Effects of drought stress and above- and below-ground microbes on multi-trophic interactions in *Solanum tuberosum* L.

Thesis Submitted for the Degree of Doctor of Philosophy

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May 2022

Abstract

Environmental stressors remain a crucial problem in agriculture. Abiotic stress conditions such as drought and biotic stress like insect pests cause extensive losses to agricultural production worldwide. Therefore, to face these challenges novel practices must be developed for improving crop health and crop protection. Microorganisms below and aboveground play key roles in agroecosystem processes and can influence interactions between crops, insect pests and their natural enemies. However, the mechanisms involved in shaping these interactions are often not sufficiently well understood to be used in agricultural practices. The aim of this thesis was to investigate the impacts of microorganisms above- and belowground, and abiotic (drought) stress, on the outcomes of multi-trophic interactions using potato *Solanum tuberosum* (L), the potato aphid *Macrosiphum euphorbiae* and its parasitoid *Aphidius ervi* as a study system. Deciphering and characterising the mechanisms involved in these complex interactions can highlight opportunities for developing and improving pest control under future climate scenarios.

The start of this thesis provides a general overview on interactions of microbes above and belowground with plants and insect pests, their natural enemies, and a biotic stressor (drought). Following this, in Chapter 2 interspecific competition between *Macrosiphum euphorbiae* and the peach-potato aphid *Myzus persicae* colonising the same plant is investigated under drought stress compared with unstressed conditions. In Chapter 3, the effect of root-colonising arbuscular mycorrhizal fungi (AMF) on plant responses to a combination of stressors (drought and aphid infestation) is investigated. In Chapter 4, the influence of aphid facultative bacterial endosymbionts on parasitoid attraction to aphids is assessed through analysis of volatile organic compounds released from aphid honeydew. In Chapter 5, parasitoid responses to aphid-infested plants grown in soils with different cultivation histories and soil microbial communities are examined. Finally, Chapter 6 discusses these findings on above-belowground interactions in relation to developing new approaches for aphid pest control under the changing climate and presents directions for further research.

Author contributions

I declare that the work here, in this thesis, is intellectually my own. Contributions were made to each chapter and the project overall by my supervisors, Dr. Alison Karley (The James Hutton Institute), Dr. Ankush Prashar (Newcastle University), and Dr. Alison Bennett (formerly at The James Hutton Institute, now at Ohio State University). Contributions were made to each chapter by my supervisors, who worked with me to co-design the experiments (as described below), as well as reviewing and providing comments on drafts of chapters in the thesis.

Chapter 2

I co-designed the experiment with guidance from Dr. Alison Karley, and Dr. Ankush Prashar. I performed the experiment; I collected the data, and I conducted all data analysis. I wrote the chapter with guidance from Dr. Alison Karley, and Dr. Ankush Prashar.

Chapter 3

I co-designed the experiment with guidance from Dr. Alison Bennett, Dr. Alison Karley, and Dr. Ankush Prashar. I performed the experiment; I collected the data and I conducted all data analysis. I wrote the chapter with guidance from Dr. Alison Bennett, Dr. Alison Karley, and Dr. Ankush Prashar.

Chapter 4

The methods for sampling the honeydew and performing SPME analysis were co-designed with Dr. Alexandre Foito (James Hutton Institute). I performed the experiment. I wrote the chapter with guidance from Dr. Alison Karley, Dr. Ankush Prashar, and Dr. Alexandre Foito.

Chapter 5

The experiment was co-designed with Dr. Davide Bulgarelli, Dr. Alison Karley, and Dr. Ankush Prashar. I conducted the experiment. I conducted the data analysis of the 16S sequencing with advice and guidance from Dr. Antonino Malacrino'. I wrote the chapter with guidance from Dr. Alison Karley, Dr. Ankush Prashar and Dr. Antonino Malacrino'.

Publications related to the thesis

The following publication was produced as part of my research in collaboration with members of the MiRA project consortium. The paper is appended to the thesis (Appendix 1).

Lee Díaz, A. S., Macheda, D., Saha, H., Ploll, U., Orine, D., & Biere, A. (2021). Tackling the context-dependency of microbial-induced resistance. *Agronomy*, *11*(7), 1293.

Dedication

To my father, my mother, and my sister, for our days in the field.

Acknowledgment

This thesis was funded by the MiRA project (grant number 765290), and conducted within The James Hutton Institute and Newcastle University, and I thank them for making this project possible and offering me the opportunity to work on a subject that I deeply love and to collaborate with amazing labs worldwide. I will be forever grateful for this exceptional opportunity.

I would like to thank my supervisor Dr. Alison Karley and Dr. Ankush Prashar for their kindness, for providing advice, constant support, and guidance throughout the project. Their enthusiasm and experience have been inspiring for developing the project and for my personal growth as a researcher and as human being (even during a pandemic!).

A special thanks to Dr. Carolyn Mitchell, for parasitoids/aphid advice and coffee chat. I would like to thank Dr. Alexandre Foito, for helping me to complete an experiment despite COVID-19 and finally to Dr. Gaynor Malloch, for teaching me everything about molecular biology. Gaynor, I would like to extend further thanks to you, working with you has been a pleasure for me. I have learnt a lot, and I will always be grateful for your kindness, help and our chat throughout my PhD.

I would like to thank, Dr. Davide Bulgarelli, for his support in the most exciting experiment that I conducted and everyone in his group, especially Dr. Carmen Escudero-Martinez for her constant support in the last experiment and to Aileen Corral for being always available for me.

At the James Hutton Institute, the glasshouse staff, especially Jim Wilde and Graham Pitkin for helping me with the plants and being always available whenever I had a question. The Ecological Sciences group, I will miss this incredibly diverse group of people.

I would also like to thank, the MiRA consortium – for this unequable experience. I would like to thank Dr. David Giron, Dr. Alison Bennett, Eric Anderson and Steve Gray for hosting me and their support in the secondments.

A special thanks to Beatriz Romano, Ana Lee Shein and Haymanti Saha for being always there in any dramatic experiment for cheering me up. I would like to thank Arjen Biere, for guidance on writing our collaborative paper. I would like to thank Saskia van Wees, for listening to me when I needed. I would also like to thank Eduardo de la Peña, for his kindness and support.

I am extremely indebted to Dr. Antonino Malacrino' for his exceptional support in statistics and in particularly in analysing sequencing data. For his patience, for his encouragement, and for being not only a good colleague but even a great friend. For this, thank you from the bottom of my heart, Nino.

All my friends: Alice, Daniel aphids (Thank you! for all the guidance in the lab and for listening all my rants through the whole journey, especially in the last period of my PhD our chats made me feel less alone and the random message was really needed), Daniel laser, Ilonka, Hao, Luca, Angela, Luna, Lea, Davide (always there for helping me with R!), Sara and Mauro (for our coffees in Dundee and for making dramatic moments in the lab and/or in the glasshouse not so dramatic…). You all made my time in Scotland nicer, and I am extremely lucky to have met all of you.

My family. I wouldn't love so much insects and nature without our days in the fields. Thank you for being always with me.

Last but not least, my partner Matthias – you have been the most beautiful, unexpected gift in these years in Scotland. Purple.

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Chapter 1. General Introduction

1.1 Rationale

Climate change is a significant and growing threat for food security. During the 20th century, the global population rose from 1.6 to 6.0 billion (Loboguerrero et al., 2019) leading in the 21st century to an immense challenge for agricultural production to be increased at least by 60% (Alexandratos and Bruinsma, 2012). In recent decades, the severity and frequency of drought stress events have increased linked with climate change. Models predict an increase of 2 °C warming of global temperature by the end of the 21st century (Williams et al., 2013). The Palmer Drought Severity Index predicts an extended drought in Europe over the next 30-90 years, resulting from diminished rainfall and increased evaporation (Dai, 2013). Drought counts as one of the most significant abiotic stresses, causing worldwide yield losses, estimated to be valued annually at US$14-19 million (Kaufmann et al., 2002). In order to enhance crop productivity under drought, it is essential to understand the physiological, biochemical and molecular mechanisms associated with plant responses and adaptation to cope with drought stress. This is especially important for the improvement of drought tolerance in crop plants, which may help many developing countries dependent on a single crop as primary source of food (Castaldi, 2009).

Nowadays, one of the major obstacles encountered by agriculture production has been insect pests. Insect pests decrease crop productivity worldwide by an average of 14 % (Johnson and Züst, 2018) and with increase in climate change severity, insect pests are anticipated to become more problematic, making even more difficult the possibility of achieving global food security (Gregory et al., 2009). Current knowledge suggests that the increasing severity of climatic factors will have profound effects on plant-insect interactions (Castex et al., 2018). Crucially, both plants and insects are extremely dependent on temperature for their successful development. In fact, global warming not only impacts plant health but increases insect pest abundance, outbreaks of insects, number of generations and development of resistant biotypes to pesticides (Gu et al., 2018). Thus, changes in temperature are predicted to be the cause of asynchrony[2] in species interactions through influencing development times and phenology, impacting interactions among species within ecosystems (DeLucia et al., 2012; McCluney et al., 2012). In 1997, Awmack predicted that plant susceptibility to aphids would be increased by climate change, and that shorter life stages for the aphid-host will decrease the rate at which natural enemies emerge and therefore impacting negatively on natural

enemies' population sizes (Yadav et al., 2019). Moreover, predators will be indirectly affected not only by changes in quantity but even in quality of their food resource (Giles et al., 2002). There is good evidence that warming temperatures can affect insects through several mechanisms such as disrupting diapause requirements, and altering spring development rates for temperate insects (Pureswaran et al., 2018).

Despite the effort of research, over the last 60 years, in studying and predicting this phenomenon, we are not able to predict accurately the effect of drought on plant-insect-natural enemy interactions (Gely et al., 2020). Thus, ecology research frequently focuses on simplistic experiments and models that often do not include several trophic levels. Drought conditions also alter parasitism attacks by the parasitoid wasps (*Aphidus ervi*) that is reported to be lower on drought stressed plants compared to control plants (Aslam et al., 2013). In 2004, Huberty & Denno, pointed out another knowledge gap. With their review, which aimed to examine how insects respond to drought, they showed that variability in insect responses is underpinned by differential responses by insect feeding guilds, e.g. sap-feeders are negatively affected by continuous water stress. However, the review noted that all the studies conducted on plant-insect interactions and drought stress were small scale and related only to a few individual plant species. Investigating the impact of drought on above-belowground interactions by encompassing plant molecular and physiological responses and their specific mechanisms driving insect responses is still poorly understood. Further investigations of these factors are important for anticipating the potential outcome of prolonged drought in agricultural systems exposed simultaneously and continuously to abiotic and biotic stress.

1.2 Bottom-up and top-down interactions

Natural agroecosystems are a dynamic and complex interactive structure where plants and microbes coexist. Beneficial microbes, such as arbuscular mycorrhizal fungi (AMF), plant growth promoting rhizobacteria (PGPR), and endophytes, live in communities and provide benefits to plants. Despite recognition of the key role played by these microbes in many functional processes that support vital ecosystem functions and services and despite their abundance, soil microbial ecology remains the largest uninvestigated sphere (Bhattacharyya et al., 2016). In fact, in both spheres,

belowground in the rhizosphere and aboveground in the phyllosphere, beneficial microbes influence and trigger crucial processes such as nutrient acquisition (Bowles et al., 2018; Thonar et al., 2017), water uptake (Bowles et al., 2016), mediating plant responses to abiotic stresses (Bauer et al., 2020; Delavaux et al., 2017), and resistance against herbivores (Papadopoulou and Dam, 2017; Pineda et al., 2020; Pineda et al., 2016). However, while the performance of individual microbial taxa (whether aboveground or belowground) is widely explored on aboveground insects, the impact of microbes on trophic interactions is still poorly understood. In fact, to date, studies have extensively addressed the effects microbes can have on herbivorous insects but rarely have investigated the effects on their natural enemies and on the entire multitrophic communities (Bell et al., 2020; Benítez et al., 2017; Gadhave and Gange, 2022). A major knowledge gap in ecology is that often plant-herbivore interactions are studied in pairwise interactions excluding the multitude of trophic interactions and synergies that occur (Strauss and Irwin, 2004). In this tripartite interaction, microbes can mediate interactions among plants, insects, and their natural enemies (Faeth and Fagan, 2002).

Natural enemies of insect pests have been estimated to account for at least 50 % of pest control, giving an essential ecosystem service which has an estimated value to the agricultural industry of $13 billion per year in the USA alone (Losey and Vaughan, 2006). The use of an organism to reduce density of another organism is called biological control and it has been used in traditional farming for about two millennia, although its use is relatively underexploited in modern agricultural pest management. In 2010, less than 230 species of invertebrate natural enemies were used in pest management worldwide (van Lenteren, 2012). Within the arthropods, four groups provide most natural enemies: Hymenoptera with 120 species, the Acari (30 species), Coleoptera (28 species) and Heteroptera (19 species). The word parasitoid identifies an organism whose juvenile stages are parasites of a single host eventually killing, sterilizing, and/or even consuming their host (Lachaud and Pérez-Lachaud, 2012). Most parasitoids are wasps (Hymenoptera) or flies (Diptera). Each predator attacks the prey in a variety of ways. For example: female wasps belonging to the order Hymenoptera use their ovipositors to lay eggs in or on host insect, eventually killing them (Godfray, 1994). Carabid beetles (Coleoptera) use their mandibles to kill and feed on prey (Lövei and Sunderland, 1996). Female of the parasitoid flies (Diptera) can lay eggs on the exterior

of their hosts (family Tachinidae), and after hatching the larvae will invade the host (Stireman et al., 2006). Family of the Pentatomidae as well as Reduviidae (Hemiptera) are well-known to use their tubular mouthparts to prey (Clercq et al., 2014).

Changes in plant-insect interactions induced by soil microbes or abiotic stress will reshape responses at higher trophic levels (natural enemies) transforming entirely our understanding of integrated pest management (IPM) programs. Tritrophic interactions could become essential tools in the context of sustainable agriculture because soil microbes have the potential to play a key role in IPM as biological control agents against natural pests (Wajnberg et al., 2016). Therefore, the response of a single trophic level will have a cascading effect through ecological networks, reshaping and modifying the composition and structure of insect communities (Sanders et al., 2016). The interactions between insect hosts and their parasitoids might be principally affected by global warming due to the fact that plant-host-parasitoid networks are results of long term co-evolutionary processes that can be altered and/or shifted by global warming (Hance et al., 2007). Unfortunately, often studies have focused on climate change effects on individual insect species and/or genotypes and have neglected to explore the outcomes for trophic interactions and the consequences for ecological networks in agricultural systems (Walther, 2010). Understanding bottom-up and top-down interactions represents an essential precursor to managing insect pests in agroecosystems under a changing climate. Microbes play a key role in mediating these interactions through processes at the soil-plant interface but also through the effects of insect endosymbionts on pest-natural enemy interactions (McLean and Godfray, 2017).

1.3 Soil microbes

In 2002 Curtis et al., estimated that around 6,000–50,000 bacterial species were present in one gram of soil and in 2004, Leake and colleagues identified up to 200 m of fungal hyphae in the same amount of soil (Leake et al., 2004). Microbes are everywhere: in the air, in the soil, in the water, in space, in glaciers, in food, and in animal intestines (Ahirwar et al., 2019). The term microbes covers diverse groups of organisms such as: bacteria, fungi, protozoa, micro-algae and viruses. The word symbiosis was introduced for the first time by Anton de Bary in 1879 for describing “a permanent association between two or more distinct organisms, at least during a part of the life cycle” (Gil et

al., 2004). Microbes belonging to specific groups have been shown to have symbiotic and mutualistic relationships with plants promoting plant growth (Finkel et al., 2017). In the past decade research has focused on the use of beneficial root-associated microbes that are an abundant natural component of the plant rhizosphere (Ahirwar et al., 2019). In fact, mutualistic microbe-plant interactions can provide a novel approach to increase agricultural productivity, providing safe, economically feasible and eco-friendly approaches (Ahirwar et al., 2019). This novel technology is a valuable tool for advances not only in crop yield enhancement, food safety or food security but also essential in meeting the current need for increasing the sustainability of agriculture.

Among the beneficial rhizosphere microbiota, PGPR and AMF have been studied extensively over the last ten years for their ecological importance in ecosystem functioning (Bhattacharyya et al., 2016; Grunseich et al., 2020; Van Der Heijden et al., 2008). Besides improving plant nutrition and plant growth promotion (Lareen et al., 2016), several studies showed that beneficial microbes can prime the plant to enhance its defence against biotic stress, pathogens and insect herbivores (Martinez-Medina et al., 2016; Prieto et al., 2017; Rashid and Chung, 2017). Notably, only a few studies have been conducted to show the direct effect of belowground organisms on natural predators (Helms et al., 2019; Jiang et al., 2020; Mejia-Alva et al., 2018) . Research on microbe-plant-insect interactions has therefore not fully explored the potential effects of beneficial microbes on higher trophic levels and the outcomes for insect pest control (Hempel et al., 2009).

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) are the most common mycorrhizal type and are classified in the fungal phylum *Glomeromycota* (Schüßler et al., 2001). The characteristic 'tree-like' structures, the arbuscules, formed within the root cells give them the name "arbuscular". Several studies, have quantified the diversity of AMF communities in soils and it has been suggested that usually five genera are present in soil, namely *Glomus, Gigaspora, Scutellospora, Acaulospora,* and *Entrophospora* (Jansa et al., 2002; Wipf et al., 2019; Basiru et al., 2021). The presence of these fungi can be identified from three important parts: the root itself, the fungal structures within and between the cells of the root, and an extraradical mycelium in the soil (Smith and

Read, 2008). Moreover, the spores formed by AM fungi are large (up to 500 μm diameter), rich in lipid, and with resistant walls containing chitin and, in some cases, *β*-1,3-glucan (Hosny et al., 2002). A huge number of nuclei, between 800 to 35 000 per cell in some species (Hosny et al., 2002), are present with a variable DNA content per nucleus. In many species, as in the *Gigasporacea*e, spores contain bacteria-like organisms (BLOs) as endosymbionts and these can be transmitted to new spore generations, but their functional significant is yet to be investigated (Cruz et al., 2008; Naumann et al., 2010). Following germination, the spores undergo nuclear division as hyphal growth is initiated, making use of carbohydrate and lipid reserves (Bianciotto et al., 1996, 2004; Bianciotto and Bonfante, 1992). Hyphal growth can cease if the host plant root is not present. When a root is encountered, the hypha adheres to the root surface and after 2- 3 days, a swollen appressoria develops and subsequently the hypha penetrates the root and forms arbuscules after another two days (Brundrett et al., 1985). Once symbiosis has been established, the growth of the mycelium continues both within the root and in the soil, leading to the formation of new multinucleate spores, which develop terminally on the hyphae (Smith and Read, 2008). Majority of terrestrial plant species establish symbiosis with AMF (van der Heijden et al., 2015). Whilst AMF are usually considered fundamental for phosphorus (P) uptake, they can also improve assimilation of other important nutrients including Zn, $NH^{+}_{4,}$ $NO_{3-,}$ Cu and K (Cavagnaro et al., 2006). In addition, AMF also provide many ecological functions. For instance, AMF influence and facilitate soil aggregation and therefore contribute to soil stability through their hyphal system (Rillig et al., 2010; Wei et al., 2019). Additionally, AMF have been estimated to use up to 20 % of fixed carbon (C) produced by the plant (Parihar et al., 2020).

How do AMF impact insect herbivores?

Several studies have documented AMF ability to affect herbivore fitness. However, variable responses by insects to AMF-colonised plants have been reported, from positive (Tomczak et al. 2016, Rasmussen et al. 2017, Malik et al. 2018), to negative (Wang et al. 2015, He et al. 2017, Rasmussen et al. 2017) and no effects (Laird and Addicott, 2008). Several authors have speculated on the processes involving the mutualistic symbiosis that might underpin their effects on plant-insect interactions: change in plant nutritional quality, activation of defensive strategies, change in gene

expression, and production of herbivore-induced plant volatile (HIPV) (Frew and Wilson, 2021; Schoenherr et al., 2018). However, the performance of herbivores is not only influenced by presence or absence of AMF but even by the type and identity of AMF colonizing the plant. For example, Goverde et al (2000), showed for the first time the positive connection between the identity of AMF communities and growth and survival of the butterfly larvae of *Polyommatus icarus* (Lycaenidae), explained by the author as possibly linked to higher leaf concentrations of P and higher leaf C/N ratio (Goverde et al., 2000) .

The potential importance of indigenous AMF for promoting abundance and diversity of foliar feeding insects under field conditions was shown using *Solanum macrocarpum* L. (Sokame et al., 2018). The study demonstrated that plants inoculated with AMF had significantly reduced pest damage on leaves despite the higher abundance and diversity of foliar feeding insect recorded on the leaves. Overall, the incidence and level of damage to leaves as well as the severity of insect attacks were reduced where AMF was applied. The authors speculate that these results were an indirect effect of AMF, which increased plant tolerance to insect herbivory, and suggested that the AMF-plant symbiosis could impact quality of food for the next trophic level.

Another important mechanism that influences the tripartite AMF-plant-herbivore interaction relates to AMF capability for priming plant defense responses. In 2019, Schoenherr et al, using the AMF *Rhizophagus irregularis* (Glomerales: Glomeraceae), and the cabbage looper (*Trichoplusia ni* Hübner) (Lepidoptera: Noctuidae) feeding on potato plants illustrated for the first time the priming of defense response genes resulting from chewing insect infestation. Four genes involved in jasmonic acid (JA) biosynthesis (AOS1, OPR3), phenylpropanoid byiosynthesis (PAL), and plant defence against insects (PI-I), were significantly upregulated in the cabbage looper-infested AMF treatment , reducing herbivore fitness after 8 days of feeding.

AMF have been shown to alter plant physiology by altering secondary metabolism pathways, such as terpenoid synthesis, and it has been demonstrated that AMF can change not only the concentration but also the composition of the terpenoid blend (Walker et al., 2012). Terpenoids are important members of the class of herbivore-induced plant volatiles (HIPVs) (Rapparini et al., 2008). Terpenoids are a large and structurally diverse group of secondary metabolites, playing different ecological and

physiological functions. Synthesis of these compounds is elicited in response to various biotic stresses, although they are not always involved directly in defence. However, terpenoids can act as repellents against herbivore antagonists (Das et al., 2013), attractants towards insect pollinators (Baldwin et al., 2006), and additionally, terpenoids are produced in response to oviposition to attract egg-parasitizing insects (Sharma et al., 2017). Of particular interest is the ability of terpenoids to attract higher trophic levels such as predators and parasitoids of herbivores, which has potential as a tool for biological control (Guerrieri et al., 2004).

How do AMF affect the third trophic level?

To date, studies of the role of AMF in regulating higher trophic levels are remarkably scarce but are still important for understanding the structure of insect communities and multitrophic interactions in both field and laboratory conditions. Addressing this knowledge gap regarding AMF effects on the outcome of multi-trophic interactions demands a community approach and opens new possibilities for the biocontrol and integrated management of pests.

The mechanisms that affect parasitoids when plants are colonized by AMF are variable. AMF-colonised plants usually show higher plant biomass and overall size, which are easier to locate by herbivore predators. For example, a study of Sorghum-sudangrass (*Sorghum x drummondii*) and fall armyworm as herbivore showed how AMF-colonised plants not only had enhanced growth (investing more in plant biomass) but also showed reduced insect damage compared with AMF-free control plants. A possible explanation is that AMF-inoculated plants invested more resources in defence against herbivores (Kaur et al., 2020). The same study also demonstrated that the AMF-inoculated plants were able to attract significantly more beneficial insects such as predators and parasitoids and so supported a lower density of harmful Hemipteran and Dipteran herbivores. To date, compared to other mechanisms, the AMF literature tends to focus on AMF alteration of volatile organic compounds (VOCs) emissions and their impact on the higher trophic level (Grunseich et al., 2020; Thirkell et al., 2017).

In another study (Schausberger et al., 2012), the strain of *Glomus mosseae* quantitatively changed the blend of volatiles emitted by bean plants, *Phaseolus vulgaris.* The blend was more attractive to the predatory mite (*Phytoseilus persimilis)*

in mycorrhizal plants and also when mycorrhizal plants were infested with the herbivore *Tetranychus urticae*, compared to the control. Moreover, the study highlighted that plant emission of three of the five compounds known to play a major role in predatory mite recruitment, β-ocimene, β-caryophyllene and methyl salicylate, were affected by the interaction between mycorrhiza and the spider mites. Thus, mycorrhiza significantly increased the emission of β -ocimene and β--caryophyllene. In contrast, methyl salicylate was increased by spider mite infestation but decreased by mycorrhiza.

In another study where *Solanum lycopersicum* (L) was used in combination with two different strains of AMF (*Funneliformis mosseae* and *Rhizophagus intraradices)*, it was shown that AMF presence increased aphid survival (*Macrosiphum euphorbiae*) and that AMF-colonised plants were more attractive toward the natural enemies (*Aphidius ervi*) (Volpe et al., 2018). Using the same AMF strains as the latter research, Prieto et al., 2016 showed that the polyphagous predator *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) exhibited more attraction towards leaves from plants inoculated with AMF. The above experiments highlighted and documented the potential role of AMF for modulating aboveground plant-arthropod interactions. Further studies are needed to better understand the mechanism underlying those interactions and how they might be used for natural pest control.

Plant growth promoting rhizobacteria (PGPR)

Since their discovery in 1683, when von Leeuwenhoek detected under the lens of his microscope minuscule ‘animals’, the use of bacteria in stimulating plant growth has been extensively exploited (Vessey, 2003). Rhizobacteria are root-colonizing bacteria. These bacteria surround the root and either live around or on the root surface. They stimulate plant growth through several mechanisms such as mobilizing nutrients in soils, producing numerous plant growth regulators (Khan et al., 2019), protecting plants from phytopathogens by controlling or inhibiting them, and improving soil structure (Ahemad and Kibret, 2014). Rhizobacteria are well known in the agricultural market and are used as bio-inoculants to promote plant growth and development under various abiotic and biotic stresses. It has been established that only 1 to 2% of bacteria promote plant growth in the rhizosphere (Beneduzi et al., 2012). Although bacteria of diverse

genera have been identified as PGPR (e.g. *Rhizobium, Mesorhizobium, Pseudomonas, Bacillus, Azotobacter,* and *Azospirillum), Bacillus* and *Pseudomonas* spp. are predominant (Podile and Kishore, 2006). Besides the promotion of plant growth and the other beneficial effects, certain strains of bacteria can cause indirect effects on plant resistance to pests (Pineda et al., 2010) by eliciting resistance to those pests in the phenomenon known as induced systemic resistance (ISR) (Pieterse et al 2002, Pineda et al 2010). Although it is appealing to apply this effect in pest control, so far it has not proved practical and research has been conducted mostly on pathogens rather than insect pests (Fahimi et al., 2014). Thus, soil chemistry and microbial diversity in the soil have a greater ability to influence plant growth and defences (Pineda et al., 2017). Growing in healthy and biologically active soils gives plants the ability to tolerate herbivory and/or to increase physical or chemical defence to limit herbivore attack (Herman et al., 2008; Kaplan et al., 2018). Thus, soil microorganisms indirectly influence plant-insect interactions. Soil conditions often vary between organic and conventional farms (Fernandez et al., 2016), differentiating in specific characteristics (chemical, physical) and microbial composition (Krey et al., 2019). This is the result of several soil management practices employed by these two farming systems. These differences between organic and conventional soils are an opportunity to understand how soil factors affected by management can influence interactions among plants and other trophic groups, providing new useful insights that could improve the management of insect pests.

How do PGPR impact insect herbivores?

PGPR modify the physiological and chemical status of their host plants and therefore can affect directly or indirectly plant defence against herbivorous insects. Two main mechanisms have been identified to regulate interactions between belowground microbes and aboveground insect herbivores. The main one that has been investigated widely over the last two decades is the enhancement of plant growth through ISR, mediated by PGPR (del Rosario Cappellari et al., 2020; Pieterse et al., 2014; Pineda et al., 2010). Induced resistance is the production of defensive compounds in response to attack by a pathogen or herbivore. Another central mechanism involved in interactions

between mutualistic microbes and herbivorous insects is the emission of herbivore-induced plant volatiles (HIPVs). However, this effect has been studied for only a few strains of rhizobacterial species to date and have reported a negative effect of volatile emission on insect fitness. Most experiments have been conducted under laboratory conditions and using only few genera of insects (mainly Lepidoptera and Hemiptera). Using a combination of *Pseudomonas* spp. and other microbial species, the microbe mixture significantly reduced the total number of cucumber beetle in field experiments compared with the insecticide treated plants (Zehnder et al., 1997). A possible explanation for these results is that PGPR induced physiological changes that lead to production and/or accumulation of plant allelochemicals acting as herbivore repellents and/or attractants of natural enemies. The same mechanisms could explain how in tomato, *Bacillus* spp. bioformulations reduced the growth of *Helicoverpa armigera* larvae (Vijayasamundeeswari et al. 2009) and *Bemisia tabaci* (Valenzuela-Soto et al. 2010). Another study, using *Pseudomonas* spp. on *Arabidopsis* plants and *Myzus persicae,* showed for the first time the ability of rhizobacteria-induced systemic resistance to an insect pests: the rhizobacterium was shown to prime *Arabidopsis* plants for increased expression of the JA-responsive gene when aphids were feeding (Pineda et al., 2012).

How do PGPR affect the third trophic level?

It is known that soil-borne non-pathogenic rhizobacteria can regulate interactions at the third trophic level by triggering biochemical changes in plant metabolism or through plant growth promotion (Benítez et al., 2017). However, up to date only few experiments have been conducted combining above-ground and belowground interactions and these complex dynamics are still poorly understood. In a recent study that used as a study system the predatory earwig *Doru luteipes* (Dermaptera: Forficulidae), the generalist herbivore *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and the specialist herbivore *Plutella xylostella (*Lepidoptera: Plutellidae) on *Eruca sativa* (Brassicaceae) plants, the authors showed that plants inoculated with PGPR and herbivore-damaged plants were more attractive towards the earwig predator in the olfactometer assay (Bell et al., 2020). Bell et al, explained their findings as a symbiotic interaction among PGPR and plants infested with herbivores that can favour the attraction of predators through the emission of VOCs; these VOCs trigger specific plant

defence pathways (Fahimi et al., 2014; McGregor and Gillespie, 2004) that have been reported to attract natural enemies and therefore indirectly representing a type of plant defence. VOC emissions and toxin production (a form of direct plant defence) are both mediated by a complex network of biochemical pathways that are under the regulation of several phytohormones including jasmonic acid (JA), which is a key regulator (Kuśnierczyk et al., 2011; Pineda et al., 2013). Silencing JA signalling pathways strongly affects plant interactions with herbivores and parasitoids and a study by Pineda et al (2012) supported further the key role of JA in mediating interactions between parasitoids and root-bacteria: by modifying the blend of herbivore-induced plant volatiles (that depend on JA signalling), root microbes could interfere with the attraction of herbivore parasitoids. To advance knowledge of processes regulating microbe-plant-insect interactions requires evaluating whether PGPR benefit plant fitness in natural communities of organisms.

1.4 Insect associated microbes

One of the most fascinating strategies adopted by many herbivore arthropods for feeding and therefore survival in nature is the association with endosymbiotic bacteria. The term symbiosis was introduced for the first time by Anton de Bary in 1879 for describing "a permanent association between two or more distinct organisms, at least during a part of the life cycle" (Gil et al., 2004). This symbiosis is estimated to have emerged hundreds of millions of years ago (Moran et. al., 1993). Endosymbionts live inside insect cells and the haemolymph, and they can be classified as primary (obligate) endosymbionts and secondary (facultative) endosymbionts. The first group is carried by all the individuals of the host species while the secondary endosymbionts are not (Brumin et al., 2011; Henry et al., 2015). However, not all the endosymbionts are strictly necessary for the survival of the population. Facultative endosymbionts have been reported to mediate the expression of several ecological traits of their insect herbivore host, for example by enhancing their thermal tolerance (Montllor et al., 2002), increasing their resistance against natural enemies such as parasitoids, and expanding their range of suitable plants (Oliver et al., 2003). In the past decades, scientists have begun to understand the hidden role played by endosymbionts and studies have demonstrated that endosymbionts may benefit their insect hosts by modifying important processes at the insect-plant interface such as plant resource

allocation, plant volatile emission, and plant defence signalling pathways (Zytynska et al., 2021). Moreover, research on insect symbionts and their effects on other trophic levels has been shown under laboratory condition. As an example, using as a model the facultative endosymbiont *Hamiltonella defensa* in *Acyrthosiphon pisum*, a study reported that the symbiont led to parasitoid extinction in laboratory conditions indicating the significant role played by endosymbionts on the stability of population dynamics (Sanders et al., 2016).

How do bacterial endosymbionts impact insect herbivores?

Roughly half of the estimated 1,200,000 species in the arthropod class *Insecta* are thought to harbour endosymbiotic bacteria. The most well characterised insect endosymbiosis is the relationship between aphids and the obligate endosymbiont *Buchnera aphidicola* (Munson et al., 1991), but similar associations have been shown in other insect families such as carpenter ants, whiteflies, leafhoppers, and weevils (Clark et al., 1992; Moran et al., 2003; Schröder et al., 1996)**.** The facultative bacterial endosymbionts found most commonly in arthropods are *Wolbachia, Rickettsia*, and *Cardinium* (Sazama et al., 2019). These endosymbionts affect their hosts in several ways. For instance, the endosymbiont *Spiroplasma* sp. can protect *Drosophila neotestacea* against parasitism by the nematode *Howardula aoronymphium* (Jaenike et al., 2010). In pea aphids, infection with *Hamiltonella defensa* results in protection from parasitism by the wasp *Aphidius ervi* (Oliver et al., 2003). Bacteria can even influence host reproduction. *Wolbachia* is described as a reproductive manipulator by causing parthenogenesis, feminisation or male-killing (Sazama et al., 2019). Thus, members of the genus *Wolbachia* have been recognised to increase fecundity of *Drosophila simulans* (Sturtevant; Weeks et al., 2007).

While the aphid facultative endosymbionts *Serratia symbiotica* and *Hamiltonella defensa* can alleviate aphid fitness under heat stress, *Rickettsia* may confer heat tolerance in the whitefly *Bemisia tabaci* (Brumin et al., 2011; Harmon et al., 2009). Another important trait that endosymbionts confer on their host is their ability to provide essential nutrients to their host that they cannot synthesise nor obtain in large quantities from their diet. For instance, essential amino acid synthesis as well as riboflavin synthesis are possible in *Acyrthosiphon pisum* (Harris) when the obligate endosymbiont *Buchnera aphidicola* is present (Douglas, 2016). Similarly, for the tsetse fly *Glossina morsitans* (Westwood) vitamins are provided by the obligate

endosymbiotic bacterium *Wigglesworthia glossinidia* (Akman et al., 2002). In the beetle genus *Megacopta*, the composition of obligate symbionts is associated with beetle food plant use, which influences insect host plant range (Hosokawa et al., 2007). When *Wolbachia* is present, the leafminer moth *Phyllonorycter blancardella* is able to manipulate the physiology of its host plant (Kaiser et al., 2010). Additionally, Su et al in 2015, demonstrated that the whitefly endosymbiont *H. defensa* can suppress induced plant defences in tomato. Feeding by symbiont-infected aphids led to decreased expression of JA-responsive genes, reducing defence-related enzyme activity in whitefly-infested tomato plants.

These findings reinforce the primary role of symbiotic bacteria in mediating insect-plant interactions altering not only insect physiology but even plant physiology and therefore plant responses to herbivory. These mechanisms are, however not completely understood. In fact, although the value of the endosymbionts has been well demonstrated under lab condition, few studies have investigated their effectiveness under field conditions. Depending on endosymbiont strain and aphid species, the studies under field conditions have shown variable degrees of parasitoid resistance (Lenhart and White, 2020; Łukasik et al., 2013a; Oliver et al., 2003). Understanding the mechanisms responsible for mediating insect-plant interactions will expand knowledge of insect biology and may aid in successful management of insect pests.

How do insect endosymbionts affect the third trophic level?

In the last decade, new evidence has emerged, mostly from laboratory experiments (McLean, 2019), showing that endosymbionts can have important effects not only on insect-plant interactions but also on other groups of arthropods such as natural enemies. Facultative endosymbionts mediate interactions between their insect hosts and parasitoids and can thus alter host–parasitoid network structure through effects on trophic links (McLean et al., 2016; Corbin et al., 2017; Yet al., 2018). Despite the advances in the last few years, most research on insect-symbiont-parasitoid interactions has focussed only on few model systems.

In 2017, Frago showed evidence for a subtle mechanism affecting insect parasitoids. The parasitoid wasps *Aphidius ervi* were less attracted toward plants infested with the pea aphid *Acyrthosiphon pisum* when aphids were infected with the facultative

endosymbiont *Hamiltonella defensa* compared with plants infested with symbiont-free aphids, and this was linked to differences in the plant VOC composition. This provides a good example of the potential for endosymbionts to modify insect population dynamics and community structure by manipulating processes at the insect-plant interface (Monticelli et al., 2019). Another example that shows how bacteria can manipulate their insect hosts and reduce attractiveness to their natural enemies has been illustrated again for pea aphid. In fact, four symbiont species (in the genera *Regiella, Rickettsia, Rickettsiella* and *Spiroplasma*) have been reported to confer in pea aphid protection from infection by the entomopathogenic fungus *Pandora neoaphidis* (Remaud and Hennert; Łukasik et al., 2013b). A comprehensive study of field populations of the grain aphid *Sitobion avenae* highlighted another knowledge gap on the effects of aphid endosymbionts on hyperparasitoid communities (Ye et al., 2018). The study found that infection frequencies of *Hamiltonella defensa* and *Regiella insecticola* were similar in living unparasitized and parasitized aphids, while the hyperparasitism frequency was lower in parasitized aphids/mummies that were endosymbiont infected. This finding supports the cascading effect of facultative endosymbionts on higher trophic levels, agreeing with the study from Rouchet and Vorburger (2012) which showed a lower abundance of hyperparasitoids in *H. defensa* infected *Aphis fabae* in field populations.

However, most research on the effects of endosymbionts on the higher trophic level and insect community dynamics is carried out in laboratory conditions and is dominated by studies of a single insect family (Aphids) preventing general conclusions to be drawn and applied to other insect groups.

1.5 Drought effects on multitrophic interactions

It has been anticipated that due to climate change, the occurrence and the length of periods of drought will increase in the coming years (Fischer et al., 2022). Drought stress can alter plant physiology, chemistry and morphology causing changes in insects performance and survival (Beetge and Krüger, 2019). This has shown to modify intrinsically multitrophic interactions (Guyer et al., 2021). In fact, changes in plants and insects physiology can lead to modifications in their interaction networks (e.g. natural enemies) (Han et al., 2019). Despite their importance for agriculture productivity, the effect of drought on the third trophic level is poorly understood and often studies focussed exclusively on bi-trophic interactions (Harvey et al., 2020; Stireman and Singer, 2018). Attack rates on the bird cherry-oat aphid, *Rhopalosiphum padi* L. were lower on barley plants under drought stress treatment (Aslam et al., 2013). Reduction in parasitoids performance was reported in multifactor experiment where two species of aphids and their parasitoids (*Diaeretiella rapae* and *Aphidius colemani)* were used under drought stress and fertiliser treatments (Shehzad et al., 2021).

Another variable that is often neglected in studies evaluating drought stress effects is how drought effects soil microbes (Kundel et al., 2020). In fact, it has been demonstrated that drought can lead to loss in nutrient cycling (Rahman et al., 2021), soil fertility (Tamburini et al., 2018) and decrease enzyme activity (Ochoa-Hueso et al., 2018). Recent investigations have shown that drought can create legacy effects on soil microbial communities (Kannenberg et al., 2020). In a 10 years long-term field experiment in the mountain where recurrent drought was tested, results indicates that the recurrent drought changed soil microbial community composition leading to modification in its functioning (Canarini et al., 2021). Further studies that include interactions between soil microbes, drought, insect pests and their natural enemies are required under field conditions, to predict and understand patterns in these interactions.

1.6 Knowledge gap

To meet the food requirements by 2050 of a global population exceeding 9 billion, crop productivity needs to increase by 70-100% (Trivedi et al., 2017). While conventional intensive agricultural practices that rely on pesticides and other synthetic chemical inputs have been successful in increasing crop yields, they have also caused loss of biodiversity, soil degradation, increased frequencies of pesticide-resistant pests and pathogens, which have important consequences for human health and food security (Tilman et al., 2002). FAO stated in 2015 that climate change threatens our ability to ensure global food security, eliminate poverty and achieve sustainable development. Climate change has both direct and indirect effects on agricultural productivity including changing rainfall patterns, flooding, drought and geographical redistribution of pests and diseases, and it is clear that there is an urgent need to develop sustainable strategies to increase food production. Despite the potential of microbial biostimulants as future tools for enhancing food production, available evidence suggested that positive results from greenhouses often fail in field conditions (Shein et al., 2021). Understanding the complexity of interactions among microbes, plants, soils, insects, and their natural enemies under a changing climate appears daunting. Those interactions have only been studied using simplified situations and mostly under laboratory conditions. For example, results of AMF research in field trials are still a relatively small proportion of the total (24%) as compared to the numerous results obtained in controlled greenhouse or growth chamber conditions (69%) (Buysens et al. 2016). Moreover, limiting observations to one genotype or clonal line of an insect species and one strain of microbe can limit our ability to detect true patterns in natural and agricultural environments. Thus, the complex nature of multi-species interactions has not yet been fully integrated into studies to understand how belowground microbes and insect microbes could affect higher trophic levels and vice-versa. Research that includes multi-level trophic interactions may give a more accurate estimation of an organism's distribution, genotype, phenotype and composition than studies examining simple pairwise comparisons (Strauss and Irwin, 2004), as a multitude of trophic interactions occur simultaneously in any given habitat.

In addition to the lack of knowledge about how microbes can affect the higher trophic level, most studies on tri-trophic interactions focus on aphids. Although aphids provide

a well described study system for studying plant-herbivore-natural enemy interactions, there is an urgent need for more experimental work including a broader taxonomic range of species. This is essential to understand the complex interactions across communities. To better elucidate the performance of these potential bottom-up and top-down mechanisms in insect communities, field experiments are needed where more complex interactions and community-level dynamics occur. Thus, employing symbiotic microbes could be one avenue to develop and improve pest control. The delicate connection between microbes, plants, insects, and their natural enemies need to be discovered and could be one of the most interesting challenges faced by researchers in the immediate future.

