agriculture* **4**: 385–389.

Phillips J, Hayman D. **1970**. Improved procedures for clearing and staining parasitic and vesicular-arbuscular mycor rhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society1* **55**: 158–161.

Pickett JA, Woodcock CM, Midega C a O, Khan ZR. **2014**. Push-pull farming systems. *Current opinion in biotechnology* **26**: 125–132.

Pineda A, Zheng S-J, van Loon JJ a, Pieterse CMJ, Dicke M. **2010**. Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends in plant science* **15**: 507–14.

R Core Team. **2013**. R: A Language and Environment for Statistical Computing.

Rapparini F, Llusià J, Peñuelas J. **2008**. Effect of arbuscular mycorrhizal (AM) colonization on terpene emission and content of *Artemisia annua* L. *Plant biology (Stuttgart, Germany)* **10**: 108–122.

Rasmann S, Agrawal A a. **2008**. In defense of roots: a research agenda for studying plant resistance to belowground herbivory. *Plant physiology* **146**: 875–80.

Rasmann S, Ali JG, Helder J, van der Putten WH. **2012a**. Ecology and evolution of soil nematode chemotaxis. *Journal of chemical ecology* **38**: 615–628.

Rasmann S, Hiltpold I, Ali J. **2012b**. The Role of Root-Produced Volatile Secondary Metabolites in Mediating Soil Interactions. Advances in Selected Plant Physiology Aspects, Dr. Giuseppe Montanaro (Ed.). Rijeka, Croatia, 269–290.

Rasmann S, Köllner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J, Turlings TCJ. **2005**. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* **434**: 732–737.

Rasmann S, Turlings TCJ. **2007**. Simultaneous feeding by aboveground and belowground herbivores attenuates plant-mediated attraction of their respective natural enemies. *Ecology letters* **10**: 926–36.

Richardson P, Edmondson R, Long S, Hart A, Willmott D. **2002**. Use of a cold-active entomopathogenic nematode *Steinernema kraussei* to control overwintering larvae of the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) in outdoor strawberry plants. *Nematology* **4**: 925–932.

Robert CAM, Erb M, Hibbard BE, Wade French B, Zwahlen C, Turlings TCJ. **2012**. A specialist root herbivore reduces plant resistance and uses an induced plant volatile to aggregate in a density-dependent manner (K Thompson, Ed.). *Functional Ecology* **26**: 1429–1440.

Robert C a M, Ferrieri RA, Schirmer S, Babst BA, Schueller MJ, Machado R a R, Arce CCM, Hibbard BE, Gershenzon J, Turlings TCJ, *et al.* 2014. Induced carbon reallocation and compensatory growth as root herbivore tolerance mechanisms. *Plant, Cell and Environment*: 2613–2622.

Rogers M, Potter D. **2002**. Kairomones from scarabaeid grubs and their frass as cues in below-ground host location by the parasitoids Tiphia vernalis and Tiphia pygidialis. *Entomologia experimentalis et applicata* **102**: 307–314.

Rowe HI, Brown CS, Claassen VP. **2007**. Comparisons of Mycorrhizal Responsiveness with Field Soil and Commercial Inoculum for Six Native Montane Species and Bromus tectorum. *Restoration Ecology* **15**: 44–52.

San-Blas E. **2013**. Progress on entomopathogenic nematology research: A bibliometric study of the last three decades: 1980–2010. *Biological Control* **66**: 102–124.

Schausberger P, Peneder S, Jürschik S, Hoffmann D. **2012**. Mycorrhiza changes plant volatiles to attract spider mite enemies. *Functional Ecology* **26**: 441–449.

Schüβler A, Schwarzott D, Walker C. **2001**. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research* **105**: 1413–1421.

Šmilauer P. **2001**. Communities of arbuscular mycorrhizal fungi in grassland: Seasonal variability and effects of environment and host plants. *Folia Geobotanica* **36**: 243–263.

Smith S, Read DJ. **2008**. *Mycorrhizal Symbiosis*. New York: Academic Press.

Smith FA, Smith SA. **1996**. Mutualism and parasitism: diversity in function and structure in the 'arbuscular' (VA) mycorrhizal symbiosis. In: Callow J, ed. Incorporating Advances in Plant Pathology. London, U.K.: ACADEMIC PRESS LTD-ELSEVIER SCIENCE LTD, 1–43.

Smith FA, Smith SE. **2013**. How useful is the mutualism-parasitism continuum of arbuscular mycorrhizal functioning? *Plant and Soil* **363**: 7–18.

Soudzilovskaia NA, Douma JC, Akhmetzhanova AA, Bodegom PM Van, Cornwell WK, Moens EJ, Treseder KK, Tibbett M, Wang Y, Cornelissen JHC. **2015**. Global patterns of plant root colonization intensity by mycorrhizal fungi explained by climate and soil chemistry. *Global Ecology and Biogeography* **24**: 371–382.

Sukarno N, Smith SE, Scott ES. **1993**. The effect of fungicides on vesicular-arbuscular mycorrhizal symbiosis. *New Phytologist* **25**: 139–147.

Taylor J, Harrier L. **2000**. A comparison of nine species of arbuscular mycorrhizal fungi on the development and nutrition of micropropagated *Rubus idaeus* L . cv . Glen Prosen (Red Raspberry). *Plant and Soil* **225**: 53–61.

Thurston GS, Yule WN, Dunphy GB. **1994**. Explanations for the Low Susceptibility of *Leptinotarsa decemlineata* to *Steinernema carpocapsae*. *Biological Control* **4**: 53–58.

