The potential of *Pseudomonas* bacteria as biocontrol agents against multiple plant pathogens

Sophie Elizabeth Clough

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Abstract

Plant pathogenic bacterium *Ralstonia solanacearum* (the causative agent of bacterial wilt), and plant parasitic nematodes *Globodera pallida* (white potato cyst nematode) and *Meloidogyne incognita* (root-knot nematode) have devastating impacts on several economically important crops globally. While these pathogens are traditionally treated with agrochemicals, their use is in decline due to legal restrictions and harmful impacts on the environment. One environmentally-friendly alternative to agrochemicals could be biocontrol, which takes advantage of naturally occurring plant growth-promoting bacteria. While recent studies have shown promising results, there is no clear screening pipeline to identify and validate successful biocontrol strains with broad activity against multiple different pathogen species.

This thesis establishes a screening method to identify effective *Pseudomonas* biocontrol agents to both bacterial and nematode pathogens using a combination of *in vitro* laboratory assays, comparative genomics, mass spectrometry and greenhouse experiments. It was found that *Pseudomonas* strains suppressed both pathogens, with strains CHA0, MVP1-4 and Pf-5 showing high activity. Several secondary metabolite clusters were identified and the suppressive role of DAPG, orfamides A and B and pyoluteorin antimicrobials were experimentally verified. Experimental evolution was used to show that *R. solanacearum* can evolve tolerance to *Pseudomonas* strains during prolonged exposure *in vitro*. However, further work is needed to test whether the evolution of tolerance could limit *Pseudomonas* biocontrol efficiency in the field. High nematode suppression by all *Pseudomonas* strains was observed in laboratory assays. Moreover, clear behavioural and developmental changes were observed in response to tested individual compounds. Despite clear nematode suppression observed *in vitro*, no protection by *Pseudomonas* was observed in greenhouse experiments. However, *Pseudomonas* strains reduced bacterial wilt disease incidence caused by one of the tested *R. solanacearum* strains *in vivo*. Despite promising *in vitro* results, more research is needed to translate *Pseudomonas* biocontrol potential into an agricultural context.

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Declaration by Author

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Chapter 1. General Introduction

1.1 Threats to food security

Food security on a global scale faces a number of threats which are worsening over time. These can be attributed to human impacts through population growth which increases the demand for food production and climate change effects that create challenges for the functioning of current food production systems. In order to increase food production, the use of simplified crop monocultures became preferable for high yields, this reduces crop diversity and leading to suitable conditions for plant pathogens to thrive (Strange and Scott, 2005; Lin, 2011; Ekroth, Rafaluk-Mohr and King, 2019). An inefficient use of pesticides and an increasing number of laws banning their use has resulted in plant pathogens becoming more difficult to control. The regions suffering from the greatest crop losses have been associated with the fastest growing human populations as well as emerging and re-emerging plant pests and diseases (Savary *et al.*, 2019; Vannier, Agler and Hacquard, 2019) leading to urgent need for effective and low-cost pathogen control methods.

1.1.1 The impacts of population growth and climate change on food security

The global population is growing at a considerable rate, putting increased pressure on food production. Global estimates predict the current human population (7.6 billion) will rise to approximately 10 billion by 2050 (Crist, Mora and Engelman, 2017). A larger population results in greater consumption, resource use and CO_2 production, all of which contribute to global warming and accelerating climate change. Climate change is a major issue which affects the supply and demand of crops (Wei, Huang, *et al.*, 2015). It can influence seasonal temperatures, rainfall levels, soil moisture, humidity and CO_2 levels resulting in crop yields becoming less predictable. It has been shown that four of the major global crops for human consumption (wheat, rice, soybean and maize) will all be negatively impacted with reduced yields due to climate change (Zhao *et al.*, 2017).

Not only does global warming affect crop yields through abiotic effects, it can also influence plant pathogen populations by increasing pathogen densities and resulting disease incidence (Hayward, 1991). Climate change effects can increase disease incidences by prolonging the growing season, allowing diseases a longer time to develop and take hold (Salinari *et al.*, 2006;

Elad and Pertot, 2014; Tomlinson *et al.*, 2014). A variety of plant pathogen populations are known to increase in these altered conditions such as, bacterial pathogen *Ralstonia solanacearum* (Hayward, 1991), plant parasitic nematodes (Jones *et al*., 2016), oomycetes including *Plasmopara viticola* (Salinari *et al.*, 2006) and fungal pathogens including *Fusarium pseudograminearum* (Vaughan, Backhouse and Ponte, 2016). Kaczmarek *et al.* (2014) addressed the concerns of climate change and its impacts on global temperatures as these are rising at elevated rates, particularly in the last 50 years since the industrial emissions have increased exponentially. They highlighted that temperature is one of the most influential factors contributing to survival and virulence of a soil-borne plant pathogen (Kaczmarek *et al.*, 2014). Examples of a widening host range of nematodes due to climate change has been observed in a variety of regions with the first ever reports of parasitic nematode infections in yam, African nightshade and sweet potato in Nigeria, Kenya and Mozambique (Coyne *et al.*, 2018) as well as in animal-infecting nematodes in colder regions such as the Arctic (Okulewicz, 2017). Okulewicz *et al*. (2017) investigated the rate of potato cyst nematode egg hatching over a range of five temperatures experienced in the UK potato growing season at different sites and found higher temperatures resulted in increased hatching. There are growing risks expected with pathogen survival in UK soils, including that the geographic host range is predicted to broaden with the changing climate, creating new challenges to the UK potato industry (Okulewicz, 2017). Host range and distribution of bacterial pathogens such as *R. solanacearum* is also expanding with the first reports of infections in previously unreported hosts including ornamental *Rosa* species in the Netherlands in September 2016 (Tjou-Tam-sin *et al.*, 2017) and in Arugala (*Eruca vesicaria)* in Brazil in 2016 (Albuquerque *et al.*, 2016). Finding ways to safely control pathogens with increasing demand of crop production is thus one of the greatest challenges for the expanding human population.

1.1.2 The widespread impacts of plant pathogens on crops

Crop pests and pathogens have been recently assessed to influence all aspects of food security from production to quality, nutritional value and stability (Savary *et al.*, 2019). Plant pathogens can fall into a number of groups such as insects, viruses, fungi, bacteria, oomycetes and nematodes (Strange and Scott, 2005; Savary *et al.*, 2019). Almost 80% of annual crop loss is due to pathogenic infection (Oerke, 2006). Crops are subject to disease both in the field and post-harvest (Strange and Scott, 2005). Key historical plant disease epidemics involving important food crops include the potato late blight in the 19th and 20th century caused by *Phytophthora infestans* (Savary *et al.* 2017). This disease swept across Europe, leading to the death or displacement of a quarter of the Irish population (Savary *et al.*, 2017). Significant factors contributing to this wide

scale impact included the high dependence on potato the main subsistence crop in Ireland, and susceptibility of the entire potato population to this pathogen species (Savary *et al.*, 2017). Other crop epidemics include wheat rusts caused by *Puccinia* spp. in the US and Asia, *Fusarium* head blight across Europe, China, US and Brazil, Brown spot of rice today in South Asia caused by *Cochliobolus miyabeanus* and coffee rust in Central America today by *Hemileia vastatrix* (Savary *et al.*, 2017). In modern agriculture, emerging antibiotic resistance to the products developed following previous epidemics is also a growing issue (Sundin and Wang, 2018). There is a clear need to improve both crop yield globally and control plant pathogenic infections.

1.1.3 A brief history of agrochemical use and the associated environmental concerns

Agrochemical practice used in the past and in the present to control these various pathogens includes pesticides, fungicides, herbicides and insecticides. In addition, integrated pest management (IPM) is a practice commonly carried out by crop producers which involves crop rotation, intercropping, organic manuring and the use of appropriate agrochemicals to prevent high disease incidences (Urwin *et al.*, 1997; Nguyen and Ranamukhaarachchi, 2010). Agrochemicals have been critical in the food production industry to ensure food security (Sparks and Lorsbach, 2017). Pesticides were first introduced in the last century with large scale production beginning in the 1940s and since then, the agricultural industry has vastly grown globally resulting in a growing dependence on agrochemicals such as fertilisers to ensure high levels of food production (Zadoks and Waibel, 2000). Classes of agrochemicals can include insecticides targeting insect pests, fungicides targeting fungal pathogens, nematicides which target nematode pathogens and herbicides which are used to constrain the growth of weeds. The first fungicide products up until the 1940s were made from combinations of chemicals including brine, arsenic, sulfur and mercury chloride (Morton and Staub, 2008). Classes of synthetic fungicides were then developed such as dithiocarbamates and phthalimides followed by triazoles and strobilurins, some of which are still used today (Morton and Staub, 2008). Herbicides such as triazines and imidazolinones were first produced as non-selective chemicals for general weed control (Reddy and Nandula, 2012). Non-selective herbicide resistance grew exponentially in the 1980-1990s and synthetic herbicides such as glyphosate then took over for greater selectivity becoming the widest-used herbicide in the world and (Gasnier *et al.*, 2009; Shaner, 2014). Agrochemicals such as zinc thiazole, bismerthiazol and saisentong have also been developed to combat bacterial plant pathogens such as *R. solanacearum* and *Xanthomonas oryzae* (Chen *et al.*, 2016b). These have mainly been used in China since the 1970s and are known to be toxic to

humans.

The introduction and subsequent bans of pesticides is a fairly common process, as negative impacts of pesticides on the environment and humans are not always immediately known. This has occurred for well-known insecticides including dichlorodiphenyltrichloroethane (DDT) and neonicotinoids. DDT was used extensively in the 1940s and 1950s to target the insect vectors of typhus. Increased insect tolerance to DDT began to reduce its effectiveness and was reported to cause acute and chronic toxicity and suspected carcinogenicity which contributed to its eventual ban (Turusov, Rakitsky and Tomatis, 2002). Neonicotinoids are highly effective broad-spectrum insecticides, but are detrimental to non-target and important insect populations such as a variety of bee species which are vital key pollinators (Goulson, 2013; Tomlinson *et al.*, 2014). Methyl bromide was a biofumigant previously used to control pathogens such as *R. solanacearum* and nematodes, however it is being phased out due to its identification as an ozone-depleting molecule (Taylor, 1994; Santos *et al.*, 2006). There have been increasing concerns regarding the use of the herbicide glyphosate and its impacts on human health as cytotoxic effects on the human endocrine system were reported (Gasnier *et al.*, 2009). Dibromochloropropane (DBCP) use was deregistered in the 1970s as it was found to cause human male sterility despite being designed as a nematicide (Fuller, Lilley and Urwin, 2008). The majority of pesticide formulations are applied with the addition of products known as adjuvants. These can enhance their properties by improving retention on and penetration into the leaf, and increased UV and temperature stability. Studies of adjuvants such as polyethoxylated tallowamine (POE-15) revealed that some of these also have toxic effects on human cells (Mesnage, Bernay and Séralini, 2013). There has been concern raised over the environmental impacts of using pesticides as they leach into aquatic systems through rivers and aquifers affecting the larger ecosystem surrounding these crop fields. In previous years, the NGO Greenpeace reported that 70% of pesticides applied in China were absorbed into the plant and subsequent run off of the remaining 30% led this to seep into the soil and groundwater. Zhai *et al*, (2018) described the impacts of the volatile nematicides used previously which damaged the ozone layer. As a result of these issues, there are fewer plant protection chemicals available which has resulted in an increased loss of crops annually, costing billions of pounds. These raised regulatory standards make it a challenge for researchers to discover agrochemicals that meet the strict requirements (Sparks and Lorsbach, 2017).

1.1.4 Emerging agrochemical resistance among plant pathogens

Another growing concern with food security is the evolution of pathogen resistance to agrochemicals (Chen *et al.*, 2016b) similar to rapid bacterial resistance to clinical antibiotics (Hawkins *et al.*, 2019). Pesticide resistance has been widely observed from insecticide resistance with over 330 cases of the neonicotinoid imidacloprid resistance in the 20 years since its launch (Bass *et al.*, 2015). Other forms of agrochemical resistance include fungicide resistance, such as *Botrytis cinerea* resistance to AnilioPyrimidines (Brent and Hollomon, 2007; Powles and Yu, 2010) and herbicide resistance to glyphosate (Shaner, 2014; Lucas, Hawkins and Fraaije, 2015). Insecticide resistance was first observed roughly 100 years ago with over 15,000 reported incidences; however resistance of weeds and plant pathogens has been slower to develop with roughly 500 cases each by 2016 (Sparks and Lorsbach, 2017). The speed at which resistance evolves can be rapid in plant pathogens taking place within several years in some cases (Hawkins *et al.*, 2019). In addition to this, as some plant pathogens become more controlled, this creates new opportunities for other pathogens to become more prominent and occupy the niches vacated (Sparks and Lorsbach, 2017). This can result in the need to control a larger range of plant pathogens. Relatively little is known about plant pathogen resistance in natural environments and within this limited knowledge, there is minimal experimental evidence exists of these occurrences.

1.1.5 Alternative options to traditional agrochemicals

Due to increased occurrences of pesticide resistance to traditional agrochemicals, a number of alternative methods have been developed over the last few decades. These can be broadly categorised into approaches that focus on (1) modifying the host plant, (2) modifying the pathogen or (3) modifying the environment in which the two interact.

1.1.5.1 Modifying the host plant

Creating transgenic plants by modifying host plant DNA to encourage plant resistance has been investigated since the advent of DNA engineering. As of 2015, farming of genetically modified crops was conducted in 28 countries - accounting for over 10% of the world's arable land (James, 2015). Transgenic modifications to plants have been observed to be effective against a variety of plant pathogens through mechanisms such as reinforced physiological barriers to prevent pathogen invasion, increased immune receptors which initiate defence responses and RNA interference which detects and combats invading pathogens (Urwin *et al.*, 1997; Deslandes *et al.*,

2002; Dong and Ronald, 2019).

Mcgarvey *et al*. (1999) found that engineering the tomato plant chromosome VI, which is known to contain a resistance locus, restricted the bacterial pathogen *Ralstonia solanacearum* movement into the xylem and up the stem during its infection process in both greenhouse and field studies. This resulted in the bacteria being unable to produce extracellular lipopolysaccharide (EPS) in the xylem and inhibited bacterial wilting symptoms (Mcgarvey, Denny and Schell, 1999). Various other plant hosts have been engineered to be resistant to numerous pathogen types. For example, tobacco plants have been modified to express the antifungal endochitinase gene from *Trichoderma harzianum* enabling plant tolerance or resistance to a number of fungal pathogens including *Rhizotonia solani* and *Botrytis cinerea* (Topper, 1998). Similarly, transgenic potato lines have been developed to combat potato cyst nematode infections by secreting a peptide that binds to nematode acetylcholine receptors and inhibits chemoreception, which could result in reduced disease incidence (Urwin *et al.*, 1997; Fuller, Lilley and Urwin, 2008; Green *et al.*, 2012).

Plasma membrane-localised pattern recognition receptors (PRRs) are conserved and recognise pathogen-associated molecular patterns (PAMPs) resulting in local and systemic plant immune responses (Boschi *et al.*, 2017). Recent investigations into transgenic crop lines include potato lines expressing the *Arabidopsis thaliana* PRR and elongation factor-Tu receptor (AtEFR) which gave rise to resistance against *R. solanacearum* (Boschi *et al.*, 2017). As PRR functioning is conserved and essential, it is suggested this will provide a more durable resistance against plant pathogens (Boschi *et al.*, 2017). Nucleotide-binding domain and leucine-rich repeat (NLR) genes also confer plant resistance to pathogens (Thomas *et al*., 2020). For example, an NLR termed 'Roq1' discovered in the plant *Nicotiana benthamiana* conferred resistance to three plant pathogens *Xanothomona*s *perforans*, *Pseudomonas syringae* and *R. solanacearum*, and transferring this to target crops is the focus of future research (Thomas *et al.*, 2020).

Tomlinson *et al.* (2014) considered the technique of "stacked" resistance genes using a new technique "SMRT RenSeq" which enables faster detection of resistance genes. Using this method, it is possible to "stack" several different resistance genes into a single host plant from its wild type relatives, resulting in a broader resistance range (Tomlinson *et al.*, 2014). Potential dual resistance could also be achieved by selecting for plants that are resistant to both types of pathogens. (Kunwar *et al.*, 2015) revealed grafting *R. solanacearum*-resistant tomato rootstocks

to new plant populations resulted in a simultaneous reduction of bacterial wilt and root knot galling incidences.

1.1.5.2 Modifying the plant pathogen to further future agrochemical alternatives

Studies investigating modifying plant pathogens can reveal new targets for controlling pathogens as an alternative to agrochemicals. Atkinson *et al.* (2013) discovered the role of the gene gp-flp-32 in migratory behaviour of nematodes. By silencing this gene, nematode J2 juvenile migratory behaviour in soil environments increased, as the nematode eggs hatch and enter the soil prematurely - resulting in their death before reaching a target plant host. This study highlighted potential targets for future nematicides. RNA interference (RNAi) was reported as a procedure to study genes associated with disease in parasitic nematodes (Urwin, Lilley and Atkinson, 2002). This occurred by using octopamine (a biologically active amine) to stimulate the uptake of double-stranded (ds)RNA targeting the cysteine proteases of two species of plant parasitic nematode (PPN) and thus reducing their disease incidence levels on host plants and has the potential to be developed as a control method. Nematode neuropeptides as transgenic nematicides have also been considered to target pathogens (Warnock *et al.*, 2017). PPNs can assimilate exogenous peptides which elicit physiological effects. The authors profiled these as model nematicides which can impact chemosensation, host invasion and stylet thrusting of *M. incognita* and *G. pallida*. These modified peptides secreted from transgenic *Bacillus* spp. and the microalaga *C. reinhardtii* could interact with PPNs and resulted in tomato infections reduced by up to 90% when compared with controls. This data paves the way for novel nematicides using novel non-food transgenic delivery systems as biocontrol.

1.1.5.3 Modifying the environment in which plant hosts and pathogens interact

A third alternative to reducing the use of agrochemicals and plant pathogen infections could be through alteration of the external environment in which plant hosts and pathogens interact. Traditionally, integrated pest management (IPM) is one recommended solution to reduce plant disease incidence. IPM includes a number of strategies such as using fertiliser, crop rotation, developing host plant resistance, using bio-pesticides, push-pull strategies and trap crops (Reuveni and Reuveni, 1998; Thomas, 1999; Cook, Khan and Pickett, 2007; Govaerts *et al.*, 2007). The addition of fertilisers to soil can have vast effects depending on the type of crop and fertiliser added. Fertiliser is a broad term, and can be manufactured from chemical components such as

nitrogen, phosphorous, potassium, calcium, sulphur and magnesium or from organic material such as manure and other waste to create nutrient-rich compost (Dordas, 2008). The addition of these can enable a plant to be more disease-tolerant to pathogens by inducing systemic resistance (Reuveni and Reuveni, 1998). Crop rotation involves altering which crops are grown each season by rotating crop species and the number of crops per year and this can reduce pathogen carryover between seasons (Govaerts *et al.*, 2007). This is important when pathogens can specifically infect one plant host such as potato cyst nematodes and so crop rotation is heavily involved in this practice to lower pathogen numbers and disease incidence (Urwin *et al.*, 1997). Using trap crops involves growing alternative plant species between the desired crop which permit infection but hinder pathogen reproduction and development within the host and stop the spread/pathogenicity from being transmitted further. This has been shown to be effective against plant parasitic nematodes (Scholte, 2000; Kooliyottil *et al.*, 2016).

Pathogen control can also be achieved by changing the structure of soil with different additives. Biochar are small particles of charcoal produced from plant matter following thermal degradation which were first created to improve soil fertility and mitigate climate change effects, but since have also been shown to have interactions with plant pathogens (Lehmann *et al.*, 2011). Biochar can affect plant root exudates in the soil which influences pathogen chemotaxis towards plants and suggests potential options to reduce host plant infection and crop loss (Gu *et al.*, 2016). Gu *et al.* (2016) tested the ability of biochar particles to reduce *R. solanacearum* infections in tomato plants. Biochar particles were able to lower disease incidence by heavily influencing the swarming motility of *R. solanacearum*.

Chen *et al.* (2016a) investigated using “nanotechnology” including silver nanoparticles in the soil to see if these reduced bacterial wilt incidence in tobacco plant infections in China. Silver nanoparticles have strong antibacterial activity and can inactivate both gram positive and gram negative bacterial pathogens, fungal pathogens and viruses. However, difficulties in previous crop applications have included agglomeration and poor diffusion through soil. The authors found certain applications had lethal effects on *R. solanacearum* in laboratory media and pot experiments by damaging cell membranes and destroying cellular proteins revealing future potential use of these nanoparticles (Chen *et al.*, 2016a). The authors highlight the nanomaterials are effective at extremely low doses well below the United States Environmental Protection Agency guidelines (USEPA, 1963), but propose future studies to monitor plant health directly.

Biofumigation is a method using plant material which involves the crushing of brassicaceous residues in soil which releases volatile compounds called isothiocyanates (ITCs) that can be toxic to plant pathogens. These have been shown to suppress plant pathogen activity including nematodes (Ngala *et al.*, 2015), bacterial pathogens (Sofrata *et al.*, 2011) and fungal pathogens e.g. *Rhizoctonia solani* (Fayzalla, El-Barougy and El-Rayes, 2009; Hansen and Keinath, 2013). Using plant phytochemicals as antagonists to pathogens has also been investigated as an alternative to agrochemicals. Chen *et al.* (2016b) tested if plant phytochemicals coumarin (found in plants such as sweet clover and lavender) and resveratrol (found in grapes, peanuts and strawberries) could reduce bacterial wilt incidences caused by the plant pathogen *R. solanacearum* in tobacco plants. Both were shown to inhibit the growth cycle, biofilm formation and swarming motility of the bacterial pathogen with resveratrol being the more effective. Būda and Čepulyte-Rakauskie, (2011) describe how different plant exudates released into the soil can cause toxicity to some plant pathogen species such as nematodes. The authors investigated one chemical, Linalool (3,7-dimethyl octa-1,1-dien-3-ol), which has been reported by earlier studies to be toxic to root knot nematodes, *Meloidogyne* spp., by inhibiting egg hatching (Ibrahim, Traboulsi and El-Haj, 2006). Būda and Čepulyte-Rakauskie, (2011) tested this on other PPN species (potato cyst nematode species) and instead of showing toxicity effects, it acted oppositely as a chemoattractant, eliciting positive responses from J2 juveniles in potato tuber soil microenvironments. This research revealed the importance of species-specificity as it showed chemicals can have different effects on different pathogen species.

Lastly, alternatives to field culturing of crops have been suggested for some crops. For example, Fujiwara *et al.* (2012) reported that growing tomato plants using organic hydroponic systems significantly reduced bacterial wilt incidence leading to higher crop yields. Hydroponic systems can also be used in combination with other agrochemical alternatives such as *Pseudomonas* bacteria which exhibit biocontrol traits (Duffy, Keel and Défago, 2004). Additionally, hydroponic systems have been developed using artificial soils made of specialist foams which chemically, physically and biologically resemble that of soil (Edmondson *et al.*, 2020). Such developments could encourage crop production in urban areas where there is little land space available for natural crop production. Some studies have also shown that altering the transplantation time of tomatoes can help to reduce losses to *R. solanacearum* pathogen due to generally cooler crop season temperatures (Wei, Huang, *et al.*, 2015). However, such practices might be pathogen-specific and hard to apply with other crops.

Changing the biotic interactions in the rhizosphere via inoculation of soil microbes that suppress plant pathogens or potentially prime the host plant immunity has also been suggested as a potential alternative to traditional agrochemicals (Weller, 1988; Vannier, Agler and Hacquard, 2019; Trivedi *et al.*, 2020). Microbial inoculants can survive in soil environments due to organic material deposits from plants in the rhizosphere and from mutualistic and antagonistic interactions between different microorganisms and with the plants themselves in the rhizosphere (Whipps, 2001). Plant growth promoting bacteria (PGPB) are able to bring beneficial effects to plants such as through biofertilization and stimulating root growth, as well as suppressing disease-causing plant pathogens through antimicrobial compound secretions, hyperparasitism and increasing competition for resources (Lugtenberg and Kamilova, 2009; Vannier, Agler and Hacquard, 2019). In this thesis, I will focus on eight *Pseudomonas* PGPBs to explore whether they can be used to develop a broad range biocontrol method against plant pathogenic *Ralstonia solanacearum* bacterium and *Globodera pallida* and *Meloidogyne incognita* nematodes.

1.2 A brief history of biocontrol

1.2.1 What is biocontrol?

Biocontrol is a method of controlling pathogens or pests using natural competitors. Kerr and colleagues discovered the first commercial biocontrol agent, *Agrobacterium radiobacter* strain K84, which was used to control crown gall disease in commercial stone fruits and roses in 1979 (Kerr, 1980; Fravel, 2005). Biocontrol has since been a growing research area during the last 30 years, becoming a more important form of pathogen biocontrol in agriculture (Weller, 1988; Droby *et al.*, 2016). Biocontrol "agents" can be a variety of different microbes ranging from fungi to bacteria and viruses (Fravel, 2005). In general, biocontrol agents are considered to pose a lesser threat to the environment compared to chemical agents as they are often derived from the natural environment. In 2005, commercial biocontrol products contributing to plant health management accounted for 1% of agricultural chemical sales (Fravel, 2005). The biocontrol market, by 2018, had a value of $1.9 billion worldwide and has grown to above 5% of agricultural sales worldwide (Robin and Marchand, 2019). Haas and Défago (2005) highlighted that especially the development of mass spectrometry technology enabling microbial metabolites detection in the rhizosphere, and genetic engineering of knockout mutants, have recently accelerated research in this area. Developing successful biocontrol agents does, however, still face a number of challenges as plant-microbe interactions and the physical environment vary dynamically in the

natural environment, which is harder to reflect these complex interactions in more controlled but simplified lab conditions (Handelsman and Stabb, 1996; Legein *et al.*, 2020).

1.2.2 Different forms of biocontrol methods

Several different microbes have been studied for their biocontrol potential including fungi, viruses and bacteria. Fungi have also been proposed as biocontrol agents to tackle plant pathogens. Arbuscular mycorrhizal (AM) fungi such as *Glomus versiforme* are able to produce phenols which can suppress multiple types of plant pathogens such as bacterial pathogen, *R. solanacearum,* and fungal pathogens such as *Rhizoctonia solani* and *Fusarium solani* (Zhu and Yao, 2004). Yang *et al.* (2016) investigated the antibacterial effects of mushroom-derived chitosan polymers on *R. solanacearum*, which have been previously shown to control *R. solanacearum* growth in plants. These were investigated in this study against *R. solanacearum* strains from five races (see Section 1.3.1 below) and were effective at enhancing membrane permeability and inhibiting the growth of the *R. solanacearum* Race 5 by 80%, the other four races were all inhibited by less than 50%. Fungal biocontrol agents such as *Verticillium suchlasporium* and *Arthrobotrys oligospora* have also been considered against nematode plant pathogens as well as bacterial pathogens but this is less popular (Lopez-Llorca and Claugher, 1990; Tunlid *et al.*, 1994; Topalović, Hussain and Heuer, 2020). The specificity of fungal biocontrol agents comes into question as fungal species can interact with a wide variety of hosts including mammals, which may have unintended negative impacts (Goettel *et al.*, 2001).

Using bacterial pathogen-specific viruses, bacteriophages, is another biocontrol alternative which has been investigated especially in case of plant pathogenic *R. solanacearum* (Yamada *et al.*, 2007; Fujiwara *et al.*, 2011; Bhunchoth *et al.*, 2015; Álvarez, López and Biosca, 2019; Wang *et al.*, 2019). Bacteriophages are viruses which infect bacteria in two ways either through causing lysis (lytic phages) or maintaining a persistent but mostly dormant infection (lysogeny) via incorporation of their genome into the host bacteria and replicating along with the growth of bacterial population (Suttle and Chan, 1994). Due to high host strain specificity, phages are often applied as “phage cocktails” to increase the infectivity range of phages against highly variable pathogens such as *R. solanacearum* (Bhunchoth *et al.*, 2015). For example, Fujiwara *et al.* (2011) investigated phage “cocktails” and found certain phages have the capacity to infect more than one *R. solanacearum* strain: one phage even infected 15 *R. solanacearum* strains belonging to multiple different biovars exhibiting a very broad infectivity range. Phage combinations have

more recently been reported to suppress pathogen infection in greenhouse and field experiments in tomato fields (Wang *et al.*, 2019). Results found increasing combinations could suppress the pathogen, whilst also having no negative impacts on the existing microbiota highlighting the potential of this method. Combinations of phages applied through irrigation systems have also proven to be effective at controlling *R. solanacearum* (Álvarez, López and Biosca, 2019).

The most studied form of biocontrol is the use of root-colonizing plant beneficial bacteria that can suppress the pathogen, or have beneficial effects for the plant (Haas and Défago, 2005). Probiotic microflora often satisfy at least one of the following characteristics: effective colonization of the ecosystem; stimulation of host defences via activation of ISR and have direct antagonistic effects against pathogens (Haas and Keel, 2003). *Bacillus amyloliquefaciens* and fluorescent *Pseudomonas* species are examples of PGPB which have been shown to effectively reduce bacterial pathogen disease incidence (Weller, 2007; Tan *et al.*, 2013). One potential challenge which could reduce the effectiveness of PGPB is other antagonistic organisms in the soil. It has been suggested that bacterial-feeding nematodes can consume biocontrol bacteria species, which results in reduced pathogen suppression. This was shown when *Burkholderia cepacia* which displays antifungal and antinematicidal biocontrol properties was consumed by nematodes which resulted in increased fungal infections of wheat highlighting a weakness of antagonistic rhizospheric interactions (Carta, 2000).

1.2.3 *Pseudomonas* as Plant Growth-Promoting Bacteria (PGPB) and biocontrol agents

1.2.3.1 Biocontrol and plant growth-promoting functions

Pseudomonas PGPB are non-pathogenic rhizobacteria that have been proven to be some of the most effective forms of biological control against soil-borne bacterial, fungal and nematode pathogens (Baehler *et al*., 2005; Haas and Defago, 2005). The desirable quality of their use as biocontrol agents is the broad range of mechanisms by which they can competitively prevent pathogens from invading its plant hosts (Haas and Keel *et al.,* 2003). By having numerous mechanisms of action, Pseudomonads could potentially target multiple types of pathogen simultaneously, making them ideal candidates for biocontrol applications. Thomashow and Weller, (1988) were the first to report *Pseudomonas*-controlled plant disease suppression by the antimicrobial “phenazine”. They highlighted some key features of *Pseudomonas* biocontrol agents

in terms of their ability to grow rapidly, ease of mass production, ability to utilise seed and root exudates, multiplication in the rhizosphere, spermosphere and within plants, production of bioactive metabolites (antibiotics, siderophores etc.), and capability to compete with bacteria and withstand various environmental stresses (Weller, 2007). The authors also highlight the potential weakness in term of inability to produce resting spores, which could limit their commercial use when developing microbial inoculant formulations. During the last few years however, liquid formulations of bacteria such as *Pseudomonas fluorescens*, have begun to be developed and used against nematode pathogens in India (Nagachandrabose, 2020). This suggests there is the potential to overcome formulation development.

1.2.3.2 Underlying mechanisms of *Pseudomonas* biocontrol

A broad repertoire of modes of action has been reported for *Pseudomonas* PGPB. These functions include the induction of the host plant's own immune response, effects on bacterial colonization on plant roots, and direct interacting with pathogens in the rhizosphere.

Pseudomonas spp. are known to elicit induced systemic resistance (ISR) in plants leading to fortification of plant cell walls and enhancement of the plants defence signalling (Preston, 2004). ISR can elicit a number of other defence mechanisms including alteration of host physiology and metabolism and activation of plant defence chemicals against pathogens and antibiotic stress factors. *Pseudomonas* strains possess flagellae which can also trigger the defence response of plants to pathogens (Preston, 2004). Flagellin recognition is mediated by FLS2 (toll receptor kinase) which elicits oxidative burst, callose deposition and synthesis of antimicrobials in a plant-enhancing protection from pathogens (Preston, 2004; Compant *et al.*, 2005).

One definition of competence of PGPB can be their ability to colonize the roots of their host plants and the ability to survive in the environment over long periods of time (long term efficacy). The O-antigens of *Pseudomonas* lipopolysaccharides (LPS) and exotoxin A released from Type III secretion systems enable efficient colonization of plant roots, reducing points of entry for pathogens and increasing their survival (Preston, 2004; Compant *et al.*, 2005). LPS and T3SS effectors can also induce the "localised induced response", also known as the hypersensitive response which can result in programmed cell death as well as enhances plant defence against pathogens, for example via activation of ISR (Preston, 2004).

Other beneficial traits include siderophore production, which enables *Pseudomonas* to compete for iron with pathogens in the soil (Haas and Défago, 2005). This is often mediated by the synthesis of two molecules: pyochelin and pyoverdine, which also give fluorescent pseudomonads their distinct yellow-green colour and has a high affinity for Fe^{3+} (Hohnadel and Meyer, 1988). Previous studies have shown that this pigment can aid PGPB in outcompeting pathogenic bacteria and fungi in soil experiments, but this is less well-understood (Haas and Défago, 2005).

Pseudomonads are also well known for their ability to secrete various antimicrobial compounds. For example, 2,4-diacetylphloroglucinol (DAPG) is an antibiotic produced by *Pseudomonas fluorescens* which is effective at inhibiting mobility of juvenile nematodes and can also reduce hatching of cyst nematodes (Cronin *et al.*, 1997). DAPG has also been shown to suppress bacterial and fungal pathogens such as *Ralstonia solanacearum* and *Pythium ultimum,* and it is controlled by the GacA/S signalling pathway responsible for the synthesis of other *Pseudomona*s metabolites (Fenton *et al.*, 1992; Haas and Keel, 2003; Ramesh, Joshi and Ghanekar, 2009). Other *Pseudomonas* antimicrobials include phenazines that produce hydroxyl radicals and inhibit electron transport chains, pyrrolnitrin shown to inhibit respiratory chains, hydrogen cyanide a potent inhibitor of metalloenzymes and pyoluteorin whose mechanisms are less well understood (Weller, 2007). The GacA/S pathway is known to be temperature sensitive and has been shown to decrease in activity as temperatures rise which is something to consider if used in countries with hotter climates (Humair *et al.*, 2009).

Various *Pseudomonas* PGPB strains can produce cyclic lipopeptides (CLPs), which are amphiphilic molecules consisting of a cyclic oligopeptide lactone ring coupled to a fatty acid tail (Ma *et al.*, 2016). They are synthesised by non-ribosomal peptide synthases (NRPS) and the primary role of lipopeptides is membrane pore formation, which leads to an imbalance in transmembrane ion fluxes and cell death (Raaijmakers *et al.*, 2010). CLPs have a broad spectrum against bacteria, fungi, protozoa and even human cell lines and have strong biocontrol potential (Gross *et al.*, 2007; Ma *et al.*, 2016). Orfamides are a type of CLP produced by some *Pseudomonas* PGPB. Raaijmakers, De Bruijn and de Kock, (2006) described 5 groups of *Pseudomonas*-derived cyclic lipopeptides: viscosin, amphisin, tolaasin, syringomycin and putisolvin. Loper and Gross, (2007) reported the first description of orfamide presence in the *Pseudomonas* PGPB strain, *Pseudomonas fluorescens* Pf-5. Orfamides are CLPs important in bacterial surface motility, attachment to surfaces and for antagonism with other microorganisms (De Bruijn *et al.*, 2007;

Gross *et al.*, 2007; Raaijmakers *et al.*, 2010). They have also been shown to cause insecticidal and fungicidal activity (De Bruijn *et al.*, 2007; Olorunleke *et al.*, 2015; Loper *et al.*, 2016; Flury *et al.*, 2017). The biosurfactant properties of orfamides makes them important in bacterial movement and growth on seed surfaces by affecting adhesion, biofilm formation and potentially affecting the plant immunity (Tran *et al.*, 2007). CLPs have also displayed antiviral activity by effects on lipid envelopes (Raaijmakers *et al.*, 2010). Orfamide A has recently been shown to trigger an increase in cytosolic Ca^{2+} by targeting calcium channels in the plasma membrane which causes the deflagellation of algal cells (Aiyar *et al.*, 2017). Such a broad activity range might potentially pose problems also by affecting other beneficial rhizobacteria.

In addition to water-dissolved chemicals, pathogen suppression can be mediated by volatile organic compounds (VOCs), which are low molecular weight and high vapour pressure compounds produced by microbes through catabolic pathways such as glycolysis (Raza *et al.*, 2016b; Raza *et al.*, 2020). VOCs released from PGPB such as the *Pseudomonas fluorescens* strain WR-1 can display a concentration-dependent bacteriostatic effect against pathogens as well as suppress their virulent traits (Raza *et al.*, 2016b). Moreover, pathogen suppression mediated by VOCs can be magnified in bacterial communities likely due to intensified competition (Raza *et al.*, 2020). VOCs have also been shown to aid plant growth and pathogen resistance against fungal species such as *Fusarium* spp. and bacterial pathogens such as *R. solanacearum* and *Pseudomonas putida*. Moreover, one *Pseudomonas* PGPB strain isolated from Antarctic soils has been shown to suppress plant pathogenic root knot nematode via the production of VOCs (Zhai *et al.*, 2018).

1.3 Introduction to *Ralstonia solanacearum* plant pathogenic bacterium

1.3.1 *R. solanacearum* history and classification

Ralstonia solanacearum is an aerobic, non-spore forming gram negative plant pathogenic bacterium. It is soil-borne and has a polar flagellar tuft. Cells are rod-shaped and 0.5-1.5 µm in length (CABI, https://www.cabi.org/isc/datasheet/45009). *R. solanacearum* causes bacterial wilt (BW) disease and has been ranked as the second most important bacterial plant pathogen worldwide (Schell, 2000; Mansfield *et al.*, 2012). It has a broad host range and has been proven to infect over 200 plant species from over 50 plant families in both tropical and sub-tropical environments in numerous locations (Hayward, 1991; Genin and Denny, 2012). This

phytopathogenic bacterium has been classified with a quarantine status since 1996 in the UK (Elphinstone *et al.*, 1996; Annex I, Part A, Section II of Directive 77/93/EEC, Directive, 1992). The genetic diversity of *R. solanacearum* strains is very high, allowing the bacterium to occupy numerous environmental niches potentially explaining its global distribution. Due to this diversity, the bacterium is often called the *R. solanacearum* species complex (RSSC) comprising three species: *R. solanacearum*, *R. pseudosolanacearum* and *R. syzygii* (Genin and Denny, 2012; Peeters *et al.*, 2013).

R. solanacearum was first named "*Bacillus solanacearum*" by Erwin F. Smith in 1896, before being reclassified as "*Pseudomonas* spp." and "Burkholderia spp.", then eventually acknowledged as "*Ralstonia*" (Yabuuchi *et al.*, 1992, 1995). *R. solanacearum* are traditionally classified based on race, phylotype and biovar. Race is determined by their host affinity, biovar based on how they utilise alcohols and carbohydrates and phylotype depending on the strains' geographical origin and is determined by which genomic island the pathogen contains (Hayward, 1991; Genin and Denny, 2012). The four phylotypes include Asia (Phylotype I), Americas (Phylotype II), Africa (Phylotype III) and Indonesia (Phylotype IV). Although strains can be classified by their geographical location, Guidot *et al.* (2009) showed that pathogenicity islands (gene clusters carrying pathogenicity determinants) could be transferred between phylotypes which could explain why strains from different geographical locations might behave similarly. The *R. solanacearum* strains studied in this thesis belong to Phylotype 2 (Race 3, biovar 2) capable of infecting potatoes (Gabriel *et al*., 2006). This class of *R. solanacearum* strains has been described as cold-tolerant with the ability to cause greater levels of disease incidence in cooler environments including the UK (Milling *et al.*, 2009).

The dissemination of *R. solanacearum* strains around the world is thought to be linked to movement along with seed potato tubers (Elphinstone *et al.*, 1996). There have been several isolated outbreaks of the disease across England in the years 1992, 1995, 1999, 2000, 2005 and 2010 in potato crops as well as outbreaks in 1997 and 1998 in tomato crops (Parkinson *et al.*, 2013). All the UK infections have originated from contaminated river water. While the first entry of *R. solanacearum* into watercourses was likely along with industrial or municipal effluents containing potato washings (Parkinson *et al.*, 2013), the pathogen can currently persist in the river network by overwintering in the roots of solanaceous, Woody Nightshade, secondary host. These non-crop plants are widely spread in the UK and predominantly located by riverbanks, enabling

easy bacterial transmission into water supplies and to the fields along with irrigation (Zou *et al.*, 2016).

1.3.2 *R. solanacearum* infection cycle

The economically significant crop hosts of *R. solanacearum* include tomato, potato, tobacco, banana and ginger (Gabriel *et al*., 2006). In addition to host plants, *R. solanacearum* can survive in different types of habitat ranging from freshwater and coastal environments to irrigation water supplies, natural forests and agricultural soil (Xue *et al.*, 2011), where it can exist in a saprophytic phase for long periods of time in the absence of hosts (Zhu and Yao, 2004; Genin, 2010).

The different stages of infection of *R. solanacearum* have been extensively described in a review by Schell, (2000) and is shown in Figure 1.1. Infection begins at the root elongation zone or sites of lateral emergence once *R. solanacearum* have attached and colonized to these surfaces. Pili and lipopolysaccharide have been suggested to play a chemotactic role in this stage and this is also the region where root exudates are released which cause trophic chemotaxis (Vasse, Frey and Trigalet, 1995). Natural infection sites and physical wounds to the plant become the point of entry where *R. solanacearum* begin to invade the intercellular spaces of the root cortex. These wounds may have been caused by nematodes, insects, agricultural equipment and destructive growth of secondary roots (Mcgarvey, Denny and Schell, 1999). After 2-3 days bacterial cells can also be found in the intercellular spaces of the inner cortex and parenchyma. By 4-5 days bacterial masses have accumulated around the stele of the plant and cellulolytic enzymes produced by *R. solanacearum* have been suggested to influence this accumulation. After 6 days post-infection the bacterial cells can be readily detected throughout the stem and after 8 days in very high numbers. Extensive wilting is then visible due to the accumulation of *R. solanacearum* blocking the flow of sap and water transport throughout the stem. *R. solanacearum* are well adapted to surviving within the nutrient-limited, low-oxygen levels in the xylem, growing to cell densities of $10^{\wedge 8}$ or $10^{\wedge 9}$ CFU/g (Jacobs *et al.*, 2012). The exopolysaccharide shedding by bacterial cells within the plant contributes to the limited sap flow by obstructing the vascular bundles which eventually leads to wilting and plant death (Schell, 2000; Siri *et al.*, 2014). Symptoms of the infection include yellowing, wilting followed by general necrosis and death (Tans-Kersten, Huang and Allen, 2001). Some *R. solanacearum* infections can also colonize hosts asymptomatically which causes latent infections. This is poorly understood and investigating this further would improve the epidemiological understanding of these strains (Genin and Denny, 2012).

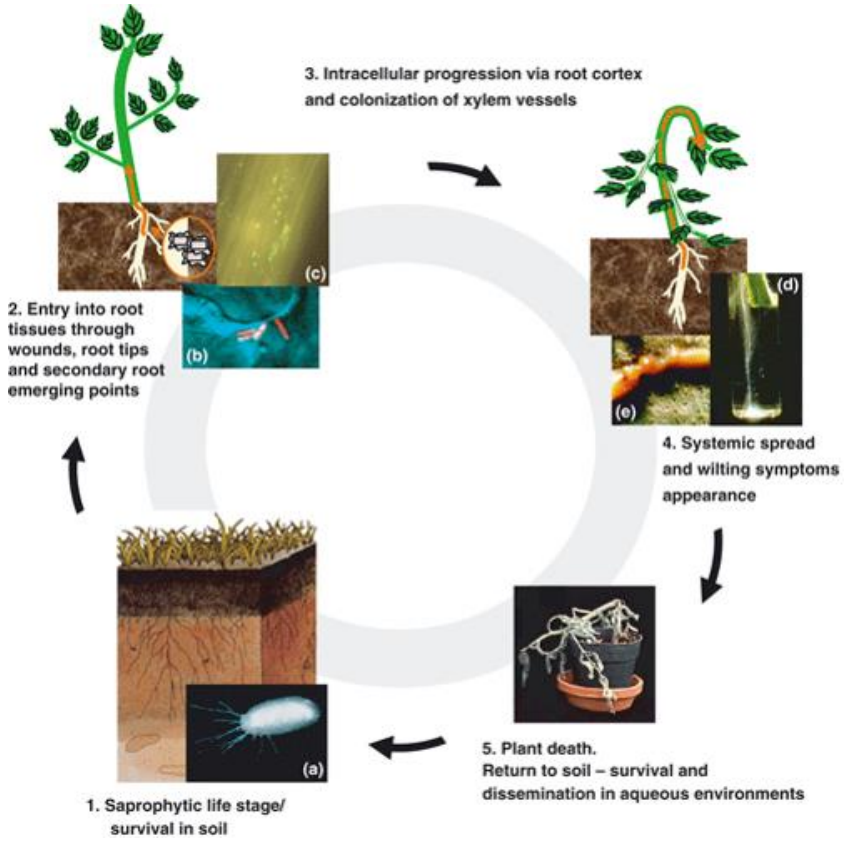

Figure 1.3.1 Life cycle of *R. solanacearum*. This begins with the pathogen in the soil (1) and attaching/penetrating host plant roots through entry points (2) where it passes through the root tissues such as the cortex and colonizes the xylem (3). EPS production occurs in the xylem which causes wilting of the plant (4) and eventual death resulting in the pathogen returning to the soil (5). This life cycle was taken from Genin (2010)

1.3.3 *R. solanacearum* virulence

Early research into *R. solanacearum* virulence in potatoes focused on the influence of abiotic factors such as temperature, light intensity, photoperiod length, soil moisture, type of soil and the presence of other pathogens (Hayward, 1991). More recent research has focussed on understanding the genetics and molecular mechanisms behind *R. solanacearum* virulence. Type III Secretion Systems are the main pathogenicity determinant for *R. solanacearum*, which help the pathogen adapt to the host plant by evading plant immune systems, through the production of a vast array of effector proteins. The specific details of the type III effectors secreted are yet to be revealed (Genin and Denny, 2012). Each *R. solanacearum* strain is suspected to carry an average of 60-75 type III effectors and half of these are specific to the species complex alone (Genin and Denny, 2012). Other virulence factors include the production of extracellular polysaccharide (EPS), plant cell-wall degrading enzymes, twitching/swimming motility, and several phytohormone

effectors such as ethylene gas and auxin which modulate the signalling of plant defence responses (Weingart, Völksch and Ullrich, 1999; Gabriel *et al.*, 2006; Valls, Genin and Boucher, 2006; Jacobs *et al.*, 2013; Peeters *et al.*, 2013).

Virulence factors of *R. solanacearum* were first studied in detail in the 1990s. Extracellular polysaccharides including acidic exopolysaccharide I (EPS1), and extracellular proteins (EXPs) such as endoglucanase (Egl), aid virulence by degrading plant cells walls, are regulated by a LysR-transcriptional regulator (phcA) and secreted through the type II secretion system (Schell, Denny and Huang, 1994; Clough *et al.*, 1997; Flavier *et al.*, 1997; Jacobs *et al.*, 2012; Yamada, 2013). EPS1 is essential for the killing and wilting of host plants. It aids colonization and results in the clogging of xylem vessels and plant wilting (Mcgarvey, Denny and Schell, 1999). The blocking of water circulation in the xylem causes host wilting and has also been suggested to cloak the bacterium from host plant recognition (Milling, Babujee and Allen, 2011). EPS1 is associated with the salicylic acid signalling pathway as mutants lacking these had lower disease incidence. PhcA is the central component to the virulence system and when mutations of this occur, virulence of *R. solanacearum* is decreased which is associated with a decrease of EPS1 by 15-fold, a 50-fold decrease in B-1,4-endoglucanase, and a 10-fold loss of pectin methylesterase (Pme) activity (Clough *et al.*, 1997). Pectinolytic enzymes function by fragmenting pectin into oligomers which can be utilised by *R. solanacearum* for growth as well as enhance its virulence by facilitating movement through pectin-rich regions of the plant host (Yamada, 2013).

Genomic analysis of *R. solanacearum* suggests that the environment within plant hosts is highly oxidative. This was inferred by the presence and upregulation of reactive oxygen species-scavenging enzymes when exposed to oxidative stress (Flores-Cruz and Allen, 2009). Oxidative burst is a mechanism of resistance of plant hosts to pathogens which has short and long-lived phases. *R. solanacearum* has evolved an efficient oxidative stress response to avoid this and maintain its successful colonization (Flores-Cruz and Allen, 2009). Nitrate levels can also be high in the tomato plant xylem (Dalsing *et al.*, 2015) and *R. solanacearum* can utilise nitrate as a terminal electron acceptor within plant hosts to aid its own survival and virulence in low oxygen conditions (Dalsing *et al.*, 2015). Mutants lacking this ability showed reduced virulence highlighting the role of anoxic growth in pathogenicity.

Flagellar motility is also important for *R. solanacearum* virulence. Motility helps *R. solanacearum* to navigate in the rhizosphere to nutrient sources, avoid toxic substances, find hosts, form biofilms and enable them to spread effectively in the soil (Tans-Kersten, Huang and Allen, 2001). Knockout mutant studies found that the level of disease incidence decreased with immotile pathogen strains (Tans-Kersten, Huang and Allen, 2001). *R. solanacearum* also displays twitching motility - a form of translocation over surfaces facilitated by the Type IV pili (Tfp) system which contributes to pathogenesis (Liu *et al.*, 2001; Wairuri *et al.*, 2012). On host surfaces during the very early infection stage, *R. solanacearum* cells divide into "rafts" of cells and develop protruding "spearheads" at the margins. *R. solanacearum* mutants that failed to develop these spearheads resulted in a failure to exhibit twitching motility and a reduction of the bacterial growth rate and disease incidence in host plants decreasing (Liu *et al.*, 2001; Siri *et al.*, 2014).