1.7 The study system

In all the kind of ecosystems there are a variety of multi-trophic interactions, which affect fitness and responses of all the organisms involved. Work in this thesis mainly focussed on the effects of microbes on plant-herbivore-natural enemy interactions aboveground using the potato aphid, *Macrosiphum euphorbiae*, and the host plant *Solanum tuberosum* (Fig.1.1). This study system is expanded further to investigate those interactions by including another aphid species and water stress in Chapter 2. The role of the aphid defensive facultative endosymbiont on aphid responses to drought stress is studied in chapter 3 and effects on the higher trophic level are studied in chapter 4. Finally, the influence of soil microbes on the higher trophic level is studied in Chapter 5.

Potato (*Solanum tuberosum* L.)

Figure 1. 1 Potato plant (*Solanum tuberosum* L.) cv Désirée used in this thesis

Potato (*Solanum tuberosum* L.) is ranked as the fourth most important food crop in the world after rice, wheat, and maize. It is grown in over 125 countries and is consumed daily by more than a billion people worldwide (Mullins et al., 2006). Potato, because of its short and flexible vegetative cycle, can be cultivated where other crops may fail (Food and Agriculture Organization of the United Nations, 2009), but it has been estimated that global drought will reduce potential potato yield by 18-32% in the years 2040-2069 (Hijmans, 2003). From a nutritional point of view, potato is a good source of proteins, vitamin C, minerals, carbohydrates and vitamin B (Obidiegwu et al., 2015), is low in anti-nutrients such as oxalates and phytates which can reduce mineral bioavailability (White et al., 2009) and can be consumed cooked or in processed forms. Although plant responses to drought depend on physiological age of the tubers and source of the seed (Steckel and Gray, 1979), potato is considered as a drought sensitive crop as its water requirements during the vegetative phase are high and its short root length limits its capacity to take up water (Gregory and Simmonds, 2011). It has been reported that early development of plant stress is most damaging to tuberisation, tuber bulking, and hence final tuber yield as a consequence of decreased rates of carbon assimilation to tubers (Obidiegwu et al., 2015) However, drought not only decreases yield, but it can also damage product quality, for instance by increasing scab incidence (Mane et al., 2008). Water availability can affect the nutrient availability, nutrient uptake by the plant and plant nutrient composition (Li et al., 2009). The presence of extensive germplasm resources in potato with different characteristics plays a key role in efforts to improve the crop's ability to cope with drought and enhance yield (Obidiegwu et al., 2015). Moreover, potato plants show decreased expansion of stems and leaves in response to drought, leading to a decline in leaf area index and dry matter content (Albiski et al., 2012; Anithakumari et al., 2012; Deblonde and Ledent, 2001; Mane et al., 2008). The UK ranks among the top 20 countries in the world that produce potato (Hijmans, 2003). Thus, potato can be found in 33 % of british meals (Adesina and Thomas, 2020). Potato breeding in the modern sense started in 1807 in England (Bradshaw and Ramsay, 2005). Up to date, late blight remains the first and the most devastating disease in potato and alongside other pathogens (cyst nematodes, and viruses) continues to menace potato production (Birch et al., 2012). Potato yields, can be affected by periods of drought and can be reduced by infestation with pests and pathogens (Beddington, 2010). The potato cultivars used throughout this thesis are :

Fontane, Innovator, Hermes and Desirée, due to being important commercial crops for human consumption.

Arbuscular mycorrhizal fungi (AMF)

AMF have been promoted as a biological tool to maintain and promote sustainable agriculture for their role as natural biofertilizers (Berruti et al., 2016). The species that this study focuses on are *Rhizophagus irregularis* and *Funneliformis mosseae. Rhizophagus irregularis* is a model species for AMF research. It is the most commonly identified AMF isolate and has been widely produced commercially in the last few decades (Renaut et al., 2020). The current knowledge of AMF diversity, biology and genomics are based on extensive research on this isolate. *Funneliformis mosseae, (sensu Glomus mosseae)* has been shown to successfully colonise roots of maize, (*Zea mays* L.), lemon grass (*Cymbopogon nardus*) (Tanwar et al., 2013) and even apple trees (Turrini et al., 2017). The use of AMF under combined stresses has been rarely investigated, as research has focused mostly on examining the effects of a single abiotic stress. Understanding how combined stressors influence AMF is important to maximise the efficacy of beneficial microbes as an alternative eco-friendly approach to overcome plant diseases.

Herbivore: Aphids

Aphids (Superfamily: Aphidoidea, Order: Hemiptera) are among the most important pests in agriculture. They are considered to be the most economically destructive pests, which mostly inhabit temperate regions (Guerrieri and Digilio, 2008). Over 3000 species of herbaceous plants and shrubs are colonized by aphids (Boivin et al., 2012). The efficient long and flexible stylets of aphids allow them to feed on phloem sap, which provides nutrients in the form of carbohydrates, amino acids, proteins, water, and other components (Guerrieri and Digilio, 2008). Aphid feeding removes nutrient resources from the plant and decreases plant fitness. The selection and identification of a proper host plant is achieved by the introduction of the stylets (by probing plant tissues) until the phloem is reached. Stylet tracks originating from probing behaviour can also be found on non-host plants (Guerrieri and Digilio, 2008). Aphid infestation

causes leaf curling, chlorosis and shoot dieback (Walgenbach, 1997). Moreover, about 50% of plant viruses are transmitted by aphids (Hooks and Fereres, 2006), causing significant damage to agronomic crops.

The potato aphid *Macrosiphum euphorbiae*

Figure 1.2 *Macrosiphum euphorbiae* (Thomas) nymphs and adult on potato leaf

Macrosiphum euphorbiae (Thomas) (Fig.1.2) originated from North America and has colonized countries throughout the world. It is highly polyphagous, feeding on more than 200 plant species from 20 families, many of which include herbaceous crops (Beetge and Krüger, 2019). In all the studies conducted on potato aphid, life-history and development were considerably changed by variation in temperature (Barlow, 1962). In *M. euphorbiae* the reproduction is mostly asexual but in cool areas cyclic parthenogenesis can occur, meaning that asexual reproduction is prevalent but sexual reproduction may occur under specific circumstances (Raboudi et al., 2012).

The green peach aphid *Myzus persicae* (Sulzer)

Figure 1.3 *Myzus persicae* (Sulzer) adults and nymphs on potato leaf.

Myzus persicae (Sulzer), (Fig.1.3) also known as peach potato or green peach aphid, is an extremely cosmopolitan aphid and present worldwide with a host range of 400 different plant species in 40 different families (Bass et al., 2014). It is an important example of a heteroecious aphid species which alternates between the primary host, *Prunus persica* L. (Rosaceae) and various secondary hosts (Kephalogianni et al., 2002). This host alternation is an important feature of the biology of *M. persicae*: in autumn, due the decreasing daylength, apterous viviparae of holocyclic clones produce female gynoparae and male morphs on a secondary host plant that migrate to the principal host *(Prunus persica L.)* where the gynoparae give birth to the oviparae (Zitoudi et al., 2001). Another important feature of *M. persicae*, is the adaptation to specific host plants. In fact, populations of *M. persicae* feeding on tobacco, *Nicotiana tabacum* L. (Solanaceae) are genetically and morphologically different from populations on other host plants (Blackman and Spence, 1992).

Facultative bacteria endosymbionts in aphids

Like many arthropods, aphids are associated with several bacterial endosymbionts that influence their interactions with the surrounding organisms. Hosted in specialized cells (mycetocytes or bacteriocytes) (Chen and Purcell, 1997), endosymbionts have enabled aphids to adapt to different host species and they have even conferred to aphids tolerance of an array of abiotic stresses (Dunbar et al., 2007; Leybourne et al., 2020a; Russell and Moran, 2006). Additionally, emerging evidence has shown that endosymbionts may suppress plant defence responses to insect feeding opening new challenges in understanding their effects on many plant species (Su et al., 2015). In the latter study, the facultative endosymbiont *Hamiltonella defensa* was shown to mediate whitefly-plant interactions through suppression of plant defence in *Solanum lycopersicum* (Miller) plants. Understanding how *H. defensa* influences aphid fitness under drought stress is important to maximise the development of effective controls in future integrated pest management programmes.

Parasitoid Wasps

Figure 1.4 *Acyrthosiphon pisum* adult "mummies" parasitized by *Aphidius ervi* (Haliday).

Several parasitoid wasps in nature exploit aphid colonies. Among them the Aphidiinae is a monophyletic subfamily of Braconidae (Hymenoptera) that specializes on aphids (Boivin et al., 2012). These parasitoids are koinobiont as the parasitoid larva (oviposited by the mother wasps) develops in a living host that continues to feed and grow. Mature third instar larva penetrates the aphid cuticle making a hole and at the same time fixing the aphid host to the surface by employing secretions from the silk glands, which generates the peculiar "mummy" (Fig.1.4). Once the larva consumes the aphid from the inside out, it leaves a brown husk. A cocoon of silk is then generated in which the parasitoid pupates. Several species of parasitoid wasps use potato aphids as a host: *Aphidius ervi* (Haliday) is used in this study. *A. ervi* is an endophagous braconid (Pennacchio et al., 1999), which parasitises various species of aphids of economic importance such as pea aphid, *Acyrthosiphon pisum* (Harris) or *Macrosiphum euphorbiae* (Thomas), both key pests of agriculturally important crops.

1.8 Aims, objectives and scope of the thesis

The overall aim of the work presented in this thesis is to investigate the effects of below-aboveground microbes on the third trophic level and responses to drought stress (Fig.1.5).

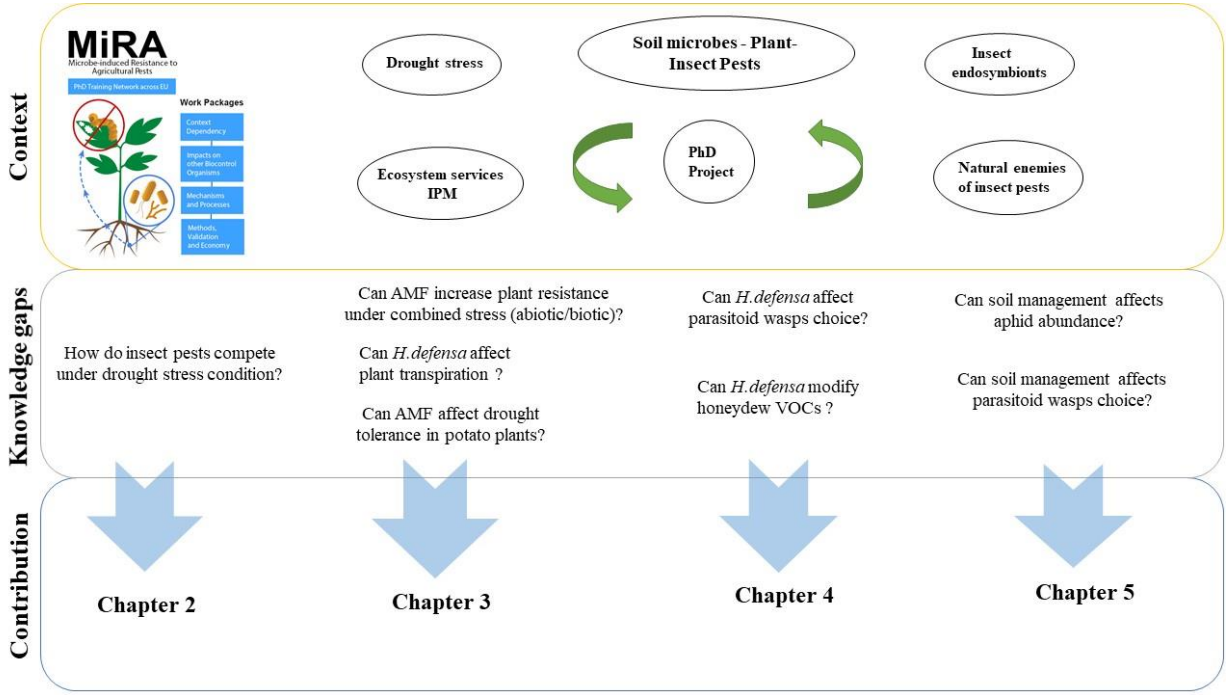

Figure 1.5 Graphical representation of the development pipeline of the PhD project. The figure describes from the top to the bottom: context of the project, knowledge gaps highlighted through the literature review (Chapter 1), and finally contributions achieved for each of the knowledge gaps previously identified.

Chapter 2 aims to investigate the effects of plant drought stress on aphid performance and colonisation. The specific objectives are: 1) to quantify interspecific competition among different species of aphids when sharing the same host plant under drought stress; 2) To investigate how drought stress and competition impact spatial distribution of two different species of aphids feeding on potato.

Chapter 3 aims to study the effects of AMF on plant tolerance of biotic (aphid) and abiotic (drought) stress. The specific objectives are: 1) to investigate the response of three different cultivars of *Solanum tuberosum* inoculated with two different species of AMF under long periods of drought stress; 2) To investigate potato aphid performance in the presence and absence of aphid facultative bacterial endosymbionts (*H. defensa*) when exposed to plants treated with AMF or drought and their interaction; 3) To investigate plant physiological responses to a combination of abiotic and biotic stressors (drought and insect pests).

Chapter 4 aims to explore the effects of aphid endosymbionts on the third trophic level. The specific objectives are: 1) to investigate if facultative endosymbiont presence affects the composition of volatile compounds emitted by aphid honeydew; 2) to investigate if *H. defensa* alters the attractiveness of aphid honeydew towards parasitoid wasps.

Chapter 5 aims to examine the outcome of different soil management on aphid biocontrol. The specific objectives are: 1) to examine potato aphid performance when exposed to soils with different biological, chemical and physical properties due to differences in their management histories; 2) to determine how soil bacterial community composition can influence dynamics aboveground (insect-parasitoid);

The discussion (Chapter 6) summarises key findings from the experimental work on multi-trophic interactions between microbes- plants-aphids and parasitoid wasps under environmental stresses and discusses how this multi-trophic approach can be broadened further to take into account the dynamic relationships inherent in nature, but also expand on tripartite interactions in an agriculturally, economically, and globally relevant system.

Chapter 2. The effect of drought stress on inter-specific competition between *Myzus persicae* (Sulzer) and *Macrosiphum euphorbiae* (Thomas) on potato (*Solanum tuberosum* L.)

Abstract
In natural communities, coexistence of host-specific herbivores on plants naturally occurs but only few studies have investigated this coexistence when the plant-host is exposed to drought stress. The potato aphid, *Macrosiphum euphorbiae* (Thomas), and the peach aphid, *Myzus persicae* (Sulzer) are common herbivores on potato, *Solanum tuberosum* L. This study, conducted under glasshouse conditions, examines the effect of drought stress on competition between *M. euphorbiae* and *M. persicae* when sharing the same host (*S. tuberosum* cv. Fontane) under drought stress conditions. When feeding alone *M. persicae* abundance was negatively influenced by drought stress, whereas *M. euphorbiae* abundance was unaffected by drought when feeding alone. The effect of drought on *M. persicae* did not change when *M. euphorbiae* was present. However, *M. euphorbiae* abundance tended to be suppressed by *M. persicae* when drought stress was applied, although this was not significant. These findings indicate that drought stress is indeed able to influence interspecies competition and that the effects of abiotic stresses should be considered in future experiments of plant-herbivore interactions. Further research in this area is required to better understand effect of abiotic stress on community structure, as such information would be valuable in the design and in the improvement of the management of insect pests.

Keywords: abiotic stress, aphid, herbivore, plant-insect interaction, potato

2.1 Introduction

An increase in the occurrence and severity of periods of drought has been anticipated to be a key characteristic over the next 50-100 years (Pfahl et al., 2017). In addition, due to the changing climate, either the duration or frequency of droughts is predicted to increase in many areas worldwide (Bedel et al., 2013). A decrease of water availability can have important consequence for plant-insect relations; by influencing the physiology of host plants, drought can alter insect biology and virulence (Deutsch et al., 2018; Maino et al., 2016). For instance, several studies have shown that increases in drought severity results in more frequent outbreaks of insect pests (Dale et al., 2001; Jactel et al., 2019). Moreover, reduced water availability by closing the stomata, a mechanism adopted by plants to protect them from prolonged water loss, leads to change in the performance of insects with potential impacts on their decision and foraging behaviour (Lenhart et al., 2015; Salgado et al., 2020).

Potato (*Solanum tuberosum* L.) is the most important non-grain crop in the world (Raymundo et al., 2018) and it is classified as the third most important food crop following rice and wheat with a global annual production of 300 million tons (FAO, 2016). The potato industry in the UK produces annually 5.6 million tonnes of potatoes which includes 17 000 ha of certified seed potatoes (AHDB, 2015). Compared to the other major crops, potato is more susceptible to drought (Zhang et al., 2018). Most potato-growing regions are already affected by an increase in frequency of drought periods (Rudack et al., 2017). It has been already anticipated that potato yields will decrease by up to 18% to 32% in the coming years due to drought (Hijmans, 2003). Therefore, understanding and increasing tolerance to drought stress is a priority for potato improvement. Yield losses can be further exacerbated by the fact that a wide range of insect pests damage potatoes (Amiri and Bakhsh, 2019). A study estimated that up to 37 % of all crop yield losses are due to pests (13% to insects, 12% pathogens, and 12% to weeds) (Paoletti and Pimentel, 2000). For potato, insect pests and diseases cause 40 % of yield losses (Beddington, 2010). Drought-tolerant potato cultivars are often more susceptible to insect pests compared to sensitive cultivars (Quandahor et al., 2021, 2019). Biotic and abiotic stresses influence plant physiology and chemistry causing bottom-up impacts on insect herbivore performance and fitness (Beetge and Krüger, 2019; Han et al., 2016). However, the mechanisms responsible for increased insect densities on stressed plants are not fully understood and may differ depending on the type of insect (Petersen and Hunter, 2001).

Sap-feeding insects are among the most economically important pests of crops. In particular, the most economically relevant group of sap-feeding insects is the aphids (Zytynska and Preziosi, 2013). Aphids cause damage in several ways: feeding, through transmission of plant viruses, and honeydew production (Fox et al., 2017). The green peach aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae) is a highly polyphagous aphid that can attack more than 400 plant species across the globe and transmit over 100 viruses to economically important crops (Brunissen et al., 2009a; Silva et al., 2012). The potato aphid, *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae), is known to feed on more than 200 plant species from 20 different families, many herbaceous and ornamental crops and it is well known for transmitting important viruses *such as* strains of Potato virus Y (PVY, genus *Potyvirus*, family *Potyviridae*), *Potato virus* A (PVA, genus *Potivirus*, family *Potyviridae*), and potato leaf roll virus (PLRV, genus *Polerovirus*, family *Solemoviridae*). These two aphid species can, in the field, share the same host and they are common on potato *(Solanum tuberosum L., Solanacea)* in the UK. Thus, *M. persicae* is consistently among the first colonizing aphids in potato crop, preceding other species, including *M. euphorbiae* (Taylor, 1962).

Phytophagous insects that share the same host can interact with each other, competing for the same source of food (Kaplan and Denno, 2007). Competition is a key element in structuring biological communities, and it is affected by both abiotic and biotic environmental stressors (Duan et al., 2017; Gergs et al., 2013). Interspecific competition among phytophagous insects is still not well documented and only some examples have been reported (Brunissen et al., 2009b; Smith et al., 2008). The current synthesis in ecology is that several factors can shape herbivore communities, including plant quality and structure (Johnson and Agrawal, 2005; Larson and Whitham, 1997), predators and parasitoids (Baer and Marquis, 2020), mutualistic interactions (Wimp and Whitham, 2001), and interspecific competition (Guilbaud and Khudr, 2020). A few studies have reported that competition occurs more frequently among sap-feeding insects, such as Hemiptera, than other feeding guilds (Lawton and Hassell 1981, Denno et al., 1995), and it has been shown that this competition becomes stronger between closely-related taxa (Denno et al., 1995; Petersen and Hunter, 2001). Thus, many interspecific competition between insect pests can be intensified by induced changes in plant nutrition (Denno et al., 1995) However, the outcome of competition varies from negative impact on both species (Jaworski et al., 2015), or negative impact on the weaker species that often migrates to other hosts (Bompard et al., 2013; Tuelher et al., 2016).

The importance of interspecific competition in shaping community structure of herbivorous insects has been not extensively investigated over the past decade, missing out critical insights on the mechanisms that can mediate competition. Moreover, the relative impact of drought stress in shaping competitive interactions remains poorly understood. To date, no information is available on competition between the two aphid species *M. persicae and M. euphorbiae* on *Solanum tuberosum.* Here, interspecific competition within *M. persicae and M. euphorbiae* was assessed under control and drought conditions. I tested whether aphid performance was altered due to competition with another aphid or due the imposed drought stress or both. It was hypothesised that (1) the different watering regimes would affect negatively both species in terms of offspring number, (2) the presence of *M. persicae* would impact negatively *M.euphorbiae* in terms of on-plant distribution (Taylor, 1962), and (3) the combination of watering regimes and the coexistence of the two species would differentially affect the abundance of *M. persicae* compared with *M. euphorbiae*, the latter having previously been shown to be negatively affected (abundance and survivorship) while feeding on severely stressed potato (An Nguyen et al., 2007) .

2.2 Materials and methods

Plant material

The experiment took place in a greenhouse at the James Hutton Institute, Invergowrie (Dundee, UK). Tubers of *Solanum tuberosum* cv. Fontane, were cut into similar size portions and rinsed using tap water. The tubers were sown in 1 L pots containing a sterile mix of 2:1 sand:loam (Washed Sand, Geddes Group, Arbroath, UK; Keith Singleton Steam Sterilized Loam, Clydesdale Trading, Lanark, UK). Plants were grown under controlled glasshouse conditions at 23°C/14°C (day/night), 16/8 h light/dark photoperiod. The plants were watered as needed. After 6 weeks of growth, water availability treatments were imposed. The well-watered plants (control) were watered regularly throughout the experiment. For the 'Drought plants', water was withheld until the end of the experiment. Aphids were placed on plants three days after the water treatments had been initiated.

Insect rearing and maintenance

Aphid clonal lines were reared on excised leaf material from 3–4-week-old potato plants (cv. Desirée) in ventilated containers comprising one Perspex cup place inside another which contained water; plant material was inserted through a c. 5 mm circular hole in the base of the inner cup and covered with a mesh lid. Plant material was refreshed weekly. *Macrosiphum euphorbiae* (clonal line MW16/48, genotype '2') and *Myzus persicae* (clonal line 229) of known genotypes were sourced from established laboratory cultures (Dr. Mark Whitehead, University of Liverpool; Dr. Gaynor Malloch, The James Hutton Institute).

Experimental protocol

The experiment comprised a randomised block design. Ten replicate blocks were staggered over a period of 5 days (two blocks per day). Ten replicates, one per block, were established for each combination of aphid species, infesting density and water treatment giving a total of 80 plants. Within each block, each plant with the assigned aphid and water treatments was positioned at random. The aphid treatments were as followed: (i) *4 adults M. euphorbiae; (ii) 4 adults M. persicae; (iii) 4 adults M. euphorbiae + 4 adults M. persicae; (iv)* aphid free plant.

Age-synchronised aphids were used on the experiment, in detail, aphids were between seven to ten days old when used in the experiment with each replicate containing aphids of the same age. Aphids were transferred into clip cages using a fine horsehair brush following the treatment described above. Clip cages were attached to the plant stem at a mid-stem position, so the cages were in an open position allowing the aphids to escape and move freely on the plants. Plants were enclosed with a perforated plastic bag to prevent aphid dispersal away from the plant. All plants had at least 6 fully expanded leaves on the day the experiment started. After 7 days, the position of the aphids on the plant was recorded and the total number of aphids (adults and nymphs) was counted for each plant identifying three parts: bottom part (first and second leaf), middle part (third and fourth leaf), and top part (fifth and sixth leaf). Subsequently, aphids were freeze-dried (Christ Alpha 1-2 LDplus, UK) and aphid mass was recorded to the nearest 1 mg using a microbalance (Mettler Toledo MX5, Mettler Toledo, UK).

2.3 Statistical analysis

All statistical analyses were carried out using R v4.1.3, with additional packages ggplot2 v.2.2.1 (Wickham, 2009), dplyr v. 3.5.3 (Mailund, 2019) , lme4 v.1.1-13(Bates et al., 2015), lubridate v. 1.7.8, (Spinu et al., 2016), car v. 3.0-7 (Fox and Weisberg, 2011), lsmeans v.2.27-62 (Lenth, 2016) and multcomp v. 1.4-8 (Hothorn, 2008). Data were checked for normality and homogeneity of variance by plotting Q-Q plots and residuals vs fitted values. Parametric models were applied where data displayed normal distribution. Non-parametric methods were used for analysis where data displayed non-normal distribution. Total number of nymphs and nymph weight were square root transformed and analysed using ANOVA. To evaluate the effects of water treatment, aphid treatment, plant part and their interaction on the square root transformed data, a linear model was applied including pot position as a random factor (variable~ Water_treatment *Aphids_treatment * Plant_part +(1|Pot)), with the function "lm" and subsequently analysis of variance (ANOVA) was performed on the model. Finally, differences between groups were assessed by Tukey's post-hoc test.

2.4 Results

Number of nymphs

To examine co-existence of the two aphid species and to investigate whether *M. euphorbiae* is more adapted to drought stress condition and interspecific competition, the effects of the watering regimes on *M. euphorbiae* and *M. persicae* are presented in Figure 2.1 and summarised in Table 2.1.

Overall, nymph production was reduced significantly by drought and was significantly lower for *M. persicae* than *M. euphorbiae* (Fig. 2.1, Table 2.1). The interaction between aphid and water treatment showed a trend towards significance (Table 2.1) due to the differential responses of the two aphid species to drought: when feeding alone, *M. persicae* nymph production after 7 days of treatment was significantly reduced by drought, whereas *M. euphorbiae* nymph production was unaffected (Fig. 2.1, Table 2.1). This pattern was also seen when the two aphid species were feeding on the same plant (Fig. 2.1), but there was a tendency for nymph production by *M. euphorbiae* to be decreased by drought when feeding on the same plant as *M. persicae*, resulting in significantly lower nymph numbers compared with the well-watered treatment (Fig. 2.1). There was no significant effect of position on plant parts on the total number of nymphs (Table 2.1).

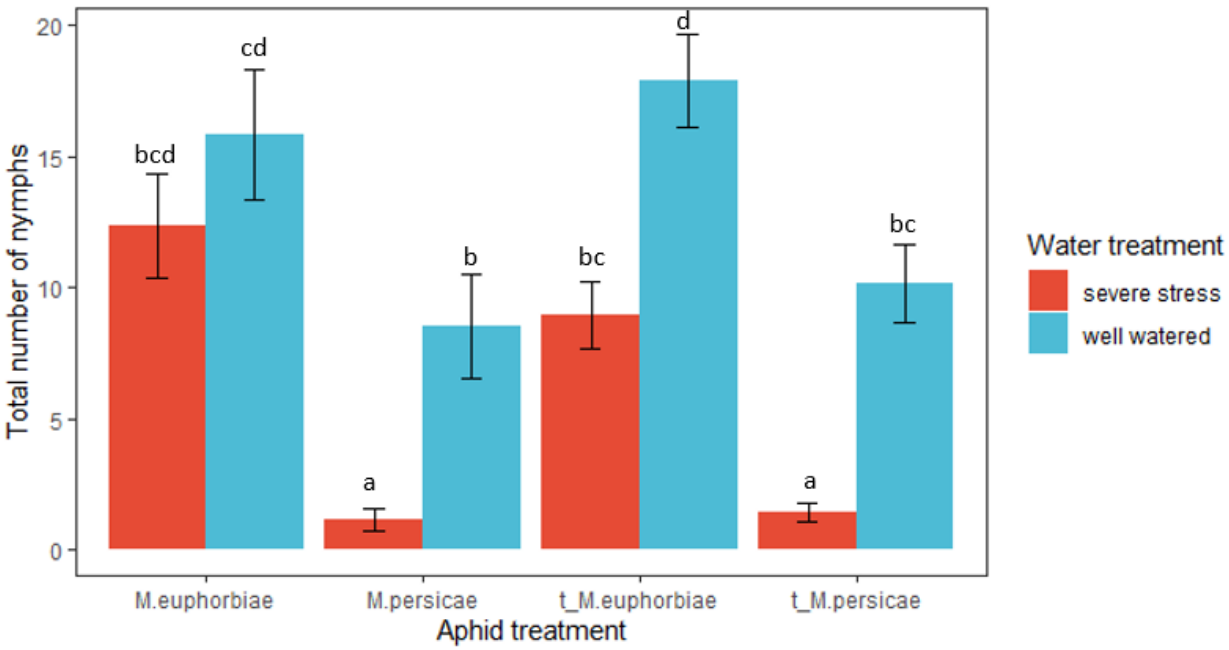

Figure 2.1 Total number of nymphs after 7 days of water and aphid treatments. The aphid treatments were 1) *M. euphorbiae* (total number of nymphs for *M.euphorbiae* alone), 2) *M.persicae* (total number of nymphs for *M.persicae* alone), 3) t_*M.euphorbiae* + *M persicae* (total number of nymphs of *M. euphorbiae* when *M. persicae* was present), 4) t_*M.persicae* + *M. euphorbiae* (total number of nymphs of *M. persicae* when *M. euphorbiae* was present). Values are means of 10 replicates. Letters indicate where values differ significantly at the 5% level (Tukey's HSD post-hoc test).

Table 2.1 Statistical results of total number of nymphs. Bold text indicates significant or near-significant p values.

Variable	**df**	***F***	***P***
Water treatment	1	61.11	**2.339e-13**
Aphid treatment	3	30.92	< **2.2e-16**
Plant part	2	0.52	0.59
Water treatment * Aphid treatment	3	3.91	**0.009**
Water treatment * Plant part	2	0.32	0.72
Aphid treatment* Plant part	6	1.13	0.34
Water treatment * Aphid treatment * Plant part	6	0.67	0.67
Residuals	216		

Nymph weight

The final weight of nymphs was significantly lower in the drought treatment compared with the well-watered treatment (d.f. = 1, p-value <0.001), and nymph weight was significantly lower for *M. persicae* compared with *M. euphorbiae* (Fig. 2.2). There was no interaction between the two treatments, indicating that nymph weight of each species was not differentially affected when aphids were feeding together compared with feeding alone (Fig.2.2).

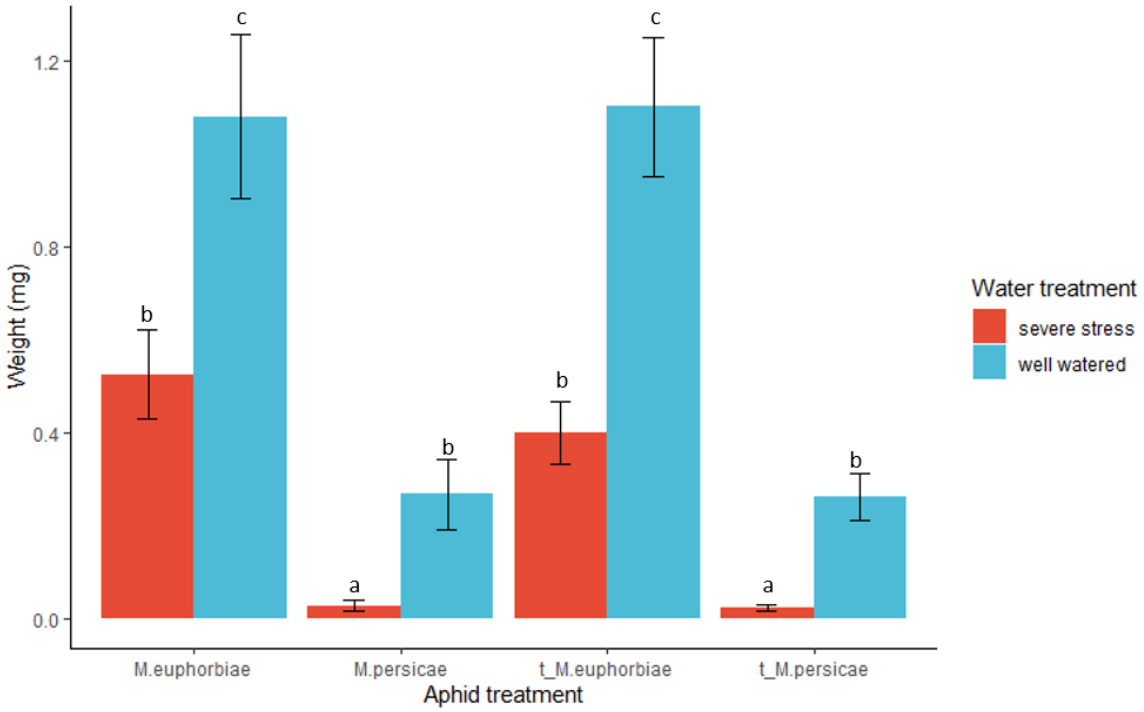

Figure 2.2 Weight of nymphs after 7 days of aphid and water treatments. The aphid treatments were 1) *M. euphorbiae* alone (nymph weight for *M. euphorbiae* alone), 2) *M. persicae* alone (nymph weight for *M .persicae* alone) , 3) t_*M.euphorbiae + M persicae* (nymph weight of *M.euphorbiae* when *M. persicae* was present), 4) t_*M. persicae + M. euphorbiae* (nymph weight of *M. persicae* when *M. euphorbiae* was present). Values are means of 10 replicates. Different letters indicate where values differ significantly at the 5% level (Tukey's HSD post-hoc test).

Table 2.2 Statistical results of nymph weight. Bold text indicates significant p values.

Variable	**df**	***F***	***P***
Water treatment	1	68.16	**1.491e-14**
Aphid treatment	3	51.46	**< 2.2e-16**
Plant part	2	0.48	0.61
Water treatment * Aphid treatment	3	0.58	0.62
Water treatment * Plant part	2	0.16	0.84
Aphid treatment* Plant part	6	1.19	0.31
Water treatment * Aphid treatment * Plant part	6	0.88	0.50
Residuals	216		

2.5 Discussion

In this chapter, the effects of drought stress on competition between aphid species were examined using two water treatments (a control treatment and water stress treatment) and two aphid species (*M. euphorbiae* and *M. persicae).* Aphids were carefully introduced to the plants and left for 7 days to settle. Next, aphid position on the plant, total number on nymphs and offspring weight was recorded. The key findings of this study were that drought stress affected nymph production in *M. persicae* to a greater extent than in *M. euphorbiae*, but that nymph production by *M. euphorbiae* was reduced by drought when it fed on the same plant as *M. persicae*. The presence of another aphid species did not influence within-plant aphid distribution. Finally, there was no interactive effect of aphid treatment and water treatment on nymph mass, although there was a trend towards significant interactive effects on nymph numbers, suggesting that *M. euphorbiae* nymph size might be differentially affected by the presence/absence of *M. persicae* under drought conditions.

Drought can shape dynamics within aphids sharing the same host

Drought affects potato plants in multiple ways, and it depends on plant development stage, cultivar, duration and intensity of stress (Pinheiro and Chaves, 2011). From a chemical and physiological perspective drought leads to stomatal closure, increased leaf sugar concentration, reduction in plant vigour and higher concentration of soluble nitrogen (Cornelissen et al., 2008; Inbar et al., 2001). It is generally accepted that drought can negatively influence the fitness of sap-feeding insects like aphids (Leybourne et al., 2021; Huberty & Denno, 2004), and previous studies have shown decreased nymph production under drought for *M. persicae* (An Nguyen et al., 2007) and *M. euphorbiae* (Simson et al., 2021) and other aphid species (Hale et al., 2013; Foote et al., 2017). I predicted that drought stress would have a greater negative impact on aphid fecundity and growth for *M. euphorbiae* than *M. persicae* because previous work showed better performance of *M. persicae* when in competition with other aphid species on stressed plants (e.g. Mezgebe and Azerefegne, 2021). By contrast, although both aphid species showed reduced fitness under drought in the pot experiment, *M. euphorbiae* was not affected to the same extent as *M. persicae*. It is possible that the higher sensitivity showed by *M. persicae* compared with *M. euphorbiae* was due to the particular genotypes of *M. persicae* and *M. euphorbiae* chosen for this study, since it has been reported that aphid-responses are dependent on genotype (Zytynska and Preziosi, 2013); a larger experiment involving several genotypes of each aphid species would confirm whether the species-specific aphid responses observed in this study are generally applicable. The difference in drought sensitivity of the two aphid

species might relate to aphid size and ability to osmoregulate: larger *M. euphorbiae* aphids can survive stressful feeding conditions for longer than smaller *M. persicae* (e.g. Alford et al., 2019). It is possible that a longer period of drought stress that was imposed in this study (10 d) would have more severe effects on *M. euphorbiae*, as observed during a 14 day treatment period in another study of *M. euphorbiae* (An Nguyen et al., 2007). Previous studies where competition amongst aphids under stress was studied indicated that generalist aphids (such as *M. persicae*) are highly affected by water stress compared with specialist aphid species (Mezgebe and Azerefegne, 2021); while *M. euphorbiae* is also considered a generalist species, its host range is smaller than *M. persicae* (Whittaker, 2015; CABI, 2021).

Coexistence between Macrosiphum euphorbiae and Myzus persicae on a shared host plant under drought stress

I postulated that the presence of *M. persicae* would negatively impact *M. euphorbiae* in terms of on-plant distribution since previous studies have shown that *M. persicae* reproduced more efficiently on *Solanum* compared with *M. euphorbiae* (Alvarez and Srinivasan, 2005) Surprisingly, the results highlighted that aphids were coexisting on the same plants without reporting any displacement of one species compared to another (i.e. 'plant part' was not a significant factor). Positional settling patterns were not observed in previous studies for *Macrosiphum euphorbiae*, whereas *Myzus persicae* has been shown to prefer the lower leaves of their host plants (Alvarez and Srinivasan, 2005; Srinivasan and Alvarez, 2011). Although this study did not track plant chemical and metabolomic profile, competition among sap-feeding insects can be induced through alteration of host quality, and this might vary between host plant and insect herbivore species, leading to species-specific changes in the secondary chemistry (Mooney et al., 2008). As this study examined only one generation of aphids, there is a possibility that the plant defence response was not induced sufficiently to create species-specific defensive responses and, therefore, there was no displacement of one aphid species by another. Another key factor that might have helped the coexistence between the two species was plant genotype. The study did not include different genotypes of potato, but it has been reported that plant genotype differences affect community structure in herbivores (Smith et al., 2008; Zytynska and Preziosi, 2013). More studies are required to further characterize possible induced changes in the secondary chemistry by competing herbivores under drought stress.

2.6 Conclusion

The research work has demonstrated that plant drought stress has a negative impact overall on aphid growth and reproduction, and that *M. euphorbiae* was less sensitive than *M. persicae*. These results, taken together, indicate that drought differently impacts aphid species that cohabit on the same plant host, which might have significant implications for community assembly of aphids sharing a common host plant. Further studies to examine aphid fitness responses in field conditions over several generations and using multiple aphid genotypes could reveal how periods of drought stress can impact pest community dynamics.

Chapter 3. Do arbuscular mycorrhizal fungi modulate plant tolerance of combined biotic/abiotic stresses?

Abstract

Plants are commonly exposed to a combination of different biotic and abiotic stresses in a complex multitrophic environment. The co-occurrence of different stresses and their interactive effects on plant fitness is not clearly understood. Moreover, plant-herbivore interactions are commonly studied in a two-way approach without the inclusion of the multitude of trophic interactions which occur naturally in the habitat. Arbuscular mycorrhizal (AM) fungi are plant root associated microorganisms that can impact plant fitness. AMF have been reported to increase plant tolerance to biotic and abiotic environmental stresses.

Here, we report on two pot experiments designed to investigate the impact of different AM fungal species on drought stress tolerance of commercial cultivars of potato. First, eco-physiological parameters and plant productivity were monitored in three cultivars of commercial potato (cv. Fontane, Innovator, Hermes) in response to three levels of water availability and two different AM fungal species. AMF colonisation of the roots was not detected, but Fontane showed the highest tolerance towards the most severe drought stress treatment showing the highest values of chlorophyll fluorescence, chlorophyll content, total number of leaves and dry root biomass compared to Innovator and Hermes. In a second experiment, Fontane was exposed to two levels of water stress (severe and ambient) and aphid herbivory (with and without secondary symbiont *Hamiltonella defensa*), in the presence or absence of one AMF species or a mixture of species. AM fungal presence (hyphae and vesicles) in the roots was observed at low colonisation frequency (<5% root length colonised). Water stress reduced shoot biomass and leaf stomatal conductance but did not affect aphid infestation. Neither AM fungi presence nor water availability affected insect performance. The results suggest the importance of plant genotype (*Solanum tuberosum* variety) on tolerance of water deficit stress and indicates that AM fungi do not have predictable effects on the outcome of plant-insect herbivore interactions under abiotic stress.

Key words*:* aphid, arbuscular mycorrhizal symbiosis, drought stress, facultative symbiosis, *Solanum tuberosum*

3. 1 Introduction

Environmental stress negatively affects plant growth and development. Drought stress is one of the most severe constraints to crop productivity (Seleiman et al., 2021) resulting in lower yield (Bhargava and Sawant, 2013). In *Solanum tuberosum,* drought seriously affects tuberization and therefore quality and number of potato tubers (Obidiegwu et al., 2015). The shallow root system limits water extraction from soil which leads potato plants to be extremely sensitive to drought stress (Gong et al., 2015). However, molecular mechanisms involved water use are not well understood, and improved knowledge could enable breeding of new varieties with drought resistance. In addition to abiotic environmental factors, crop plants often are exposed to biotic challenges such as herbivorous insect pests (Mittler, 2006).

The potato aphid, *Macrosiphum euphorbiae* (Thomas), is found throughout the world (Pompon et al., 2010). It is an economic pest of important herbaceous crops, particularly on potato plants (Le Roux et al., 2007). Maternally inherited bacterial symbionts are widespread within herbivorous insect pest populations (Wagner et al., 2015), particularly in aphids (Goggin, 2007). *Hamiltonella defensa,* is a secondary endosymbiont found commonly infecting Hemiptera (Oliver et al., 2005). In the pea aphid (*Acyrthosiphon pisum*), *H. defensa* provides resistance against attack from parasitic wasps (Donald et al., 2016). Several studies have described their effects on aphid fitness, reporting how the presence of endosymbionts result in changes of aphid probing (Leybourne et al., 2020b), suppression of jasmonic acid (JA) related anti-herbivore-induced defences (Schausberger, 2018), and plant volatile emissions (Frago et al., 2017). However, no attempts have been made to elucidate how the presence of *H.defensa* could alter aphid-plant interactions under drought stress.