Titus JH, Lepš JAN. **2000**. The response of arbuscular mycorrhizae to fertilization, mowing, and removal of dominant species in a diverse oligotrophic wet meadow. *American Journal of Botany* **87**: 392–401.

Van Tol RWHM, van Bexooijen J, Ketelaars TACM. **1998**. Searching behaviour of entomopathogenic nematodes: roots and soil temperature determine success of black vine weevil (*Otiorhynchus sulcatus*) control. *Insect Pathogens and Insect parasitic Nematodes IOBC Bulletin* **21**: 187–191.

Van Tol R, van der Sommen ATC, Boff MIC, van Bezooijen J, Sabelis MW, Smits PH. **2001**. Plants protect their roots by alerting the enemies of grubs. *Ecology Letters* **4**: 292–294.

Trejo-aguilar D, Lara-capistrán L, Maldonado-mendoza IE, Zulueta-rodríguez R, Sangabriel-W. **2013**. Loss of arbuscular mycorrhizal fungal diversity in trap cultures during long-term subculturing. *IMA Fungus* **4**: 161–167.

Treseder KK. **2013**. The extent of mycorrhizal colonization of roots and its influence on plant growth and phosphorus content. *Plant and Soil* **371**: 1–13.

Turlings TCJT, Tumlinson JHJ, Lewis WJ. **1990**. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* **250**: 1251.

Vannette RL, Hunter MD. **2009**. Mycorrhizal fungi as mediators of defence against insect pests in agricultural systems. *Agricultural and Forest Entomology* **11**: 351–358.

Vannette RL, Hunter MD. **2013**. Mycorrhizal abundance affects the expression of plant resistance traits and herbivore performance. *Journal of Ecology* **101**: 1019–1029.

Varma A, Schuepp H. **1994**. Infectivity and effectiveness of *Glomus intraradices* on micropropagated plants. *Mycorrhiza* **5**: 29–38.

Vierheilig H, Coughlan A, Wyss U, Piché Y. **1998**. Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Applied and Environmental Microbiology* **64**: 5004–5007.

Villani M, Allee L, Diaz A. **1999**. Adaptive strategies of edaphic arthropods. *Annual review of*.

Walder F, Niemann H, Natarajan M, Lehmann MF, Boller T, Wiemken A. **2012**. Mycorrhizal Networks: Common Goods of Plants Shared under Unequal Terms of Trade. *Plant Physiology* **159**: 789–797.

Werner GDA, Kiers ET. **2014**. Order of arrival structures arbuscular mycorrhizal colonization of plants. *The New phytologist* **205**: 1515–1524.

West HM. **1995**. Soil Phosphate Status Modifies Response of Mycorrhizal and Nonmycorrhizal *Senecio vulgaris* L to Infection by the Rust, *Puccinia lagenophorae* Cooke. *New Phytologist* **129**: 107–116.

White GF. **1927**. A method for obtaining infective nematode larvae from cultures. *Science* **66**: 302–303.

Whittingham J, Read DJ. **1982**. vesicular-arbuscular mycorrhiza in natural vegetation systems III. Nutrient transfer between plants with mycorrhizal interconnections. *New Phytologist* **90**: 277–284.

Wilson M, Nitzsche P, Shearer PW. **1999**. Entomopathogenic nematodes to control black vine weevil (Coleoptera: Curculionidae) on strawberry. *Journal of economic entomology* **92**: 651–657.

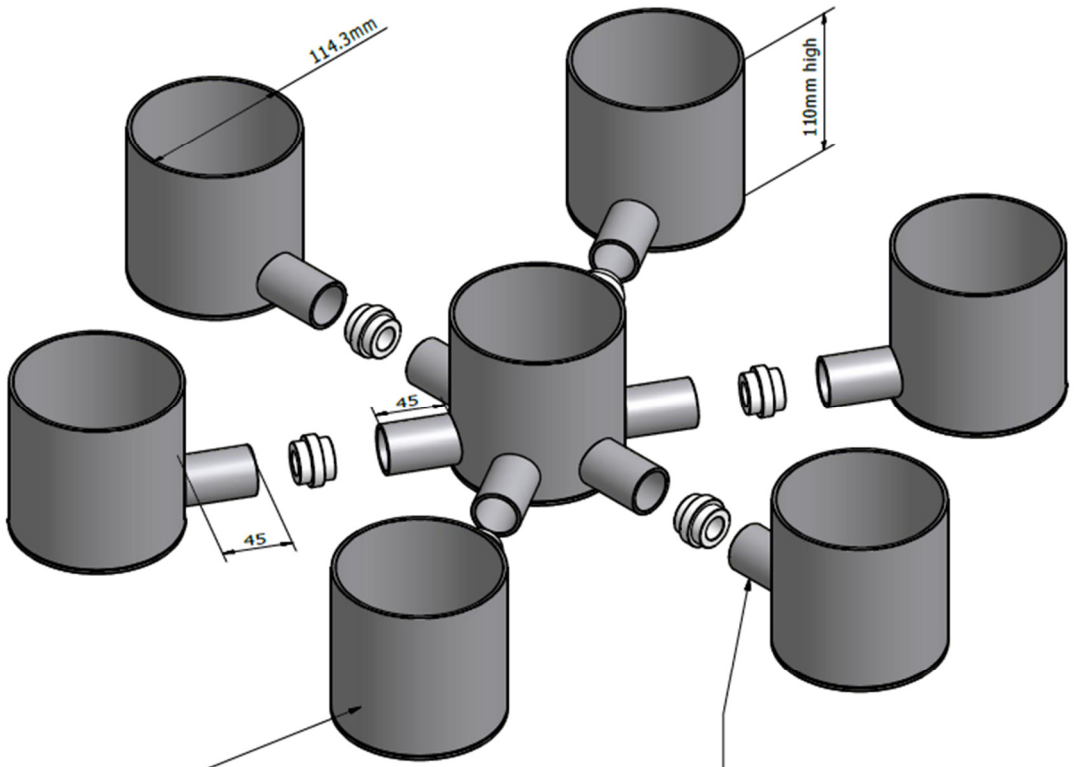
Wolfe BE, Husband BC, Klironomos JN. **2005**. Effects of a belowground mutualism on an aboveground mutualism. *Ecology Letters* **8**: 218–223.