Yao and Allen (2006) describe the chemotaxis behaviour of *R. solanacearum* as it is attracted to a diverse range of organic molecules and amino acids in plant root exudates, especially in host tomato plants. Knockout studies of chemotactic signal transduction proteins, CheA and CheW, in *R. solanacearum* revealed their key role in chemotaxis as mutant strains lost their virulence. Yao and Allen (2007) further revealed aerotaxis as another mechanism for *R. solanacearum* allowing it to locate optimal intracellular oxygen levels leading to increased levels of host colonization. As a result, both chemotaxis and aerotaxis are important virulence mechanisms contributing to the pathogenicity of *R. solanacearum.*

1.3.4 Biocontrol of *R. solanacearum* using *Pseudomonas* PGPB

Pseudomonas-R. solanacearum interactions have been investigated in the last two decades to test whether *Pseudomonas* strains could potentially be used as biocontrol agents. Ramesh, Joshi and Ghanekar (2009) reported on the ability of *Pseudomonas* isolates to reduce wilting incidences in aubergines and other plant hosts infected by *R. solanacearum* in India. Greenhouse experiments revealed wilting was non-existent in the first few weeks of PGPB application, but 20% wilting was still visible after 6 weeks of treatment (Ramesh, Joshi and Ghanekar, 2009). It was suggested that DAPG production was the most important underlying mechanism for the *Pseudomonas* biocontrol effect. Authors also noted that other antibiotics (pyrrolnitrin and phenazine-1-carboxylate) and siderophores were also produced which could have affected biocontrol activity. The other PGPBs tested in this study included *Enterobacter* and *Bacillus* isolates but these proved to be less effective (Ramesh, Joshi and Ghanekar, 2009).

Pseudomonas-mediated upregulation of systemic resistance in tomato plants has also been shown to reduce *R. solanacearum* pathogenicity (Vanitha *et al.*, 2009; Vanitha and Umesha, 2011). The authors found that *Pseudomonas* pre-treatment of tomato seedlings resulted in the increased upregulation of plant defence genes encoding enzymes such as phenylalanine ammonia (PAL), guaiacol peroxidase (POX), polyphenol oxidase (POL) and lipoxygenase (LOX) which reached maximum activities 9-12 h after *R. solanacearum* inoculation (Vanitha and Umesha, 2011). The enzymes are involved in the production of lignin and the oxidation of phenolic compounds and clearly play a role in aiding plant resistance to *R. solanacearum* (Vanitha and Umesha, 2011).

Kurabachew and Wydra (2013) screened 150 rhizobacterial isolates for *in vitro* antibiosis effects against *R. solanacearum* using plate assays in the lab. The authors identified 13 bioactive isolates belonging to *Pseudomonas*, *Serratia* and *Bacillus cereus* species. These species inhibited the *R. solanacearum* growth and also harboured plant growth promoting traits (siderophore, HCN and indole acetic acid production) and the ability to communicate via quorum sensing. The selected isolates significantly reduced bacterial wilting incidence in both pot and split root experiments with both *P. putida* and *B. cereus* strains showing strong potential as biocontrol strains. The authors suggested the host plant from which bacteria were isolated influenced their biocontrol success as the bacteria were better adapted to that host plant environment (Kurabachew and Wydra, 2013).

Raza *et al.* (2016b) considered *Pseudomonas* effects on *R. solanacearum* strains focusing on volatile organic compounds (VOCs) released by *Pseudomonas fluorescens* WR-1 strain. This paper revealed that VOCs showed a concentration-dependent bacteriostatic effect against *R. solanacearum* and inhibited multiple virulence traits including swarming, motility, chemotaxis, biofilm formation, root colonization and reduced polygalacturonase activity.

Hu *et al.* (2016) explored the effect of *Pseudomonas* communities with varying diversity on *R. solanacearum* in tomato rhizosphere. It was hypothesized that increasing the community diversity of PGPB could lead to increased host plant protection via intensified resource and interference competition. The authors found that pathogen density and plant disease incidence decreased along with increasing *Pseudomonas* community diversity, suggesting that diverse

Pseudomonas communities could be effective in the biocontrol of *R. solanacearum*.

The literature highlights the potential of *Pseudomonas* to control *R. solanacearum* which have been isolated in other regions (China and India). It is yet to be revealed whether *Pseudomonas* have the same biocontrol success against pathogenic strains isolated from the UK. Additionally, there have been few studies looking at the potential of resistance to biocontrol arising over time. This thesis will explore the biocontrol potential in the context of pathogens isolated from the UK and look at long term biocontrol potential through experimental evolution. Other desirable traits of a biocontrol agent are to successfully control different types of plant pathogens, particularly those which interact with each other. This is the case with *R. solanacearum* and plant parasitic nematodes (PPNs), and thus this thesis will also investigate the biocontrol potential of *Pseudomonas* and PPNs.

1.4 Introduction to plant parasitic nematodes (PPNs)

Plant parasitic nematodes (PPNs) are microscopic non-segmented worms that are obligate plant parasites which can live off the nutrients obtained from plant cell cytoplasm (Williamson and Hussey, 1996; Cronin *et al.*, 1997). PPNs fall into two groups: ecto-parasites and endo-parasites. Ectoparasitic nematodes e.g. *Trichodorus similis* spend their entire life cycle outside of the plant hosts and typically feed on root epidermal cells in the soil. Whereas endoparasitic nematodes, such as *Globodera pallida*, are highly adapted to spending part or all of their life cycle within the plant host itself (Jones *et al.*, 2013). The sedentary endoparasites of the *Heteroderidae* family cause the greatest economic damage to crops worldwide and comprise two main groups: potato cyst nematodes (PCNs, including *Globodera* and *Heterodera* genera) and root-knot nematodes (RKNs, including *Meloidogyne* genus) (Williamson and Hussey, 1996). Two of the major cyst nematode species parasitise potato: white potato cyst nematode (PCN) *Globodera pallida* and yellow PCN *Globodera rostochiensis* (Eves-Van Den Akker *et al.*, 2016).

Worldwide annual losses reach into the billions of dollars in terms of crop loss (Sasser and Freckman, 1987; Eves-Van Den Akker *et al.*, 2016) and PPNs are responsible for approximately 12.3% reduction in crop yield annually which equates to around $80 billion loss worldwide (Warnock *et al.*, 2017). *Meloidogyne incognita* is the most economically significant PPN globally (Zhai *et al.*, 2018). It can infect the roots of over 2000 different plant species in tropical and

subtropical regions (Niebel, Gheysen and Van Montagu, 1994). PCNs cause the greatest threats in temperate regions and are the main threats to the UK potato industry (Niebel, Gheysen and Van Montagu, 1994). Approximately, 65% of potato-growing field sites in the UK are infested by PPNs and 92% of these cases were associated with the presence of *G. pallida*. In the UK alone, PCN causes annual costs in excess of £50 million and threaten the future economic viability of many potato growers (Haydock and Evans, 1998).

1.4.1 PPN infection cycles

Cyst and root knot nematodes have different infection cycles, which involve the pathogen utilising approximately 500 secreted effector proteins which are secreted from their cuticle, oesophageal, amphids and rectal glands through their stylets to establish feeding sites within the plant host to enable their survival (Fuller, Lilley and Urwin, 2008; Rehman, Gupta and Goyal, 2016; Mejias *et al.*, 2019; Vieira and Gleason, 2019). Nematode interactions with hosts generally last longer than a month and result in morphological changes to both the host cells and parasite (Fuller, Lilley and Urwin, 2008). The host and the type of the environment can also influence the length of these interactions (Favery *et al.*, 2016). Simplified life cycles of both RKN and PCN in Figure 1.2 were taken from Abad and Williamson (2010).

The life cycle of cyst nematodes involves 5 developmental stages: four juvenile stages (J1-J4), which are separated by moults where the cuticle is replaced, and an adult stage (Fuller, Lilley and Urwin, 2008; Eves-Van Den Akker *et al.*, 2016; Kooliyottil *et al.*, 2016). Sexual dimorphism occurs between J2 and J3 (Fuller, Lilley and Urwin, 2008). The infective stage of the life cycle occurs when the motile, second stage juveniles (J2) emerge from eggs within a cyst and migrate towards to penetrate the plant. Wyss and Zunke (1986) showed second stage juveniles (J2) entered roots via the epidermis and migrate through the cortex by piercing and rupturing walls with their stylet which can cause cellular damage and necrosis. Upon penetrating the endodermis, J2s pierce a procambial cell and inject secretions which physically alter the cell to become a feeding site. Here the cytoplasm becomes dense and the protoplasts of neighbouring cells fuse forming a larger feeding site known as a “syncytium” that allows the nematode to undergo numerous life cycle stages. The syncytium can recruit up to 200 surrounding cells in this process (Fuller, Lilley and Urwin, 2008). The nematode then becomes sedentary and undergoes three moults where it develops into the adult form. Adult females, whose bodies have broken through the root surface, are fertilised by motile adult males that leave the roots. Egg production by the

female begins within 3-6 weeks after the initial infection, which is species and environment dependent. The cyst nematode adult female body swells to become spherical (*Globodera*) or lemon shaped (*Heterodera*) and become filled with embryonated eggs. Upon their death, the body wall hardens to form a protective enclosure around the eggs which can keep them viable for many years (Fuller, Lilley and Urwin, 2008). Once PCNs become established in a field they are difficult to eradicate as their cysts can remain viable for up to two decades. The US Department of Agriculture (USDA) have classified yellow PCN as potentially more dangerous than any insect or disease affecting the potato industry (Aphis USDA 12/09/2015). It is also on the European plant pest quarantine lists.

The life cycle of root knot nematodes (RKNs) has five stages separated by moults (with cuticle replacement) with four juvenile stages and one adult, similar to that of the PCN (Favery *et al.*, 2016). Eggs are laid in the soil and protected by a gelatinous matrix. The first moult occurs within the egg and then nematodes hatch from the egg as J2 juveniles. The J2 begins host plant infection close to the root tip and migrates intercellularly towards the zone of cell division where they are able to form feeding sites by encouraging cell differentiation in the vascular parenchymal cells. Secretions from the stylet help encourage this cell re-differentiation. The cells become metabolically active and enlarged forming what are called 'giant' cells. Numerous rounds of mitosis, in the absence of cytokinesis, occur within this giant cell as well as cellular division of the surrounding cortical and pericycle cells. This result in a localized swelling which is the characteristic formation of the gall or "root knot". Once feeding sites are established, the J2 becomes sedentary, which feeds from the giant cells and undergoes three moults into the adult form. The sexual dimorphism into females and vermiform males is associated with this sedentary state (Favery *et al.*, 2016). Once the female nematode matures inside the plant, they deposit their eggs in gelatinous matrices (up to several hundred per matrix) outside of the root. From there, the larvae hatch and J2s migrate through the soil where they can become attracted to plant roots and the cycle continues (Niebel, Gheysen and Van Montagu, 1994). The mode of reproduction of nematodes in the *Meloidogyne* genus varies, however the most widespread species including *M. incognita* and *M. javanica* reproduce by mitotic (apomictic) parthenogenesis (Favery *et al.*, 2016).

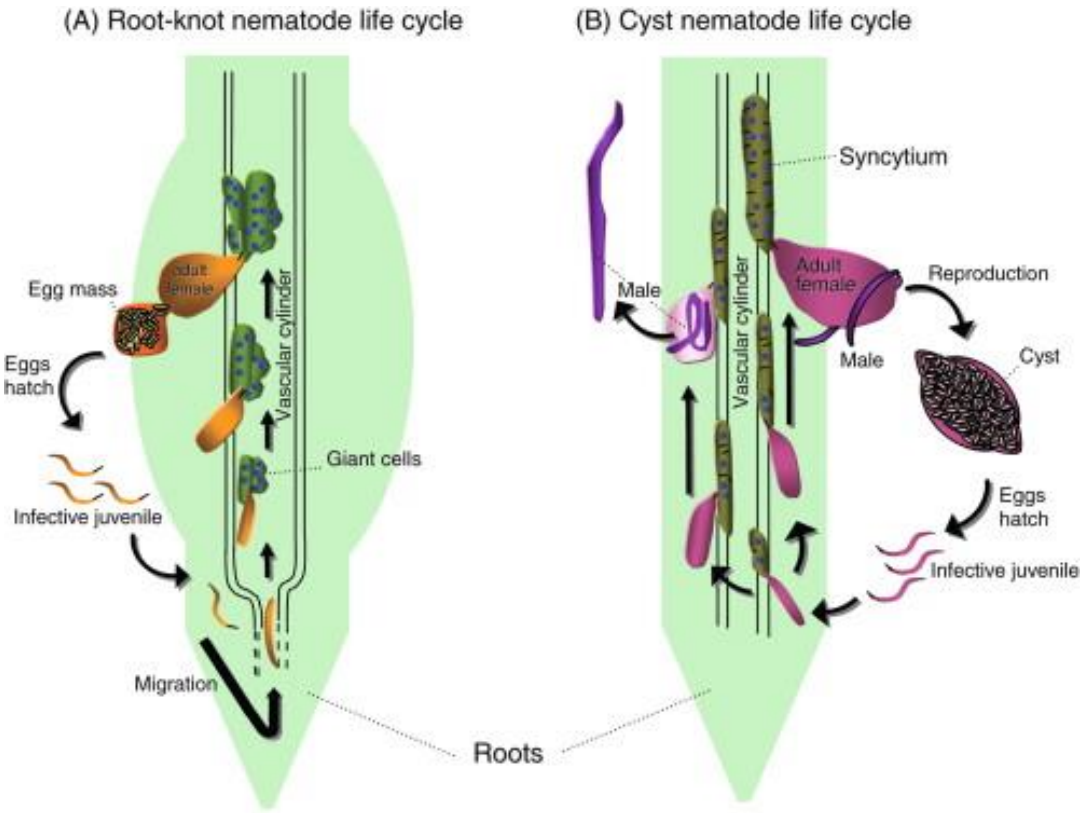

Figure 1.4.1 Schematic of the life cycle and feeding site development of both types of PPN in plant hosts, (A) in RKN and (B) in PCN. The clear differences between the two life cycles are the types of feeding sites with the giant cell on in panel (A) for the RKN and the development of the syncytium as the feeding site for the PCN in (B). This figure was taken from Abad and Williamson (2010)

1.4.2 PPN virulence

Both cyst nematodes and root knot nematodes have a number of tissues and organs that secrete proteins into their plant hosts such as amphids, their hypodermis and vulva. Nematode secretions, or "effectors", involved in parasitism originate mainly from the oesophageal glands and are important in penetration and migration through roots, feeding site development and suppressing host defences (Hussey, 1989; Williamson and Hussey, 1996). There are two sub-ventral and one dorsal oesophageal gland and these produce effectors such as cell wall degrading enzymes. The effectors produced by these glands are secreted from the nematode via the hollow, protrusible stylet located in the oral cavity of the nematode, which is used to penetrate plant cell walls, inject secretions and withdraw host cell nutrients (Davis, Hussey and Baum, 2004). Identifying specific effectors produced by PPNs has been a challenge faced by many researchers. However, understanding their function is important for determining PPN virulence. The key virulence factors are endoglucanases, pectate lyase and venom allergen-like proteins (VAPs).

Lozano-Torres *et al.* (2014) reported nematode secretions which can inhibit plant damage-triggered immune responses. VAPs suppress surface-localised immune receptors. Knocking out Gr-VAP1i gene leads to a clear decrease in pathogenicity in *G. rostochiensis* in plant hosts. The delivery of VAPs coincides with enzymatic breakdown of plant cell walls by migratory J2-juvenile nematodes, which suggests that VAPs are utilised by nematodes to suppress defence activation of damaged host tissue during infection and also play a role in syncytium establishment. The Gr-VAP1 shares roughly 30% similarity to previously reported VAP secretory proteins produced by *M. incognita* which suggests a potential mode of action for a biocontrol target that could affect multiple PPNs (Ding *et al.*, 2000).

One plant cell wall degrading enzyme identified in PCNs, chorismate mutase, which plays important roles in various plant-host interactions at different stages of infection: invasion, migration, suppression of host defences and induction of the feeding site (Popeijus *et al.*, 2000a; Chen *et al.*, 2005). Popeijus *et al.* (2000b) used an expressed sequence tagging (EST) technique to look at over 1000 expressed genes from cyst nematodes: *G. pallida* and *G. rostochiensis*. Numerous genes were considered to play a role in plant parasitism including those that code for serine protease, cysteine protease, pectate lyase, matrix metalloprotease, peroxiredoxin, glutathione peroxidase and others (Popeijus *et al.*, 2000a). Many of these genes have been verified to play a role in the parasitism of animals by nematodes and are expected to have similar roles in their plant hosts by aiding infection by providing protection from plant defences. Some PCN effectors are also able to mimic ligands which host plant proteases can process. These interact with plant CLE receptors and influence plant nematode and parasitism e.g. ligand mimic GrCLE1 from *G. rostochiensis* (Guo *et al.*, 2011).

RKNs damage plant hosts in a number of ways through root malformation by galling as well as plant wilting and the stunting of plant growth (Perry and Moens, 2013). Polygalacturonase is expressed in the subventral gland cells of RKN and these are suggested to be involved in cell wall degradation during the nematode migration process (Jaubert *et al.*, 2002; Moens, Perry and Starr, 2009). Secreted effectors from the saliva through the stylets of *M. incognita* are known to play a vital role in the establishment of feeding sites and PPN infections (Favery *et al.*, 2016). Peroxidase enzyme activity by *M. incognita* has been described to be essential in the establishment and/or maintenance of the “giant” cells (Hussey, Sasser and Huisingh, 1972).

The number of PPN effectors must be significantly greater than those described so far for *R. solanacearum* and there is much more to discover. Effects differ between species and are essential to their survival. The infection cycles of root knot and cyst nematodes differ, adult cyst nematodes are more destructive to the plant host during its invasion pathway than the RKN (Fuller, Lilley and Urwin, 2008). This damage occurs during the nematodes' life cycle where the enlarged female containing the eggs dies within the root and breaks through the root surfaces to form a hard cyst attached to the root surface.

1.4.3 Biocontrol of PPNs using *Pseudomonas* PGPB

Previous studies exploring the potential role of Pseudomonads as biocontrol agents of PPNs found that *Pseudomonas* can directly inhibit the nematodes as well as influence the activation of plant defences against PPNs (Imran A. Siddiqui and Shaukat, 2003; Zhai *et al.*, 2018). Siddiqui and Shaukat (2003) tested the biocontrol ability of *P. fluorescens* strain CHA0 against the RKN *M. javanica*. It was found that egg hatching and juvenile mortality were affected by the supernatant containing secondary metabolites DAPG and pyoluteorin. Another form of biocontrol activity was observed in terms of VOC production by *Pseudomonas* which exhibited *in vitro* killing effects on *M. incognita* juveniles (Zhai *et al.*, 2018). Siddiqui, Haas and Heeb (2005) focused further on CHA0 and the underlying genetic mechanisms involved in biocontrol activity. Knockout studies verified that alkaline metalloproteinase "AprA" protease which can degrade proteins, was involved in causing biocontrol effects against *M. incognita* nematode (Siddiqui, Haas and Heeb, 2005). These nematicidal effects have been revealed to specifically cause damage to intestinal tissues (Guo *et al.*, 2016). A study conducted by Zhao *et al.* (2018) screened over 860 PGPB against RKNs and selected five isolates to focus on, including *P. fluorescens*. These were tested for juvenile and egg mortality effects *in vitro* and *in vivo* in tomato pot experiments. Each of the five isolates displayed plant growth promoting traits by increasing plant biomass and reducing galling incidences which the authors recommend require further investigation.

Pseudomonas interactions with PCNs have also been investigated with *P. fluorescens* strain F113 (Cronin *et al.*, 1997), where biocontrol potential and DAPG production was tested against yellow PCN, *Globodera rostochiensis*. *In vitro* exposure of PCN cysts to the bacteria resulted in increased egg hatching rates (Cronin *et al*., 1997). Nematode juveniles can survive for up to 12 days in the soil in the absence of a plant host, by initiating hatching of eggs in the soil prior to crops being sown this could be an effective biocontrol method (Cronin *et al.*, 1997). The

authors also found a reduction in the motility of juvenile nematodes both *in vitro* and in the soil. By creating a knockout of DAPG in the *P. fluorescens* strain F113, these effects were diminished highlighting the importance of DAPG for these effects. The *in vitro* results were also replicated using a synthetic form of DAPG, highlighting that this metabolite was likely to be the contributing mechanism to effects observed (Cronin *et al.*, 1997). In another study, *P. fluorescens* liquid formulations were applied as a seed treatment and soil drench application against natural populations of *G. pallida* and *G. rostochiensis* (Nagachandrabose, 2020). The seed treatment was shown to suppress nematode activity by reducing egg densities which affected the multiplication rate of the PPNs and successful root penetration (Nagachandrabose, 2020). The split root experiments in this study also supported the findings from previous studies that DAPG was also involved in triggering systemic resistance in tomato plants (Imran A. Siddiqui and Shaukat, 2003; Nagachandrabose, 2020). It was found that upon adding Fe^{3+}, plant protection is reduced, which suggests siderophore activity might play an important role in plant protection.

1.4.4 *Caenorhabditis elegans* as a PPN nematode model: differences and similarities with PPNs

Caenorhabditis elegans is a free-living nematode which has been a popular model for genetic studies of development and behaviour due to its ease of culture, short life cycle, small genome and hermaphrodite mode of reproduction (Byerly, Cassada and Russell, 1976; Félix and Braendle, 2010; Clark and Hodgkin, 2014; Hunt, 2017). Adult *C. elegans* reach a length of 1-2 mm (Félix and Braendle, 2010). They have been termed as "soil nematodes" yet can be found in other microbe-rich habitats in temperate climates wherever decaying plant matter such as rotting fruit is available (Félix and Braendle, 2010; Clark and Hodgkin, 2014). The standard wild-type laboratory strain, N2, was first discovered in a compost heap in Bristol (UK), in 1957 (Nicholas, Dougherty and Hansen, 1957; Dallière *et al.*, 2017). It is also used as a model nematode species for PPNs (Niu *et al.*, 2010; Kearn *et al.*, 2014; Zhai *et al.*, 2018).

1.4.4.1 *C. elegans* life cycle

In the laboratory, *C. elegans* populations are maintained at 20 ^{o}C on petri dishes containing nutrient agar with a lawn of *Escherichia coli* OP50 strain as a food source (Brenner, 1974; Félix and Braendle, 2010). The *C. elegans* life cycle is similar to PPNs with four moulting stages (Byerly, Cassada and Russell, 1976). In lab conditions the life cycle takes 3.5-4 days with a short embryonic period followed by four juvenile stages termed L1-L4 which are separated by moulting. The newly

hatched larvae are approximately 0.25 mm long (Dallière *et al.*, 2017). Development can occur at slower and faster rates between 8-27 ^{o}C before coming to a complete halt (Félix and Braendle, 2010; Gómez-Orte *et al.*, 2017). Under stressful conditions, the L3 stage larvae can enter a dauer larvae stage which can be resistant to stress and survive for up to 3 months without food, whereas adults can only survive for 2 weeks without food (Félix and Braendle, 2010). Adult hermaphrodites lay around 300 eggs and which can survive for up to 20 days (Dallière *et al.*, 2017). Male *C. elegans* occur at a low frequency of 0.1-0.2% (Ward and Carrel, 1979).

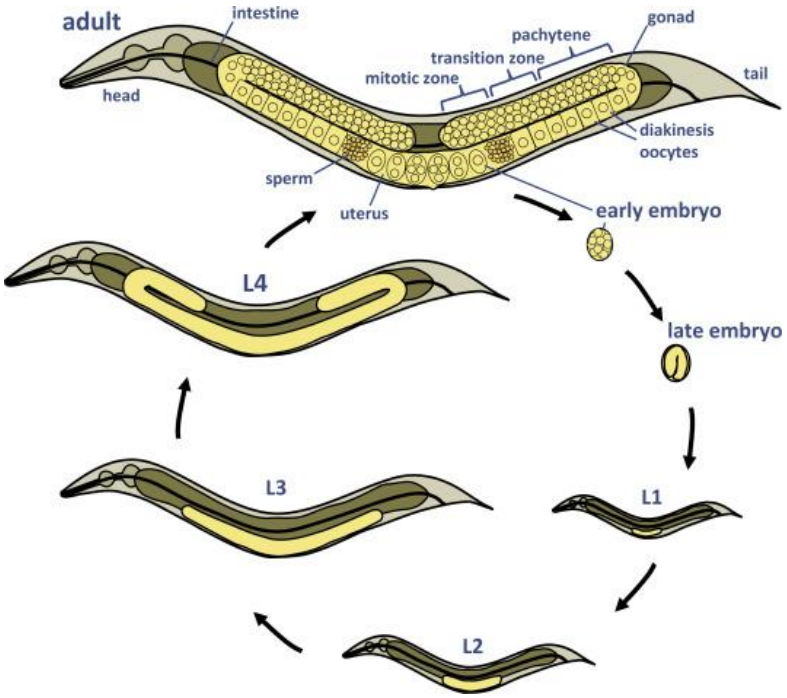

Figure 1.4.2 Life cycle of *C. elegans*. Outline the various stages from embryonic development, through the juvenile stages separated by moults to the fully developed adult form. Image taken from Lans and Vermeulen (2015)

1.4.4.2 *C. elegans* feeding behaviour

C. elegans feeds on bacteria and small eukaryotes (Félix and Braendle, 2010; Clark and Hodgkin, 2014) mediated by pharyngeal pumping, which enables bacteria to pass through the pharynx to the gut (Clark and Hodgkin, 2014; Dallière *et al.*, 2017). Bacterial metabolites have been shown to influence *C. elegans* lifespan and fat storage in both positive and negative ways (Zhang, Lu and Bargmann, 2005). Different soil bacteria can be harmful or beneficial to *C. elegans* (Abada *et al.*, 2009; Gómez-Orte *et al.*, 2017). As a result, *C. elegans* often shows food preference for distinct bacteria (Burlinson *et al.*, 2013). Choice feeding assays have suggested that chemosensation plays

a role in this (Abada *et al.*, 2009). External factors such as oxygen and carbon dioxide levels have also been shown to influence *C. elegans* feeding and food leaving behaviours (Milward *et al.*, 2011).

1.4.4.3 *C. elegans* interactions with PGPBs

C. elegans can be used as a model system for nematodes to study microbial pathogenesis (Niu *et al.*, 2010). Olfactory chemotaxis towards food-associated odours is a robust behaviour, and based on olfactory discrimination, nematodes can choose to feed or leave bacteria untouched to avoid pathogens. Some pathogens have adapted to attract nematodes, by using volatile organic compounds. For example, Niu *et al.* (2010) reported a "trojan horse" mechanism whereby soil bacteria *Bacillus nematocida* produce VOCs to lure the *C. elegans* nematodes towards them and upon consumption by the nematode, can enter the nematode intestines. Inside the intestines, *B. nematocida* secretes proteases with broad mechanisms that lead to nematode death (Niu *et al.*, 2010). *C. elegans* can share the same ecological niches as bacteria such as *Pseudomonas* and can act as their host, which can be visualised in real time through the use of fluorescent markers such as green fluorescent protein (Clark and Hodgkin, 2014). It is known that *Pseudomonas* can also produce VOCs, which could trigger similar Trojan horse effects as described above. Direct inhibition of PPNs by *Pseudomonas putida* VOCs has also been found by Zhai *et al.* (2018), while no effect was observed with *C. elegans*. These results highlight that PGPB VOC effects can vary depending on the bacterial and nematode species.

Secondary metabolites including DAPG and HCN produced by *P. fluorescens* strain CHA0, have also been reported to have nematicidal effects on *C. elegans* (Neidig *et al.*, 2011). Cyclic lipopeptides produced by bacteria also affect *C. elegans* (Pradel *et al.*, 2007). For example, Serrawettin lipopeptide produced by *Serratia marcescens* has been reported to act as a repellent to *C. elegans* (Pradel *et al.*, 2007). As it is known some *Pseudomonas* PGPB have the ability to produce CLPs, this may suggest a potential impact on *C. elegans*. Nandi *et al.* (2015) reported biocontrol effects of *Pseudomonas chlororaphis* PA23 against *C. elegans* using pyrrolnitrin and HCN production. This strain is known to express biocontrol properties against *Sclerotinia sclerotiorum*, a fungal pathogen. It produces pyrrolnitrin, phenazine and HCN as well as degradative enzymes. Nandi *et al*. (2015) found pyrronitrin and HCN contributed to fast- and slow-killing of *C. elegans* despite pyrrolnitrin having no prior definition as a nematicide. These compounds also behaved as repellents and reduced nematode viability and hatching rates. Moreover, gene expression of the biocontrol metabolites mentioned above were upregulated

when the bacteria were co-cultured with *C. elegans* which is encouraging if *P. chlororaphis* was to be applied as a biocontrol to soil (Nandi *et al.*, 2015). Some bacterial species can be more nematicidal such as *P. brassicacearum* DF41, which has been reported to be able to kill *C. elegans* via formation of a biofilm in the nematode head — blocking the buccal activity and exposing the nematode to toxic metabolites (Nandi *et al.*, 2016).

1.5 Consideration of the potential antagonisms with biocontrol

We have described a number of traits which *Pseudomonas* PGPB possess that have the potential to suppress plant pathogens. However, there are other interactions which can occur that can influence the effectiveness and long term success of a biocontrol agent interaction with a plant pathogen. This can be due to other antagonistic microbial interactions occurring at the same time, the plant host itself, or evolutionary implications such as the development of antimicrobial tolerance or resistance to the biocontrol bacteria.

In vitro studies of plant pathogens and biocontrol agents are not representative of the interactions in the natural rhizosphere as soil environments are diverse with numerous microorganisms present. It can be a challenge for the biocontrol bacteria to compete with already established native bacteria (Hu *et al.*, 2016). In addition to being able to compete with the native bacteria, the gene expression of *Pseudomonas* PGPB secondary metabolites such as DAPG can also be influenced by which host plant rhizosphere the bacteria are interacting, as well as the age of the plant (Notz *et al.*, 2001; Siddiqui and Shaukat, 2003). Properties of the soil itself such as soil moisture, temperature, clay content and pH can also influence the success of the biocontrol (Van der Putten *et al.*, 2006). The presence of bacterial feeders such as flagellates and nematodes in the soil could also influence biocontrol effects by consuming the biocontrol agent before it suppresses the pathogen (Pedersen *et al.*, 2009). However, some *Pseudomonas* strains which are closely related to known PGPB biocontrol strains have been reported to repel bacterial-feeding nematodes such as *C. elegans* which highlights that bacterial-feeder impacts are strain-specific (Burlinson *et al.*, 2013).

The long term success of a biocontrol agent can be affected by the plant pathogen evolving resistance or tolerance to it. Previous control strategies against bacterial plant pathogens included the application of the antibiotics such as streptomycin. This was used to control *R.*

solanacearum and led to the development of antibiotic-resistance (Xue *et al.*, 2013). It is desirable to investigate the potential evolution of tolerance of pathogens to the biocontrol prior to it being developed to have a better understanding of its long term success. *R. solanacearum* has been shown to easily adapt to new environments with ease both *in vitro* and *in vivo* (Riley *et al.*, 2001; Guidot *et al.*, 2014).

1.6 Development of general biocontrol strategies against both *R. solanacearum* and PPNs

Multiple plant pathogens commonly coexist in the same environment because they can all infect the same host (e.g. potato), which can result in naturally occurring mutualistic behaviours. Hayward (1991) and Mcgarvey, Denny and Schell (1999) have suggested that a synergistic relationship between nematodes and *R. solanacearum* increases plant infection. Evidence showed that a higher presence of nematodes causing root knot infections increased the number of open wounds on the roots leading to the facilitation of *R. solanacearum* invasion into host tissues giving a double effect of bacterial wilt and root knot gall formation resulting in increased levels of death rates of plant hosts. This has been observed in numerous crops including potatoes and tomatoes and the author highlighted the importance of developing joint biocontrol against both nematodes and *R. solanacearum to* increase crop production.

Barua and Bora (2008) considered the biocontrol effects of *P. fluorescens* and *Trichoderma harzianum* against both root knot nematode *M. incognita* and *R. solanacearum* in aubergine (termed “brinjal” in South Asia, and “eggplant” in USA). They found that *Trichoderma* was more successful in suppressing *M. incognita*, whereas *Pseudomonas* caused the greatest reduction in *R. solanacearum* cell numbers. Elsharkawy *et al.* (2015) treated tomato plants with a combination of the bacteria *Bacillus thuringiensis* and actinomycetes *Streptomyces avermectinius* to investigate the biocontrol potential against *R. solanacearum* and *M. incognita.* Their findings revealed a reduction in bacterial wilt and root-knot gall incidences, which suggests that this biocontrol combination could provide dual control against these plant pathogens. Dual biocontrol strategies could be achieved by using a combination of different biocontrol agents, or single biocontrol agents that show broad activity range to multiple different pathogens.

Despite some research beginning to consider the potential bacterial biocontrol of both *R. solanacearum* and PPNs, there is a need to take this further. This has not been done with a focus on pathogens isolated from the UK. There has been some focus on the underlying mechanisms in these biocontrol scenarios, but confirming the active expression of secondary metabolites with the use of LC-MS has not been conducted in the majority of these studies. The evolution of tolerance of pathogenic bacteria to biocontrol agents has also not been reported with UK *R. solanacearum* isolates hence the need to address this.

1.7 Project Aims and Objectives

This research aimed to screen a number of *Pseudomonas* plant growth-promoting bacterial strains for potential biocontrol effects against two types of plant pathogens: *Ralstonia solanacearum* bacterium and *Meloidogyne incognita* and *Globodera pallida* plant parasitic nematodes.

In this research, I aimed to answer following four key questions:

1. Can the same *Pseudomonas* strains suppress different types of plant pathogens?

The motivation behind this was to see whether the biocontrol strains used in this study could display biocontrol abilities against pathogens which have been reported to form mutualistic detrimental infections on plant hosts. This is important to consider if a biocontrol agent can be used broadly or if it is species-specific. To study the role of bacterial pathogen variation, I used seven different *R. solanacearum* strains isolated from the UK and Poland and tested to what extent they could be suppressed by eight different *Pseudomonas* strains. Similarly, *Pseudomonas* efficacy was tested against the *C. elegans* nematode model and two plant parasitic nematodes. It was found that *Pseudomonas* suppression of all pathogens was possible to an extent. *Pseudomonas* strain CHA0 appeared the most suppressive against *R. solanacearum*. CHA0 and Pf-5 were the most suppressive against *C. elegans*. There was less variation of *Pseudomonas* effects on PPNs during *in vitro* experiments alone.

2. What are the underlying mechanisms behind the suppression observed? Is it consistent with differing plant pathogens?

The motivation behind this was to consider if the same underlying mechanisms of biocontrol bacteria could have an impact on numerous plant pathogens. This is important to understand whether there is one dominant mechanism of suppression or if numerous metabolic pathways play a role in biocontrol. To investigate the underlying potential mechanisms involved in biocontrol effects, comparative genomics was conducted of the *Pseudomonas* genomes to identify potential secondary metabolite clusters of interest. Four metabolites were selected and purchased for direct *in vitro* assays to see if these would replicate direct *Pseudomonas* effects. DAPG was chosen as it is universally produced by all *Pseudomonas* strains in this study, as well as unique metabolites pyoluteorin and orfamide A and B produced by CHA0 and Pf-5 as these strains were consistently effective in studies. I also extracted my own cyclic lipopeptides termed "CLP extracts" to test these on the plant pathogens. Mass spectrometry analysis of *Pseudomonas* supernatants was also conducted to confirm whether or not these metabolites were being expressed in the growth conditions used in these studies. Pyoluteorin was the most suppressive metabolite against *R. solanacearum* and *G. pallida* PPN. *M. incognita* was found to be less susceptible to the metabolites. Mass spectrometry analysis only confirmed the presence of DAPG in CHA0 when cultured in LB broth and Orfamide A when cultured in CPG broth which highlighted that not all *Pseudomonas* metabolites were expressed.

3. Can pathogens evolve tolerance to *Pseudomonas* suppression?

The motivation behind this is to understand whether the biocontrol of a pathogen can remain robust, or it over time the pathogen can overcome suppression. This is important to consider the long lasting effectiveness of a biocontrol agent. Tolerance evolution could limit the long term efficacy of biocontrol. I tested this with *R. solanacearum* by conducting a selection experiment and exposing liquid culture populations of *R. solanacearum* to fresh *Pseudomonas* supernatant (containing secondary metabolites every three days for three weeks). I found that *R. solanacearum* can evolve tolerance *in vitro*. Mechanistically, this was mediated by tolerance to orfamides A and B.

4. Can *in vitro* suppression be replicated *in vivo*?

To successfully bridge the gap between lab and field biocontrol effects, it is important to consider the reproducibility of *in vitro* laboratory results in *in vivo* settings. It is unclear whether biocontrol results observed in the laboratory environment can be extrapolated to natural environments. This

will give a more representative insight into the likelihood for biocontrol success. To test this, *in vivo* glasshouse screening of *Pseudomonas* strains against all pathogen species (*R. solanacearum* bacterium and both species of parasitic nematode *G. pallida* and *M. incognita*) was conducted in separate studies using tomato plant hosts. It was found that *Pseudomonas* strain CHA0 could suppress one *R. solanacearum* strain of the two strains tested *in vivo* to an extent. *Pseudomonas* suppression of PPNs *in vivo* was less successful, however, there was evidence of plant growth promoting effects and a non-significant trend in suppression o*f G. pallida in vivo*.

Thesis chapter outline

This thesis includes the following chapters which are presented in the form of research papers:

Chapter 2: General methods

This chapter is not in the form of a research paper, rather it outlines general methods that were used in each of the three results chapters to avoid repetition. The specific methods used in individual chapters are outlined within their respective chapters and general methods cited accordingly throughout the thesis.

Chapter 3: *In vitro* and *in vivo* screening of efficient *Pseudomonas* biocontrol strains against *Ralstonia solanacearum* pathogen and investigation of potential underlying mechanisms of inhibition

In this chapter, the biocontrol effects of eight *Pseudomonas* PGPB strains were screened against seven plant pathogenic *Ralstonia solanacearum* bacterial strains. Direct and indirect interactions with *R. solanacearum* were first measured using bacterial agar plate assays and the use of *Pseudomonas* culture supernatant. Comparative genomic analysis was carried out and a number of secondary metabolite clusters identified which encoded for DAPG, pyoluteorin and orfamides. This led to direct metabolite assays using these compounds which gave insight into mechanistic effects of biocontrol. Lastly, *in vivo* tomato infection assays were carried out using selected bacterial strains to consider whether biocontrol effects could be observed in an agricultural setting. Screening experiments identified *Pseudomonas* strain CHA0 as a potential biocontrol strain from *in vitro* studies and revealed metabolites such a pyoluteorin to possibly contribute to this. *In vivo* studies strengthened these findings as CHA0 displayed the strongest biocontrol effects against *R. solanacearum* strain #7.

Chapter 4: Plant pathogenic *Ralstonia solanacearum* bacterium can rapidly evolve tolerance to antimicrobials produced by *Pseudomonas* biocontrol bacteria

Following on from Chapter 3, the long-term biocontrol effects of the eight *Pseudomonas* PGPB strains were tested against seven *R. solanacearum* strains using an experimental evolution approach. *R. solanacearum* populations were cultured in high and low concentrations of *Pseudomonas* supernatant (containing secondary metabolites) over a period of three weeks with 1% of the population being serially transferred into freshly prepared supernatant every three days. Fitness assays were conducted to investigate whether any pathogen tolerance or resistance evolution had occurred at the phenotypic level, and mass spectrometry carried out to explore which metabolites were expressed in the supernatant. Tolerance evolution to identify candidate substance was measured using commercially available chemical standards. It was found that *R. solanacearum* populations were able to evolve increased tolerance to *Pseudomonas* strains over time, and more specifically, this was linked to tolerance to orfamide cyclic lipopeptides.

Chapter 5: Screening and identification of effective *Pseudomonas* biocontrol strains against plant parasitic nematodes and testing their efficacy *in vitro* and *in vivo*

In this chapter, the efficacy of *Pseudomonas* strains were screened against *C. elegans* and two plant parasitic nematodes: *Globodera pallida* and *Meloidogyne incognita*. Feeding preference assays were first investigated as *C. elegans* are larger than PPNs and bacterial feeders. Direct and indirect assays with all three nematode models and *Pseudomonas* followed this. Mass spectrometry was conducted to reveal any metabolite expression which might have played a role in observed results. Single and combined metabolite studies were then conducted and lastly, three greenhouse experiments took place to look at the long-term more complex and representative dynamics between PPNs and *Pseudomonas* strains CHA0 and Pf-5. It was found that *Pseudomonas* strains CHA0 and Pf-5 observed killing effects on *C. elegans* and that all eight *Pseudomonas* strains rendered the PPNs inactive. Genome mining helped to identify what compounds might be important for biocontrol. *In vivo* studies revealed similar trends to *in vitro* results, which were however non-significant. More experiments are thus required to study how *in vitro* biocontrol effects can be translated *in vivo*.

Chapter 6: General Discussion

A general discussion considering the results and contexts of all chapters together. The potential future directions that could progress this area of research and successfully translate *in vitro* biocontrol outcomes to agricultural environments will also be discussed.

The Appendix information for each chapter is shown at the end of each thesis chapter. References are provided at the end of the thesis.

Chapter 2 General Methods

All routine methods and biological material used across the three research chapters are addressed here in the general methods chapter to avoid repetition.

2.1 Biological materials

Plants:

Tomato – *Solanum lycopersicum*, cultivar 'Ailsa craig'

Nematodes:

Free-living nematode - *Caenorhabditis elegans,* N2 (wild-type)

Root knot nematode - *Meloidogyne incognita* (populations: VW6, Africa and Brazil)

Potato cyst nematode - White potato cyst nematode – *Globodera pallida* population Lindley (Pa2/3 pathotype)

Bacterial strains:

-*Pseudomonas* spp., 9 isolates originating from a variety of locations (Table 2.1)

-*Ralstonia solanacearum*, six UK isolates from FERA annual river sampling and one Polish isolate (Table 2.2)

-*Escherichia coli* OP50 (Brenner, 1974)

Table 2.1 *Pseudomonas* strains used in these experiments, and their geographical origin.

***Pseudomonas* strains**	***Pseudomonas* strains' origin**
CHA0 *(P. protegens)*	Tobacco, Switzerland (Natsch *et al.*, 1994)
Pf-5 *(P. protegens)*	Cotton, USA (Howell and Stipanovic, 1979)
Q2-87 *(P. fluorescens)*	Wheat, USA (Bangera and Thomashow, 1999)
Q8R1-96 (*P. brassicacearum)*	Wheat, USA (Raaijmakers and Weller, 1998)
1M1-96 *(P. fluorescens)*	Wheat, USA (Raaijmakers and Weller, 2001)
MVP1-4 *(P. fluorescens)*	Pea, USA (Landa *et al.*, 2002)
F113 *(P. fluorescens)*	Sugar Beet, Ireland (Shanahan *et al.*, 1992)
Ph11C2 *(P. fluorescens)*	Tomato, France (Govaerts *et al.*, 2007)
Sbw25 (*P. fluorescens* - **only used during *in vitro R. solanacearum* experiments, Chapter 3**)	Sugar beet, England (Thompson *et al.*, 1995)

Table 2.2 *Ralstonia solanacearum* strains used in these experiments, and their geographical origin.

***Ralstonia* strains**	**FERA sample collection number**	***Ralstonia* strains' origin**
#1	21415687	River Loddon, UK (2014)
#2	21517183	Ex River Loddon, UK (2015)
#3	21517184	Ex River Loddon, UK (2015)
#4	21314705	River Jubilee, UK (2013)
#5	21422327	Commercial culture, Poland (used for verification tests) (2014)
#6	21314706	River Jubilee, UK (2013)
#7	21415697	River Loddon, UK (2014)

2.2 Culturing and maintenance of bacterial strains

Nine fluorescent Pseudomonad strains (CHA0, Pf-5, Q2-87, Q8R1-96, 1M1-96, MVP1-4, F113, Ph11C2 and Sbw25) and seven *Ralstonia solanacearum* strains (#1-7) were used in the experiments. Further information on these bacterial strains is listed in Table 2.1 and Table 2.2. For *C. elegans* work, bacterial strain *E. coli* 0P50 was required as a nematode food source. *C. elegans* populations were maintained on nematode growth medium (NGM) agar plates seeded with *E. coli* OP50. Stocks of *E.coli*-NGM plates were stored at 4 ^{o}C. Long-term *C. elegans* stocks were stored in soft agar freezing solution at -80 ^{o}C. All bacterial stocks were stored in 20% glycerol stocks at -80 ^{o}C. Prior to experiments, bacterial starting cultures were prepared as follows: frozen samples were inoculated in 5 mL of LB, CPG or NB broth (media recipes described in Table 2.3) and incubated with shaking at 200 rpm at 28 ^{o}C (or 37 ^{o}C for *E. coli*). *Pseudomonas* strains were grown for 24 h prior to experiments. These methods were carried out at the start of every experiment in a similar way unless stated otherwise.

Table 2.3 Media recipes used in these experiments.

Media recipes	
LB Broth (1 Litre)	10 g Tryptone (pancreatic digest of casein) 5 g Yeast extract 5 g NaCl (+15 g agar for solid media)
Nutrient Broth (1 Litre)	5 g Peptic digest of animal tissue 3 g Beef extract - final pH 6.9 +/- 0.2 (+ 15g agar for solid media)
CPG Broth (1 Litre)	1 g Casamino acids (casein hydrolysate) 10 g Peptone

	5 g Glucose (+17 g agar for solid media)
Kings Medium B Broth (1 Litre)	20 g Proteose peptone 1.5 g K_2HPO_4 1.5 g $MgSO_4 \bullet 7H_2O$ 10 mL Glycerol
Nematode Growth Media (NGM) agar (1 Litre)	3 g NaCl 2.5 g Peptone 17 g Agar + 972mL dH_2O Once cooled to below 55°C: 1 mL 5 mg/ml Cholesterol (made up in ethanol) 1 mL 1 M $MgSO_4$ 1 mL 1 M $CaCl_2$ 25 mL 1M KPO_4 buffer (pH 6.0)
Pseudomonas selective agar (500 mL)	*Pseudomonas* Agar Base CM0559 (Oxoid) containing: Gelatin Peptone 16 g/l Casein hydrolysate 10 g/l Potassium sulphate 10 g/l Magnesium chloride 1.4 g/l Agar 11 g/l *Pseudomonas* C-N Selective Supplement SR0102E (Oxoid): C-N supplement: Cetrimide 100 mg Sodium nalidixate 7.5 mg 24.2 g Agar base 5 mL Glycerol Once cooled to below 55°C: 1 bottle of C-N supplement suspended in 2 mL 50% ethanol
Soft agar freezing solution for freezing *C. elegans* stocks	0.58 g NaCl 0.68 g KH_2PO_4 30 g Glycerol 0.56 mL 1 M NaOH 0.4 g Agar
Alkaline hypochlorite solution	8.25 mL dH_2O 3.75 mL 1 M NaOH 3 mL of bleach (sodium hypochlorite)
M9 buffer	3 g K_2PO_4 6 g Na_2HPO_4 5 g NaCl 1 mL of 1 M $MgSO_4$ (pH 6.0)
Cryomedia for freezing bacterial stocks	50% v/v glycerol

2.3 *Pseudomonas* genome sequences and preliminary analysis

The genomes of the eight *Pseudomonas* strains used in this study were received from collaborators at Utrecht University (Sequenced using Illumina Miseq platform), Netherlands. *De novo* assembled genomes were analysed to identify potential secondary metabolic clusters linked

with antibiosis using AntiSMASH5.0 pipeline (https://antismash.secondarymetabolites.org, Blin *et al.*, 2017, 2019), which allows rapid genome-wide identification and in-depth analysis of secondary metabolite biosynthesis gene clusters based on several open source databases. Fasta files were uploaded to AntiSMASH5.0 bacterial version with detection strictness set to "relaxed" and all search features included to maximise potential metabolitic clusters identified. Based on this analysis, we identified four potential antimicrobial compounds which could potentially be produced by our *Pseudomonas* strains. One of these compounds, 2, 4-diacetylphloroglucinol (DAPG), is a common antimicrobial produced by all eight fluorescent *Pseudomonas* strains used in our experiments. Moreover, we identified three specific gene clusters for Pyoluteorin (an antimicrobial with unknown mechanisms of action; Kidarsa *et al.*, 2011) and orfamide A and B (cyclic lipopeptides that can cause membrane pore formation; Ma *et al.*, 2016), which were found only in CHA0 and Pf-5 *Pseudomonas* strains (Chapter 3, Methods 3.3.6).

2.4 Confirming the identity of specific *Pseudomonas* secondary metabolite presence in the bacterial supernatant through mass spectrometry

To test if the identified secondary metabolite clusters were active in the growth conditions we used in our experiments, we used mass spectrometry to measure the production of identified compounds by CHA0 and Pf-5 *Pseudomonas* strains against chemical standards. We also used untargeted analysis to identify potential novel antimicrobials comparing against existing databases. The strains were grown in triplicate in LB, CPG and NB media with 200 rpm shaking at 28 °C for 24 h. Following incubation, bacterial densities of all samples were normalised to an optical density of 0.1 (OD 600nm) after cultures were centrifuged for 10 minutes at 4000 *g.* The supernatant was then filtered using 0.2 µm filters to separate bacterial cells and fragments from secondary metabolites present in the supernatants. A total of 20 samples were produced (two *Pseudomonas* strains in three growth conditions in triplicate, as well as two samples of unfiltered supernatant for comparisons). 200 µL of each sample was provided to the Centre of Excellence in Mass Spectrometry (CoEMS) at the University of York for mass spectrometry analysis.