Arbuscular mycorrhizal fungi (AMF) occur in almost all terrestrial ecosystems forming symbiotic beneficial associations with most land plants (van der Heijden et al., 2015, 1998). The symbiosis plays a key role in ecosystem nutrient cycling and in plant defence against environmental stresses, including drought and salinity (Pivonia et al., 2008). Potential mechanisms underlying AMF-associated increases in drought tolerance include: 1) change in stomatal regulation, which may be influenced by abscisic acid (ABA) levels in the xylem; 2) control of turgor level even at low tissue water potential and changes in cell wall elasticity (Davies et al., 2002); and 3) improved plant water status by increased hydraulic conductivity

through a greater capacity for water absorption by the external hyphae (Augé et al., 2007). In addition, AMF-colonised plants recover from drought stress faster than non-mycorrhizal plants (Al-Karaki and Clark, 1998). Although several studies show clear effectiveness of AMF in improving plant resistance to drought, in the last year a growing number of studies have also reported their unpredictability (Salomon et al., 2022). Presence of AMF has also been shown to affect aphids feeding on the same plant (Wang et al., 2020). The impacts on aphids, however, can range from negative to positive (Koricheva et al., 2009). In turn, aphids may counterattack through top-down effects on AMF via the host plant (Wilkinson et al., 2019). Although the outcome of AMF symbiosis on plant interactions with aphids have been already reported (Babikova et al., 2014; Volpe et al., 2018) scarce information is available on these interactions under abiotic stress condition such as water stress in *Solanum tuberosum*. Moreover, little attention has been given to the presence or absence of aphid secondary endosymbionts, such as *Hamiltonella defensa,* in aphid-plant interactions and how this could affect or modulate physiological responses of the plant when exposed to stress.

Here a multidisciplinary approach has been used involving ecophysiological and morphometric analyses. The present study was conducted to explore the effect of AM fungal colonisation on plant physiological parameters. Using the potato aphid, *Macrosiphum euphorbiae*, feeding on *Solanum tuberosum* and exposed to different levels of water availability, two experiments have been designed and performed to test firstly, the hypothesis that AM fungal colonisation leads to microbe-induced benefits for plant performance under abiotic (drought) and biotic (insect herbivore) stress. Secondly, to evaluate potato aphid performance in the presence and absence of an aphid facultative bacterial endosymbiont (*Hamiltonella defensa*) when exposed to plants treated with AMF or drought or their combination. Specifically, four questions were asked: 1) Does the presence of AMF affect the tolerance to drought stress in potato?; 2) Do three *Solanum tuberosum* cutivars respond similarly to drought stress?; 3) Do two different AMF modulate plant tolerance when two stresses (abiotic (water) and biotic (aphid infestation)) are combined? 4) Can AMF alter *M. euphorbia*e fitness and nymph density when the facultative endosymbiont (*Hamiltonella defensa*) is present?

3.2Materials and Methods

Experiment 1

In October 2018, tubers of *Solanum tuberosum* cv. Fontane, Innovator and Hermes, were cut into similar size portions and surface-sterilised by soaking in 0.1% (v/v) sodium hypochlorite for 1 min then rinsed using tap water. The tubers were sown in 2 L pots containing sterile soil 2:1 (sand: loam) (Keith Singleton Steam Sterilized Loam, Clydesdale Trading, Lanark, UK) mixture that had been autoclaved twice at 121 °C for 4 h, with an interim overnight cooling period. Plants were grown under controlled glasshouse conditions at 23°C/14°C (day/night), 16/8 h light/dark photoperiod. The three cultivars were exposed to three water treatments (control, moderate and severe stress) and three AM fungal inocula treatments (inoculation with one of two AM fungal species and a no-AMF control). The experiment was set up as 3x3x3 randomised block design with 10 replicate blocks, giving a total of 270 plants (Fig. 3.1).

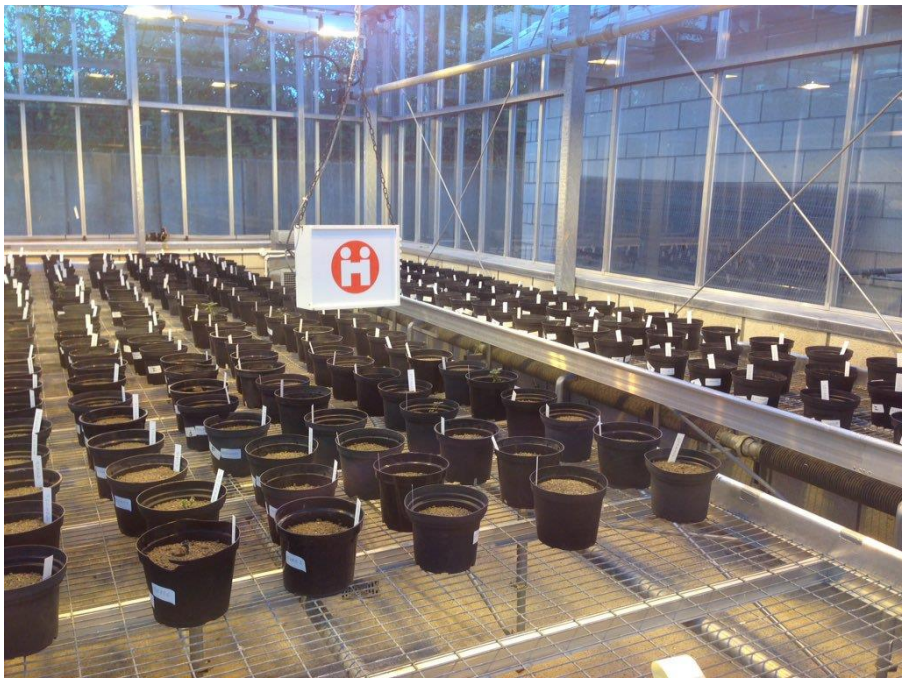

Figure 3.1 Performance assay used for experiment 1 in a glasshouse cubicle at the James Hutton Institute

For each experimental block of plants, AMF treatments were applied as follows. AM fungal spores were extracted from the supplied inoculum by wet sieving and sucrose density centrifugation (Daniels & Skipper, 1982). Two different arbuscular mycorrhizae species were used: *Rhizophagus irregularis* [Ri] and *Funneliformi mosseae* [Fm] respectively QS-81 (2017) and D54 (2017) (INOQ GmbH-Mykorrhiza für alle Pflazen). A microbial wash was prepared by vacuum filtering 4 mL of the AM fungal inoculum through Whatman filter paper grade 1 in order to exclude fungal spores and hyphae. Half of the volume of the spore solution or the microbial wash was sterilized by autoclaving. Each pot received both 1 mL of live or sterile AM fungal spore inocula and microbial wash. The live inoculum was composed of live AM fungal spore solution and autoclaved microbial wash. The abundance of the spore was checked and counted in three 1 mL samples of the live spore solution. Following inoculation, the plants were watered twice a week with tap water and once a week with a modified Hoagland nutrient solution (1 mmol/L KNO_3 and 0.5 mmol/L NH_4NO_3). After *c.* 7 weeks of growth, when AM fungal colonisation should have established, water availability treatments were imposed. These comprised a control (no stress, 480 mL water per pot twice per week), moderate stress [Ms] (330 mL per pot twice per week) and a severe stress [Ss] (270 mL per pot twice per week). Plants were monitored for a further 6 weeks. Every 14 days the following growth parameters were measured on each plant: chlorophyll content of the most recently expanded leaf with a portable chlorophyll meter (SPAD-502, Minolta, Japan), number of leaves, length of the main stem, soil moisture recorded with (ML3 ThetaProbe, Delta T Devices, Burwell, UK) and chlorophyll fluorescence parameters of the most recently expanded leaf using a pulse-modulated fluorimeter (FMS-2, Hansatech Instruments Ltd., Norfolk, UK). For the latter measurement, leaves were dark-adapted for 20 min prior to generating light response curves. Leaf relative water content (RWC) was measured twice during the experiments, after 13 days of stress and after 6 weeks. For each time point, plants from five randomly selected blocks were used. The most recently expanded leaf was excised at the base of lamina, and leaves were immediately sealed within plastic bags and quickly transferred to the laboratory. Fresh weights were determined within 2 h after excision. Turgid weights were obtained after soaking leaves in water in petri dishes for 24 hours at 4 ^{o}C in the dark. After soaking, leaves were carefully blotted dry with tissue paper and weighed to determine turgid weight. Dry weights were obtained after oven drying the leaf samples for 72 h at 70°C. Leaf RWC was calculated using the equation of Schonfeld et al. (1988):

$$RWC\ (\%) = (FW - DW) / (TW - DW) \times 100$$

Where, FW = Fresh weight DW = Dry weight TW = Turgid weight.

At the end of the experiment, belowground parts of the plant were washed free of soil and separated into roots, stolons and tubers. All plant fractions were dried at 70 °C and weighed. Samples of dry roots were taken and assessment of roots colonization by AM fungi determined. To verify AM fungal colonisation, root subsamples were boiled in 3% KOH for 15 minutes, rinsed in tap water for 5 minutes and immersed in 2% HCl for 30 minutes. Finally they were boiled in 1:1:1 Lactic acid:water:glycerol with 0.05% (w/v) Trypan blue for 20 minutes. Roots were rinsed in water for a further 5 minutes before being placed in destain solution (10:9:1 glycerol:water:HCl (1%)) at 5˚ C for 2 days. Root samples were then placed onto slides and 100 root fragments were scored for presence of AM fungal hyphae, arbuscules, vesicles, spores and non-AM fungi (McConigle et al., 1990).

Experiment 2

For this experiment, in May 2019 tubers of *Solanum tuberosum* cv. Fontane were used. Tubers were cut and sterilized (see above). In June 2019, four weeks after sowing, plants were transplanted into 1 L pots containing sterilised soil mixed with sand (1:1 sand:loam; Keith Singleton Steam Sterilized Loam, Clydesdale Trading, Lanark, UK) that had been autoclaved twice at 121 °C for 4 h, with an interim overnight cooling period. The experiment was set up as 3x2x3 randomised block design with three levels of AMF treatment (no-AMF control or one of two AMF inocula), one of two water treatments (unstressed control or water stress), one of three aphid treatments (no aphid control, or one of two potato aphid types) and 6 replicates, giving a total of 108 plants.

For each experimental block of plants, AM fungal spores were extracted from the supplier's inoculum by wet sieving and sucrose density centrifugation (Daniels & Skipper, 1982). Two different arbuscular mycorrhizal inocula were used: *Rhizophagus irregularis* (00101SP, Symplanta GmbH & Co. KG, Germany) and an uncharacterized mix of arbuscular mycorrhiza fungi, rootgrow-™ Professional (RGPro) (PlantWorks Limited, Kent Science Park, UK). A microbial wash was prepared, and sterile AM fungal spore and live AM fungal spore preparations were made and inoculated into each pot (see above). Plants were grown under controlled glasshouse conditions at 23 °C/ 14 °C (day/night) and 16/8 h light/dark, and were watered two or three times weekly with tap water. *Macrosiphum euphorbiae* (Thomas), lines DM18/13 and MW16/48 were reared on excised *S. tuberosum* (cv. Desiree) leaves in ventilated

cups and maintained at 18 ± 20 C and 16h:8h (day:night). Prior to experimentation, lines were genotyped and characterised for the presence of the facultative endosymbiont *Hamiltonella defensa* (Moran et al., 2005). After 5 weeks, in July 2019, when AMF colonisation should have been established, the water treatments were initiated: drought stress was applied by withholding water from plants for a total of 14 days, while the control plants continued to receive water 2-3 times per week. After one week of drought stress, four adults of *M. euphorbiae* genotype 2 infected with *Hamiltonella defensa* (DM18/13) and genotype 2 without *H.defensa* infection (MW16/48), were transferred with a soft brush onto the underside of the most recently expanded leaf, using a clip cage which confined the aphids to a 15 mm diameter area of leaf and allowed them to probe and feed. Plant measurements were taken every three days during the experimental period: chlorophyll content of the most recently expanded leaf using a portable chlorophyll meter (SPAD-502, Minolta, Japan), number of leaves per plant, length of the main stem, and leaf stomatal conductance of the most recently expanded leaf using a diffusion porometer (Model AP4, Delta-T Devices, Burwell,Cambridge, UK), and soil moisture was recorded (using the ML3 ThetaProbe, Delta T Devices, Burwell, UK). After one week of aphid infestation, aphids were removed and the total numbers of adults (with and without wings) and nymphs per plant were recorded. Subsequently aphids were freeze-dried, and dry weight was determined. Prior to plant harvest, the apical most fully expanded leaf was sampled to determine leaf relative water content following the method of Schonfeld et al. (1988) (described above).

After 14 days of drought stress plants were harvested, and shoots were separated into stem and leaf fractions. Belowground parts were washed free of soil and separated into roots, stolons and tubers. All plant fractions were dried at 70 °C and weighed. Samples of dry roots were taken and root colonization by AM fungi was assessed according to the method of McConigle et al., 1990 (described above). Phosphorus concentration in leaves was determined using 50 mg samples of dried milled leaf material. Samples were acid digested as described by (Subramanian and Arts, 2011) and concentrations of P in diluted digests were determined spectrophotometrically by reaction with malachite green (Irving and MacLaughlin, 1990). Absorbance values were converted to amounts of P using a calibration curve and expressed per unit of leaf dry mass.

3.3 Data and statistical analysis

All statistical analyses were carried out using R v4.1.3, with additional packages ggplot2 v.2.2.1 (Wickham, 2009), dplyr v. 3.5.3 (Mailund, 2019) , lubridate v. 1.7.8, (Spinu et al., 2016), car v. 3.0-7 (Fox and Weisberg, 2011), lsmeans v.2.27-62 (Lenth, 2016) and multcomp v. 1.4-8 (Hothorn, 2008). Data were checked for normality and heteroscedasticity and log-transformed [i.e. log10(x+1)] where appropriate. Root colonisation data (% root colonised with vesicles) was square root transformed. Three-way ANOVA was used to evaluate the effect of different factors, and differences between group were assessed by Tukey's post-hoc test.

3.4 Results

Experiment 1

Arbuscular mycorrhizal colonisation

No arbuscular colonization of the roots was detected in the two AMF treatments, *Rhizophagus irregularis* and *Funneliformi mosseae*. No colonization was found in the control plants.

Biometric parameters and ecophysiology

Soil moisture

Soil moisture content varied significantly between water treatments and was highest in the control water treatment and lowest in the severe water stress treatment (Fig. 3.2). Soil moisture content also varied significantly between cultivars and was higher for Innovator than for Hermes and Fontane in all three treatments (Fig. 3.2, Table 3.1). There were not significant interactions between cultivar and water treatment (Table 3.1).

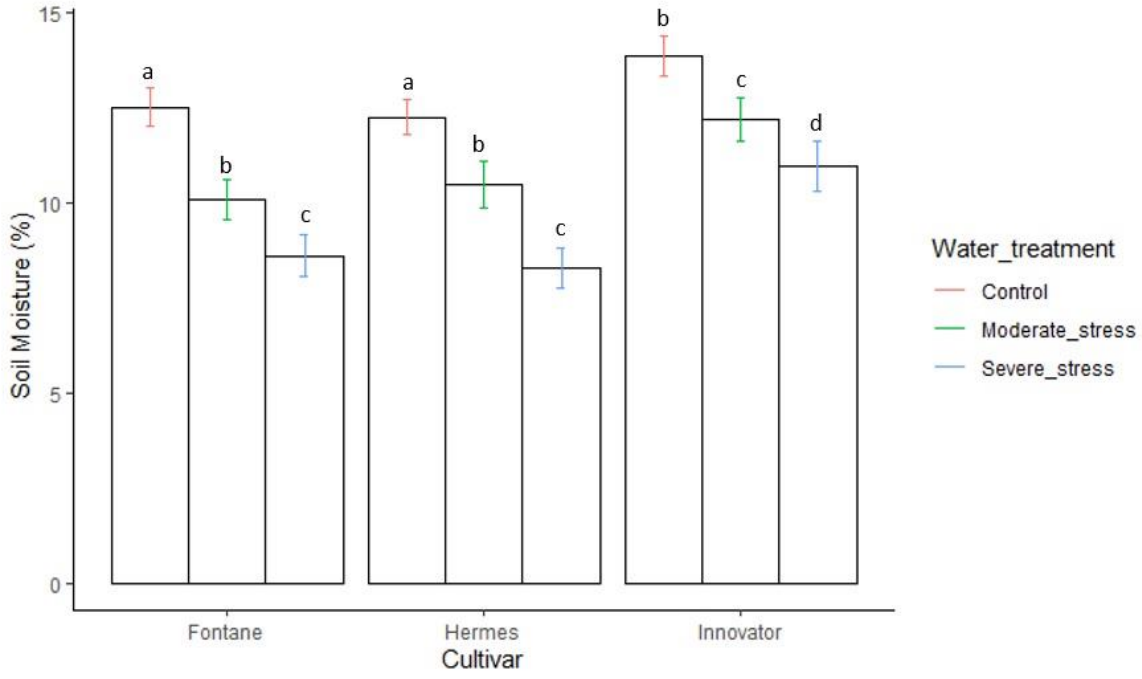

Figure 3.2 Soil Moisture Content for the three potato cultivars in response to different water treatments. Values are means (±s.e..m) of 27 replicate plants. Different letters indicate significant differences ($p < 0.05$) between treatments after three-way ANOVA followed by Tukey's post hoc test

Leaf Chlorophyll fluorescence

Leaf chlorophyll fluorescence measured as F_v/F_m varied significantly among the three cultivars, with lowest values for Hermes (Fig. 3.3, Table 3.1). Significant differences were observed between water treatments (Table 3.1). Under well-watered conditions, the mean value of F_v/F_m was higher compared with the moderate and the severe stress, and the value reduced with more severe stress for all three cultivars (Fig.3.3).

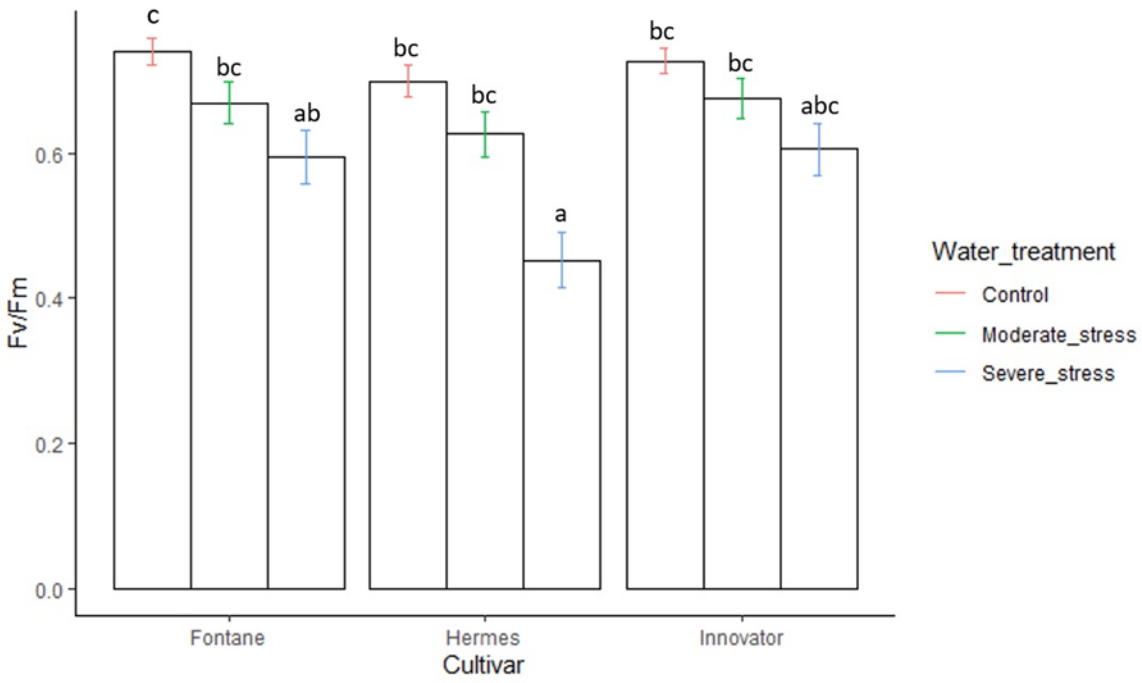

Figure 3.3 Leaf Chlorophyll fluorescence (F_v/F_m) measured during water treatments in the three different potato cultivars. Values are means (±s.e..m) of 27 replicates. Different letters indicate significant differences ($p < 0.05$) between treatment after three-way ANOVA followed by Tukey's post hoc test.

Leaf Chlorophyll Content Index (CCI)

Leaf chlorophyll content CCI measurements provide an index of leaf chlorophyll concentration. The three cultivars showed differences in CCI, with lowest values in Hermes (Fig. 3.4). Water treatment did not affect CCI values.

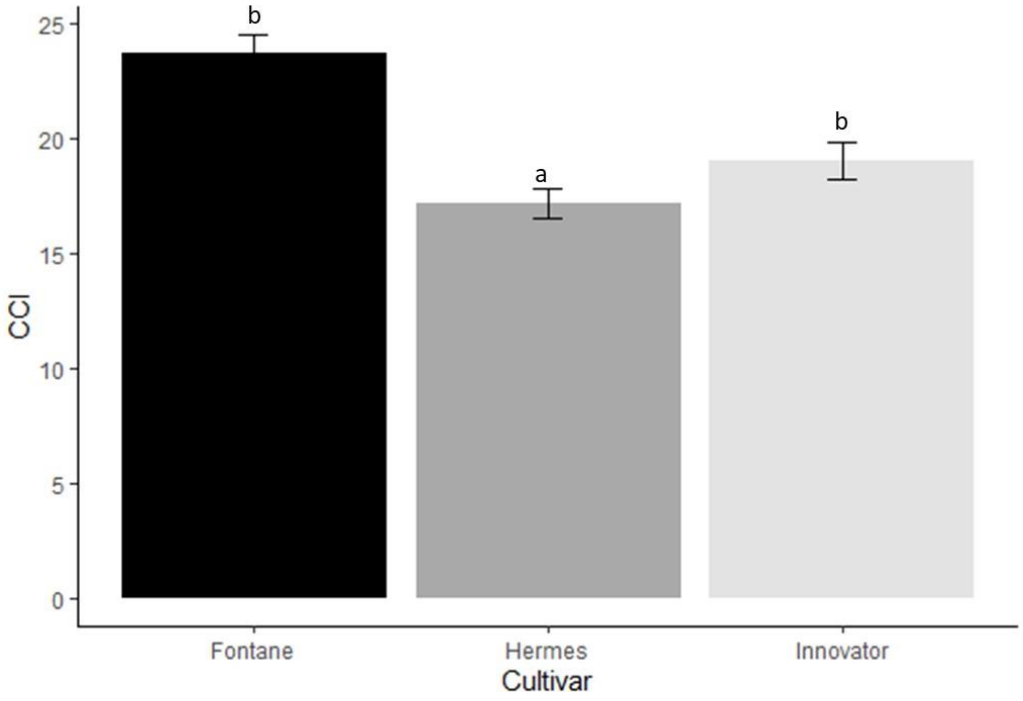

Figure 3.4 Chlorophyll content index (CCI) for leaves of potato cultivars Fontane, Hermes, Innovator. Values are means (±s.e..m) of 27 replicate plants. Different letters indicate significant differences ($p < 0.05$) between treatment after three-way ANOVA followed by Tukey's post hoc test

Leaf Relative Water Content (RWC)

At the first time point, 25 days after the beginning of the water stress treatments, there were no significant differences in RWC between cultivars or between water stress levels. Hermes tended to maintain higher leaf RWC values than Fontane and Innovator in the severe stress treatment while Innovator tended to maintain the highest values in the moderate stress treatment (Table 3.1). At the second time point, after 5 weeks (35 d) of water stress treatment, a different pattern was observed for RWC. The relative water content varied significantly between cultivars, Table 3.1, full statistical output is displayed in Table 3.2.

Table 3.1 Statistical summaries of three-way ANOVA for the physiological variables quantified in the experiment. Significant differences are highlighted in bold.

Variable	**F_v / F_m**			**Chlorophyll content (CCI)**			**Soil Moisture (%)**			**Relative water content at 25 days[a]**			**Relative water content at 35 days [a]**		
	df	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**
Block	9	2.28		9	0.98		9	6.99		4	1.74		4	4.40	
Cultivar	2	3.14	**0.046**	2	27.10	**<.001**	2	19.13	**<.001**	2	0.74	0.480	2	2.99	**0.001**
Water treatment	2	21.73	**<.001**	2	0.26	0.774	2	44.71	**<.001**	2	0.11	0.892	2	0.37	0.9696
AMF	2	1.33	0.266	2	0.65	0.522	2	0.07	0.937	2	0.49	0.614	2	3.20	0.5436
Cultivar*Water treatment	4	0.42	0.793	4	0.35	0.844	4	0.65	0.629	4	1.29	0.280	4	4.03	0.9703
Water treatment* AMF	4	1.17	0.327	4	0.09	0.986	4	1.36	0.248	4	1.44	0.227	4	3.89	0.7968
Cultivar*AMF	4	0.13	0.973	4	0.50	0.732	4	1.34	0.258	4	0.63	0.639	4	2.25	0.5794
Cultivar*AMF*Water treatment	8	1.36	0.217	8	0.76	0.636	8	0.88	0.531	8	1.09	0.378	8	12.81	0.792

[a] Relative water content measure at 25 days and 35 days of water treatment

Table 3.2 Table showing the mean and standard error for the RWC at 35 days for each cultivar and water treatment.

Cultivar	Water treatment	Mean	Standard Error
Fontane	Control	34.606	5.49
Hermes	Control	12.975	6.08
Innovator	Control	10.054	4.96
Fontane	Moderate stress	26.919	5.55
Hermes	Moderate stress	17.263	5.69
Innovator	Moderate stress	15.336	5.95
Fontane	Severe stress	28.951	3.99
Hermes	Severe stress	12.714	5.81
Innovator	Severe stress	12.671	5.08

Total number of leaves

Leaf number per plant throughout the experimental period varied significantly between cultivars, mainly due to low values for Innovator (9 leaves per plant, on average) compared with Fontane and Hermes, which did not differ (Fig. 3.5). Plants grown under the control water treatment had generally higher numbers of leaves.

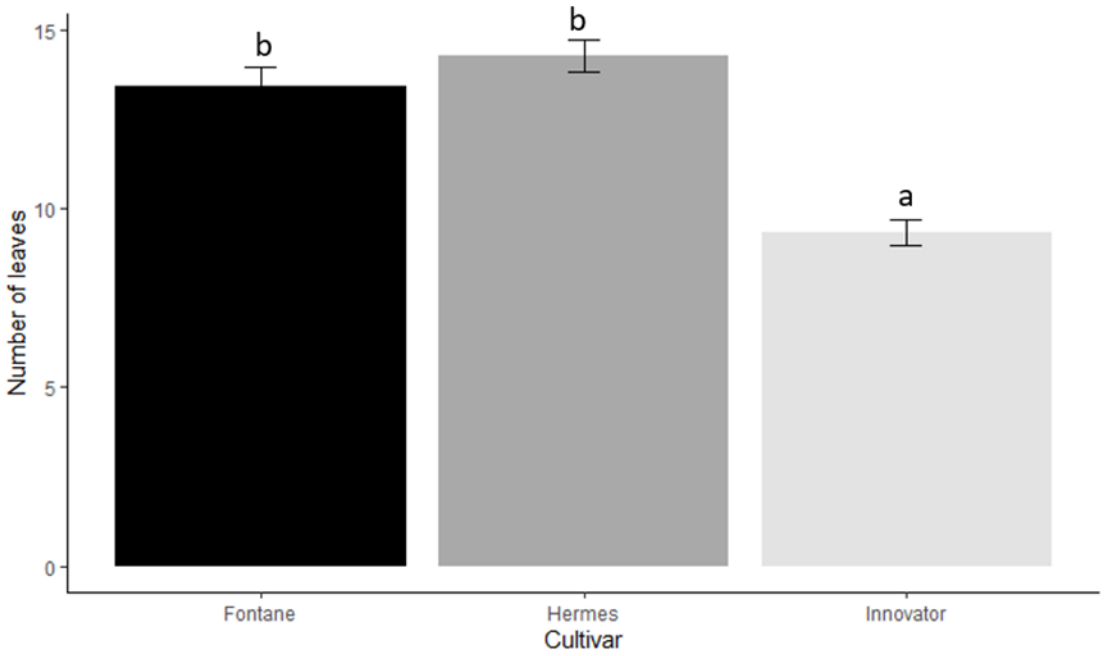

Figure 3.5 Total leaf number per plant for three potato cultivars under three levels of water stress. Values are means (+s.e..m) of 27 replicate plants. Different letters indicate significant differences ($p < 0.05$) between treatments after three-way ANOVA followed by Tukey's post hoc test.

Plant mass

Root mass, fresh and dry, varied significantly between cultivars, and root dry mass also varied significantly between water and AMF treatments (Table 3.3). Hermes developed the largest dry root biomass (0.472 g/plant), whilst Innovator had lowest values of root dry biomass (0.210 g/plant) (Fig. 3.6). In the AMF treatment, root dry mass was higher than in the uninoculated control (data not shown). The significant effect of water treatment was largely driven by much larger dry root mass in Hermes in the control treatment compared with the severe stress treatment (Fig. 3.6), leading to a significant interaction between cultivar and water treatment (Table 3.3). Tuber fresh and dry weight also decreased with increasing water stress intensity (data not shown; Table 3.3).

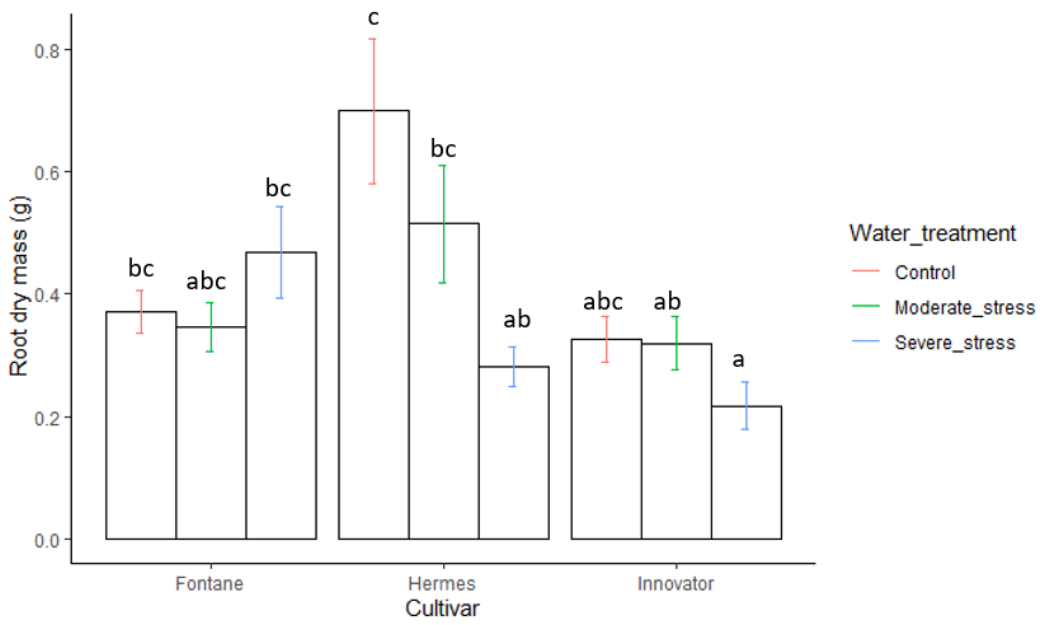

Figure 3.6 Root dry mass of three cultivars of potato in response to three water treatments. Values are means (±s.e..m) of 27 replicate plants. Different letters indicate significant differences ($p < 0.05$) between treatment after three-way ANOVA followed by Tukey's post hoc test.

Table 3.3. Statistical summaries of three-way ANOVA for plant productivity measurements. Significant results are highlighted in bold.

Variable	**Number of leaves**			**Number of stems**			**Fresh root mass (g)**			**Dry root mass (g)**			**Fresh Tuber mass (g)**			**Dry Tuber mass (g)**		
	df	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**
Block	9	15.46		9	1.58		9	3.15		9	2.50		9	1.09		9	5.19	
Cultivar	2	29.87	**<.001**	2	0.37	0.692	2	13.96	**<.001**	2	7.41	**<.001**	2	1.49	0.228	2	2.54	0.081
Water treatment	2	2.49	0.085	2	0.37	0.693	2	2.33	0.100	2	3.54	**0.031**	2	4.75	**0.010**	2	5.0	**0.008**
AMF	2	1.47	0.233	2	2.52	0.083	2	1.62	0.201	2	3.74	**0.025**	2	1.12	0.329	2	2.16	0.118
Cultivar*Water treatment	4	0.91	0.456	4	0.88	0.479	4	1.14	0.339	4	4.88	**<.001**	4	0.10	0.981	4	0.09	0.985
Water treatment* AMF	4	0.74	0.565	4	0.71	0.587	4	0.28	0.890	4	1.34	0.256	4	1.54	0.193	4	1.37	0.245
Cultivar*AMF	4	1.04	0.388	4	0.85	0.497	4	4.51	0.002	4	3.23	0.014	4	2.04	0.090	4	1.34	0.257
Cultivar*AMF*Water treatment	8	0.69	0.696	8	1.52	0.151	8	2.14	0.034	8	0.80	0.603	8	0.69	0.700	8	0.73	0.669

Experiment 2

Arbuscular mycorrhizal colonization

A low level of arbuscular mycorrhizal infection was detected in the roots of AMF treated plants of < 5 % on average. Overall, for the species *Rhizophagus irregularis* (R), root colonisation levels by hyphae were 3.3% on average compared with 2% colonisation for the PlantWorks (PW) mixture (Fig.3.7). Similarly, low levels were recorded for vesicles (Ri 1.3 %, PW 0.3 %; Fig. 3.8). No AMF colonization was found in the roots of control plants. There was a significant interaction effect between AMF, endosymbionts, and water treatments (Fig.3.9; Fig 3.10; Table 3.4). However, due to low colonisation level of AMF in the roots it was not possible to determine if the interactions were biologically significant.

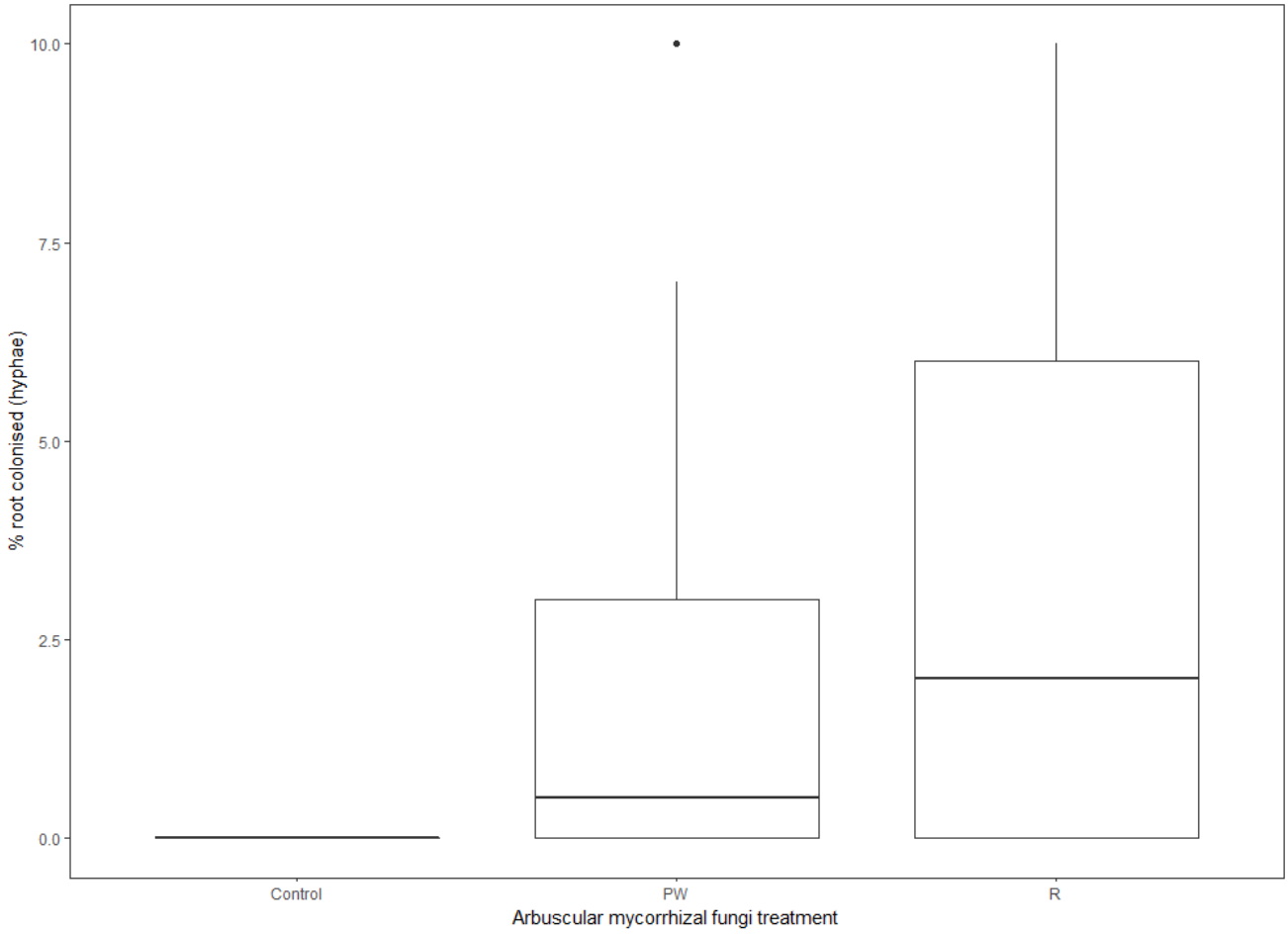

Figure 3.7 Boxplots comparing root colonisation by hyphae of arbuscular mycorrhizal fungi (%root colonised) in control and AMF treatments. Treatments: Control: Control, PW: PlantWorks, R: *Rhizophagus irregularis*.

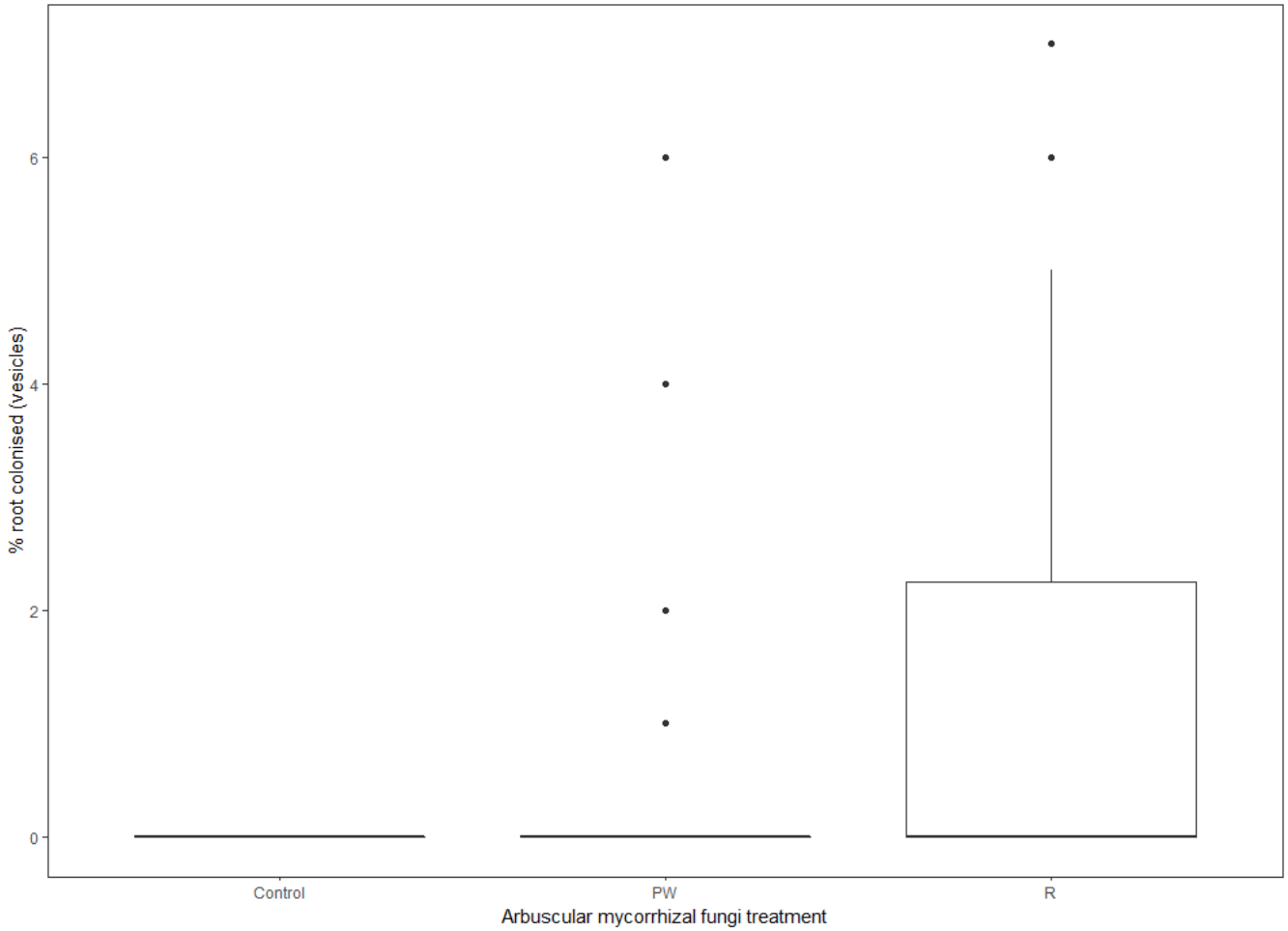

Figure 3.8 Boxplot comparing root colonisation by AMF vesicles in two AMF treatments compared with untreated control potato plants. Treatments: Control: Control, PW: PlantWorks, R: *Rhizophagus irregularis*.

Table 3.4. Summary statistics for percentage of roots colonised by AMF vesicles and hyphae Significant differences ($p<0.05$) are highlighted in bold.