Wooley SC, Paine TD. **2007**. Can intra-specific genetic variation in arbuscular mycorrhizal fungi (*Glomus etunicatum*) affect a mesophyll-feeding herbivore (*Tupiocoris notatus* Distant)? *Ecological Entomology* **32**: 428–434.

Zhu YG, Smith SE, Barritt AR, Smith FA. **2001**. Phosphorus (P) efficiencies and mycorrhizal responsiveness of old and modern wheat cultivars. *Plant and Soil* **237**: 249–255.

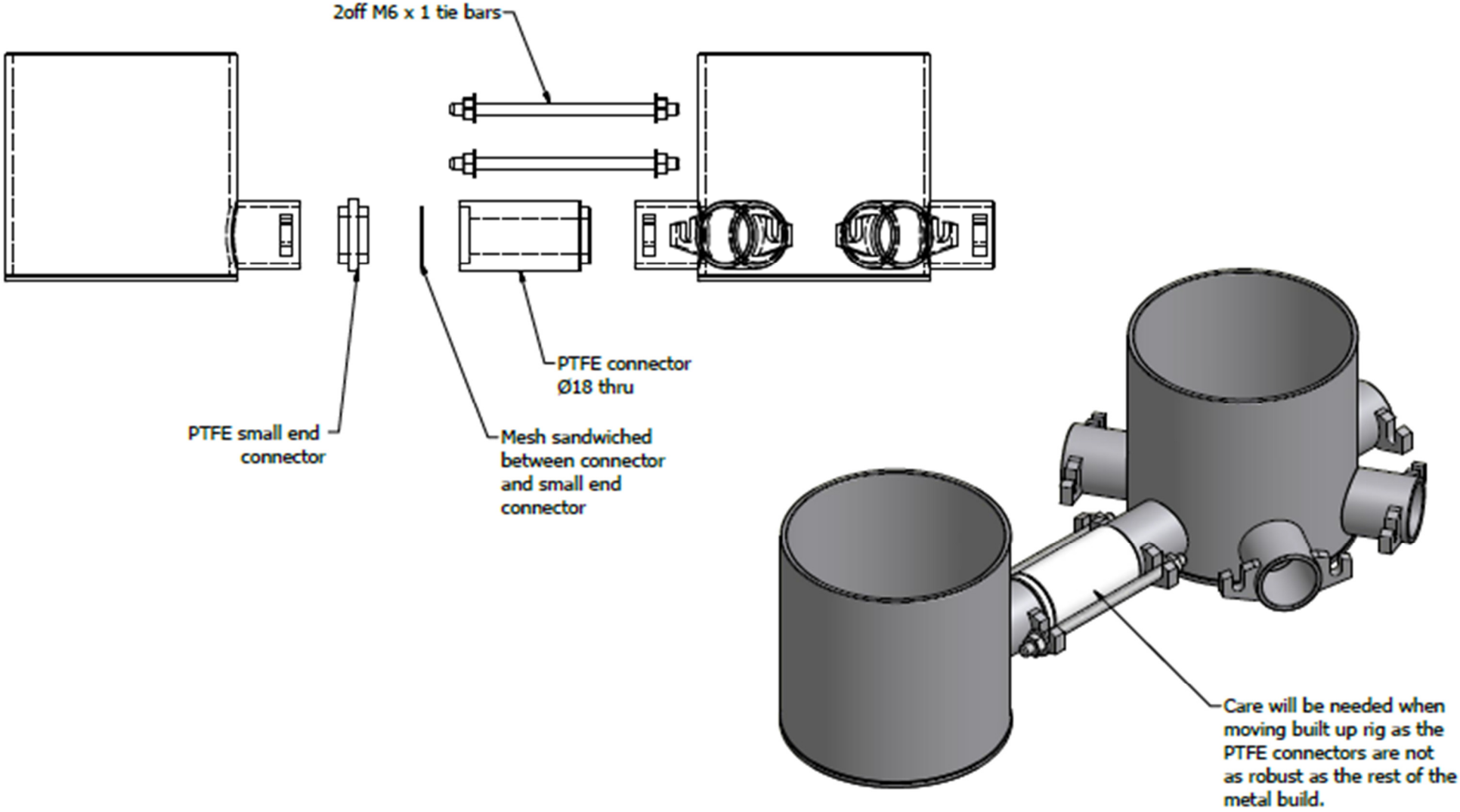
Zvereva EL, Kozlov MV. **2012**. Sources of variation in plant responses to belowground insect herbivory: A meta-analysis. *Oecologia* **169**: 441–452.