Tony Larson and Swen Wagner of the CoEMS group performed *Pseudomonas* metabolite identification and semi-quantification of select target compounds by ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) analysis. The LC separation was performed by an Acquity UPLC I class system (Waters, Elstree, UK), on a BEH C18 100x2.1, 1.7u column (Waters). The mobile phase A was water with 0.1% formic acid (which also served as weak wash), mobile

phase B acetonitrile with 0.1 % formic acid (also served as strong wash). The gradient started at 10% B, increased to 100 % B over 10 min, remaining there for 2 min, decreased to 10% B at 12.1 min and remained there for 1.9 min, leading to an overall gradient duration of 14 min. Column temperature was 40 ^{o}C and sample temperature 10 ^{o}C. Injection volume was 7.5 µL. The MS end was a Synapt G2S-Si QTOF (Waters) mass spectrometer. The instrument was operated in positive ESI, resolution mode, using the HDMS^{E} acquisition technique (alternating scans of MS and MS^{2} acquisitions (fragmentation by CID) alongside travelling wave ion mobility separation). Voltages in the low energy function (MS) were 4 V both in trap and transfer cell; and, in the high energy function (MS^{2}), 4 V in trap and a 20 -100 V ramp in transfer cell. The source was operated with capillary set to 3 kV, source temperature 150 ^{o}C, desolvation temperature 450 ^{o}C, cone gas flow 20 L/h, desolvation gas flow 450 L/h, nebuliser 6.5 bar. Trap gas flow was 2 mL/min, helium cell flow 180 ml/min, IMS gas flow 80 mL/min. Mass range was 100-1000m/z, scan time 0.2 sec, lock mass was leucine-enkephalin at m/z 556.2771.

Data was collected using MassLynx Software Version V4.2 SNC983 (Waters) and analysed using UNIFI 1.9 (Waters) for semi-quantitative analysis of target analytes (Orfamide A, Orfamide B, Pyoluteorin and DAPG). Calibration curves were constructed for target analytes at 10, 50, 100, 300 and 500 nM in three media types (LB, NB, CPG); the MS response for all analytes was linear in this range. Analytes were quantified using the calibration curve in the appropriate medium. Blanks, standards, and samples were injected in technical triplicates from the same vial. Progenesis QI v.2.0 (Waters) was used for untargeted analysis of metabolomics data: putative compound identification was based on a score comprising MS match of exact mass of the compound precursor (ChemSpider database search with 10 ppm mass tolerance) and a match to *in silico* MS^{2} fragmentation pattern. Further data processing and downstream analysis were performed using Bioconductor package XCMS in R, combined with a database (LipidMaps) search.

Untargeted analyses were also conducted to compare collected datasets with Knapsack database (http://www.knapsackfamily.com/KNApSAcK/) Briefly, Waters.raw files were converted to zlib compressed 32 bit precision .mzML files using ProteoWizard MSConvert [1] version 3.0.19172, using the combineIonMobilitySpectra filter, and spectra centroided using the qtofpeakpicker with resolution set to 20000 and threshold 1. Using custom scripts in R 3.0.0 operating in a linux 64 bit environment, .mzML files were further processed using the mzR package [1] to lockmass correct spectra against leucine enkephalin ($[M+H]^{+}$ = 556.27568, $[M-H]^{-}$ = 554.26202). Lockmasses were identified within a +/- 0.1 Da m/z window, and the running mean of lockmasses in a 60s moving window used to adjust the m/z values for every scan. Across files,

feature detection was achieved using the xcmsSet() function from the xcms package [2], using the following parameters: method = 'centWaveWithPredictedIsotopeROIs', ppm = 10, snthresh = 10, peakwidth = c(3, 20), prefilter=c(3, 1000), integrate = 2, mzdiff = -0.1, firstBaselineCheck = FALSE. Custom scripts were used to retain gaussian features, which were then aligned and missing values recalculated across samples using the xcms group() and fillPeaks() functions, respectively.Feature areas were adjusted by subtracting the mean + 3 x the standard deviation of blank samples, with adjusted values < 0 being set to 0. The feature list was then filtered to retain only the most intense monoisotope belonging to a single compound as identified by CAMERA [3], with areas above 0 in at least 90% of blank-subtracted samples. These filtered features were reported as masstags (unique m/z and retention time pairs) and annotated where possible against authentic standards or putative compounds from literature or databases based on exact mass. The Progenesis QI v.2.0 (Waters) was also used to search for compounds by their monoisotopic mass which have been reported to be present in *Pseudomonas* genomes from the literature. The potential metabolites were identified if their observed monoisotopic mass matched with the accurate known ppm within a range of +/- 10 ppm. However without a chemically verified standard for definitive identity matches, this can only be considered speculative.

2.5 Stocks of DAPG, Pyoluteorin, Orfamide A and Orfamide B

Chemically verified purified forms of Orfamide A (https://www.scbt.com/p/orfamide-a-939960-34-6), Orfamide B (https://www.scbt.com/p/orfamide-b-939960-35-7), Pyoluteorin (https://www.trc-canada.com/product-detail/?CatNum=P840375) and 2,4-diacetylphloroglucinol (DAPG) (https://www.scbt.com/p/2-4-diacetylphloroglucinol-2161-86-6) were purchased to test their efficacy against all plant pathogen strains. 10 mM stocks were made in 100% DMSO and stored at -20 °C, with the exception of DAPG for which a 100 mM stock was prepared in 100% methanol and stored at -20 °C.

2.6 Extraction and isolation of *Pseudomonas* lipopeptide antimicrobials

This protocol was adapted from Ma *et al.* (2016) to extract cyclic lipopeptide orfamides from two of the *Pseudomonas* strains. We chose CHA0 as this strain is known to produce orfamides, and F113 as an alternative which may or may not produce orfamides. Based on the Ma *et al.* protocol, seed cultures of CHA0 and F113 were grown in 250 mL flasks containing 50 mL of King's Broth growth medium at 28 °C with shaking at 200 rpm. This was then inoculated into 2 L flasks containing 500 mL of liquid media and shaken at 150 rpm for 48 h. *Pseudomonas* supernatant was collected via centrifugation at 10,000 *g* for 10 minutes (J25 XP series centrifuge). The supernatant

was then acidified to a pH of 2.0 using 8 M HCl and stored overnight at 4 ^{o}C. The precipitate was collected after centrifugation at 10,000 *g* for 20 minutes and extracted with 1 mL of 100% methanol. At this stage in the centrifuge tube there were two distinguishable precipitates, one at the bottom of the tube and one close to the top. It was decided to collect these separately and call them 'A' and 'B' as it was unknown if they would have different or similar properties. The extracted "crude" precipitate that was soluble in methanol was collected in 1.5 mL sterile microcentrifuge tubes. The organic phase was collected by spinning samples until dry in a vacuum concentrator (Savant svc SpeedVac 100h concentrator) and samples then dissolved in 300 μL of DMSO to make our cyclic lipopeptide (CLP) extract stocks.

Table 2.4 Colour observations of final CLP extracts

Growth Media	*Pseudomonas* Strain	Colour of dissolved precipitate in 100% DMSO
King's Broth	CHA0 A	Dark Green/Brown
King's Broth	Pf-5 A	Deep Red
King's Broth	CHA0 B	Pale Green
King's Broth	Pf-5 B	Red

2.7 Data processing, figure generation and statistical programming

All raw data generated in this thesis were processed for statistical analysis and figure generation using various R packages (RCore, 2016; Wickham, 2016). Packages included: ggplot2, readr, plyr, magrittr, dplyr, reshape2, tidyverse, nlme, multcomp, RColorBrewer, lmerTest and plotly.

Chapter 3 *In vitro* and *in vivo* screening of efficient *Pseudomonas* biocontrol strains against *Ralstonia solanacearum* pathogen and investigation of potential underlying mechanisms of inhibition

3.1 Abstract

Traditionally, plant pathogens are controlled using agrochemicals such as mandipropamid and prothioconazole. The use of agrochemicals is, however, in decline due to their toxic effects on the environment, which may lead to a ban on their use. An alternative possibility to agrochemicals are plant-growth promoting bacteria that could be used to control pests and pathogens. However, identifying effective biocontrol agents that work in natural conditions has been challenging. The aims of this study were to conduct *in vitro* and *in vivo* screening of efficient *Pseudomonas* biocontrol strains against *Ralstonia solanacearum* pathogen – the causative agent of bacterial wilt – and to investigate potential underlying mechanisms of inhibition. We first tested the effectiveness of eight *Pseudomonas* strains for pathogen suppression *in vitro* both directly and indirectly using soft agar and supernatant inhibition assays. Results revealed that the inhibitory effects were *Pseudomonas* strain-specific, with strain CHA0 showing the highest levels of pathogen suppression. Genomic screening was conducted to identify potential secondary metabolite clusters involved in the inhibition (2, 4-diacetylphloroglucinol (DAPG), pyoluteorin and orfamides A and B), and their antimicrobial activity was directly validated by using chemical standards in lab assays. Finally, the biocontrol potential of CHA0 and Pf-5 *Pseudomonas* strains was tested against two *R. solanacearum* strains *in vivo* in the tomato rhizosphere. It was found that all *Pseudomonas* strains showed similar levels of biocontrol efficiency alone and in combination. However, bacterial wilt incidence was only reduced in the case of one of the tested *R. solanacearum* strains. Together, these findings demonstrate an efficient screening pipeline for identifying potentially beneficial biocontrol agents both *in vitro* and *in vivo*. However, more detailed understanding of *Pseudomonas* biocontrol activity in the plant rhizosphere is required to translate *in vitro* findings to agricultural applications.

3.2 Introduction

The use of traditional chemical pesticides has declined in recent years due to elevated costs, environmental toxicity and stricter legislation (Chen *et al.*, 2016b). As a result, new methods and approaches are required to control plant pathogens in order to ensure future food security in the face of expanding human population and global change (Hayward, 1991; Kaczmarek *et al.*, 2014; Wang *et al.*, 2019). One alternative to traditional agrochemicals is biocontrol, which often relies on the use of natural competitors, such as other microorganisms, to control soil-borne plant pathogens. Overall, biocontrol poses a lower environmental threat compared to traditional chemical agents, as biocontrol agents can be found naturally in the natural environment. Furthermore, biocontrol agents have been shown to exert a broad range of activity, showing effectiveness against bacterial, fungal, viral and nematode pathogens (Sofrata *et al.*, 2011; Hansen and Keinath, 2013; Wang *et al.*, 2019; Nagachandrabose, 2020). One example of a potential biocontrol species is naturally occurring soil-borne *Pseudomonas* plant growth promoting bacteria (PGPB), known for efficient and broad range biocontrol activity against plant pathogenic bacteria, fungi and nematodes (Pierson *et al.*, 1998). Biocontrol outcomes are however often variable and unsuccessful in field conditions, despite clear inhibition observed under laboratory conditions. One example for this could be that biocontrol agents fail to produce *in vitro* effects during *in vivo* settings as was observed in case of *Pseudomonas*-mediated protection of *Eucalyptus* trees against *R. solanacearum* infections in China (Ran *et al.*, 2005). Mechanistically, this could be due to various different factors linked with survival, colonisation or expression of genes responsible for the biocontrol activity (Ran *et al*., 2005). Alternatively, even though biocontrol agents can show broad range effects against different types of pathogens, they might not be able to inhibit different genotypes of the same pathogen (Xue *et al.*, 2013). As a result, considering genotype-genotype interactions, and the mechanisms by which inhibition is mediated, is important for developing functionally robust biocontrol methods. It is unknown whether *Pseudomonas* PGPB can biocontrol *R. solanacearum* isolated from the UK, and which underlying mechanisms might be causing this. It is also unknown whether the biocontrol effects observed *in vitro*, can be replicated *in vivo*. Here we used a model system consisting of the plant pathogen *R. solanacearum* and *Pseudomonas* PGPB to specifically test the activity of eight biocontrol strains of two *Pseudomonas* species (*P. fluorescens* and *P. protegens*) against seven pathogen isolates originating from the UK (N=6) and Poland (N=1).

The plant pathogenic bacterium, *R. solanacearum* causes vascular wilt disease. It is found globally across the world and has a quarantine status in many countries meaning it is monitored regularly

to prevent further spread (Plant Health Directive, EU, 1995). *R. solanacearum* is ranked the second most important bacterial plant pathogen globally and it possesses numerous virulence mechanisms that allows it to infect more than 250 plant species from 54 plant families (Hayward, 1991; Genin and Denny, 2012; Mansfield *et al.*, 2012; Nion and Toyota, 2015). *R. solanacearum* strains display high variation in their susceptibility to different antimicrobials by different soil bacteria and this has been suggested to be a consequence of the high genetic diversity within the species (Genin and Denny, 2012). Between-strain variability stems from their diversity of pathogenicity-related genes, such as the presence of type III secretion effectors, which can reside on both the chromosome and megaplasmid within its genome (Genin and Denny, 2012). There is also evidence of horizontal gene transfer between different pathogen strains, which can result in exchange of virulence traits between different *R. solanacearum* genotypes (Guidot *et al.*, 2009). *R. solanacearum* strains in Europe are considered to be clonal and have been reported to originate from one introduction event (Hayward, 1991). However, it is unknown whether different UK isolates will been equally suppressed by biocontrol bacteria, hence the need to investigate gene x gene interactions.

Developing a biocontrol agent begins in the lab by screening for and identifying desirable biocontrol traits *in vitro* (Fravel, 2005). *Pseudomonas* biocontrol efficiency is often mediated by the variety of secreted secondary metabolites such as antimicrobials and siderophores (Becker *et al.*, 2012). Some biocontrol characteristics are found universally within *Pseudomonas* species, including the ability to produce 2, 4-diacetylphloroglucinol (DAPG) - an effective antimicrobial against bacterial, fungal and nematode plant pathogens (Cronin *et al.*, 1997; Compant *et al.*, 2005; Haas and Défago, 2005; Humair *et al.*, 2009). Other biocontrol properties can be strain-specific, such as the production of hydrogen cyanide (HCN) by *P. protegens* CHA0 strains (Haas and Keel, 2003), and production of cyclic lipopeptides such as orfamides by *P. protegens* CHA0 and Pf-5 strains (Ma *et al.*, 2016). Screening of bacterial biocontrol agents against *R. solanacearum* can include exposing pathogenic bacterial strains to biocontrol bacterial culture or supernatant and measuring pathogen growth or survival as a function of time (Fravel, 2005). However, the successful identification of biocontrol strains *in vitro* does not always translate to successful biocontrol outcomes *in vivo*. Nutrient availability, competition with other well-established rhizospheric bacteria, degradation and adsorption of secreted antimicrobials and likelihood of successful colonization can all affect the efficacy of disease suppression in soil (Weller, 2007). Therefore, it is vital that *in vitro* screening is coupled with *in vivo* validation of biocontrol effectiveness to develop successful biocontrol applications with translational potential.

Here we used such an approach to screen and identify efficient *Pseudomonas* biocontrol bacterial strains against six UK and one Polish *R. solanacearum* isolates. To achieve this, we first tested the direct and indirect interactions between *R. solanacearum* and eight plant growth promoting *Pseudomonas* strains *in vitro* (all *Pseudomonas* strains were previously shown to have antimicrobial activity against *R. solanacearum* strains (Hu *et al.*, 2016). We then conducted genomic screening of *Pseudomonas* strains to identify potential secondary metabolite clusters and directly tested if these compounds had antimicrobial activity against *R. solanacearum* under laboratory conditions. Finally, the biocontrol potential of a subset of *Pseudomonas* strains was tested against two *R. solanacearum* strains in the tomato rhizosphere. Results revealed that antimicrobial effects of *Pseudomonas* were strain-specific, and the strongest inhibitory effects were mediated by *P. protegens* strain CHA0. Mechanistically, pyoluteorin antibiotic had the strongest inhibitory effect followed by DAPG and orfamides A and B. *In vivo* tests using the same *Pseudomonas* strains revealed that strain CHA0 also had a greater biocontrol potential over Pf-5 in the tomato plant rhizosphere, but that CHA0 biocontrol efficiency depended on the *R. solanacearum* strain. Together, these results show that *in vitro* screening can reliably identify potential, highly active *Pseudomonas* biocontrol strains for the control of *R. solanacearum*. However, more work is still needed to understand the *in vivo* activity of biocontrol strains in the plant rhizosphere.

3.3 Methods

3.3.1 Measuring inhibition of *R. solanacearum* by *Pseudomonas* strains in direct contact

Soft agar overlay assays were used to test direct inhibition of *R. solanacearum* by *Pseudomonas* strains. Bacterial strains were prepared as follows: *Pseudomonas* strains were grown for 24 h in 5 mL of 100% LB broth at 28 °C with shaking at 200 rpm and *R. solanacearum* strains were grown for 30 h under the same conditions. In depth details of the bacterial strains used in this study and their geographical origins can be found in Chapter 2, General Methods, Table 2.1. Soft agar overlay plates were prepared by first filling sterile 90 mm petri dishes with a layer of hard LB agar (made to the normal agar recipe, Chapter 2: General Methods, Table 2.2) which was left to solidify. 200 µL of each *R. solanacearum* strain was then mixed with 20 mL of cooled (below 55 °C) liquid soft agar (half of the agar concentration of the normal recipe, Chapter 2: general methods), and poured on top of the hard agar layer. As *R. solanacearum* grows more slowly than *Pseudomonas*, plates were left to incubate for ~ 8 hours prior to spotting the *Pseudomonas* cultures on top of the soft agar overlay as follows. Each plate was divided evenly into quarters and 2 µL of a *Pseudomonas* strain (approximately 1.0×10^6 CFU/mL) was spotted on the centre of each quarter of the plate (example shown in Figure 3.3.1). Plates were then incubated upside down at 28°C and observed daily after they had been incubated for 72 h which is when zones of inhibition could be visualised and quantified. The distance of the inhibition zones was measured from the outer edge of the *Pseudomonas* spot to the *R. solanacearum* lawn (in millimetres with a ruler). Each strain combination treatment was carried out in triplicate.

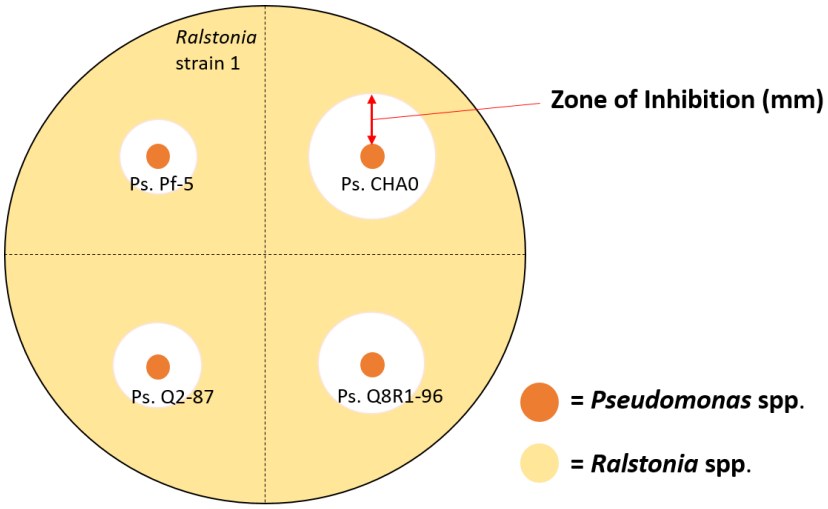

Figure 3.3.1 Experimental design quantifying direct inhibition of *R. solanacearum* by *Pseudomonas* strains using soft agar overlay assay

3.3.2 Measuring inhibition of *R. solanacearum* by *Pseudomonas* strains indirectly using supernatant assays

To investigate *R. solanacearum* inhibition by *Pseudomonas* in the absence of direct contact, we exposed *R. solanacearum* strains to supernatants of each *Pseudomonas* species in pairwise supernatant cultures. The *Pseudomonas* supernatants included all secondary metabolites excreted when *Pseudomonas* strains were grown alone in LB media and were prepared as follows. All *Pseudomonas* strains were first cultured individually in 20 mL of LB broth (Table 2) for 24 h with shaking at 200 rpm. Supernatant was then prepared by centrifuging cultures for 10 minutes at 4000 *g* before separating bacterial cells and fragments from soluble material including secondary metabolites using 0.2 µm filters. The inhibition was measured using flat bottomed 96-well plates in 50:50 *Pseudomonas* supernatant to LB mixtures. As a negative control *R. solanacearum* was grown in 50:50 LB to sterile water mixtures. At the start of the experiment, every supernatant mixture was inoculated with 2 µL of each *R. solanacearum* strain (approximately $1.0x10^6$ CFU/mL) and microplates were then incubated at 28 ^oC for three days and their bacterial densities were recorded as optical density at 24 and 72 h (OD 600 nm; Tecan Infinite spectrophotometer). All pairwise combinations were replicated four times and control treatments three times.

3.3.3 Investigating *Pseudomonas* secondary metabolite biosynthesis gene clusters using AntiSmash5.0

The methods for this are in Chapter 2: General methods, section 2.4.

3.3.4 Determining the effects of identified *Pseudomonas* metabolites on *R. solanacearum* growth in single-compound and multi-compound mixtures

Based on AntiSMASH predictions, synthetic stocks of DAPG, pyoluteorin, orfamide A and orfamide B were acquired to validate their inhibitory effects on *R. solanacearum* directly (Purchasing information can be found in Chapter 2: general methods). The effect of DAPG was measured by inoculating 2 µL of each *R. solanacearum* strain (approximately $1.0x10^6$ CFU/mL) to 100% LB broth with DAPG at the following concentrations: 1000, 500, 100, 50 and 0 µM (control). Due to a relatively high costs of pyoluteorin compared to other chemical standards, only two *R. solanacearum* strains (#1 and #7) were used to test the susceptibility to pyoluteorin (100 µM concentration only) and only strain #1 susceptibility was tested with orfamides A and B (100 µM

concentration only) using the same methods as with the DAPG. To explore the combined effects of different metabolites, we selected 100 µM metabolite concentrations of DAPG, Pyoluteorin and Orfamides A and B. Stocks were made in 100% LB broth and 1-, 2-, 3- and 4-way combinations of each metabolite in equal volumes to a total volume of 200 µL (resulting in final concentrations of 100, 50, 33 and 25% of each metabolite, respectively). All treatments were replicated three times and inoculated with 2 µL of *R. solanacearum* strain #1. In all assays, bacterial densities were recorded as optical density at 0 h and 24, 48 and 72 h after inoculation with a spectrophotometer (OD 600 nm; Tecan Sunrise spectrophotometer). Additionally, to monitor whether the age of these stock concentrations affected their ability to suppress *R. solanacearum*, DAPG stocks were made 72, 48, 24 and 0 h prior to beginning of the experiment and their effect tested in independent experiments.

3.3.5 Testing the effect of isolated *Pseudomonas* cyclic lipopeptides on *R. solanacearum* growth

Cyclic lipopeptides (CLPs) were extracted from *P. protegens* CHA0 and *P. fluorescens* F113 following the protocol by Ma *et al.* (2016) (General methods: Chapter 2, Section 2.5). The strain CHA0 was chosen because it has previously been reported to produce cyclic lipopeptides such as orfamides (Ma *et al.*, 2016) and was identified to contain an orfamide biosynthesis cluster, whereas no orfamide cluster was observed in F113 strain based on literature. Hence, no orfamide production was expected to be observed in the case of F113 strain. Again, due to limited amount of CLP extracts, only *R. solanacearum* strains #1 and #7 were used. Inoculant cultures were grown in 5 mL of LB broth overnight (Chapter 2, General Methods, Section 2.2) and 2 µL of each *R. solanacearum* strain was inoculated to 1% CLP extracts in LB broth (200 µL final volume). Bacterial densities were recorded at 24, 48 and 72 h (OD 600 nm; Tecan Infinite spectrophotometer) and each treatment was carried out in triplicate.

3.3.6 Testing *Pseudomonas* biocontrol efficiency *in vivo* using a tomato plant model

To explore the translational potential of *Pseudomonas* CHA0 and Pf-5 biocontrol strains *in vivo*, we tested if any of the inhibitory effects observed *in vitro* could protect tomato plants from *R. solanacearum* infections. Two experiments were conducted to test the potential biocontrol efficacy against *R. solanacearum* strains #1 and #7. In the first experiment, the infectivity of *R. solanacearum* strains were tested with tomato in the absence of *Pseudomonas* strains. In the second experiment, *R. solanacearum* infectivity was tested in the presence of CHA0 and Pf-5

Pseudomonas biocontrol, and non-biocontrol *P. fluorescens* Sbw25 strain was used as a control (does not produce pyoluteorin or orfamides based on AntiSMASH analysis). In both experiments, tomato (*Solanum lycopersicum* 'Microtom') plants were grown in an incubated light chamber at 28 ^{o}C with 16:8 h light:dark conditions with regular watering. Seeds were sown in 7 cm seedling trays in 35 g of autoclaved compost where they remained for the entire experiment. Experiments were conducted between July and September 2019 in temperature-controlled plant growth chambers (Sanyo MLR-352) at the University of York.

Experiment 1 – Testing the infectivity of two *R. solanacearum* strains on tomato plants

Ralstonia solanacearum strains #1 and #7 were cultured in 30 mL of CPG broth (General methods: Chapter 2) for 48 h with shaking at 200 rpm at 28 ^{o}C. CPG broth was chosen to keep consistent with other *in vivo* experimental chapters (Chapter 5). To mimic natural pathogen entry points in the field, the root systems of 4-week old tomato plants were cut using a sterile scalpel in the same directional diagonal movement prior to bacterial inoculation. 1 mL of bacterial culture (approximately 0.4 OD 600 nm) was inoculated to the base of the visible stem above the soil. All plants were watered one hour prior to monitoring infection to be certain that wilting was not due to dehydration. Bacterial wilting incidence was monitored every other day by scoring the plants as "uninfected" and "infected" when plants showed clear wilting symptoms: the stem was bending over and the plants were leaning with the leaves looking shrivelled (Figure 3.3.2). The experiment was terminated after three weeks of pathogen inoculation. The entire plant above soil was removed and plant aboveground dry weight recorded after placing individual plants packaged in separate envelopes in a drying cabinet. Six replicate plants were used for each treatment.

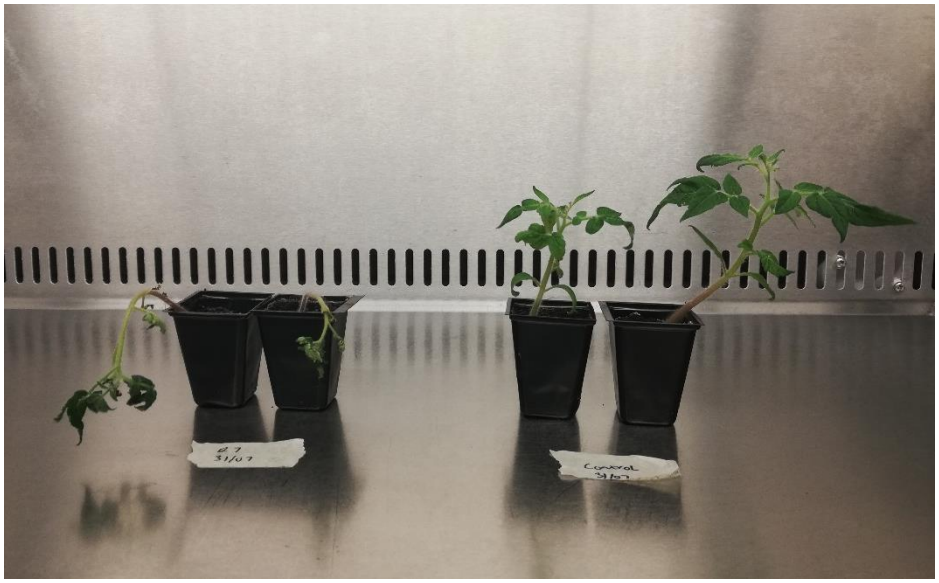

Figure 3.3.2 Example of bacterial wilting symptoms caused by *R. solanacearum* strain #7 on Microtom tomato plant cultivars. The two replicates on the left side display clear wilting symptoms, while the two plants on the right show two healthy control replicates.

Experiment 2 – Testing CHA0 and Pf-5 *Pseudomonas* biocontrol efficacy individually and in combination

To test the biocontrol efficacy of CHA0 and Pf-5 strains both individually and in combination, another tomato infection experiment was conducted in a plant growth chamber. Similar to the first experiment, *P. fluorescens* SBW25 strain was used as a negative control. *Pseudomonas* cultures were prepared by inoculating 100 mL of CPG broth with 100 μL of frozen stock bacterial cultures in 300 mL glass jars. These were grown for 24 h at 28 ^{o}C with shaking at 200 rpm. Because it was uncertain whether the nutrients present in the inocula had positive effects on plant growth during the first experiment, we removed them in the second experiment. The bacterial cultures were then washed of the nutrient media they were grown in by centrifuging the cultures at 4000 *g* for 10 minutes and resuspended in sterile dH2O. Washing of cells was important to prevent the bacterial growth media influencing results via effects on bacterial or tomato growth. *Pseudomonas* was inoculated onto the soil of 4-week old seedlings one week prior to *R. solanacearum* strains to allow time for the biocontrol bacteria to effectively colonise the soil and roots. *Pseudomonas* cell densities were adjusted to an OD 600 nm of 0.25 (approximately $1.0x10^{9}$ CFU/mL) and 6 mL was poured into each pot, soaking through the soil and roots and filling the saucer below. Roots of tomato seedlings were cut prior to *R. solanacearum* inoculation using a sterile scalpel to mimic natural pathogen entry points in the field. *R. solanacearum* strains were prepared as described in Experiment 1, adjusted to an OD600 nm of 0.25 and 1 mL of each *R. solanacearum* strain was inoculated at the base of the visible stems following root cuttings one

week following *Pseudomonas* inoculation. Bacterial wilting symptoms were recorded for four weeks as described above. Plant aboveground dry weights were recorded as previously described in experiment 1 at the end of the experiment. Nine replicate plants were used for each treatment.

3.3.7 Statistical analyses

We used repeated measures ANOVA with between-subject and random effects to analyse mean differences in pathogen densities for all experiments including temporal data. Two-way ANOVA was used to analyse the mean differences between treatments when only one time point was collected followed by post hoc Tukey tests with 95% confidence levels. Tukey contrasts with Bonferroni-corrected p-values were used for pairwise comparisons. Poisson glm models with chi squared tests were used to analyse the binomial *in vivo* tomato infection data. All statistical tests were carried out using RStudio.v.3.4.4, for details on packages used see Chapter 2, General Methods, Section 2.7.

3.4 Results

3.4.2.1 Measuring direct inhibition of *R. solanacearum* by *Pseudomonas* strains using soft agar assays

The presence of inhibition zones on each lawn indicated that to an extent, each *Pseudomonas* strain could inhibit *R. solanacearum* growth. Inhibition zone sizes differed depending on the identities of interacting *Pseudomonas* and *R. solanacearum* strains (*Ralstonia*Pseudomonas*: $F_{42, 112} = 2.075$, $P = 0.0013$, Figure 3.4.1). This variation appeared to be mainly driven by *Pseudomonas* strains, as post hoc analyses revealed only small and non-significant variation between *R. solanacearum* strains (*Ralstonia*: $F_{6, 161} = 0.4951$, $P = 0.8114$, Figure 3.4.1), which suggests that they responded in very similar way overall. In contrast, higher variation was observed between *Pseudomonas* strains (*Pseudomonas*: $F_{7, 160} = 118.031$, $P = 0.001$, Figure 3.4.1), and post hoc analyses revealed that CHA0 was the most inhibitory strain followed by Q8R1-96 and MVP1-4 that showed modest levels of inhibition (Tukey: $P < 0.05$). The strains 1M1-96 and Ph11C2 caused the smallest zones of inhibition on *R. solanacearum* lawns (Tukey: $P < 0.05$). The size of inhibition zones increased over time in general (*Time*: $F_{14, 320} = 26.537$, $P < 0.001$, Figure 3.4.1) and this was most clear with the CHA0 and Pf-5 *Pseudomonas* strains.

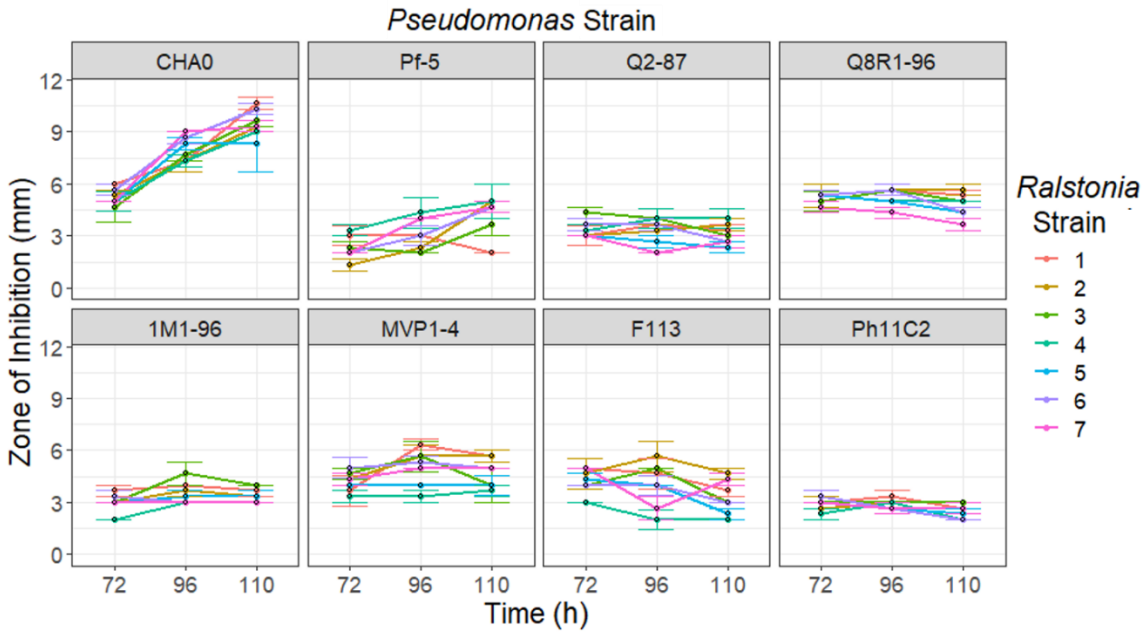

Figure 3.4.1 Direct inhibition of *R. solanacearum* by *Pseudomonas* strains using soft agar assays. Each panel represents a different *Pseudomonas* strain and the coloured lines denote the average size of the inhibition zones for different *R. solanacearum* strains over time on soft agar lawns. All bars show the standard error of the mean (+/-1 SEM) based on three replicates

3.4.2.2 Measuring indirect inhibition of *R. solanacearum* by *Pseudomonas* strains using supernatant assays

Almost all *Pseudomonas* supernatants displayed suppression of *R. solanacearum* growth compared to no-supernatant control treatments (*Supernatant treatment*: $F_{8, 236} = 3.697$, $P = 0.01567$, Figure 3.4.2), and in general, pathogen suppression increased slightly over time (*Time*: $F_{2, 488} = 77.831$, $P < 0.001$, Figure 3.4.2). The data was also analysed in terms of *Ralstonia* growth reduction in the *Pseudomonas* supernatant relative to the LB broth control treatment. In contrast to agar plate assays, *R. solanacearum* strain variation was more evident and post hoc analyses revealed that the growth reduction of strains #2 and #3 was significantly lower compared to other strains, indicative of innate tolerance (*Ralstonia*: $F_{6, 217} = 31.8795$, $P < 0.001$, Figure 3.4.2) the other five *R. solanacearum* strains did not differ from each other in their susceptibility overall. When comparing the suppression by the supernatant of *Pseudomonas* strains, CHA0 caused the greatest reduction in *R. solanacearum* growth overall followed by Ph11C2 and MVP1-4, which showed intermediate growth reduction (*Pseudomonas strain*: $F_{7, 216} = 7.202$, $P < 0.001$, Figure 3.4.2). *Pseudomonas* strains 1M1-96, Q8R1-96 and Pf-5 showed relatively smallest growth reduction. Together, these findings support the direct inhibition assay (Figure 3.4.1) highlighting the relatively strong pathogen growth suppression by *Pseudomonas* CHA0 strain.

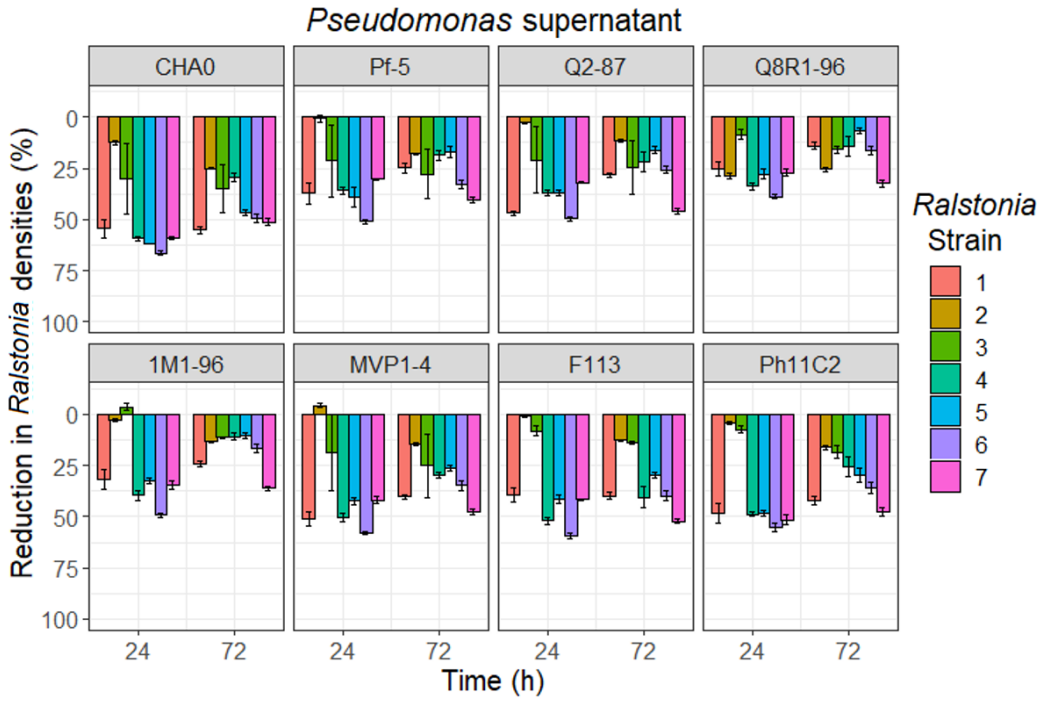

Figure 3.4.2 Indirect inhibition of *R. solanacearum* by *Pseudomonas* strains in supernatant assays. The bars in each panel represent the growth reduction in *R. solanacearum* densities compared to the control (LB broth) treatment at 24 and 72 h, and values below zero denote growth reduction in the supernatant. Each panel represents the different *Pseudomonas* supernatants in which the pathogen populations were exposed to. All bars show the standard error of the mean (+/-1 SEM) and are based on 4 replicates.

3.4.3 Comparative genomic analysis reveals variation in the presence of secondary metabolic clusters amongst different *Pseudomonas* strains

Comparative genomic analysis based on the AntiSMASH output identified between 11-17 metabolic clusters in each of the eight *Pseudomonas* genomes (Tables 3.1-2). Table 3.1 describes the types of clusters recognised with examples of the range of metabolites matched with the AntiSMASH database, while Table 3.2 provides specific details of the cluster diversity within each *Pseudomonas* genome. Non-ribosomal peptide synthetase (NRPS) clusters were the most abundant, which was expected as these can account for a number of common antimicrobials produced by fluorescent *Pseudomonas* strains. As expected, DAPG metabolite (belonging to the T3PKS cluster) as well as the pyoverdine siderophore (NRPS cluster) metabolite clusters were found in all strains. Overall, the highest number of clusters (17) were detected in CHA0 and Pf-5 strains. Also, these strains harboured some unique metabolite clusters such as the T1PKS metabolic cluster, which encodes pyoluteorin antimicrobial, and CDPS cluster, which encodes unknown metabolites. CHA0 and Pf-5 also had the greatest number of NRPS clusters which was expected as these are also the only two of the eight strains which can produce the cyclic

lipopeptides known as orfamides which belong to the NRPS cluster. Other *Pseudomonas* strains also possessed some unique clusters such as ectoine metabolic cluster found in Q2-87 strain. Appendix Tables A.1-8 show a more in-depth insight into the clusters and the percentage similarity with various *Pseudomonas* and other bacterial genomes based on the AntiSMASH database. The important clusters which were investigated further were 95-100% matched to characterised clusters increasing the certainty of the products identity as well as had been recognised in the literature.

Table 3.1 AntiSMASH5.0 cluster types recognised in eight *Pseudomonas* genomes including a brief definition of their function

Cluster	Description	Range of metabolites in cluster
NRPS	Non-ribosomal peptide synthetase cluster	Orfamide, putisolvin, pyochelin, salicyclate, lipopeptide, viscosin, syringopeptin, rhizomide, delfibactin, pyoverdine
NRPS-like	NRPS-like fragment	Mangotoxin, lankacidin
Bacteriocin	Other unspecified ribosomally synthesised and post-translationally modified peptide product (RiPP) cluster	Unknown
CDPS	tRNA-dependent cyclodipeptide synthases	Unknown
T1PKS	Type I PKS (Polyketide synthase)	Pyoluteorin
T3PKS	Type III PKS	DAPG
NAGGN	N-acetylglutaminylglutamine amide	Unknown
Arylpolyene	Aryl polyene cluster	APE Vf
Butyrolactone	Butyrolactone cluster	Unknown
Betalactone	Beta-lactone containing protease inhibitor	Fengicynin, mycocubtilin
Ectoine	Ectoine cluster	Unknown
Lanthipeptide	Lanthipeptide cluster	Putative Class II
LAP	Linear azol(in)e-containing peptides	Unknown
Other	Unknown	Pyrrolnitrin

Table 3.2 AntiSMASH5.0 cluster types recognised of the eight *Pseudomonas* genomes used in this study

Pseudomonas Strain	Total no. of clusters	NRPS	NRPS-like	Bacteriocin	CDPS	T1PKS	T3PKS	NAGGN	Arylpolyene	Butyrolactone	Betalactone	Ectoine	Lanthipeptide	LAP	Other
CHA0	17	8		2	1	1	1	1	1	1					1
Pf-5	17	7	1	2	1	1	1	1	1		1				1
Q2-87	15	4	1	3			1	1	1	1	1	1	1		
Q8R1-96	13	6	1	1			1	1	1	1	1				
1M1-96	12	3	1	1			1	1	1	1	1		2		
MVP1-4	13	4	1	1			1	1	1	1	1		1	1	
F113	11	2	2	1			1	1	1	1	1		1		
Ph11C2	14	4	1	3			1	1	1	1	1		1		

3.4.5.1 Testing the inhibitory effects of detected candidate secondary metabolites on *R. solanacearum* growth individually and in combination

We identified gene clusters encoding the biosynthesis of metabolites in the genomes of the eight *Pseudomonas* strains and selected four metabolites of CHA0 and Pf-5 to test on *R. solanacearum*. To test the potential inhibitory role of a subset of common and unique metabolites of CHA0 and Pf-5, the effects of DAPG, pyoluteorin and orfamides A and B were first tested individually against *R. solanacearum* strains using commercially available chemical standards. DAPG was selected as this metabolite can be produced by all eight of the Pseudomonas strains, whereas orfamdies and pyoluteorin are unique to these two strains. The effect of DAPG was tested against all *R. solanacearum* strains, while the effect of orfamides and pyoluteorin were only tested against a subset of strains due to high costs of chemical standards (*Ralstonia* strain #1 for orfamides, and *R. solanacearum* strains #1 and #7 for pyoluteorin).

It was found that DAPG suppressed every *R. solanacearum* strain in a concentration-dependent manner, and all tested strains were unable to grow at the two highest concentrations (500 µM and 1000 µM: *Concentration*: $F_{4, 200} = 238.2599$, $P < 0.001$, Figure 3.4.3 A). Across the entire dataset, the effect of DAPG was not dependent on the *Ralstonia* strain identity (*Ralstonia*: $F_{6, 98} = 0.724$, $P = 0.6305$, Figure 3.4.3 A). However, excluding the two highest concentrations, where all growth was inhibited by DAPG (500 µM and 1000 µM), showed that the response to DAPG varied between different *Ralstonia* strains (*Ralstonia*: $F_{6, 56} = 3.355$, $P = 0.0068$, Figure 3.4.3 A) similarly between the two lowest DAPG concentrations (*Ralstonia*Concentration*: $F_{12, 42} = 0.8892$, $P = 0.5643$, Figure 3.4.3 A). For a more in-depth understanding of strain variation, we considered the growth reduction in DAPG relative to the growth in the control treatment at the final time point. *Ralstonia* growth reduction was greater in 100 µM DAPG than 50 µM (*Concentration*: $F_{1, 124} = 46.8$, $P < 0.001$, Figure 3.4.3 A - variation most evident at the final time point). Also, *R. solanacearum* strain growth reduction varied between strains at both 100 µM (*Ralstonia*: $F_{6, 56} = 5.671$, $P < 0.001$, Figure 3.4.3 A - variation most evident at the final time point) and 50 µM (*Ralstonia*: $F_{6, 56} = 3.856$, $P = 0.00273$, Figure 3.4.3 A - variation most evident at the final time point). Post hoc analyses revealed that strains #5 and #6 were the least susceptible to DAPG, while strains #2 and #3 were more susceptible.

Both orfamides A and B reduced the growth of *R. solanacearum* strain #1 and this effect became more distinct over time (*Metabolite:* $F_{2, 6} = 460.7$, $P < 0.001$; *Metabolite*Time*: $F_{4, 12} = 45.806$, $P = 0.0359$, Figure 3.4.3 B). However, no difference was found between orfamide A and B ($P = 0.1164373$). No visible growth was observed when either *R. solanacearum* strains #1 or #7

were exposed to pyoluteorin (*Ralstonia*: $F_{2, 16} = 48.596$, $P < 0.001$, Figure 3.4.3 C) suggesting that both strains were highly susceptible to this compound. It was also confirmed that DAPG was not inactivated, e.g. through natural degradation, during the 72h growth period as the 'aged' stocks were equally effective as the 'fresh' stocks (*Stock age*: $F_{2, 249} = 0.42078$, $P = 0.657$, Figure 3.4.4).

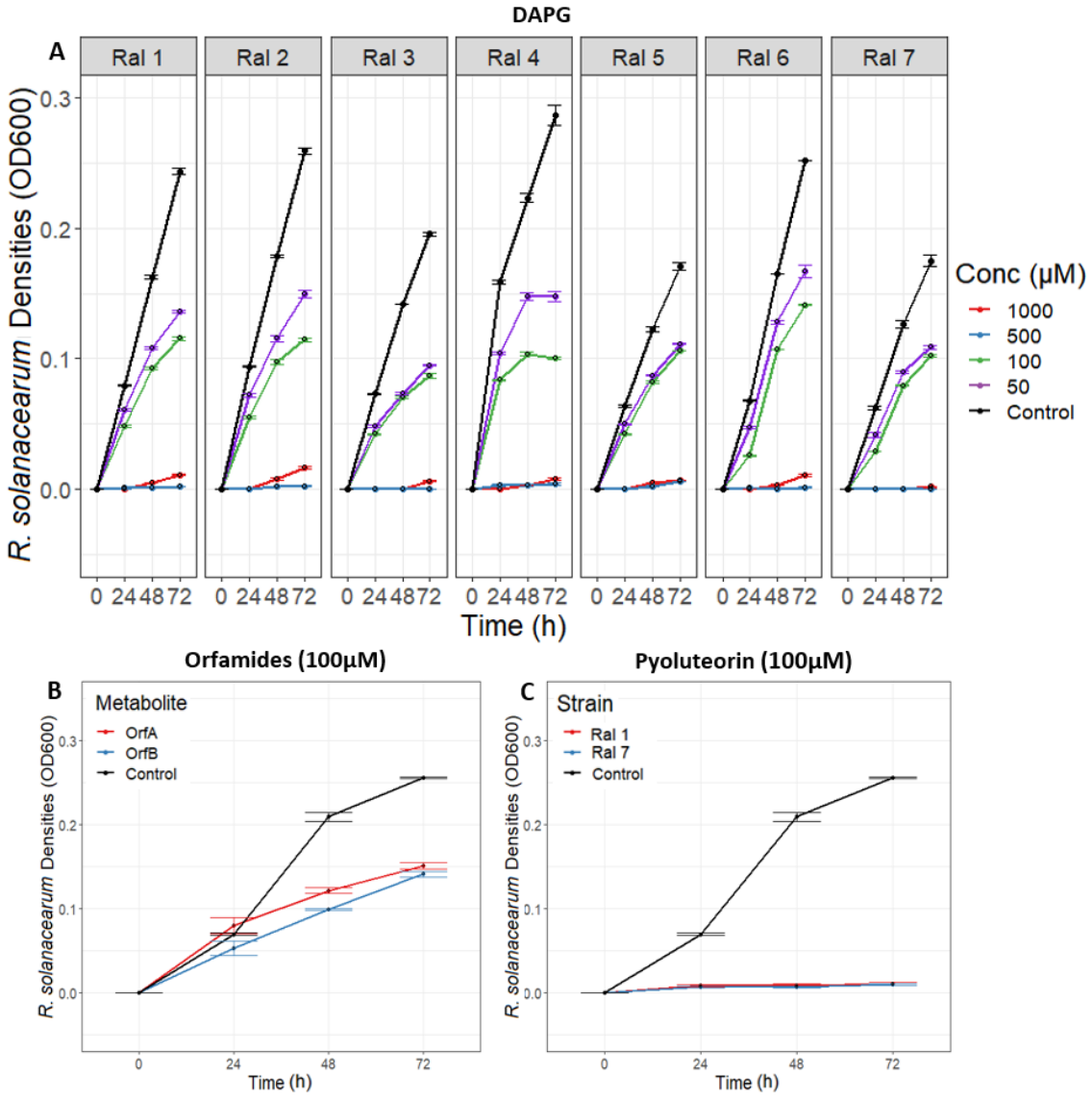

Figure 3.4.3 Testing the individual inhibitory effects of detected candidate secondary metabolites *on R. solanacearum* growth. Panel (A) shows the growth of seven *R. solanacearum* strains (in different panels) in various DAPG concentrations over time (1000 µM – red, 500 µM – blue, 100 µM – green, 50 µM – purple and 0 µM LB broth (control) - black). Panel (B) shows *R. solanacearum* strain #1 growth in the absence and presence of 100 µM of orfamides A and B. Panel (C) shows the combined growth of *R. solanacearum* strains #1 and #7 in the absence and presence of 100 µM of pyoluteorin. In all panels, bars show the standard error of the mean (+/-1 SEM) based on 3 replicates.