Variable	**Vesicle (%root)**			**Hyphae (%root)**		
	df	**F**	**p**	**df**	**F**	**p**
Block	5	1.23		5	0.64	
Water treatment	1	2.17	0.144	1	0.14	0.705
Endosymbiont presence	2	2.92	0.059	2	1.22	0.300
AMF product	2	13.84	**<.001**	2	19.75	**<.001**
Water treatment* Endosymbiont presence	2	1.44	0.242	2	0.66	0.519
Water treatment* AMF product	2	0.72	0.490	2	0.34	0.716
Endosymbiont presence*AMF product	4	4.35	**0.003**	4	4.05	**0.005**
Water treatment* Endosymbiont presence* AMF product	4	2.53	**0.046**	4	0.31	0.871

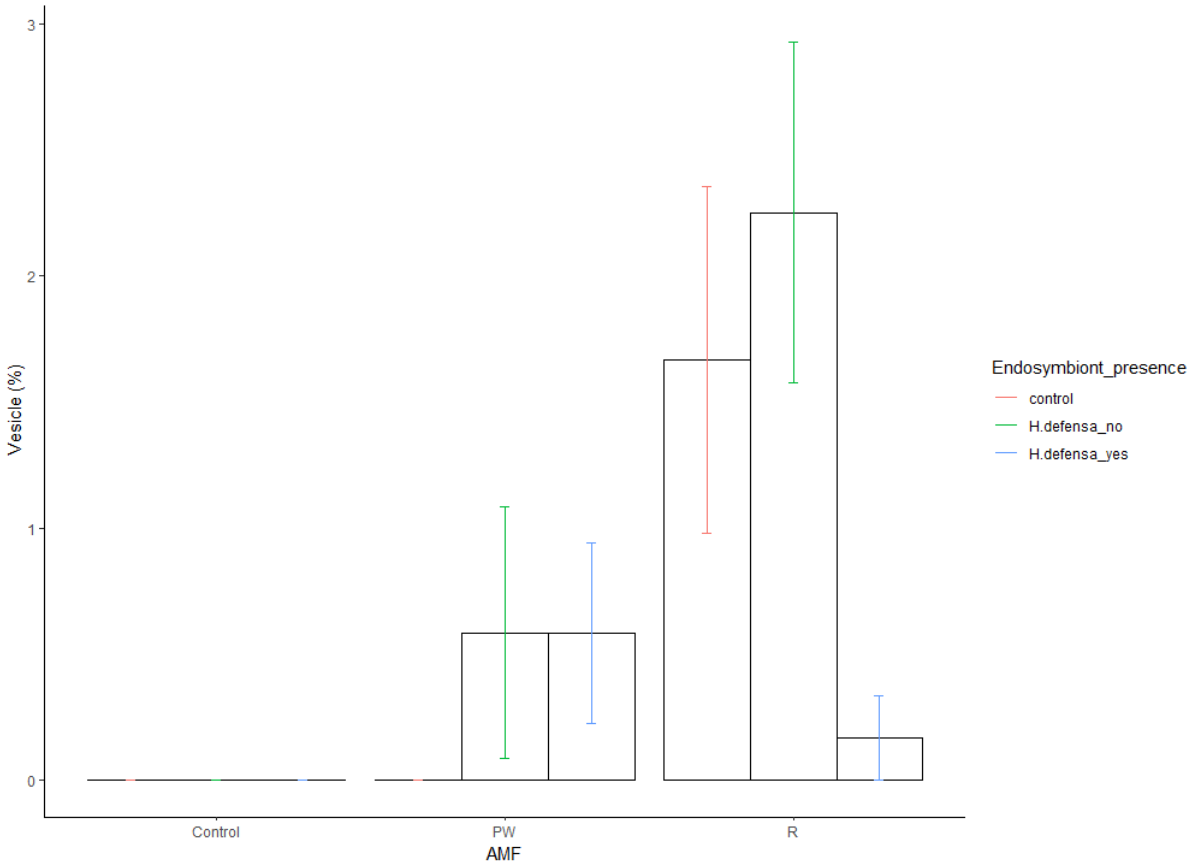

Figure 3.9 Vesicle presence (% root colonised) in response to different AMF treatments: (Control: Control, PW: PlantWorks, R: *Rhizophagus irregularis*) and endosymbiont presence *(H. defensa no* = absence; *H. defensa yes* = presence, control = no aphids).

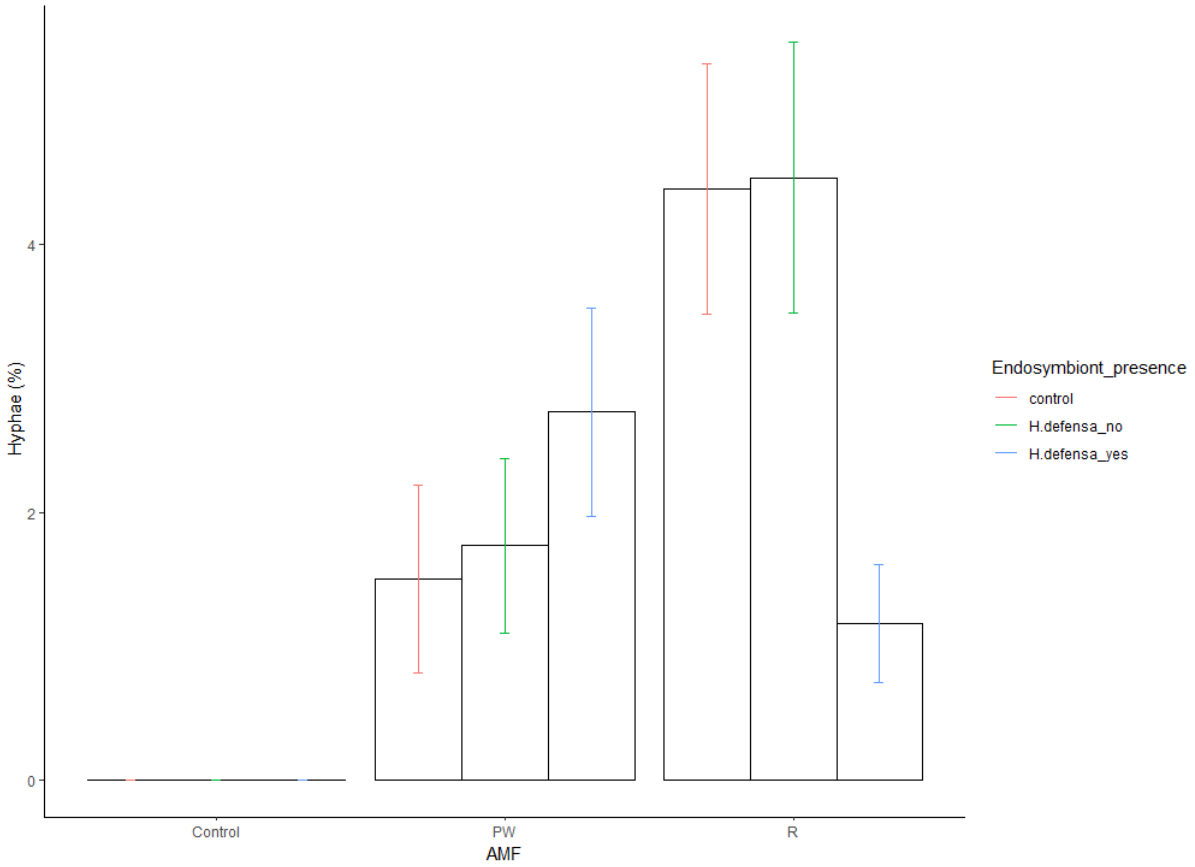

Figure 3.10 Hyphae presence (%root colonised) in response to different AMF treatments: (Control: Control, PW: PlantWorks, R: *Rhizophagus irregularis*) and endosymbionts presence *(H. defensa no* = absence; *H. defensa yes* = presence, control = no aphids).

Stomatal conductance

The lack of water had a significant effect on stomatal conductance (g_s), which was lower in the water stress treatment. Well-watered plants showed rapid increased in stomatal conductance in the first week of treatment followed by a general decrease in conductance (Fig. 3.11; Fig. 3.12). Interestingly, water treatment and its interaction with endosymbiont presence and AMF treatment showed significant effects on stomatal conductance (Table 3.5).

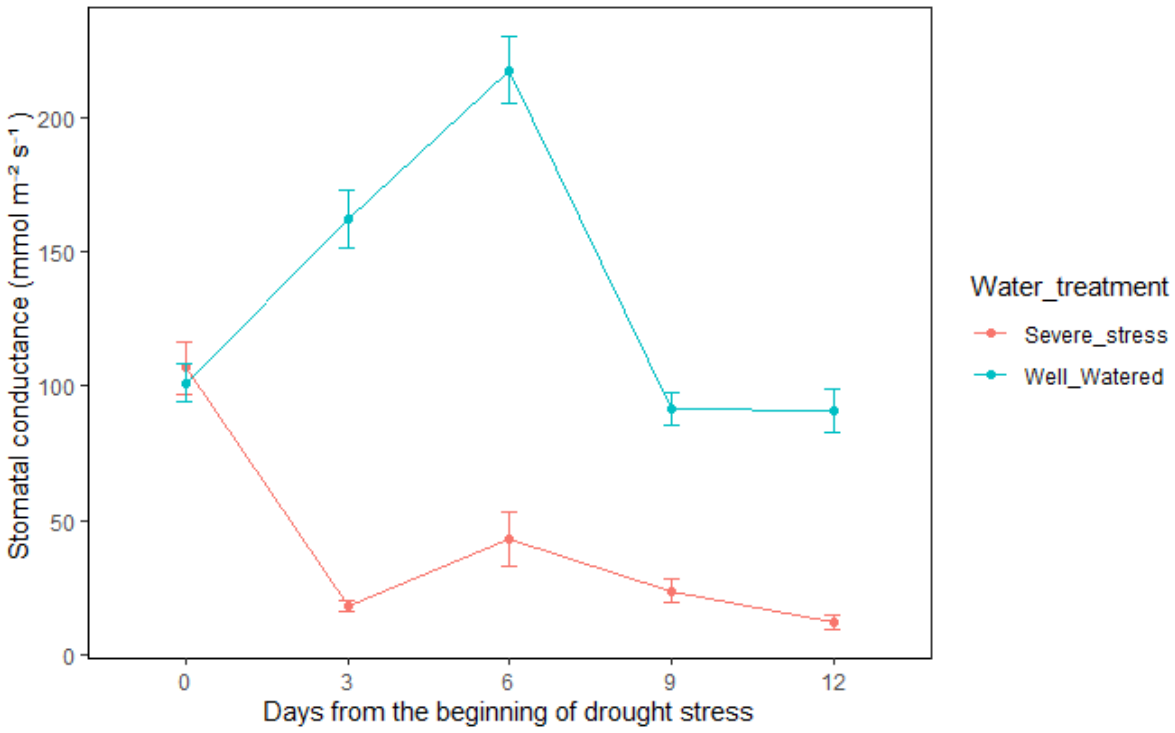

Figure 3.11 Stomatal conductance (mmol m^{-2} s^{-1}) in water stressed potato plants compared with well-watered control plants during a 12 day treatment period.

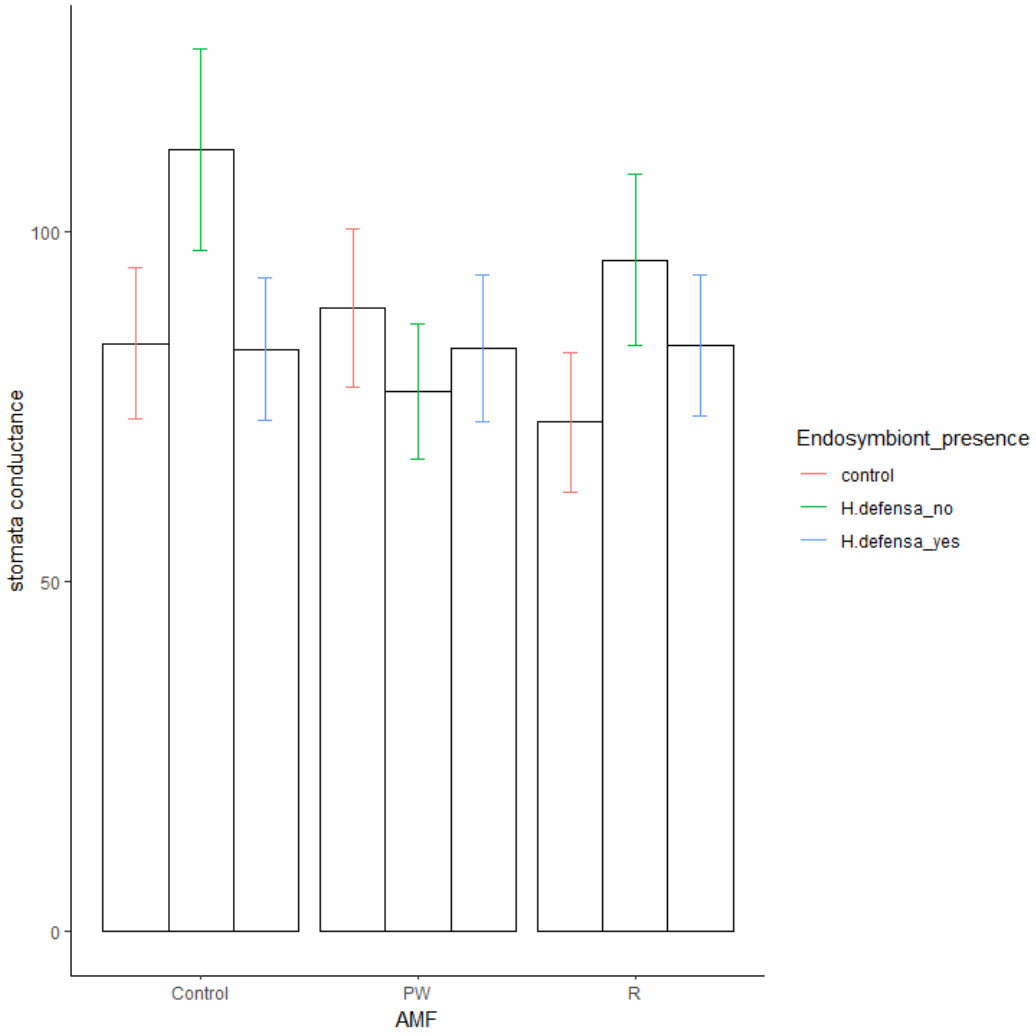

Figure 3.12 Stomatal conductance (mmol m^{-2} s^{-1}) in response to different AMF treatments: (Control: Control, PW: PlantWorks, R: *Rhizophagus irregularis*) and endosymbionts presence *(H. defensa no* = absence; *H. defensa yes* = presence, control = no aphids) during a 12-day treatment period.

Soil moisture and leaf traits

There was a significant effect of water treatment on soil moisture, however there were no significant interactions between water treatment, endosymbionts presence and AMF (Table 3.5). Despite the effect of water treatment on soil moisture, leaf relative water content (RWC) was unaffected (data not shown). Chlorophyll content index was unaffected by water regimes, AMF treatments and endosymbionts presence (data not shown). The AMF species used in this study did not have a significant effect on leaf phosphate (P) concentrations; average values for leaf P concentration are reported in Table 3.6. Neither water deficit nor endosymbiont presence showed significant effects on leaf P content (Table 3.5).

Table 3.5. Statistical summaries of three-way ANOVA for the plant physiological variables quantified in the experiment. Significant differences ($p<0.05$) are highlighted in bold.

Variable	**Stomatal conductance (mmol mm^{-2} s^{-1})**			**Soil Moisture (%)**			**Chlorophyll content (CCI)**			**Relative water content (%)**			**P concentration (µg P/mg DW)**		
	df	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**
Block	5	6.08		5	1.35		5	2.24		5	0.60		5	1.71	
Water treatment	1	641.74	**<.001**	1	22.88	**<.001**	1	0.07	0.798	1	0.01	0.923	1	0.26	0.614
Endosymbiont presence	2	5.09	**0.008**	2	1.63	0.201	2	0.31	0.734	2	0.74	0.481	2	0.17	0.847
AMF product	2	1.48	0.232	2	0.81	0.447	2	0.14	0.869	2	0.36	0.702	2	0.36	0.697
Water treatment *Endosymbiont presence	2	6.19	**0.003**	2	1.00	0.372	2	2.79	0.067	2	0.86	0.429	2	1.18	0.311
Water treatment* AMF	2	1.06	0.351	2	1.12	0.333	2	1.19	0.308	2	0.51	0.601	2	1.63	0.201
Endosymbiont presence*AMF product	4	3.80	**0.007**	4	1.53	0.201	4	0.51	0.731	4	1.24	0.300	4	1.17	0.330
Water treatment* Endosymbiont presence* AMF product	4	3.79	**0.007**	4	1.54	0.199	4	0.76	0.552	4	1.08	0.369	4	0.51	0.727

Table 3.6. Average values for leaf P concentration.

AMF treatment	Water treatment	Endosymbiont presence	ug/g dry mass of leaf tissue
Rhizophagus irregularis	Well-watered	- *H.defensa*	8.56
Rhizophagus irregularis	Well-watered	+ *H.defensa*	8.86
Rhizophagus irregularis	Well-watered	control	8.66
Rhizophagus irregularis	Severe stress	- *H.defensa*	11.65
Rhizophagus irregularis	Severe stress	+ *H.defensa*	9.01
Rhizophagus irregularis	Severe stress	Control	8.58
PlantWorks	Well-watered	- *H.defensa*	6.89
PlantWorks	Well-watered	+ *H.defensa*	9.82
PlantWorks	Well-watered	Control	9.45
PlantWorks	Severe stress	- *H.defensa*	7.69
PlantWorks	Severe stress	+ *H.defensa*	7.87
PlantWorks	Severe stress	Control	9.70
Control	Well-watered	- *H.defensa*	8.98
Control	Well-watered	+ *H.defensa*	9.88
Control	Well-watered	Control	10.50
Control	Severe stress	- *H.defensa*	8.19
Control	Severe stress	+ *H.defensa*	9.08
Control	Severe stress	Control	6.95

Fresh and dry above-below ground biomass

Aboveground fresh and dry mass was significantly smaller in plants under drought stress than in the well-watered control (Fig. 3.13). This was mainly driven by the effect of water deprivation on leaf fresh and dry mass (Fig.3.14, Table 3.7). There was no effect of AMF treatment on shoot or leaf fresh or dry mass. Belowground fresh mass of roots and tubers were significantly reduced by water treatment (Supplementary Table 3.1), and AMF had a significant effect on the final dry weight of tubers due to the higher tuber mass in the PW treatment (Supplementary Table 3.1). There was a significant interaction between water treatment and endosymbiont presence on the final dry weight of roots (Fig.3.15; Table 3.8). Full statistical results for significant parameters are displayed in Supplementary Table 3.1.

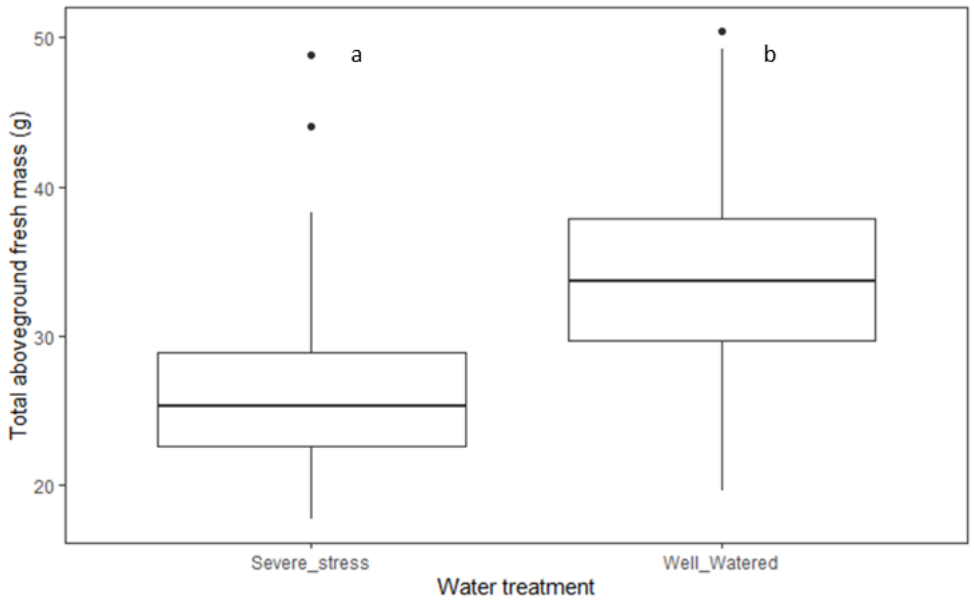

Figure 3.13 Boxplots comparing the total aboveground mass (g) of potato plants under different water treatments. Different letters indicated their means were different by Tukey's Honest Significant Difference test (p value < 0.05)

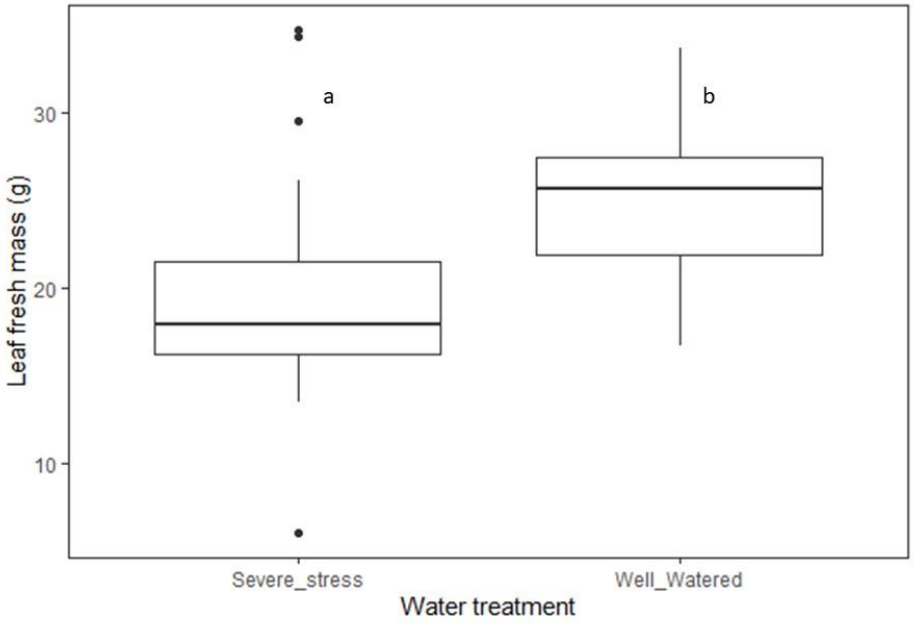

Figure 3.14 Leaf fresh mass (g) of potato plants under two water stress treatments. Different letters indicated their means were different by Tukey's Honest Significant Difference test (p value < 0.05)

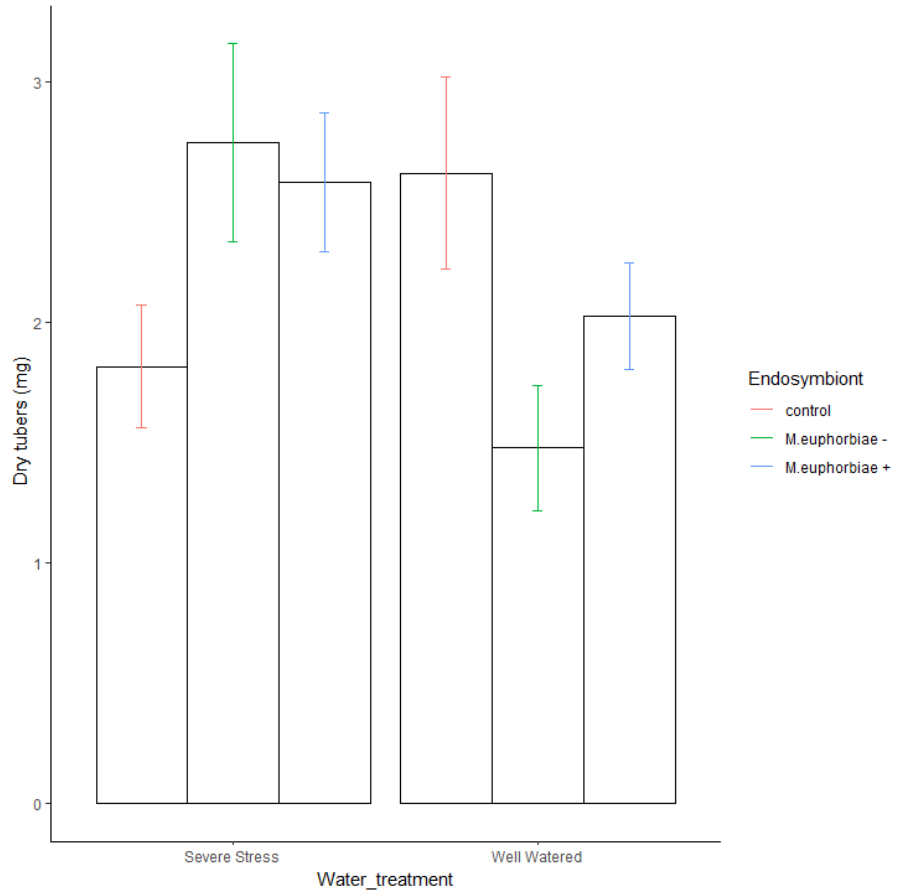

Figure 3.15 Tuber dry mass (mg) in response to two different water treatments (Well-Watered and Severe Stress) and endosymbiont presence *(M.euphorbiae* - = absence of *H.defensa* ; *M.euphorbiae* + = presence of *H.defensa*, control = no aphids).

Table 3.7. Statistical summaries of three-way ANOVAs for plant productivity (fresh or dry mass in g per plant) quantified in the experiment. Significant differences ($p<0.05$) are highlighted in bold.

Variable	**Stem fresh**			**Leaves fresh**			**Stem dry**			**Leaves dry**			**Aboveground biomass fresh**			**Aboveground biomass dry**		
	df	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**
Block	5	1.83		5	1.86		5	1.46		5	0.39		5	2.26		5	0.24	
Water treatment	1	4.54	**0.036**	1	53.75	**<.001**	1	2.50	0.117	1	12.14	**<.001**	1	37.07	**<.001**	1	12.29	**<.001**
AMF product	2	0.35	0.705	2	0.28	0.758	2	0.12	0.890	2	0.11	0.899	2	0.02	0.979	2	0.05	0.951
Endosymbiont presence	2	0.20	0.821	2	0.44	0.645	2	0.03	0.972	2	1.0	0.372	2	0.39	0.676	2	0.77	0.467
Water treatment* AMF product	2	1.47	0.235	2	1.18	0.313	2	0.23	0.793	2	0.65	0.523	2	1.64	0.200	2	0.70	0.499
Water treatment* Endosymbiont presence	2	0.81	0.448	2	0.67	0.514	2	0.32	0.725	2	0.16	0.855	2	0.11	0.899	2	0.21	0.807
Endosymbiont presence*AMF product	4	1.43	0.230	4	1.24	0.302	4	0.74	0.567	4	0.76	0.555	4	1.64	0.171	4	0.89	0.476
Water treatment* Endosymbiont presence* AMF product	4	1.94	0.111	4	2.80	0.031	4	1.80	0.136	4	0.86	0.491	4	2.67	0.037	4	1.31	0.272

Table 3.8. Statistical summaries of three-way ANOVAs for plant productivity (mass in g per plant) quantified in the experiment. Significant differences ($p<0.05$) are highlighted in bold.

Variable	**Fresh Roots**			**Fresh Tubers**			**Dry Roots**			**Dry tubers**			**Belowground biomass fresh**			**Belowground biomass dry**		
	df	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**
Block	5	0.97		5	0.92		5	2.51		5	2.13		5	0.33		5	2.76	
Water treatment	1	8.67	**0.004**	1	6.16	**0.015**	1	0.6	0.552	1	1.98	0.163	1	10.82	**0.001**	1	0.01	0.940
AMF product	2	1.38	0.258	2	1.63	0.203	2	0.97	0.382	2	3.95	**0.023**	2	0.76	0.473	2	0.85	0.431
Endosymbiont presence	2	2.08	0.131	2	1.52	0.224	2	1.09	0.342	2	0.02	0.976	2	0.86	0.427	2	1.01	0.368
Water treatment* AMF product	2	0.14	0.867	2	1.05	0.355	2	0.97	0.382	2	1.92	0.154	2	0.01	0.990	2	0.55	0.577
Water treatment* Endosymbiont presence	2	0.65	0.526	2	2.87	0.062	2	0.13	0.563	2	5.31	**0.007**	2	2.83	0.065	2	1.88	0.158
Endosymbiont presence*AMF product	4	0.42	0.796	4	0.86	0.489	4	0.75	0.174	4	0.42	0.791	4	0.52	0.719	4	1.10	0.362
Water treatment* Endosymbiont presence* AMF product	4	1.55	0.195	4	1.36	0.256	4	1.63		4	0.24	0.914	4	1.74	0.149	4	1.44	0.226

Aphid infestation

Overall, the number of aphid nymphs was not significantly affected by the treatments (Table 3.9). However, the effect of AMF treatment on the number of nymphs was close to significance. On average, aphids in the Ri treatment produced 22.3 nymphs per plant compared with aphids in the PW treatment which produced 17.6 nymphs per plant, while the number of nymphs for the sterile control was 30.1.

Table 3.9. Statistical summaries of three-way ANOVA for total number of nymphs per plant.

Variable	**Total number of nymphs**		
	df	**F**	***p***
Block	5	2.35	
Water treatment	1	0.90	0.350
AMF product	2	3.21	0.053
Endosymbiont presence	1	0.40	0.529
Water treatment* AMF product	2	0.38	0.690
Water treatment* Endosymbiont presence	1	0.05	0.819
Endosymbiont presence*AMF product	2	1.30	0.287
Water treatment* Endosymbiont presence* AMF product	2	2.50	0.098

3.6 Discussion

The two experiments were successful in establishing water stress treatments, shown by decreased soil moisture content and reduced stomatal conductance. AMF colonisation of the roots was not observed in the first experiment, and there was partial evidence of AMF colonisation in the second experiment, which limits our ability to state clearly the potential

benefits of AMF for plant stress alleviation. We summarise our findings in relation to the two main questions outlined in the study aims.

Do AMF modulate the tolerance of drought stress in plant?

Potato is sensitive to drought stress. Water irrigation is therefore essential to achieve good yields (Aliche et al., 2018). The drought stresses imposed in this study were successful in imposing physiological stress on plants, as shown by reduced leaf chlorophyll fluorescence (F_V/F_M), which was particularly evident in the Severe Stress treatment (Table), a finding that agrees with other studies (Schapedndonk et al., 1989). Regarding chlorophyll content, surprisingly, the water stress imposed did not decrease the leaf chlorophyll content in the drought treatment but differed considerably between cultivars, although this finding was not observed in previous studies. However, few studies showed that chlorophyll content in potato increased with the intensification of water deficit stress (Khosravifar et al., 2015). Moreover, there was no significant effect of water treatment on leaf Relative Water Content (RWC) after 25 days.

The three *Solanum tuberosum* cultivars varied considerably in their leaf chlorophyll content, dry matter growth and allocation to different parts of the plants. Hermes accumulated more mass in roots compared with Fontane and Innovator under control conditions. However, Hermes also showed a significant decrease in root dry mass in response to drought, whereas Innovator and Fontane did not, and Hermes and Fontane developed larger numbers of leaves per plant than Innovator. Previous studies have shown that Fontane is more efficient at transporting photo assimilates under environmental stress (Lahijani et al., 2018). Fontane and Innovator showed no decrease in root dry mass in response to increasing water stress indicating greater tolerance of water stress compared with Hermes. Despite low levels of AMF colonisation, there was a significant influence of AMF on root growth resulting in larger root dry mass, which is in agreement with previous studies (Fan et al., 2011; Karley et al., 2017). Increased root biomass, induced by AMF, has been attributed by others to increased uptake of P due to the ability of AMF to explore a larger soil volume for nutrients (Wu et al., 2021). Effects of AMF on plant species can be multidirectional and may vary in different environmental conditions. It is also possible that the formation of AMF associations may impact root responses to soil bacteria as well as leading to modifications of secondary metabolite synthesis in plants resulting in increased root biomass (Yang et al., 2014). The effect of AMF on root growth is intriguing given the limited presence of root colonisation and hints

that the addition of AMF inoculant might have acted as a biostimulant; further research has been recommended to explain this phenomenon (Shein et al., 2021).

Does AMF colonisation of roots modulate plant tolerance of combined abiotic (drought) and biotic (aphid infestation) stress?

In the second experiment, plants showed strong physiological responses to water stress through reduced stomatal conductance. The reduction in leaf water loss by stomatal closure is an adaptive response exhibited by plants to maintain a high tissue water potential under drought. AM fungi showed low levels of root colonization, indicating that AM fungi colonisation was either unsuccessful for both products or that the symbiosis was degraded soon after establishment. Probably as a result, AM fungi did not affect leaf P concentration as has been observed in previous studies (Turkmen et al., 2008). Although AMF types showed different levels of root colonization, a significant impact of these AMF treatments was not detected on plant or insect performance. However, previous studies showed significant effects of AM fungi on *Solanum tuberosum* growth (Bennett et al., 2016) but not on insect fitness (Bennett et al., 2016; Karley et al., 2017). Several studies have reported that the compatibility of AM fungi with potato plants could play a key role in the symbiosis since they often generate very weak and inconsistent root colonization especially under field conditions (Ocampo and Hayman, 1980; Vosátka and Gryndler, 2000). Furthermore, it has been highlighted that some potato cultivars could be resistant to colonization by AM fungi (Bhattarai and Mishra,1984). Tuber dry mass was increased by AMF inoculation in line with the findings of previous studies (Hijri, 2016; Lu et al., 2015; Wu et al., 2013).While aphids are often positively influenced by AMF colonisation of their host plants in terms of aphid growth (Whitham, 2002), in this study, despite the low level of AMF colonisation in the roots, there was a trend towards a negative effect of AMF colonisation on the number of nymphs produced. Decreased insect performance in response to AMF has been reported in chewing and sucking insects, which has been attributed to changes in herbivore-induce plant defence triggered by AMF colonisation (Dong et al., 2017). These contrasting effects of AMF on insect herbivore development indicate that AMF colonisation effects are highly dependent on the investigated study system and/or insect species.

An interesting focus, which could open future work, would be to elucidate the role of facultative endosymbiont-associations in the aphid-plant interactions. Our experiment showed

that aphid feeding increased the total dry mass of tubers under drought compared with well watered conditions and that endosymbiont presence affected plant stomatal conductance. These observations might have resulted from plant physiological changes induced by aphid feeding and suggests that aphid symbiont infection could be important in altering the mechanism of interactions between plants and aphids. Plants with higher root conductivity are able to tackle long period of drought stress, probably because roots of stressed plants tend to spread into deep soil for obtain resources (Creelman et al., 1990). In this study, root and tuber dry mass increased with aphid feeding under drought stress conditions. Similar results were shown in potato as well as for *Oryza sativa* and *Sorghum bicolor* and could reflect root proliferation as a plant stress response to mine a larger volume of the substrate for moisture and nutrients (Kato et al., 2006; Quandahor et al., 2021; Singh et al., 2011).By contrast, *H. defensa*-infected aphids are known to reduce dry matter allocation to roots (Hackett et al., 2013; Bennett et al., 2016), which might affect leaf gas exchange relations through changes in photosynthetic rate. Moreover, recent studies have shown that facultative endosymbiotic bacteria infection can reduce volatile emission by aphid-infested plants (Frago et al., 2017), and increase the speed and frequency of aphid probing at the plant leaf surface (Leybourne et al., 2019), showing that aphid symbionts are capable of triggering changes in mechanisms at the plant-aphid interface, which might underpin the observed shifts in plant resource allocation below-ground.

3.7 Conclusion

The results of this study showed that the three *Solanum tuberosum* cultivars demonstrated phenotypic differences between potato cultivars in morphology (leaf number) and physiology (leaf relative water content and chlorophyll content). There was limited evidence for the cultivars showing differences in tolerance of water stress: only root dry mass showed differential responses, being higher in Hermes under control water supply and showing a larger reduction in root mass in this cultivar when under severe water stress compared with Innovator and Fontane. Even though levels of AMF colonization were low or absent, and there was no evidence for expected AMF effects on plant physiology (leaf P concentrations), AMF treatment resulted in larger root mass compared with the control plants, particularly with the PlantWorks inoculum, suggesting that AMF had a biostimulatory effect on root growth even at low levels of colonisation. Surprisingly, drought stress did not affect aphid nymph production. Instead, AMF-treated plants showed a lower number of nymphs compared with the control, contrary to previous studies of aphids feeding on potato. All these results reveal that the effects on plant growth of root association with AMF are variable and depend on several factors (biotic/abiotic)

that need to be further investigated to achieve a practical method for the use of AMF in protecting *Solanum tuberosum* against environmental stresses.

3.8 Supplementary material

Supplementary Table 3.1 Table showing the mean and the standard error for aboveground biomass fresh and dry and belowground biomass fresh and dry. Treatments: Control: Control, PW: PlantWorks, R: *Rhizophagus irregularis*.

Stem fresh				
Water treatment	**AMF**	**Endosymbiont presence**	**Mean**	**Standard Error**
Severe stress	Control	control	5.568	0.425
Severe stress	Control	-*H.defensa*	8.223	0.902
Severe stress	Control	+ *H.defensa*	5.693	0.648
Severe stress	PW	control	7.530	1.063
Severe stress	PW	-*H.defensa*	6.641	0.848
Severe stress	PW	+ *H.defensa*	10.370	2.401
Severe stress	R	control	7.645	0.586
Severe stress	R	-*H.defensa*	7.316	0.751
Severe stress	R	+ *H.defensa*	7.290	1.000
Well-Watered	Control	control	9.876	1.713
Well-Watered	Control	-*H.defensa*	9.645	1.548
Well-Watered	Control	+ *H.defensa*	7.591	0.907
Well-Watered	PW	control	7.351	1.055
Well-Watered	PW	-*H.defensa*	10.310	1.582

Well-Watered	PW	+ *H.defensa*	7.610	0.814
Well-Watered	R	control	8.878	0.844
Well-Watered	R	-*H.defensa*	7.168	1.463
Well-Watered	R	+ *H.defensa*	8.721	1.742
Leaves Fresh				
Severe stress	Control	control	16.475	0.817
Severe stress	Control	-*H.defensa*	22.973	1.891
Severe stress	Control	+ *H.defensa*	16.846	1.212
Severe stress	PW	control	22.311	2.713
Severe stress	PW	-*H.defensa*	17.363	1.224
Severe stress	PW	+ *H.defensa*	18.935	3.718
Severe stress	R	control	20.446	1.031
Severe stress	R	-*H.defensa*	17.860	1.305
Severe stress	R	+ *H.defensa*	18.240	1.346
Well-Watered	Control	control	27.476	1.765
Well-Watered	Control	-*H.defensa*	26.578	2.333
Well-Watered	Control	+ *H.defensa*	24.761	1.487
Well-Watered	PW	control	21.205	1.145
Well-Watered	PW	-*H.defensa*	25.468	1.970
Well-Watered	PW	+ *H.defensa*	25.213	1.722
Well-Watered	R	control	25.225	0.780
Well-Watered	R	-*H.defensa*	25.363	1.837
Well-Watered	R	+ *H.defensa*	25.808	2.110

Stem dry				
Severe stress	Control	control	0.830	0.098
Severe stress	Control	-*H.defensa*	1.262	0.145
Severe stress	Control	+ *H.defensa*	0.886	0.117
Severe stress	PW	control	0.987	0.195
Severe stress	PW	-*H.defensa*	0.929	0.160
Severe stress	PW	+ *H.defensa*	1.167	0.250
Severe stress	R	control	1.150	0.118
Severe stress	R	-*H.defensa*	1.004	0.181
Severe stress	R	+ *H.defensa*	1.196	0.208
Well-Watered	Control	control	1.466	0.277
Well-Watered	Control	-*H.defensa*	1.054	0.221
Well-Watered	Control	+ *H.defensa*	1.107	0.202
Well-Watered	PW	control	0.995	0.130
Well-Watered	PW	-*H.defensa*	1.472	0.306
Well-Watered	PW	+ *H.defensa*	1.074	0.174
Well-Watered	R	control	1.221	0.109
Well-Watered	R	-*H.defensa*	1.078	0.250
Well-Watered	R	+ *H.defensa*	1.245	0.218
Leaves dry				
Severe stress	Control	control	3.114	0.359
Severe stress	Control	-*H.defensa*	3.155	0.616
Severe stress	Control	+ *H.defensa*	3.120	0.644

Severe stress	PW	control	3.039	0.506
Severe stress	PW	-*H.defensa*	2.792	0.436
Severe stress	PW	+ *H.defensa*	3.952	0.394
Severe stress	R	control	2.986	0.570
Severe stress	R	-*H.defensa*	3.378	0.443
Severe stress	R	+ *H.defensa*	3.944	0.101
Well-Watered	Control	control	4.858	0.300
Well-Watered	Control	-*H.defensa*	4.058	0.727
Well-Watered	Control	+ *H.defensa*	4.335	0.348
Well-Watered	PW	control	3.979	1.317
Well-Watered	PW	-*H.defensa*	5.216	1.136
Well-Watered	PW	+ *H.defensa*	4.268	0.366
Well-Watered	R	control	3.533	0.530
Well-Watered	R	-*H.defensa*	3.614	0.617
Well-Watered	R	+ *H.defensa*	4.807	0.301
Aboveground biomass fresh				
Severe stress	Control	control	22.043	1.227
Severe stress	Control	-*H.defensa*	31.196	2.644
Severe stress	Control	+ *H.defensa*	22.539	1.548
Severe stress	PW	control	29.841	3.505
Severe stress	PW	-*H.defensa*	24.005	1.752
Severe stress	PW	+ *H.defensa*	29.305	4.102

Severe stress	R	control	28.091	1.168
Severe stress	R	-*H.defensa*	25.176	2.046
Severe stress	R	+ *H.defensa*	25.530	2.279
Well-Watered	Control	control	37.353	3.328
Well-Watered	Control	-*H.defensa*	36.223	3.858
Well-Watered	Control	+ *H.defensa*	32.353	2.351
Well-Watered	PW	control	28.556	1.995
Well-Watered	PW	-*H.defensa*	35.778	3.075
Well-Watered	PW	+ *H.defensa*	32.823	2.401
Well-Watered	R	control	34.104	1.221
Well-Watered	R	-*H.defensa*	32.531	3.118
Well-Watered	R	+ *H.defensa*	34.530	3.458
Aboveground biomass dry				
Severe stress	Control	control	3.944	0.387
Severe stress	Control	-*H.defensa*	4.418	0.521
Severe stress	Control	+ *H.defensa*	4.007	0.719
Severe stress	PW	control	4.027	0.523
Severe stress	PW	-*H.defensa*	3.722	0.496
Severe stress	PW	+ *H.defensa*	5.119	0.580
Severe stress	R	control	4.137	0.509
Severe stress	R	-*H.defensa*	4.383	0.584
Severe stress	R	+ *H.defensa*	5.141	0.232