Appendix 1



Tube dia is 114.3mm
Wall thickness 3.05mm
Base made from 3mm plate

Connector arms made from
33.4mm OD tube with a 2.77mm wall
drawn at 45mm long.
Full miter cut and welded to pots


2off M6 x 1 tie bars
PTFE connector
Ø18 thru
PTFE small end
connector
Mesh sandwiched
between connector
and small end
connector
Care will be needed when
moving built up rig as the
PTFE connectors are not
as robust as the rest of the
metal build.

Appendix 2

Manuscript Resubmitted after comments, to the Journal of Chemical Ecology.

DEVELOPING SOIL MICROBIAL INOCULANTS FOR PEST MANAGEMENT: CAN ONE HAVE TOO MUCH OF A GOOD THING?

KIRAN R. GADHAVE*, JAMES E. HOURSTON AND ALAN C. GANGE

School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK

*Author for correspondence

Tel. 01784 443188

Fax. 01784 414224

Email: PVBA211@live.rhul.ac.uk

Running title- Soil microbial inoculants in pest management: patterns, mechanisms and recommendations

Abstract- Soil microbes present a novel and cost-effective method of increasing plant resistance to insect pests and thus a sustainable opportunity to reduce current pesticide application. However, the use of microbes in integrated pest management programmes is still in its infancy. This could be primarily attributed to the variations in microbial inoculum performance in laboratory and field conditions. Soil inoculants containing single, indigenous microbial species have shown promising results in increasing plant resistance to foliar feeding insects. Conversely, commercial inoculants containing multiple species often show no effects. We present a simple model that endeavours to explain the

discrepancies in results when microbial inoculants containing single and multiple species are used under both controlled and field conditions. Furthermore, we discuss how this knowledge can be applied to manipulate soil microbial species and develop 'tailored' microbial inoculants that could be used in plant protection against antagonists.

Key Words- Microbial inoculants, tritrophic interactions, pest management, insect herbivores.

MICROBE-MEDIATED ABOVE- AND BELOW-GROUND INTERACTIONS IN THE FIELD

The rhizosphere is the most diverse and dynamic ecosystem in nature (Hinsinger et al. 2009). Microbial diversity within this zone is colossal and is critical for plant growth, and ultimately in the maintenance of life on earth (Bakker et al. 2013). The influence of microbes associated with roots can be exerted on higher trophic levels such as insects, both above- and below-ground. A number of studies have shown how plant growth promoting (PGP) rhizobacteria or arbuscular mycorrhizal (AM) fungi can influence the growth and performance of foliar-feeding insects [bacteria (Pineda et al. 2010); endophytes (Jaber and Vidal 2010); mycorrhizas (Koricheva et al. 2009)] and diversity and abundance of plant pathogens (Weller et al. 2002) and nematodes (Kerry 2000). The majority of the effects on foliar-feeding insects appear to be negative, although many are also context-dependent, being greatly influenced by abiotic factors (Gange et al. 2012).

The fact that soil microbes can have both direct and indirect beneficial effects on plants has led to much research into the development of commercial inocula, that aim to improve plant growth and yield (Herrmann and Lesueur 2013). Invariably, inocula that contain PGP rhizobacteria or AM fungi comprise a mixture of species (Trabelsi and Mhamdi 2013). The most successful of these types of inocula has been the range of products containing *Rhizobium* for legumes (Brockwell and Bottomley 1995) and those containing mycorrhizal fungi (Ceballos et al. 2013). However, a feature of all microbial inoculants, even those containing *Rhizobium*, is that they frequently appear to have no effects when applied in field conditions (Corkidi et al. 2004; Herrmann and Lesueur 2013) despite controlled laboratory experiments being positive. This is often put down to poor quality of product, but such effects can still be seen even when the products applied are of high quality (Herrmann and Lesueur 2013). Such null results could result in a lack of sales and product development, which from a sustainable agriculture point of view, would be most unfortunate.

SINGLE VS MULTIPLE SPECIES

A second feature in the literature, which appears common across both bacteria and mycorrhizal studies, is that controlled experiments in which one species of microbe is added to a plant seem to show much greater effects on insect performance than when two or more species are added. Single species of PGP rhizobacteria, when applied to plants in controlled experiments, are likely to be more effective in offering plant resistance to a variety of insects including chewers (Zehnder et al. 1997; Van Oosten et al. 2008) and phloem feeders (Valenzuela-Soto et al. 2010) than multiple PGP bacterial species (Herman et al. 2008; Boutard-Hunt et al. 2009). Experiments with combinations of

arbuscular mycorrhizal fungi often show little or far less of an effect on insects than do single species additions (Gange 2001; Gange et al. 2005; Vannette and Hunter 2013). Two separate experiments were conducted using soil bacteria and mycorrhiza on insect herbivores serve to illustrate these differences (Fig. 1). Any of three individual species of PGP *Bacillus* were found to be more effective in reducing field infestations of the specialist aphid, *Brevicoryne brassicae* than a mixture of the same species (Fig. 1a). Similarly, colonization of strawberry by two individual *Glomus* spp. significantly reduced the growth and survival of black vine weevil (*Otiorhynchus sulcatus*) larvae, but the combination of the same species did not (Gange 2001) (Fig. 1b). These experiments contrast with the backdrop of root bacterial and mycorrhizal communities showing high diversity (Bakker et al. 2013) and lead to three important questions: (i) why is it that single species additions often show negative effects on insects, while combinations do not?; (ii) are microbial inoculants that contain a consortium of species likely to be of use in pest control? and (iii) if 'less is more' when it comes to the enhancement of plant resistance, why have root communities with few species not been selected for in nature?