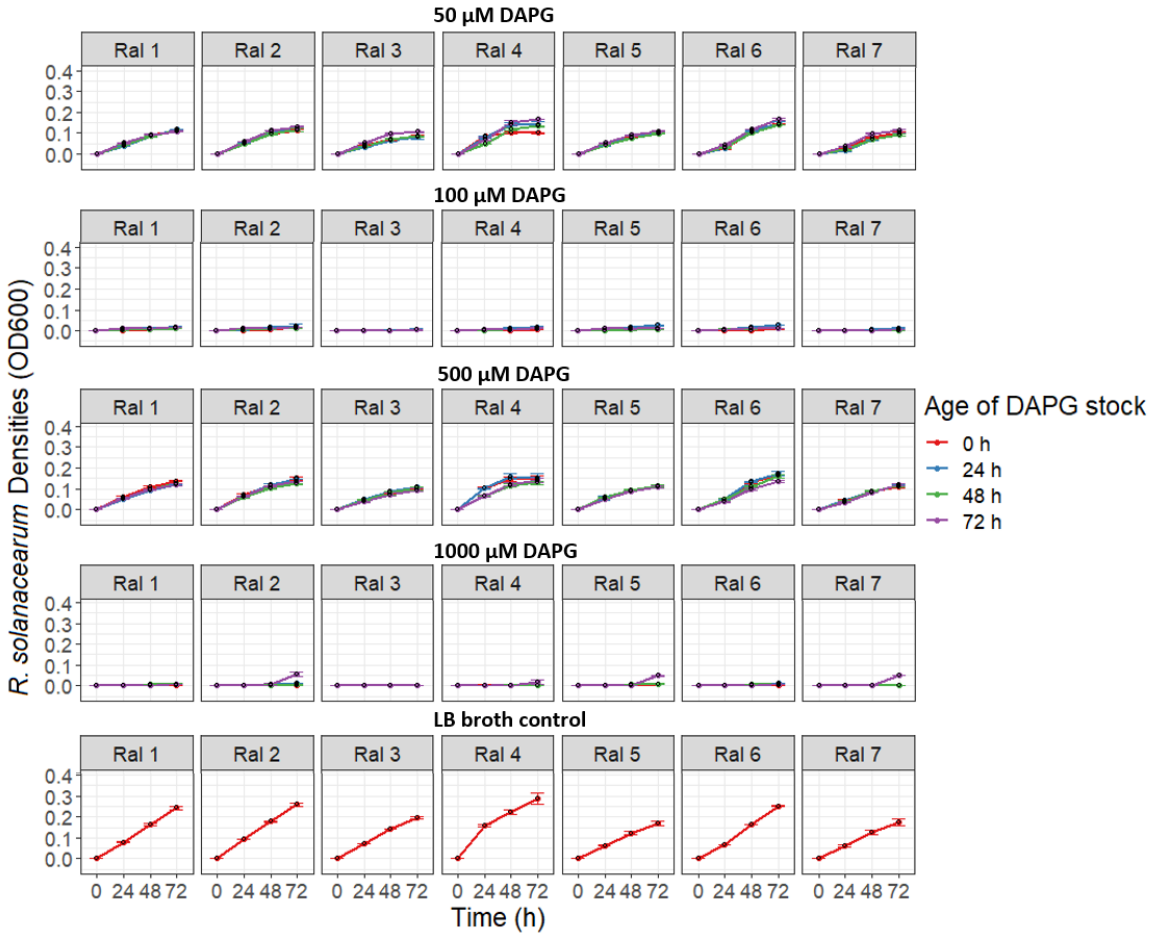

Figure 3.4.4 Testing different aged DAPG stocks on *R. solanacearum* strains 1-7. The line graphs show the ability of the seven *R. solanacearum* strains to grow in a number of DAPG concentrations with stocks prepared at different time intervals (0 h DAPG stock - red, 24 h DAPG stock – blue, 48 h DAPG stock – green, 72 h DAPG stock – purple). Each column represents an individual *R. solanacearum* strain and each row of growth rates represents which concentration of DAPG the *R. solanacearum* isolates were exposed to. All bars show the standard error of the mean (+/-1 SEM).

To test the combinatory effects of candidate secondary metabolites, we prepared 1-, 2-, 3- and 4-way combinations of DAPG, pyoluteorin and orfamides A and B in equal volumes. The effect of these combinations was tested only on *R. solanacearum* strain #1 due to limited availability of chemical standards.

All metabolite combinations had a suppressive effect on *R. solanacearum* growth in comparison to the control treatment (*Treatment:* $F_{1, 34} = 20.032$, $P < 0.001$, Figure 3.4.5). Individual metabolite effects were similar to the previous experiment (*Metabolite:* $F_{10, 22} = 50.4923$, $P < 0.001$, Figure 3.4.5 A), with pyoluteorin suppressing *R. solanacearum* growth to a significantly greater extent than DAPG ($P < 0.001$), Orfamide A ($P < 0.001$) and Orfamide B ($P < 0.001$), which all had similar effects on pathogen suppression ($P > 0.05$, Figure 3.4.5 A). In the case of two-way metabolite combinations, treatments containing pyoluteorin caused a significantly greater suppression of *R. solanacearum* growth compared to 'DAPG + OrfA' ($P < 0.001$, Figure 3.4.4 5) and 'DAPG + OrfB' ($P < 0.001$, Figure 3.4.5 B) combinations, which did not differ from each other ($P >0.05$, Figure 3.4.4 5). Similarly, only 3-way combinations that contained pyoluteorin showed high suppression of *R. solanacearum* growth compared to 'OrfA and OrfB' ($P < 0.001$, Figure 3.4.5 C) combination. The combination with all four metabolites (Figure 3.4.5 D) also resulted in strong suppression of *R. solanacearum* growth, which was likely due to the presence of pyoluteorin. Finally, we used the last measurement time points to compare how the dilution of pyoluteorin (only 50%, 33% or 25% used in combinations) affected the suppression of pathogen growth. No significant differences were found, which suggests pyoluteorin was highly effective at suppressing *R. solanacearum* growth even when diluted down to 25 µM concentration (*Combination:* $F_{4, 10} = 0.7333$, $P = 0.567$, Figure 3.4.5 D). Together, these results show that inhibitory compounds did not show synergistic or additive effects when applied in combinations.

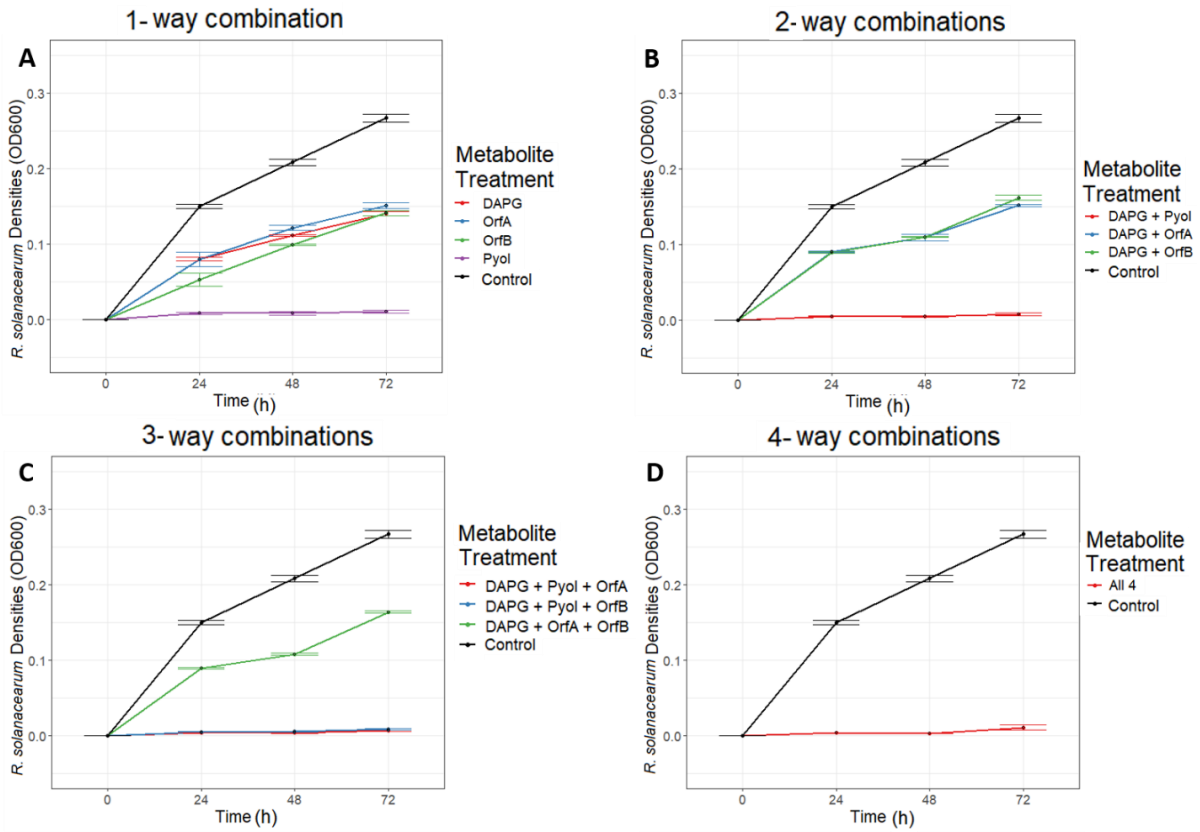

Figure 3.4.5 Testing the combinatory inhibitory effects of detected candidate secondary metabolites *on R. solanacearum* growth. The black line in each panel denotes *R. solanacearum* density in the absence of the compounds over time (control). Each panel represents *R. solanacearum* strain #1 growth rates upon exposure to combinations of: Orfamide A (OrfA), Orfamide B (OrfB), Pyoluteorin (PyoI) and DAPG in single (A) and 2- (B), 3- (C) and 4-way combinations (D). All error bars show the standard error of the mean (+/-1 SEM) based on 3 replicates.

3.4.5.2 Testing the inhibitory effects of cyclic lipopeptides isolated from *Pseudomonas* CHA0 and F113 strains on *R. solanacearum* growth

Cyclic lipopeptides were isolated from *Pseudomonas* CHA0 and F113 strains as described in Chapter 2, General Methods, Section 2.6. The effects of CLP extracts were tested against two *R. solanacearum* strains #1 and #7 due to limited quantities of extracted compounds (Figure 3.4.6). The growth of both *Ralstonia* strains was constrained by all CLP extracts except for 'F113 B' (*Ralstonia*Treatment*: $F_{4, 20} = 0.07241$, $P = 0.9974$, Figure 3.4.6), and the growth suppression by orfamides became visible only after 48h measurement time point during the assays (*Treatment*Time*: $F_{8, 50} = 50.927$, $P < 0.0001$, Figure 3.4.6). At the final time point, there were clear differences between CLP extract effects on both strain #1 (*Treatment*: $F_{4, 10} = 324.6$, $P < 0.001$, Figure 3.4.6) and strain #7 (*Treatment*: $F_{4, 10} = 48.55$, $P < 0.001$, Figure 3.4.6). Post hoc analyses revealed treatment effects were more varied against strain #1 with every treatment being distinct from the control and from one another and, with the two CHA0 treatments being the most

suppressive of these (Tukey: $P < 0.05$). Post hoc analyses for strain #7 effects showed the growth densities in 'CHA0 A', 'CHA0 B' and 'F113 A' treatments were all significantly different to that of the control and 'F113 B' treatments, but not from each other (Tukey: $P < 0.05$).

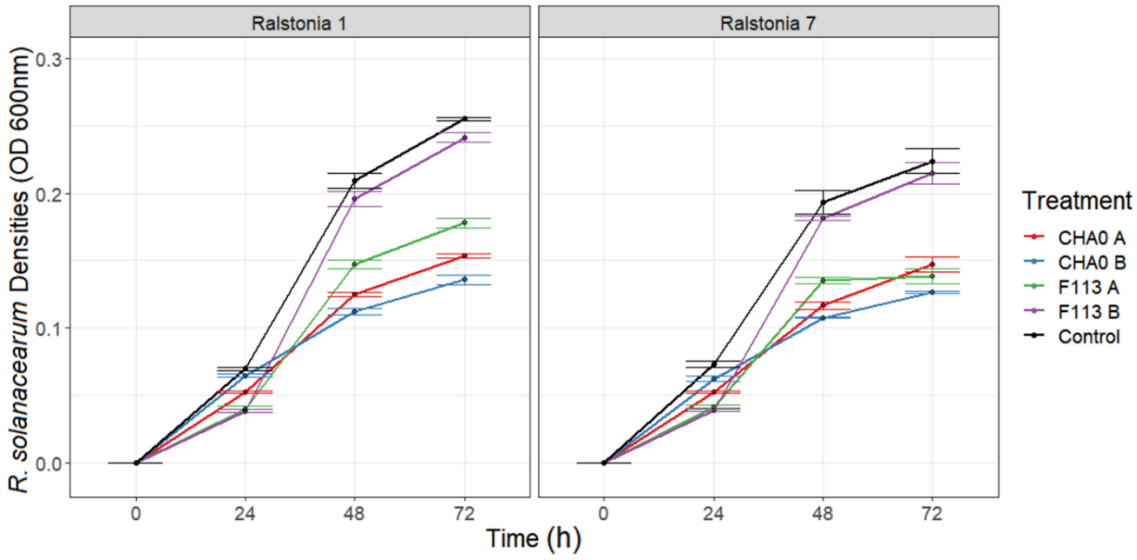

Figure 3.4.6 Testing the inhibitory effects of CLP extracts isolated from *Pseudomonas* CHA0 and F113 strains on *R. solanacearum* growth. The black lines denote *R. solanacearum* density in the absence of the CLP extracts over time (control), while coloured lines shows the growth in the presence of CLP extracts isolated from CHA0 (red and blue) and F113 (green and purple) *Pseudomonas* strains (A and B refers to two different fractions isolated separately during the extraction process). All error bars show the standard error of the mean (+/-1 SEM) based on 3 replicates.

3.4.6 Testing *Pseudomonas* biocontrol efficacy *in vivo* in tomato system

Firstly, the two *R. solanacearum* strains (#1 and #7) were tested for their ability to infect tomato plants in a plant growth chamber experiment (Figure 3.4.7). Results showed that both *R. solanacearum* strains were able to infect the tomato plants and caused similar levels of bacterial wilt symptoms (Treatment: $F_{1, 12}$= 17.878, P = 0.7967, Figure 3.4.7 A, around 50% of plants wilted). In terms of above ground plant biomass, a reduction was observed when plants were infected by *R. solanacearum* (Treatment: $F_{2\ 17}$= 7.715, P = 0.00413 Figure 3.4.7 B) and that this reduction was driven by the strain #7 (P = 0.002), while the effect of strain #1 was non-significant (P = 0.243).

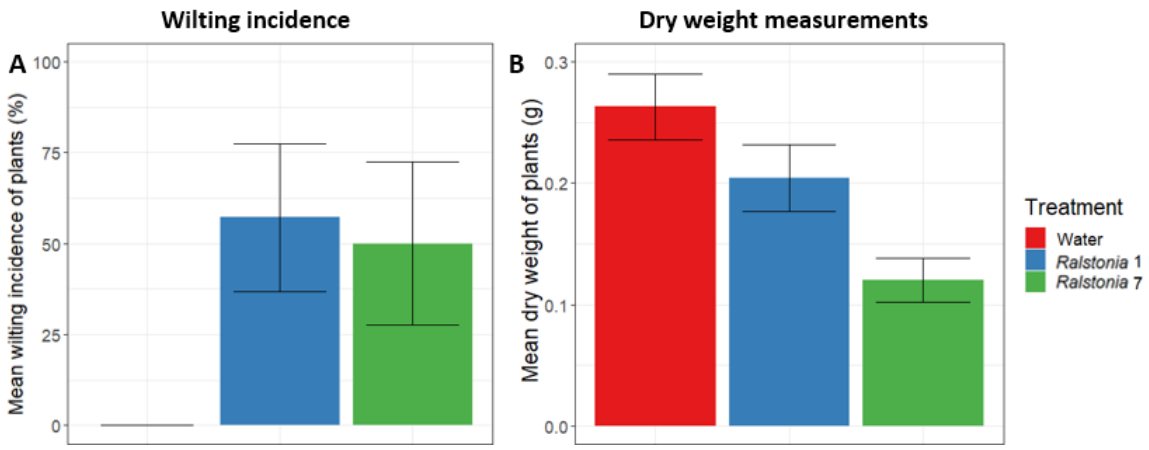

Figure 3.4.7 Tomato plant infections by *R. solanacearum* strains #1 and #7 in the absence of *Pseudomonas* strains. Panel (A) displays the percentage of wilted plants (wilting incidence) in three different treatments. Panel (B) shows differences in plant aboveground dry weight (biomass) at the end of the experiment. All error bars show the standard error of the mean (+/-1 SEM) based on 6 replicates.

Secondly, the biocontrol potential of CHA0 and Pf-5 *Pseudomonas* strains was tested alone and in combination (Figure 3.4.8). *Pseudomonas* strain Sbw25 was also used as an additional control lacking secondary metabolite clusters for orfamide and pyoluteorin biosynthesis. It was first investigated if *R. solanacearum* disease incidence levels in strain #1 had higher wilting incidence compared to strain #7 (*Ralstonia:* $F_{1, 88}$= 117.17, *P* = 0.01999, Figure 3.4.8 A). *Pseudomonas* strains, or their combinations, had no effect on wilting incidence in comparison with the water control treatment in the case of *Ralstonia* strain #1 (*Pseudomonas:* $F_{1, 42}$= 60.628, *P* = 0.9456, Figure 3.4.8 A). However, all *Pseudomonas* treatments reduced wilting incidence in the case of *Ralstonia* strain #7 with similar efficiencies (*Pseudomonas:* $F_{1, 44}$= 50.091, *P* = 0.01114, Figure 3.4.8 A). Despite a trend suggesting CHA0 may be the most suppressive *Pseudomonas* strain, no significant differences were found between different *Pseudomonas* treatments with *Ralstonia* strain #7 (*Treatment:* $F_{3, 33}$= 37.279, *P* = 0.7162, Figure 3.4.8 A).

We next compared the effects of inoculated bacteria on the aboveground dry weight of tomatoes (Figure 3.4.7 B). *Pseudomonas* treatments overall did not have significant effects on the plant growth compared to the no-bacteria water control treatments (*Ralstonia:* $F_{1, 88}$= 0.963, *P* = 0.329, Figure 3.4.8 B). There was a significant reduction in the dry weights of tomatoes treated with *Ralstonia* strain #1 compared to strain #7 (*Ralstonia:* $F_{1, 88}$= 3.961, *P* = 0.0497, Figure 3.4.8 B) with post hoc analyses revealing the dry weights of strain #7 to be greater, indicative of biocontrol effects (*P* = 0.049). Crucially, none of the *Pseudomonas* treatments had an effect on tomato dry weights in the case *Ralstonia* strain #1 (*Pseudomonas:* $F_{3, 31}$= 0.77, *P* = 0.52, Figure 3.4.8 B), or #7

(*Pseudomonas:* $F_{3, 31}$= 0.921, *P* = 0.441, Figure 3.4.8 B). Together, these results suggest that all *Pseudomonas* strains showed some levels of biocontrol efficacy, and that these effects were only observed with *Ralstonia* strain #7.

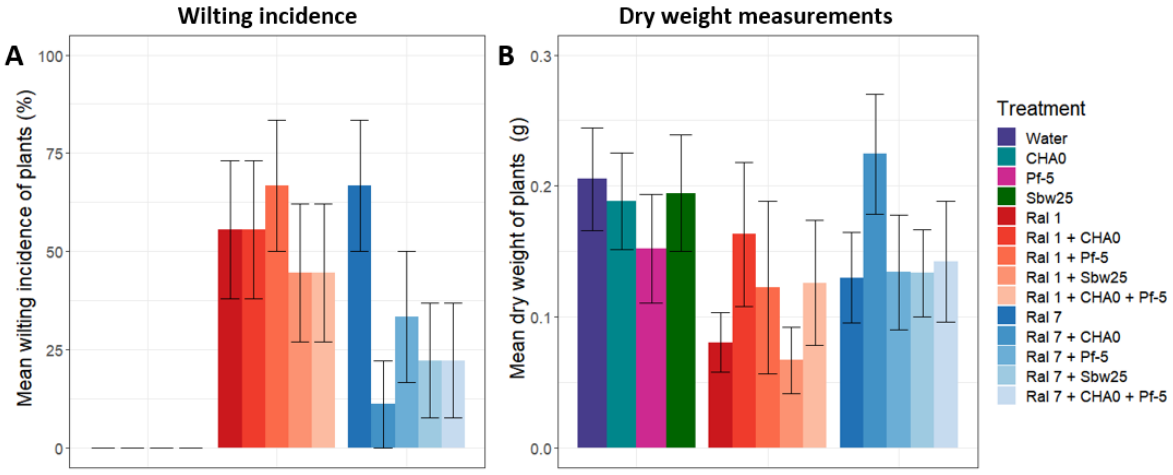

Figure 3.4.8 Tomato plant infections by *R. solanacearum* strains #1 and #7 in the absence and presence of *Pseudomonas* strains. Panel (A) displays the percentage of wilted plants (mean wilting incidence) in all 14 treatments and panel (B) shows the mean aboveground dry weight of individual plants at the end of the experiment. All error bars show the standard error of the mean (+/-1 SEM) based on 3 replicates (every replicate consisting of nine plants).

3.5 Discussion

3.5.1 Chapter Summary

The aims of this study were to screen and identify effective *Pseudomonas* bacterial biocontrol strains against the bacterial pathogen *Ralstonia solanacearum*, understand underlying mechanisms of inhibition, and to validate their efficacy *in vivo* in the tomato rhizosphere. *Pseudomonas* strain CHA0 was the most suppressive strain in both direct and indirect *R. solanacearum* inhibition assays. Comparative genomic analysis highlighted that metabolite clusters encoding DAPG, pyoluteorin and orfamides A and B were unique for the most suppressive CHA0 *Pseudomonas* strain. The effects of these metabolites were tested directly against a subset of *R. solanacearum* isolates and all of them were found to have negative effects on *R. solanacearum* growth with pyoluteorin being the most suppressive compound. *In vivo* tests revealed that CHA0 was effective at reducing bacterial wilt incidence, but to no greater extent than other *Pseudomonas* strains or their combinations. Moreover, reduction in disease incidence *in vivo* was only observed in the case of one of the two tested *R. solanacearum* strains. Despite successful identification of potential *Pseudomonas* biocontrol species, more work is needed to harness their biocontrol activity in the plant rhizosphere.

3.5.2 *In vitro* screening of *Pseudomonas* bacteria identified strain CHA0 as a highly antagonistic biocontrol candidate

Direct and indirect screening of biocontrol strains against pathogens *in vitro* is the first stage towards identifying potentially effective biocontrol agents (Fravel, 2005). Baehler *et al.* (2005) describe how *Pseudomonas* biocontrol strains can produce multiple metabolites with some being universally produced across Pseudomonads (such as DAPG), while others being more strain-specific, such as orfamides that have only been reported in CHA0 and Pf-5 (Ma *et al.*, 2016). When screening for the most effective *Pseudomonas* strains against *R. solanacearum* through direct and indirect *assays*, *P. protegens* strain CHA0 showed the most suppressive activity against all the tested *R. solanacearum* strains. This result is in line with previous studies demonstrating the high biocontrol activity of CHA0 against various plant pathogens (Hu *et al.*, 2016). Although multiple *Pseudomonas* strains have been described in the literature with biocontrol abilities, CHA0 is one of the most well-established, studied and successful biocontrol agents (Weller, 1988). Its biocontrol abilities have been tested at different temperatures (Humair *et al.*, 2009), against

multiple plant pathogens such as plant parasitic nematode pathogens (Imran A. Siddiqui and Shaukat, 2003; Siddiqui and Shaukat, 2004), fungal pathogens (Jamali *et al.*, 2009). It has also been considered for its insecticidal properties (Flury *et al.*, 2017) and showing antagonism towards protists (Jousset *et al.*, 2006) and *C. elegans* (Neidig *et al.*, 2011). In this study CHA0 also displayed a broad-spectrum efficacy by suppressing all *R. solanacearum* strains to a similar degree *in vitro*. Moreover, two *R. solanacearum* strains studied in more detail responded very similarly to the presence of four identified candidate metabolites in our lab assays. This suggests that *R. solanacearum* diversity had a smaller effect than *Pseudomonas* diversity had in the genotype-genotype interactions. One explanation for this is that all but one *R. solanacearum* isolates originated from a relatively small geographical area (the UK) and were thus likely similar to each other. In general, European *R. solanacearum* strains are thought to be very similar compared to the *R. solanacearum* species complex globally (Timms-Wilson, Bryant and Bailey, 2001) belonging to a clonal lineage of Phylotype 2b strains adapted to grow in cold climate (Hayward, 1991). In contrast, investigated *Pseudomonas* biocontrol strains included different species (e.g. *P. protegens* and *P. fluorescens*) originating from different countries across Europe and America. Hence, *Pseudomonas* strains were likely genetically more dissimilar compared to *R. solanacearum* strains. Genomic analysis of *R. solanacearum* strains would however be required to verify these hypotheses.

3.5.3 The inhibition of *R. solanacearum* by *Pseudomonas* was associated with the presence of certain secondary metabolite clusters with high inhibitory activity

Comparative genomic analyses revealed that *Pseudomonas* strains CHA0 and Pf-5 had the greatest number of secondary metabolic clusters with both strains possessing 17 clusters and the other six strains ranging from 11-15 metabolic clusters. Based on previous literature, CHA0 and Pf-5 can produce a similar range of secondary metabolites such as pyoluteorin and cyclic lipopeptides, which could potentially explain why CHA0 exhibited the greatest inhibitory effects amongst the *Pseudomonas* strains (Haas and Keel, 2003; Loper and Gross, 2007; Ma *et al.*, 2016). When testing these candidate metabolites on *R. solanacearum* growth, pyoluteorin stood out as the most suppressive compound leading to poor bacterial growth. Only *Pseudomonas* strains CHA0 and Pf-5 were found to harbour the T1PKS metabolic cluster, which encodes for pyoluteorin. CHA0 and Pf-5 strains also had the highest amount of NRPS clusters (8 and 7 respectively), which could be responsible for the production of a wide variety of metabolites (orfamide, putisolvin, pyochelin, salicyclate, lipopeptide, viscosin, syringopeptin, rhizomide,

delfibactin and pyoverdine) and could also have contributed to their biocontrol efficacy. However, the strain Pf-5 was less suppressive compared to CHA0 strain *in vitro* even though it encoded the same metabolic clusters. This suggests that, despite Pf-5 having the genes to produce pyoluteorin and other metabolites, these metabolic clusters were potentially not activated in the growth conditions used in the experiment as it has been well-established that growth media can significantly influence the production of secondary metabolites including antimicrobials (Jamali *et al.*, 2009; Heidari-Tajabadi *et al.*, 2011; Yang *et al.*, 2018). Alternatively, it is possible that the relatively high suppression of CHA0 was triggered by activity of another secondary metabolite cluster. However, we found no evidence for synergistic or additive effects between four tested candidate metabolites. To study the potential role of actual metabolites produced by *Pseudomonas* strains, we also isolated cyclic lipopeptides (CLPs) from strains CHA0 and F113, which could have shown variation in their efficacy, or potentially include additional antimicrobial compounds. We found that both CLP A and B isolated from CHA0 strain were highly inhibitory, while CLP A, but not B, isolated from F113 strain showed inhibitory effect. This leads us to speculate that the strain F113 could actually contain a cyclic lipopeptide secondary metabolite cluster or a different secondary metabolite, which has not been identified yet and contributed to the effects observed. In general, this result suggest that the actual metabolic diversity of *Pseudomonas* is likely to be much greater than predicted based on the presence of known secondary metabolite clusters, and that CHA0 genomes could potentially contain certain clusters that are not found in Pf-5 explaining the difference in their suppressiveness. Mass spectrometry analyses could potentially reveal further information on unidentified and novel secondary metabolites that could be isolated and tested in more detailed studies in the future such as novel cyclic lipopeptides which have been discovered recently (Geudens and Martins, 2018). Mass spectrometry can also reveal which clusters are being expressed in the experimental conditions of this study as metabolite production varies depending on the environment the bacteria are cultured in (Deveau *et al.*, 2016; Köhl, Kolnaar and Ravensberg, 2019).

3.5.4 Biocontrol activity of *Pseudomonas* strains in tomato model

It is important to validate *Pseudomonas* efficacy *in vivo*, because pathogen suppression observed *in vitro* might not robustly reflect the biocontrol activity in more natural greenhouse or field conditions (Weller, 1988). We first validated that both selected *R. solanacearum* strains were infective against the tomato plants leading to around 50% bacterial wilt disease incidence. Such variation is typical for *R. solanacearum* both in the field and greenhouse experiments as its

virulence is determined by a combination of host immunity and environmental conditions, that can vary considerably even in controlled greenhouse experiments (Hu *et al.*, 2016; Wei *et al.*, 2019). Surprisingly, no significant variation in pathogen suppression was observed between *Pseudomonas* biocontrol and the Sbw25 control strain lacking key secondary metabolite clusters. Similarly, using strains in combinations had no effect on their biocontrol efficacy. However, bacterial wilt incidence was clearly reduced in the presence of *Pseudomonas* strains in the case of *R. solanacearum* strain #7. These results are in contrast to the *in vitro* results that showed that CHA0 was the relatively more inhibitory strain compared to Pf-5 strain. Similarly, *R. solanacearum* strains #1 and #7 did not show differences in their response to DAPG, Pyoluteorin or CLPs produced by CHA0 strains, but clearly differed in their response to *Pseudomonas* strains in the tomato infection experiment.

These results reinforce the importance of acknowledging genotype-genotype (G x G) interactions for the biocontrol efficiency *in vivo*. This is an important factor as it shows different biocontrol strains are more effective than others, and this depends on which pathogen they are interacting with. A better understanding of this will result in a more tailored biocontrol response for greater control of a pathogen (Raaijmakers, Vlami and De Souza, 2002). Investigating these interactions in plant disease systems can also drive selection pressures and evolved resistance as those which are less controlled by a biocontrol will have a greater likelihood to overcome the suppression (Bose and Schulte, 2014). Considering the G x G interactions is not only being more widely used in plant disease systems but in human disease systems to detect genetic defects of complex diseases (Ferrario and König, 2018). While it is difficult to explain these results without conducting follow-up experiments, they suggest that pathogen-bacteria interactions are altered in the presence of plants (Milling, Babujee and Allen, 2011). It is possible that the relatively higher suppressiveness of *Ralstonia* strain #7 was driven by the combined effect of *Pseudomonas* inhibition and plant immune responses. Alternatively, it is possible that *Ralstonia* strain #1, was poorer at colonising tomato roots, failed to express genes required for protection against *Pseudomonas* inhibition (e.g. efflux pumps or other deactivation of antimicrobials) or was not able to activate virulence gene expression (Ran *et al.*, 2005). The lack of difference between *Pseudomonas* strains suggests that production of antimicrobial secondary metabolites was not necessarily the key mechanism of suppression in the rhizosphere. As a result, the potential role of resource competition (Wei, Yang, *et al.*, 2015; Yang *et al.*, 2016, 2017) and siderophores (Gu *et al.*, 2020a; Gu *et al.*, 2020b) in by these *Pseudomonas* strains should be investigated in the future.

Despite all *Pseudomonas* strains reducing disease incidence, some plants were not protected. This is in line with previous studies showing variation in *Pseudomonas* biocontrol efficiency (Hu *et al.*, 2016). It is known that *R. solanacearum* infections vary considerably in field conditions and this irregularity has been linked with variation in environmental conditions, interactions with rhizosphere microbiota and activation of plant immune systems (Xue *et al.*, 2013). All these factors could affect pathogen densities in the soil, which could further affect the likelihood of plant infection via knock-on effects on quorum-sensing controlled virulence gene expression (Szentes *et al.*, 2013). It is also possible that the conditions used in tomato experiments may not have been optimal for *R. solanacearum*, as the 'microtom' tomato species has very small root systems, which could make it harder for the *Pseudomonas* strains to colonise the plant roots and therefore provide protection against *R. solanacearum*. Moreover, autoclaving of the soil prior to *Pseudomonas* inoculation could have influenced soil properties having consequences for the growth and secondary metabolite production by *Pseudomonas* strains. For example, it has previously been reported that the zinc and iron composition in soil can aid the production of DAPG and HCN metabolites (Jamali *et al.*, 2009). Alternatively, autoclaving the soil may result in the release of a flush of nutrients into the soil which might affect microbial metabolism as it has been shown that heating soil to over 120 ^{o}C can result in an increased level of ammonium and nitrogen in the soil (Serrasolsas and Khanna, 1995). Together, these findings highlight the difficulties of translating *in vitro* results to *in vivo* applications, pinpointing the importance of studying biocontrol effects in semi-realistic *in vivo* conditions.

3.5.5 Conclusions and wider perspective

We have shown through an *in vitro* and *in vivo* screening process that *Pseudomonas* biocontrol of the *R. solanacearum* pathogen occurs in a genotype-genotype manner. The underlying mechanisms potentially related to this include pyoluteorin production as well as other metabolites (DAPG and orfamides). In contrast to previous studies, we did not find increased efficacy when using biocontrol strain combinations, perhaps because only two strains were used together, whereas it has been shown biocontrol efficacy can be vastly improved with eight *Pseudomonas* strains combined (Hu *et al.*, 2016) or when used in combination against fungal pathogens (De Vrieze *et al.*, 2018). This inconsistency may have been due to both biocontrol strains combined being very similar in their metabolic repertoire and thus not having additive effects, or it might be on strain could establist itself better in the rhizosphere. Additionally, not only may a high metabolic repertoire of combined *Pseudomonas* strains aid pathogen suppression and their ability to evolve resistance/tolerance, but it might also aid the *Pseudomonas* ability to

establish itself amongst native bacteria for example, siderophore production could allow them to improve their iron acquisition (Bose and Schulte, 2014; Hu *et al.*, 2016). To develop biocontrol research further, it is important to identify potentially novel secondary metabolite clusters using a combination of genome analysis to identify metabolic clusters, mass spectrometry to identify and quantify specific metabolites, and direct testing using chemical standards and CLP extracts such as we have highlighted to check whether these metabolites are being produced *in vitro* (Keel *et al.*, 1992; Yasmin *et al.*, 2017). As pyoluteorin was the most suppressive metabolite, perhaps screening for *Pseudomonas* biocontrol strains which produce this could be a more efficient way to screen for biocontrol strains in the future. Screening methods such as this have been done previously for instance screening for siderophore production when creating biocontrol agents against rice fungal pathogens (Chaiharn, Chunhaleuchanon and Lumyong, 2009). Biocontrol effects could also be studied in more realistic *in vivo* microbial communities where both pathogen and *Pseudomonas* species face interactions with other microbes. Soil transcriptomics or mass spectrometry could then be conducted to detect compounds and whether desired biocontrol genes are active. Together, these findings increase our understanding of potential underlying mechanisms of pathogen suppression and gives further ideas on how model biocontrol experiments can be optimized in the future. Another benefit of this study was developing an effective low-cost screening process *in vitro* for a number of biocontrol bacteria and pathogen strains highlighting the significance of genotype-genotype interactions for successful biocontrol outcomes.

Chapter 4 Plant pathogenic *Ralstonia solanacearum* bacterium can rapidly evolve tolerance to antimicrobials produced by *Pseudomonas* biocontrol bacteria

4.1 Abstract

Soil-borne plant pathogens are a significant threat to crop production. One way to improve the control of disease outbreaks without using agrochemicals is to use naturally occurring plant growth-promoting bacteria that can biocontrol pathogens via the production of antimicrobials. However, it is still unclear if pathogenic bacteria can evolve tolerance to the antimicrobials produced by biocontrol bacteria, which could constrain the long-term effectiveness of such strategies. Here we used an experimental evolution approach *in vitro* to investigate if pathogenic *Ralstonia solanacearum* bacterium —a causative agent of bacterial wilt — is able to evolve tolerance to antimicrobials produced by *Pseudomonas* bacteria. We also explored if these adaptations were specific to certain *R. solanacearum* and *Pseudomonas* combinations, and what were the underlying mechanisms behind tolerance evolution. We found that while all *R. solanacearum* strains could initially be inhibited by *Pseudomonas* bacteria, this effect became weaker over time. Direct fitness assays revealed that, while the majority of pathogen strains evolved high levels of tolerance to multiple *Pseudomonas* strains, some *Pseudomonas* strains retained their antimicrobial activity throughout the experiment. Mechanistically, tolerance evolution was linked with improved tolerance to orfamide lipopeptide and pyoluteorin antimicrobials secreted by certain *Pseudomonas* strains in our experimental conditions. Together, these results suggest that *R. solanacearum* can rapidly evolve more tolerance to *Pseudomonas* antimicrobials, which could potentially affect the variability and long-term efficacy of biocontrol outcomes in the field.

4.2 Introduction

The use of traditional chemical pesticides has declined in recent years due to cost, environmental toxicity and legislation (Chen *et al.*, 2016b). As a result, new methods are needed to control plant pathogens and to ensure food security in the face of expanding human population (Hayward, 1991; Kaczmarek *et al.*, 2014). Biocontrol is a means of using natural competitors, such as naturally existing microorganisms, to control the growth and damage caused by plant pathogens. One example of a potential biocontrol agent is naturally occurring soil-borne *Pseudomonas* plant growth promoting bacteria (PGPB) that can limit the growth of different plant pathogens including bacteria, fungi and nematodes (Pierson *et al.*, 1998) via resource and interference competition. While multiple *Pseudomonas* species with high biocontrol potential have been identified, it is still unclear how broadly they can inhibit the growth of different strains of one given pathogen species.

Understanding the range of *Pseudomonas* biocontrol agent activity is important because plant pathogen populations are often genetically highly variable even within agricultural fields (Xue *et al.*, 2013). This variation can be linked to pathogen susceptibility and tolerance to different biocontrol agents and therefore, considering such genotype-genotype interactions is key for developing successful biocontrol treatments. Biocontrol efficiency can also differ between different *Pseudomonas* strains, or genotypes, due to variation in the secondary metabolites they can produce (Becker *et al.*, 2012). Such differences could be seen, for example, in number of different secondary metabolites produced by different strains that can range from different antimicrobials to iron-scavenging siderophores. Some biocontrol characteristics can be more universal, such as the production of 2, 4-diacetylphloroglucinol (DAPG), which is an effective antimicrobial against bacterial, fungal and nematode plant pathogens (Cronin *et al.*, 1997; Compant *et al.*, 2005; Haas and Défago, 2005; Humair *et al.*, 2009). In contrast, other biocontrol characteristics are strain-specific such as the production of hydrogen cyanide (HCN) by *P. protegens* CHA0 strain (Haas and Keel, 2003), or the production of cyclic lipopeptide antibiotics by *P. protegens* CHA0 and Pf-5 strains, known to be active against multiple pathogens including bacteria and fungi (Nielsen *et al.*, 2002; Geudens and Martins, 2018). It is also possible that different strains produce varying amounts of given secondary metabolites due to differences in gene expression, which could further affect their biocontrol efficacy (Landa *et al.*, 2002).

Another important characteristic of a biocontrol agent is for it to remain effective over a long period of time without the risk of the pathogen evolving tolerance. This is especially a concern in the case of plant pathogenic bacteria, which have been shown to rapidly evolve in response to their growth environment in the lab (Riley *et al.*, 2001), or to the internal environment within their host plant (Guidot *et al.*, 2014). Similar to the evolution of antibiotic resistance in clinical settings, plant pathogenic bacteria could evolve resistant to different antimicrobial produced by biocontrol bacteria in the rhizosphere due to comparable selection – most clinical antibiotics originate from soil bacteria such as *Streptomyces* who are responsible for over two thirds of the clinical antibiotics including neomycin, cypemycin, grisemycin, bottromycins and chloramphenicol (Kieser *et al.*, 2000; Gillespie, 2002). It is thus possible that *Pseudomonas* biocontrol agents could potentially lose their efficacy in the long-term if pathogens are able to evolve more tolerant or resistant.

Here we used a model system consisting of the plant pathogenic bacterium *R. solanacearum* and eight different *Pseudomonas* plant growth-promoting biocontrol bacterial strains (Chapter 2, General Methods, Table 2.1). *Ralstonia solanacearum* is a globally spread quarantine pathogen which causes vascular wilt disease (Plant Health Directive, EU, 1995). It possesses numerous virulence mechanisms that enable the efficient infection of more than 200 plant species from 50 plant families (Hayward, 1991; Genin and Denny, 2012; Mansfield *et al.*, 2012; Nion and Toyota, 2015). *R. solanacearum* is highly variable genetically and this variation has previously been linked with its susceptibility to antimicrobials produced by different soil bacteria (Xue *et al.*, 2013). The eight *Pseudomonas* species selected for this study, have previously been shown to have a range of plant growth-promoting properties that can contribute to *R. solanacearum* pathogen suppression (Haas and Défago, 2005; Becker *et al.*, 2012; Hu *et al.*, 2016; Ma *et al.*, 2016). However, it is unclear if the efficacy of *Pseudomonas* strains varies depending on the *R. solanacearum* isolate identity (genotype), what are the underlying mechanisms of *R. solanacearum* inhibition, and if *R. solanacearum* can evolve more tolerant to *Pseudomonas* in the long-term. To experimentally study these questions, we exposed seven *R. solanacearum* strains to the supernatants of eight *Pseudomonas* species in pairwise combinations in a selection experiment lasting 21 days. The supernatants contained all potential secondary metabolites produced in the growth media and two concentrations were used (50% and 80%) to test the strength of selection by unidentified *Pseudomonas* antimicrobials. Evolved *R. solanacearum* populations were serially transferred into freshly prepared supernatants every 72 h and, bacterial densities were quantified and populations cryopreserved throughout the experiment. Following the selection experiment, fitness assays

were performed to explore if any strains had developed a tolerance to *Pseudomonas* supernatants. Moreover, a combination of comparative genetic analyses, mass spectrometry and microbiological assays were used to identify and test the inhibitory effects of candidate metabolites secreted by the most inhibitory *Pseudomonas* strains. It was found that all *R. solanacearum* populations were able to overcome *Pseudomonas* inhibition over time and the evolution of tolerance was verified in direct fitness assays. Based on genome mining, the antimicrobial activity by two inhibitory strains (Pf-5 and CHA0) was shown to be potentially mediated by DAPG, pyoluteorin and orfamides. The importance of orfamide and pyoluteorin tolerance was directly verified using additional fitness assays with chemical standards. Together, these findings suggest that the potential risks of pathogen tolerance evolution should be considered when developing *Pseudomonas* biocontrol applications.

4.3 Materials and methods

4.3.1 Bacterial strains and growth media

We used seven strains of *R. solanacearum* provided by NCPPB (National Collection of Plant Pathogenic Bacteria) and Fera Science Ltd (York, UK – bacterial strains described in Chapter 2, General Methods, Table 2.1). Six of the strains were isolated during annual river sampling surveys (2013-2015) from various locations in the UK, while the 7th strain was originally isolated in Poland. All these strains were found to be virulent in a tomato model at Fera Science Ltd. Eight fluorescent Pseudomonad strains (CHA0, Pf-5, Q2-87, Q8R1-96, 1M1-96, MVP1-4, F113, and Ph11C2) were used as *Pseudomonas* biocontrol bacteria (bacterial strains described in Chapter 2, General Methods, Table 2.1). Prior to experiments, bacterial starting cultures were prepared as follows: previously frozen samples were inoculated in 5 mL of LB broth (media recipes described in Chapter 2, General Methods, Table 2.2) and incubated with shaking at 200 rpm at 28 ^{o}C. *Pseudomonas* strains were grown for 24 h prior to experiments and *R. solanacearum* strains for 48 h (to give approximately 1.0x10^{6} CFU/mL). All strains were stored at -80 ^{o}C in 20% glycerol in LB media. These methods were carried out with every experiment unless stated otherwise.

4.3.2 The selection experiment and quantification of pathogen population density dynamics

To investigate pathogen tolerance evolution to *Pseudomonas* antimicrobials, we exposed each *R. solanacearum* strain to the supernatants of each *Pseudomonas* species in pairwise pathogen and *Pseudomonas* supernatant combinations in a 21 day long selection experiment.

The *Pseudomonas* supernatants included all secreted secondary metabolites produced when *Pseudomonas* strains were grown alone in LB media – a method that has previously been shown *Pseudomonas* antimicrobial activity against Chinese *R. solanacearum* strain (Hu *et al.*, 2016). *Pseudomonas* supernatants were prepared as follows. All *Pseudomonas* strains were first cultured individually in 20 mL of LB broth (Table 2) in 50 mL falcon tubes for 24 h at 28 ^{o}C with shaking at 200rpm and centrifuged for 10 minutes at 4000 *g*. The supernatant was then filtered using a 0.2 μm filter to separate bacterial cells and fragments from secondary metabolites present in the supernatants. We used flat bottomed 96-well plates with a maximum volume of 250 μL for the selection experiments and used two supernatant-LB dilutions for each *Pseudomonas* strain: low (1:1, i.e. 50%) and high (4:1, i.e. 80%). Controls included *R. solanacearum* grown in low (1:1, i.e. 50%) and high (4:1, i.e. 80%) LB broth diluted with sterile H_2O (LB broth: H_20). All supernatant

combinations were replicated four times and control treatments three times. At the start of the experiment, each supernatant and control combination was inoculated with 2 µL of each *R. solanacearum* strain (approximately $1.0x10^6$ cells mL^{-1}). Microplates were incubated without shaking (to enable natural population structure) at 28 °C for the whole duration of the experiment and bacterial densities measured every 72 h as optical density (OD 600 nm; Tecan Infinite spectrophotometer) after the first reading at 24 h time point. Subsamples of bacterial cultures were serially transferred to fresh supernatant and control treatments every three days. Before the transfer, bacterial cultures were gently mixed using a pipette, after 20 µL of mixed cultures were transferred to new wells with corresponding supernatant treatments. All populations were cryopreserved in 20% glycerol-LB media (media recipes described in Chapter 2, General Methods, Table 2.2) every second transfer (2^{nd}, 4^{th}, 6^{th} and 8^{th} transfer), and the last sample was used for fitness assays.

4.3.3 Fitness assays for determining evolution of antimicrobial tolerance

To quantify potential evolution of antimicrobial tolerance and its associated growth costs, the relative fitness of ancestral *R. solanacearum* strains were compared with the control ("LB-exposed") and evolved ("supernatant-exposed") *R. solanacearum* populations at the final time point of the selection experiment (21 days equalling approximately 300 *R. solanacearum* generations; estimated as 1 generation per 90 minutes). Ancestral and LB-evolved populations were grown in both the absence and presence of ancestral *Pseudomonas* supernatants and in LB broth dilutions without supernatants. The evolution of tolerance was only tested in the presence of *Pseudomonas* supernatant to which the specific pathogen strain had previously been exposed during the selection experiment. For example, if the pathogen strain had been exposed to *Pseudomonas* CHA0 strain, its resistance was quantified only in the presence of the ancestral *Pseudomonas* CHA0 supernatant. Similarly, tolerance was measured only in the same supernatant concentration populations had been exposed to during the selection experiment (low in low and high in high). Ancestral, supernatant-exposed and LB-exposed cryopopulations were regrown for 24 h prior to assays in 200 µL of 100% LB broth at 28 °C. *Pseudomonas* supernatants were derived by growing ancestral *Pseudomonas* strains in 20 mL of growth media in 50 mL falcon tubes to create low and high supernatant dilutions as described previously. Finally, the ancestral, LB-exposed control and supernatant-exposed evolved populations were inoculated into these wells using a stainless steel flame-sterilised cryoreplicator (~1-2 µL transfer volume, Boekel). Bacterial growth was recorded at 24 h intervals for 3 days (OD 600 nm; Tecan Sunrise spectrophotometer)

and tolerance estimated as the growth reduction of evolved *R. solanacearum* strains in *Pseudomonas* supernatant relative to the ancestral strains.

4.3.4 Investigating *Pseudomonas* secondary metabolite biosynthesis gene clusters based on genome information

The methods for this are in Chapter 2: General methods, Section 2.3.

4.3.5 Confirming the identity of specific *Pseudomonas* secondary metabolite presence in the bacterial supernatant through mass spectrometry

The methods for this are in Chapter 2: General methods, Section 2.4.

4.3.6 Determining *R. solanacearum* tolerance to identified *Pseudomonas* secondary metabolites experimentally

To directly test if the supernatant-exposed *R. solanacearum* populations had evolved tolerance to identified candidate antimicrobials, we measured the growth of ancestral and evolved bacteria isolated from the low supernatant concentration in the presence of single compounds using chemical standards. The details of stocks and suppliers can be found in Chapter 2 (General Methods, Section 2.5). To investigate tolerance evolution to DAPG, ancestral strains, supernatant-exposed and LB-exposed control populations were grown for 24 h at 28 ^{o}C in 100% LB broth and then inoculated (1x10^{6} CFU/mL) into LB broth at the following DAPG concentrations: 1000 µM, 500 µM, 100 µM, 50 µM and 0 µM (LB broth control). Bacterial densities were recorded as optical density at 0 h and 24, 48 and 72 h after inoculation with a spectrophotometer (OD 600 nm). Due to the relatively high price of other synthetic antimicrobials, only a subset of the *R. solanacearum* strains (#1 and #7) were used to test tolerance evolution to pyoluteorin and orfamides A and B. As these metabolites were predicted to be produced by *Pseudomonas* CHA0 and Pf-5 strains, we only tested the evolved *R. solanacearum* populations, which had been exposed to the supernatants of these two strains in 50% low supernatant concentration. All *R. solanacearum* populations were revived and grown as described previously and tolerance measured in 100 µM concentrations of pyoluteorin and each orfamide in 100% LB broth. Bacterial densities were recorded as optical density at 0 h and 24 h, 48 h and 72 h after inoculation with a spectrophotometer (OD 600 nm).

4.3.7 Statistical analyses

We used repeated measures ANOVA with between subject and random effects to analyse changes in pathogen densities during the selection experiment and with other datasets with temporal structure. Tukey contrasts with Bonferroni-corrected p-values were used for pairwise comparisons. In additional selection experiment analysis, datasets were simplified by quantifying the supernatant inhibition by subtracting OD values observed in the supernatant treatments from the LB-control treatments. Two-way ANOVA was used to analyse the mean differences between treatments at the final time point of the selection experiments, and for all fitness assay data, pairwise treatment comparisons were analysed using post-hoc Tukey tests with 95% confidence levels. All statistical tests were carried out using RStudio.v.3.4.4, for details on packages using see Chapter 2, General Methods, Section 2.7.