Well-Watered	Control	control	6.324	0.546
Well-Watered	Control	-*H.defensa*	5.113	0.897
Well-Watered	Control	+ *H.defensa*	5.443	0.471
Well-Watered	PW	control	4.974	1.345
Well-Watered	PW	-*H.defensa*	6.689	1.292
Well-Watered	PW	+ *H.defensa*	5.343	0.502
Well-Watered	R	control	4.754	0.559
Well-Watered	R	-*H.defensa*	4.693	0.803
Well-Watered	R	+ *H.defensa*	6.053	0.423
Fresh roots				
Severe stress	Control	control	26.715	1.767
Severe stress	Control	-*H.defensa*	38.111	6.989
Severe stress	Control	+ *H.defensa*	28.754	4.615
Severe stress	PW	control	36.153	7.456
Severe stress	PW	-*H.defensa*	24.023	5.015
Severe stress	PW	+ *H.defensa*	28.561	2.465
Severe stress	R	control	29.120	2.307
Severe stress	R	-*H.defensa*	29.325	2.783
Severe stress	R	+ *H.defensa*	25.243	1.531
Well-Watered	Control	control	44.665	3.380
Well-Watered	Control	-*H.defensa*	34.925	8.032
Well-Watered	Control	+ *H.defensa*	38.850	4.600
Well-Watered	PW	control	37.816	4.321

Well-Watered	PW	-*H.defensa*	36.615	2.616
Well-Watered	PW	+ *H.defensa*	31.136	3.751
Well-Watered	R	control	40.030	6.345
Well-Watered	R	-*H.defensa*	32.996	6.541
Well-Watered	R	+ *H.defensa*	28.390	3.389
Fresh tubers				
Severe stress	Control	control	9.427	2.633
Severe stress	Control	-*H.defensa*	25,786	7.333
Severe stress	Control	+ *H.defensa*	18.876	5.101
Severe stress	PW	control	16.630	1.856
Severe stress	PW	-*H.defensa*	22.551	4.013
Severe stress	PW	+ *H.defensa*	20.585	1.875
Severe stress	R	control	15.398	3.595
Severe stress	R	-*H.defensa*	18.541	4.485
Severe stress	R	+ *H.defensa*	16.666	2.464
Well-Watered	Control	control	23.429	4.665
Well-Watered	Control	-*H.defensa*	15.350	5.609
Well-Watered	Control	+ *H.defensa*	22.435	2.537
Well-Watered	PW	control	26.970	6.596
Well-Watered	PW	-*H.defensa*	27.925	6.023
Well-Watered	PW	+ *H.defensa*	23.461	3.638
Well-Watered	R	control	28.164	5.852
Well-Watered	R	-*H.defensa*	19.898	5.623

Well-Watered	R	+ *H.defensa*	19.496	3.755
Dry roots				
Severe stress	Control	control	4.055	0.732
Severe stress	Control	-*H.defensa*	5.976	1.267
Severe stress	Control	+ *H.defensa*	4.788	1.076
Severe stress	PW	control	5.285	1.128
Severe stress	PW	-*H.defensa*	3.610	1.069
Severe stress	PW	+ *H.defensa*	5.526	1.457
Severe stress	R	control	6.395	0.699
Severe stress	R	-*H.defensa*	4.413	0.505
Severe stress	R	+ *H.defensa*	4.133	0.444
Well-Watered	Control	control	6.362	1.330
Well-Watered	Control	-*H.defensa*	4.830	0.909
Well-Watered	Control	+ *H.defensa*	7.222	2.244
Well-Watered	PW	control	6.293	1.441
Well-Watered	PW	-*H.defensa*	4.789	0.652
Well-Watered	PW	+ *H.defensa*	3.862	0.874
Well-Watered	R	control	4.858	0.535
Well-Watered	R	-*H.defensa*	4.326	0.886
Well-Watered	R	+ *H.defensa*	4.305	0.938

Dry tubers				
Severe stress	Control	control	1.195	0.284
Severe stress	Control	-*H.defensa*	2.843	0.828
Severe stress	Control	+ *H.defensa*	2.836	0.696
Severe stress	PW	control	2.300	0.612
Severe stress	PW	-*H.defensa*	2.854	0.691
Severe stress	PW	+ *H.defensa*	2.775	0.494
Severe stress	R	control	1.955	0.281
Severe stress	R	-*H.defensa*	2.543	0.745
Severe stress	R	+ *H.defensa*	2.134	0.232
Well-Watered	Control	control	2.074	0.446
Well-Watered	Control	-*H.defensa*	0.980	0.303
Well-Watered	Control	+ *H.defensa*	1.639	0.253
Well-Watered	PW	control	3.429	0.821
Well-Watered	PW	-*H.defensa*	2.047	0.610
Well-Watered	PW	+ *H.defensa*	2.777	0.430
Well-Watered	R	control	2.362	0.744
Well-Watered	R	-*H.defensa*	1.406	0.333
Well-Watered	R	+ *H.defensa*	1.668	0.285
Belowground biomass fresh				
Severe stress	Control	control	36.142	2.289
Severe stress	Control	-*H.defensa*	63.898	10.204

Severe stress	Control	+ *H.defensa*	47.630	3.725
Severe stress	PW	control	52.783	7.591
Severe stress	PW	-*H.defensa*	46.575	7.085
Severe stress	PW	+ *H.defensa*	49.146	3.997
Severe stress	R	control	44.518	2.526
Severe stress	R	-*H.defensa*	47.866	7.213
Severe stress	R	+ *H.defensa*	41.910	2.962
Well-Watered	Control	control	68.085	7.049
Well-Watered	Control	-*H.defensa*	50.275	10.371
Well-Watered	Control	+ *H.defensa*	61.285	6.787
Well-Watered	PW	control	64.786	10.282
Well-Watered	PW	-*H.defensa*	64.540	5.759
Well-Watered	PW	+ *H.defensa*	54.598	5.966
Well-Watered	R	control	68.195	10.048
Well-Watered	R	-*H.defensa*	52.895	10.113
Well-Watered	R	+ *H.defensa*	47.886	5.641
Belowground biomass dry				
Severe stress	Control	control	5.251	0.834
Severe stress	Control	-*H.defensa*	8.819	1.663
Severe stress	Control	+ *H.defensa*	7.624	0.881
Severe stress	PW	control	7.586	1.340
Severe stress	PW	-*H.defensa*	6.464	1.520

Severe stress	PW	+ *H.defensa*	8.302	1.873
Severe stress	R	control	8.351	0.790
Severe stress	R	-*H.defensa*	6.957	0.930
Severe stress	R	+ *H.defensa*	6.268	0.627
Well-Watered	Control	control	8.437	1.573
Well-Watered	Control	-*H.defensa*	5.811	0.975
Well-Watered	Control	+ *H.defensa*	8.861	2.437
Well-Watered	PW	control	9.722	1.101
Well-Watered	PW	-*H.defensa*	6.837	1.067
Well-Watered	PW	+ *H.defensa*	6.639	0.678
Well-Watered	R	control	7.220	1.028
Well-Watered	R	-*H.defensa*	5.732	0.980
Well-Watered	R	+ *H.defensa*	5.973	0.912

Chapter 4. Do aphid endosymbiotic bacteria impact host-searching behaviour of parasitoid through VOC emissions by aphid honeydew?

Abstract
Bacterial endosymbionts have facilitated aphids to tolerate and adapt to a wide range of environmental stressors but their effects on the other trophic levels are not well understood. *Macrosiphum euphorbiae* (Thomas), the potato aphid, is an important herbivorous pest worldwide especially for the *Solanum* family and it forms a facultative association with the bacterial endosymbiont *Hamiltonella defensa.* Parasitoid wasps can be effective at regulating aphid populations in natural and managed vegetation. Volatile compounds emitted from honeydew, the sugar-rich excretion of phloem-sap feeders, have been shown to act as cues for parasitoids to locate their aphid prey. However, little research has investigated the influence of endosymbionts on volatiles emitted by honeydew and the attractiveness to parasitoid wasps. Two experiments were performed to address this knowledge gap by comparing clonal lines of *M. euphorbiae* with and without *H.defensa* infection using two different aphid genotypes. Firstly, volatile compounds of honeydew were extracted by SPME technique and analysed by GC-MS. Secondly, the parasitoid wasp *Aphidius ervi*, was given a choice of honeydew collected from uninfected and *H. defensa* infected aphids. In both experiments, honeydew production was significantly lower for one of the two aphid genotypes. In the choice assay, but not in the VOC collection experiment, aphids infected with *H.defensa* produced larger quantities of honeydew compared with uninfected aphids and parasitoid wasps were more likely to be attracted towards a higher abundance of honeydew. Volatile analysis showed little difference in honeydew volatile composition from infected and uninfected aphids. Further research in this area is required to better understand the role of facultative endosymbionts in aphid honeydew production and prey location by natural enemies.

Keywords: *Aphidius ervi*, biological control, *Hamiltonella defensa*, *Macrosiphum euphorbiae*, *Solanum tuberosum*, volatile organic compounds

4.1 Introduction

While feeding, aphids secrete products of phloem ingested from plants in the form of a sugar-rich excretion: honeydew. This substance consists of an aqueous mixture of different chemical compounds such as sugars, amino acids, organic acids, inorganic ions, proteins and lipids (van Neerbos et al., 2020). Many factors contribute to variation in the composition of honeydew including aphid species (Woodring et al., 2004), environmental conditions (Fischer et al., 2002), seasonal changes (Sandström and Moran, 2001), host plant species as well as nutritional status of the host plant, and aphid developmental stage (Arakaki and Makoto, 1998; Leroy et al., 2011b; Shaaban et al., 2020). Although it is widely accepted that the primary endobacterial symbiont of aphids - *Buchnera aphidicola* - plays a key role in mediating aphid metabolism (Douglas, 1995; Wilkinson et al., 1997), the role of facultative endobacteria in nutrient metabolism in these insects is not well understood.

Hamiltonella defensa is a facultative bacterial endosymbiont of aphids that confers certain advantages to their aphid hosts, such as improving aphid fitness (Koga et al., 2003), tolerance to high temperature (Montllor et al., 2002), manipulation of plant defensive responses (Li et al., 2019), and crucially provides resistance to natural enemies (Leybourne et al., 2020). Little is known about how the presence of *H. defensa* affects honeydew composition. To date, a single study investigating the influence of facultative endosymbionts (*H. defensa* and *Regiella insecticola*) showed that endosymbiont presence was associated with lower concentrations of amino acids in the honeydew of *Aphis fabae* compared with uninfected aphids (Schillewaert et al., 2017).

In agroecosystems, nectar (floral and extrafloral) and honeydew (produced by Homoptera) are the most common sources of carbohydrates for hymenopteran parasitoids (Wäckers and Steppuhn, 2003). Adult parasitoids depend on these carbohydrates as sources of energy to increase life expectancy (Lee et al., 2004). Amongst aphid parasitoids, the braconid wasp *Aphidius ervi* Haliday (Hymenoptera: Aphidiidae) is recognized as a common and efficient generalist parasitoid. This species is a solitary koinobiont that lays eggs inside the aphid body, and uses aphids as hosts for their developing offspring. A single adult female *A.ervi* can parasitise more than 300 aphids in her lifetime (He and Wang, 2006). Other aphid species used as hosts by *A. ervi* include: *Aulacorthum solani, Rhopalosiphum padi, Myzus persicae, M. euphorbiae and Sitobion avenae* (Starý, 1974; Takada and Tada, 2000). Therefore, *A. ervi* is considered as an important biological control agent in agricultural systems in which these aphids can be found (Powell and Pickett, 2003). To locate the appropriate aphid host stage for

oviposition, the females follow physical and chemical stimuli from the aphid host including volatile organic compounds (VOCs) emitted from honeydew (Lenaerts et al., 2016), which act as a host-location kairomone and oviposition stimulus for parasitoids. For instance, a study by Shaltiel and Ayal (1998) showed the parasitoid wasp *Diaretiella rapae* actively used the honeydew emitted by its host, the cabbage aphid *Brevicoryne brassicae*, as a kairomone. In another study, where honeydew from *Acyrthosiphon pisum* was used as a model system, volatile organic compounds (VOCs) produced by bacteria associated with the honeydew (*Staphylococcus sciuri)* were able to attract natural enemies (Leroy et al., 2011a).

However, research into the effect of aphid honeydew on parasitoid searching behaviour has not considered yet whether aphid endosymbionts could modify honeydew kairomone effects on parasitoids through changes in honeydew composition. In this context, the aims of this study are, first, to investigate whether *H. defensa* affects the volatile composition of honeydew of *Macrosiphum euphorbiae* and, second, to examine the attractiveness of honeydew volatiles to the generalist parasitoid wasp *Aphidius ervi* in the presence or absence of aphid symbiont infection. Honeydew composition was examined using solid phase microextraction (SPME) and GC-MS techniques to collect and characterise honeydew volatiles. The hypothesis that *H. defensa* infection influences wasp selection of aphids was tested by collecting honeydew from clonal lines of aphids that were symbiont-free or naturally infected with *H. defensa,* and a choice experiment was performed.

4.2 Material and Methods

Plant material

Plants were grown from tubers (*Solanum tuberosum* cv Désirée) in commercially produced insecticide-free compost (sand-perlite-peat mix containing N:P: K 17:10:15; William Sinclair Horticulture LTD, Lincoln, UK) in a glasshouse with supplementary light (16:8 h light and 20:15°C day:night) and watered daily.

Insect rearing and maintenance

Aphid clonal lines were reared on excised leaves from 3-week-old potato plants (*Solanum tuberosum* cv Désirée) contained in ventilated cups. These comprised two Perspex cups (5 cm width x 15 cm depth) placed one inside the other; the stem of the potato leaves was inserted through a *c.* 5mm circular hole in the base of the inner cup, and the cup surface was sealed with a mesh-ventilated lid. Plant material was refreshed weekly. Using this set up, age-synchronised apterous adults were produced for the experiments. All the insect cultures were maintained at 18 ± 2 °C and 16h:8h (day:night).

Mummies of the Braconid wasp, *Aphidius ervi* Haliday, supplied by Fargro (West Sussex, UK), were transferred to plastic ventilated boxes and supplied with a ball of cotton wool soaked in a 50 % (v/v) honey solution on which to feed; this was held within a small plastic lid secured with Blu-Tack (Bostik, Stafford, UK), and was replaced daily to prevent fermentation of the honey. Emerging wasps (5-7 days old) were transferred to *Acyrthosiphon pisum* (pea aphids: clonal line LL01) infested bean plants enclosed in a fine mesh cage. This clonal line harbours no known secondary endosymbionts and has a high reproductive output. After 10 days, mummies were carefully collected from the plant and transferred into ventilated boxes until hatching. All the parasitoid cultures and the pea aphid cultures on which they were reared were maintained in growth cabinets at 20± 1 °C, 70 % humidity and with a light regime of 16 h light:8 h dark.

Macrosiphum euphorbiae genotyping

Characterised laboratory cultures of potato aphids of known genotype were used in the experiment, using the genotype classifications defined in Clarke (2013). Clonal lines were used belonging to genotype 6 with or without *H. defensa* infection (MW17/08 and DM18/8,

respectively) and genotype 3 with or without *H. defensa* infection (MW17/25 and DM18/16, respectively). Genotyping was performed using seven microsatellite loci for confirming clonal lines used in the experiment (Me1, Me6, Me7, Me9, Me10, Me11, Me13) (Raboudi et al., 2005). Firstly, DNA was extracted from frozen homogenised tissue of 3 aphids per clonal line using NucleoSpin ® Tissue Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. Extracted DNA was quantified using Nanodrop ND-1000 (ThermoFischer Scientific, UK), then stored at -20 ̊C. Multiplex PCR was carried out using Illustra^{TM} puReTaq Ready-To-Go^{TM} PCR beads using the forward and reverse primers as shown in Supplementary Table 4.1. The Master Mix comprising 22 µl of sterile water, 1 µl of forward primer and 1 µl of reverse primer, was then added to each of the PCR beads with 1 µl of DNA template. The reaction was carried out using a Prime Thermal Cycler (Techne, Staffordshire, UK) using a touchdown program PMS2 (Sloane et al., 2001) with conditions as described in Supplementary Table 2. To prepare the amplified products for genotyping, 12µl of LIZ marker (Applied Biosystems, UK) was added to a 1ml aliquot of thawed Hi-Di™ formamide and 9µl of the resulting hi-di formamide-lizmarker mixture was added to each well in a 96-well genotyping plate. 1µl of each PCR product was then added to a well. Samples were sequenced by the Genome Technology facility at the James Hutton Institute, using a capillary-based Applied Biosystems AB3730 system, and Peak Scanner Software v1.0 was used to calculate product sizes and confirm genotype classifications.

Facultative Endosymbiont Detection

A diagnostic PCR screen was used targeting universal eubacterial 16S rDNA and the 16-23S rDNA as positive controls for the presence of bacteria and facultative endosymbiont bacteria, respectively (Darby and Douglas, 2003; Fukatsu and Nikoh, 2000; Sandström et al., 2001) along with the specific 16S rDNA target sequence for *Hamiltonella defensa* (Darby and Douglas, 2003). DNA was extracted from frozen homogenised tissues of 3 aphids per clonal line, sampled from live cultures, using the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. First, aphids were washed in 96-100% ethanol (Sigma-Aldrich, UK) for five minutes then rinsed with sterile distilled water; samples were then flash-frozen in liquid nitrogen and homogenised using micropestles. Extracted DNA was quantified using Nanodrop ND-1000 (ThermoFischer Scientific, UK). PCR was carried out using Illustra^{TM} puReTaq Ready-To-Go^{TM} PCR beads using the forward and reverse

primers shown in Supplementary Table 4.1 for *H. defensa* detection. The Master Mix comprising 22 µl of ultraPure DNase/RNase-Free Distilled Water (Fisher Scientific, Loughborough, UK), 1 µl of forward primer and 1 µl of reverse primer, was then added to each of the PCR beads with 1 µl of DNA template. The reaction was carried out using a Prime Thermal Cycler (Techne, Staffordshire, UK) with conditions as described in Supplementary Table 4.2. An aliquot (10 µl) of the amplified product was separated and visualised on 1.5% agarose gel using SYBR Safe® DNA staining agent. In positive reactions, the residual 15 µl of amplified product was purified using the QIAquick PCR Purification Kit (Qiagen, UK) following the manufacturer's protocol. Purified products were quantified and analysed for quality using a Nanodrop ND-1000 (ThermoFischer Scientific, UK) and aliquots were prepared for sequencing using Sanger methodology. Sequencing of products was carried out using a 36cm capillary array on a 48 capillary ABI 3730 system (ThermoFisher Scientific, UK). Samples were sequenced by the Genome Technology facility at the James Hutton Institute. Sequenced data were subjected to a BLAST search, using the NCBI online database, to check hits of the sequences of known aphid endosymbionts. Sequences were compared against known prokaryotic sequence data held in the server and *H. defensa* presence was therefore confirmed for each genotype.

Experimental design

For investigating the aims stated in the introduction, two experiments were conducted. Experiment one, to characterise honeydew volatile composition, was devoted to analysing volatiles released from honeydew. Experiment two, insect choice assay, was conducted to test parasitoid wasps preference for honeydew produced by different aphid clonal lines. One batch of plants for each experiment was generated with the same methodology as described in plant material section (Fig. 4.1).

Experiment 1 consisted of a randomised block design. Three replicate blocks were staggered over a period of 7 days. The treatment factors were aphid genotype (two levels: genotype 3 and 6) and symbiont presence (two levels: with and without *H. defensa*). Within each replicate block, the treatment combination (genotype x symbiont presence/absence) was assigned at random. Ten leaves on each plant each had a separate clip cage attached and contained aphids feeding on the leaf, along with a piece of tin foil to collect honeydew (see below for details of honeydew collection). Aphid-free controls were also included to provide a baseline for

background VOCs, giving a total of 4 aphid treatments plus one aphid-free treatment totalling 5 treatments x 3 replicates = 15 plant units.

Experiment 2 assessed insect behaviour through a choice test and comprised a randomised block design. Sixteen replicate blocks were staggered over a period of 8 days (two blocks per day). The treatment factors were aphid genotype (two levels: genotype 3 and 6) and symbiont presence (two levels: with and without *H. defensa*), with treatments assigned at random to plants in each block, summing to 2 x 2 x 16 = 64 plants per block.

Honeydew collection

Disks of tin foil were first washed in 96 % Ethanol (Sigma-Aldrich, UK) and subsequently rinsed with deionized water three times before being used. On the day of the experiment, 8 age synchronized apterous adult aphids were placed inside 10 Perspex clip cages per plant, to contain the aphids onto the underside of fully expanded leaves; then 10 tin foil disks (one for each clip cage) were weighed and placed inside the cages, beneath the aphids. After 36 h, the tin foil disks were retrieved, weighed again to quantify the mass of the collected honeydew, and immediately placed in vials for volatile collection (experiment 1) or to test parasitoid behaviour (experiment 2). The numbers of aphids that survived were counted to take into account how many aphids had successfully settled on the plant and therefore had contributed to the production of honeydew. Total number of aphids survived was subsequently used to standardize honeydew mass (i.e. per aphid) recorded among treatments.

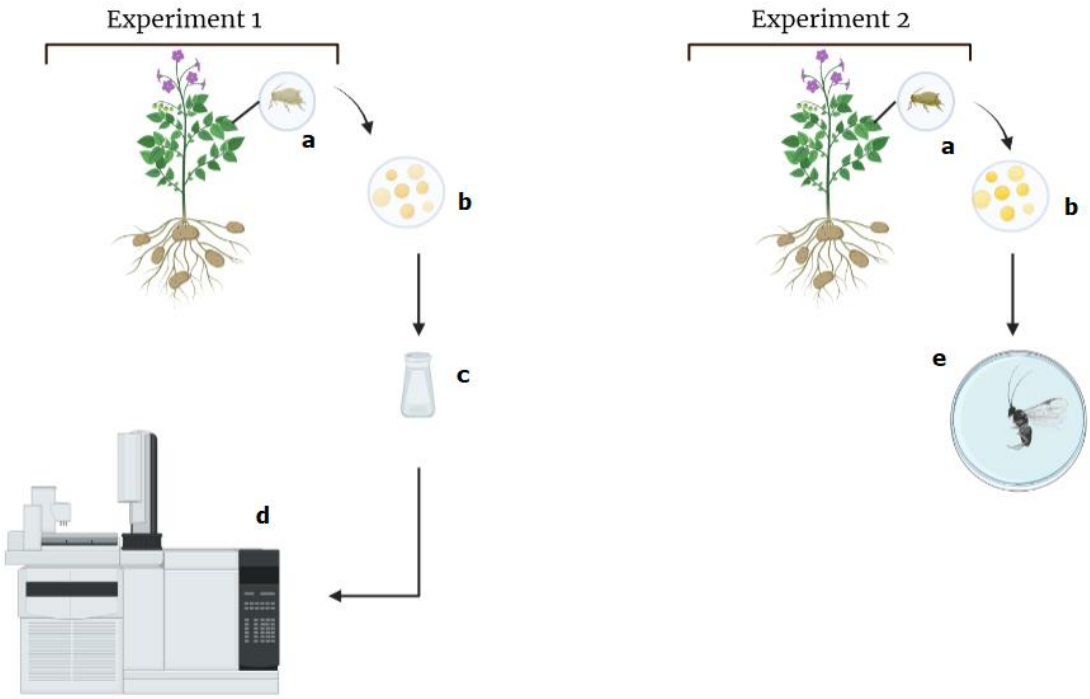

Figure 4.1 Graphical representation of the honeydew collection methodology used for experiment 1 and 2. **(a)** Aphids (8) were enclosed in a clip cage underneath disk of tin foil and attached to a leaf. For each plant 10 clip cages were used and 10 tin foil disks. **(b)** After 36 hours tin foil disks were retrieved, and honeydew mass and aphid number were recorded. Subsequently the 10 tin foil disks were enclosed in a vial **(c)** for volatile collection using SPME fibres and analysed by GC-MS **(d**). In experiment two, tin foil disks were positioned randomly in a petri dish and a parasitoid wasp **(e)** was released inside for the choice test experiment.

Honeydew volatile composition: experiment 1

The volatile analysis was performed by SPME on the following different samples: tin foil with no honeydew collected (control), tinfoil with honeydew collected from genotype 3 aphids (clonal lines with or without *H. defensa*), tinfoil with honeydew collected from genotype 6 aphids (clonal lines with or without *H. defensa*), giving a total of 5 treatments. For each volatile analysis two extra controls were used, an empty vial (blank) and a vial filled with 10 clean disks of tinfoil (tinfoil control), to account for possible contamination and/or VOC background. For each replicate sample, 10 tin foil-lined clip cages were attached to the plant, with three replicate plants in each treatment. For VOC analysis, the 10 tin foil discs were removed from

the clip cage, placed inside a 20 mL screw neck headspace sampling vial (Supelco, Sigma-Aldrich, UK, catalogue no. 20069637) and closed with a PTFE-lined screwcap (Supelco, Sigma-Alrdrich, UK, catalogue no. SU860101) and the weight of the sample (weight of honeydew collected) was recorded. Honeydew volatiles were collected using a single fibre type, 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) with a 23 gauge needle (Supelco, Sigma-Aldrich, UK, catalogue no. 57293-U). Fibres were conditioned at 250 °C for 30 min in a flow of dry nitrogen according to manufacturer's guidelines before VOC collection using a fibre conditioning station. Sampling vials were preincubated at 55°C for 2 min. Absorption of headspace volatiles to the SPME fibre was achieved by exposing the fibre to the sample headspace volatiles for 30 min at 55°C with a vial penetration of 22 mm and injection penetration of 54 mm. Volatiles were then analysed by GC-MS using a Trace DSQ™ II Series Quadrupole system (Thermo Electron Corporation, Hemel Hempstead, UK), fitted with a CTC CombiPAL autosampler configured for SPME with an attached SPME fibre conditioning station supplied with nitrogen purge gas at a fixed flow of 6 mL/min (CTC Analytics, Switzerland). The volatiles were desorbed at 250 °C by exposure of the fibre for 2 min within a programmed temperature vaporising (PTV) injector operating in constant temperature splitless mode and fitted with a Merlin Microseal™ High Pressure Septum and a Siltek™ deactivated metal PTV liner (120 mm × 1 mm internal Ø x 2.75 mm external Ø, Thermo Scientific, UK). On completion of desorption the fibre was reconditioned automatically within the fibre conditioning attachment. Separation of volatiles was achieved on a DB-1701 GC column (30 m × 0.25 mm internal Ø × 0.25 μm film thickness; Agilent Technologies, UK) using helium carrier gas at a flow rate of 1.5 mL/min in constant flow mode. The GC temperature programme was 40 °C for 2 min, 10 °C/min to 240 °C then isothermal at 240 °C for 10 min. The GC-MS interface temperature was 250 °C. After a 1 min delay, mass spectra were acquired at 6 scans/s over the mass range of 35 to 400 u (atomic mass unit) under electron ionisation (EI) conditions at 70 eV(electronvolt), with a source temperature of 200 °C. The GC-MS was tuned daily using perfluorotertiarybutyl amine (PFTBA) with the instrument's autotune function (Deasy et al., 2016).

Data were acquired and analysed using Xcalibur™ 2.0.7 (Thermo Electron Corporation, Hemel Hempstead, UK). Specific ions characteristic of each compound in the samples were selected by examination of the mass spectrum of each component in the total ion chromatogram (TIC) of several raw data files, representative of each stage of the experiments, using Xcalibur™. These ions, to be used for compound identification and measurement of raw abundance, were

selected on the basis that they should have a high relative abundance, should be unique to the compound and/or the compound should be well resolved chromatographically from other compounds with ions with the same *m*/*z* (mass to charge ratio). A defined time window centred on the chromatographic peak apex, along with the selected characteristic ions were used for compound detection and abundance measurement in a processing method created in Xcalibur™. A summed selected ion chromatogram (SIC) for all of the chosen ions within the appropriate time window was then generated and integrated. This value constituted the raw abundance of each compound. Processed data were checked for correct peak assignment and adjusted where necessary. Compounds were identified by comparison of their mass spectra and retention times with those of reference standards are indicated in Supplementary Table 4.3. Tentative identification of the remaining compounds was made by comparison with entries in MS libraries (Palisade 600 k, Palisade Corporation, Ithaca, NY, USA; NIST05, National Institute of Standards, Gaithersburg, MD, USA).

Insect choice: experiment 2

Two-way choice tests were conducted to compare parasitoid searching behaviour when exposed to volatiles from honeydew collected from aphids of a given genotype with or without *H. defensa* infection. Experiments were performed under laboratory conditions between 10:00 h and 12:00 h. Petri dishes (140 mm diameter) were used as choice chambers and were divided in half by marking the underside of the dish with a black marker pen. For each choice test, 10 tin foil disks were collected from a single plant of each treatment (i.e., from clip cages attached to leaves with aphids of a given genotype and symbiont infection status) and weighed to record honeydew mass and immediately placed into each half of the petri dish chamber, randomly. The position of the treatments to each half of the petri dish was assigned at random for each assay. A single *A. ervi* female (3-5 days old), presumed mated, which had been starved for a minimum of two hours, was placed in the centre of the petri dish and the position was noted (i.e. in either half of the chamber) every minute for a 10 min observation period. Wasps were only used once. Petri dishes were cleaned after each choice test using a dilute solution of Teepol™, then rinsed with deionised water and dried before being used again. A total of 16 assays were conducted for each aphid genotype (32 assays in total).

4.3 Statistical analyses

All statistical analyses were carried out using R v4.1.3 with additional packages ggplot2 v.2.2.1 (Wickham, 2009), dplyr v. 3.5.3 (Mailund, 2019), lubridate v. 1.7.8, (Spinu et al., 2016), car v. 3.0-7 (Fox and Weisberg, 2011), lsmeans v.2.27-62 (Lenth, 2016) and multcomp v. 1.4-8 (Hothorn, 2008). Data were checked for normality and homogeneity of variance by plotting Q-Q plots and residuals vs fitted values. Parametric models were applied where data displayed normal distribution. Non-parametric methods were used for analysis where data displayed non-normal distribution. Total mass of collected honeydew (mg), for both experiments 1 and 2, was firstly standardized, divided by total number of live aphids recorded at the end of the honeydew collection period, and then square root transformed. Two-way ANOVA was used to evaluate the effects of aphid genotype and *H. defensa* infection, and their interaction, on the square root transformed data. Differences between groups were assessed by Tukey's post-hoc test. For the choice test experiment, the effect of symbiont infection on the proportion of times (out of ten observations) spent by the wasp in each half of the assay arena (+/- *H. defensa*) was analysed using generalized linear mixed effect models using an additional package "lme4" (Bates et al., 2022). Specifically, the model was as follows: choice~genotype+hdratio+(1|block), using block as random factor. For volatile analysis, the blank and the tin foil was used to screen possible contaminants (related to fibre chemistry and impurities - as an example, plasticisers) and/or background VOCs. A final table with the sample-specific VOCs detected was then created with the associated retention time and peak areas. Subsequently, the peak areas of all the compounds were firstly standardized with the total mass of honeydew collected and then normalized by adding +1 and log_10-transformed to stabilise residual variance. Principal components analysis (PCA) plots for the three first principal components were performed using prcomp package (Sigg and Buhmann, 2008). Subsequently, data were analysed for each volatile compound using ANOVA for the two main effects and their interactions. Specifically, the model was as following: aov(datavolatile1 [[m]] ~ h.defensa*genotype + Error(block), using block as random factor.

4.4 Results

Experiment 1: honeydew volatile composition

To investigate volatile composition differences between the four clonal lines of *M. euphorbiae*, firstly, honeydew was collected, and total mass measured. Honeydew production varied between genotypes ($F_{1,116}$ = 9.29, P = <0.01; Fig.4.2; Table 4.1), but was not significantly affected by *H. defensa* infection (Table 4.1)**.**

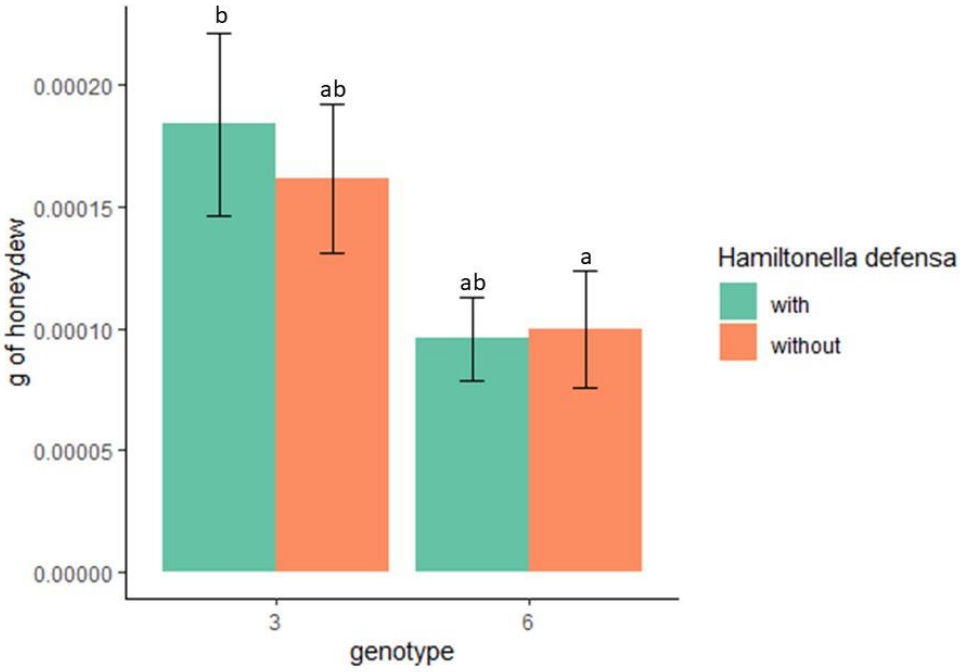

Figure 4.2 Total mass of honeydew (g) per aphid collected after 36 h period for genotype 3 and 6 aphids of M. euphorbiae infected with *H. defensa* (with) and uninfected (without). Bars that share the same letter are not significantly different from each other. Values are means (± SEM) of n=3.

Table 4.1 Statistical results of ANOVA test of total mass (mg) of honeydew collected (n= 3) for SPME analysis. Bold text indicates significant p values.

Variable	**df**	***F***	***P***
Genotype	1	9.298	**<0.01**
Hamiltonella defensa	1	0.642	0.424
Genotype * *Hamiltonella defensa*	1	0.002	0.959
Residuals	116		

In order to detect volatile compounds from honeydew samples SPME technique was used. By employing the SPME PDMS fibre assembly, a total of 26 volatiles were detected. Significant effects of aphid genotype and *H. defensa* infection were detected only for compound, 10-18-bisnorabieta-8-11-13-triene, and no differences were found for the remaining 25 compounds as shown in the Supplementary Table 4.4.

The principal component analysis (PCA) was carried out in order to elucidate differences or grouping within the detected volatiles on the first three principal components. No significant grouping with respect to aphid genotype or *H. defensa* infection was observed (Fig.4.3; Fig.4.4; Fig.4.5).

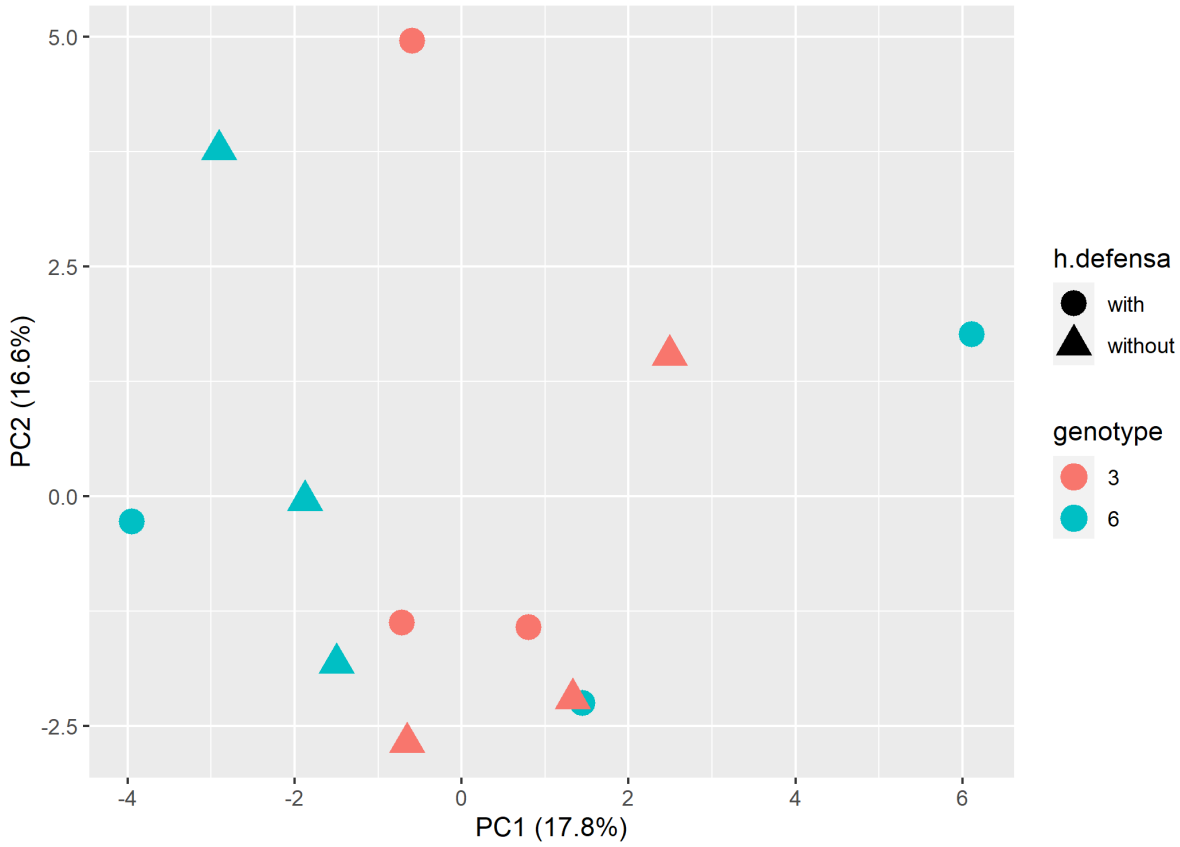

Figure 4.3 Principal components analysis (PCA) showing the composition of volatiles emitted by the honeydew of *Macrosiphum euphorbiae* aphids of genotype 3 and genotype 6 with and

without *H. defensa* infection. Percentage values indicate the %variation explained by the first two principal components.

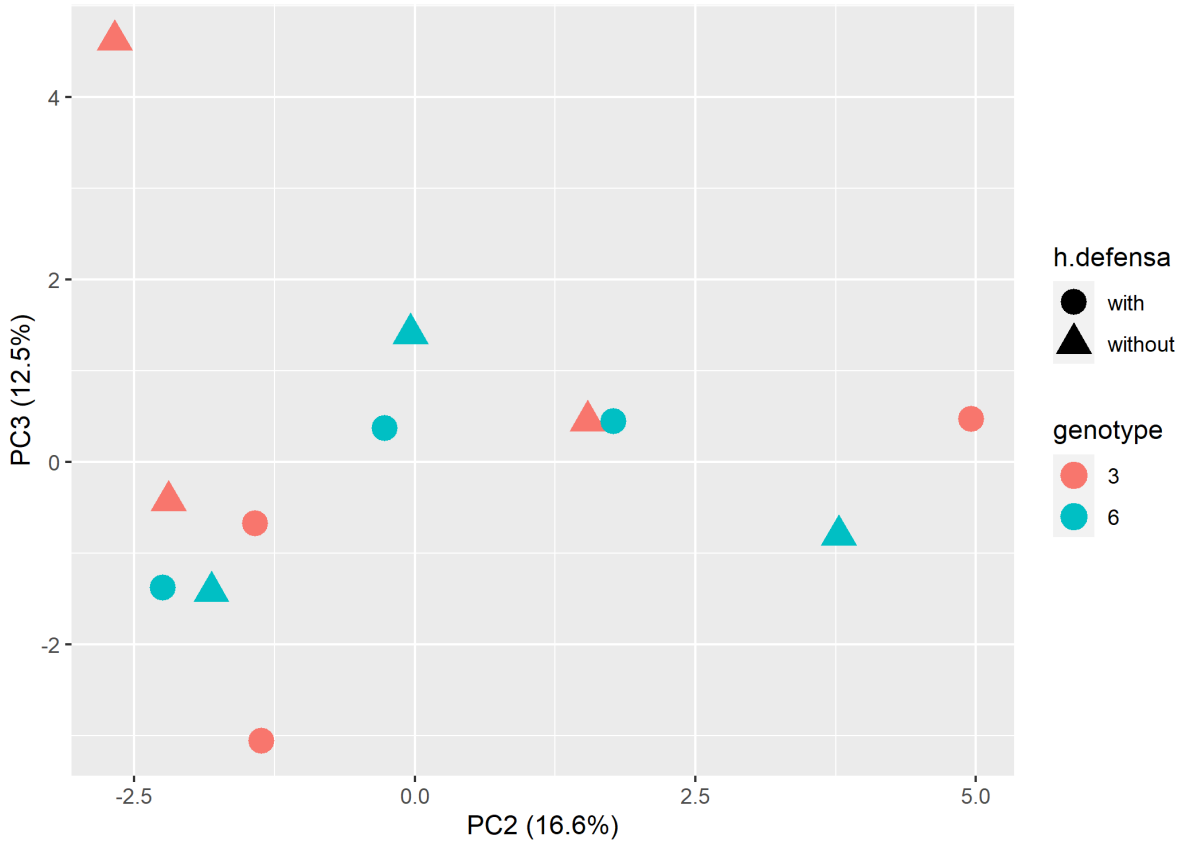

Figure 4.4 Principal components analysis (PCA) showing the composition of volatiles emitted by the honeydew of *Macrosiphum euphorbiae* aphids of genotype 3 and genotype 6 with and without *H. defensa* infection. Percentage values indicate the %variation explained by the second and third principal components.