MECHANISTIC INSIGHTS INTO TRITROPHIC INTERACTIONS

A critical feature of microbial inoculants is that it is generally unknown if the species contained within them are present in the soil to which they are applied. Generally, there is likely to be a reasonable amount of overlap because the inoculum species are usually easy to culture, having been previously extracted from field soils. However, there will never be a perfect overlap, as a large proportion of the soil microbial community is unculturable (Amann et al. 1995). This uncertainty will always lead to variable inoculum performance in different field soils and crops.

Single microbial species scenario To answer question (i), we propose a simple model (Fig. 2), based on the compatibility of species in the inoculum and the rhizosphere. Imagine a very simple, three species root microbial community. Of course, rhizosphere communities are far more complex, but we limit the species richness in this figure for the sake of clarity. The dynamics of the community may be relatively stable over time, assuming that abiotic factors remain constant. We then add a single species inoculum, as happens in many laboratory experiments. If this species is present in the root community, its population will increase, and through a process of antagonism, that of the other two species will decrease (Fig. 2a). Priority effects may also play a part here, as it has been observed that established mycorrhizal fungi will supress invader species not originally present (Werner and Kiers 2014). However, it is unlikely that resources (i.e. plant derived carbon) will be so limiting as to allow one species to go extinct. The system will, therefore, return to its original state after a further period of competition. The critical point is the sudden change in microbial populations relative to each other, which will be registered by the plant (Bakker et al. 2013). There is evidence that much of plant biochemistry derives from microbial interactions within the tissues (Kloepper and Ryu 2006). These changes will elicit chemical signals in plant tissues that affect an insect feeding on that plant negatively, as reported by earlier studies (Gange et al. 2012). For instance, *B. amyloliquefaciens* FZB42 persists in the rhizosphere over 5 weeks (Kröber et al. 2014), changes foliar glucosinolate levels and suppresses insect populations (K.R. Gadhave, et al, unpublished). Soil bacteria and fungi prime

plants against insects and the ‘microbe induced priming’ is a likely mechanism by which this occurs.

Microbial mediators and plant defensive chemistry Both single as well as multiple microbial species change the levels of constitutive defence compounds produced by plants against herbivores (Gange et al. 2012). For instance, earlier studies showed that two single rhizobacterial species alter the glucosinolate and cucurbitacin profiles in *Arabidopsis* and cucumber plants respectively (Zehnder et al. 1997; Brock et al. 2013). Microbe mediated induction of plant defences, including those involving the emission of herbivore induced plant volatiles (HIPVs) that attract natural enemies of insect herbivores, is facilitated primarily by an interplay of jasmonic acid, salicylic acid and ethylene pathways [e. g. bacteria (Koornneef and Pieterse 2008); fungi (Van der Ent et al. 2009; Jung et al. 2012)]. Single PGP rhizobacterium; *Pseudomonas fluorescens* and AM fungus; *G. intraradices* have been shown to change the proportion of terpenes and sesquiterpene produced as volatiles respectively (Fontana et al. 2009; Pangesti et al. 2015). Such changes in volatile emissions have been shown to attract natural enemies to herbivore infested plants (Hoffmann et al. 2011; Schausberger et al. 2012) suggesting important implications for indirect plant defence. Kniskern et al (2007) showed that salicylic and jasmonic acid defence pathways reduce natural bacterial diversity in *Arabidopsis*. Thus, it is probable that plants harbouring less diverse bacteria are able to invest more in defence signalling against plant antagonists, which partially explains why single species microbial inocula could be more effective against herbivores than those with multiple species.

The induction of systemic resistance in plants involves the recognition of Microbes Associated Molecular Patterns (MAMPs) such as flagellin, lipopolysaccharides, siderophores, antibiotics and biosurfactants in bacteria (Van Wees et al. 2008; Van der Ent et al. 2009), and chitin, endopolygalacturonases and ergosterol in fungi (Klemptner et al. 2014; Zhang et al. 2014) by plant receptors. Thus, it is likely that the MAMPs associated with single and multiple microbial species may differ in their ability to mount defence against pests. A literature on the effects of single vs mixed microbial species on plant defensive metabolites is not robust to make any generalizations. However, it is plausible that microbial species number and composition in inocula can have far-reaching impacts on plant signalling pathways and MAMPs, which are the key determinants of induced defences of plants. ..

Mixed microbial species scenario If the inoculum contains multiple species that are in common with those in the root system this will simply increase all their populations in the rhizosphere (Fig. 2b). The relative abundance of one species to the others remains the same, and no chemical changes are elicited in the plant. Thus, no effect of inoculation is seen on plant antagonists. An example may be shown by results of Roger *et al*. (Roger et al. 2013) who showed that *Spodoptera littoralis* caterpillars preferentially fed on plants inoculated with combinations of two isolates of the AM fungus; *Rhizophagus irregularis* rather than on plants inoculated with only one isolate. Perhaps the worst case scenario may be if the inoculum contains no species that are present in the rhizosphere. We suggest that this is the most likely reason for some field applications having little or no measurable effects. In most cultivated soils, it is likely that the local adaptation of communities has occurred, and the added ‘exotic’ species will fail to establish. A good example of this is the responses exhibited by *O. sulcatus* larvae feeding on *Rubus idaeus* plants treated with a mycorrizal mix extracted from *R. idaeus* soils, and a commercial mycorrizal

species mixture (Fig. 1c). The larval performance was severely reduced on plants treated with the native species mix, compared with those treated with a commercial inoculum (J. E. Hourston, unpublished).