4.4 Results

4.4.1 *Ralstonia solanacearum* densities increased over time in the presence of *Pseudomonas* supernatants

We first explored the mean growth of all *R. solanacearum* strains in the presence of two supernatant concentrations averaged over all *Pseudomonas* strains during the selection experiment (Figure 4.4.1 A-B). *Ralstonia solanacearum* growth was lower in both *Pseudomonas* supernatant concentrations relative to the control treatments (*Treatment*: $F_{1, 488} = 142.752$ $P < 0.001$, Figure 4.4.1), and on average, *R. solanacearum* densities were lower in the high supernatant concentration (*Supernatant concentration*: $F_{1, 488} = 53.878$, $P < 0.001$, Figure 4.4.1 A-B). A similar result was observed when we analysed the pathogen growth dynamics as the growth reduction relative to control treatment: *R. solanacearum* populations exposed to the high supernatant concentration experienced a greater reduction in bacterial growth compared to the low supernatant concentration (*Supernatant concentration*: $F_{1, 446} = 143.66$, $P < 0.001$, Figure 4.4.1 A-B). The reduction of *R. solanacearum* growth changed over time during the selection experiment in the low supernatant concentration as pathogens reached similar densities to the control treatment at the end of the selection experiment (*effect of time within low supernatant concentration*: $F_{7, 1561} = 81.972$, $P < 0.001$, Figure 4.4.1 A). This was also the case in the high supernatant concentration, where the growth reduction in contrast increased over time during the experiment (*effect of time within high supernatant concentration*: $F_{7, 1561} = 18.31$, $P < 0.001$, Figure 4.4.1 B).

We next compared how the pathogen growth reduction was affected by *R. solanacearum* and *Pseudomonas* strains' identities (Figure 4.4.1 C-D). The growth reduction differed significantly between *Ralstonia* strains (*Ralstonia*: $F_{6, 441} = 78.601$, $P = P < 0.001$, Figure 4.4.1 C-D). Post hoc analyses revealed that strains #2 and #3 were reduced to a lesser extent relative to the other five strains. The growth reduction of *R. solanacearum* strains was not influenced by supernatant concentration (*Supernatant concentration*Ralstonia*: $F_{6, 434} = 0.693$, $P = 0.6554$, Figure 4.4.1 C-D). Similar to above, the overall growth reduction by *Pseudomonas strains* varied significantly during the experiment (*Pseudomonas*: $F_{7, 440} = 8.95$, $P < 0.001$, Figure 4.4.1 E-F) with the strain CHA0 being more suppressive compared to Q2-87, Q81R-96, F113 and Ph11C2 strains. The mean suppression by *Pseudomonas* strains was not affected by the supernatant concentration (*Supernatant concentration*Pseudomonas*: $F_{7, 432} = 0.3566$, $P = 0.9268$, Figure 4.4.1 E-F). Together these results suggest that pathogen growth suppression was relatively stronger in high

supernatant concentration and depended on both *R. solanacearum* and *Pseudomonas* strain identities.

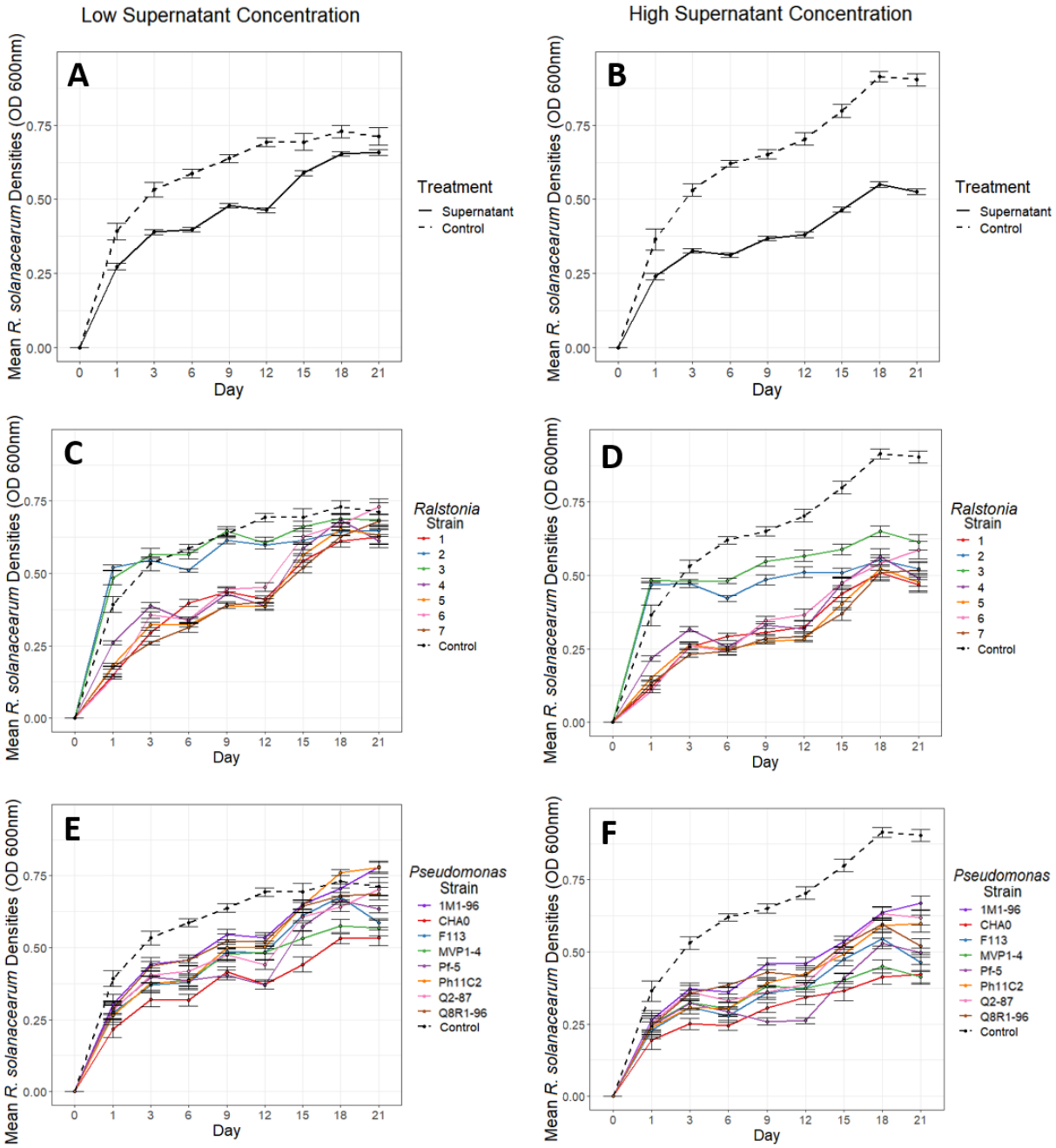

Figure 4.4.1 *R. solanacearum* population density dynamics in low (panels A, C and E) and high (panels B, D and F) *Pseudomonas* supernatant concentrations. Panels (C) and (D) show the effect of *R. solanacearum* strain variation (averaged over *Pseudomonas* strains), and panels (E) and (F) the effect of *Pseudomonas* strain variation (averaged over *R. solanacearum* strains) on *R. solanacearum* densities in low (C and E) and high supernatant (D and F) concentrations. The dashed black line in each panel denotes *R. solanacearum* density in the absence of *Pseudomonas* supernatants (control). All bars show the standard error of the mean (+/-1 SEM).

4.4.2 *Ralstonia solanacearum* strains evolved tolerance to *Pseudomonas* antimicrobials

Fitness assays were conducted after the selection experiment to compare the growth of ancestral, LB-exposed and supernatant-exposed *R. solanacearum* populations in LB broth and ancestral *Pseudomonas* supernatants (past supernatant exposure defined as 'Evolutionary history'). The growth densities of all *R. solanacearum* populations was significantly lower in the presence of *Pseudomonas* supernatant, as opposed to the LB broth (*Treatment*: $F_{1, 4478} = 700.3$, $P < 0.001$, Figure 4.4.2 A-B).

We then explored the general effect of evolutionary history by averaging our data over *R. solanacearum* and *Pseudomonas* strains and comparing the percentage growth reduction by supernatants relative to *R. solanacearum* growth in LB media without supernatants. The growth of all *R. solanacearum* populations was reduced irrespective of their evolutionary history when exposed to *Pseudomonas* supernatants (*identity effect for Ralstonia strain*: $F_{6, 2233} = 4.502$, $P = 0.00153$, Figure 4.4.2 A-B). The growth of ancestral *R. solanacearum* populations were reduced most clearly, followed by LB-exposed control and supernatant-exposed *R. solanacearum* populations, which were reduced the least (*Evolutionary history*: $F_{2, 1117} = 124.6$, $P = 0.00103$). Post hoc analyses showed the reduction of the LB-exposed and ancestral *R. solanacearum* populations was not significantly different ($P = 0.252$), but both ancestral (Tukey: $P < 0.05$) and LB-exposed control (Tukey: $P < 0.05$) populations differed significantly from the supernatant-exposed *R. solanacearum* populations. There is some evidence of facilitation in the control population as these were reduced to a lesser extent than the ancestral population, despite having no previous history of exposure to the *Pseudomonas* supernatant.

Overall, the reduction in *R. solanacearum* growth was greater in the high *Pseudomonas* supernatant concentration (*Supernatant concentration*: $F_{1, 2238} = 16.53$, $P < 0.0001$, Figure 4.4.2 B), which is in line with the population density dynamics observed during the selection experiment. However, this effect was mainly driven by ancestral strains whose growth was clearly reduced more by *Pseudomonas* supernatant in high concentrations (*Supernatant concentration*Evolutionary history*: $F_{2, 1117} = 124.6$, $P < 0.001$, Figure 4.4.2 B), whereas concentration effects were not observed in the case of LB-exposed control or supernatant-exposed evolved populations ($P > 0.05$). This indicates that *R. solanacearum* populations evolved more tolerance to *Pseudomonas* supernatants during the selection experiment and that this effect was similar in both supernatant concentrations.

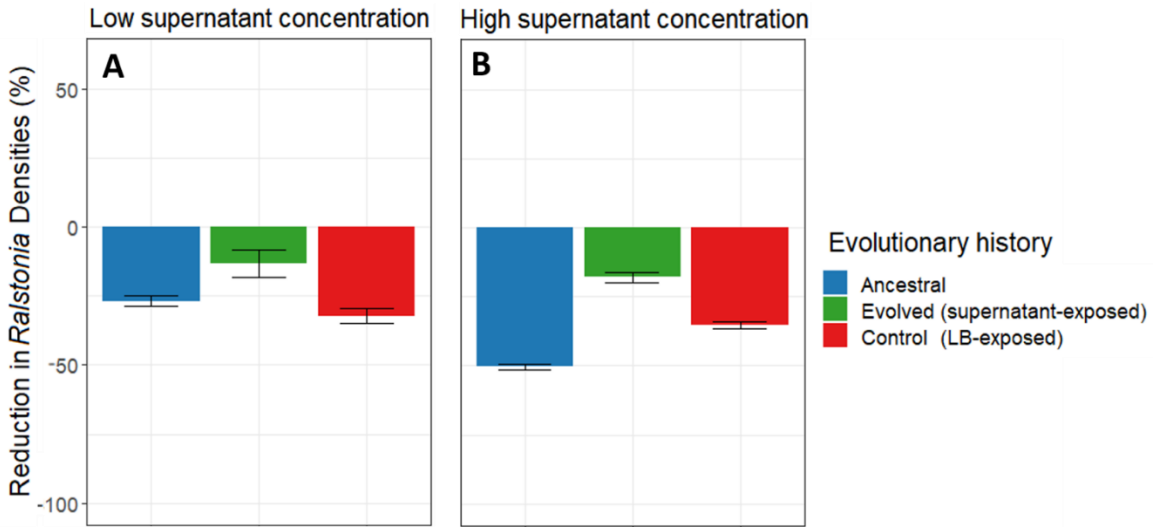

Figure 4.4.2 Evolution of tolerance in terms of pathogen growth reduction in the presence of *Pseudomonas* supernatants compared to supernatant-free growth media. Tolerance was measured only in the same supernatant concentration *R. solanacearum* strains had evolved during the selection experiment and only against the *Pseudomonas* strain they had previously been exposed to. Bar plots show the percentage reduction in density of Ancestral (blue), Evolved (green) and Control (red) *R. solanacearum* selection lines when grown in *Pseudomonas* supernatant compared to the control treatment with standard error of the mean (+/-1 SEM). Panel (A) and (B) show populations exposed to low and high supernatants, respectively. All data was averaged over *R. solanacearum* and *Pseudomonas* strains across two measurement time points during the fitness assays (24h and 72h).

4.4.3 The effect of *R. solanacearum* strain identity for the evolution of tolerance

We next explored the role of *R. solanacearum* strain identity on the tolerance evolution against *Pseudomonas* supernatants in both concentrations (Figure 4.4.3). The growth of all ancestral *Ralstonia* strains was reduced in the presence of *Pseudomonas* supernatant in general, but this effect varied between strains (*identity effect for Ralstonia*: $F_{6, 2233} = 4.502$, $P = 0.000153$, Figure 4.4.3). To explore this in more detail, we compared the responses of ancestral *Ralstonia* strains independently in low and high supernatant concentrations.

In the case of the ancestral *R. solanacearum* strains, it was found that the pathogen growth reduction was greater in the high *Pseudomonas* supernatant concentration (*identity effect for ancestral Ralstonia in high supernatant concentration*: $F_{1, 670} = 114.2$, $P < 0.001$, Figure 4.4.3 A+D). More variability was observed in the high supernatant concentration with the growth densities of strains #5 and #6 being reduced the most and strain #7 the least (*identity effect for ancestral Ralstonia* : $F_{6, 329} = 39.41$, $P < 0.001$, Figure 4.4.3 D). No variation was observed amongst ancestral strains grown in the low supernatant concentration (*identity effect for ancestral Ralstonia*: $F_{6, 329} = 1.938$, $P = 0.0742$, Figure 4.4.3 A).

In the case of LB-exposed control populations, no *R. solanacearum* strain variation was observed in low (*identity effect for control Ralstonia*: $F_{6, 329} = 3073$, $P = 0.338$, Figure 4.4.2 C) or high (*identity effect for control Ralstonia*: $F_{6, 329} = 1109.6$, $P = 0.0614$, Figure 4.4.2 F) supernatant concentrations and all strains showed similar levels of tolerance as the ancestral populations.

In contrast, supernatant-exposed evolved populations showed a higher degree of strain-specific tolerance evolution (*identity effect for evolved Ralstonia*: $F_{6, 889} = 3.105$, $P = 0.00514$, Figure 4.4.3 B+E). While no clear differences were found in the low supernatant concentration (*identity effect for evolved Ralstonia:* $F_{6, 441} = 0.651$, $P = 0.689$, Figure 4.4.3 B), strains #2 and #6 showed higher levels of tolerance in the high supernatant concentration (*identity effect for evolved Ralstonia*: $F_{6, 441} = 15.36$, $P < 0.0001$, Figure 4.4.3 E). Together, these results suggest that the identity of *R. solanacearum* strains had only a minor effect on the tolerance evolution.

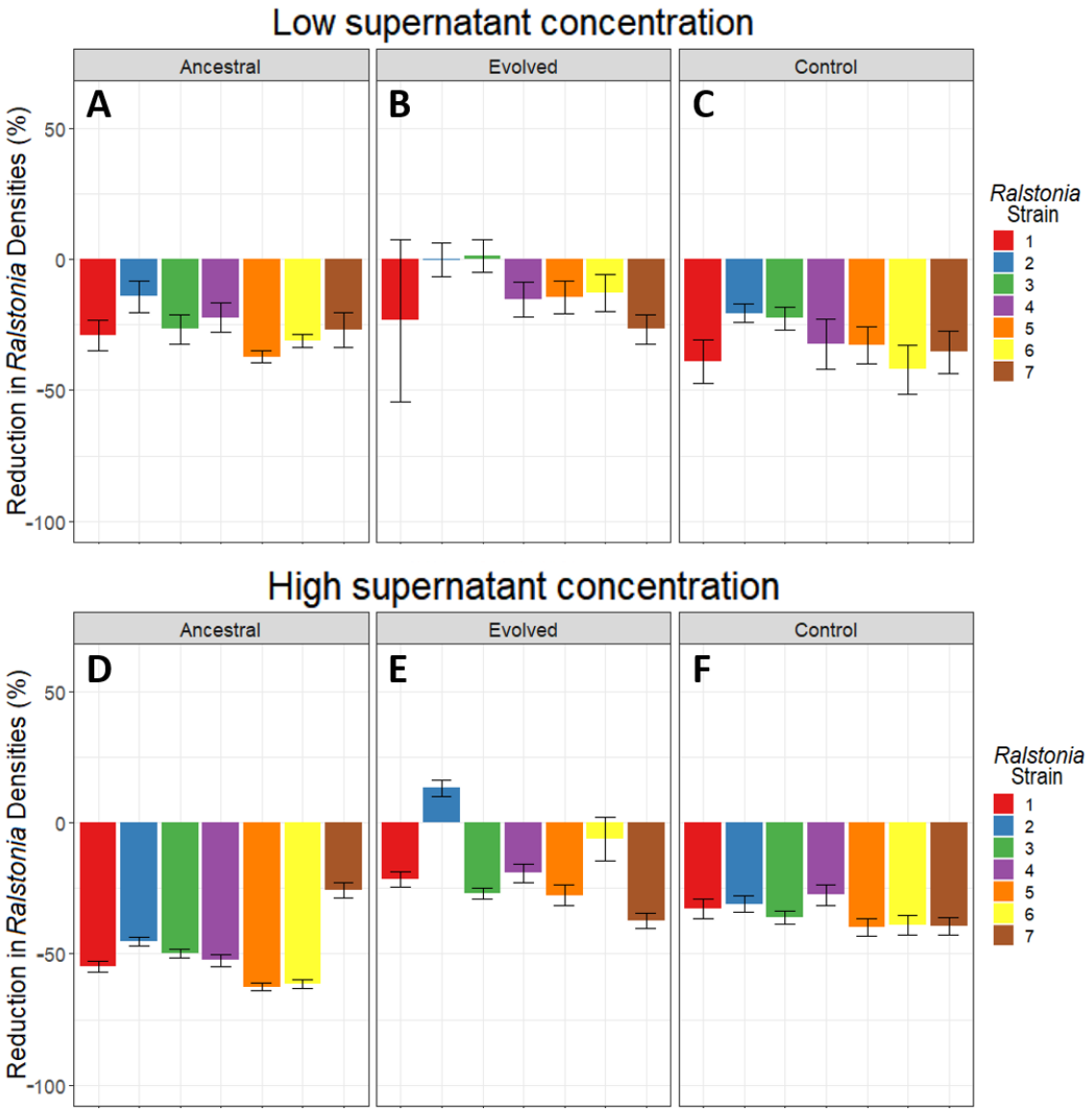

Figure 4.4.3 The significance of *R. solanacearum* strain identity for the evolution of tolerance. Tolerance was measured as pathogen growth reduction in the presence of *Pseudomonas* supernatants compared to supernatant-free growth media. Only the same supernatant concentration and *Pseudomonas* strains *R. solanacearum* strains had been exposed to during the selection experiment were used for the analysis. Bar plots show the percentage reduction in density of Ancestral (A and D), supernatant-exposed Evolved (B and E) and LB- exposed Control (C and F) *R. solanacearum* selection lines when grown in *Pseudomonas* supernatant compared to the control treatment with standard error of the mean (+/-1 SEM). Panels (A-C) and (D-F) show populations exposed to low and high supernatants, respectively. All data was averaged over *Pseudomonas* strains across two measurement time points during the fitness assays (24h and 72h).

4.4.4 The effect of *Pseudomonas* strain identity for the evolution of tolerance

We next analysed if *R. solanacearum* tolerance evolution was affected by the *Pseudomonas* strain identity to which it had been exposed during the selection experiment (Figure 4.4.4). *Pseudomonas* variation influenced *Ralstonia* growth reduction in general (*identity effect for*

Pseudomonas: $F_{6, 2233}$ = 18.2, P < 0.001 Figure 4.4.4). While all *Pseudomonas* strains were equally effective at suppressing Ancestral populations at the low supernatant concentration (*identity effect for Pseudomonas:* $F_{7, 328}$ = 0.81, P = 0.58, Figure 4.4.4 A), *Pseudomonas* strain MVP1-4 showed higher growth reduction relative to other *Pseudomonas* strains in the high supernatant concentration (*identity effect for Pseudomonas:* $F_{7, 328}$ = 3.424, P = 0.00151, Figure 4.4.4 D).

In the case of LB-exposed control populations, variability was observed in the low supernatant concentration (*identity effect for Pseudomonas:* $F_{7, 328}$ = 21.31, P < 0.001, Figure 4.4.4 C) with post hoc analyses revealing a clear increase in tolerance to Ph11C2 and CHA0 strains, while *Ralstonia* strains remained highly susceptible to MVP1-4, F113 and Q8R1-96 strains. This was also the case in the high *Pseudomonas* supernatant concentrations (*identity effect for Pseudomonas:* $F_{7, 328}$ = 30.97, P < 0.001, Figure 4.4.4 F), with post hoc analyses highlighting the relatively high susceptibility to MVP1-4, Pf-5 and Ph11C2 strains and less clear tolerance evolution to Ph11C2 and CHA0 strains.

Similar to above, supernatant-exposed evolved populations showed high variability in their tolerance evolution to different *Pseudomonas* strains (*identity effect for Pseudomonas:* $F_{7, 888}$ = 6.941, P < 0.001, Figure 4.4.4 B+E), and this variation was greater at low supernatant concentration (*supernatant concentration*Pseudomonas:* $F_{7, 440}$ = 8.393, P < 0.001, Figure 4.4.4 B). Specifically, *R. solanacearum* evolved increased tolerance to Ph11C2, Q2-87, CHA0 and Pf-5 strains, but remained susceptible to MVP1-4, 1M1-96 and Q8R1-96 strains in low supernatant concentration (*identity effect for Pseudomonas:* $F_{7, 440}$ = 8.393, P < 0.001, Figure 4.4.4 B). Qualitatively similar patterns were observed in high supernatant concentration (*identity effect for Pseudomonas:* $F_{7, 440}$ = 5.181, P < 0.001, Figure 4.4.4 E), but the level of tolerance evolution was lower in general. Together, these results show that tolerance evolution was clearly affected by the *Pseudomonas* strain identity being generally weakest against MVP1-4 strain and strongest against Ph11C2, CHA0, Pf-5 and Q2-87 strains.

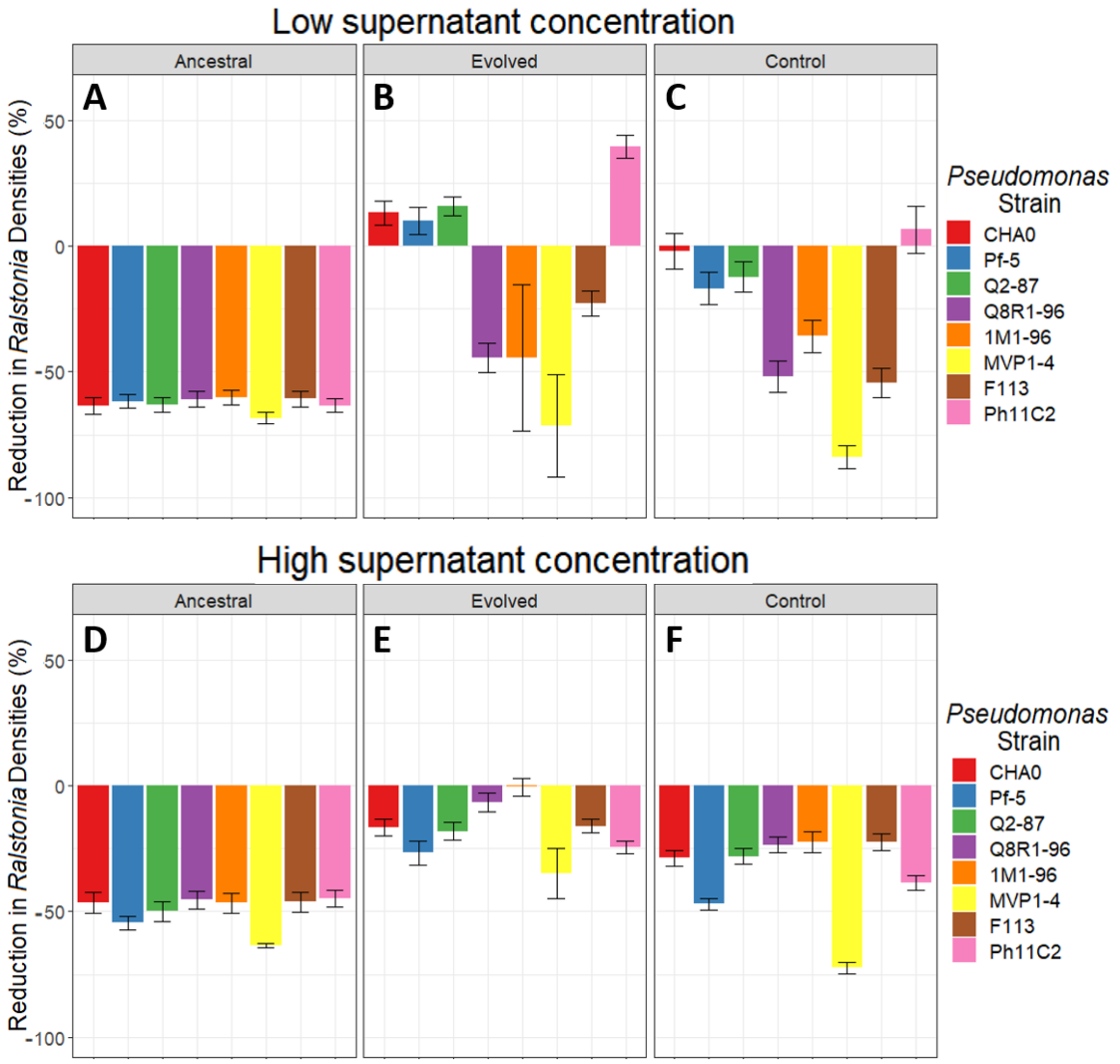

Figure 4.4.4 The significance of *Pseudomonas* strain identity for the evolution of tolerance. Tolerance was measured as pathogen growth reduction in the presence of *Pseudomonas* supernatants compared to supernatant-free growth media. Only the same supernatant concentration of *Pseudomonas* strains which *R. solanacearum* strains had been exposed to during the selection experiment were used. Bar plots show the percentage reduction in density of Ancestral (A and D), Evolved (B and E) and Control (C and F) *R. solanacearum* selection lines when grown in *Pseudomonas* supernatant compared to the control treatment with standard error of the mean (+/-1 SEM). Panels (A-C) and (D-F) show populations exposed to low and high supernatants, respectively. All data was averaged over *R. solanacearum* strains across two measurement time points during the fitness assays (24h and 72h).

4.4.5 Comparative analysis of *Pseudomonas* genomes reveals variation in the presence and type of secondary metabolic clusters among strains

The results of comparative genomic analyses can be found in Chapter 3, Section 4.3.

4.4.6 Confirming the production of *Pseudomonas* secondary metabolites in LB using mass spectrometry

Our genome screening results suggested that biocontrol activity may have been due to the presence of DAPG which was found in all genomes or certain less common compounds, such as pyoluteorin and orfamides that were identified only in CHA0 and Pf-5 strains. Due to the presence of these two unique secondary metabolite clusters, CHA0 and Pf-5 strains were chosen for more detailed study to mechanistically understand *R. solanacearum* tolerance evolution against specific secondary metabolites. To verify the production of these compounds in LB media, the supernatant produced by CHA0 and Pf-5 grown in the same experimental conditions as those used in our assays were analysed using mass spectrometry. Analysis was conducted in two ways: (1) by comparing peaks potentially corresponding to antimicrobial compounds against existing compound databases and (2) investigating the mass spectra against four chemical standards: DAPG, Pyoluteorin, Orfamide A and Orfamide B. Detailed methods describing this analysis can be found in (Chapter 2, General methods, 2.4).

The number of observed metabolites that matched with Knapsack database of 104 *Pseudomonas*-related metabolites is shown in Appendix S.1. The overall number of identified metabolites was higher with *Pseudomonas* CHA0 strain than Pf-5 - 72 and 70 metabolites identified, respectively (Figure 4.4.5). A number of these metabolites were found only in very low concentrations and were discarded as they could not be distinguished from blank media-only control reads (yellow bars, Figure 4.4.5). However, the production of some metabolites accounted for over 15% of the entire sample (red and blue bars, Figure 4.4.5) which shows that some of the unidentified metabolites were produced at very high levels. Based on matching monoisotopic masses, a list of putative metabolites including pyochelin, rhizoxin and syringomycin A present in the *Pseudomonas* supernatants was determined using Progenesis QI v.2.0 (Waters) analysis (Table 4.1). Of the four metabolites tested which we had validated standards for, it was found that only orfamide A production by CHA0 strain was detected in LB supernatant with an average concentration of 7.5 mg/mL (Figure 4.4.6). In contrast, none of the four candidate compounds were detected in Pf-5 supernatant in LB media. These results suggest a number of speculative secondary metabolites were produced by the *Pseudomonas* strains when cultured in LB media as ~70 unidentified metabolites were accounted for in the samples, but only the production of orfamide A was confirmed.

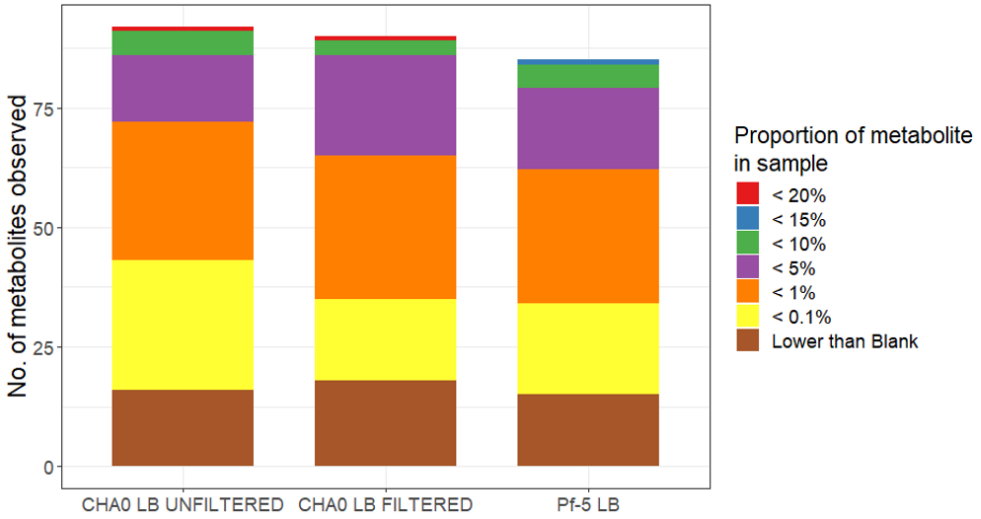

Figure 4.4.5 Mass spectrometry analyses identifying potential *Pseudomonas* CHA0 and Pf-5 secondary metabolites produced when grown in LB broth. The different colour bars show the no. of metabolites in the mass spectra of *Pseudomonas* supernatants that matched with compounds present in *Pseudomonas* Knapsack database and the percentage of the sample (as determined by peak area) it accounted for. It also contains a bar representing unfiltered *Pseudomonas* supernatant as described in Chapter 2, General Methods, Section 2.5.

Table 4.1 Putative *Pseudomonas* metabolites tentatively identified using Progenesis software

Candidate compound	Metabolite function	Present in CHA0 supernatant	Present in Pf-5 supernatant
Indole-3-acetic acid	Auxin	YES	YES
Pyochelin	Siderophore	YES	
Rhizoxin	Interferes with mitosis by binding to *Beta*-tubulin affecting microtubule dynamics	YES	YES
Pseudophomin B	Cyclic lipodepsipeptide – forms ion channels, promotes ion fluxes and causes cell necrosis	YES	YES
Syringomycin A	Lipodepsinonapeptide phytotoxin – pore forming toxin also causing ion fluxes	YES	

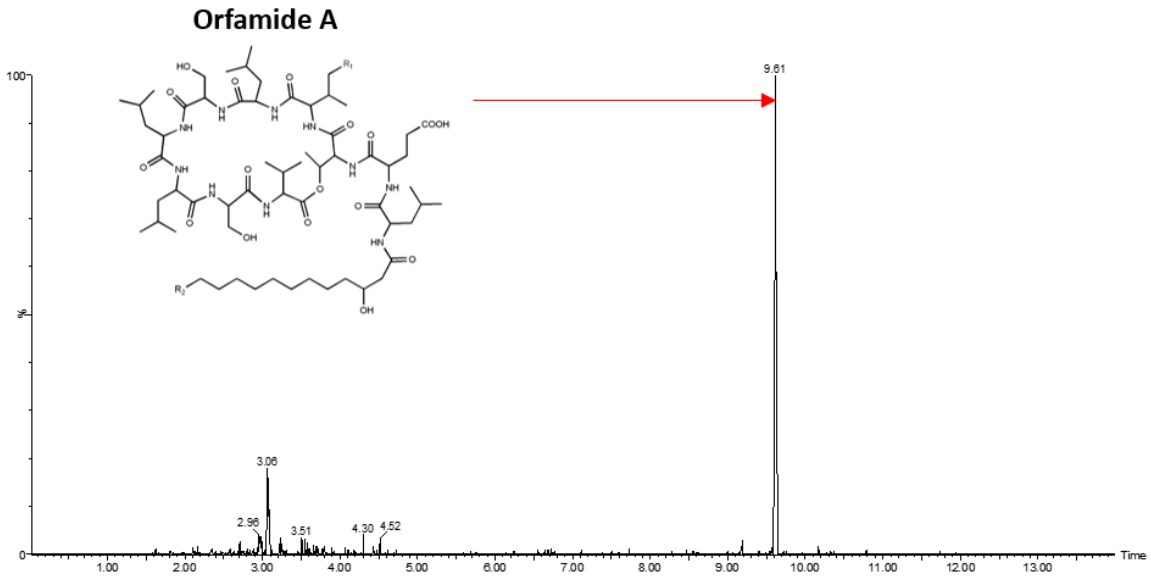

Figure 4.4.6 Mass spectrometry analyses indicating the presence of *Pseudomonas* metabolite orfamide A in the supernatant of strain CHA0. Orfamide A is identified with a monoisotopic mass (m/z) of 1317.826 [M+Na]+ and a retention time (t_R) of 9.61 seconds at 15 ppm. This spectrum represents the replicate with the strongest peak out of the three replicates.

4.4.6 The evolutionary history influenced *R. solanacearum* ability to grow in the presence of DAPG, Orfamides A and B and Pyoluteorin antimicrobials

To test the potential role of four candidate secondary metabolites produced by *Pseudomonas* (DAPG, orfamides A and B and Pyoluteorin) for *R. solanacearum* tolerance evolution, we compared the growth of ancestral and evolved bacterial populations in the presence of commercially available chemical standards. Only the evolved populations originating from the low supernatant concentration were used in these analyses, and while the effects of DAPG were tested with all *R. solanacearum* strains in multiple concentrations, only a subset of *Ralstonia* strains were used to analyse the effects of other candidate compounds. Tolerance to DAPG was explored by averaging data over *R. solanacearum* and *Pseudomonas* strains and comparing the percentage growth reduction by DAPG relative to *R. solanacearum* growth in LB media (more detailed growth trajectories for each *Ralstonia* strain over time can be found in Figure 4.4.9).

It was found that DAPG suppressed all *R. solanacearum* populations in a concentration-dependent manner, with the largest growth reduction observed in the two highest concentrations: 1000 µM and 500 µM (*Concentration*: $F_{3, 4644} = 2234$, $P < 0.001$, Figure 4.4.7). The growth reduction was also affected by the evolutionary history and this effect varied along with DAPG concentrations (*Evolutionary history*Concentration*: $F_{6, 4636} = 71.96$, $P < 0.001$, Figure 4.4.7). Interestingly, in the two lowest DAPG concentrations (50 µM and 100 µM), the growth of both LB-

exposed control and supernatant-exposed evolved populations were reduced relatively more compared to ancestral population, which indicates that these populations evolved increased susceptibility to DAPG (*Evolutionary history in 50 µM DAPG*: $F_{2, 1117} = 80.33$, $P < 0.001$, Figure 4.4.6; *Evolutionary history in 100 µM DAPG*: $F_{2, 1117} = 95.83$, $P < 0.0001$, Figure 4.4.6). While no difference between LB-exposed control and supernatant-exposed evolved populations was found at 50 µM ($P = 0.717$) and 100 µM ($P = 0.380$) DAPG concentrations, the supernatant-exposed evolved populations were slightly less reduced than the LB-exposed control population ($P = 0.024$) at 500 µM DAPG concentration, which may be indicative of tolerance to relatively higher levels of DAPG.

We next explored if the *R. solanacearum* and *Pseudomonas* strain identity affected the DAPG tolerance. *Ralstonia* strains did not differ in their innate tolerance to DAPG, (*identity effect for ancestral Ralstonia*: $F_{6, 1506} = 1.598$, $P = 0.144$, Appendix Figure 4.4.8 A). The growth reduction of LB-exposed control populations was slightly affected by *R. solanacearum* strain identity, with strains #6 and #4 being the most susceptible (*identity effect for control Ralstonia*: $F_{6, 1337} = 9.98$, $P < 0.001$, Figure 4.4.8 A). In the case of supernatant-exposed evolved *R. solanacearum* strains, more variation was found (*strain identity for evolved Ralstonia*: $F_{6, 1785} = 12.22$, $P < 0.001$, Figure 4.4.8 A). Specifically, the growth of strains #2, #3 and #7 was most reduced by DAPG (unable to grow to the highest densities), while strains #1, #4, #5 and #6 were the least susceptible to DAPG. The *Pseudomonas* supernatant species identity also had an effect on *Ralstonia* growth reduction with those populations that had been exposed to MVP1-4 experiencing the smallest growth reduction (*strain identity of Pseudomonas strain*: $F_{7, 1784} = 2.409$, $P < 0.001$, Figure 4.4.8 B).

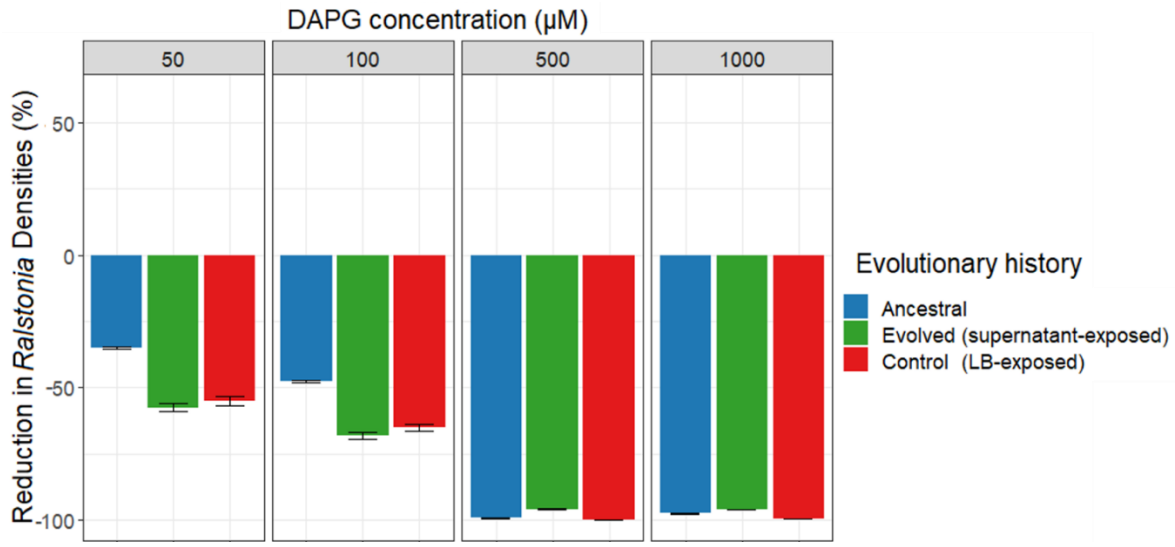

Figure 4.4.7 Evolution of tolerance in terms of pathogen growth reduction in the presence of synthesised DAPG compared to DAPG-free growth media (LB). Bar plots show the percentage reduction in density of Ancestral (blue), Evolved (green) and Control (red) *R. solanacearum* selection lines when grown in four DAPG concentrations (1000 µM, 500 µM, 100 µM and 50 µM compared to the control treatment grown in the absence of DAPG with standard error of the mean (+/-1 SEM). All data was averaged over *R. solanacearum* and *Pseudomonas* strains across two measurement time points during the fitness assays (24h and 72h).

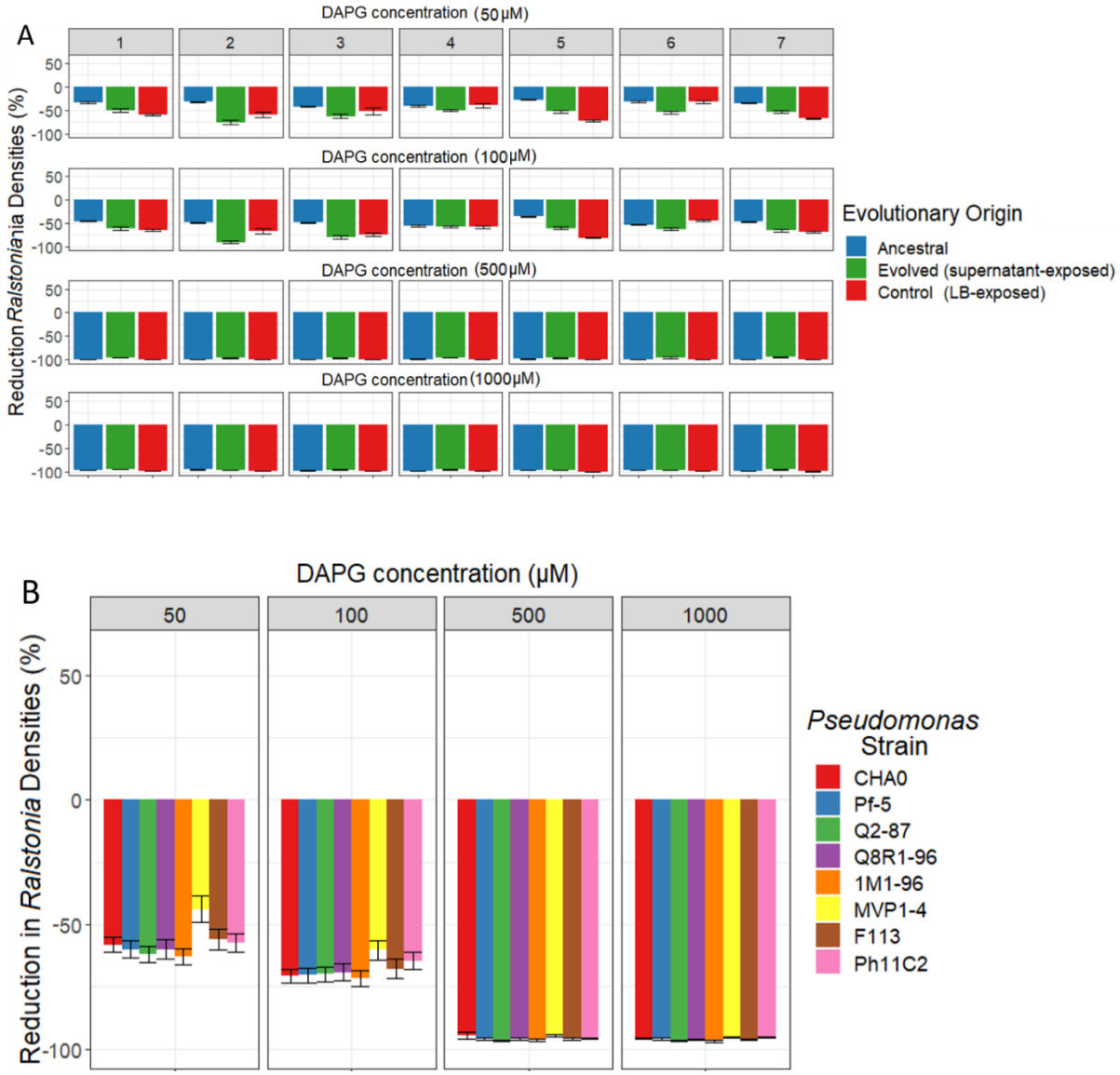

Figure 4.4.8 Evolution of tolerance in terms of pathogen growth reduction in the presence of synthesised DAPG an antimicrobial of *Pseudomonas* compared to antimicrobial-free growth media. Panel (A) shows the percentage reduction in density of Ancestral (blue), Evolved (green) and Control (red) *R. solanacearum* selection lines when grown in four DAPG concentrations (1000 µM, 500 µM, 100 µM and 50 µM compared to the control treatment. The data was averaged over *Pseudomonas* strains with standard error of the mean (+/-1 SEM). Panel (B) displays the data of the supernatant-exposed Evolved populations averaged over the *Ralstonia* strains. All data was averaged across two measurement time points during the fitness assays (24h and 72h).

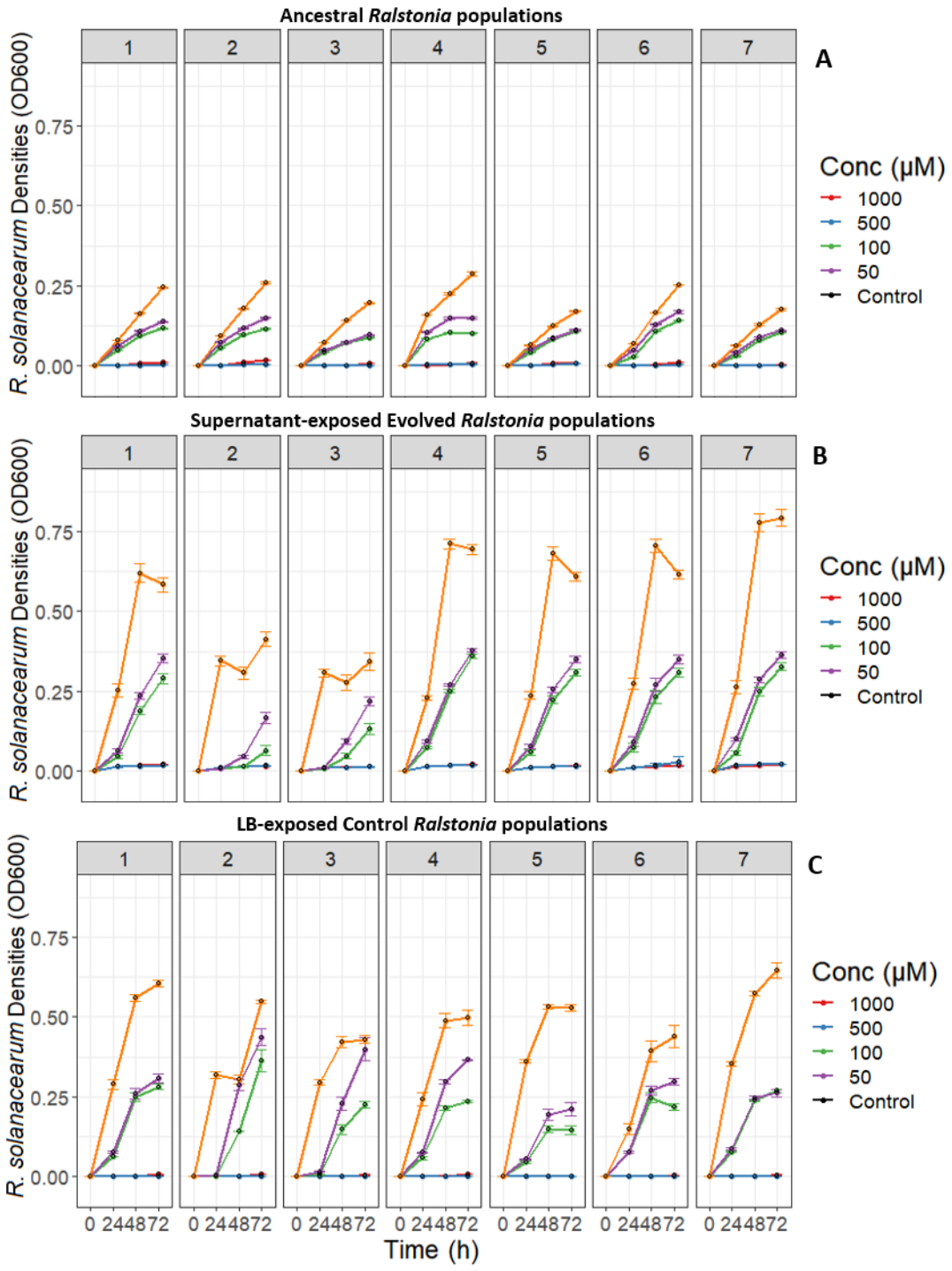

Figure 4.4.9 Evolution of tolerance in terms of comparing Ancestral and supernatant-exposed Evolved and Control *Ralstonia* populations in varying concentrations of DAPG. The line graphs show the ability of Ancestral (A) and Evolved (B) and Control (C) *Ralstonia* selection lines to grow in the presence of various DAPG concentrations (1000 µM – red, 500 µm – blue, 100 µm – green, 50 µm – purple and 0 µm LB broth (control) – orange) over time. The data in panel B was averaged over the 8 *Pseudomonas* strains.