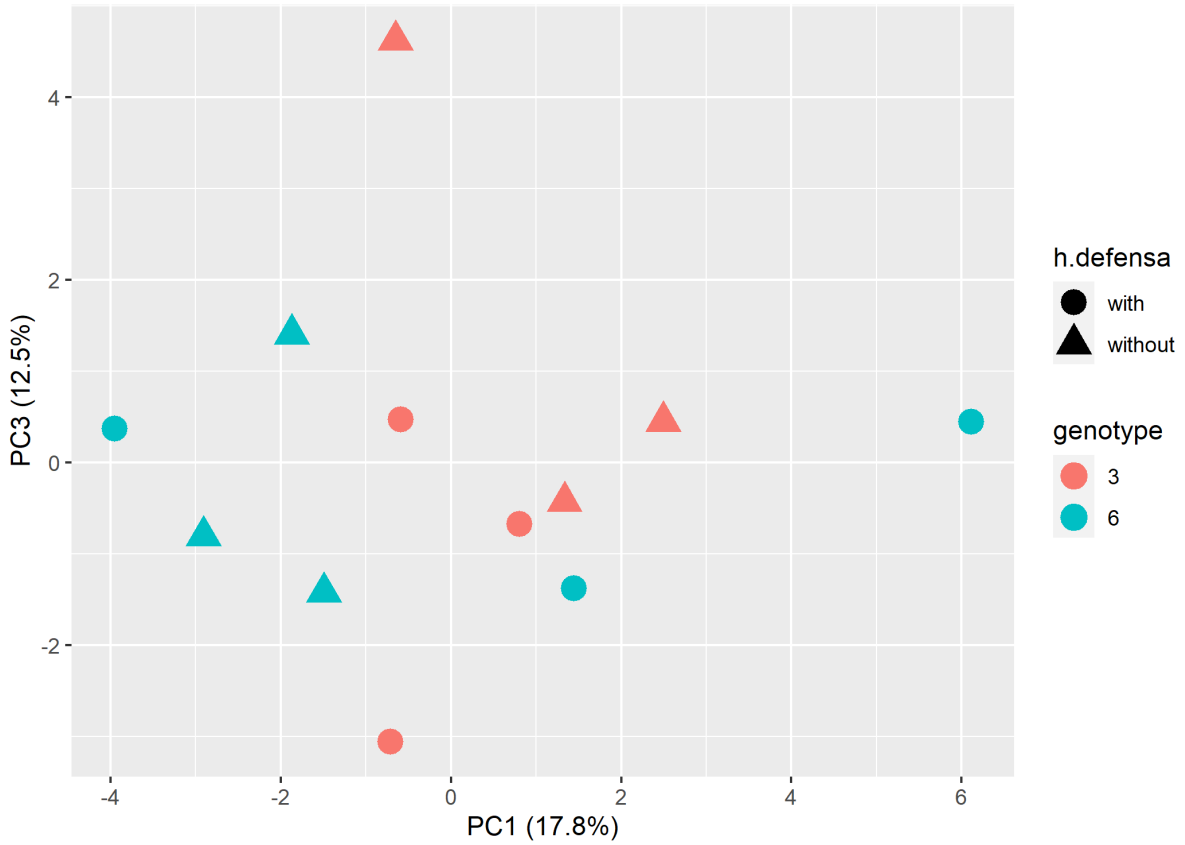

Figure 4.5 Principal components analysis (PCA) showing the composition of volatiles emitted by the honeydew of *Macrosiphum euphorbiae* aphids of genotype 3 and genotype 6 with and without *H. defensa* infection. Percentage values indicate the %variation explained by the first and third principal components.

Experiment 2: insect choice test

Regarding the total amount of honeydew produced for the choice test experiment, there was a significant interaction between aphid genotype and +/- *H. defensa* infection on the final amount of honeydew (g) recorded (Fig.4.6, Table 4.2). Tukey's HSD post-hoc analysis showed significantly more honeydew was produced by genotype 3 infected with *H. defensa* and significantly less honeydew was produced by uninfected genotype 6 aphids (Fig.4.6).

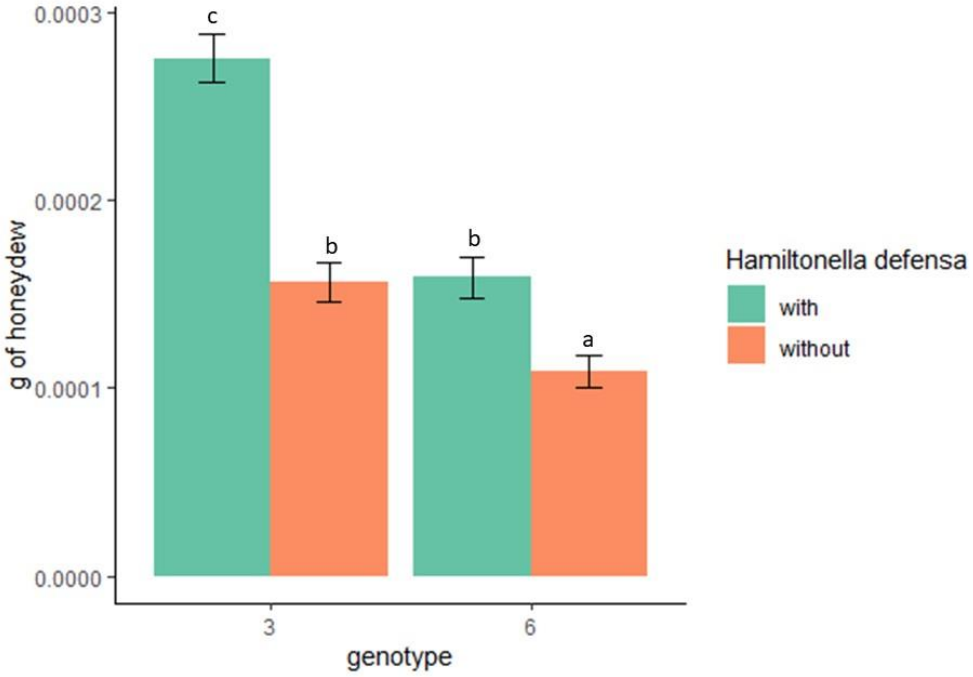

Figure 4.6 Total mass of honeydew (g) per aphid collected after 36 h period for genotype 3 and 6 infected with *H. defensa* (with) and uninfected (without). Bars that share the same letter are not significantly different from each other. Values are means (± SEM) of n=16.

Table 4.2 Statistical results of ANOVA test of total mass (g) of honeydew per aphid collected (n= 16) for the choice test. Bold text indicates significant p values.

Variable	**df**	***F***	***P***

Genotype	1	52.9544	**<0.001**
Hamiltonella defensa	1	62.5321	**<0.001**
Genotype* *Hamiltonella defensa*	1	8.5483	**<0.01**
Residuals	636		

Aphidius ervi did not show any significant preferences when exposed to honeydew from genotype 3 or genotype 6 infected and uninfected with *H. defensa (*Fig 4.7) with GLMER for infected vs uninfected honeydew: $z=3.469$, $p<0.001$. In both cases, the intercept was positive for both genotypes suggesting that the effect is being driven by the honeydew quantity GLMER for ratio of honeydew produced by infected/uninfected aphids: $z= -3.379$, $p<0.001$ (Fig.4.7).

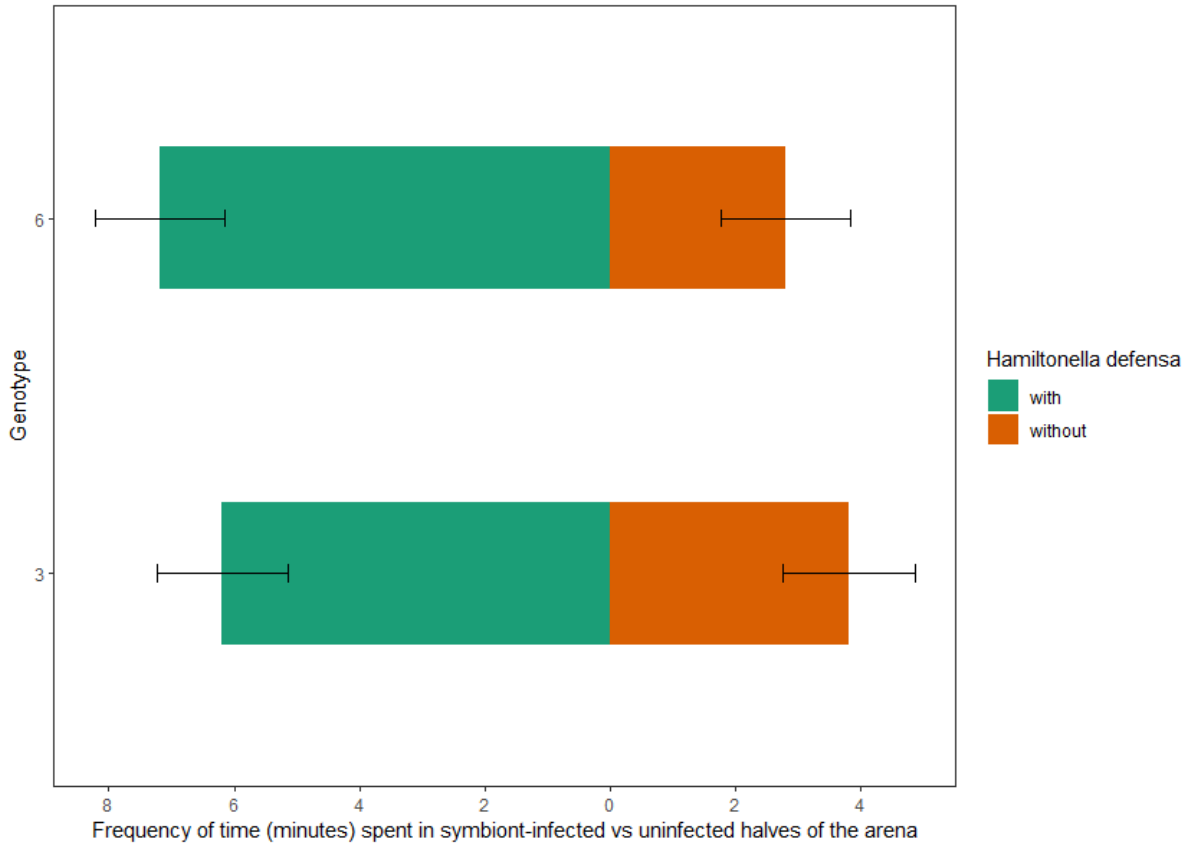

Figure 4.7 The frequency (each minute, in a ten minute observation period) spent by individual *Aphidius ervi* wasps in each half of the arena when given a choice between honeydew collected from potato aphids infected with *H. defensa* and from uninfected aphids in two aphid genotypes. Values are means (± SEM) of n = 16 for all choice tests.

4.5 Discussion

The present study is the first work undertaken to define and investigate the role of the facultative endosymbiont, *Hamiltonella defensa,* on the composition of volatiles emitted by aphid honeydew in the potato aphid, *Macrosiphum euphorbiae,* and its attractiveness towards its natural enemy *Aphidius ervi*. Honeydew was collected from two aphid genotype clonal lines each with and without *H. defensa* infection and their volatile composition was analysed. Next, parasitoid preference for honeydew produced by aphids with or without *H. defensa* infection was monitored. The key findings of this study the total quantity of honeydew produced by potato aphids differed between two aphid genotypes, and honeydew quantity was larger for endosymbiont infected aphids in one of the two experiments. There were no effects of aphid symbiont infection on parasitoid choice, although differences were observed between the two aphid genotypes. Neither aphid genotype nor symbiont presence nor their interaction affected the composition of honeydew volatiles, indicating the amount of honeydew was the primary driver of parasitoid choice.

Genotype and infection with secondary endosymbiont affect honeydew production

The observation that *H. defensa* infection was associated with lower honeydew production, at least in one experiment, aligns with previous studies where effects of aphid facultative endosymbionts on aphid-plant interactions have been observed. Endosymbionts play a key role in acquisition of nitrogen by their aphid hosts (Baumann et al., 1998), and there is some evidence for symbionts affecting aphid honeydew composition. In a study of the effect of secondary endosymbionts (*Hamiltonella defensa* and *Regiella insecticola)* on honeydew composition of the black bean aphid, *Aphis fabae*, it was revealed that symbiont-infected aphids did not impact the final amount of carbohydrates produced. Symbiont-infected aphids, however, produced honeydew containing lower concentrations of total amino acids, suggesting a physiological cost of harbouring endosymbionts (Schillewaert, 2017). The present study detected larger amounts of honeydew produced by symbiont infected aphids, which might result from an energetic cost of symbiont infection that stimulates aphid feeding.

Honeydew comprises plant phloem sap that has been processed by the aphid gut and is dominated by oligosaccharide sugars synthesised by aphids (Auclair, 1963). Few studies have explored honeydew quantities, and these studies are typically focussed on the context of mutualistic relationship with ants, (Detrain et al., 2010), showing that ant-tended aphids typically produce more honeydew in the presence of ants than when ants are absent (Fischer

and Shingleton, 2001). These studies, however, also highlight aphid age and size-specific patterns in honeydew production (Mittler, 1958). Experiments with five different age classes of aphids revealed that older age classes (adult stage) produced twice as much honeydew per hour as younger nymphal stages (Fischer et al., 2002). Other studies have highlighted that aphid species and clonal lines could influence the production of honeydew (Monticelli et al., 2020). For example, in the experiment of Völkl et al. (1999), four different aphid species feeding on the same plant species produced different quantities of honeydew, which also differed in composition. Clonal variation in aphid growth rate, development time and insect mass is commonly observed in studies of aphid fitness, and these factors affect aphid physiological processes that result in variation in feeding and honeydew production (e.g. Karley et al., 2002), as observed in the present study.

Another factor that might influence honeydew production is plant structural part, which is not well explored. In the experiment conducted by Maxwell and Palinter (1959), honeydew deposition by greenbug (*Toxoptera graminum* (Rondani)) and spotted alfalfa aphid (*Therioaphis maculata* (Buckton) were compared in relation to several parameters including different feeding sites: stem, petiole and leaf. In this study honeydew production of alfalfa aphids was significantly impacted by the feeding site on the plant (higher excretion of honeydew was observed for aphids feeding on the leaf and stems compared with the amount recorded from petioles), by leaves yellowed by age, and by different varieties of plants. Further research, however, would be required to determine which of these aphid and plant factors explain greater production of honeydew quantities by some aphid genotypes and in the presence of symbiont infection.

Honeydew amount is a key determinant in wasps searching behaviour

In 1956, Zoebelein, reported a broad number of parasitic Hymenopteran species visiting honeydew produced by aphids. Since then, this carbohydrate source has been shown to provide a supplementary food source for predators and parasitoids, influencing insect longevity and fecundity (Hogervorst et al., 2007; Lenaerts et al., 2016). Thus, honeydew acts as a foraging cue for natural enemies (Purandare and Tenhumberg, 2012). In this study *Aphidius ervi* spent more time foraging in the arena half with the larger amount of honeydew. The results are align with similar findings by Bouchard and Cloutier (1984) although the authors used a different parasitoid species, *Aphidius nigripes*. Their study showed that *A. nigripes* searched significantly longer in plants where larger quantities concentrations of honeydew from three

different aphid species (*Aphis nasturtii*, *Macrosiphum euphorbiae*, or *Myzus persicae)* were applied on the plants. Moreover, the authors hypothesised that parasitoid responsiveness might decrease below a specific threshold quantity of honeydew, obtained through calculation under laboratory conditions with heavily infested plants (~ 2.94 mg/cm^2); however, to our knowledge this hypothesis has never been tested and such concentrations are unlikely to occur under field conditions due the variability of aphid infestation. In a recent study, adult hoverflies of *Episyriphus balteatus* were used as predators of the pea aphid *A. pisum*, and it was shown for the first time that the strain of *Staphylococcus sciuri*, a host-associated bacterium in the aphid gut-flora and present in the aphid honeydew, produced odorant volatiles that significantly affected syrphid foraging and oviposition behaviour in a wind tunnel assay (Leroy et al., 2011a), in line with previous findings on the same strain of bacteria (Schulz et al., 2007). A further key finding of the present study is related to indirect effects of endosymbionts on aphid fitness. Here, aphids infected with *H. defensa* produced more droplets of honeydew leading to a higher attractiveness toward *A. ervi*. It has been already reported that the facultative endosymbiont *H. defensa* provides little protection to *M. euphorbiae* against parasitism by *A. ervi* (Clarke et al., 2017; Oliver et al., 2005). The indirect cost of harbouring *H. defensa* shown in the present study (indicated by reduced honeydew production) could be further investigated for its potential use in improving biological control of aphids, for example by developing parasitoid strains more efficient at detecting honeydew from aphids. Experimental work using different predators and aphid species would be a useful next step to identify mechanisms by which honeydew volatiles could be used to attract natural enemies and improve biological control of aphid pests.

Detection of volatile compounds using SPME technique

The solid-phase microextraction (SPME) technique is a fast, simple and solvent-free method that, thanks to the appearance of different types of adsorbents with a wide range of polarities, can trap compounds from diverse substrates (Arthur and Pawliszyn, 1990). In this experiment, the SPME fibres trapped 26 different volatiles released by *M. euphorbiae* honeydew from symbiont-infected and uninfected aphids, including aldehydes, terpene, and sesquiterpenes. Several interesting peaks were found at later retention times shown in Supplementary Table 3, although identification was not possible because some compounds were not present in the reference library. No differences in honeydew VOCs were detected between the treatments

(aphid genotype or symbiont presence/absence). It is, however, quite likely that small differences in VOC composition were undetectable due to the low levels of replication (n=3) combined with the high degree of biological variability between samples.

Additionally, compounds detected in our experiment, such as *β*-Farnesene, are important chemical cues that mediate trophic interactions (Francis et al., 2004). Specifically (*E*)-β-Farnesene, can have two origins: from aphids or produced by attacked plants to attract natural enemies (Francis et al., 2005; Wang et al., 2022). Volatile compounds detected in this study are not in accordance with previous research (Leroy et al., 2012, 2011a), where the volatile compounds identified were dominated by degraded or modified products of honeydew sugars. However, overall studies on volatiles are still challenging due to the complexity of the biological samples associated with the different methods and the availability of comprehensive reference libraries for volatile compounds. The latter factors can impact detectability of compounds and it might be possible that among our unidentified compounds primary insect volatiles were present.

4.6 Conclusion

In conclusion, the results reported here contribute to the field of research on endosymbiotic bacteria of aphids and their indirect effects on the behaviour of aphid natural enemies. I hypothesized that *H.defensa* infection of aphids would affect honeydew volatile composition, but I found that no significant variation was detected overall in the volatile blends from two aphid genotypes with and without symbiont infection. My study did, however, show that one aphid genotype (genotype 3) produced more honeydew than another (genotype 6), and that symbiont infected aphids sometimes produced more honeydew than uninfected aphids. As parasitoid wasps were more attracted to larger quantities of honeydew produced by genotype 3 compared with genotype 6, likely due to the greater quantity of VOCs released rather than differences in volatile composition, these findings suggest that aphid genotype and symbiont infection could interact to influence aphid susceptibility to parasitoid attack. Further experimental investigation is required to understand the underpinning mechanisms and the implications of these findings in the context of aphid biological control.

4.7 Supplementary Material

Supplementary Table 4.1: Primer names, targets, 5’ – 3’ sequence, use and source for all primers used in this study for genotyping *M. euphorbiae* lines using microsatellite markers and for facultative endosymbiont screens.

Primer name	Target	Sequence (5'-3')	Primer Source
Me1F	*Locus*	TTCGCGAAAAACTTTATGACC	Raboudi, et al., 2005.
Me1R	*Me1*	(NED)-TCGCTGCGTTCCTATACTACC	
Me6F	*Locus*	ACTCATTCAAACAAACACGC	
Me6R	*Me6*	(6FAM)- CGTGAAAAGAATTCAATGTTTG	
Me7F	*Locus*	TTAAGTCACTGCCGGTTCG	
Me7R	*Me7*	(VIC)- ATTAGCTCGAGCTCGTAC	
Me9F	*Locus*	AGCGAAACCTCCCCTAATAG	
Me9R	*Me9*	(NED)-GCACAAATAAGCTCGAGTGC	
Me10F	*Locus*	TCGCTGCGAGACTCGTATTG	
Me10R	*Me10*	(VIC)-GACGACGACGTGTACAATG	
Me11F	*Locus*	CGTTTTCTACCCAAAGGAGG	
Me11R	*Me11*	(6-FAM)- ATTGTCCGTATACCACGACG	
Me13F	*Locus*	GAACTCACTCAGACTCGTGTGG	
Me13R	*Me13*	(6-FAM)- CAGCCGGAATACCAAGAGC	

16SA1	*16S rDNA (Positive for aphid primary endosymbiont)*	AGAGGTTGATCMTGGCTCAG	Fukatsu & Nikoh (2000)
16SB1		TACGGYTACCTTGTTACGACTT	
PABSF	*Hamiltonella defensa 16S rDNA*	AGCGCAGTTTACTGAGTTCA	Darby & Douglas (2003)
1507R		TACCTTGTTACGACTTCACCCCAG	Sandström et al., 2001
16SB1		TACGGYTACCTTGTTACGACTT	Fukatsu & Nikoh (2000)

Supplementary Table 4.2: Thermocycling conditions for genotyping and for diagnostic PCR of aphid facultative endosymbionts.

Target	Time	Temperature (°C)	Cycle	Action
Genotyping	2 minutes	94	1 cycle	Denaturation
	30 s	55		Annealing
	45 s	72		Extension
	15 s	94	1 cycle	Denaturation
	30 s	53		Annealing
	45 s	72		Extension
	15 s	94	1 cycle	Denaturation
	30 s	51		Annealing
	45 s	72		Extension
	15 s	94	1 cycle	Denaturation
	30 s	49		Annealing
	45 s	72		Extension
	25 s	94	30 cycles	Denaturation
	30 s	47		Annealing
	2 minutes	72		Extension
16S rDNA	5 minutes	95	-	Initial denaturation
	30 s	95	35 cycles	Denaturation
	45 s	60		Annealing
	45 s	72		Extension
	7 minutes	72	-	Final extension
16S rDNA for *H. defensa*	2 minutes	95	-	Initial denaturation
	30 s	95	35 cycles	Denaturation
	30 s	55		Annealing
	3 minutes	72		Extension
	7 minutes	72	-	Final Extension

Supplementary Table 4.3: Compounds identified in honeydew volatiles samples by SPME-GC-MS. RT and m/z stands respectively for retention time mass and charge number of ions.

No.	Compound	RT(min)	*m/z*
1	Hexanal	5.23	39, 41, **44**, 56, 67, 72, 82
2	Ethylbenzene	5.73	44,77,**91**,106
3	Ethylbenzene	6.38	**91**, 106
4	Heptanal	7	41,43,68,**70,** 71,81,96
5	2-heptanone, 3-methyl	7.75	**43**, 57, 72
6	Benzaldehyde	8.65	51.10,74.03,**77.08**,78.09,**105.07, 106**
7	Octanal (CAS)	8.74	**41.07**,43.09,57.10,69.12,82.13,84.14,85.14,95.09,110.11
8	1-hexanol-2-ethyl	9.43	45.14,**57.13**,70.11,**72.11,**73.10,83.11,98.12,104.12,133.04,341.09
9	Unknown 9.78	9.78	41.10,56.15,**57.12**,67.11,81.10,82.14,83.14,96.15,109.14,124.20
10	Benzeneacetaldehyde	10.17	39.08,63.06,65.10,89.08,**91.07**,92.11,120.07
11	Benzeneacetaldehyde	10.19	39.08,63.06,65.08,89.10,**91.06**,92.12,120.08
12	Nonanal	10.34	41.07,55.10,**57.11**,70.12,82.15,98.14,114.12,124.16
13	Unknown 10.5	10.5	51, 65, 77, **79**, 105, 107, 108
14	Unknown 10.57	10.57	51.05,77.07,**79.09**,91.05,107.07,108.09,125.99,193.01,250.96,267.00,283.06

15	Cyclopentane.butyl-	11.02	41, 43, 55, **69**, 70, 83, 97
16	Decanal (CAS)	11.13	41.06,43.08,57.09,**69.12**,82.13,95.12,96.14,110.11,123.10
17	Decanal (CAS)	11.31	41.07,57.13, **69.14,**82.13,95.09,110.15,123.13,128.16,138.17
18	2-Nonenal	11.57	41.09,43.10,55.08,69.14,**70.12**,83.13,97.13,207.05
19	Decanal(CAS)	11.83	**41.04**,43.10,67.10,70.13,82.13,95.09,112.14,128.12,138.12
20	Unknown 12.47	12.47	38.99,55.06,67.12,**69.11**,71.11,82.18,83.16,96.20,109.12,111.13,137.08,194.35,325.34
21	Unknown 12.84	12.84	41.05,57.09,67.09,70.09,81.11,95.13,97.09,**123.09**,124.17,141.06
22	Undecanal(CAS)	13.24	**41.06**,43.10,57.09,67.09,71.13,82.14,96.12,97.13,110.13,126.11,142.17
23	Ethanol, 2-phenoxy-	13.377	44.0,51.02,64.93,77.02,**94.04**,95.08,107.15,138.15,139.34,
24	Beta-elemene	13.72	**40.18,**44.05,65.20,67.13,68.11,79.15,93.11,107.45,121.05,133.44,147.21,161.31,175.44,189.34,255.18
25	Alpha-gurjunene	13.77	**40.01**,42.97,67.20,91.00,105.15,119.10,133.11,147.22,147.22,161.14,175.01,189.18,204.20,205.23
26	Unknown 13.887	13.887	43.03,51.21,60.13,**73.30,**87.28,98.63,129.20,189, 192.96
27	Dodecanal	14.08	41.09,44.06,57.16,**69.10,**82.14,**95.16**,97.16,109.12,119.05
28	trans-Caryophyllene	14.17	41.08,55.16,65.08,67.10,69.09,79.10,91.10,**93.06**,105.10,107.16,133.12,148.16,161.13,175.15,189.22,204.19
29	*β*-sesquiphellandrene	14.34	44.03,65.07,67.12,**69.10**,77.03,91.11,105.13,119.10,133.12,147.17,**161.17**,204.21
30	Beta-farnesene	14.43	41.06,67.10,**69.09**,79.09,93.09,107.12,120.11,133.08,161.13,204.21
31	Propanoic acid 2-methyl- 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	14.51	**71**, 83, 98
32	Dodecanal	14.56	43.09,57.11,67.12,68.15,**82.13,**96.16,97.17,110.15,111.13,140.16,166.14

33	Propanoic acid 2-methyl- 3-hydroxy-2,4,4-trimethylpentyl ester	14.76	**71**, 89, 143, 173
34	trans-β-Farnesene	14.87	41.08,55.11,67.08**,69.12**,91.08,93.11,105.11,120.09,133.12,161.16,204.23
35	(Z,E)-α-farnesene	14.91	**41.05**,67.12,69.08,79.10,93.07,107.13,119.06,120.07,161.29
36	GERMACRENE-D / alpha muurolene	15.04	39.17,44.05,57.49,65.14,79.13,91.10,**105.11**,119.13,133.10,147.18,161.17,204.22
37	Unknown 15.29	15.29	**44.09**,55.15,67.25,79.13,119.29,133.21,161.18,162.46,204.95
38	trans-geranylacetone	15.316	**43**, 67, 69, 80, 105, 136, **151**
39	*β*-sesquiphellandrene	15.41	69, 93, 105, **133,** 204
40	1-Dodecanol (CAS)	15.48	41, 43, 68, **69**, 83, 97, 111, 112, 140
41	Hexadecane(CAS)	15.69	43.12,57.06**,71.06**,85.12,99.20,126.80,155.14,
42	Benzene, (1-pentylhexyl)-	16.56	55.92,71.04,83.06,**91.06**,105.14,111.13,122.07,140.93,161.10,162.18,203.67,232.37,281.11
43	Benzene, (1-butylheptyl)-	16.61	40.02,44.03,**91.08,**105.13,147.34,175.21,232.29
44	Benzene, (1-propyloctyl)-	16.74	39.94,43.89,77.19,**91.05,**105.09,119.12,133.04,134.29,189.31,232.29
45	Unknown 16.8	16.8	43, 57, **71**, 85, 91, 99
46	Propanoic 2-methyl.1-(1,1-dimethylethyl)2-methyl-1,3-propanediyl ester	16.95	43.08,**71.09**,72.07,111.14,159.11,243.16
47	Benzene, (1-ethynonyl)-	16.99	44.07,71.09,**91.07**,105.21,119.06,203.19,232.24
48	VERIDIFLOROL /viridiflorol	17.1	**43.06,**54.97,67.03,69.10,81.07,93.07,105.08,107.12,109.12,122.11,133.11,149.34,161.09,189.16,204.20
49	Benzene, (1-butyloctyl)-	17.69	39.99,69.08,**91.08**,105.08,119.10,147.14,189.23,207.17,246.38
50	Benzene, (1-ethyldecyl)-	18.1	**44.11,**55.08,68.92,82.26,91.08,105.10,119.16,150.84,217.19,246.18

51	Benzene, (1-pentyloctyl)-	18.67	39.16,55.12,67.18,**91.11**,105,119.01,105.09,119.01,149.18,161.23,206.43,260.38
52	Benzene, (1-butylnonyl)-	18.74	**91,** 147, 173, 203, 260
53	Unknown 18.79	18.79	**40.00**,55.18,69.16,91.11,96.00,173.11,203.31
54	Unknown 19.08	19.08	43.98,67.16,69.06,79.09,95.10,109.08,**122.20**,123.20,149.08,152.31,164.00,213.22,248.20
55	Unknown 19.27	19.27	40.00,55.07,67.10,81.12,95.08,96.15,**122.**14,124.20,151.10,152.27,248.16
56	Unknown 19.59	19.59	**44.10**,51.18,77.15,82.22, 97.14,108.21,136.27,207.07
57	Unknown 19.61	19.61	41.12,55.06,69.11,76.97,91.11,**95.20**,110.11,123.21,137.66,150.15,150.15,179.43,248.82,260.30
58	Unknown 19.65	19.65	**40.04**,67.10,121.15,165.19,191.20,246.29
59	Unknown 20.26	20.26	**43**, 55, 67, 81, 95, 109, 121
60	Unknown 20.4	20.4	**40.21**,43.43.10,78.09,81.12,97.15,121.15,165.19,191.34,207.34,246.25
61	Unknown 20.63	20.63	44.10,67.05,79.12,91.10,107.11,**109.09**,110.12,149.08,207.14,216.18
62	2-propanol, 1-chloro-,phosphate (3:1)	20.71	44.01,67.07,81.13,95.13,98.97,109.06,121.10,125.03,149.03,156.81,159.12,175.11,201.07,207.11, 241.26,**277**.00,279.00
63	10,18-bisnorabieta-8,11,13-triene	21.42	44, 81, 143, 207, **227**, 242
64	Unknown 22.05	22.05	44.05,67.04,91.09,119.09,134.15, **135**; 163.11,203.39,270.32
65	Unknown 22.075	22.075	41.24,44.07,66.03,79.10,91.14,104.01,117.13,134.16,**135.12,**161.13,201.18,270.36
66	Unknown 22.082	22.082	44.06,77.45,79.09,93.10,105.15,**134.11**,148.17,209.14,270.92,281.09
67	Unknown 22.18	22.18	**44.04**,66.11,79.12,91.12,93.15,105.04,134.11,135.17,136.22,207.11,281.07
68	Unknown 22.34	22.34	44.05,68.08,77.14,79.22,95.09,107.03,**135**.43,149.30,161.34,201.29
69	Unknown 22.42	22.42	**44.01**,67.12,79.13,93.07,107.13,134.16,163.19,174.20,201.25,207.07,281.08
70	2,2'biadamantanylidene	22.74	43.93,65.10,81.21,105.11,107.15,**135.20,**160.24,174.24,200.22,253.17, **268**

71	Unknown 23.06	23.06	41.05,66.10,67.08,79.12,91.09,93.09,105.08,133.13,134.17,160.17,199.20,201.22,227.22,240.25,**268.27**,269.25
72	Unknown 23.15	23.15	66.10, 77.15, **91.09,** 117.11, 133.13, 135.18, 161.10, 200.23, 207.07
73	Unknown 23.41	23.41	**44**, 67, 79, 91

Supplementary Table 4.4: Anova results for the interaction between aphid genotype and *H. defensa* infection status on the abundance of 26 volatile compounds, standardised per unit mass of honeydew, emitted by aphid honeydew detected using SPME fibres. Significant values are reported in bold. *The letter p, in the annotation, stands for point.

Compound	**Decanal_A**			**Unknown_12p47**		**Unknown_13p887**		**Unknown_14p08**		**Trans-caryophyllene**	
Variable	df	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
h.defensa	1	0.642	0.449	0.018	0.896	0.736	0.419	1.177	0.314	0.310	0.595
genotype	1	0.339	0.579	2.174	0.143	0.755	0.414	0.001	0.918	0.295	0.604
h.defensa:genotype	1	0.549	0.483	3.021	0.126	1.006	0.349	1.320	0.288	0.374	0.560
Residuals	7										
Compound	**B-farnesene**			**Trans-b-farnesene**		**z-e-alpha-farnesene**		**Germacrene-d-alpha-muurolene**		**Beta-sesquiphellandrene**	
Variable	df	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
h.defensa	1	0.553	0.481	0.056	0.820	2.897	0.133	0.908	0.372	0.000	0.998
genotype	1	0.383	0.555	0.231	0.645	0.007	0.936	0.910	0.372	0.001	0.977
h.defensa:genotype	1	2.244	0.178	0.106	0.755	0.000	0.991	1.005	0.349	0.000	0.990
Residuals	7										
Compound	**Viridiflorol**			**Unknown_19p08**		**Unknown_19p27**		**Unknown_19p61**		**Unknown_20p26**	
Variable	df	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
h.defensa	1	0.046	0.836	0.210	0.660	0.172	0.691	0.143	0.716	0.875	0.381
genotype	1	1.017	0.347	0.452	0.523	0.178	0.685	0.132	0.727	0.875	0.381
h.defensa:genotype	1	1.124	0.324	3.227	0.115	2.561	0.154	0.109	0.751	0.875	0.381
Residuals	7										
Compound	**Unknown_20p63**			**10-18-bisnorabieta-8-11-13-triene**		**Unknown_22p05**		**Unknown_22p075**		**Unknown_22p082**	
Variable	df	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
h.defensa	1	0.788	0.4040	10.893	**0.0131**	0.004	0.95	0.000	0.997	1.086	0.332
genotype	1	1.174	0.3144	5.588	**0.0501**	0.014	0.91	0.035	0.856	1.062	0.337
h.defensa:genotype	1	5.099	0.0585	0.002	0.9702	3.338	0.11	3.308	0.112	1.357	0.282
Residuals	7										
Compound	**Unknown_22p34**			**Unknown_22p42**		**2-2-biadamantanylidene**		**Unknown_23p6**		**Unknown_23p15**	
Variable	df	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
h.defensa	1	0.390	0.5521	0.875	0.381	0.000	0.990	0.813	0.397	0.160	0.7014
genotype	1	3.818	0.0916	0.875	0.381	1.764	0.226	0.594	0.466	0.226	0.6488
h.defensa:genotype	1	4.339	0.0758	0.875	0.381	0.062	0.811	0.345	0.575	4.103	0.0824
Residuals	7										
Compound	**Unknown_23p41**										
Variable	df	F	*p*								

h.defensa	1	0.013	0.912
genotype	1	0.002	0.966
h.defensa:genotype	1	0.013	0.914
Residuals	7		

Chapter 5. Does the soil microbiota impact aphid biocontrol on potato*?*

Abstract
Agriculture practices can change the composition of soil microbiome communities and affect the ecosystem services delivered by soil microbes. Soil organisms can influence the performance of insect herbivores feeding aboveground, but only a few studies have investigated their effects on the third trophic level – the natural enemies of insect herbivores. We examined soil-mediated effects on herbivore performance by growing potato plants (*Solanum tuberosum* L.) in the field soil that had been managed either conventionally with high input levels or with fewer inputs and integrated management. Firstly, population increase of the potato aphid *Macrosiphum euphorbiae* (Thomas) was assessed on plants grown in each soil in a glasshouse experiment. Secondly, the parasitoid wasp *Aphidius ervi* was given a choice of aphid-infested plants grown in conventional soil and integrated soil. Finally, soil chemical properties were analysed, and the soil microbial community was characterised using 16S rRNA amplicon-sequencing. Total number of nymphs did not differ between the two soil treatments. In the choice assay, parasitoids wasps were more attracted toward plants grown in integrated soil. For the soil chemical profile, only total organic carbon was affected by aphid presence, but no other differences were detected. Using 16s rRNA sequencing, differences in the soil microbial community were found between conventional and integrated soils. Taken together these results suggest that soil management strategies can improve an important ecosystem service, aphid biocontrol, in potato crops.

Keywords Aboveground-belowground interactions, *Aphidius ervi*, integrated pest management, potato aphid, 16s rRNA, soil microbiome, *Solanum tuberosum*

5.1 Introduction

Modern agriculture faces critical challenges in containing losses caused by insect pests and, therefore, novel and sustainable strategies are needed. In recent years, there has been increasing interest in using soil beneficial microbes as biocontrol agents for achieving sustainable agriculture (Nath et al., 2020; Zytynska, 2021). Soil microbial communities are primary drivers of plant productivity and diversity (Lukac et al., 2017) and they play a key role in regulating ecosystem services such as pathogen and pest regulation through direct and indirect mechanisms (Schmidt-Jeffris et al., 2021), and in the restoration of degraded terrestrial ecosystem (Harris, 2009). Alongside driving nutrient cycling of major elements such as carbon (C), nitrogen (N) and phosphorus (P) (Alegria Terrazas et al., 2016; Tkacz and Poole, 2015) which impacts the structure and the function of soil ecosystems (Aislabie and Julie R. Deslippe, 2018), the soil microbiota is responsible for organic matter decomposition (OM) (Cordero et al., 2020) and for physical modification of the soil which can influence moisture content (Saleem et al., 2018). Pivotal soil processes such as nitrogen fixation, denitrification and nitrification are exclusively mediated by soil microbes (Lukac et al., 2017). The effect of soil microbial diversity and composition on plant nutrient uptake is dependent on soil chemistry and nutrient availability, and both interact to alter plant nutritional quality and defensive chemistry in response to environmental stressors, including insect pests (Coban et al., 2022; Pineda et al., 2017).

Multitrophic interactions linking above- and below-ground organisms are capable of shaping the composition and diversity of natural communities (Van Der Putten et al., 2001). For instance, the composition and activity of soil microbes associated with plant roots (=the root microbiome) has been reported in several studies to influence insect herbivores (Shikano et al., 2017; Tao et al., 2017). As an example, inoculation of soil with the bacterium *Bacillus velezensis* decreased growth and feeding rates of *Myzus persicae* aphids on *Arabidopsis thaliana* (Harun-Or-Rashid et al., 2017). In another study, soil inoculation with *Bacillus subtilis* induced resistance against an important agricultural insect pest, *Bemisia tabaci,* in tomato plants (*Solanum lycopersicum)* (Valenzuela-Soto et al., 2010). Large differences in inhibition of herbivore feeding (*Trichopulsia nia* larvae*)* on *Arabidopsis thaliana* were detected when plants were grown in soils that strongly differed in soil microbial community (Badri et al., 2013). Moreover, density of *Aphis jacobaea* feeding on *Senecio jacobaea* depended on the composition of the soil microbial community and thus associated with changes in the

concentration of phloem amino acids (Kos et al., 2015). Finally, another multifactorial experiment demonstrated that conditioning of soil with particular forb and grass species led to the soils harbouring different soil microbiomes that induced resistance towards thrips (*Frankliniella occidentalis)* feeding on *Chrysanthemum*, but not against the spider mites (Ana Pineda et al., 2020).

To date, most of the studies on above-belowground interactions have focused predominantly on the impact of the rhizosphere microbiome on above-ground insect herbivores, while only very few studies have explored influences on the third trophic level and most of these manipulated and correlated single groups of soil organisms such as species of fungi and bacteria (Heinen et al., 2018). As an example, a field study exploring the impact of *Bacillus* spp. on broccoli showed that inoculated plants had fewer ladybird and syrphid visits compared with control plants. Nonetheless, in the same study, percentage of cabbage aphids (*Brevicoryne brassicae)* parasitised by *Diaraetiella rapae* was higher in plants inoculated with either *Bacillus cereus* and *B. subtilis* compared with those that had been inoculated with a mixture of *Bacillus* species (Gadhave et al., 2016). Several studies using a different soil organism, such as arbuscular mycorrhizal fungi (AMF), exhibited high context dependency. Using *Rhizophagus irregularis* on sweet pepper plants both in the field and in a greenhouse experiment showed that the density of ladybird predators was not influenced under greenhouse conditions by AMF symbiosis whereas significantly higher predator density was observed under field conditions where the AM fungal mutualism was present (Balog et al., 2017). However, due to the scarcity of studies, is still difficult to elucidate mechanisms and patterns involved in the interactions between soil microbial communities and the third trophic level.

Another fundamental factor that affects soil community diversity and composition is the farming system and soil management (Martínez-García et al., 2018). For instance, distinct microbiomes are harboured under long-term conventional and organic farming systems (Hartmann et al., 2015; Krey et al., 2019). Organic compost amendment stimulates distinct groups of microorganisms compared with conventional synthetic fertilizers (Islam et al., 2011; Lupatini et al., 2017). Furthermore, off-target effects of pesticides have been shown to affect the soil microbiome and microbes in the phyllosphere (Cernava et al., 2019). These practices influence soil organic matter content, microbial enzymatic activities and nutrient availabilities, which are critical in the cycling of nutrients in soil (Bandick & Dick, 1999). For example, tissue

nitrogen concentrations of plants in organic farms are generally lower compared with those grown in conventional systems (Power and Doran, 1984). Moreover, agriculture practices have been shown to influence the aboveground fauna and the third trophic level (Garratt et al., 2011). As an example, pest populations, in organic management, are generally reduced over the longer term as a consequence of practices that increase predator biodiversity and natural enemy abundance (Zehnder et al., 2007). Specifically, in a meta-analysis where conventional and organic system studies were compared, natural enemy abundance and activity were positively affected by organic management, although the *Coleoptera* group did not follow this pattern (Garratt et al., 2011).

This study aims to unravel the complex interaction of soil chemical factors and microbiome composition with insect herbivores and their natural enemies, mediated through the plant. The study objectives are threefold. Firstly, to investigate how different soil types and their microbial communities mediate the performance of shoot feeding aphids (*Macrosiphum euphorbiae*) on potato plants. Secondly to determine the attractiveness of aphid-infested plants grown in different soils to the generalist parasitoid wasp, *Aphidius ervi*. Finally, to relate insect responses to soil conditions by quantifying soil chemical composition and microbial community using 16S rRNA sequencing. The goal was to identify soil characteristics that can influence the third trophic level.

5.2 Materials and Methods

Soil sampling

The soil used in this study was collected from the Centre for Sustainable Cropping Platform (CSC) based at the James Hutton Institute's Balruddery Farm (Dundee, Scotland). The CSC platform was established in 2009 as long-term platform for interdisciplinary research on sustainable farming (https://csc.hutton.ac.uk/). The CSC platform is a 42-ha contiguous block of six-fields of ~6 ha each in rotation with winter wheat, potato, beans, spring barley, winter OSR and winter barley. In each field, one-half is randomly assigned under conventional crop management practices and in the other field-half an integrated management approach is applied (details related to the management are reported in Table. 5.1). For this experiment, soil samples were taken from a depth of 0-15 cm from the Middle East field, planted with oilseed rape, with coordinates for the conventional treatment - NO 30677 32774 and for the integrated treatment - NO 30882 32784. Soil samples were stored separately at 4 °C after being sampled. Subsequently, soil was sieved to < 3 mm to remove rocks and large debris and homogenize the sample. A 200g fraction of each soil type was stored at −20°C for 16S rRNA analysis of microbial community composition at time zero (T0). Thereafter, 600 ml of each soil type was used to fill sterile square pots (9 x 9 x 10 cm) for the experiment.