We propose that the answers to questions (ii) and (iii) lie in the nature of the soil microbial community itself. It is well known that plant roots in soil are linked by a Common Mycelial Network (CMN) of mycorrhizas (Walder et al. 2012). This network allows the inter-plant transfer of nutrients and signals of insect attack (Babikova et al. 2013). The analogous networks through which soil bacteria facilitate plant growth and interlink with CMNs are sparsely explored. However, recent studies on root-microbial communication revealed the roles of an array of bacterial molecules that enable intra and interspecies communication in the rhizosphere through biofilm formation and quorum sensing (Faure et al. 2009). We propose that these ubiquitous bacterial attributes help PGP bacteria maintain diversity and stable contact with roots, and derive common benefits to plants. Diverse rhizosphere communities are thus far more likely to connect with such networks than depauperate communities. Such connections provide advantages to both the microbes and the plants.

Thus, a microbial inoculant that contains many species is likely to contain at least some species that will be in common with those in the soil networks. Given that complete overlap of inoculum with the soil community can be discounted, the scenario depicted in Fig. 2a is more likely, and thus effects should be observed if one or a few species are in common. Whether the multiple non-indigenous species trigger synergistic functional responses in plants is poorly understood. It is more plausible that the addition of multiple non-resident microbial species in the rhizosphere will encourage competition within added species and between benign indigenous microflora for nutrients and niches. This may favour the least beneficial microflora in the rhizosphere and potentially reduce the magnitude of plant growth promotion which would otherwise have been realised if a single native species was present in an inoculum. For example, Conn & Franco (Conn and Franco 2004) showed that the addition of a non-adapted commercial mixed inoculant in the soil disrupted the resident actinobacterial endophyte community in wheat by reducing the diversity from 40 genera to 21 and colonization of detectable root microbiota by half. Conversely, the addition of a single actinobacterial endophyte species increased its colonization level without any adverse effects on the diversity and colonization of the indigenous endophyte population. Thus, the presence of the non-indigenous microbiota is more likely to disrupt the established rhizosphere bacteria and/or CMN and produce antagonistic effects on indigenous microbial communities.

Despite the many listed advantages of single species inocula on indigenous rhizo-bacterial communities and plant fitness, their addition would be far too risky economically, because the chance of one species matching with the indigenous population is lower than if there are many species being added. To overcome this trade-off, we recommend the use of inocula that contain only a few species, to reflect native soil conditions. Individual species of bacteria or fungi added to potted plants and grown under controlled conditions are a poor mimic of field conditions mainly because of their inability to compete in a more diverse and dynamic rhizosphere in the field. The challenge is now to better understand the structure of soil microbial communities, such that inocula can be produced that are tailored to local conditions. Integrated pest management systems, including sustainable components such as tailored microbial

inoculants could be a promising resource to increase agricultural productivity and protection of the environment through a lessening of the reliance on pesticides.

CONCLUDING REMARKS

Microbial inoculants are becoming a common feature in agriculture and horticulture, but are often mistrusted by growers due to their inconsistent results. We suggest that careful matching of some species within inocula to those occurring naturally will overcome this inconsistency. This careful tailoring of inocula would not only have the potential to improve plant performance including fitness against insects, but also reduce the unknown effects of introducing entirely alien microbial species to an area (Schwartz et al. 2006). It is unlikely that a universal soil microbial inoculant could be developed, as the communities within different soils and beneath certain crops differ (Hortal et al. 2013). However, a better understanding of microbial community structure will enable more sustainable products to be developed. A few 'tailored' species in such products would not only represent a good thing, but also make sense from both an economic and ecological point of view.

Acknowledgements

We are grateful to Andreas Ebertz for his help in producing artwork and to Royal Holloway, University of London and the Natural Environment Research Council for providing the financial support to carry out field studies.

References

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143-169

Babikova Z et al (2013) Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack. Ecol Lett 16:835-843

Bakker PA, Berendsen RL, Doornbos RF, Wintermans PC, Pieterse CM (2013) The rhizosphere revisited: root microbiomics. Front Plant Sci. doi: 10.3389/fpls.2013.00165.

Boutard-Hunt C, Smart CD, Thaler J, Nault BA (2009) Impact of plant growth-promoting rhizobacteria and natural enemies on *Myzus persicae* (Hemiptera: Aphididae) infestations in pepper. J Econ Entomol 102:2183-2191

Brock AK, Berger B, Mewis I, Ruppel S (2013) Impact of the PGPB *Enterobacter radicincitans* DSM 16656 on growth, glucosinolate profile, and immune responses of *Arabidopsis thaliana.* Microb Ecol 65:661-670

Brockwell J, Bottomley PJ (1995) Recent advances in inoculant technology and prospects for the future. Soil Biol Biochem 27:683-697

Ceballos I, Ruiz M, Fernández C, Peña R, Rodríguez A, Sanders IR (2013) The in vitro mass-produced model mycorrhizal fungus, *Rhizophagus irregularis*, significantly increases yields of the globally important food security crop cassava. PLoS One 8:e70633. doi: 10.1371/journal.pone.0070633

Conn VM, Franco CM (2004) Effect of microbial inoculants on the indigenous actinobacterial endophyte population in the roots of wheat as determined by terminal restriction fragment length polymorphism. Appl Environ Microbiol 70:6407-6413