Only a subset of *R. solanacearum* populations were used for analysing the inhibitory effects of orfamides and pyoluteorin. In the case of orfamide tolerance, only the *R. solanacearum* strain #1 populations, which had been exposed to *Pseudomonas* CHA0 and Pf-5 supernatants in the selection experiment (in addition to ancestral and LB-exposed control populations of the same strain) were used. As the effect of *Pseudomonas* strain identity was non-significant (*Pseudomonas:* $F_{1, 32} = 0.261$, $P = 0.6128$, Figure 4.4.10 A-B), growth data was pooled over this factor. It was found that *R. solanacearum* growth did not vary between orfamide A and orfamide B showing that the two metabolites had similar effects on the pathogen growth (*Pseudomonas:* $F_{1, 32} = 1.72$, $P = 0.199$, Figure 4.4.10 A-B). However, the evolutionary history influenced *R. solanacearum* growth trajectories (*Evolutionary* history*:* $F_{2, 31} = 59.9079$, $P < 0.001$ Figure 4.4.10 A) with supernatant-exposed evolved populations reaching greater densities compared to LB-exposed control ($P < 0.001$) or ancestral ($P < 0.001$) populations when grown in 100 µM orfamide concentrations (A and B with similar effects). Post hoc analyses also revealed there was no difference in *R. solanacearum* growth between the ancestral and LB-exposed control populations, suggesting these populations showed similar susceptibility to orfamides.

In the case of pyoluteorin, *R. solanacearum* strains #1 and #7 were selected for tolerance analysis at 100 µM pyoluteorin concentration. These populations had also been exposed to *Pseudomonas* CHA0 and Pf-5 supernatants in the selection experiment, and similarly to orfamide assays above, growth data was pooled over *Pseudomonas* strains due to non-significant effect (*Pseudomonas:* $F_{1, 32} = 0.0211$, $P = 0.8854$, Figure 4.4.10 C). It was found that *R. solanacearum* growth did not vary between the two *Ralstonia* strains tested (*identity effect for Ralstonia:* $F_{1, 32} = 0.370$, $P = 0.546$, Figure 4.4.10 C). However, the evolutionary history had a clear effect on pyoluteorin tolerance (*Evolutionary history:* $F_{2, 31} = 149.39$, $P < 0.001$ Figure 4.4.10 C) with LB-exposed control and supernatant-exposed evolved and populations both reaching relatively higher densities compared to ancestral strains (no difference was found between LB-exposed control and supernatant-exposed evolved populations, $P > 0.05$). Together these results suggest that *R. solanacearum* tolerance evolution was linked to improved growth in the presence of orfamides and pyoluteorin antimicrobials, although it has only been confirmed that orfamide A is present in the supernatant of *Pseudomonas* strain CHA0.

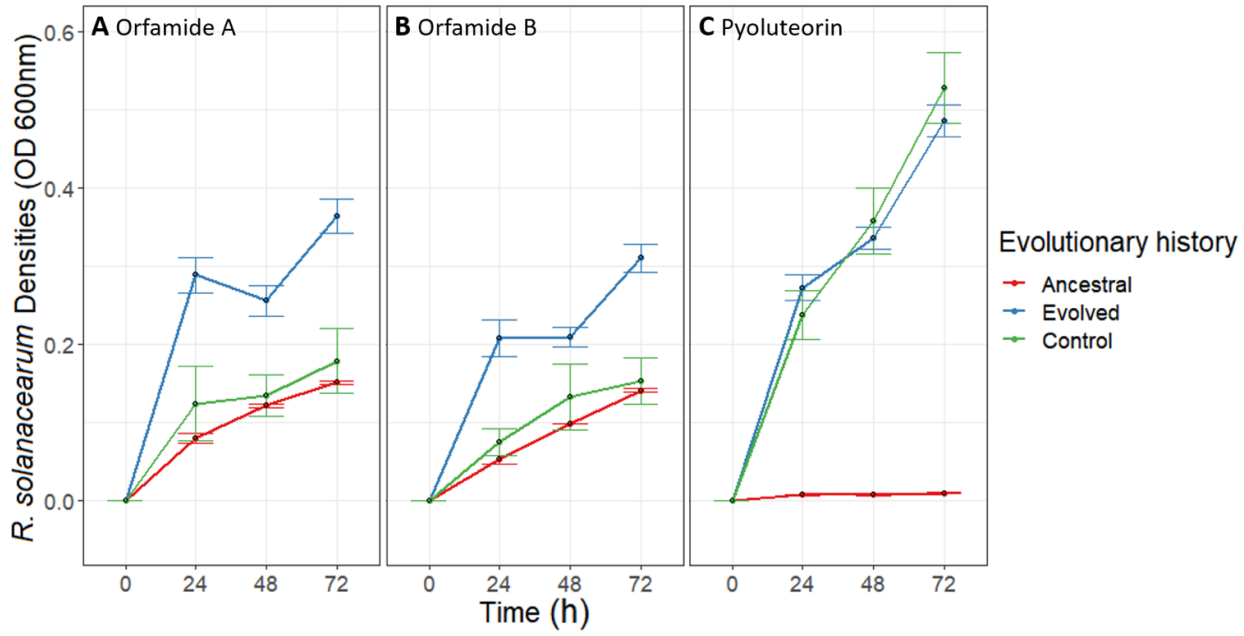

Figure 4.4.10 Evolution of tolerance in terms of comparing the growth of ancestral LB-exposed control and supernatant-exposed evolved *R. solanacearum* populations in 100 µM of orfamide A (A), orfamide B (B) and pyoluteorin (C) concentrations. The line graphs show the ability of Ancestral (red line) and Evolved (blue line) and Control (green line) *R. solanacearum* selection lines to grow in the presence of selected metabolites over time. The evolved selection lines in each panel are averaged over the two *Pseudomonas* strains (CHA0 and Pf-5), and in panel C, each line is additionally averaged over two *R. solanacearum* strains (1 and 7).

4.5 Discussion

4.5.1 Chapter Overview

The aims of this study were to investigate if the bacterial plant pathogen *R. solanacearum* was able to develop tolerance to antimicrobials produced by *Pseudomonas* biocontrol bacterial agents, if this is dependent on the *R. solanacearum* and *Pseudomonas* strain identities and to unveil some of the potential underlying mechanisms. We found that all *Pseudomonas* strains were initially able to suppress pathogen growth, with some strains causing greater suppression than the others. Comparisons between ancestral, LB-exposed control and supernatant-exposed evolved populations revealed potential pathogen tolerance *to Pseudomonas* antimicrobials. While tolerance evolution was little affected by the *R. solanacearum* strain identity, it was more variable depending on the *Pseudomonas* strain identity with MVP1-4 strain showing consistent growth suppression with no clear evidence for tolerance evolution. Orfamides and pyoluteorin antimicrobials were linked to tolerance evolution with evolved populations growing to higher densities in the presence of these, and orfamide A was found to be produced by CHA0 strain in the experimental growth conditions. Together these results suggest that the evolution of tolerance to orfamides could potentially reduce the long-term efficiency of *Pseudomonas*-based biocontrol.

4.5.2 Pathogen population dynamics under *Pseudomonas* antimicrobial selection

In general, our results showed that all eight of our *Pseudomonas* strains had the ability to suppress all seven *R. solanacearum* isolates. These effects were mainly bacteriostatic constraining *R. solanacearum* growth in a concentration-dependent manner. Similar results have been previously reported by (Raza *et al.*, 2016a) who conducted agar and soil assays with one *Pseudomonas* strain and one *R. solanacearum* strain pair revealing similar concentration-dependent suppressive effects of antimicrobial volatile organic compounds. We found that MVP1-4 and CHA0 were the most suppressive strains against *R. solanacearum* growth. This is in line with previous research as it is one of the most well documented and highly effective *Pseudomonas* PGPB based on the literature (Haas and Keel, 2003; Jousset *et al.*, 2006; Hu *et al.*, 2016). Interestingly, strain MVP1-4 was also an effective strain. As the suppressiveness and secretion of MVP1-4 *Pseudomonas* antimicrobials is unclear, further investigation is required and suggests there are still secondary metabolites to discover. Alternatively, it might be the expression of

known metabolites produced by MVP1-4 is greater than that of other strains, which could be confirmed with mass spectrometry (Yasmin *et al.*, 2017). In contrast, CHA0 and Pf-5 are the most well studied *Pseudomonas* strains, which could also partly explain their relatively largest metabolic repertoire reported (Haas and Keel, 2003; Gabriel *et al*., 2006). Some *Pseudomonas* metabolites such as DAPG are universally produced by these bacterial species (Haas and Défago, 2005), which could explain why all the *Pseudomonas* strains used in this study showed pathogen suppression, however DAPG expression was not observed directly. In contrast, the high suppressiveness of certain *Pseudomonas* strains was likely linked to unique secondary metabolite clusters such as orfamides and pyoluteorin in case of strain CHA0. By considering the one-sided evolution in this study with a focus on the pathogen (*R. solanacearum*) growth dynamics in *Pseudomonas* supernatant, we are not observing direct *Pseudomonas* biocontrol effects. It is known that when bacteria are directly interacting, bacterial warfare occurs which might activate alternative biocontrol mechanisms only when in the presence of *R. solanacearum* (Granato, Meiller-Legrand and Foster, 2019).

Despite being relatively smaller, variation was observed in *R. solanacearum* strains' growth during the selection experiment. For example, *R. solanacearum* strain #6 grew to the highest densities by the end of the selection experiment, which suggests that it had the greatest innate tolerance to the *Pseudomonas* antimicrobials – a result, which was also confirmed in separate fitness assays. It was also found that *R. solanacearum* strains isolated from the same geographical location followed the same growth dynamics, leading us to speculate that they might be genetically closely related. It must be noted that *R. solanacearum* strains used in this study all belong to the same bacterial species mostly isolated from one small geographical area (UK), whereas *Pseudomonas* biocontrol strains included *Pseudomonas protegens* and *Pseudomonas fluorescens* species. As a result, *Pseudomonas* strains were likely more distantly related compared to *R. solanacearum* strains, which could partly explain why they had more varied effects.

4.5.3 The evolution of tolerance depended on the interactions between *R. solanacearum* and *Pseudomonas* genotypes

Acknowledging the evolutionary dynamics between the pathogen and its biocontrol agents is important, because pathogen tolerance evolution could weaken biocontrol efficacy in the long-term for example over several crop seasons as reported by Ramesh *et al.* (2009). We found that

the ancestral and LB-exposed control *R. solanacearum* populations were more susceptible to *Pseudomonas* antimicrobials compared to the supernatant-exposed evolved *R. solanacearum* strains. This indicates that continued antimicrobial exposure can select for tolerance to *Pseudomonas* antimicrobials in just weeks in laboratory conditions. We can define this as evolved tolerance as the supernatant exposure has remained the same and not been diminished, yet the evolved *Ralstonia* populations are able to grow without the fitness costs observed to the ancestral population (Roy and Kirchner, 2000). Levin-Reisman *et al.* (2017) investigated the relationship between tolerance and resistance further to report that tolerance preceded resistance and gave rise to the evolution of resistance, showing these interactions can influence the evolutionary history of a pathogen, such as *R. solanacearum* to *Pseudomonas* antimicrobials. As no extinctions were observed in any *R. solanacearum* populations over the selection experiment, our results suggest that *Pseudomonas* supernatants had only bacteriostatic effects, or alternatively, consistent production by directly interacting bacteria would have been required for more effective killing (Fravel, 1988). Bacterial tolerance to antimicrobials has also been described in human bacterial pathogen interactions such as between *Staphylococcus aureus* and *Pseudomonas aeruginosa* – both opportunistic human pathogens which exist in polymicrobial chronic cystic fibrosis infections (Michelsen *et al.*, 2014).

Tolerance could evolve via horizontal gene transfer, which has previously been reported amongst *R. solanacearum* strains (Guidot *et al.*, 2009; Shehreen *et al.*, 2019). However, as our selection lines were started from clonal *R. solanacearum* populations, it is more likely that the tolerance evolution was driven by positive selection for *de novo* resistance mutations. Such rapid evolution has been shown to take place previously in *R. solanacearum* evolution within plants (Guidot *et al.*, 2014). This study experimentally evolved *R. solanacearum* on three "original" and "distant" plant hosts for 300 generations. Guidot *et al*. (2014) found the pathogen increased fitness on all hosts over time and through genome sequencing detected 1-3 independent parallel mutations that contributed to increased fitness. In the future, a subset of evolved clones could be sequenced to identify potential parallel mutations linked with elevated antimicrobial resistance in our evolved populations.

Surprisingly, some LB-exposed control populations showed increased levels of tolerance compared to ancestral strains despite having never been exposed to antimicrobials during the selection experiment, which can also be described as facilitation (Bronstein, 2009; Zélé *et al.*, 2018). One explanation for this is that the negative effects of antimicrobials were potentially

compensated for by the LB-exposed pathogen adapting to grow better in our experimental conditions (LB broth). Alternatively, it could be an example of cross feeding where the pathogen is enable to utilise products also present in the supernatant other than the secondary metabolites, which enhances its ability to grow in the supernatant (Bronstein, 2009). It has been well-documented that *Escherichia coli* adaptation to growth medium alone can occur spontaneously over time and result in large variations on the fitness of a population (Blount, Borland and Lenski, 2008; Tenaillon *et al.*, 2016). Similarly, it has been shown that *P. fluorescens* Sbw25 strain rapidly adapts in KB growth media during laboratory experimental evolution (Scanlan *et al.*, 2015). Riley *et al.* (2001) conducted a selection experiment on 18 populations of *R. solanacearum* strain for 1000 generations in minimal media. Their key results revealed diversity was mostly driven in phenotypic traits rather than competitive fitness, and strong phenotypic *R. solanacearum* divergence in the absence of strong selection pressure, which is similar to our evolutionary result observed in the LB-exposed control populations. It has also been shown previously that antibiotic resistance can evolve even in the absence of antibiotics by pleiotropic effects in the presence of specific growth media (Knöppel, Näsvall and Andersson, 2017) and such correlated evolutionary outcomes could also partly explain our results. The increased population densities of the evolved *R.solanacearum* pathogen population compared to that of the ancestral and control populations in our own study infer evidence of the pathogen becoming more virulent. Bacterial wilting effects are density-dependent and thus with increasing pathogen load, this is likely to increase wilting symptoms *in vivo*. One way to investigate this further could be to conduct *in vivo* host plant fitness assays comparing the wilting incidences caused by ancestral and evolved *R. solanacearum* populations. It is suspected the evolved populations could cause greater wilting incidences.

4.5.4 *R. solanacearum* strains evolved tolerance to specific *Pseudomonas* secondary metabolites

Tolerance evolution was most clear with evolved *R. solanacearum* populations that reached considerably higher densities than both the LB-exposed control and ancestral *R. solanacearum* populations when re-exposed to supernatants in the fitness assays. Similar effects were also observed in direct metabolite assays with the subset of *R. solanacearum* strains. In the case of DAPG, we only saw evidence of tolerance at 500 µM concentration, where evolved populations grew slightly better compared to ancestral and LB-exposed populations. In contrast, both LB-exposed and evolved supernatant-exposed populations did worse than ancestral strains in 50 µM

and 100 µM concentrations indicative of reduced DAPG tolerance. One explanation for this is that different mechanisms were responsible for the tolerance to different secondary metabolites, and as no DAPG production was observed in LB media, selection could have favoured loss of this trait, especially if it was costly to maintain. Alternatively, DAPG tolerance could have been traded-off with the tolerance to orfamides potentially due to antagonistic pleiotropy. DAPG has previously been shown to have concentration-dependent effects also on the algal plant pathogen, *Pythium ultimum* (De Souza *et al.*, 2003). Similar to DAPG, other *Pseudomonas* secondary metabolites (massetolide A, viscosin, syringomycin lipopeptides and syringopeptins) have also been shown to have concentration-dependent effects on fungal pathogens *Phytophthora infestans* and *Agaricus bysporus* (Tran *et al.*, 2007; D'aes *et al.*, 2010; Arseneault and Filion, 2017). One potential concentration-dependent resistance mechanism in *R. solanacearum* could be linked to pumping out antimicrobials using efflux pumps or degradation of antimicrobials via production of degrading enzymes (Brown, Swanson and Allen, 2007).

Mass spectrometry results only detected orfamide A in the supernatant of *Pseudomonas* strain CHA0, indicative of its importance for tolerance evolution. In line with this, the supernatant-exposed evolved populations had the greatest tolerance to orfamides A and B compared to the ancestral and LB-exposed control populations. This result highlights the specific importance of orfamides in explaining the observed tolerance evolution. Orfamides A and B are known to be associated with the same operon which could explain the similar fitness assay results in the case of both orfamides (Ma *et al.*, 2016). The tolerance by *R. solanacearum* strains to the orfamides could be explained via multiple mutually nonexclusive resistance mechanisms such as degradation of antibiotics, molecular changes in the binding target of the antibiotic or increased activity of efflux pumps. It has been suggested *R. solanacearum* uses multidrug efflux pumps as a form of protection against plant host defences (Brown, Swanson and Allen, 2007). Polymyxin B is an antimicrobial peptide produced by soil bacteria *Paenibacillus polymyxa* which bacterial pathogens such as *Burkholderia* spp. - very closely related to *R. solanacearum* - have developed resistance to (Loutet and Valvano, 2011; Hsu *et al.*, 2017). *R. solanacearum* species are almost always resistant to polymyxin B and it is used as one important component of *R. solanacearum* selective plates (Elphinstone *et al.*, 1996). As orfamides are also lipopeptides, it might be their defence mechanisms are similar to that of polymyxin B.

In the case of pyoluteorin assays, no difference was observed between the control and evolved populations even though they both were clearly more tolerant compared to the ancestral

strains. This suggests, that this adaptation could have been driven by media adaptation or some other unknown factors not related to *Pseudomonas* supernatant selection. Pyoluteorin is a chlorinated polyketide antibiotic and its production is regulated by the DAPG precursor, monopacetylphloroglucinol (Kidarsa *et al.*, 2011). The closely regulated biosynthetic gene clusters of DAPG and pyoluteorin are thus thought to have a close evolutionary history (Kidarsa *et al.*, 2011). Interestingly, we saw clear decreases in DAPG tolerance of LB-exposed control and supernatant-exposed populations, which was however correlated by improved growth in the presence of pyoluteorin indicative of trade-off between the tolerance to these two compounds. This pattern is difficult to explain without further detailed experiments and better understanding of the link between antimicrobial tolerance and primary (growth) metabolism, which could be achieved for example via transcriptomics studies. Genome resequencing could also be used to identify potential mutations and mechanisms underlying orfamide and pyoluteorin tolerance and DAPG susceptibility.

4.5.5 Suggestions towards more effective long-term pathogen biocontrol

Results from this study have provided insights into the need for research into pathogen and biocontrol genotype-genotype interactions at the evolutionary timescale to develop more robust biocontrol agents. Investigating the potential of a pathogen to evolve tolerance or resistance to a biocontrol agent is important for increasing the long-term efficacy of pathogen biocontrol. An optimal biocontrol agent would be one that exerts a broad range of efficacy against different, potentially co-occurring pathogen species and multiple different genotypes within each pathogen species. There is a clear need to identify biocontrol agents that do not drive strong resistance evolution on pathogens. While it might be difficult to identify generalist *Pseudomonas* strains that suppress multiple strains of pathogens over a long period of time, the antimicrobial activity range of biocontrol applications could be broadened by using combinations of biocontrol agents that show varying efficacy against different pathogen species and genotypes as demonstrated by Hu *et al*. (2016). Additionally, high biocontrol agent diversity could potentially aid the *Pseudomonas* to establish and colonise the rhizosphere for example via elevated siderophore production and improved iron acquisition (Hu *et al.*, 2016). One novel future area of research would be to investigate the combinatory effects on an evolutionary timescale. For example, it has previously been shown that phage and bacterial biocontrol agents can be more effective in combination compared to when applied alone due evolutionary trade-offs that can constrain the emergence of generalist resistance evolution (Wang *et al.*, 2017). For example, using the strain MVP-14 in

combination with CHA0 *Pseudomonas* strain might be an effective way to constrain *R. solanacearum* growth over longer time periods as overall only very weak tolerance evolution against MVP1-4 was observed. Moreover, *Pseudomonas* species could be also combined with *R. solanacearum*-specific phages or highly suppressive *Bacillus* bacteria to improve their efficacy and biocontrol efficiency range (Wang *et al.*, 2017). Lastly, it is not clear if similar evolutionary outcomes would take place in more natural environments in the soil or the plant rhizosphere. Studying the tolerance and resistance evolution of pathogens in the natural environment is thus required to develop a greater understanding of the role of rapid evolution for biocontrol outcomes. For example, Landa *et al.* (2002) investigated the long-term colonization ability and secondary metabolism production of various *Pseudomonas* strains in the pea plant rhizosphere over eight months. They noted clear differences in long-term colonization success between biocontrol strains, which could have resulted from evolutionary changes. Moreover, *R. solanacearum* has been shown to evolve in response to selection by bacteriophages or antibiotics produced by *B. amyloliquefaciens* bacterium in the tomato rhizosphere previously (Wang *et al.*, 2017, 2019). Considering long term effects *in vivo* is therefore a crucial step to understand whether the biocontrol can be successful at long-term pathogen suppression.

4.5.6 Final conclusions

Our research has shown that *R. solanacearum* strains were able to evolve tolerance to the supernatants (containing antimicrobials) of our *Pseudomonas* biocontrol strains within a 21-day time period. The observed tolerance was most clear to the orfamide antimicrobials produced by two of the strains whereas strains such as MVP1-4 remained the most 'tolerance-proof' throughout the study. In the future, considering combinations of *Pseudomonas* and other microbial strains might be a potentially useful avenue for reducing the likelihood of tolerance evolution (Wang *et al.*, 2017, 2019). Studying resistance evolution in a more representative field conditions, as well as looking at biocontrol effects against multiple pathogens which can also occupy the same rhizospheric environments as *R. solanacearum*, would be interesting future avenues of research.

Chapter 5 Screening and identification of effective *Pseudomonas* biocontrol strains against plant parasitic nematodes and testing their efficacy *in vitro* and *in vivo*

5.1 Abstract

Soil-borne pathogens, such as plant parasitic nematodes (PPNs), are a significant threat to crop production. One way to control PPNs using a more environmentally friendly approach than agrochemical applications is to use naturally occurring plant growth promoting bacteria that can suppress nematodes via production of secondary metabolites, which include 2,4-diacetylphloroglucinol (DAPG), pyoluteorin and orfamide lipopeptides to name a few. We investigated the inhibition efficiency of eight rhizospheric *Pseudomonas* strains on the *Caenorhabditis elegans* N2 wild-type model *in vitro* and on two PPNs, *Globodera pallida* (potato cyst nematode) and *Meloidogyne incognita* (root knot nematode), both *in vitro* and *in vivo* with tomato. *In vitro* lab experiments revealed that direct exposure to *Pseudomonas* strains imposed strong mortality on *C. elegans*, with CHA0 and Pf-5 *Pseudomonas protegens* strains displaying the strongest inhibition. Direct *Pseudomonas* exposure also increased PPN mortality. Genome mining highlighted several metabolites of interest and mass spectrometry was used to confirm that these bacteria constitutively excreted DAPG to the growth media which we experimentally identified to *C. elegans* mortality. Further experiments revealed that pyoluteorin and orfamides secondary metabolites synthesised by CHA0 and Pf-5 strains had clear effects on *G. pallida* while DAPG had less of an impact. *M. incognita* was not affected by these secondary metabolites. Lastly, we tested the biocontrol potential of CHA0 and Pf-5 strains in the tomato rhizosphere *in vivo* on both *M. incognita* and *G. pallida*. We found only weak biocontrol trends suggesting that inhibitory effects observed *in vitro* could not be produced *in vivo*. Together, these results suggest that *Pseudomonas* strains show promise for secondary metabolite-mediated biocontrol activity against nematodes via production of DAPG, pyoluteorin and orfamides. However, additional research is needed to understand how these bacterial functions are regulated and activated in the plant rhizosphere to translate these findings into agricultural context.

5.2 Introduction

Plant parasitic nematodes (PPNs) are microscopic non-segmented worms, the majority of which act as endoparasites by invading plant roots and feeding from plant cell contents (Williamson and Hussey, 1996; Cronin *et al.*, 1997; Okulewicz, 2017). The Heteroderidae nematode family causes the greatest economic damage worldwide and comprise of two main nematode groups; cyst (inc. potato cyst nematodes, *Globodera* spp.; PCN) and root-knot nematodes (*Meloidogyne* spp.; RKN) (Williamson and Hussey, 1996; Eves-Van Den Akker *et al.*, 2016). Once established in a field, cyst nematodes are especially difficult to eradicate as their cysts can remain viable for up to two decades. The US Department of Agriculture (USDA) have classified yellow PCN as potentially more dangerous than any insect or disease affecting the potato industry (Aphis USDA 12/09/2015) and it is also included on the European plant pathogen quarantine lists (Jones *et al.*, 2013).

Control of PPN infections is generally conducted through integrated pest management (IPM) which involves crop rotation, the use of chemical nematicides and resistant plant cultivars (Urwin *et al.*, 2001; Robin and Marchand, 2019). However, this management strategy is facing new challenges as the integral part — the use of chemical nematicides — is on the decline due to their toxicity to the environment. Because of this, the costs of controlling nematodes are expected to double without alternatives (Ngala *et al.*, 2015). One potential new solution is biocontrol, a method of controlling pathogens or pests using natural competitors such as microorganisms which have the potential to play an important role in agriculture (Weller, 1988; Nagachandrabose, 2020; Topalović, Hussain and Heuer, 2020).

One example of a potentially useful group of biocontrol agents is naturally occurring soil-borne plant growth promoting bacteria (PGPB), such as *Pseudomonas* species that have broad range of activity against different plant pathogens including bacteria, fungi and nematodes (Pierson *et al.*, 1998; Siddiqui, Haas and Heeb, 2005; Nagachandrabose, 2020). Pathogen suppression by bacteria is often thought to be mediated via secretion of antimicrobial secondary metabolites. Some *Pseudomonas* PGPB biocontrol characteristics are found universally within the species including the secretion of the secondary metabolite 2,4-diacetylphloroglucinol (DAPG), which has been shown to influence egg hatching and juvenile mortality in *Meloidogyne javanica* and *Globodera rostochiensis* nematodes as well as free-living nematodes including *C. elegans* (Cronin *et al.*, 1997; Imran A. Siddiqui and Shaukat, 2003; Compant *et al.*, 2005; Haas and Défago, 2005; Neidig *et al.*,

2011). Other *Pseudomonas* metabolites are more strain-specific such as antimicrobial cyclic lipopeptides known as "orfamides", which are produced by *Pseudomonas protegens* strains CHA0 and Pf-5 (Loper and Gross, 2007; Ma *et al.*, 2016). These compounds are also known to have effects on non-bacterial plant pathogens through inhibition of fungal hyphae growth (Raaijmakers *et al.*, 2010; Ma *et al.*, 2016). Orfamides have been suggested to have the potential to influence PPNs as anthelmintics but this has not been directly investigated thus far (Greenberg, 2014).

Development of a biocontrol agent begins in the laboratory with screening and identifying desirable biocontrol traits *in vitro* (Handelsman and Stabb, 1996; Fravel, 2005). Screening for bacterial biocontrol agents against PPNs can include exposing them to bacterial cultures or supernatants and monitoring effects on PPN survival, or behaviours such as development, growth rate, locomotion, feeding and egg laying and hatching (Siddiqui and Shaukat, 2003). Discovery of potential desirable traits *in vitro* does not, however, ensure that biocontrol agents will work *in vivo,* which is a common issue in biocontrol research. Nutrient availability and competition with other well-established rhizosphere bacteria can constrain biocontrol agent colonization and survival in the rhizosphere, while secondary metabolites can be quickly degraded or adsorbed preventing efficient disease suppression (Weller, 2007). Therefore, combining *in vitro* screening with *in vivo* validation of the biocontrol agent effectiveness is a vital step for developing functionally robust biocontrol applications with translational potential.

Here we employ such methods to screen and identify effective *Pseudomonas* biocontrol bacterial strains against plant parasitic nematodes and validate their efficacy *in vivo* in the tomato rhizosphere. *Caenorhabditis elegans* was used initially as a nematode model and the same assays were then reproduced *in vitro* with two important PPN species, *Meloidogyne incognita* and *Globodera pallida*. We first tested how the direct interaction with the bacteria affected nematode mortality and behaviour *in vitro*. We then conducted genomic screening to identify potential secondary metabolite clusters responsible for inhibitory activity and used mass spectrometry and *in vitro* testing to directly evaluate the production and anti-nematode activity of three candidate compounds (DAPG, orfamides and pyoluteorin) on *C. elegans* and PPNs. Finally, the biocontrol potential of two *Pseudomonas* strains was tested in three glasshouse experiments in the tomato rhizosphere. The tomato model was selected as it is susceptible to PPNs and can grow faster than the potato. Results revealed that nematicidal effects of *Pseudomonas* were strain and growth media-specific, and nematicidal activity was mediated by DAPG, pyoluteorin and orfamides. In general, *C. elegans* was more sensitive to *Pseudomonas* than PPNs, which also showed

behavioural changes in response to secondary metabolites. Despite promising biocontrol effects observed *in vitro,* no beneficial effects were observed *in vivo*. Further research is required to understand the regulation and expression of bacterial biocontrol activity in the plant rhizosphere.

5.3 Materials and methods

5.3.1 Cultivation and maintenance of nematode populations

5.3.1.1 Plate cultivation of *C. elegans* stocks

For *C. elegans* work, the common N2 nematode strain was used and populations were maintained on nematode growth media (NGM) agar plates seeded with lawns of *E. coli* 0P50 (Chapter 2, General Methods, Section 2.1). Nematode populations were synchronised by treating adults with alkaline hypochlorite solution and collecting the eggs inside them using the protocol by Sulston and Hodgkin (1988) and nematode-occupied NGM plates were stored at 20 °C. A section of agar containing nematodes was transferred to a fresh *E. coli*-NGM plate every 3 days, using a flame-sterilised scalpel, to maintain populations for regular use. Long term stocks of *C. elegans* were also kept in soft agar freezing solution at -80 °C.

5.3.1.2 Cultivation of *Globodera pallida*

Globodera pallida (Chapter 2, General Methods, Section 2.1) was previously maintained on susceptible plants of potato 'Desiree' grown in a mix of 50:50 sand:loam soil. Dry soil containing cysts of *G. pallida* was kept in long term storage at 4 °C.

5.3.1.3 Extraction of *Globodera pallida* cysts from soil and hatching of J2s

Globodera pallida cysts were obtained using a flotation method by washing infested soil through a Fenwick can (Fenwick, 1940). Cysts were collected in tap water and then transferred to a 1.5 mL microcentrifuge tube. Cysts were washed in 1 mL of 0.1% malachite green solution and left to rotate at room temperature for 90 minutes. The malachite green solution was then removed and cysts were washed four times using autoclaved tap water. 1 mL of 0.1% chlorhexidine digluconate and 0.5 mg/mL cetyltrimethyl ammonium bromide (CTAB) was then added and the cysts incubated on the rotator for an additional 30 minutes. Following four more washing stages with autoclaved tap water, the cysts were transferred into an autoclaved hatching chamber and immersed in 10-12 mL of filter-sterilised potato root exudate. The chamber was then wrapped in foil and incubated at 20 °C. Exudate was changed every 3-4 days and collected in 15 mL plastic tubes. Hatched second-stage (J2) nematodes were allowed to settle, then collected from the bottom 1 mL of the tube, washed in sterile tap water, and stored at 10 °C until use.

5.3.1.4 Cultivation of *Meloidogyne incognita*

Stocks of *M. incognita* (Chapter 2, General Methods, Section 2.1) were maintained on tomato plants. Tomato (*Solanum lycopersicum* 'Ailsa Craig') plants were grown in compost in a glasshouse at 24 °C with additional lighting to provide 16:8 h light:dark conditions with regular watering.

5.3.1.5 Extraction of *Meloidogyne incognita* J2s from tomato roots

M. incognita J2s were collected from 8-weeks post-infected tomato plants. The above ground tissue was removed at the base of the stem after the roots were thoroughly, but carefully, washed clean of compost. Roots were separated into sections and supported on nylon mesh covered with a single layer of tissue paper over funnels that were placed into a misting chamber. Roots were exposed to a warm tap water mist to encourage hatching of J2s, which were collected in 50 mL polypropylene tubes under the funnels. The tubes were changed every 1-2 days and after being left to settle, the bottom 1 mL of water containing J2s was collected using a glass Pasteur pipette, pooled together, washed in autoclaved tap water and stored at 10 °C.

5.3.2 *In vitro* experiments testing *Pseudomonas* biocontrol efficacy in the lab

5.3.2.1 *C. elegans* feeding preference assays

We first studied nematode feeding preferences using a model laboratory nematode species, *Caenorhabditis elegans* (Chapter 2, General Methods, Section 2.1). It is important to highlight that *C. elegans* and PPNs have very different ecologies and physiological differences. For example, *C. elegans* has the ability to feed on bacteria, while the stylet (feeding apparatus) of PPNs is much narrower preventing them ingesting bacteria. As a result, PGPBs that show feeding-independent killing are required for the biocontrol of PPNs. *Pseudomonas* bacterial starter cultures (Chapter 2, General Methods, Table 2.1) were grown in 5 mL of NB and CPG broth in 15 mL tubes in growth conditions as described in the general methods, with their optical densities measured and normalised to 0.1 OD 600 nm (Chapter 2, General Methods, Section 2.2). 90 mm petri dishes filled with 15 mL of NB or CPG agar were then divided into eight equal sized sections and 20 µL of each desired strain were spotted at equidistant points from the centre of the plate. The plates were then incubated at 28 °C for 24 h. After incubation, ten larval stage 4 (L4) nematodes were individually transferred from *E. coli*-NGM plates to the centre of the plates. These plates were then incubated at 20 °C for the remainder of the experiment. The nematode feeding choices were monitored by counting the number of nematodes on each different *Pseudomonas* spot on the plate at 24, 48 and 72 h by observations with a Motic Light Microscope with a 5x objective and

10x eyepiece. Each treatment was repeated five times.

5.3.2.2 *C. elegans* pharyngeal pumping assays measuring feeding activity

Bacterial starter cultures of the eight *Pseudomonas* strains and *E. coli* were set up as described in the general methods (Chapter 2, General Methods, Section 2.2). Bacterial densities were measured using a Tecan Infinite spectrophotometer and normalised to an OD of 0.1 600 nm using 100% CPG or NB broth (equalling approximately $1x10^6$ CFU/mL). 50 µL of each bacterial strain were spread as an even lawn on 60 mm petri dishes, which were filled with 6 mL of NB or CPG agar (Chapter 2, General Methods, Table 2.2). Plates were then allowed to air dry next to a Bunsen burner in the laminar flow cabinet before being incubated at 28 ^oC (for *Pseudomonas* plates) or 37 ^oC (for *E. coli*) for 24 h. The following day, fifteen adult hermaphrodite nematodes were transferred from *E. coli*-NGM plates to the lawns of *Pseudomonas* strains and left to acclimatise for 1 h at 20°C. Following incubation, pharyngeal pumping was monitored for 30 seconds using a DinoLite DinoEye Microscope Camera recording via a VWR inverted light microscope using a 40x objective with a 10x eyepiece magnification. The pharyngeal pumping method was adapted from Boehnisch *et al.* (2011). After recording, each worm was removed from the petri dish to avoid counting the same individual twice. This experiment was carried out in triplicate

5.3.2.3 Comparing *C. elegans* survival upon direct and indirect exposure to different *Pseudomonas* strains

To study the effect of *Pseudomonas* strains on nematode survival, *C. elegans* was exposed to a variety of bacterial lawns cultured in either CPG or NB broth and their survival monitored for 72 h. Two different culture media were used to see if this would affect biocontrol efficacy. Bacterial starter cultures were grown in 5 mL of NB and CPG broth in 15 mL tubes in growth conditions as described in the general methods, with their optical densities measured and normalised to 0.1 OD 600 nm (Chapter 2). Three lawn treatments were designed for each *Pseudomonas* strain: (1) 50 µL of each *Pseudomonas* monoculture, (2) a 50:50 mix of each *Pseudomonas* strain and *E. coli* culture (25 µL:25 µL) and (3) a 50:50 mix of each *Pseudomonas* supernatant and *E. coli* culture (25 µL:25 µL). The *Pseudomonas* supernatants comprised growth medium containing any secondary metabolites, including antimicrobials, secreted from the bacteria during growth. Lawns of 50 µL of *E. coli* OP50 monoculture were also created as control treatments (this is known to be their preferred food source which can the continuous growth and life cycles of C*. elegans* populations

for weeks in optimal conditions).

Each of these treatments was replicated 6 times for bacterial cultures in both CPG and NB media and incubated for 24 h at 28 °C prior to *C. elegans* transfer. The *Pseudomonas* supernatant (representing secondary metabolites including antimicrobials) was collected by centrifuging the *Pseudomonas* strains for 10 minutes at 4000 *g* and filtering the supernatant via 0.2 μm filters (Eppendorf, Centrifuge 5810R model). After 24 h of incubation, half of the replicates within each treatment were heat-treated in a water bath at 65 °C for 30 minutes to kill the bacteria. Bacterial lawns were then seeded with 15 L4-stage *C. elegans* and monitored using a Motic Light Microscope with a 5x objective and 10x eyepiece. Nematode activity was recorded by monitoring which nematodes were still alive or considered dead at 0, 1, 3, 8, 24, 48 and 72 h. Those classified as "dead" did not react to being touched with a platinum wire. The experiment was terminated after 72 h as the second-generation adult nematodes (developed from eggs laid on lawns) became indistinguishable from the first generation adults.

5.3.2.4 Comparing PPN survival upon exposure to different *Pseudomonas* strains

Pseudomonas strains were cultured in 5 mL of CPG broth in 15 mL tubes in growth conditions as described in the general methods, with their ODs measured and normalised to 0.1 OD 600 nm (Chapter 2, General Methods, Section 2.2). 140 μL of each of these bacterial strains were added to a 48-well microtiter plate with 6 replicates of each *Pseudomona*s strain. The control for this experiment was 140 μL of autoclaved tap water. Ten μL of *M. incognita* or *G. pallida* J2 suspensions were added and following counts at 0 h, nematode activities were recorded at: 1, 3, 24 and 48 h using a light microscope (Wild Heerbrugg M5A 25x objective and 10x eyepiece).

5.3.2.5 Viability testing of PPNs using NaOH

When conducting *in vitro* PPN experiments, an irregular morphology was observed. The nematodes would appear immobile, but curled in a C-shaped position, making it difficult to confirm if the nematode was alive or not. To determine whether plant parasitic nematodes which appeared to be inactive, but showed irregular morphologies were living or dead, 1.5 μL of 1M NaOH was added to each well and observation of nematode activity was carried out for several minutes. Those that were living would begin to twitch and coil for up to 30 minutes after NaOH addition. NaOH treatment was applied at the end of every *in vitro* PPN experiment where this irregular morphology was observed. This method was adapted from reports by Xiang and

Lawrence (2016).

5.3.3 Determining biocontrol properties through genomic and metabolomics analysis

5.3.3.1 Investigating *Pseudomonas* secondary metabolite biosynthesis gene clusters based on genome information with AntiSMASH5.0 and determining insight into the effects observed in *C. elegans in vitro* experiments

Due to *Pseudomonas* supernatant showing the ability to suppress PPN and *C. elegans* activity, the genomes of the eight *Pseudomonas* strains used in this study were run through AntiSMASH5.0 (https://antismash.secondarymetabolites.org) to identify potential secondary metabolic clusters linked with antibiosis. Detailed methods of this process can be found in Chapter 2, General Methods, Section 2.3.

5.3.3.2 Mass spectrometry of *Pseudomonas* supernatants in CPG and NB growth media gives insight into the effects observed in *C. elegans in vitro* experiments

Although genome analyses can give insight on the potential of bacteria to produce a number of metabolites, all identified metabolites might not be produced in given growth conditions used in specific experiments. Mass spectrometry was thus conducted to investigate the production of identified *Pseudomonas* supernatants in more detail. The full methodology for these assays is described in Chapter 2, General Methods, Section 2.4.

5.3.4 Investigating indirect mechanisms of inhibition using observed *Pseudomonas* metabolites

5.3.4.1 Testing effects of candidate inhibitory compounds on *C. elegans* survival

To investigate the indirect effects of *Pseudomonas* metabolites on *C. elegans*, each chemically validated *Pseudomonas* metabolite and our own isolated cyclic lipopeptides were tested against *C. elegans*. 100 μM stocks of orfamide A, orfamide B and pyoluteorin were diluted from the original 10 mM and 100 mM stocks described in Chapter 2, General Methods, Section 2.5). These were prepared in water. As a greater amount of DAPG was available, we tested its effects in three concentrations: 1000, 500 and 100 μM. The control for this experiment was 140 μL of autoclaved tap water for all experiments. Each treatment was conducted with 150 μL volume of each metabolite in 48-well microtitre plates in triplicate. Ten L4-stage *C. elegans* were transferred from *E. coli*-NGM stock plates and the activity of the first generation was monitored using a Motic Light Microscope with a 5x objective and 10x eyepiece at: 0, 1, 2, 4, 8, 24, 30, 48, 72 h. The egg

hatching and development of juveniles was also monitored up until 96 h. This was done by observing egg laying, hatching and noting the developmental stages reached by the hatched nematodes at 96 h.

5.3.4.2 Testing individual effects of candidate inhibitory compounds on *G. pallida* and *M. incognita* survival at a range of concentrations

Dilutions from 10 mM stocks of Orfamide A, B and Pyoluteorin were made in water to concentrations of 100, 50, 25, 10, 5, 2 and 1 μM. DAPG dilutions were created from a 100 mM stock and made to concentrations of: 1000, 500, 100, 50, 10, 5, and 1 μM DAPG. Experiments were set up with 140 μL of each compound concentration in 48-well plates with six replicates per treatment. The control for these experiments was 140 μL of autoclaved tap water. Ten μL of 20-30 *M. incognita* or *G. pallida* J2s suspended in autoclaved tap water was then added to each well. Nematode activity was recorded as described in section 5.3.2.4.

5.3.4.3 Testing individual effects of isolated *Pseudomonas* cyclic lipopeptides on *G. pallida* and *M. incognita*

The extraction method through which these treatments were obtained can be found in Chapter 2, General Methods, Section 2.6. As the concentrations of these products were unknown, a pilot study exploring a dilution series was carried out which revealed little effect on J2 nematodes. As a result, we decided to test only one concentration of isolated CLP extracts. Each CLP extract sample was prepared by diluting the stocks to 1% in 140 μL then inoculated with ten μL of 20-30 *M. incognita* or *G. pallida* J2s in triplicate. Nematode activity was recorded as described in section 5.3.2.4.

5.3.4.4 Testing combinatorial effects of candidate inhibitory compounds on *G. pallida* and *M. incognita survival*

Metabolite effects were also tested in combination to study if stronger effects on PPN survival could be observed. This experiment was carried out with the highest concentration of each metabolite tested previously as at their highest concentrations, these all still had subinhibitory effects on PPNs (1000 μM for DAPG and 100 μM for orfamides A-B and pyoluteorin). The combinations were tested in 2-, 3- and 4-way combinations. Ten μL of 20-30 *M. incognita* or *G. pallida* J2s suspended in autoclaved tap water was then added to each well. Nematode activity was recorded as described in 5.3.2.4.

5.3.5 *In vivo* PPN glasshouse experiments

5.3.5.1 Growth and maintenance of tomato plants before and during the glasshouse experiments

To prepare plants for *in vivo* tests, tomato (*Solanum lycopersicum* 'Ailsa Craig') plants were grown in a glasshouse at 24 ^{o}C with additional lighting of 16:8 h light:dark conditions and regular watering. Three glasshouse experiments were conducted between October 2018-April 2019:

(1) *Meloidogyne incognita* tomato plant infection (short term - ST)
(2) *Globodera pallida* tomato plant infection (short term - ST)
(3) *Meloidogyne incognita* tomato plant infection (long term - LT)

Seeds were initially sown in compost in 6 inch shallow pots for 2-3 weeks and seedlings were then transferred into individual pots for the remainder of the experiment. The seedlings used for *M. incognita* infections were transferred into 4 inch pots with 500 g of autoclaved 50:50 sand and loam mix. To avoid risks of wilting observed in our first experiment, we opted to use larger pots for *G. pallida* short-term (2) *and M. incognita* long-term (3) experiments. The seedlings in (2) and (3) experiments were grown in 5 inch pots with 1000 g of the same autoclaved sand:loam soil mix. The decision to use 5 inch pots for the second and third experiments was made to provide the plants greater space and stability for their root systems. A more consistent soil moisture level could also be maintained with the increased soil volume.

5.3.5.2 *Pseudomonas* inoculations of tomato plants

One week following the re-potting of seedlings, the soil and roots of tomato plants were inoculated with *Pseudomonas* bacterial cultures. We chose to focus on the two *Pseudomonas* strains that appeared to have the strongest inhibitory effects on nematodes in previous experiments: Pf-5 and CHA0. In total, 6 tomato treatments were used:

(1) Water, no nematodes (control)
(2) Water + nematodes
(3) *Pseudomonas* Pf-5, no nematodes
(4) *Pseudomonas* Pf-5 + nematodes
(5) *Pseudomonas* CHA0, no nematodes
(6) *Pseudomonas* CHA0 + nematodes

Bacterial inoculation cultures were prepared by mixing 10 mL of CPG broth with 10 μL of overnight bacterial cultures in 50 mL tubes. Bacteria were then grown with shaking for 24 h at 28

°C at 200 rpm and then inoculated into 500 mL of CPG broth in 2 L Erlenmeyer flasks and grown for another 24 h at 28 °C with 200 rpm shaking. The bacterial cells were then washed by centrifuging and the 500 mL cultures were spun at 6000 *g* for 10 minutes (J25 XP series centrifuge) and resuspended in dH_2O. Washing of cells was important to prevent the bacterial growth media from influencing bacterial growth in the rhizosphere. Bacterial cell densities were adjusted to an OD 600 nm of 0.5 (around $5x10^8$ CFU/mL), diluted to twice the volume with dH_2O, and finally, 100 mL of inoculant was poured into each pot, soaking through the soil and roots and filling the saucer below. Control treatments involved pouring 100 mL of water onto tomato plants. For *G. pallida* short-term (2) and *M. incognita* long-term (3) experiments, the cultures were grown in twice the volumes to use 200 mL of inoculants as the pots had twice the amount of soil. The number of replicates varied slightly between treatments (7-10 plants) as some plants were lost naturally and the germination rate varied. Replicate numbers are described in detail in Table.5.1.

Table 5.1 The number of replicate plants included in treatments in the three glasshouse tomato experiments.

Treatment	ST *M. incognita* (1)	ST *G. pallida* (2)	LT *M. incognita* (3)
Water, no nematodes	8	10	8
Water + nematodes	8	8	8
Pseudomonas Pf-5, no nematodes	8	8	7
Pseudomonas Pf-5 + nematodes	8	8	7
Pseudomonas CHA0, no nematodes	8	8	7
Pseudomonas CHA0 + nematodes	7	7	7

5.3.5.3 Serial dilutions to confirm *Pseudomonas* inoculation densities on tomato plants

To confirm bacterial numbers used to inoculate tomato plants, colony forming unit (CFU) counts of each bacterial treatment were carried out. Twenty μL of each bacterial culture was diluted to 1 in 10 in 180 μL of dH_2O in 96-well plates. 10% of this population was then transferred to an adjacent well also containing 180 μL of dH_2O, this was repeated to make several serial dilutions. Twenty μL was spotted onto the centre of 90mm plates containing ~15 mL of CPG agar. Fifteen sterile glass beads were added to the plate and then the agar plate, in a face-up orientation, was shaken in several directions for 20 seconds to spread the bacteria evenly over the plate. Glass beads were then removed and plates were incubated at 28 °C and colony counts performed after 24 h. Table 5.2 summarises the CFU counts observed. The amount of bacteria applied did vary

between the experiments with a range of 1-6x10^9 CFU/mL. However, as these are all in a high concentration this is unlikely to have had large effect on results.

Table 5.2 Colony forming unit counts for CHA0 and *Pseudomonas* strains prior to rhizosphere inoculations for the three tomato experiments conducted.

Tomato experiment	*Pseudomonas* Strain	CFU/mL
Short-term *G. pallida*	CHA0	6.32 x10^9
	Pf-5	6.05 x10^9
Short-term *M. incognita*	CHA0	3.60 x10^9
	Pf-5	1.46 x10^9
Long-term *M. incognita*	CHA0	3.65 x10^9
	Pf-5	4.03 x10^9

5.3.5.4 *M. incognita* and *G. pallida* inoculations

Juvenile nematodes were inoculated onto tomato plant roots one week after *Pseudomonas* inoculations. Three 1 mL pipette tips were inserted at a diagonal angle towards the stems of the tomato plants to a depth of ~3 cm and removed to form holes. Approximately 2000 J2 *M. incognita* or *G. pallida* per plant were applied through the holes and washed through in 2 mL of water (~666 µL per hole). Control treatments received 2 mL of water in a similar manner. The holes were then covered back over with surface soil from each pot. All plants were left one day before being watered to allow the nematodes to navigate through the soil. Following nematode inoculation, *M. incognita*-infected plants remained in the glasshouse at 24 ^oC with additional lighting with 16:8 h light:dark conditions. Plants infected with *G. pallida* were transferred to a 20 ^oC glasshouse to provide more suitable conditions for nematode growth.

5.3.5.5 Chlorophyll fluorescence measurements

Dark-adapted chlorophyll fluorescence measurements were taken from the 3rd leaves up from the cotyledons at weekly intervals throughout the experiment using a chlorophyll fluorometer (Opti-Sciences 0S-30p+ model). Fv/Fm represents the ratio of variable fluorescence to maximal fluorescence and reports the maximum quantum efficiency of photosystem II in plants (Kitajima and Butler, 1975; Baker and Oxborough, 2004). For the long-term *Meloidogyne* experiment, measurements were terminated five weeks post-nematode inoculation as the decision was made to give each plant additional nutrients to survive the extended time of the experiment, which in turn altered their chlorophyll recordings. The additional nutrients provided were soluble feed (Vitafeed 1:1:1).

5.3.5.6 Plant height and above and below ground plant dry weight measurements

Plant heights were measured from the cotyledons at the base of the stem to the highest leaf node before the uppermost flowering part of the plant at the end of the experiment. At harvest, all above ground tissue was transferred to paper bags and placed in a 65 °C incubator until completely dry, then weighed. The root system dry weights were also measured. Control and nematode-infected root systems treatment roots were also dried in paper bags processed similar as described above after thorough nematode quantification.