Table 5.1 Details of fields preparations, fertilisation and herbicide applications for both conventional and integrated management for the six crops in rotations at the CSC.

	Potato		**Winter Wheat**		**Beans**		**Spring Barley**		**Winter Oilseed**		**Winter Barley**	
	conventional	integrated	conventional	integrated	conventional	integrated	conventional	integrated	conventional	integrated	conventional	integrated
Cultivation timing	Nov-Mar	Mar-Apr	Oct	Oct	Feb-Mar	Mar	Feb-Apr	Mar-Apr	Aug-Sep	Aug-Sep	Aug-Sep	Aug-Sep
Cultivation method	conv	conv	conv	conv	conv	non-inv	conv	non-inv	conv	non-inv	conv	non-inv
Sowing/Planting 1 Sowing/Planting 2	Apr	Apr	Oct	Oct	Mar-Apr	Mar-Apr	Mar-Apr	Mar-Apr clover undersow (Apr)	Aug-Sep	Aug-Sep	Sep	Sep oil radish cover (Aug)
Fertiliser 1 product rate (kg/ha)		compost 3500		compost 3500		compost 3500		compost 3500		compost 3500		compost 3500
Fertiliser 2 product rate (kg/ha)	14-14-21 1400	14-14-21 1050	30-0-0 300	30-0-0 225	0-20-30 200	0-20-30 150	30-0-0 380	30-0-0 285	17-17-17 170	17-17-17 120	0-20-30 300	0-20-30 225
Fertiliser 3 product rate (kg/ha)			30-0-0 300	30-0-0 225			0-0-60 160	0-0-60 120	30-0-0 300	30-0-0 230	30-0-0 300	30-0-0 225
Fertiliser 4 product rate (kg/ha)			30-0-0 300	30-0-0 225					30-0-0 300	30-0-0 230	30-0-0 300	30-0-0 225
Herbicide (pre-em) product rate (per ha)			Liberator 0.6 L	Liberator 0.3 L					Katamaran turbo 2.5 L	Katamaran turbo 1.25 L	Liberator 0.6 L	Liberator 0.3 L
Herbicide (post-em) product rate (per ha)	Stomp, Artist, Reglone, Shark 2.9 L, 2.5 kg, 2 L, 0.3 L	Reglone, Shark 2 L, 0.3 L	Traton SX, Charge 0.04 kg, 1L	Traton SX, Charge 0.04 kg, 1L	Stomp 3 L	Stomp 1.65 L	Traton SX, Charge 30 g, 1 L	Headland spruce 4.5 L	Panarex 1L	Panarex 1L	Traton SX, Charge 0.045 kg, 1 L	Tomahawk Charge 0.045 kg, 1 L
Harvest	Sep-Oct	Sep-Oct	Sep (straw baled)	Sep (straw chopped and incorporated)	Sep-Oct	Sep-Oct	Aug-Sep (straw baled)	Aug-sep (straw chopped and incorporated)	Aug	Aug	Aug (straw baled)	Aug (straw chopped and incorporated)

Plant and insect rearing

Tubers of *Solanum tuberosum* cv Désirée were cut into similar size portions and surface-sterilised by soaking in 0.1% (v/v) sodium hypochlorite for 1 min then rinsed three times using tap water. The tubers were sown in pots containing either conventional or integrated field soil. Plants were grown in a glasshouse with supplementary light (16 h light:8 h dark and 20:15 ℃ day:night), watered daily and no nutrients were added.

Aphids of a clonal line of *Macrosiphum euphorbiae* (DM18/16) were reared on excised leaves of 3-week-old potato plants (*Solanum tuberosum* cv Désirée) contained in ventilated cups. These comprised two Perspex cups (5 cm width x 15 cm depth) placed one inside the other; the petiole of the potato leaves was inserted through a *c.* 5mm circular hole in the base of the inner cup, and the cup opening was sealed with a mesh-ventilated lid. Plant material was refreshed weekly. Using this set up, age-synchronised apterous adults were produced for the experiments. All the insect cultures were maintained at 18 ± 2 °C and 16h:8h (day:night).

Mummies of the Braconid wasp *Aphidius ervi* Haliday, supplied by Fargro (West Sussex, UK), were transferred to plastic ventilated boxes supplied with a ball of cotton wool soaked in a 50 % (v/v) honey solution on which to feed; this was held within a small plastic cup secured to the base of the box with Blu-Tack (Bostik, Stafford, UK), and was replaced daily to prevent fermentation of the honey. Emerging wasps (5- 7 days old) were transferred to *Acyrthosiphon pisum* (pea aphids: clonal line LL01) infested bean plants enclosed in a fine mesh cage. This clonal line harbours no known secondary endosymbionts and has a high reproductive output. After 10 days mummies were collected and transferred back to the ventilated boxes until hatching. All the parasitoid cultures and the pea aphid cultures on which they were reared were maintained in growth cabinets (at 20 °C ± 1 °C, 70 % humidity and with a light regime of 16 h light:8 h dark).

Experimental design

The experiment comprised a 2x2 randomised block design with treatment factors of soil type (conventional or integrated) and aphid (presence or absence). Twelve replicate blocks were staggered over a 7-day period (four blocks per day) giving a total of 48 plants. The first part of the experiment consisted of infesting half of the plants with aphids to test soil treatment effects on aphid fitness and plant and soil responses to aphids. Aphid-infested plants (24 in total) were subsequently used for parasitoid choice tests for a total of 12 choice assays, comparing the two soil treatments. All soil samples were analysed for pH, gravimetric soil moisture content and available phosphorus using the Olsen-P method. For logistical reasons, only the first six replicate blocks were analysed for soil microbial community composition and for dissolved organic carbon (DOC), total nitrogen (TN), total organic nitrogen (TON), ammonium (NH_4^+) and nitrite (NO_2).

Aphid infestation

After 10 weeks of growth, half of the plants were infested with two age-synchronised 7-10 d old adult apterous aphids of *M. euphorbiae* transferred using a fine paintbrush. Subsequently, infested and uninfested plants were enclosed in a microperforated plastic bag (Sealed Air, UK) that allowed ventilation while preventing aphid escape. Seven days following infestation, the microperforated plastic bags were gently removed and total number of aphids (nymphs, adult dead/alive) was noted. Thereafter plants were enclosed in polyethylene terephthalate bags (PET) secured around the pot rim with a rubber band and left for 3 hours, allowing VOCs to accumulate.

Insect choice tests

Two-way choice tests were conducted with adult parasitoid wasps. Experiments were performed between 10:00 and 12:00 h using a two-armed olfactometer, with each arm connected to one of the two plants grown in either the two soil treatments, using the set up described in Slater et al., 2019. Each arm of the olfactometer was inserted into a small hole cut into the corner of the PET bag and sealed using polytetrafluoroethylene (PTFE) tape (Fig. 5.1). The olfactometer chamber was marked on the external surface with a dividing line half-way between the two arms attached to each plant. After three hours, a female parasitoid wasp (2-5 d old) which was starved for a minimum of two hours was placed in the centre of the olfactometer chamber and the position (i.e. in either half of the olfactometer) was noted every minute for a 10-minute observation period. Parasitoid wasps were only used once.

Olfactometers were cleaned after each choice test using a dilute solution of Teepol^{TM}, then rinsed with deionised water.

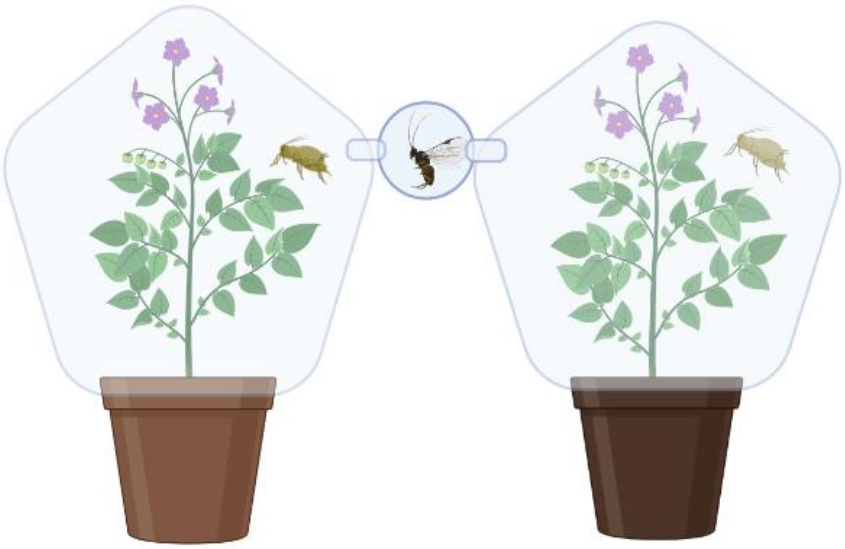

Figure 5.1 The olfactometer set up for choice tests. Plants were covered in PET bags to collect volatiles. Volatiles from the plants passively diffuse into the olfactometer. The parasitoid wasp is introduced to the olfactometer and its position is noted during the observation period. This image was prepared in © BioRender -biorender.com

Rhizosphere fractionation and sampling of soil-grown potato plants and bulk soil

Soil samples were collected for amplicon sequencing following established protocols (Robertson-Albertyn et al., 2017; Alegria Terrazas et al., 2020*). S*oil was sampled from plant pots used in the choice assays, and bulk soil collected at T0 for each soil treatment. For T0 samples, 6 x 250 g aliquots of bulk soil were collected from each soil type after sieving and prior to filling pots. These samples were packed into sterile falcon tubes and stored at -20 ^{o}C. Rhizosphere soil samples were collected from potato plants at the end of the parasitoid choice experiments, when plants were 10 weeks old. Plants were carefully removed from the soil (Fig.5. 2) and the roots were gently shaken to remove loosely bound soil particles from the root system; tightly adhered soil particles, defined as rhizosphere soil, was collected from roots excised from the uppermost 6 cm of the seminal root system of each plant. This root material

was transferred into a sterile 50 mL falcon tube containing 15 mL of phosphate buffered saline solution (PBS). Samples were then vortexed for 30 s, the soil sedimented for 2-3 mins, and the roots transferred into a new 50 mL falcon tube with 15 mL PBS, in which the samples were vortexed again for 30 s to separate the remaining rhizosphere soil from roots. The roots were discarded, the liquid and soil from the two falcon tubes were combined into a single falcon tube, and this rhizosphere soil fraction was centrifuged at 1,500 g for 20 mins. After centrifugation, the supernatant was discarded, and the pellet immediately stored at -80°C. Bulk soil samples (taken at T0) were extracted in the same way.

Figure 5.2 Representative example of potato plant (*Solanum tuberosum*) cv Désirée, used in this study, at the time of sampling for the rhizosphere fraction

DNA extraction from rhizosphere and bulk soil

Total DNA was extracted from the rhizosphere and bulk soil sample pellets using FastDNA™ SPIN kit for soil (MP Biomedicals, Solon, USA) following the manufacturer's instructions. Two DNA extraction controls comprising 15 mL of PBS solution were included to account for background contamination from instruments, reagents, and extraction buffer. To assess the concentration and the purity (260/280 nm and 260/230 nm ratios) of the extracted DNA, a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was used. Aliquots (60 µl) at a final DNA concentration of 10 ng mL^{-1} were prepared using sterilized deionized water, and were stored at −20 ˚C prior to analysis.

16S rRNA gene amplicon library construction

The amplicon library was generated via a selective PCR amplification of the hypervariable V4 region of the 16S rRNA gene using the PCR primers 515F (50 -GTGCCAGCMGCCG CGGTAA-30) and 806R (50 -GGACTACHVGGGTWTCTAAT-30) as previously described (Robertson-Albertyn et al., 2017; Alegria Terrazas et al., 2020). Briefly, PCR primer sequences were fused with Illumina flow cell adapter sequences at their -5' termini and the 806R primers contained 12-mer unique 'barcode' sequence to enable the multiplexed sequencing of several samples in a single pool (Caporaso et al., 2012). For each individual bulk (T0), rhizosphere and control DNA extracts, 50 ng of DNA was subjected to PCR amplification using the Kapa HiFi HotStart PCR kit (Kapa Biosystems, Wilmington, USA).

The individual PCR reactions were performed in 20 µL final volume and contained: 4 µL of 5X Kapa HiFi Buffer; 10 µg Bovine Serum Albumin (BSA) (Roche, Mannheim, Germany); 0.6 µL of a 10 mM Kapa dNTPs solution; 0.6 µL of 10 µM solutions of the 515F and 806R PCR primers and 0.25 µL of Kapa HiFi polymerase. Reactions were performed in a G-Storm GS1 thermal cycler (Gene Technologies, Somerton, UK) using the following programme: 94 ◦C (3 min), followed by 35 cycles of 98 ˚C (30 s), 50 ◦C (30 s) 72 ˚C (1 min) and a final step of 72 ˚C (10 min). For each 515F-806R primer combination, a no template control (NTC) was subjected to the same process. To minimize potential biases originating during PCR amplifications, individual reactions were performed in triplicate and two independent sets of triplicate reactions per barcode were performed. To check the amplification and detect any possible contamination, prior to purification, 6 µL aliquots of individual replicates and the corresponding NTCs were inspected on 1.5% agarose gel. Only samples that display the expected amplicon size and no detectable contamination in NTCs were retained for library

preparation. Individual PCR amplicons replicates were then pooled in a single plate, moving each sample to a specific position according to their barcode. Subsequently, samples were purified using Agencourt AMPure XP Kit (Beckman Coulter, Brea, USA) with 0.7 µL AmPure XP beads per 1 µL of sample. Following purification, the DNA concentration of 6 µL of each sample was quantified using PicoGreen (Thermo Fisher Scientific, Watham, USA). Once quantified, individual barcode samples were pooled to a new tube in an equimolar ratio to generate amplicon libraries. All library QC and processing was carried out in the Genome Technology lab at the James Hutton Institute, Dundee. Illumina-compatible library pools were quality checked using a Bioanalyzer (High Sensitivity DNA Chip; Agilent Technologies) and quantified using both Qubit and qPCR (Kapa Biosystems, Wilmington, United States). Amplicon libraries were supplemented with 15% of 4 pM phiX solution. High-quality libraries were run at 10 pM final concentration on an Illumina MiSeq system with paired-end 2 × 150 bp reads following established protocols for FASTQ file generation (Caporaso et al., 2012).

Raw reads processing

Reads were processed using the DADA2 v1.22 (Callahan et al., 2016) pipeline implemented in R v4.1.2 to remove low-quality data, identify ASVs (Amplicon Sequence Variants) and remove chimeras. Taxonomy was assigned using SILVA v138 (Quast et al., 2013). Reads identified as chloroplasts were removed from the downstream analyses.

Soil chemical analysis

Gravimetric soil moisture content was determined after drying soil samples at 105 °C until dry mass was constant. Soil pH was measured using 10 ± 0.05 g of air-dried soil in a 50 mL centrifuge tube with 20 mL of 0.1 M $CaCl_2$. The suspension was stirred intermittently for 30 min and left to settle for 1 h. pH was read with a calibrated bench top FiveEasy™ Mettler Toledo with a glass electrode probe. Soil P concentration was determined using the $NaHCO_3$ extraction method (Olsen, 1954). Briefly, in 50 mL centrifuge tubes, 0.5 g of wet soil and 5 mL of 0.5 M $NaHCO_3$ solution were shaken for 60 min. The tubes were then centrifuged for 5 min at 5100 rpm. After centrifugation, the supernatant was stored at 4°C until analysis. Phosphate concentrations were determined spectrophotometrically using Malachite Green reagent (Irving and McLaughlin, 1990). Absorbance values were converted to amounts of P using a calibration curve and expressed per unit dry mass. Ammonia-N, NO_3 –N, and total organic C (TOC) were measured using the KCl extraction method. To prepare the extract, 5 g

of wet soil and 25 mL of 1 M KCl were placed in 50 mL centrifuge tubes, and the tubes were shaken (45 rpm) for 45 min and then centrifuged for 5 min at 5100 rpm. The supernatant was stored at 4°C until analysis. Ammonia-N and NO_3 –N were determined spectrophotometrically using a Konelab Aqua 20 Discrete Analyzer, (Thermo Electron Corporation). Total organic carbon (TON) was determined using The Skalar San++ Continuous Flow Analyser (CFA).

5.3 Statistical analysis

All statistical analyses were carried out using R v4.1.3 with additional packages ggplot2 v.2.2.1 (Wickham, 2009), dplyr v. 3.5.3 (Mailund, 2019) , lme4 v.1.1-13 (Bates et al., 2015), lubridate v. 1.7.8, (Spinu et al., 2016), car v. 3.0-7 (Fox and Weisberg, 2011), lsmeans v.2.27-62 (Lenth, 2016) and multcomp v. 1.4-8 (Hothorn, 2008). Data was checked for normality and homogeneity of variance by using Q-Q plots and residuals vs fitted values. Parametric models were applied where data displayed normal distribution. Non-parametric methods were used for analyses where data displayed non-normal distribution.

Aphids, choice test, and soil statistical analysis

Total number of nymphs per plant was analysed by one-way ANOVA. For the choice test experiment, the effect of soil management on the proportion of times (out of ten observations) spent by the wasp in each half of the assay arena (conventional/integrated) was analysed using generalized linear mixed effect models using an additional package "lme4" (Bates et al., 2022). Specifically, the model was as following: no_successes ~ soil_treatment +(1|block), using block as random factor. Subsequently, ANOVA was performed on the model. P in soil was analysed by two-way ANOVA. Gravimetric soil moisture and soil pH were $\log_{10}$ transformed followed by two-way ANOVA. Ammonia-N, NO_3 –N and total organic nitrogen (TON) were $\log_{10}$ transformed and two-way ANOVA was subsequently performed.

16S rRNA statistical analysis

For the 16S rRNA analysis, additional packages used were *phyloseq* (McMurdie and Holmes, 2013) and *picante* (Kembel et al., 2010). Shannon index was calculated using the package *vegan* (Dixon, 2003) and comparison among treatments was performed through linear model, using the function *lm* () specifying the formula ~ Soil * Herbivores. Thus, a multivariate approach was used to study the effects of each factor on the structure of the microbial communities. For this, differences between pairs of samples at level of community composition were visualized with Non-metric Multi-dimensional Scaling (NMDS). PERMANOVA multivariate analysis (999 permutations, stratified at block level) was used to test differences, and pairwise contrasts were subjected to FDR method. Package *DESeq2* (Love et al., 2014) was used to search differentially abundant ASVs between treatments and control. ASVs that were differentially abundant between groups were identified by filtering the contrast table using |log2FC|>1 and Padj<0.05. This choice was dictated by the fact that simulated and real data indicate that *DESeq* is more accurate and less prone to false positive (than other

parametric, non-parametric and compositional approaches) when the number of replicates is relatively limited as in my experimental design (e.g.,Weiss et al., 2017). Finally, to gain further insight on the rhizosphere community composition, ASVs were split into rare and abundant: ASVs were classified as common or rare based on the relative abundance mean across all samples of one treatment, with the threshold for rare set at <0.1% relative abundance.

5.4 Results

Total number of nymphs

After seven days of infestation, there were no significant differences in the number of *Macrosiphum euphorbiae* nymphs on plants grown in the integrated soil compared with the conventional soil (Fig.5.3).

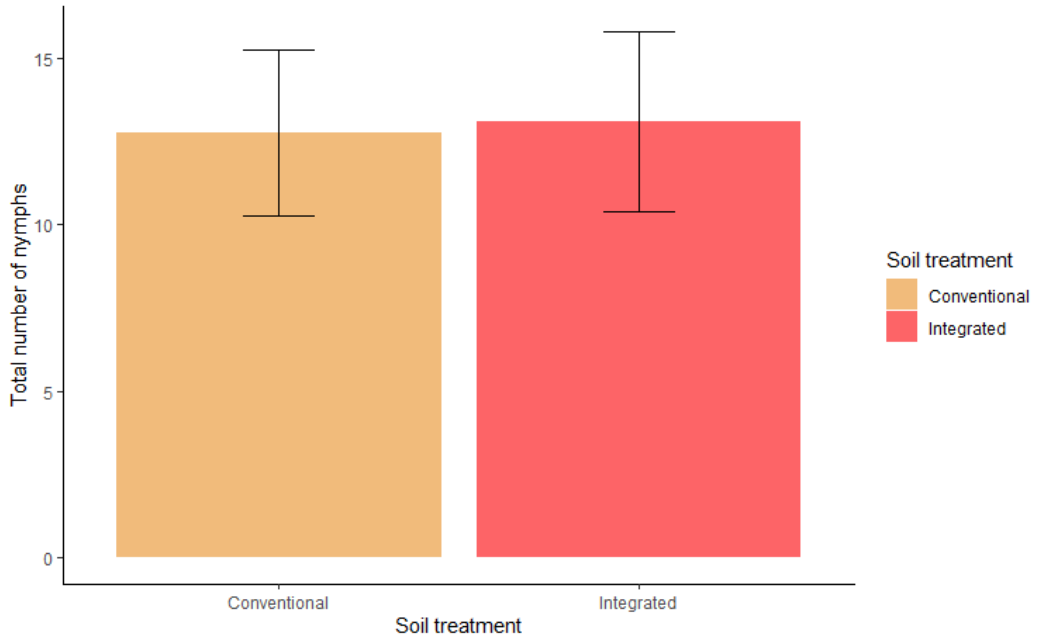

Figure 5.3 Total number of nymphs after 7 days of infestation of potato plants grown in conventional or integrated soils. Values are means (±SEM) of n=12 replicates.

Insect choice test

Female *Aphidius ervi* wasps spent significantly more time (Fig.5.4) in the olfactometer chamber half associated with the aphid-infested plants grown in integrated soils compared with the plants infested with aphids grown in conventional soil (*P*= 5.44, *DF* =1, *Pr* =0.01964).

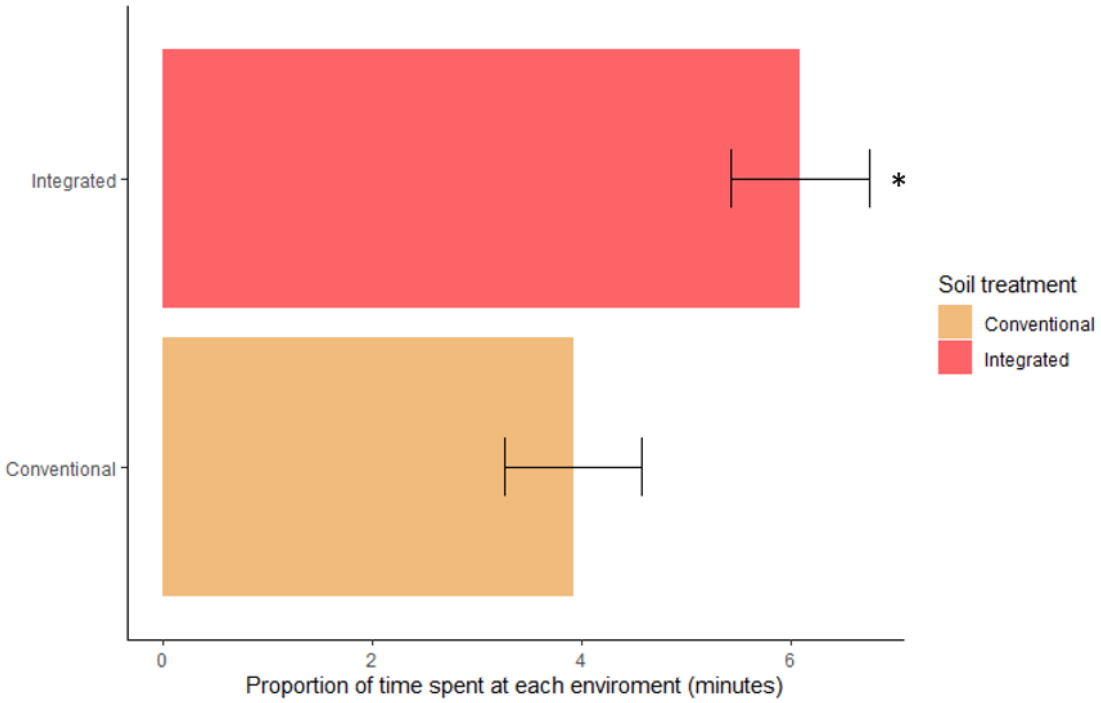

Figure 5.4 Time spent in the arena by individual *Aphidius ervi* wasps when given a choice between plants infested with aphids grown in either conventional soil or integrated soil. Values are means (± SEM) of n = 12 for all choice tests. * Signifies bars are significantly different.

Soil chemical composition

Regarding soil chemical characteristics, the presence or absence of herbivores and the different soil regimes and their interactions had no effect on overall soil chemical composition (Table 5.2). On the contrary, the presence of the aphid led to higher values of soil total organic carbon ($F_{1,20}$, P= 0.05) as shown in Table 5.2 and Supplementary Table 5.1.

Table 5.2 Statistical results of soil chemical properties. Bold values are statistically significant. Values with * were log10 transformed prior to statistical analysis.

P Olsen	**df**	***F***	***P***
Soil treatment	1	1.2611	0.2675
Herbivores	1	0.4979	0.4841
Soil treatment:Herbivores	1	1.8090	0.1855
Residuals	44		
pH*	**df**	***F***	***P***
Soil treatment	1	0.0026	0.9598
Herbivores	1	0.0522	0.8203
Soil treatment:Herbivores	1	0.8055	0.3743
Residuals	44		
Soil gravimetrical moisture*	**df**	***F***	***P***
Soil treatment	1	0.239	0.627
Herbivores	1	0.178	0.676
Soil treatment:Herbivores	1	0.203	0.654
Residuals	44		

Total organic nitrogen*	**df**	***F***	***P***
Soil treatment	1	1.147	0.297
Herbivores	1	0.031	0.861
Soil treatment:Herbivores	1	0.736	0.401
Residuals	20		
Ammonium*	**df**	***F***	***P***
Soil treatment	1	1.147	0.297
Herbivores	1	0.031	0.861
Soil treatment:Herbivores	1	0.736	0.401
Residuals	20		
Nitrate*	**df**	***F***	***P***
Soil treatment	1	0.378	0.545
Herbivores	1	1.684	0.209
Soil treatment:Herbivores	1	0.186	0.671
Residuals	20		
Total organic carbon*	**df**	***F***	***P***
Soil treatment	1	0.657	0.4270
Herbivores	1	4.644	**0.0435**
Soil treatment:Herbivores	1	0.028	0.8689
Residuals	20		
Total nitrogen*	**df**	***F***	***P***
Soil treatment	1	1.0432	0.3193
Herbivores	1	0.0121	0.9136
Soil treatment:Herbivores	1	0.5424	0.4700
Residuals	20		

Rhizosphere bacterial community

The metabarcoding analysis identified overall 1826 different ASVs in the soil samples within different soil regimes and with the presence or absence of aphids. To test diversity among different samples, Shannon diversity index (Fig.5.5) was used, subsequently, linear modelling showed that the diversity only varied significantly between soil treatments and not between plants with and without aphids (Table 5.3). To test the influence of soil management and aphid infestation on the rhizosphere microbial community, a multivariate approach was used (PERMANOVA); this showed a significant difference between soil treatments ($F_{1,20}$ = 4.83; P = 0.001) (Table 5.4). The NMDS plots indicated a clustering of ASVs related to soil treatments (Fig.5.6). The rhizosphere microbiome composition did not differ between plants infested with aphids and without infestation (Table 5.4). To gain insight regarding the differences in soil treatments previously detected, single differentially abundant ASVs were analysed. This approach detected differences at bacterial *Genus* level between soil management with or without insect herbivores. For each treatment the log2 fold change values were used to quantify unique characteristic differentially abundant ASVs. We found (Supplementary Table 5.2) 26 differentially abundant ASVs in the conventional soil with aphid infestation, and 34 differentially abundant ASVs in the conventional soil without aphids. In the integrated soil, 11 differentially abundant ASVs were associated with aphid infestation, and 10 differentially abundant ASVs were associated with uninfested plants. Finally, the samples at time zero (T0) were tested as described above and in total we found that conventional soil presented 113 differentially abundant ASVs compared with integrated soil where only 85 differentially abundant ASVs were identified. Finally, the relative abundance of ASVs that were common and rare (> 0.1 % and < 0.1 %, respectively) was significantly affected by soil management (p=0.001; Figure 5.7a,b), but not by aphid herbivory nor the interaction of aphid herbivory with soil type.

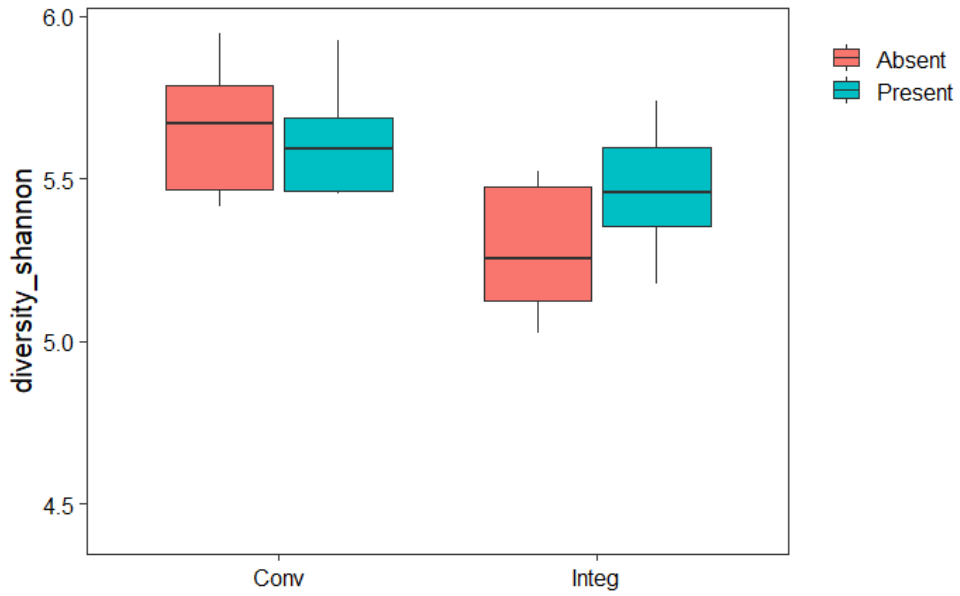

Figure 5.5 Diversity index (Shannon diversity) of the potato rhizosphere soil microbiome, based on 16S rRNA sequencing, from plants grown in conventional and integrated soils. Absent and present refers to aphids.

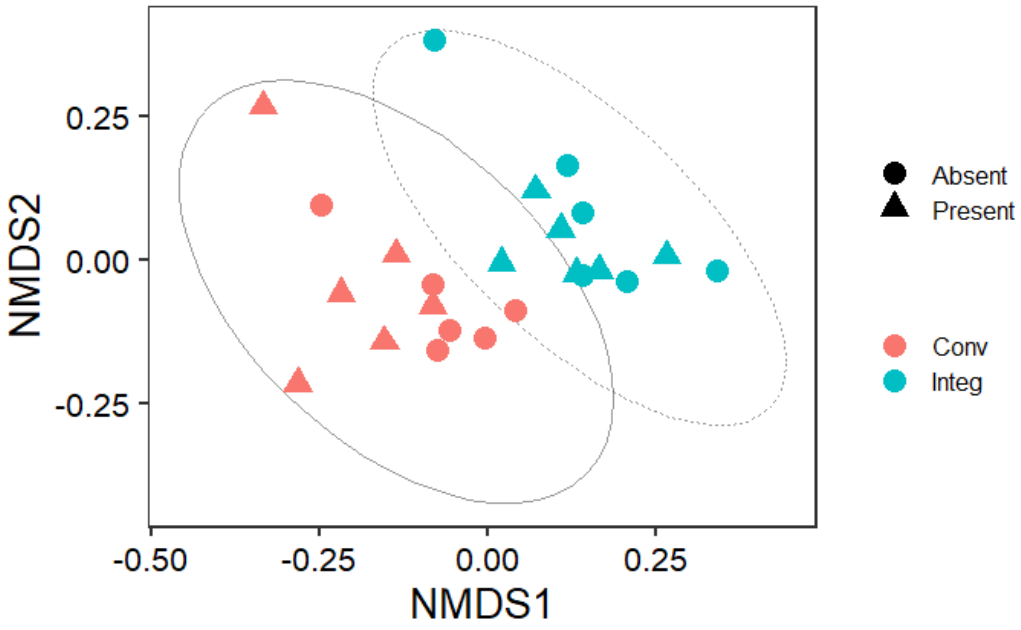

Figure 5.6 NMDS (Non-metric Multi-Dimensional Scaling) charts of the potato rhizosphere soil microbiome, based on 16S rRNA sequencing, from plants grown in conventional and integrated soils, with and without aphid infestation.

Table 5.3 Results from the linear model testing the significance of Shannon diversity index against soil, herbivores and their interactions. Bold values are statistically significant.

Variable	**df**	***F***	***P***
Soil	1	3.6786	**0.06951**
Herbivores	1	1.1510	0.29611
Soil:Herbivores	1	1.0767	0.31183
Residuals	20		

Table 5.4 Results from PERMANOVA analysis testing the effects of soil type, herbivore presence and their interactions on structuring the potato rhizosphere bacterial communities. Bold values are statistically significant.

Variable	**df**	**R^2**	***F***	***P***
Soil	1	0.18092	4.8390	**0.001**
Herbivores	1	0.03639	0.9731	0.391
Soil:Herbivores	1	0.03475	0.9293	0.445
Residuals	20	0.74793		

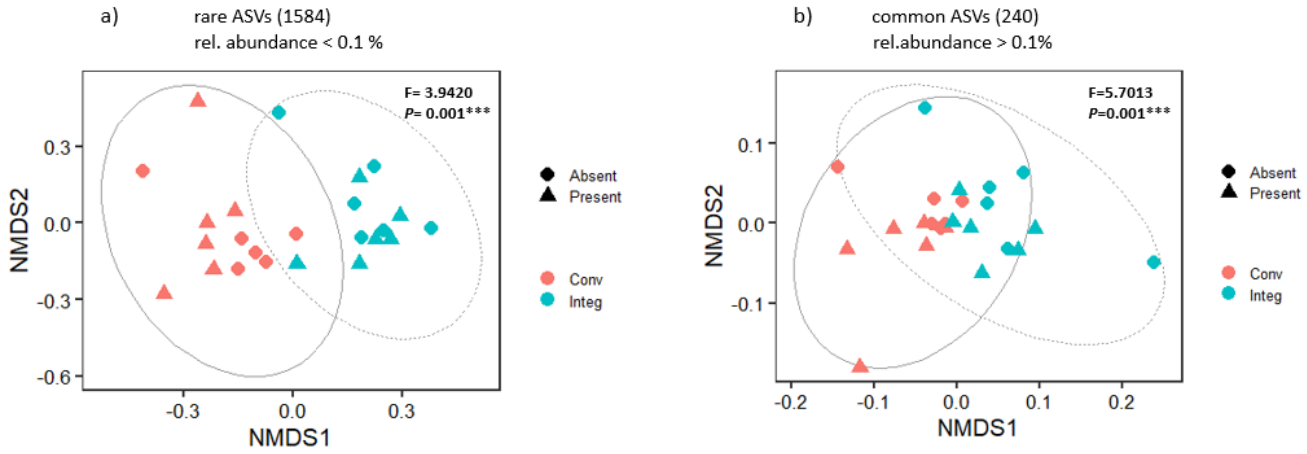

Figure 5.7 Potato (*Solanum tuberosum*) rhizosphere microbiome profiles of ASVs groups that were common (a) and rare (b) in relation to soil type and aphid presence. ASVs were classified as common (>0.1%) or rare (<0.1%) based on the relative abundance mean across all the samples.

5.5 Discussion

The current study is the first work, to the best of our knowledge, attempting to explore the impact of different soil managements on the third trophic level using *Solanum tuberosum* (L), *Macrosiphum euphorbiae* and its natural enemy *Aphidius ervi* as a study system. The main goal was, however, to identify a set of soil characteristics (chemical and biological) that can impact insect herbivores and the third trophic level for improving aphid biocontrol. Firstly, potato plants were infested with the potato aphid, *Macrosiphum euphorbiae*, and subsequently a choice test was performed through a passive diffusion olfactometer method with its natural enemy *Aphidius ervi*. Next, soil chemical characteristics were analysed, and Illumina MiSeq was used to define soil microbial community. The key findings of this study were that parasitoid wasps showed a preference for plants grown on soil from integrated soil management, and that different soil management regimes presented different soil microbial communities. By contrast, neither soil chemical characteristics nor number of aphid nymphs were influenced by soil management, indicating that soil management can promote the ecosystem service of biocontrol without influencing insect pest infestation levels.

Soil management effects on insect pests and their natural enemies

Soil management has been reported to influence insect pests (Altieri et al., 2012). Surprisingly in our experiment there were no differences among total number of aphids on plants grown in two different soils, which is in contrast to previous studies using other study systems. For

example, in a multifactorial experiment conducted using peach-potato aphids (*Myzus persicae,* Sulzer) and Colorado beetles (*Leptinotarsa decemlineata,* Say) on potato plants grown in soil collected from either conventional or integrated farms, peach-potato aphids were not affected by soil regime but aphid abundance was negatively influenced by Colorado beetle presence, leading to a 50% suppression in aphid colony growth. By contrast, Colorado beetles were not affected by aphid presence, but the conventional soil suppressed Colorado beetle population growth. This could be partially explained by soil-mediated plant defense (Krey et al., 2019), and is in line with several greenhouse studies that reported strong effects of soil legacy on aboveground herbivore feeding (Kos et al., 2015).

The main core finding in this study is related to the positive response of *Aphidius ervi* toward plants grown with integrated soil. This result is in agreement with the current literature, despite the fact that diverse study systems and beneficial microbes have been used (Guerrieri and Digilio, 2004). Although we did not measure volatile emission, in our experiment we demonstrated that different soil microbial community affected the third trophic level. It has been already reported that various groups of soil microbes can positively influence the attraction of natural enemies through their effects on herbivore-induced plant volatile production (HIPVs) (Schausberger et al., 2012). As an example, increased attraction of the parasitoid *Microplitis mediator* to caterpillar infested-plant was showed when rhizobacterium *Pseudomonas fluorescens* WCS417r was inoculated in *Arabidopsis thaliana* (Pangesti et al., 2015). Interestingly, in the same study qualitative changes on volatile blend release were detected rather than quantity difference as hypotheses by the authors, when caterpillar infest-plant were inoculated with rhizobacterium. Studies that integrated biochemical and molecular analysis could reveal important mechanisms involved in the production of HIPVs and highlight their ecological function among soil microbes-plant-insect- parasitoids.

Nevertheless, natural enemies presence and abundance it depends on multiple factors. Generally, organic farming systems have a positive effect on the abundance of natural enemies (Garratt et al., 2010, 2011). Absence of chemicals pesticides is a key determinant of this response. Thus, organic farming can promote soil microbial community evenness and natural pest control (Crowder et al., 2010). Other key factors that can encourage natural enemies include use of cover crops over winter. Cover crops have been shown to directly and indirectly affect the third trophic level, increasing biodiversity (Norris and Kogan, 2005). In fact, cover crops can provide favourable micro-habitats for a broad range of parasitoids and predators such as spiders, ladybirds, predatory ants, and parasitoid wasps (Gurr et al., 2017). At the same time,

presence of cover crops can enlarge number of arthropods feeding on them, providing food sources for the natural enemies (Bugg et al., 1987). In another study where the influence of soil management in rose cultivation was investigated, revealed that the use of green manure decrease pests rate and on the contrary increase natural enemies occurrence (Carvalho et al., 2013). However, up to date research involving the third trophic level is still scarce and therefore experimental work using different insect pests and their predators would be a fruitful next step to identify and disentangle mechanisms that might be exclusively related to soil-mediated effects from insect-mediated responses.

Top-down and bottom-up factors that can drive and influence soil microbial composition

A huge milestone in characterising the identity of organisms and the composition of communities has been the development of DNA barcoding (Abdelfattah et al., 2018). It has enabled research to investigate the microbial worlds (Piper et al., 2019). Metabarcoding analysis represents a novel tool for integrated pest management in the search for new solutions in controlling insect pests. Most studies have focussed on individual factors that can alter soil microbial community and only few connect soil microbiome changes and effects on higher trophic levels (Malacrino et al., 2021; Vescio et al., 2021). In the current study, soil chemical analysis did not show any differences on soil chemical profile between the two different soil regimes. This is in contrast with a huge body of research that has previously investigated extensively this subject (Henneron et al., 2015) and this finding is surprising given the substantial dissimilarity of soil management methods used at the CSC in terms of conservation tillage, cover cropping, and carbon amendments. However, our results are in line with another study (Krey., et al 2019), where the authors hypothesised that the period of organic transition of the study site had been insufficient to build up soil chemical differences. An interesting finding of the soil chemical analysis is related to the effect of insect herbivores on DOC. This is agreement with other studies where higher level of DOC were detected under aphid infestation (Potthast et al., 2022). Sap-feeding insects, such as aphids, can indirectly alter aboveground and belowground biota, affecting nutrient cycling to different degrees (Potthast et al., 2022; Van Dam and Heil, 2011).

Despite the absence of detectible chemical differences between the two different soil systems, soil microbial diversity and composition varied between the two soil types. Analysis of differentially expressed ASVs showed higher numbers of ASVs in the conventional soil

compared with the integrated soil. This finding is not consistent with the a large body of work that shows how soil microbial community biodiversity is higher in organic compared with conventional systems (Crowder et al., 2010). It is unclear why diversity in bacterial communities appeared to be higher in the conventional farming system. One possibility is that this reflects differences in organic input matter, as results of different farming management. A further reason could be linked to legacy effects due to the previous crops present in that portion of the field that could have boosted the soil microbiome. Further, aphid infestation did not affect the potato rhizosphere community composition, which contrasts with previous studies Malacrino et al., 2021a,b), although it might result from the longer period of aphid herbivory (3 weeks) compared with the present study (7 days). Studies to uncover the effects of the rhizosphere microbiome on multitrophic interactions are key to understanding their role in plant tolerance of variable abiotic and abiotic conditions and could be important for developing robust IPM strategies (e.g. Sanchez-Mahecha et al., 2022).

5.6 Conclusion

This study represents a contribution to the field of biocontrol of insect pests using soil management for enhancing parasitoid wasp attraction. Specifically, the results showed that the parasitoid wasps were more attracted to plants grown on soil from a field with integrated soil management compared to plants grown on soil from conventional soil management conditions. This indicates that the use of tillage approach, organic matter amendments, lower synthetic fertiliser input and cover cropping, which are key features of the integrated management system, can impact the third trophic level through indirect effects of soil biological, chemical and physical characteristics on the physiology of the crop and its infesting aphids. This finding highlights novel opportunities to use soil management as a tool for improving aphid biocontrol. Although the soil properties driving these effects and the underpinning molecular mechanisms are still not clear, this study indicates that the soil microbiome might play a role and presents a foundation for further work to explore bottom-up interactions, which can be used to better develop and design Integrated Pest Management (IPM) programs for aphid pests. Further work that includes varying individual soil properties and analysis of plant physiological, molecular and metabolomic responses could identify which soil properties are key for recruiting aphid natural enemies to infested plants in integrated management farm systems.