Corkidi L, Allen EB, Merhaut D, Allen MF, Downer J, Bohn J, Evans M (2004) Assessing the infectivity of commercial mycorrhizal inoculants in plant nursery conditions. J Environ Hortic 22:149-154
Faure D, Vereecke D, Leveau JH (2009) Molecular communication in the rhizosphere. Plant Soil 321:279-303
Fontana A, Reichelt M, Hempel S, Gershenzon J, Unsicker SB (2009) The effects of arbuscular mycorrhizal fungi on direct and indirect defense metabolites of *Plantago lanceolata* L. J Chem Ecol 35:833-843
Gange AC (2001) Species-specific responses of a root-and shoot-feeding insect to arbuscular mycorrhizal colonization of its host plant. New Phytol 150:611-618
Gange AC, Brown VK, Aplin DM (2005) Ecological specificity of arbuscular mycorrhizae: evidence from foliar-and seed-feeding insects. Ecology 86:603-611
Gange AC, Eschen R, Schroeder V (2012) The soil microbial community and plant foliar defences against insects. In: Iason GR, Dicke M, Hartley SE (eds) The Ecology of Plant Secondary Metabolites: From Genes to Global Processes. Cambridge University Press, UK, pp 170-188
Herman MAB, Nault BA, Smart CD (2008) Effects of plant growth-promoting rhizobacteria on bell pepper production and green peach aphid infestations in New York. Crop Prot 27:996-1002
Herrmann L, Lesueur D (2013) Challenges of formulation and quality of biofertilizers for successful inoculation. Appl Microbiol Biotechnol 97:8859-8873
Hinsinger P, Bengough AG, Vetterlein D, Young IM (2009) Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant Soil 321:117-152
Hoffmann D, Vierheilig H, Schausberger P (2011) Arbuscular mycorrhiza enhances preference of ovipositing predatory mites for direct prey-related cues. Physiol Entomol 36:90-95
Hortal S, Bastida F, Armas C, Lozano Y, Moreno J, García C, Pugnaire F (2013) Soil microbial community under a nurse-plant species changes in composition, biomass and activity as the nurse grows. Soil Biol Biochem 64:139-146
Jaber LR, Vidal S (2010) Fungal endophyte negative effects on herbivory are enhanced on intact plants and maintained in a subsequent generation. Ecol Entomol 35:25-36
Jung SC, Martinez-Medina A, Lopez-Raez JA, Pozo MJ (2012) Mycorrhiza-induced resistance and priming of plant defenses. J Chem Ecol 38:651-664
Kerry B (2000) Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-parasitic nematodes. Annu Rev Phytopathol 38:423-441
Klemptner RL, Sherwood JS, Tugizimana F, Dubery IA, Piater LA (2014) Ergosterol, an orphan fungal microbe-associated molecular pattern (MAMP). Mol Plant Pathol 15:747-761
Kloepper JW, Ryu C-M (2006) Bacterial endophytes as elicitors of induced systemic resistance. In: Schulz, BJE, Boyle CJC, Sieber TN (eds) Microbial Root Endophytes. Springer, Verlag, Berlin, Heidelberg, pp 33-52

Kniskern JM, Traw MB, Bergelson J (2007) Salicylic acid and jasmonic acid signaling defense pathways reduce natural bacterial diversity on *Arabidopsis thaliana.* Mol Plant Microbe Interac 20:1512-1522
Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiol 146:839-844
Koricheva J, Gange AC, Jones T (2009) Effects of mycorrhizal fungi on insect herbivores: a meta-analysis. Ecology 90:2088-2097
Kröber M et al. (2014) Effect of the strain *Bacillus amyloliquefaciens* FZB42 on the microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing. Front Microbiol (in press). doi: 10.3389/fmicb.2014.00252
Pineda A, Zheng SJ, van Loon JJA, Pieterse CMJ, Dicke M (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. Trends Plant Sci 15:507-514
Roger A, Getaz M, Rasmann S, Sanders IR (2013) Identity and combinations of arbuscular mycorrhizal fungal isolates influence plant resistance and insect preference. Ecol Entomol 38:330-338
Schausberger P, Peneder S, Juerschik S, Hoffmann D (2012) Mycorrhiza changes plant volatiles to attract spider mite enemies. Funct Ecol 26:441-449
Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501-515
Trabelsi D, Mhamdi R (2013) Microbial inoculants and their impact on soil microbial communities: a review. BioMed Res Int. doi: 10.1155/2013/863240
Valenzuela-Soto JH, Estrada-Hernandez MG, Ibarra-Laclette E, Delano-Frier JP (2010) Inoculation of tomato plants (*Solanum lycopersicum*) with growth-promoting *Bacillus subtilis* retards whitefly *Bemisia tabaci* development. Planta 231:397-410
Van der Ent S, Van Wees S, Pieterse CMJ (2009) Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. Phytochemistry 70:1581-1588
Van Oosten VR, Bodenhausen N, Reymond P, Van Pelt JA, Van Loon LC, Dicke M, Pieterse CMJ (2008) Differential effectiveness of microbially induced resistance against herbivorous insects in *Arabidopsis.* Mol Plant Microbe Interact 21:919-930
Van Wees S, Van der Ent S, Pieterse CMJ (2008) Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol 11:443-448
Vannette RL, Hunter MD (2013) Mycorrhizal abundance affects the expression of plant resistance traits and herbivore performance. J Ecol 101:1019-1029. doi:10.1111/1365-2745.12111
Walder F, Niemann H, Natarajan M, Lehmann MF, Boller T, Wiemken A (2012) Mycorrhizal networks: common goods of plants shared under unequal terms of trade. Plant Physiol 159:789-797
Weller DM, Raaijmakers JM, Gardener BBM, Thomashow LS (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol 40:309-348
Werner GDA, Kiers ET (2014) Order of arrival structures arbuscular mycorrhizal colonization of plants. New Phytol. doi:10.1111/nph.13092