5.3.5.7 Quantifying *Pseudomonas* rhizosphere colonisation at the end of *in vivo* experiments

To quantify the level of bacterial colonization of the roots at the end of each *in vivo* experiment, soil wash samples were collected and CFU counts performed on *Pseudomonas*-selective agar plates (Oxoid). Roots were shaken gently and washed in a 60 mL universal containing 50 mL of tap water to collect the rhizosphere soil containing bacteria (Figure 5.3.1). Each soil wash sample was then shaken thoroughly for ~10 seconds, left to settle for 10 seconds (to allow most of the soil to settle) and the top 750 μL was collected and mixed with 500 μL of 50% glycerol and stored in 1.5 mL microcentrifuge tubes at -80 ^{o}C. Samples were completely thawed prior to CFU counts, then vortexed for 10 s, left to settle for 10 s (for any remaining soil in the sample to settle) and 1 in 10 serial dilutions in PBS were set up in 96-well microtiter plates. 15 μL of each dilution was spotted onto *Pseudomonas*-selective agar plates and incubated at 28 ^{o}C prior to counts. Four samples from each treatment were tested in triplicate. Following CFU counts, a variety of colony morphologies were observed which were subsequently plated onto new *Pseudomonas*-selective agar to confirm that they were *Pseudomonas*.

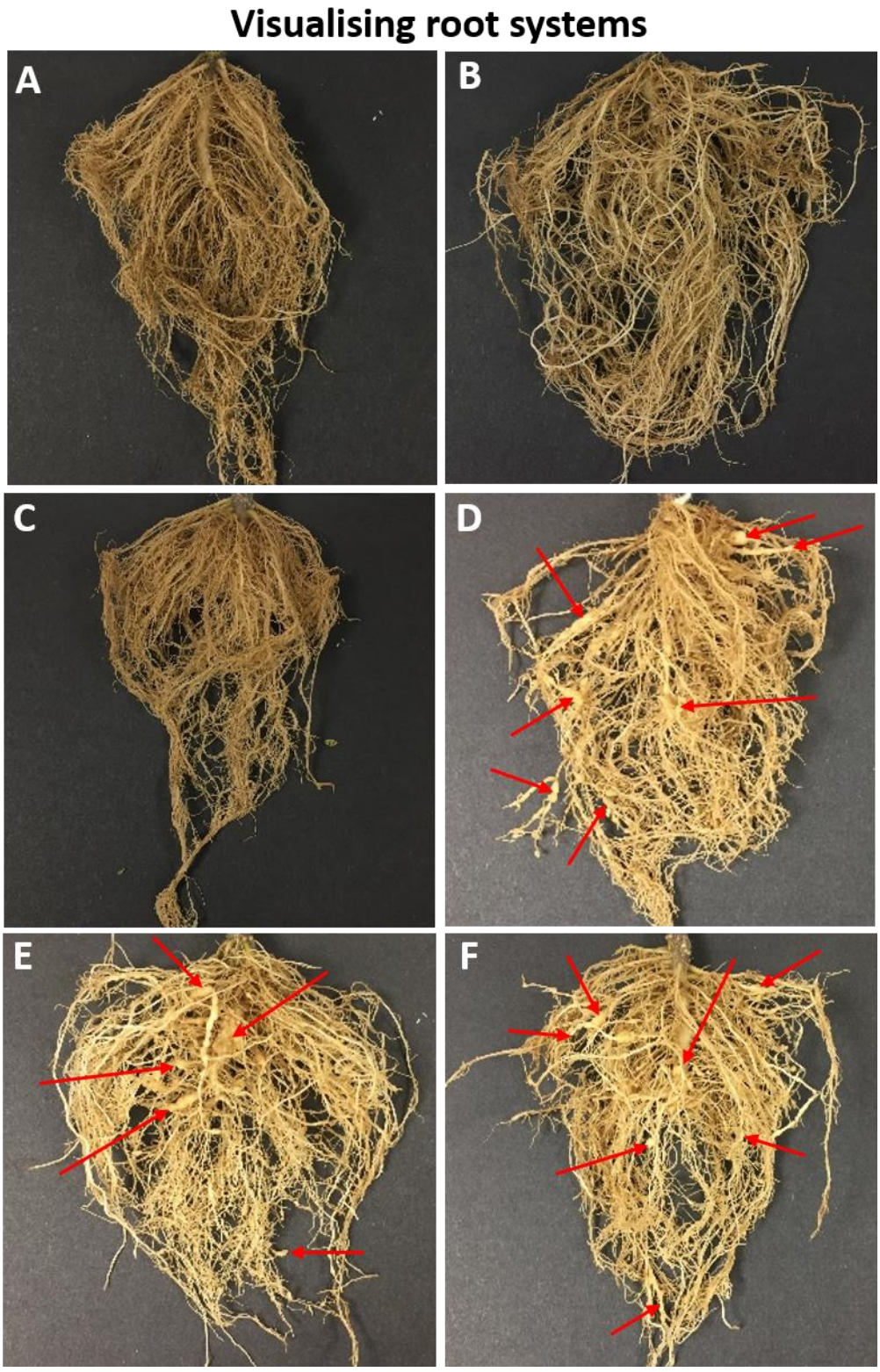

Figure 5.3.1 Examples of the washed root systems from each of the six tomato treatments during the *M. incognita* ST experiment. Each figure is one of the replicate root systems after washing prior to acid fuchsin staining. (A-C) are images of the uninfected three root systems (A) water, (B) Pf-5-treated and (C) CHA0-treated. (D-F) are examples of root galling (indicated by red arrows) observed in each nematode-infected treatment: (D) water, (E) Pf-5-treated and (F) CHA0-treated.

5.3.5.8 Acid fuchsin staining and visual quantification of PPN infection levels

Acid fuchsin staining was used to visualise nematode infections in root tissue. Roots were thoroughly washed and soaked in sodium hypochlorite solution (with 1% available chlorine) for two minutes. The roots were then rinsed for five minutes in several beakers of water and then transferred to a beaker containing boiling acid fuchsin (0.035% (w/v) acid fuchsin, 2.5% (w/v) glacial acetic acid) for two minutes. Roots were very briefly rinsed in water, placed in petri dishes and soaked in acidified glycerol (1 drop of acetic acid/100 mL glycerol) to enable de-staining. Plates were stored at 4 ^{o}C prior to nematode infection quantification. Acid fuchsin-stained roots were visualised under a light microscope (Olympus SZX9 x22 eyepiece). Approximately 25% of the roots were removed from the system to quantify infection and then counts were multiplied to account for the whole root system. For *M. incognita*-infected roots as shown in Figure 5.3.2, some galls contained one nematode (A) whereas others contained several (B). *G. pallida* do not create galls during their infections so only the number of nematodes was recorded. Panel (D) gives an example of the female PPN emerging from the root surface.

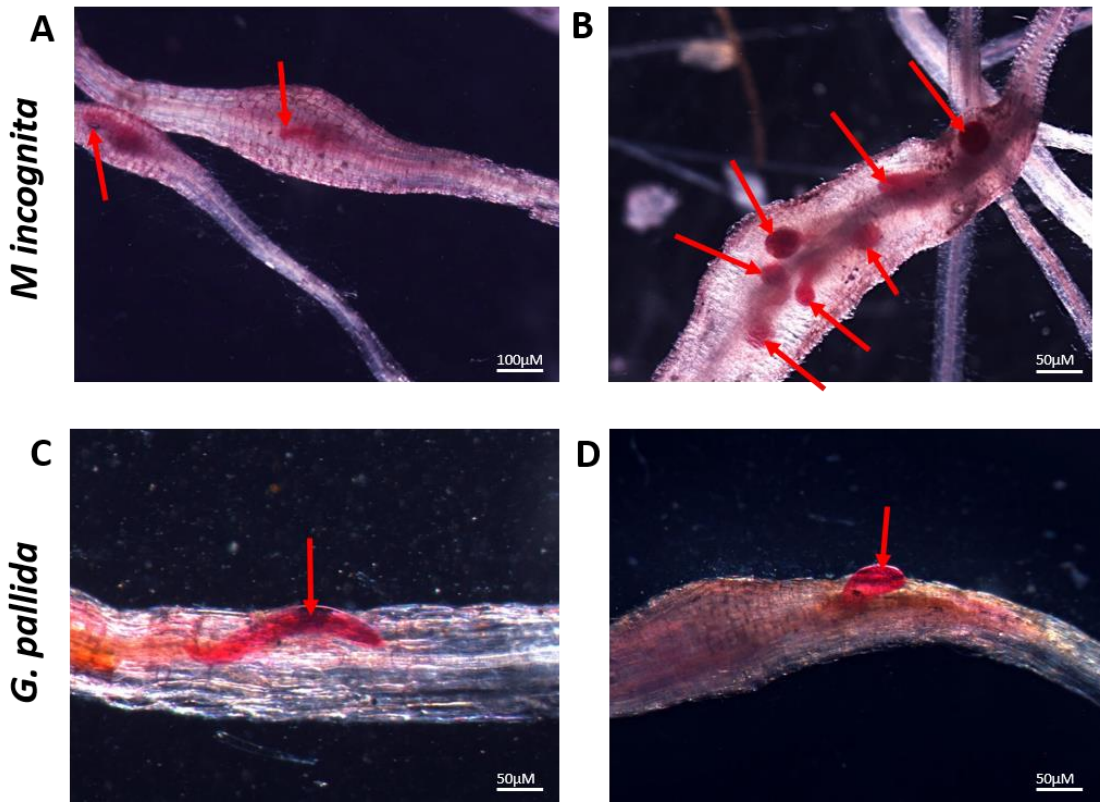

Figure 5.3.2 Example of PPN infections following acid fuchsin staining. The red arrows indicate individual PPN Infection sites in tomato roots at varying magnifications and stages of infection. The top row displays microscope images from the *M. incognita* infections (A-B) and the bottom row of *G. pallida* infections (C-D). The first image indicates two individual infection sites with the red arrows touching the acid fuchsin-stained J2/J3 stage nematodes (A). Panel (B) gives an example of the galling structures where multiple *M. incognita* nematodes have established infection sites in the same region of the root resulting in the large root knot "gall". *G. pallida* nematodes in (C-D) all have individual sites of infection with panel (C) highlight a post-parasitic J2 which has entered the plant. Panel (D) reveals a more advanced stage of *G. pallida* development with the arrow pointing to a J4 female nematode emerging through the root tissue where it will remain on the root surface developing eggs and eventually forming the characteristic cyst.

5.3.5.9 *Meloidogyne incognita* egg counts

Analysis of nematode multiplication for the LT *M. incognita*-infected plants was achieved by recovering eggs from galled roots using hypochlorite treatment. This dissolves the gelatinous matrix of the egg masses to release eggs. The galled roots were cut into sections and shaken in a sealed plastic container in 1% sodium hypochlorite solution for four minutes. The hypochlorite solution containing released eggs was then poured over nested 150, 63 and 25 µm-pore sieves and rinsed thoroughly with water. The eggs were carefully washed in ~20 mL of water and collected into 50 mL tubes and stored at 4 ^{o}C prior to counting of eggs. Just before quantification, each tube was shaken well to mix the settled eggs and loaded into a Peters counting slide using a sterile Pasteur pipette to fill the 1 mL chamber over the gridded area for egg counts under a Leica

DMRB light microscope (Leica, Germany).

5.3.6 Statistical analysis

Two-way ANOVA tests were conducted to analyse the mean differences between treatments. For more detailed analyses these were followed by post-hoc Tukey tests with 95% confidence levels. Tukey contrasts with Bonferroni-corrected p-values were used for pairwise comparisons. For all experiments conducted over a length of time repeated measures ANOVA tests were conducted with between-subject and random effects to analyse mean differences in pathogen densities. All statistical tests were carried out using RStudio.v.3.4.4, for details on packages using see Chapter 2, General Methods, Section 2.7.

5.4 Results

5.4.1 *In vitro* screening of inhibitory *Pseudomonas* strains using *C. elegans* as a model nematode species

5.4.1.1 Nematode feeding preference and activity

To screen for inhibitory *Pseudomonas* bacterial strains, we first conducted feeding preference and activity assays (Figure 5.4.1-5.4.2). It was found that *C. elegans* feeding preference varied depending on the growth media in which *Pseudomonas* strains were cultured with stronger feeding preferences observed when grown in CPG media (*Growth Media*Pseudomonas*: $F_{7, 144}$ = 6.06, $P < 0.001$, Figure 5.4.1 A-B). Feeding preference for strains grown in CPG showed high variation with strains 1M1-96 and F113 being the most preferred and strains CHA0, Pf-5 and Q2-87 the least preferred strains or strains which the nematodes chose to avoid (*CPG Media*Pseudomonas*: $F_{7,72}$= 13.43, $P < 0.001$ Figure, 5.4.1 A). *C. elegans* feeding preferences were less varied when *Pseudomonas* strains were cultured in NB growth media as no strain was chosen significantly more often compared to others (*NB Media*Pseudomonas*: $F_{7,72}$= 2.247, P = 0.0399, Figure 5.4.1 B).

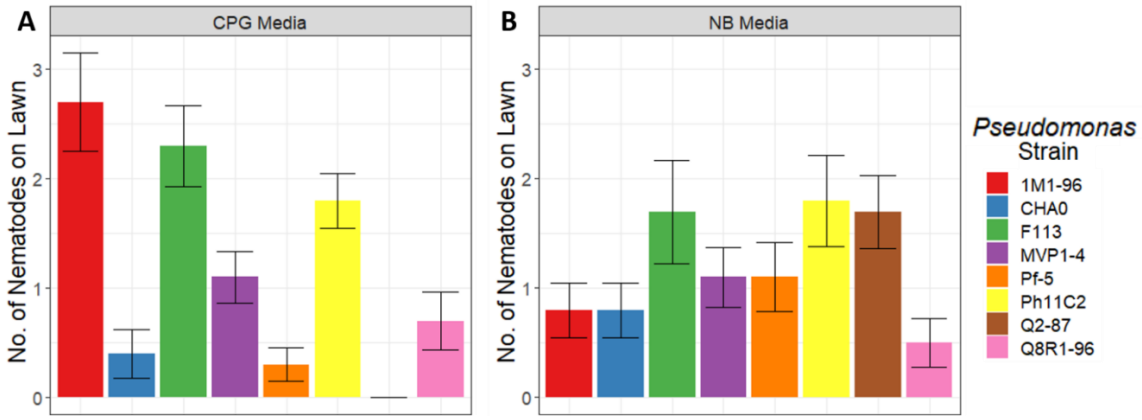

Figure 5.4.1 *C. elegans* feeding preference between eight *Pseudomonas* strains. The bars show the average feeding preference (in terms of number of nematodes observed on bacterial lawns) between eight *Pseudomonas* strains grown in CPG (A) and NB (B) media averaged over 72 h after nematode inoculation. All error bars show the standard error of the mean (+/-1 SEM) based on 3 replicates.

To confirm *C. elegans* were feeding on bacteria, pharyngeal pumping activity — positive indication of feeding, (Boehnisch *et al.*, 2011) — was recorded after one hour of exposure on bacterial lawns. Pumping varied depending on the growth media in which strains were cultured with rates being significantly faster in NB (Growth media: $F_{1, 808}$ = 637.6, $P < 0.001$, Figure 5.4.2 A-B). Post hoc analyses showed that pharyngeal pumping on all NB lawns (Figure 5.4.2 B) was

greater than rates on CPG lawns and that the highest rates were observed on *E. coli*, CHA0 and 1M1-96 bacterial strains (*Growth media*Pseudomonas*: $F_{8, 792}$ = 151.9, P < 0.001, Figure 5.4.2 A-B). Additional post hoc analyses revealed that pumping rates were more varied on *Pseudomonas* lawns cultured in CPG media (*CPG media*Pseudomonas*: $F_{8, 396}$ = 236.29, P < 0.001, Figure 5.4.2 A). Specifically, strains 1M1-96, MVP1-4, Q2-87 and Q8R1-96 were associated with moderate pumping rates by *C. elegans*, while the pharyngeal pumping rates for CHA0, Pf-5 and Ph11C2 were negligible. In these treatments, nematodes were found to die or remain alive but not actively feed, which suggests that these strains could potentially be toxic for *C. elegans.* In general, pharyngeal pumping activity suggests that *Pseudomonas* strains, which were not preferred in choice feeding assays, were not actively fed upon with the exception of strain Q2-87. Together these results suggest the growth media strongly influenced feeding preference, feeding activity and *Pseudomonas* strain-specific killing of *C. elegans* with CPG media containing higher amounts of glucose having a strong influence on nematode activity. .

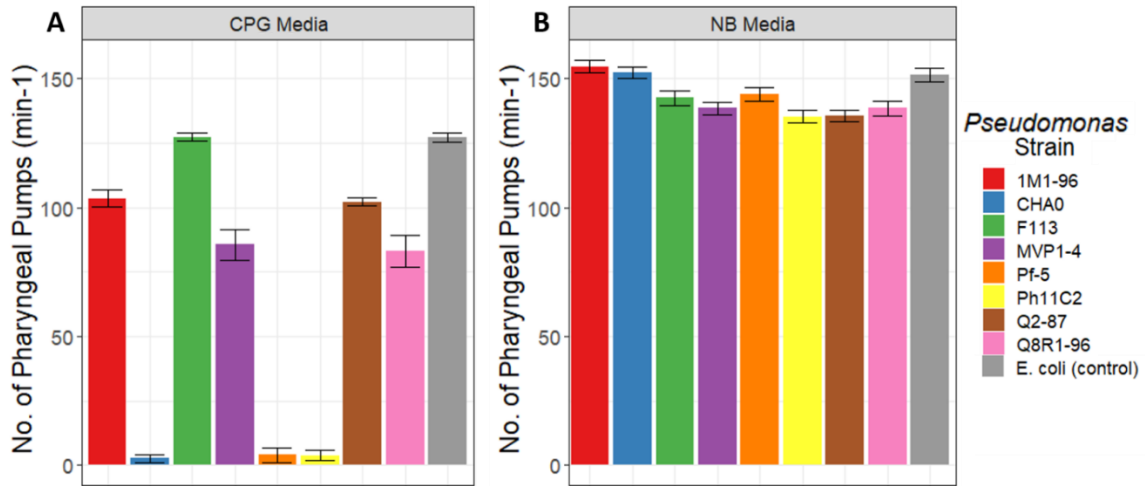

Figure 5.4.2 Pharyngeal pumping rates of *C. elegans* indicative of feeding on different *Pseudomonas* strains. The bars show the average *C. elegans* pumping rates on eight *Pseudomonas* strains and *E. coli* control strain grown in CPG (A) and NB (B) agar media. All error bars show the standard error of the mean (+/-1 SEM) based on 3 replicates.

5.4.1.2 Comparing *C. elegans* survival when exposed to different *Pseudomonas* strains

To study the effect of *Pseudomonas* on nematode survival, *C. elegans* were exposed to a variety of bacterial lawns cultured in either CPG or NB broth and their survival was monitored for 72 h. Three types of lawn treatments were used: (1) 100% *Pseudomonas* or *E. coli* (control treatment) lawns, (2) 50:50 *Pseudomonas* and *E. coli* co-culture lawns and (3) 50:50 *Pseudomonas*-extracted

supernatant and *E. coli* co-culture lawns. Together, 6 replicates were used and half of the replicates in each treatment were heat-treated (65°C for 30 min) prior to nematode transfer, to observe if nematicidal effects required active production of secondary metabolites or other inhibitory activity by living bacteria.

Pseudomonas strains reduced *C. elegans* survival rates to significantly lower levels than the *E. coli* control treatment only when cultured in CPG growth media *(Growth media*: $F_{1, 148}$ = 27.3371, $P < 0.001$ Figure 5.4.3) and no observable effects between *Pseudomonas* strains were found in NB media (*Pseudomonas strain:* $F_{8, 66} = 0.49$, $P = 0.8593$, Figure 5.4.3 A). In contrast, nematode survival was highly dependent on *Pseudomonas* strain identities in CPG media with strains CHA0 and Pf-5 causing 100% nematode mortality within 1-3 h. *Pseudomonas* strain Ph11C2 also increased nematode mortality but to a lower extent (*Pseudomonas strain:* $F_{8, 66}$ = 8.8047, $P < 0.001$, Figure 5.4.3 B). *Pseudomonas* mortality effects were not reduced by the presence of *E. coli* as a food source verifying that nematode mortality was unlikely due to starvation (*E. coli Treatment:* $F_{1, 49} = 185.78$, $P = 0.9206$, Figure 5.4.3 B). Moreover, mortality caused by *Pseudomonas*-CPG lawns was not reproduced when the lawn treatment was made of *Pseudomonas* supernatant as opposed to direct bacterial cultures (*Lawn treatment:* $F_{2, 72} = 8.4373$, $P < 0.001$, Figure 5.4.3 B). To further separate the need for active *Pseudomonas* to induce *C. elegans* mortality, all treatments were replicated with an additional step of heat-treating every bacterial lawn prior to *C. elegans* transfer onto them. It was found that *C. elegans* survival was not impacted by any of the *Pseudomonas* heat-treated lawns from either growth media type, and thus, this data has not been visualised (*Heat Treatment:* $F_{1, 298} = 27.261$, $P < 0.001$). As a result, *Pseudomonas* had to be alive to have nematicidal effects on *C. elegans*.

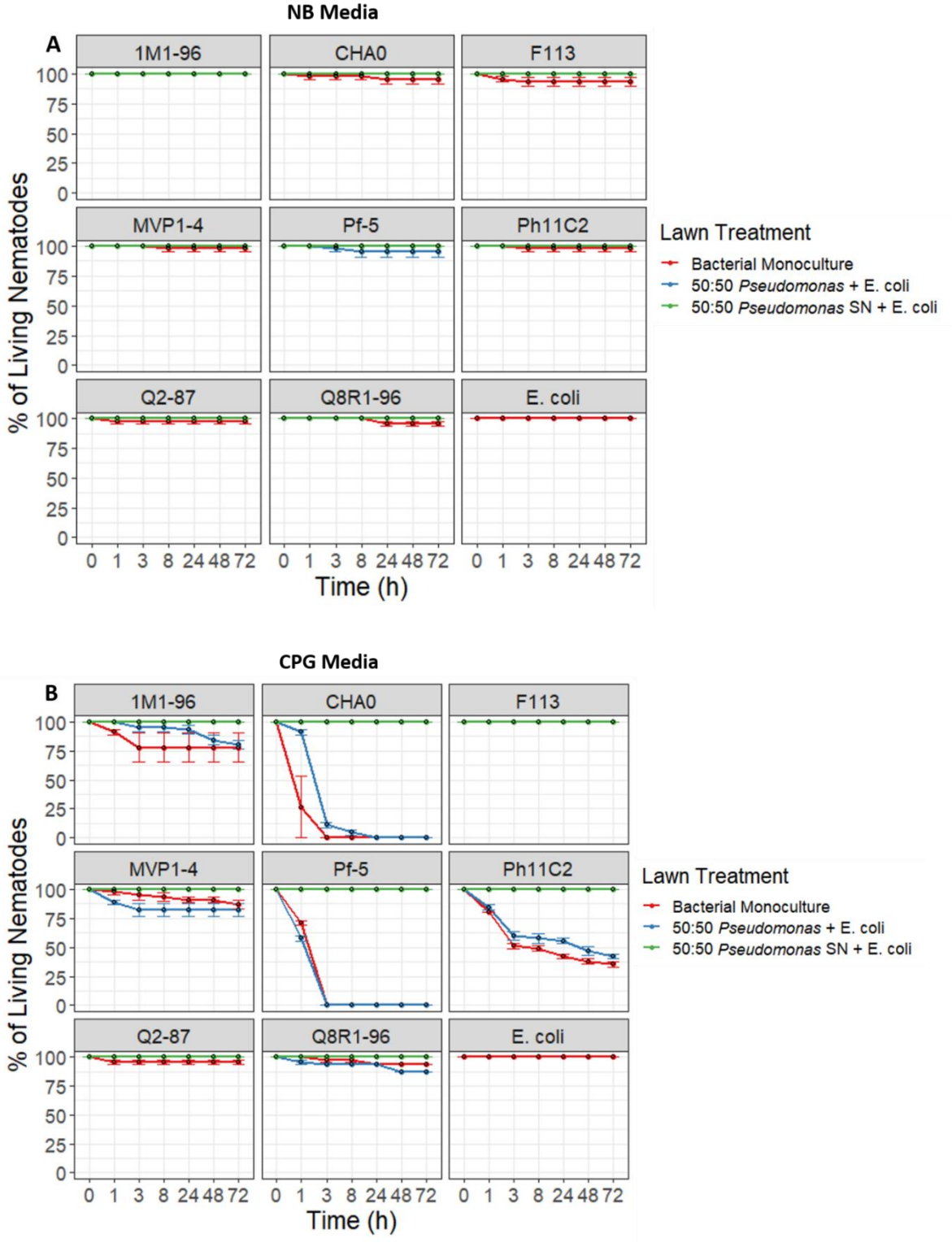

Figure 5.4.3 *C. elegans* survival rates when exposed to mono- and co-culture lawns of *Pseudomonas* strains and *E. coli* (control treatment). Panels (A) and (B) show the effects on *C. elegans* survival when exposed to bacterial lawns in monocultures (red line) and co-cultures (blue line) in NB and CPG agar plates, respectively. The green line shows the effects of 50:50 *Pseudomonas* supernatant-*E. coli* on *C. elegans* when grown on supernatant-supplemented (SN) *E. coli* mono-culture lawns. All bars show the standard error of the mean (+/-1 SEM) and include 3 replicates.

5.4.2 *In vitro Pseudomonas* effects on plant parasitic nematode activity in CPG media

To test if the same *Pseudomonas* strains showed inhibitory activity against both *C. elegans* and PPNs, we exposed PPNs to all *Pseudomonas* monocultures in CPG media, where we had found clear nematicidal effects against *C. elegans*. As the PPN species investigated do not thrive on agar plates, we conducted these assays in liquid media and determined PPN survival in terms of the loss of visible mobility that could not be reactivated with chemical stimulus (see Section 5.3.2.5).

It was found that PPN survival decreased over time upon exposure to all *Pseudomonas* strains which all differed significantly in comparison to the water control treatment (*Pseudomonas*Time:* $F_{12, 528} = 475.2982$, $P < 0.001$, Figure 5.4.4 A-B). Post hoc Tukey analyses revealed *Pseudomonas* effects were rapid with survival being significantly lowered after just one hour of exposure ($P < 0.001$), whereas PPN mobility in the control treatment remained near 100% for the entire experiment. All *Pseudomonas* treatments appeared to have similar effects on mobility of both of the PPN species (*PPN Species:* $F_{1, 106} = 0.0831$, $P = 0.7738$, Figure 5.4.4 A-B).

Focussing on strain variation, all of the *Pseudomonas* treatments differed significantly from the control (water) treatment in which nematodes maintained almost 100% mobility throughout the experiment (*Pseudomonas Strain:* $F_{8,99} = 39.6501$, $P < 0.001$, Figure 5.4.4 A-B). *Pseudomonas* strain differences were driven by strain F113 which appeared to affect mobility at a slower pace than the other strains ($P = 0.009$).

NaOH addition after 48 h —which results in observable twitching and coiling of live PPNs — was conducted to distinguish between immobilisation and PPN death (grey shaded area in figure 5.4.4). It was observed all *Pseudomonas* strains killed *G. pallida* J2s as no activity was observed following NaOH addition compared to the control treatment (*G. pallida *Pseudomonas strain:* $F_{8,45} = 4430$, $P < 0.001$, Figure 5.4.4 A). The *Pseudomonas* effects were similar for *M. incognita* except for F113 treatment where approximately 10% of *M. incognita* J2s regained activity suggesting that this *Pseudomonas* strain had slightly weaker nematicidal activity against *M. incognita (M. incognita*Pseudomonas strain:* $F_{8,45} = 925$, $P < 0.001$, Figure 5.4.4 B). Together these results suggest that all *Pseudomonas* strains were generally highly toxic for J2s of both PPNs.

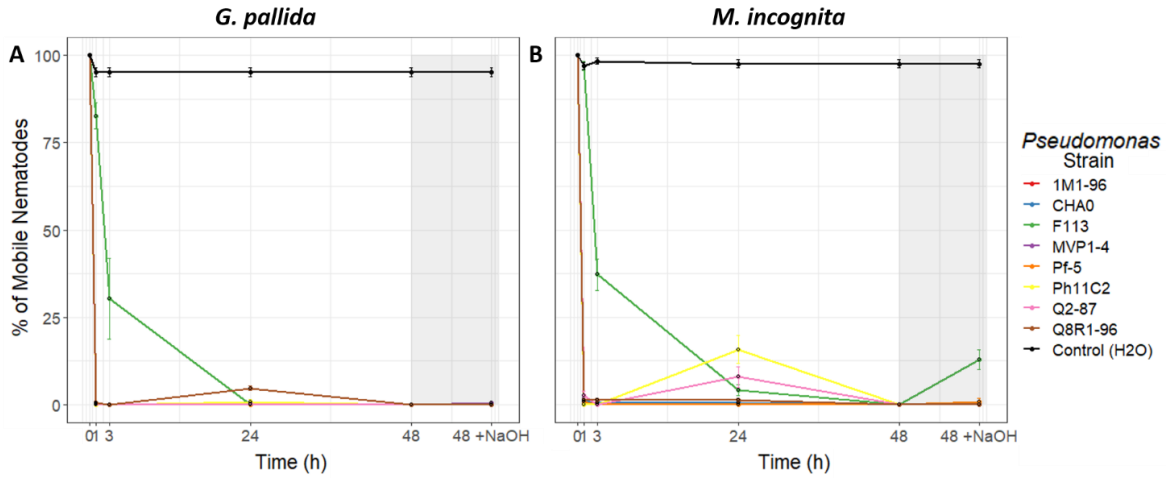

Figure 5.4.4 Percentage of mobile plant parasitic nematodes when exposed to different *Pseudomonas* strains before (white area) and after (grey area) chemical stimulus. Each line represents PPN J2 survival upon exposure to different *Pseudomonas* monocultures or the water control. The panel (A) shows the effects on *G. pallida* and the panel (B) on *M. incognita* PPN species. The shaded areas on the right side of the panels represent the NaOH application to distinguish between dead and living and responding nematodes. All bars show the standard error of the mean (+/-1 SEM) based on 3 replicates.

5.4.3 Using mass spectrometry to identify *Pseudomonas* secondary metabolites with potential nematicidal activity

Pseudomonas and *C. elegans* direct culture assays revealed a high sensitivity of the nematodes to CHA0 and Pf-5 *Pseudomonas* strains and these strains were chosen for more detailed analysis. Our results from AntiSMASH (Chapter 3, Section 3.4.3) genome screening suggested that this activity may have been due to the presence of DAPG, pyoluteorin and orfamide secondary metabolite clusters that were present in both CHA0 and Pf-5 strains. To verify this, the mass spectra produced of CHA0 and Pf-5 grown in the same conditions as those for the nematode assays were explored and analysed. Analysis was conducted in two ways: (1) comparing potential peaks based on existing databases and (2) investigating the mass spectra against four chemical standards: DAPG, Pyoluteorin, Orfamide A and Orfamide B. Detailed methods describing this process can be found in (Chapter 2, General Methods, Section 2.4).

The number of metabolites observed that matched with the Knapsack database of 104 *Pseudomonas*-related records is plotted in Figure 5.4.5. The overall number of identified metabolites was higher in the CPG compared to NB supernatants which supports our earlier data and possibly explains why *Pseudomonas* strains grown in CPG had stronger effects on nematodes (Figure 5.4.5). A number of these metabolites were found only in very low concentrations and were discarded as they could not be distinguished from blank media-only control reads (yellow

bars, Figure 5.4.5). The expression of some metabolites accounted for over 10% of the entire sample based on peak area (red and blue bars, Figure 5.4.5), which shows some metabolites were produced at high levels. The initial analysis revealed there was a high number of metabolites detected in the supernatant but did not specify what these metabolites were. Based on matching monoisotopic masses, a list of putative metabolites including pyochelin, rhizoxin and phenazine present in the *Pseudomonas* supernatants was determined using Progenesis QI v.2.0 (Waters) analysis (Table 5.3). Of the four standard compounds tested, DAPG was the only clear peak detected and was present in the CPG-grown supernatant of CHA0 strain with an average concentration of 16.2 mg/mL (Figure 5.4.6). Interestingly, none of the standards were detected in Pf-5 strain supernatant or in any NB supernatant samples in case of both strains. Together these results suggest that the identified secondary metabolite clusters were not fully functional or not induced in the absence of nematodes.

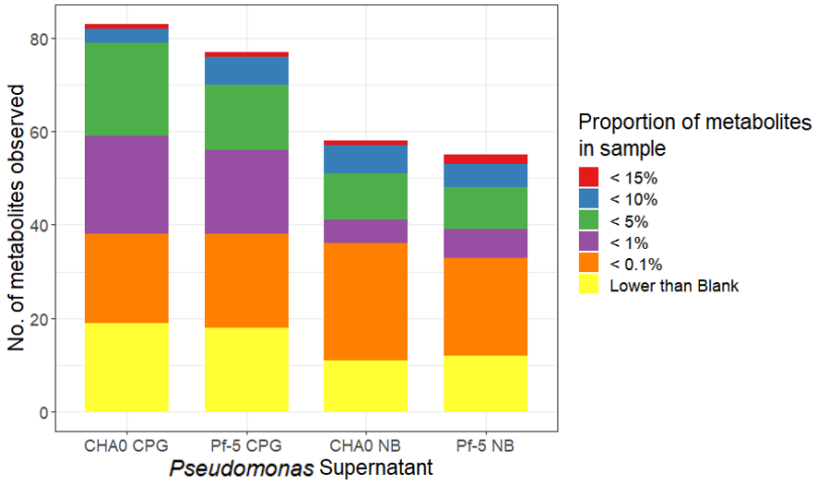

Figure 5.4.5 Mass spectrometry analyses identifying potential *Pseudomonas* CHA0 and Pf-5 secondary metabolites produced when cultured in CPG and NB broth media. The bars indicate number of metabolites in the mass spectra of *Pseudomonas* supernatants that matched with compounds present in *Pseudomonas* Knapsack database and the percentage of the sample (determined by peak area) it accounted for.

Table 5.3 Putative *Pseudomonas* metabolites identified using Progenesis software

Suspected compound	Metabolite function	Present in CPG supernatant	Present in NB supernatant
Pyochelin	Sideophore	Both CHA0 and Pf-5	Both CHA0 and Pf-5
Phenazine	Promotes biofilm development	Both CHA0 and Pf-5	
Rhizoxin	Interferes with mitosis by binding to *Beta*-tubulin affecting microtubule dynamics	Both CHA0 and Pf-5	
Indole-3-acetic acid	Auxin		Both CHA0 and Pf-5
Pseudophomin A	Cyclic lipodepsipeptide – forms ion channels, promotes ion fluxes and causes cell necrosis		Both CHA0 and Pf-5
Pseudophomin B	Cyclic lipodepsipeptide – forms ion channels, promotes ion fluxes and causes cell necrosis	Both CHA0 and Pf-5	
Syringotoxin B	Lipodepsinonapeptide phytotoxin – pore forming toxin also causing ion fluxes		Both CHA0 and Pf-5
Syringomycin A	Lipodepsinonapeptide phytotoxin – pore forming toxin also causing ion fluxes	Only in CHA0	
Syringomycin E	Lipodepsinonapeptide phytotoxin – pore forming toxin also causing ion fluxes	Both CHA0 and Pf-5	Both CHA0 and Pf-5

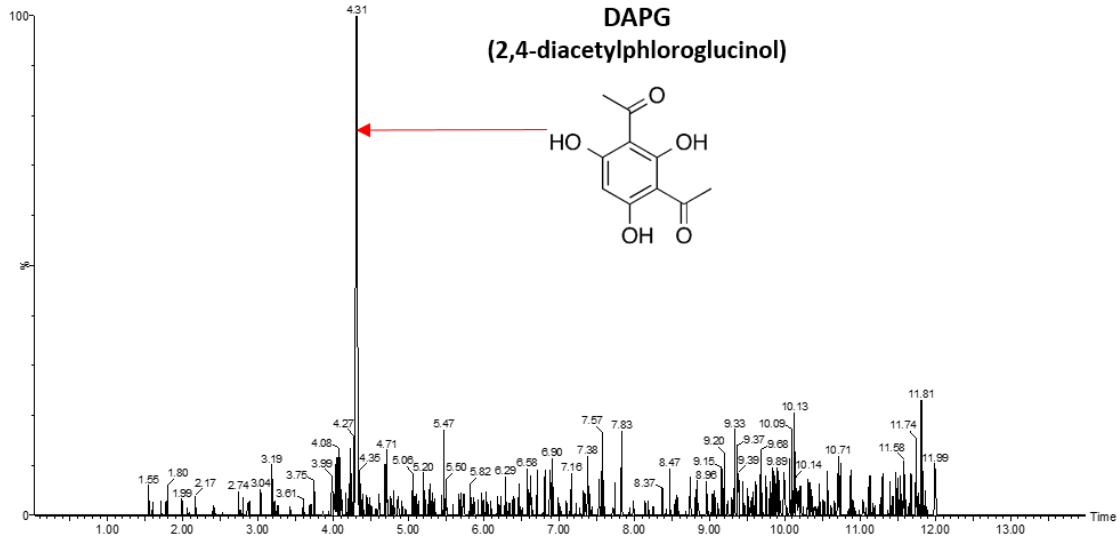

Figure 5.4.6 Mass spectrometry analyses identifying potential *Pseudomonas* CHA0 and Pf-5 secondary metabolites produced when cultured in CPG and NB broth media. The chromatogram displays a clear peak indicating the presence of the *Pseudomonas* metabolite DAPG in the supernatant of strain CHA0 when cultured in CPG growth media. DAPG is identified with a monoisotopic mass (m/z) of 175.04 and a retention time (t_R) of 4.31 seconds at 25 ppm. Shown spectrum represents one of the three replicates with the strongest DAPG peak.

5.4.4 Nematicidal activity of candidate compounds on *C. elegans*

The nematicidal activity of identified secondary metabolites was tested on *C. elegans* in terms of loss of activity and survival and changes in development (egg hatching and post-hatching nematode development). In addition to DAPG, pyoluteorin and orfamides A and B, also CLP extracts that were isolated from the supernatants were tested (Chapter 2, General Methods, Section 2.6).

C. elegans activity decreased over time in every treatment with the majority of nematodes being inactive (no movement) after 48 h (*Time:* $F_{8, 256} = 168.813$, $P < 0.001$, Figure 5.4.7 A-B). As no food supply was provided for nematodes in this experiment, a clear decrease in *C. elegans* activity was also observed in the water control treatment. *C. elegans* activity varied depending on which metabolite it was exposed to (*Metabolite:* $F_{10, 22} = 4.1771$, $P = 0.0025$, Figure 5.4.7 B). Post hoc Tukey analyses revealed that the CLP CHA0 A and CLP F113 A reduced *C. elegans* activity the most (CHA0 A, $P = 0.00489$; F113 A, $P < 0.001$) and that this suppression was much greater compared to the purified orfamides potentially due to higher concentration, which could not be determined as well as potential cocktail effects. In the case of DAPG, no significant difference between the tested concentrations was observed (*Time:* $F_{1, 2} = 0.023$, $P = 0.7961$, Figure 5.4.7 A-B).

In the case of developmental effects (Table 5.4), egg laying was observed during the first

hour of the experiment in every treatment. However, complete development to the adult *C. elegans* stage was only observed in the control treatment, which suggests all treatments slowed down *C. elegans* development. Moreover, compounds that had the most suppressive effect on *C. elegans* activity were also linked with the greatest reduction in nematode development and hatching.

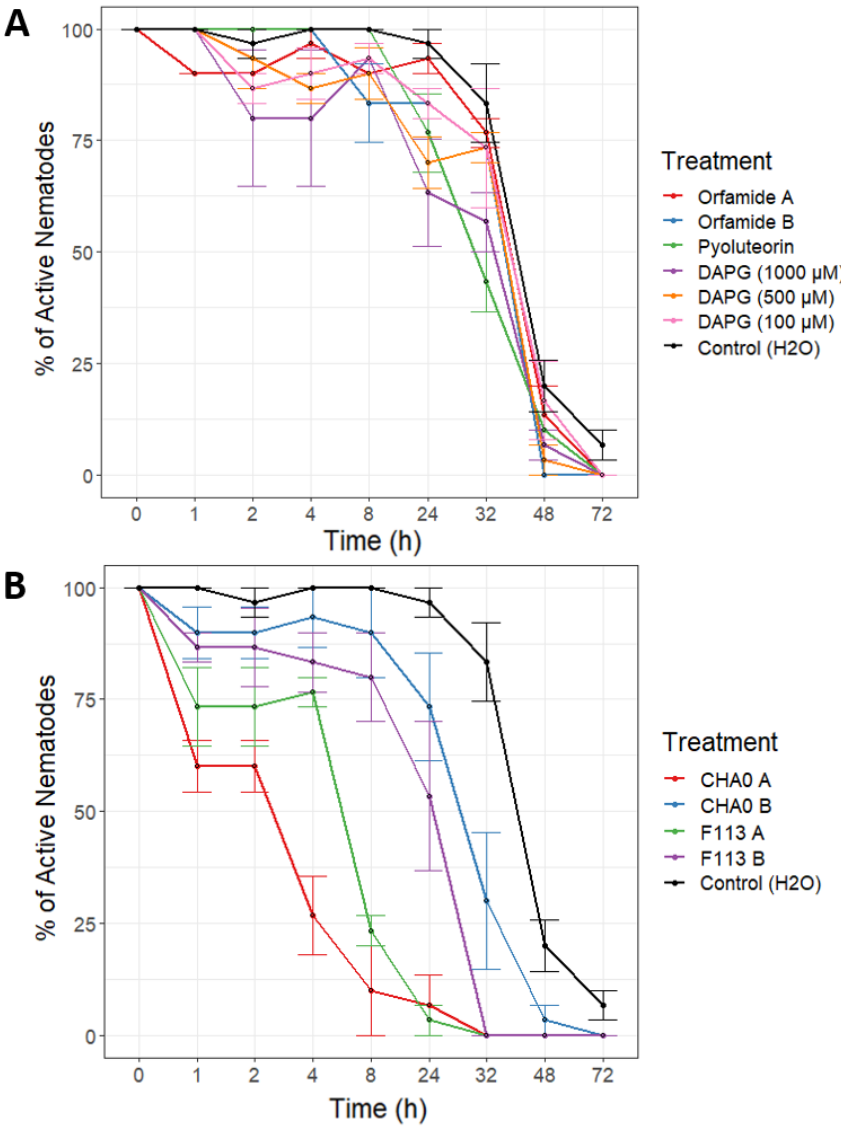

Figure 5.4.7 *C. elegans* activity in the presence of synthetic (A) and extracted (B) *Pseudomonas* secondary metabolites. The lines denote *Pseudomonas* metabolite variation and concentration effects on *C. elegans* activity over time. In Panel (A) the lines denotes activity in 100 µM Orfamide A (red), 100 µM Orfamide B (blue), 100 µM pyoluteorin (green), 1000 µM DAPG (purple), 500 Mm, DAPG (orange), 100 µM DAPG (pink) and water (control) – (black). In Panel (B) the lines denote activity in CHA0 A CLP extract (red), CHA0 B CLP extract (blue), F113 A CLP extract (green), F113 B CLP extract (purple) and water (control) – (black). All bars show the standard error of the mean (+/-1 SEM) based on 3 replicates

Table 5.4 Effects of purified- and extracted-*Pseudomonas* secondary metabolites on *C. elegans* development 96 h after egg hatching.

C. elegans most advanced developmental stage observed	Orfamide A	Orfamide B	CLP extract: CHA0 A	CLP extract: F113 A	CLP extract: CHA0 B	CLP extract: F113 B	Pyoluteorin	DAPG (1000 μM)	DAPG (500 μM)	DAPG (100 μM)	Water (control)
No Hatching			**X**								
L1				**X**		**X**					
L2	**X**				**X**						
L3		**X**					**X**	**X**	**X**	**X**	
L4											
Adult											**X**

5.4.5. Nematicidal activity of candidate compounds on *G. pallida* and *M. incognita*

Concentration-dependent nematicidal activity of the same four synthetic metabolites, and CLP extracts, was tested with two PPN species in terms of loss of J2 activity. In addition, we also investigated if metabolite combinations were more effective than individual compounds. NaOH treatment was also used when necessary to distinguish between dead and inactive nematodes.

5.4.5.1 The concentration-dependent effects of single candidate compounds on J2s of *G. pallida* and *M. incognita*

Of all four tested synthetic compounds, the activity of *M. incognita* J2s was influenced very little compared to *G. pallida,* with 100% nematode mobility observed in each treatment (*PPN species:* $F_{1, 386} = 391.644$, $P < 0.001$, Figure 5.4.8). As a result, in all further analyses we focussed on metabolite effects on *G. pallida* as it was more clearly influenced by different compounds.

A time effect was observed in all metabolite treatments with the percentage mobility of *G. pallida* J2s decreasing at each time point until NaOH addition after a clear increase in activity was observed again (*Time*Treatment:* $F_{1, 904} = 151.671$, $P < 0.001$, Figure 5.4.8). This suggests that over the tested time course all metabolites reduced nematode activity, but no effect on nematode mortality was observed as demonstrated by a reactivation following the application of NaOH.

When treated with pyoluteorin, all metabolite concentrations significantly reduced *G. pallida* mobility over time in a dose-dependent manner (*Concentration:* $F_{1, 7} = 4.9158$, $P < 0.001$, Figure 5.4.8 E). Post hoc Tukey analyses of this revealed the lowest concentration of pyoluteorin caused the weakest, but still significant, effect on *G. pallida* ($P = 0.002$). Following NaOH addition at the final time point, nematodes in each pyoluteorin concentration displayed similar levels of reactivition. However, only a certain level of activity was regained indicative of some levels of mortality (*Concentration:* $F_{7, 38} = 20.7925$, $P < 0.001$, Figure 5.4.8 E).

Concentration-dependent effects that were somewhat non-linear were also observed in the other three *Pseudomonas* metabolite treatments: Orfamide A - (*Concentration:* $F_{7, 40} = 2.415$, $P = 0.0368$, Figure 5.4.8 A), Orfamide B (*Concentration:* $F_{7, 40} = 3.338$, $P = 0.0068$, Figure 5.4.8 C) and DAPG (*Concentration:* $F_{7, 40} = 4.481$, $P < 0.001$, Figure 5.4.8 G). Moreover, within the time-frame of the experiment, the effects were observed at the level of mobility with no effect on nematode mortality: Orfamide A - (*Concentration:* $F_{7, 40} = 42.42$, $P = 0.204$, Figure 5.4.8 A), Orfamide B (*Concentration:* $F_{7, 40} = 75.37$, $P = 0.0713$, Figure 5.4.8 C) and DAPG (*Concentration:* $F_{7,}$

$_{40}$ = 59.17, *P* = 0.489, Figure 5.4.8 G). This suggests that only pyoluteorin affected both *G. pallida* J2 activity and viability, while other compounds only reduced nematode activity.

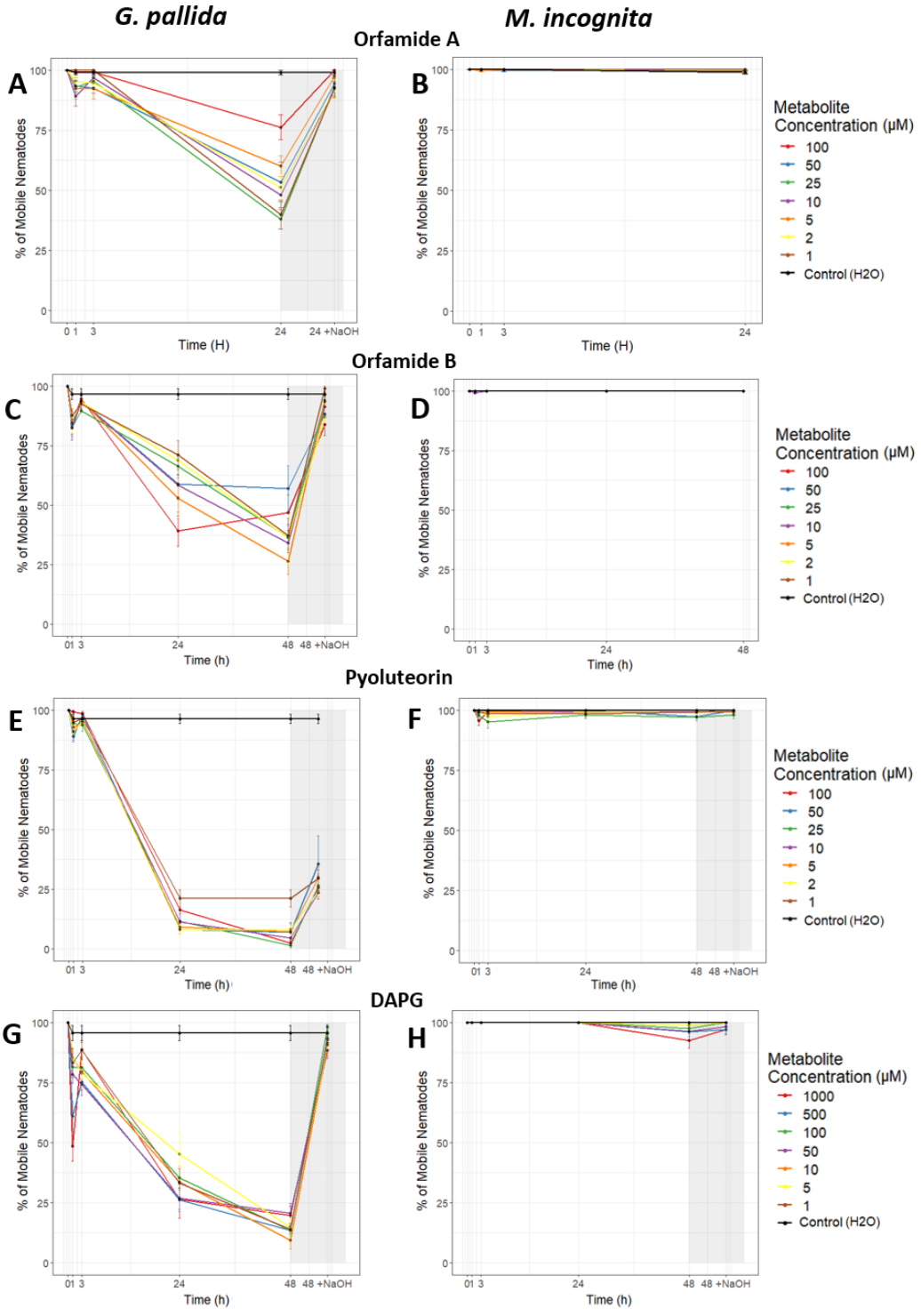

Figure 5.4.8 Percentage of mobile plant parasitic nematodes when exposed to different synthesised *Pseudomonas* metabolites before (white area) and after (grey area) chemical stimulus. The left column of panels show the effects on J2s of *G. pallida* and the right column of panels on J2s of *M. incognita*. Each row of graphs represents J2 mobility upon exposure to: Orfamide A (A-B), Orfamide B (C-D), Pyoluteorin (E-F) and DAPG (G-H). The lines denote the range of concentrations PPN species were exposed to, with the DAPG panels differing in concentration range. The shaded areas on the right side of the panels represent the NaOH application to distinguish between dead and living and responding nematodes. All bars show the standard error of the mean (+/-1 SEM) based on 6 replicates.