5.7 Supplementary Material

Supplementary Table 5.1 Table showing the mean and the standard error for each soil chemical properties on the rhizosphere

P Olsen *(µg/g soil)*			
Soil treatment	**Herbivores**	**Mean**	**Standard Error**
Conventional	With	44.339	2.204
Conventional	Without	40.419	1.974
Integrated	With	43.915	1.939
Integrated	Without	45.137	1.447
pH*			
Soil treatment	**Herbivores**	**Mean**	**Standard Error**
Conventional	With	6.090	0.089
Conventional	Without	6.003	0.086
Integrated	With	6.019	0.043
Integrated	Without	6.073	0.067
Soil gravimetrical moisture (soil water as % soil dry-mass)			
Soil treatment	**Herbivores**	**Mean**	**Standard Error**
Conventional	With	42.420	22.045
Conventional	Without	22.393	4.721
Integrated	With	21.014	2.970
Integrated	Without	30.904	12.547
Total organic nitrogen(mg/g)			
Soil treatment	**Herbivores**	**Mean**	**Standard Error**
Conventional	With	0.0019	0.0005
Conventional	Without	0.0010	0.0002
Integrated	With	0.0022	0.0004
Integrated	Without	0.0075	0.0005
Ammonium(mg/g)			
Soil treatment	**Herbivores**	**Mean**	**Standard Error**
Conventional	With	0.0004	8.426937e-0
Conventional	Without	0.0002	6.817415e-05
Integrated	With	0.0007	1.876531e-0
Integrated	Without	0.0007	5.570444e-0
Nitrate(mg/g)			
Soil treatment	**Herbivores**	**Mean**	**Standard Error**
Conventional	With	0.0019	0.0005
Conventional	Without	0.0090	0.0002
Integrated	With	0.0022	0.0004
Integrated	Without	0.0036	0.0002
Total organic carbon(mg/g)			
Soil treatment	**Herbivores**	**Mean**	**Standard Error**
Conventional	With	0.2942	0.0035
Conventional	Without	0.0212	0.0033
Integrated	With	0.0267	0.0054

Integrated	Without	0.0172	0.0036
Total nitrogen(mg/g)			
Soil treatment	**Herbivores**	**Mean**	**Standard Error**
Conventional	With	0.003	0.0006
Conventional	Without	0.002	0.0003
Integrated	With	0.004	0.0009
Integrated	Without	0.006	0.0045

Supplementary Table 5.2 Differentially abundant ASVs at *Genus* level between different soil regimes (conventional and integrated) with and without herbivores, and at time zero (T0).

Soil treatment	***Herbivore***	***Genus***	***rn***	***log2FoldChange***	***padj***
conventional	present	Cytophaga	ASV_261	3.782792282	0.03557916
conventional	present	Unidentified Gemmatimonadaceae	ASV_402	3.665212029	0.008258417
conventional	present	Chthoniobacter	ASV_418	5.989981023	0.005689286
conventional	present	Unidentified AKYH767	ASV_562	3.493230945	0.03494089
conventional	present	Pseudorhodoferax	ASV_584	5.204107404	0.006268407
conventional	present	Terrimonas	ASV_633	5.342846737	0.005689286
conventional	present	Unidentified NS11-12 marine group	ASV_644	3.872365303	0.03557916
conventional	present	Unidentified Myxococcaceae	ASV_741	3.770118803	0.030422286
conventional	present	Unidentified Gemmatimonadaceae	ASV_753	3.819401784	0.03557916
conventional	present	OLB12	ASV_912	3.573807663	0.043789796
conventional	present	Unidentified env.OPS 17	ASV_953	4.490132609	0.017366512
conventional	present	Puia	ASV_1012	3.362918995	0.040072717
conventional	present	Rhodoferax	ASV_1027	4.023518032	0.020123961
conventional	present	Pedosphaera	ASV_1033	3.423276785	0.04933951
conventional	present	Unidentified Pedosphaeraceae	ASV_1138	4.282092633	0.021459351
conventional	present	Aetherobacter	ASV_1221	4.027972369	0.030371656
conventional	present	Pseudosphingobacterium	ASV_1283	3.921819701	0.032723711
conventional	present	YC-ZSS-LKJ147	ASV_1295	4.004493594	0.032405638
conventional	present	Dyadobacter	ASV_1421	3.645204171	0.040072717
conventional	present	Rudaea	ASV_1529	3.474824496	0.04843116
conventional	present	Unidentified Sandaracinaceae	ASV_1536	3.419791188	0.041479312
conventional	present	Agromyces	ASV_1593	3.59784533	0.045561996
conventional	present	Citrobacter	ASV_32	6.071358227	0.003639662
conventional	present	Ellin6067	ASV_846	4.091382239	0.030371656
conventional	present	Unidentified Nostocaceae	ASV_900	4.739126219	0.015143174

conventional	present	Opitutus	ASV_1556	3.428812652	0.049389274
conventional	absent	Unidentified Solirubrobacteraceae	ASV_134	-2.600065482	0.030371656
conventional	absent	Flavisolibacter	ASV_193	-4.258339103	0.017366512
conventional	absent	Unidentified Oxalobacteraceae	ASV_219	-6.648304704	7.54889E-07
conventional	absent	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	ASV_258	-4.042827112	0.030422286
conventional	absent	Unidentified Pedosphaeraceae	ASV_307	-3.906998848	0.023952066
conventional	absent	Unidentified Comamonadaceae	ASV_319	-4.926042236	0.005689286
conventional	absent	Lapillicoccus	ASV_347	-3.540557089	0.045561996
conventional	absent	Adhaeribacter	ASV_366	-3.931388142	0.017366512
conventional	absent	Gemmatimonas	ASV_401	-3.647786803	0.041479312
conventional	absent	Unidentified Micromonosporaceae	ASV_472	-4.799728412	0.006268407
conventional	absent	Candidatus Solibacter	ASV_589	-3.4854141	0.03557916
conventional	absent	Aetherobacter	ASV_702	-3.464326398	0.049389274
conventional	absent	ADurb.Bin063-1	ASV_759	-3.7003918	0.040072717
conventional	absent	Unidentified Myxococcaceae	ASV_765	-4.344914392	0.020123961
conventional	absent	Paenibacillus	ASV_777	-3.98647365	0.017648258
conventional	absent	Gemmatimonas	ASV_779	-2.75205297	0.03494089
conventional	absent	Streptomyces	ASV_828	-3.698602992	0.040072717
conventional	absent	Unidentified A4b	ASV_835	-3.699996413	0.030422286
conventional	absent	Jatrophihabitans	ASV_869	-3.657601632	0.031251838
conventional	absent	Gemmatimonas	ASV_951	-4.468239371	0.019051833
conventional	absent	Subgroup 10	ASV_969	-3.702849034	0.020123961
conventional	absent	Unidentified Xanthomonadaceae	ASV_1074	-4.189623977	0.017366512
conventional	absent	Gemmatimonas	ASV_1110	-4.089607893	0.031251838
conventional	absent	Ellin517	ASV_1189	-4.068275786	0.020123961
conventional	absent	Duganella	ASV_1191	-4.095047789	0.030371656
conventional	absent	Unidentified Roseiflexaceae	ASV_1238	-3.871834764	0.036607243
conventional	absent	Bryobacter	ASV_1335	-3.647819964	0.041479312
conventional	absent	Sphingobium	ASV_1465	-3.492207815	0.04933951

conventional	absent	Gemmatimonas	ASV_1591	-3.272972944	0.045160674
conventional	absent	Streptomyces	ASV_293	-6.000033348	0.005689286
conventional	absent	Marmoricola	ASV_385	-4.187526491	0.010496577
conventional	absent	Candidatus Solibacter	ASV_731	-4.916787389	0.010002142
conventional	absent	Sphingomonas	ASV_899	-3.666287763	0.031251838
conventional	absent	Gemmata	ASV_1241	-3.248392157	0.045561996
integrated	present	Unidentified Gemmatimonadaceae	ASV_90	4.278139218	0.036727193
integrated	present	Lysobacter	ASV_145	7.043633503	0.002268839
integrated	present	Unidentified Xanthobacteraceae	ASV_377	4.358617246	0.033883642
integrated	present	Unidentified Microscillaceae	ASV_493	3.890720286	0.005125598
integrated	present	Singulisphaera	ASV_495	4.06169774	0.048150559
integrated	present	Ellin517	ASV_565	4.739879565	0.008800242
integrated	present	ADurb.Bin063-1	ASV_759	4.180583181	0.02481078
integrated	present	Unidentified Vampirovibrionaceae	ASV_798	3.871336291	0.040189439
integrated	present	Unidentified WD2101 soil group	ASV_946	3.840317142	0.009717702
integrated	present	Unidentified Pedosphaeraceae	ASV_1554	3.348617873	0.033883642
integrated	present	Unidentified Oxalobacteraceae	ASV_367	6.160647854	0.005125598
integrated	absent	Massilia	ASV_37	-5.589081898	0.000275827
integrated	absent	Rhodoferax	ASV_312	-5.635386305	0.006492601
integrated	absent	Unidentified Xanthobacteraceae	ASV_377	4.358617246	0.033883642
integrated	absent	Streptomyces	ASV_399	-5.139224265	0.006492601
integrated	absent	Aeromicrobium	ASV_522	-4.54421829	0.009717702
integrated	absent	Corallococcus	ASV_583	-3.976673669	0.048150559
integrated	absent	Lechevalieria	ASV_762	-4.878404283	0.006492601
integrated	absent	Unidentified Pedosphaeraceae	ASV_1264	-4.044249248	0.048150559
integrated	absent	Streptomyces	ASV_881	-4.713807042	0.015127255
integrated	absent	Phenylobacterium	ASV_971	-3.458520722	0.04421645
conv_time_zero	not_present	Holophaga	ASV_35	4.023843418	1.48051E-06
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_68	2.860813066	0.042226041
conv_time_zero	not_present	Bryobacter	ASV_84	1.138586135	0.031432915

conv_time_zero	not_present	Gemmatimonas	ASV_99	1.078293759	0.037173811
conv_time_zero	not_present	ADurb.Bin063-1	ASV_105	5.44624932	1.48051E-06
conv_time_zero	not_present	Flavobacterium	ASV_126	1.471264333	0.004741397
conv_time_zero	not_present	Unidentified Anaerolineaceae	ASV_127	2.048458931	0.014000019
conv_time_zero	not_present	Uliginosibacterium	ASV_180	4.596927692	0.000350852
conv_time_zero	not_present	Unidentified Pedosphaeraceae	ASV_184	1.214377762	0.022428828
conv_time_zero	not_present	Unidentified Micropepsaceae	ASV_185	5.887520642	7.92524E-11
conv_time_zero	not_present	Unidentified WD2101 soil group	ASV_194	5.17790891	8.68174E-05
conv_time_zero	not_present	Rhodanobacter	ASV_212	4.523555474	0.00019153
conv_time_zero	not_present	Candidatus Solibacter	ASV_227	5.292774727	4.01242E-06
conv_time_zero	not_present	RB41	ASV_230	5.67806477	4.2941E-07
conv_time_zero	not_present	RB41	ASV_231	3.137498857	0.003828495
conv_time_zero	not_present	Cytophaga	ASV_261	3.749339605	0.010593066
conv_time_zero	not_present	Unidentified Microscillaceae	ASV_269	2.695991929	8.68174E-05
conv_time_zero	not_present	Unidentified Anaerolineaceae	ASV_354	2.085033623	0.015336756
conv_time_zero	not_present	Candidatus Udaeobacter	ASV_372	4.364564607	0.001500721
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_397	5.053102692	0.000128827
conv_time_zero	not_present	Nitrospira	ASV_404	5.655344768	4.2941E-07
conv_time_zero	not_present	UTBCD1	ASV_449	2.263031906	0.011146284
conv_time_zero	not_present	Gemmatimonas	ASV_450	4.560708176	0.000219408
conv_time_zero	not_present	Gemmatimonas	ASV_454	3.635584481	0.00027979
conv_time_zero	not_present	Ellin517	ASV_461	3.405986019	0.017015563
conv_time_zero	not_present	Rhodanobacter	ASV_462	4.972685806	2.8933E-05
conv_time_zero	not_present	ADurb.Bin063-1	ASV_469	4.972687413	1.72183E-06
conv_time_zero	not_present	Candidatus Solibacter	ASV_477	4.596929883	2.82446E-05
conv_time_zero	not_present	Unidentified Caulobacteraceae	ASV_492	4.15379682	0.005063771
conv_time_zero	not_present	Terrimonas	ASV_513	4.392311158	0.000285674
conv_time_zero	not_present	Cellvibrio	ASV_523	4.590096901	0.000469842
conv_time_zero	not_present	Unidentified A21b	ASV_545	4.981847308	1.89684E-06
conv_time_zero	not_present	Chitinophaga	ASV_556	2.641540495	0.037407742

conv_time_zero	not_present	Unidentified Roseiflexaceae	ASV_558	4.13749505	0.005063771
conv_time_zero	not_present	Flavobacterium	ASV_569	5.536041325	0.000128939
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_596	4.990947836	3.25038E-05
conv_time_zero	not_present	Rhodoferax	ASV_612	5.596927867	9.84216E-07
conv_time_zero	not_present	Unidentified Anaerolineaceae	ASV_634	3.253983491	0.008523036
conv_time_zero	not_present	Nitrospira	ASV_638	5.536047983	1.06826E-08
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_648	4.972684048	0.000170302
conv_time_zero	not_present	Unidentified SC-I-84	ASV_673	4.523555468	0.00019153
conv_time_zero	not_present	Flavobacterium	ASV_713	5.357539567	0.000427999
conv_time_zero	not_present	Unidentified SC-I-84	ASV_756	3.02045863	0.015350745
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_770	3.2927757	0.019838437
conv_time_zero	not_present	Terrimonas	ASV_794	3.29277568	0.020035396
conv_time_zero	not_present	Unidentified Steroidobacteraceae	ASV_876	4.446249912	0.000229279
conv_time_zero	not_present	Unidentified Anaerolineaceae	ASV_883	4.963465544	0.000170302
conv_time_zero	not_present	OLB12	ASV_912	4.104329388	0.002845408
conv_time_zero	not_present	Unidentified WD2101 soil group	ASV_934	4.18586049	0.000674665
conv_time_zero	not_present	Parafilimonas	ASV_937	3.201628844	0.015336756
conv_time_zero	not_present	Unidentified Micropepsaceae	ASV_944	3.536047088	0.008094229
conv_time_zero	not_present	Mycobacterium	ASV_956	3.405986018	0.017015563
conv_time_zero	not_present	Unidentified WD2101 soil group	ASV_961	2.963469621	0.021968044
conv_time_zero	not_present	RB41	ASV_989	4.378503964	0.001236557
conv_time_zero	not_present	Unidentified WD2101 soil group	ASV_1100	3.104331068	0.02599592
conv_time_zero	not_present	Bryobacter	ASV_1115	4.217223331	0.00199473
conv_time_zero	not_present	Haliangium	ASV_1183	4.33627436	0.003552444
conv_time_zero	not_present	Unidentified Chitinophagaceae	ASV_1226	3.744155799	0.002622186
conv_time_zero	not_present	Bryobacter	ASV_1251	3.78658997	0.005063771
conv_time_zero	not_present	Polycyclovorans	ASV_1279	2.84799206	0.035334484
conv_time_zero	not_present	Unidentified Vicinamibacteraceae	ASV_1294	4.121006972	0.005210902
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_1307	3.350491865	0.01143843
conv_time_zero	not_present	Minicystis	ASV_1311	4.087454462	0.005633569

conv_time_zero	not_present	Ellin516	ASV_1347	2.807350231	0.036534739
conv_time_zero	not_present	Ferruginibacter	ASV_1360	3.999993088	0.003364469
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_1389	3.944851626	0.003828495
conv_time_zero	not_present	Ferruginibacter	ASV_1414	2.887521	0.023153305
conv_time_zero	not_present	SM1A02	ASV_1435	2.963469679	0.021189719
conv_time_zero	not_present	Haliangium	ASV_1496	3.765527329	0.009319141
conv_time_zero	not_present	Unidentified Diplorickettsiaceae	ASV_1512	3.744155592	0.003082612
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_1513	3.744154839	0.005254072
conv_time_zero	not_present	Haliangium	ASV_1542	3.700433555	0.005680328
conv_time_zero	not_present	Phenylobacterium	ASV_1546	2.722461556	0.04183548
conv_time_zero	not_present	Granulicella	ASV_1564	3.169920032	0.016311135
conv_time_zero	not_present	Candidatus Solibacter	ASV_1574	3.201628862	0.015192157
conv_time_zero	not_present	Unidentified A4b	ASV_1639	2.45065838	0.028262424
conv_time_zero	not_present	Unidentified	ASV_1651	3.536047118	0.008033585
conv_time_zero	not_present	Terrimonas	ASV_1659	3.232656358	0.008367286
conv_time_zero	not_present	Unidentified Fibrobacteraceae	ASV_1676	3.292776572	0.01282049
conv_time_zero	not_present	Unidentified 37-13	ASV_1727	3.405986027	0.016964865
conv_time_zero	not_present	Unidentified KF-JG30-B3	ASV_1865	3.232654877	0.021257493
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_1869	2.887520364	0.033503762
conv_time_zero	not_present	Opitutus	ASV_1905	3.169919298	0.022791618
conv_time_zero	not_present	ADurb.Bin063-1	ASV_1928	3.137497882	0.023922283
conv_time_zero	not_present	Chthoniobacter	ASV_1950	3.104331136	0.024942495
conv_time_zero	not_present	Unidentified A4b	ASV_1990	3.070383907	0.026181324
conv_time_zero	not_present	Flavobacterium	ASV_2069	2.963468954	0.031336861
conv_time_zero	not_present	Nitrospira	ASV_2081	2.632264493	0.034232526
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_2134	2.887520363	0.033503762
conv_time_zero	not_present	Methylotenera	ASV_2267	2.722462123	0.030325818
conv_time_zero	not_present	SM1A02	ASV_2349	2.632264514	0.033951609
conv_time_zero	not_present	Unidentified Anaerolineaceae	ASV_153	1.062395732	0.035334484
conv_time_zero	not_present	RB41	ASV_221	3.14438242	0.021165097

conv_time_zero	not_present	Unidentified SC-I-84	ASV_222	4.035615719	0.005852219
conv_time_zero	not_present	Rhizobacter	ASV_337	5.285393395	4.60622E-05
conv_time_zero	not_present	Rhodanobacter	ASV_489	2.887520326	0.033827699
conv_time_zero	not_present	Candidatus Solibacter	ASV_530	4.744151038	0.001385173
conv_time_zero	not_present	Ellin6067	ASV_564	2.31549797	0.030160649
conv_time_zero	not_present	Candidatus Udaeobacter	ASV_604	3.963467086	0.004067472
conv_time_zero	not_present	Unidentified Pedosphaeraceae	ASV_665	5.053102655	0.000128939
conv_time_zero	not_present	Pseudomonas	ASV_705	4.700429658	0.001601937
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_744	4.916469769	4.09481E-05
conv_time_zero	not_present	Acidibacter	ASV_803	4.48541735	0.002676871
conv_time_zero	not_present	Streptomyces	ASV_829	2.584957034	0.047290665
conv_time_zero	not_present	Terrimonas	ASV_901	4.485420341	0.000229279
conv_time_zero	not_present	Flavobacterium	ASV_902	4.700429728	0.001569118
conv_time_zero	not_present	Unidentified Methylophilaceae	ASV_906	4.104330787	0.000833219
conv_time_zero	not_present	Methylotenera	ASV_921	4.887519939	3.51202E-06
conv_time_zero	not_present	Candidatus Nitrocosmicus	ASV_960	4.13749617	0.002676871
conv_time_zero	not_present	Unidentified Latescibacteraceae	ASV_1105	4.1858591	0.00239045
conv_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_1197	4.292772934	0.003828495
conv_time_zero	not_present	Ellin6067	ASV_1483	3.786588887	0.009186451
conv_time_zero	not_present	Unidentified Pedosphaeraceae	ASV_1635	3.560708053	0.01410288
integ_time_zero	not_present	Unidentified 67-14	ASV_19	-3.369229741	0.013565176
integ_time_zero	not_present	Terrabacter	ASV_80	-1.833538983	0.033951609
integ_time_zero	not_present	Gemmatimonas	ASV_108	-2.969622121	0.045719634
integ_time_zero	not_present	Methylotenera	ASV_120	-2.969622574	0.033503762
integ_time_zero	not_present	Dyella	ASV_139	-4.882639023	3.51202E-06
integ_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_162	-4.142951912	0.005223034
integ_time_zero	not_present	Chthoniobacter	ASV_165	-3.587068724	0.000105025
integ_time_zero	not_present	Gemmatimonas	ASV_167	-3.196392465	0.03382342
integ_time_zero	not_present	Nakamurella	ASV_217	-4.63420109	0.000157222
integ_time_zero	not_present	Nitrospira	ASV_249	-3.087458834	0.028632273

integ_time_zero	not_present	Unidentified Sporichthyaceae	ASV_308	-2.107189066	0.034131707
integ_time_zero	not_present	Acidibacter	ASV_320	-3.874463577	0.008523036
integ_time_zero	not_present	Unidentified Saprospiraceae	ASV_344	-2.757313229	0.023236251
integ_time_zero	not_present	Gaiella	ASV_384	-4.357546382	0.001601937
integ_time_zero	not_present	Unidentified Pedosphaeraceae	ASV_446	-2.182275978	0.02373505
integ_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_505	-5.409386891	9.15926E-08
integ_time_zero	not_present	Edaphobaculum	ASV_516	-3.790072139	0.005913204
integ_time_zero	not_present	Conexibacter	ASV_531	-3.890765996	0.005063771
integ_time_zero	not_present	Lapillicoccus	ASV_587	-3.392312818	0.018744019
integ_time_zero	not_present	Nocardioides	ASV_597	-4.285397481	0.000677193
integ_time_zero	not_present	Acidibacter	ASV_607	-4.700434842	0.000105025
integ_time_zero	not_present	Unidentified Bacillaceae	ASV_621	-5.247923451	3.39398E-07
integ_time_zero	not_present	Nocardioides	ASV_650	-3.7369609	0.006599171
integ_time_zero	not_present	Candidatus Udaeobacter	ASV_712	-4.614704988	0.000157222
integ_time_zero	not_present	Knoellia	ASV_721	-3.906885398	0.005696609
integ_time_zero	not_present	Unidentified A21b	ASV_735	-3.700435723	0.003823
integ_time_zero	not_present	Solirubrobacter	ASV_782	-4.297674177	0.004067472
integ_time_zero	not_present	Geobacter	ASV_832	-2.47031868	0.04227826
integ_time_zero	not_present	Unidentified Nitrososphaeraceae	ASV_845	-3.459427266	0.012874836
integ_time_zero	not_present	Labrys	ASV_891	-3.029744133	0.017413142
integ_time_zero	not_present	Unidentified 67-14	ASV_892	-3.772584882	0.005680328
integ_time_zero	not_present	Desulfuromonas	ASV_940	-3.403720135	0.00117009
integ_time_zero	not_present	Unidentified WD2101 soil group	ASV_952	-3.502495537	0.016087519
integ_time_zero	not_present	Candidatus Udaeobacter	ASV_973	-3.790070854	0.016087519
integ_time_zero	not_present	Unidentified Sutterellaceae	ASV_987	-3.790072891	0.002702956
integ_time_zero	not_present	Flavisolibacter	ASV_1001	-3.523557695	0.009319141
integ_time_zero	not_present	Unidentified Verrucomicrobiaceae	ASV_1014	-3.564780306	0.008523036
integ_time_zero	not_present	Terrimonas	ASV_1015	-3.954190635	0.007853956
integ_time_zero	not_present	Conexibacter	ASV_1031	-3.273013582	0.03099763
integ_time_zero	not_present	Pedosphaera	ASV_1033	-3.523556462	0.022236362

integ_time_zero	not_present	Mycobacterium	ASV_1159	-3.50249489	0.022791618
integ_time_zero	not_present	Opitutus	ASV_1171	-4.115470391	0.009319141
integ_time_zero	not_present	Unidentified Pedosphaeraceae	ASV_1175	-3.938595046	0.00239045
integ_time_zero	not_present	Unidentified Pedosphaeraceae	ASV_1203	-4.029740698	0.011139864
integ_time_zero	not_present	Unidentified Anaerolineaceae	ASV_1215	4.263028204	0.000522298
integ_time_zero	not_present	Subgroup 10	ASV_1253	-3.392312232	0.026286666
integ_time_zero	not_present	Pseudaminobacter	ASV_1257	-3.523557711	0.009194515
integ_time_zero	not_present	Unidentified Fimbriimonadaceae	ASV_1304	-2.617382453	0.018606337
integ_time_zero	not_present	Unidentified Pirellulaceae	ASV_1398	-3.681818176	0.018606337
integ_time_zero	not_present	Mucilaginibacter	ASV_1442	-3.604856306	0.021189719
integ_time_zero	not_present	Flavisolibacter	ASV_1480	-3.544316779	0.005063771
integ_time_zero	not_present	Chthoniobacter	ASV_1481	-3.544314928	0.022236362
integ_time_zero	not_present	Galbitalea	ASV_1494	-3.523556465	0.022236362
integ_time_zero	not_present	Unidentified TRA3-20	ASV_1495	-3.523557028	0.016311135
integ_time_zero	not_present	Rhodoplanes	ASV_1522	-3.481121896	0.016858226
integ_time_zero	not_present	Rhodoplanes	ASV_1596	-3.369229268	0.019067376
integ_time_zero	not_present	Unidentified AKYH767	ASV_1631	-3.321923024	0.030101154
integ_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_1632	-3.321923645	0.020479048
integ_time_zero	not_present	Unidentified Gemmataceae	ASV_1648	-3.029743878	0.022094983
integ_time_zero	not_present	Iamia	ASV_1679	-3.247923215	0.022236362
integ_time_zero	not_present	Unidentified A4b	ASV_1681	-3.247922653	0.031882951
integ_time_zero	not_present	Ellin6067	ASV_1682	-3.247923124	0.023310347
integ_time_zero	not_present	Massilia	ASV_1723	-3.169920315	0.034254621
integ_time_zero	not_present	Parafilimonas	ASV_1794	-3.087458796	0.029583707
integ_time_zero	not_present	Citrifermentans	ASV_1833	-3.029743	0.04183548
integ_time_zero	not_present	Unidentified KD3-93	ASV_1861	-2.999996076	0.03382342
integ_time_zero	not_present	Unidentified Rhizobiales Incertae Sedis	ASV_1862	-2.999996137	0.032687988
integ_time_zero	not_present	Unidentified Verrucomicrobiaceae	ASV_2012	-2.807351782	0.031882951
integ_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_176	-5.303776805	1.33751E-07
integ_time_zero	not_present	Caenimonas	ASV_315	-3.718812311	0.017413142

integ_time_zero	not_present	Sphingomonas	ASV_340	-2.596786807	0.030101154
integ_time_zero	not_present	Nitrospira	ASV_423	-5.357546859	2.45651E-06
integ_time_zero	not_present	RB41	ASV_512	-5.142951734	8.68174E-05
integ_time_zero	not_present	Sphingomonas	ASV_631	-3.604856388	0.020363567
integ_time_zero	not_present	Unidentified Gemmatimonadaceae	ASV_675	-4.984885964	0.000519153
integ_time_zero	not_present	RB41	ASV_975	-2.544317928	0.04183548
integ_time_zero	not_present	Sphingomonas	ASV_1040	-3.662959209	0.018744019
integ_time_zero	not_present	Ellin6067	ASV_1181	-4.087457013	0.005676355
integ_time_zero	not_present	Unidentified Anaerolineaceae	ASV_1185	-3.681819538	0.006877366
integ_time_zero	not_present	Mycobacterium	ASV_1190	-4.073243019	0.005934884
integ_time_zero	not_present	Candidatus Solibacter	ASV_1206	-3.321924095	0.014988925
integ_time_zero	not_present	Actimicrobium	ASV_1269	-3.922825743	0.013296592
integ_time_zero	not_present	RB41	ASV_1270	-3.922826514	0.008033585
integ_time_zero	not_present	Unidentified Anaerolineaceae	ASV_1369	-3.736959625	0.017015563
integ_time_zero	not_present	Ferruginibacter	ASV_1745	-3.142953326	0.035416377

Chapter 6. General discussion and future perspectives

6.1 Introduction

In agricultural ecosystems, multiple trophic interactions occur simultaneously, and their outcomes depend on the balance between top-down and bottom-up effects. Microbes, plants, and insects are the principal actors in community dynamics and despite the current state of research, we still have only a basic understanding of the factors regulating the outcomes of their interactions. In the General Introduction (Chapter 1), I reviewed the relevant literature and highlighted several key areas requiring greater research focus. I address those gaps in my research, using a multitrophic approach to examine the individual and combined effects of abiotic and biotic environmental factors on microbe-plant-insect interactions both aboveground and belowground (Fig.6.1). This was accomplished by employing physiological and molecular techniques under greenhouse and laboratory conditions to explore processes and mechanisms underpinning the outcome of trophic interactions. Below, firstly I outline the general limitations and after, I summarize the results from these four chapters. Finally, I briefly outline key findings of the thesis and explore future directions.

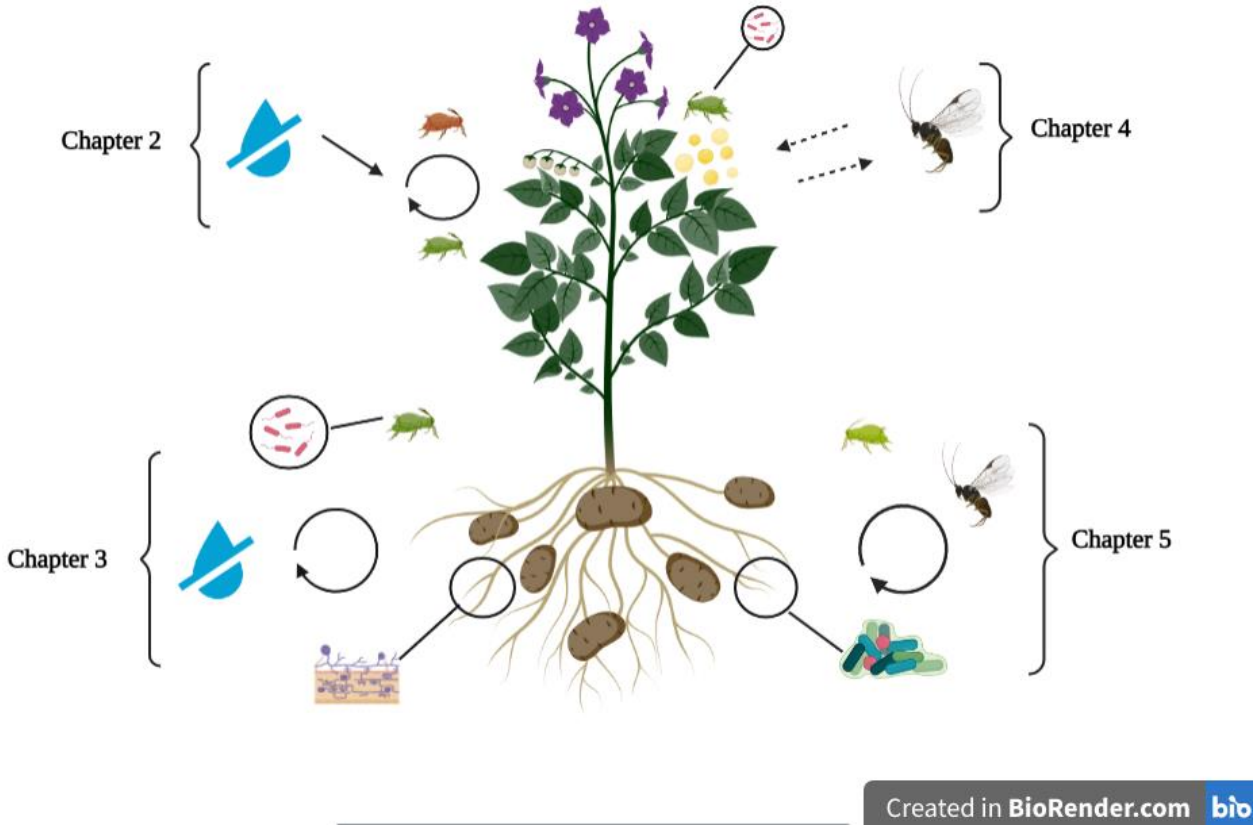

Figure 6.1 Graphical representation for describing each chapter in the thesis. This image was made in © BioRender - biorender.com

6.2 Limitations

I previously stated the specific limitations of certain methodologies used in my project, highlighted by the experiment performed with arbuscular mycorrhizal fungi, in Chapter 3, and the methodology for detecting VOCs in Chapter 4. Firstly, the main limitation encountered in this project was due to the low or non-existent colonization of the potato roots by AMF. Despite the effort to improve the protocols related to AMF inoculation and to use different suppliers and strains of AMF inoculum, as described in Chapter 3, I was not able to establish symbiosis in experiment 1, and in experiment 2, I detected only 5% of root colonization. Overall, it is generally accepted that good establishment of AMF is a key prerequisite for inducing resistance in plants (Khaosaad et al., 2007; Pozo and Azcón-Aguilar, 2007). The minimum level of root colonization for inducing resistance responses in plants is widely recognized to be around 30%. However, the context-dependency of root colonisation that I found in my experiments is in line with a growing number of studies where the unpredictability of AMF colonisation is highlighted (Salomon et al., 2022; Shein et al., 2021). Another limitation, discussed in Chapter 4, is the ability to detect VOCs and identify the "unknown" compounds captured by SPME fibres. Additionally, the limited number of replicates (n = 3) used for capturing the honeydew VOCs may have also contributed to the variable detection of volatile compounds and the subsequent variability between samples, making it difficult to determine whether there was a significant treatment effect (in this case, aphid symbiont infection). Analysis of volatile compounds remains a challenge due to the lack of sensitivity of some techniques, the difficulty in collecting representative samples and the time-intensive process of compound identification, which often limits the number of samples that can be processed (Brown et al., 2021; Wardencki et al., 2007).

6.3 Abiotic and biotic environmental factors driving interactions between species within communities

Ecological communities are complex, and each species can be directly and indirectly influenced by and connected to each other (Wootton, 1994). Abiotic factors, such as drought stress, can affect those relations directly and indirectly through the plant host (Leybourne et al., 2021). Despite the economic importance of aphids as insect pests of potato plants and the increasing incidence of drought as the climate changes, little attention had been paid to how different species of aphids interact when their host plant undergoes severe drought stress. This was investigated, comparing two common pests in potato, *Macrosiphum euphorbiae* and *Myzus persicae* in Chapter 2. The results indicated the suppression of *Myzus persicae* and *Macrosiphum euphorbiae* by drought stress, but limited evidence of interspecific competition.

A comprehensive review focused on herbivorous insect responses to drought stress on trees, highlighted interesting findings. The study reported that very few studies analysed the impact of drought on insect assemblages, focusing on the effects of drought stress only on one or few species (mostly leaf-feeding). Hence, the evidence related to the effect of drought on the third trophic level are extremely scarce (Gely et al., 2020). Further work is needed to establish exact mechanisms governing the outcome of interspecific competition under drought stress in community interactions. Studies estimating the effect of drought stress on generalist and specialist pests and their natural enemies could provide a better understanding of how communities are altered by drought stress. Drought-insect-plant dynamics were further examined in Chapter 3.

6.4 The importance of endosymbionts in multitrophic interactions

In the last 10-20 years there has been growing recognition of the importance of endosymbionts as crucial contributors to the ecological or evolutionary success of insects (McCutcheon et al., 2019). As aphid endosymbionts mediate interactions between plants and insects, as discussed in Chapter 1, a novel area of study involves investigating their potential effects on below-aboveground interactions. A comprehensive study, where AMF, aphids with and without *H. defensa* infection, and *Aphidus ervi* were used, reported that plants infested with *Macrosiphum euphorbiae* with *H.defensa* showed higher allocation of plant mass to below-ground plant parts (Karley et al., 2017). The effect of *H. defensa* on above-belowground interactions was explored

in Chapter 3 and Chapter 4 using *Macrosiphum euphorbiae* feeding on potato. A significant effect on aboveground plant mass was not detected in Chapter 3, but tuber dry mass was lower in plants under well watered conditions when infested with symbiont-free aphids. Further, stomatal conductance was significantly lower when the endosymbiont was present. This result suggests that *H. defensa* infection might indirectly mediate plant responses to water availability. Generally, drought stressed plants are inferior hosts for aphids (Davis et al., 2015; Huberty and Denno, 2004), as successful feeding by aphids requires adequate plant cell turgor pressure which is mediated by plant water content (Archer et al., 1995, Taiz and Zeiger 2002). The findings that aphid infection with *H.defensa* differentially affected tuber dry mass allocation in response to water availability, and also reduced stomatal conductance, suggests that the symbiont affects mechanisms at the aphid-plant interface (e.g. through aphid feeding or saliva composition) that lead to changes in plant physiology. Exactly how this happens, and the potential consequences for plant tolerance of drought stress requires further investigation. Although molecular mechanisms were not investigated here, these are highlighted as a potential avenue for future investigation.

Aphid honeydew is known to be a potential food source for parasitoids (Tena et al., 2018), and parasitoids have been shown to use honeydew presence as a cue for searching (Wäckers et al., 2008). Few studies, however, have investigated the VOCs emitted from honeydew to assess which compounds might influence parasitoid searching behaviour (Leroy et al., 2012, 2011a). In Chapter 4, an experiment was carried out to gain insight into potential differences among volatiles released from honeydew of aphids infected with *H. defensa* compared with symbiont-free aphids. The results showed that parasitoid wasps were more attracted by honeydew quantity rather than presence or absence of endosymbionts, although aphids harbouring the symbiont often produced more honeydew; this might also relate to the effects of symbiont presence on plant mass allocation (see above). Although no significant effect was found of endosymbiont presence or aphid genotype on volatile compounds in the experiment, several potential candidate VOCs were detected with the SPME method that might influence wasp behaviour. Plants under attack by insect pests can release volatiles for attracting natural enemies (Röse et al., 1998). Simultaneously, many aphid species can release alarm pheromone, such as (E)- *β-farnesene* for warning colony members of imminent dangers. However, this pheromone can act as a kairomone and attract natural enemies (Hatano et al., 2008). In my samples, several terpenes were detected such as *β-farnesene, Trans-b-farnesene, Z-e-alpha-farnesene* and *Germacrene-d-alpha-muurolene.* It would be interesting to examine each

semiochemical compound detected in the experiment for the effects on wasp behaviour. However, improvements in the methodology for detecting volatile compounds would allow for more detailed understanding of their role in regulating interactions between insect pests, their endosymbionts and aphid natural enemies, and highlight opportunities for using VOCs as another tool for attracting aphid natural enemies as a component of integrated pest management.

6.5 Soil microorganism effects are context dependent, but soil management play a key role in bottom-up dynamics

Soil microorganisms are known to have affected the evolution of plants (Lyu et al., 2021), influencing plant resistance and susceptibility towards abiotic and biotic factors, and are responsible for supporting many ecosystem services associated with soil and vegetation (Bender et al., 2016). Therefore, it is widely acknowledged that soil microbes represent a "green" technology for achieving sustainable agriculture. For instance, AMF have been reported to increase parasitism attack of insect pests. In *Phleum pratense L*., inoculation with *Glomus intraradices* increased by 140% the frequency of parasitism of *Aphidius ervi* on *Rhopalosiphum padi* (Hempel et al., 2009). Another multifactorial experiment using *Solanum tuberosum* inoculated with AMF, reported that the presence of AMF promoted parasitism of the aphid *Macrosiphum euphorbiae* by its parasitoid *Aphidius ervi* (Bennett et al., 2016). However, several factors should be improved in AMF technology to have reliable outcomes in IPM programs and agricultural sustainability. For example: 1) characterise and select specific strains that are suitable for a target pest and environment; 2) increase quantity of spores of propagate in the final product, for optimizing higher percentage of root colonization; 3) conduct more research in field conditions and under different environments, for reducing the context-dependency ; 4) identify native AMF communities *in situ* that could be isolated and used for specific ecosystem.

Farming management approach is the major factor affecting agrobiodiversity and the delivery of ecosystem services from agricultural land (Kremen and Miles, 2012). The results of Chapter 5 support these findings, showing that parasitoids invested more time in searching for plants grown in soil from an integrated farming system compared with soil managed conventionally. Soil microbiome composition could be an important factor affecting higher trophic levels indirectly through their effects on the plant-insect herbivore interaction, and it would be

interesting to further investigate the mechanisms underpinning these plant-mediated bottom effects on aphid natural enemies. Specifically, studies combining VOCs analysis, metabolomic analysis and metabarcoding of microbial communities would allow for a more detailed and comprehensive understanding of the species-level responses within the system. For example, long term experiments testing soil microbial community composition in response to aphid infestation could elucidate how root microbes assemble when plants are under attack and transcriptomic analysis could identify genes expressed in this interactive process. Hence, field work involving different farming systems over a full growing season could test natural abundance of parasitoids in the fields. Holistic experiments that combine several variables and conducted "in situ" could provide valuable insight for increasing parasitoid presence/activity and therefore improve IPM of aphid pests.

6.6 Conclusion and future directions

This study provides a first step towards evaluating mechanisms by which microbes in the soil and aboveground (in insect herbivores) can influence the outcome of multitrophic interactions and identifying how this knowledge could be used to improve insect pest management. The research findings have raised further questions, and the methodological limitations discussed here represent opportunities that future studies should aim to address.

In summary, the findings of this thesis provide five areas for future research:

1) The effect of drought stress in mediating insect pest and natural enemy community dynamics;
2) Elucidating which mechanisms are involved in mediating AMF colonization for improving their reliability as an IPM tool;
3) Disentangle the mechanisms underpinning aphid endosymbiont effects on aphid-plant aphid-parasitoid outcomes;
4) Establish protocols to better detect VOCs from different biological samples;
5) Investigating whether soil management practices can be used to improve insect pest control.

Ultimately, much more work is needed to determine mechanisms affecting multitrophic interactions below and aboveground with future research incorporating field experiments,

performed in multiple locations, and combining a holistic approach. This would extend our knowledge and awareness of how communities interact in natural agroecosystems and can be managed to improve pest control with reduced reliance on agrochemical inputs.

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