Zehnder G, Kloepper J, Yao C, Wei G (1997) Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera: Chrysomelidae) by plant growth-promoting rhizobacteria. J Econ Entomol 90:391-396
Zhang L et al. (2014) Fungal endopolygalacturonases are recognized as microbe-associated molecular patterns by the arabidopsis receptor-like protein RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1. Plant Physiol 164:352-364

Figures

Fig. 1. Effects of inoculation of individual vs. multiple microbial species and commercial vs. non-commercial inoculant on insect herbivores: individual species of **(a)** *Bacillus* most effectively reduced *B. brassicae* field infestation; **(b)** *Glomus* significantly reduced *O. sulcatus* larval survival, than the mixtures of the same species. **(c)** Non-commercial inoculant containing indigenous mycorrhizal species reduced *O. sulcatus* larval mass more than the commercial mycorrhizal inoculant. The notations; *B. c.*, *B. s.*, *B. a.*, *G. m.*, *G. f.*, CI and NCI represent *B. cereus*, *B. subtilis*, *B. amyloliquefaciens*, *G. mosseae*, *G. fasciculatum*, commercial (mixed) and non-commercial (mixed) inoculants respectively. In each case, the Y axis represents the percent reduction in insect performance on treated plants, compared to control (untreated plants).

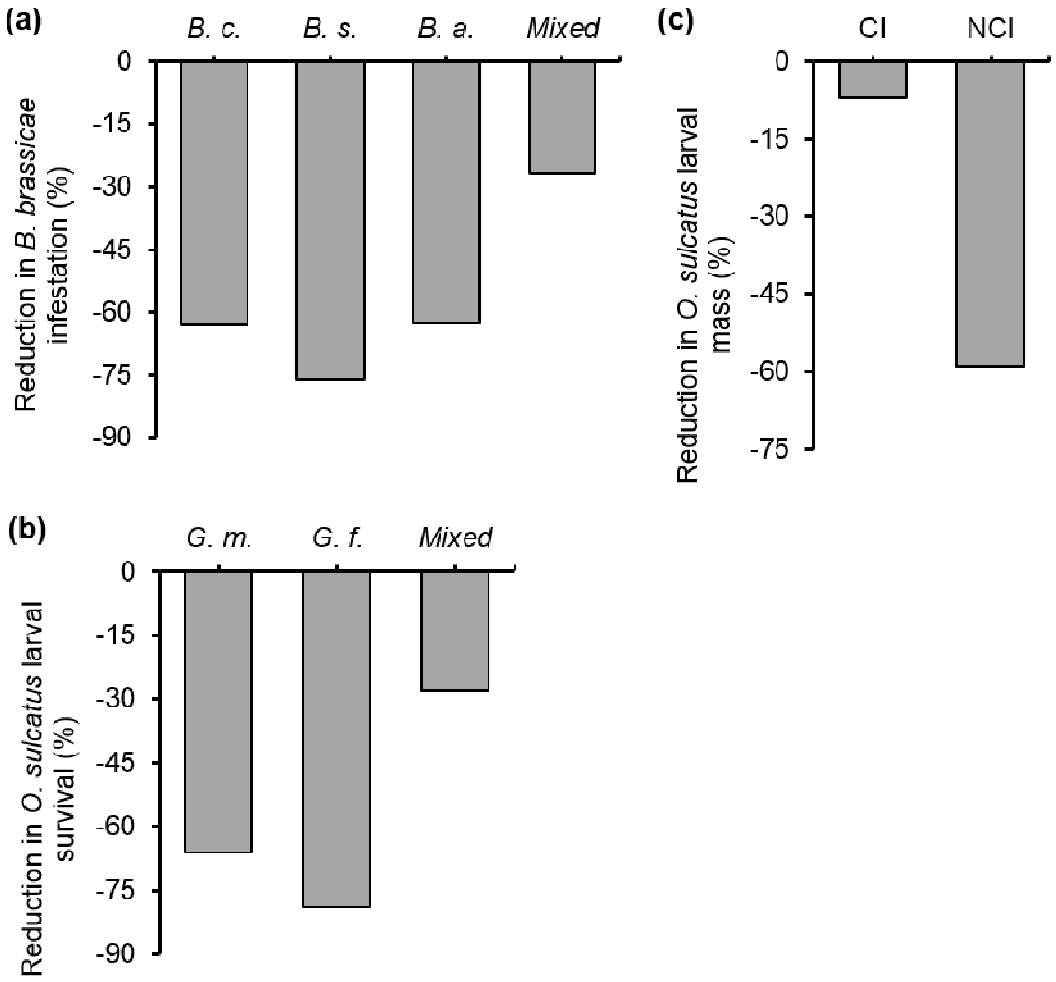


Fig. 2. A model explaining the potential effects of applications (depicted by arrows) of single vs. multiple microbial species on the rhizosphere microbial community and insect herbivores: **(a)** the addition of single microbial species will increase its population and decrease the abundance of others, possibly less beneficial, species through antagonism, which will prime the plant for systemic defence and reduce insect infestation; **(b)** the addition of multiple species will increase their populations, with the unchanged relative abundance in the rhizosphere. This will not elicit any chemical changes in plant tissues and so will

not significantly influence insect growth or population dynamics.