5.4.5.2 Nematicidal activity of cyclic lipopeptide extracts on J2s of *G. pallida* and *M. incognita*

We next tested whether the isolated CLP extracts had effects on PPN activity (Figure 5.4.9). While the CLP extracts appeared to affect both PPN species, they clearly had a stronger effect on *G. pallida* as observed in the previous experiments (*PPN species:* $F_{1, 28}$ = 23.4112, P < 0.001, Figure 5.4.9 A-B). Similarly, while the mobility of both PPN species decreased over time, nematode mobility was fully regained following NaOH addition (*Time:* $F_{4,116}$ = 20.5253, P < 0.001, Figure 5.4.9 A-B). Only CLP extract CHA0 A had some long-lasting effects on *M. incognita* as the nematode activity was only partially regained in these treatments (*Metabolite*PPN:* $F_{4, 10}$ = 4.522, P < 0.001, Figure 5.4.9 B).

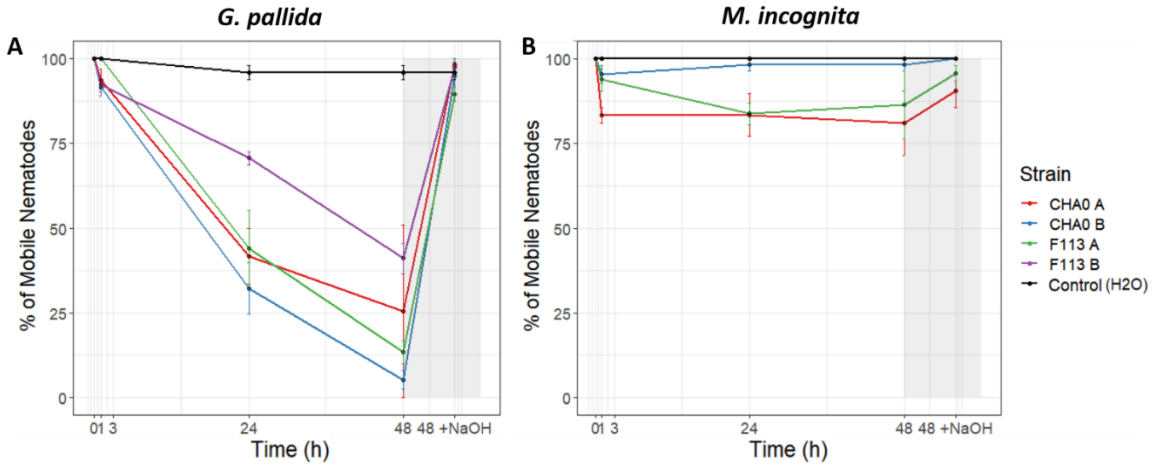

Figure 5.4.9 Percentage of mobile plant parasitic nematodes when exposed to isolated *Pseudomonas* cyclic lipopeptide extracts before (white area) and after (grey area) chemical stimulus. The panel (A) shows the effects on *M. incognita* J2s and the panel (B) on *G. pallida* J2s. Each line denotes the CLP extract the PPNs were exposed to which were isolated from two of our *Pseudomonas* strains: CHA0 and F113. The shaded areas on the right side of the panels represent the NaOH application to distinguish between dead and living and responding nematodes. All bars show the standard error of the mean (+/-1 SEM) based on 3 replicates.

5.4.5.3 Nematicidal effects of candidate inhibitory compound combinations on J2s of *G. pallida* and *M. incognita*

As *Pseudomonas* strains could produce a number of different inhibitory metabolites at the same time, we finally tested if identified metabolites might have a greater influence on PPN activity in combination. Comparisons between metabolite effects on the two PPN species reflect earlier observations with *M. incognita* mobility being influenced very little by metabolite exposure compared to the control treatment, even when using a combination of all four metabolites (*Metabolite presence:* $F_{1, 92}$ = 187056, P = 0.0836 Figure 5.4.10). In contrast, *G. pallida* mobility was

strongly inhibited by all metabolite combinations (*Metabolite presence:* $F_{1, 92}$= 500.1321, P = 0.0319, Figure 5.4.10).

Similar to previous experiments, the effects on *G. pallida* were mainly transient and activity was regained following NaOH addition (*Time:* $F_{5, 189}$ = 146.9097, P < 0.001, Figure 5.4.10). However, while these time effects showed a similar pattern in all combinations (*Combination:* $F_{3, 90}$ = 1.7364 P = 0.1652, Figure 4.4.10), clear differences were observed in the reactivation between treatments following NaOH addition, and in contrast to individual compounds, some combinations caused significant mortality (*Combination:* $F_{15, 32}$ = 63.143, P < 0.001, Figure 5.4.10). More specifically, the presence of pyoluteorin was consistently linked with reduced *G. pallida* mobility in every combination (*Combination*Pyoluteorin:* $F_{15, 932}$ = 692.9824, P = 0.1652, Figure 5.4.10), and in line with previous experiments, DAPG (P = 0.9261), Orfamide A (P = 0.901) and Orfamide B (P = 0.4947) had no effects on nematode mortality.

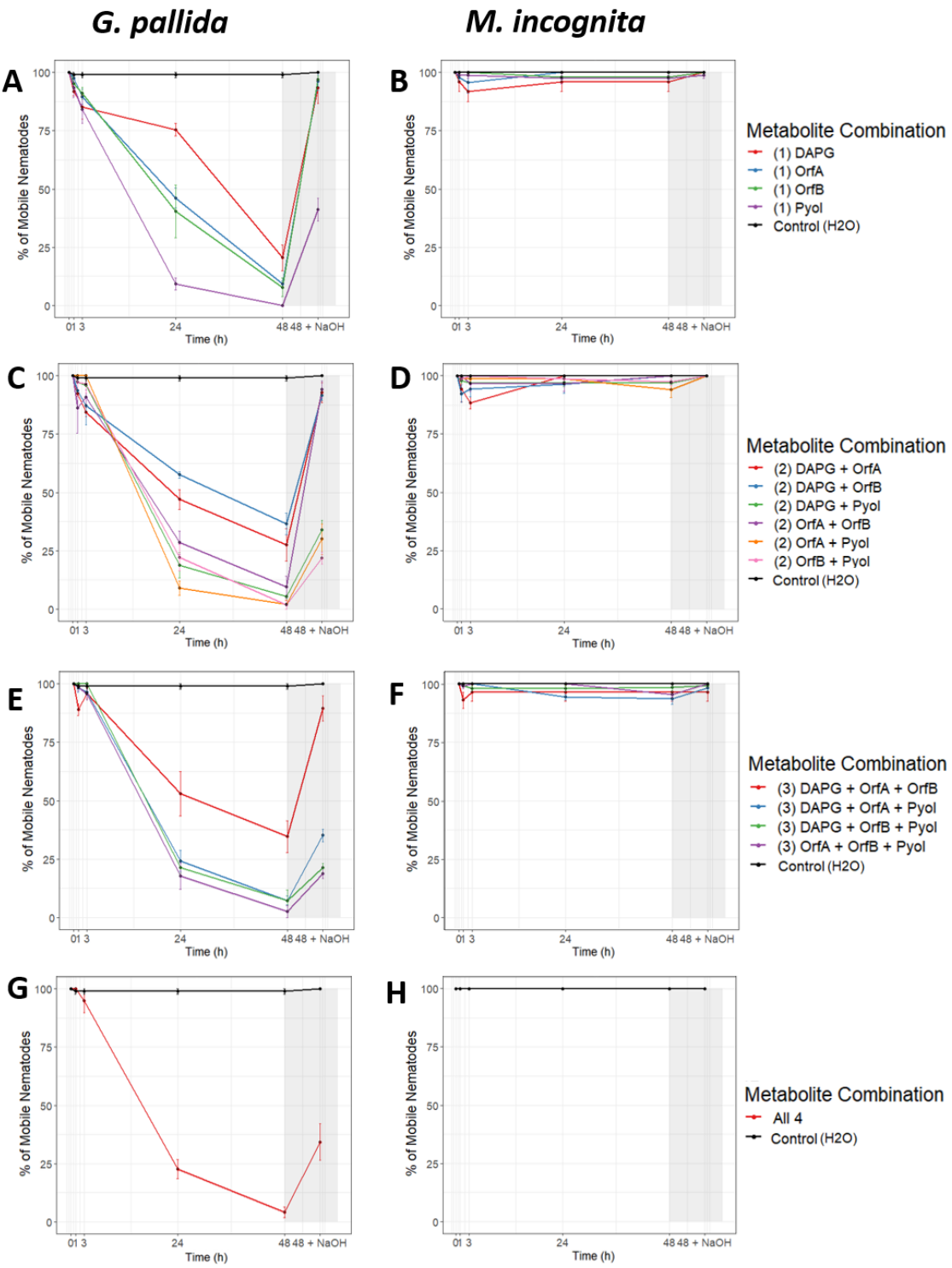

Figure 5.4.10 Percentage of mobile plant parasitic nematodes when exposed to *Pseudomonas* metabolite combinations before (white area) and after (grey area) chemical stimulus. The left column of panels show the effects on *M. incognita* J2s and the right column of panels on *G. pallida* J2s. Each row represents PPN mobility upon exposure to combinations of: 100 µM Orfamide A, 100 µM Orfamide B, 100 µM Pyoluteorin and 1000 µM DAPG in single (A-B) and 2- (C-D), 3- (E+F) and 4-way interactions (G+H). The shaded areas on the right side of the panels represent the NaOH application to distinguish between dead and living and responding nematodes. All bars show the standard error of the mean (+/-1 SEM) based on 3 replicates.

5.4.6 *In vivo* PPN greenhouse experiments

In the last part of this chapter we tested if any of the inhibitory effects shown by *Pseudomonas* CHA0 and Pf-5 strains could protect tomato plants from PPN infections *in vivo* greenhouse experiment.

5.4.6.1 *Pseudomonas* effects on PPN infection rates during greenhouse experiments in tomato

Following acid fuchsin staining, nematode infection levels were quantified in tomato plant roots. Despite an observed trend, the *Pseudomonas* treatments did not appear to significantly reduce nematode infection levels compared to the control treatment in either the short-term (ST) *G. pallida* (*Experiment:* $F_{2\ 21}$= 2.242, P = 0.131, Figure 5.4.11 A) or the ST *M. incognita* experiments (*Experiment:* $F_{2,20}$ = 0.623, P = 0.546, Figure 5.4.11 B). Similarly, *Pseudomonas* treatments in the long-term (LT) *M. incognita* greenhouse experiment did not significantly reduce nematode multiplication compared to the control water treatment. In contrast, multiplication of *M. incognita* on plants that were treated with *Pseudomonas* Pf-5 strain appeared to be considerably higher than on the control plants (*Experiment:* $F_{2,63}$=10.83, P < 0.001, Figure 5.4.11 C).

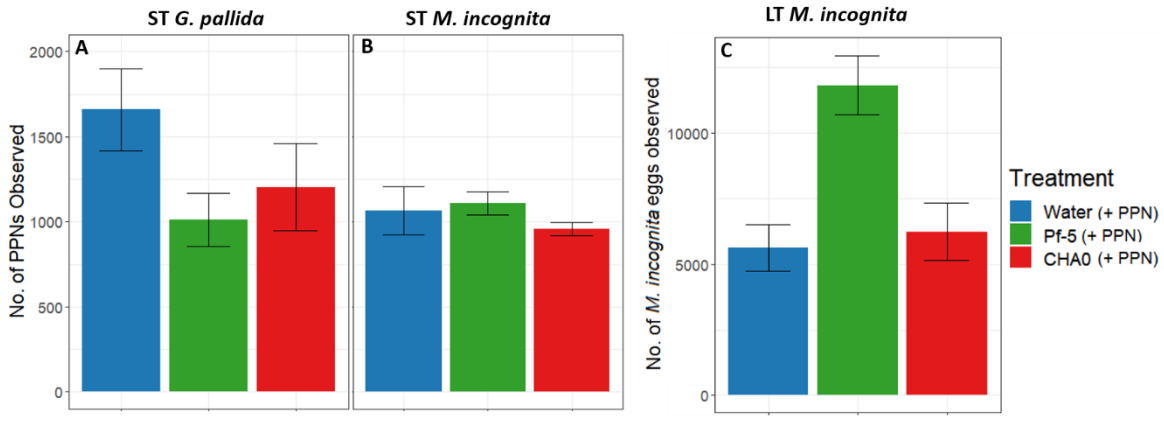

Figure 5.4.11 Plant parasitic nematode infection levels on tomato plant root systems in all greenhouse experiments. The panels displays the observed number of PPNs quantified per gram of root tissue in the (A) short-term *G. pallida* and (B) short-term *M. incognita* greenhouse experiments. Panel (C) displays the quantity of *M. incognita* eggs isolated per root system of tomato plants in the long term *M. incognita* greenhouse experiment. All bars show the standard error of the mean (+/-1 SEM) based on 7-9 replicates.

5.4.6.2 Quantification of *Pseudomonas* rhizosphere colonization

To test if the lack of protective effect by *Pseudomonas* strains was due to poor colonisation, bacterial numbers were determined at the end of each greenhouse experiment (Figure 5.4.12). Recovered *Pseudomonas* numbers were significantly lower than the initial inoculated *Pseudomonas* densities in the short-term *G. pallida* (*Recovery:* $F_{1, 132}$= 4.4 x10^{11}, $P < 0.001$, Figure 5.4.12 A), short-term *M. incognita* (*Recovery:* $F_{1, 132}$= 5.8x10^{11}, $P < 0.001$, Figure 5.4.12 B) and long-term *M. incognita* (*Recovery:* $F_{1, 132}$= 2.7 x10^{11}, $P < 0.001$, Figure 5.4.12 C) experiments. Moreover, recovery of bacteria was extremely variable across the three experiments (Experiment*:* $F_{2, 198}$= 43.773, $P < 0.001$, Figure 5.4.12). *Pseudomonas* was also present in similar densities in the control treatments in both *M. incognita* ST and LT experiments. Recovery from CHA0 treatment was significantly higher in the ST *G. pallida* experiment (*Experiment:* $F_{5, 66}$= 6.255, $P < 0.001$, Figure 5.4.12 A) and LT *M. incognita* experiment (*Experiment:* $F_{2, 198}$= 2.443, $P = 0.0431$, Figure 5.4.12 C), and not in the ST *M. incognita* experiment (*Experiment:* $F_{5, 66}$= 1.32, $P = 0.267$, Figure 5.4.12 B).

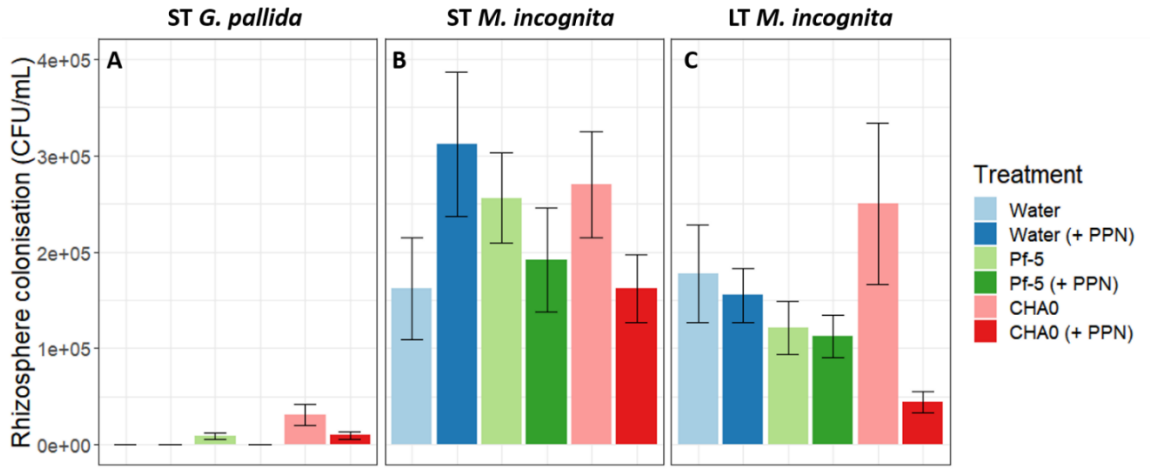

Figure 5.4.12 *Pseudomonas* densities in the tomato rhizosphere at the end of the three greenhouse experiments. The three panels display *Pseudomonas* CFU/mL counts recorded during the (A) short-term *G. pallida*, (B) – short-term *M. incognita* and (C) long-term *M. incognita* greenhouse experiments. All bars show the standard error of the mean (+/-1 SEM) based on 7-9 replicates.

5.4.6.3 *Pseudomonas* effects on plant chlorophyll fluorescence during the greenhouse experiments

To observe whether nematode infection or *Pseudomonas* treatment influenced plant health we monitored chlorophyll fluorescence as an indication of plant health. Chlorophyll fluorescence was monitored for the entire short-term *G. pallida* experiment but no differences were observed between treatments (*Treatment:* $F_{3, 49}$= 8.6, $P = 0.7372$, Figure 5.4.13 A). Fluorescence of plants

was only recorded at the final time point for the short-term *M. incognita* experiment (Figure 5.4.13 B). However, no difference between treatments was observed (*Treatment:* $F_{1, 18}$ = 1.15, *P* = 0.371, Figure 5.4.13 B). Chlorophyll fluorescence decreased significantly over time only in the long-term *M. incognita* greenhouse experiment (*Treatment:* $F_{4, 72}$= 8.6, *P* = 39.93, Figure 5.4.13 C). Post hoc Tukey analyses at the final week revealed the "Water + PPN" treatment resulted in significantly lower fluorescence than the other treatments (*P* = 0.0117), which suggest that *Pseudomonas* strains had some positive effects on the plant health.

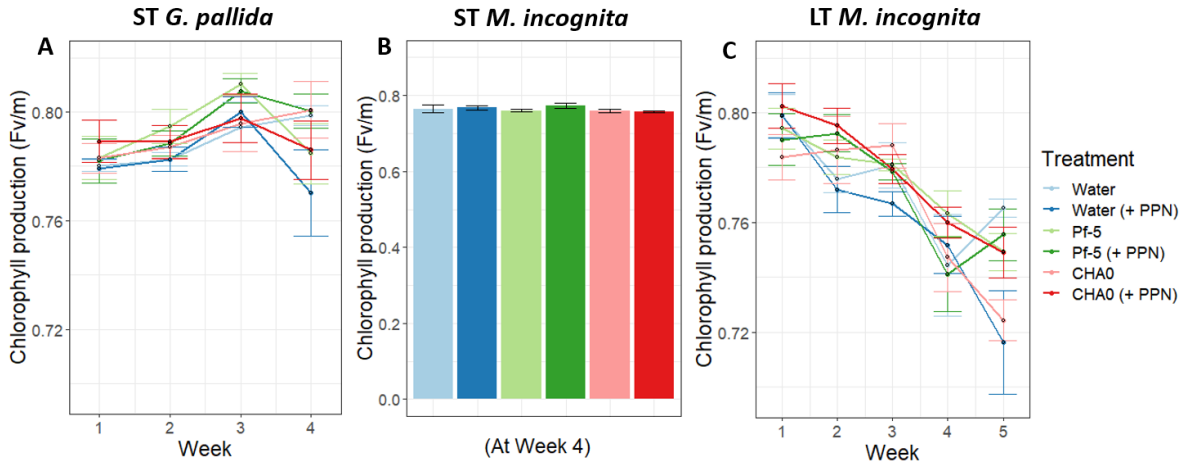

Figure 5.4.13 Chlorophyll fluorescence measurements recorded during three greenhouse experiments. The three panels display the chlorophyll fluorescence measurements recorded during the (A) short-term *G. pallida*, (B) - short-term *M. incognita* and (C) long-term *M. incognita* greenhouse experiments. Each line or column show the standard error of the mean (+/-1 SEM) based on 7-9 replicates.

5.4.6.4 *Pseudomonas* effects on plant growth during the greenhouse experiments

Phenotypic measurements of the plants were recorded at the end of each greenhouse experiment including root biomass, above ground plant biomass and plant height (Figure 5.4.14). Considering the probiotic effects of *Pseudomonas* treatment, a positive effect on root biomass was found in the absence of PPNs. CHA0 treatment resulted in a greater root biomass compared to the control treatment in both the ST *M. incognita* (*ST M. incognita:* $F_{2, 19}$= 4.343, *P* = 0.028, Figure 5.4.14 B) and the LT *M. incognita* (*LT M. incognita:* $F_{2, 21}$= 5.152, *P* = 0.0151, Figure 5.4.14 C) experiments. However, this effect was not visible in the presence of PPNs. Root biomass dry weights were significantly higher in the infected root systems in the short term experiments (*Experiment*PPN infection:* $F_{2, 135}$= 85.13, *P* < 0.001, Figure 5.4.14 A-C). The infected root systems could be heavier due to nematode infection, particularly the root galling in *M. incognita*.

Moreover, roots were also preserved in glycerol which may have influenced the dry weight mass as not all glycerol can be removed by washing which makes comparisons between the weights of PPN-infected roots not a good proxy for plant health. In support for this, post hoc analyses revealed that biomasses were not higher for infected roots in the LT *M. incognita* experiment where the roots were not stained with glycerol (P = 0.97).

The above ground biomass of tomato plants did not differ significantly between six treatments in the presence or absence of PPNs in either the ST *G. pallida* (*ST G.pallida:* $F_{5, 44}$= 1.128, P = 0.36, Figure 5.4.14 D) or the ST *M. incognita* experiments (*ST M. incognita:* $F_{5, 41}$= 1.496, P = 0.212, Figure 5.4.14 E). In the LT *M. incognita* experiment, there was a trend of all four *Pseudomonas*-inoculated treatments having a greater biomass in the absence of nematodes but this was not statistically significant (*LT M. incognita:* $F_{5, 38}$= 2.266, P = 0.0673, Figure 5.4.14 F).

In the case of plant height, significant variation between treatments was only observed in the *G. pallida* experiment with the CHA0 and water treated plants growing significantly taller than those in the Water (+ PPN) treatment (ST *G. pallida:* $F_{5, 44}$= 2.561, P = 0.0.0405, Figure 5.4.14 F). In conclusion, no clear beneficial effects of *Pseudomonas* strains were observed *in vivo.*

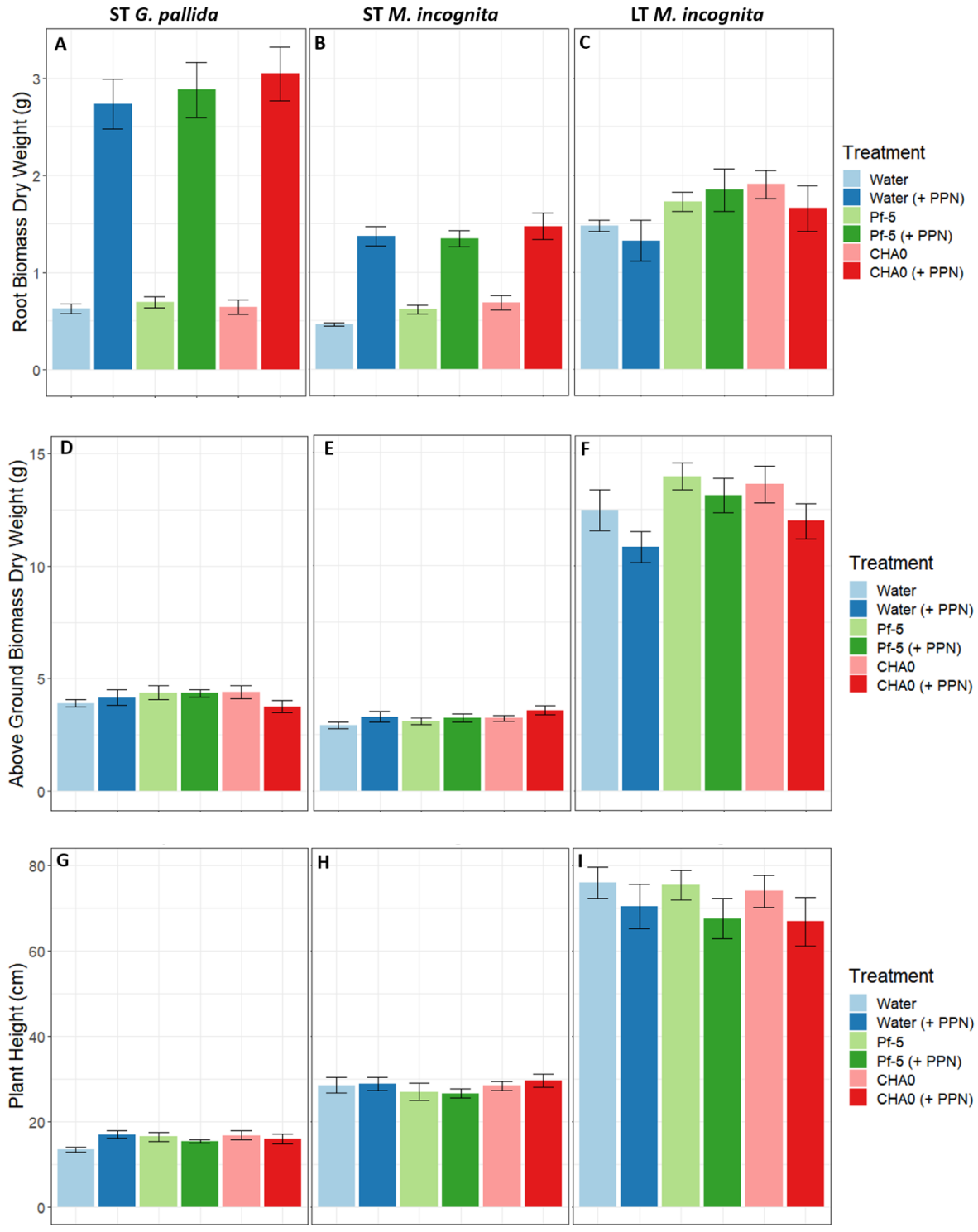

Figure 5.4.14 Tomato plant biomass and height measurements during three tomato experiments. The three rows display dry weight biomass (g) measurements (A-C), above ground biomass (g, D-F) and plant height (cm, G-I). Each column represents data from the short-term *G. pallida* (left), short-term *M. incognita* (middle) and long-term *M. incognita* (right) greenhouse experiments. All bars show the standard error of the mean (+/-1 SEM) based on 7-9 replicates.

5.5 Discussion

5.5.1 Chapter Overview

The aims of this study were to screen and identify effective *Pseudomonas* biocontrol bacterial strains against plant parasitic nematodes in order to subsequently validate their efficacy *in vivo* in the tomato rhizosphere. Initial experiments used *C. elegans* as a nematode model followed by assays with two plant parasitic nematode species (*Meloidogyne incognita* and *Globodera pallida*). *C. elegans* interactions with *Pseudomonas* were strain-dependent whereas all PPN species were susceptible to all *Pseudomonas* cultures. Genomic screening of the *Pseudomonas* strains identified potentially important secondary metabolite clusters including DAPG, pyoluteorin and orfamides. Mass spectrometry confirmed the presence of DAPG in culture supernatant which could be responsible for antagonism observed and it also revealed other putative metabolites which may contribute to biocontrol. Further experiments to validate the efficacy of these secondary metabolites against all three nematode models found that *G. pallida* was more sensitive to *Pseudomonas* metabolites, and that the pyoluteorin was the most inhibitory compound tested along with the CLP extracts. Two *Pseudomonas* strains were selected to investigate their biocontrol potential in the tomato rhizosphere *in vivo*. *In vivo* results reinforced the challenges faced when reproducing biocontrol effects in more complex systems. More work is thus needed to understand bacterial biocontrol activity in the plant rhizosphere.

5.5.2 Using *C. elegans* as a model species to screen inhibitory *Pseudomonas* species and linking nematicidal activity with the mechanisms of inhibition

We chose to first conduct *Pseudomonas* biocontrol screening with the *Caenorhabditis elegans* nematode model. Using *C. elegans* as a model species highlighted potential *Pseudomonas* biocontrol strains such as CHA0 and Pf-5. This was evident in both feeding assays and survival assays. These results also showed that the growth media in which bacterial strains were cultured had a large effect on their interactions with *C. elegans*. When *Pseudomonas* strains were cultured in CPG growth medium, which contains a larger concentration of glucose, the bacteria showed much greater antagonism to the survival of *C. elegans*. The data is in agreement with other authors who have reported that secondary metabolite production is heavily influenced by composition and concentration of culture media (Köhl, Kolnaar and Ravensberg, 2019). For example, *Pseudomonas* biocontrol activity against the fungal pathogen *Sclerotinia sclerotiorum* is enhanced in the presence of glucose (Heidari-Tajabadi *et al.*, 2011). Glucose has also been shown

to affect other biocontrol bacteria (Yang *et al.*, 2019). Moreover, various carbon resources utilised by *Bacillus amyloliquefaciens* can influence its antimicrobial activity and suppression of the bacterial pathogen *R. solanacearum* (Yang *et al.*, 2019). *C. elegans* also have reduced preference for *Pseudomonas* strains that produce higher levels of pyoluteorin which can act as a repellent (Neidig *et al.*, 2011). This may have influenced feeding behaviour as was also observed in this study. Mass spectrometry investigations into the secondary metabolite content showed detected DAPG concentrations were higher in the supernatant of strains grown in CPG providing further evidence that *Pseudomonas* strains produce higher amounts of metabolites in CPG nutrient environments.

C. elegans interactions with commercially available *Pseudomonas* metabolites including DAPG, pyoluteorin and orfamides were less detrimental than the direct *Pseudomonas* screening assays. However, all *Pseudomonas* metabolites suppressed nematode activity to an extent. DAPG has been previously reported to influence *C. elegans* egg laying and fertility (Meyer *et al.*, 2009; Neidig *et al.*, 2011) and our results have built on this to suggest that other *Pseudomona*s metabolites can also influence egg laying and fertility. The slowest developmental rates occurred for eggs hatched in the presence of CLP extracts. The CLP extracts may have been the most effective as they could potentially contain more than one individual type of cyclic lipopeptide and/or other metabolites and could also be in a higher concentration. It was reported in the general methods (Chapter 2) that these CLP samples had very visible colouration, whereas the purchased synthesised orfamides appear as a white powder, which suggests other metabolites might have also been isolated in these samples. Flury *et al.* (2017) reported how different orfamides in *Pseudomonas* strain CHA0 can act synergistically with other defence mechanisms such as hydrogen cyanide, one behaving as a toxin and the other acting as a surfactant, against insect larvae. Loper *et al.* (2016) also reported multiple metabolites including orfamides in *Pseudomonas* strain Pf-5 contributed to an increased oral toxicity against *Drosophila melanogaster* as an insecticide. In the current study, the high nematicidal activity of eCLP extracts could have thus been due to presence of multiple different compounds, which could be studied using mass spectrometry in the future. The mechanisms behind the *Pseudomonas* and *C. elegans* interactions have not yet been investigated in detail and DAPG is the only metabolite that has been previously tested on nematodes (Imran A. Siddiqui and Shaukat, 2003). Neidig *et al.* (2011) also describe how some *Pseudomonas* strains such as *P. protegens* Pf-5 possess type VI secretion systems, which function by secreting toxins such as peptidoglycan hydrolase and phospholipase to kill competing bacteria (Decoin *et al.*, 2014). This indicates that they have mechanisms other than

the secretion of secondary metabolites that could have nematicidal activity and therefore explain why the metabolite effects we observed were not as powerful as anticipated. Further tests could also be conducted to investigate which specific biocontrol properties could suppress PPNs. For example, the negative effects of volatile organic compounds (VOCs) on nematodes could be reduced by using activated charcoal, which suggests that nematicidal effects can be triggered by gaseous antimicrobials produced by biocontrol bacteria (Zhai *et al.*, 2018).

5.5.3 Testing screened *Pseudomonas* strains from earlier *C. elegans* studies against plant parasitic nematodes to determine any *in vitro* biocontrol effects

We chose to focus on *Pseudomonas* cultured in CPG growth medium to study PPN interactions due to the minimal inhibitory effects observed against *C. elegans* compared to when strains were cultured in NB growth media. Exposing J2s of the plant parasitic nematodes *G. pallida* and *M. incognita* to our eight *Pseudomonas* strains grown in CPG broth resulted in 100% mortality in every treatment. Strain F113 was the only strain to have a slower effect on viability, which is consistent with it also being a weaker antagonistic strain against *C. elegans* also. These mortality effects on PPNs differ to *C. elegans* in that all the strains appeared to be highly effective at killing PPNs, suggesting the biocontrol strain variation was less important. PPNs cannot ingest the *Pseudomonas*, yet their mortality rates were high in a short period of time suggesting that the *Pseudomonas* strains were all able to produce a product which increased nematode mortality. The nematicidal effects were much clearer with *G. pallida* and it is not yet clear as to why this is the case. It is well understood that the parasitic processes and feeding site establishment of the two nematode species differ during plant root invasion (Fuller, Lilley and Urwin, 2008). However differences in the sensitivity of the two species to *Pseudomonas* remains to be studied in greater detail. Fluensulfone is a newly registered nematicide and was tested for its nematicidal activity against both the *C. elegans* model and *M. incognita* (Kearn *et al.*, 2014). The authors revealed *C. elegans* was similarly susceptible to the irreversible effects of fluensulfone on egg hatching and survival that were also observed during *M. incognita* exposure. The effects of fluensulfone appeared to be distinct from previous nematicide modes of action which might be why *C. elegans* and *M. incognita* results were more comparable (Kearn *et al.*, 2014). Investigating physical interactions of *Pseudomonas* and all nematode models is vital for a greater understanding in biocontrol efficacy. Of the *Pseudomonas* metabolites tested against the PPNs, pyoluteorin appeared to be the only metabolite to impose any mortality on *G. pallida* nematodes. Whereas the CLP extracts had the strongest effects on *M. incognita* PPN populations, they did not significantly influence their mortality. As mentioned in Chapter 3 (Section 3.5.3), without further

investigation through mass spectrometry it cannot be certain what metabolites were present following the crude extraction process.

It was hypothesised that using *C. elegans* as the initial nematode model would predict similar outcomes in PPNs, however, this was not entirely the case when making comparisons between the *Pseudomonas* direct culture or metabolite experiments. PPNs in general were more susceptible to *Pseudomonas* culture than *C. elegans* were, which puts into question whether *C. elegans* is a good model in PPN studies. Results suggested that *Pseudomonas* toxicity effects on *C. elegans* were not feeding-related which validates that the structural differences in feeding does not account for differences is biocontrol effects. One possible explanation could be that PPNs were more susceptible as they are half the size of *C. elegans* and that effects of secondary metabolites would have a greater effect on them e.g. by crossing the cuticle more quickly. Kearn *et al.* (2014) found that the nematicidal effects of fluensulfone which were observed on PPNs also occurred with *C. elegans* but only when applied at greater doses. They suggested this might be due to varied metabolism or sensitivity to the nematicide (Kearn *et al.*, 2014). In the metabolite experiments, the *Pseudomonas* CLP extracts in particular all displayed varying long-lasting inhibitory effects on *C. elegans* whereas suppression against PPNs was relatively much smaller. They also affected *C. elegans* development the most which has yet to be investigated in PPNs. *C. elegans* studies made it easier to screen for specific strains of interest which highlighted key metabolites that proved to be effective to an extent against PPNs. Additionally, as *C. elegans* is a free-living nematode – as opposed to the biocontrol target parasitic nematodes –this poses the question of whether *Pseudomonas* supernatants and/or their metabolites would have similar impacts on non-parasitic microorganisms in soil communities and therefore their suitability to perform as successful biocontrol agents.

5.5.4 *In vivo* validation of screened *Pseudomonas* biocontrol strains with tomato plants and PPNs highlighted limitations of upscaling experiments from growth chambers to glasshouses

Clear validation of *in vitro* observations of *Pseudomonas* biocontrol effects against PPNs was not achieved during *in vivo* tomato experiments. This was particularly the case for *M. incognita* infections in both short and long term glasshouse experiments. *G. pallida* remained the more susceptible PPN species to *Pseudomonas* during *in vivo* experiments, which was also previously observed *in vitro,* with a general trend of reduced nematode infection levels in *Pseudomonas*-treated root systems. However, these results were not significant. A number of factors could have

hindered the *in vivo* biocontrol success of the selected *Pseudomonas* strains. It was evident that there was a variation in *Pseudomonas* densities recovered from the root systems of the tomato plants. The same dosage of *Pseudomonas,* based on a previous study by Hu *et al.* (2016), was applied in each greenhouse treatment. This dosage showed some effectiveness against the bacterial plant pathogen *Ralstonia solanacearum* in Chapter 3, though those plants were much smaller in size. It is also known that *Pseudomonas* biocontrol effects could vary between pathogens (Hayat *et al.*, 2010). The level of colonization of *Pseudomonas* in terms of CFU on plant roots directly affects the amount of DAPG produced. (Haas and Keel, 2003) describe how antibiotic concentrations produced by bacteria such as *Pseudomonas* in culture media are one or two orders of magnitude higher than that observed in soil studies. This is one explanation for why *Pseudomonas* and DAPG effects *in vitro* were not replicated *in vivo*. Variable root colonization of *Pseudomonas*, despite high inoculation densities, may result in the failure of bacteria to activate secondary metabolite gene expression which might have been why there was no clear suppression of PPN infections. Bacterial recovery was particularly low from the *G. pallida* experiment, even though this experiment showed the greatest trend of PPN suppression. It might be that colonization was more successful than observed during recovery due to the wetness of the soil when terminating the experiment. The *G. pallida* experiment was conducted in a cooler glasshouse than the *M. incognita* experiments which resulted in the soil drying out more slowly and being more wet at the end of the experiment. This might have affected how much *Pseudomonas* remained attached on the plant roots prior to washing the root systems and quantifying recovered bacteria.

In addition to the factors mentioned, we opted to use autoclaved soil in these treatments so we could focus directly on the interactions specifically between *Pseudomonas* and our pathogens. Jamali *et al.* (2009) reported abiotic factors influencing biocontrol e.g. the presence of zinc in the soil is required for DAPG production and iron is important in HCN production. As we autoclaved the soil, this may have influenced the composition which therefore might have affected the *Pseudomonas* inoculant's ability to produce secondary metabolites. Köhl, Kolnaar and Ravensberg (2019) compared nutrient concentrations in growth media and the rhizosphere and reported how those in media are 100x richer than the soil. This also adds to the explanation of why *in vitro* results were not replicated *in vivo.* Each experiment, despite having 7-10 biological plant replicates, was only conducted once because of time constraints, and a larger number of experiments would yield a firmer conclusion. Would we have had more replicates and time it would have been interesting to inoculate treatments with different *Pseudomonas* doses to

investigate if this would have made a difference. The addition of fertilizer may also aid bacterial colonization and plant protection. This was added in the long-term *M. incognita* experiment and could possibly explain why *Pseudomonas* was recovered by higher numbers. *Pseudomonas* seed treatment studies against PCNs was shown to effectively suppress nematode activity in a number of ways such as egg density, multiplication rate and root penetration (Nagachandrabose, 2020). They also showed that the recovery of bacteria was significantly higher in seed treatment applications than in soil drench applications. This supports our own results where bacterial recovery varied greatly (Nagachandrabose, 2020).

Successfully translating biocontrol effects from *in vitro* to *in vivo* applications may also have been less apparent as biocontrol effects are more efficacious over a longer time period (Siddiqui and Shaukat, 2003). *Pseudomonas* biocontrol effects against bacterial pathogens have been shown to be more effective after 6 weeks of infection in bacterial pathogen studies (Ramesh, Joshi and Ghanekar, 2009). Although this was not observed in our long term *M. incognita* experiment, all *Pseudomonas* experiments and metabolites had stronger effects on *G. pallida* than *M. incognita*. Perhaps this would have been more evident in a long term *G. pallida* experiment although nematode infections are not directly comparable to bacterial diseases. Bacterial density would increase over the course of the infection and so a later biocontrol effect could still slow disease progression, whereas once *G. pallida* J2s have invaded the roots there is less opportunity for the *Pseudomonas* to have an effect on numbers. It might be that allowing a longer time between *Pseudomonas* application and PPN inoculation might increase biocontrol efficacy. Another possibility for future study is the use of combinations of biocontrol strains; Hu *et al.* (2016) used combinations of *Pseudomonas* strains when inoculating plants and this appeared to significantly reduce disease incidence. Jamali *et al.* (2009) also mention synergistic effects of *Pseudomonas* strain combinations which can increase the expression of secondary metabolites such as DAPG in the rhizosphere. *In vivo* studies can be conducted in different ways, and there is a trade-off when choosing to consider direct interactions or having a natural representative experimental setting. The choice to autoclave the soil prior to conducting a rhizosphere experiment could potentially reduce background interactions with the natural soil community to solely focus on the biocontrol and pathogen interactions. Un-autoclaved soil would contain other organisms which could influence the biocontrol-pathogen interactions and make establishing clear effects more difficult. However, removing the underlying soil community results in an environment that is less representative of the natural agricultural settings where the biocontrol

needs to be applied. Were time and study size greater, it would be ideal to study interactions in both settings which you could then make comparisons between.

5.5.5 Conclusions

In conclusion, screening for *Pseudomonas* biocontrol strains against PPNs – first using a *C. elegans* model – revealed some variation amongst strains and suggested potential biocontrol strains. We discovered that some *Pseudomonas* metabolites were more effective than others at inhibiting the nematodes, and this appeared to be species-specific. Pyoluteorin in particular is a strong candidate of interest for future research as it was highly effective at suppressing the potato cyst nematode, *Globodera pallida*. Lastly, we highlighted the difficulty of translating results *in vitro* in the lab to *in vivo* glasshouse experiments. Although these experiments have built on the complexity of our biocontrol investigations against PPNs, and unveiled some potential mechanistic insight into *Pseudomonas* effects, it is still not entirely representative of natural rhizospheric interactions. Future research with larger greenhouse experiments are vital for a better understanding of the biocontrol potential which *Pseudomonas* PGPB possess. Understanding how to upregulate the synthesis of biocontrol metabolites such as pyoluteorin to aid secretion in field conditions could unlock a greater biocontrol potential for *Pseudomonas* bacteria.

Chapter 6 General Discussion

6.1 Overview

In the face of increasing demand to control plant pathogens and to improve food security, alternatives to agrochemicals are urgently required. The aims of this thesis were to screen for potential *Pseudomonas* bacterial biocontrol strains which would be effective in controlling two types of plant pathogens: *Ralstonia solanacearum* bacterium and plant parasitic *Meloidogyne incognita* and *Globodera pallida* nematodes by using a combination of *in vitro* lab and *in vivo* greenhouse experiments.

This research has progressed the understanding of *Pseudomonas* as potential biocontrol agents against these plant pathogens and suggest that CHA0, Pf-5 and MVP1-4 strains might be potentially effective in controlling both bacterial and nematode pathogens. CHA0, Pf-5 and MVP1-4 from initial *R. solanacearum* and nematode screening appeared to be the most promising biocontrol strains. With the nematodes this was strongly apparent with the initial *C. elegans* model, but *Pseudomonas* strain variation was not as impactful with the two plant parasitic nematode species. Identification of secondary metabolite genes based on genomics, and validation of their presence using mass spectrometry, revealed that not all candidate inhibitory metabolites were produced or actively expressed in high concentrations, at least in the growth conditions used in these experiments. Four chemical standards representing metabolites produced by *Pseudomonas* CHA0 and Pf-5 (DAPG, pyoluteorin and orfamides A and B) were selected for detailed direct *in vitro* experiments. Results showed that orfamides and pyoluteorin displayed antagonistic effects against *R. solanacearum* and plant parasitic nematodes, with pyoluteorin in particular showing long lasting effects on the mobility of *G. pallida*. This effect has not previously been reported. The long term biocontrol efficacy of *R. solanacearum* was also tested using experimental evolution, where pathogens were discovered to develop tolerance to *Pseudomonas* supernatants during a 21-day study. Specifically, *R. solanacearum* evolved tolerance to orfamides A and B, which were produced by CHA0 in the growth environment. However, several *Pseudomonas* strains were able to reduce the bacterial wilting incidence in a greenhouse experiment indicative of generalised inhibitory strategy in addition to orfamides. Interestingly, plant protection depended on the *R. solanacearum* strain identity and was only observed with one of the tested strains. With nematodes, only non-significant trends were observed in plant

protection. This suggests that *Pseudomonas* might have a biocontrol effect on *G. pallida*, but that these could be subtle at least in the used in *in vivo* greenhouse conditions. The key advances in this thesis include a more in-depth understanding of the metabolite-mediated interactions between *Pseudomonas* and bacterial and nematode plant pathogens both *in vitro* and *in vivo*. The evolution of tolerance to orfamides by *R. solanacearum* has not been reported before. Long-lasting suppression of both *R. solanacearum* and *G. pallida* by pyoluteorin also suggest that it is possible to identify secondary metabolites that are active against two largely differing plant pathogens. Such compounds thus show promise for novel biocontrol strategies in the future.

6.2 The importance of *Pseudomonas* PGPB genotypic variation when screening for biocontrol activity against *R. solanacearum*

It is important to understand how specific or generalist different biocontrol agents are and if they can effectively control a large variety of pathogen genotypes. This is necessary to ensure pathogens will be effectively controlled by the biocontrol treatment (Symondson, Sunderland and Greenstone, 2001). It will also give insight into whether a biocontrol has the ability to control multiple plant pathogens which is a desirable trait. The screening of *Pseudomonas* biocontrol strains against different strains of *R. solanacearum* revealed that all pathogen isolates – despite being isolated from various locations across England and one from Poland – responded in a similar manner to the *Pseudomonas* strains. Some genotype-genotype effects were observed when comparing the biocontrol effectiveness of the eight *Pseudomonas* strains against seven *R. solanacearum* isolates with *Pseudomonas* strain CHA0 being the most suppressive. This was evident from both direct and indirect *in vitro* assays and against all *R. solanacearum* isolates. Variation in biocontrol activity of *Pseudomonas* was predicted as the eight strains chosen originated from a study by Jousset *et al.* (2011) which had selected them specifically to cover all main genomic subgroups of *Pseudomonas*.

Pseudomonas has already been reported to be antagonistic with *R. solanacearum* previously, and this antagonism has been linked to several secondary metabolites such as DAPG, siderophores and phenazine (Ramesh, Joshi and Ghanekar, 2009), volatile organic compounds (Raza *et al*., 2016b), HCN and indole acetic acid (Kurabachew and Wydra, 2013). In addition to direct effects on the pathogens, these metabolites can induce systemic resistance in host plants enabling pre-regulation of their own defence enzymes such as phenylalanine ammonia lyase,

guaiacol peroxidase and lipoxygenase (Vanitha and Umesha, 2011). It is, however, possible that some *Pseudomonas* metabolites can become phytotoxic in large quantities, which could have negative effects on the plant health. An example of this is hydrogen cyanide produced by *Pseudomonas* PGPB, which have been shown to affect potato yields (Dowling and O'Gara, 1994). The role of biocontrol strain variation on the biocontrol activity has been tested relatively rarely (Nagarajkumar, Bhaskaran and Velazhahan, 2004; Fujiwara *et al.*, 2011). Our results showed that all *Pseudomonas* strains were effective at suppressing all UK *R. solanacearum* populations to an extent. However, some of the strains were more suppressive overall to all pathogen strains, while some showed suppression that was specific to certain *R. solanacearum* strains. Our results are in line with other reports investigating *Pseudomonas* biocontrol effects against *R. solanacearum* (Hu *et al.*, 2016), however few studies have focused on *R. solanacearum* isolated from the UK. These results show novelty by expanding on studies which generally tend to focus on one pathogen strain and show how biocontrol effects can be varied across different UK *R. solanacearum* strains.

The potential role of certain secondary metabolites identified in genome screening were tested to see if they could explain observed *Pseudomonas-R. solanacearum* interactions. *Pseudomonas* strain CHA0 was one of the two strains with the greatest number of secondary metabolite clusters identified through genome mining, which could be indicative of its high suppressiveness against *R. solanacearum.* Four metabolites DAPG, pyoluteorin and orfamides A and B all produced by NRPS clusters were tested against *R. solanacearum*. All compounds were found to suppress *R. solanacearum* growth with pyoluteorin being the most suppressive. Interestingly, while *Pseudomonas* strain Pf-5 contained the same metabolic clusters as CHA0, the CHA0 strain showed greater biocontrol ability in *in vitro* studies. This suggests that although CHA0 and Pf-5 both contain the genes for all four secondary metabolites tested, there were differences in the expression of these which could explain the discrepancies observed. A likely explanation for this difference is that Pf-5 failed to produce these compounds in the used growth media and only orfamide A production was detected by CHA0 strain based on mass spectrometry. Nagarajkumar, Bhaskaran and Velazhahan (2004) tested fourteen different *P. fluorescens* isolates for their antifungal biocontrol activity against *Rhizoctonia solani*. They saw a clear difference between strains in their antifungal activity with a variation in the amount of cell wall degrading enzyme, HCN and salicyclic acid produced. This reflects a variation in secondary metabolite expression between *Pseudomonas* stains, as observed in this study. Interestingly, not much is known about pyoluteorin-mediated suppression of *R. solanacearum*, which proved to be one of the most inhibitory compounds in this research. Further work on this compound is hence required. Also,

other potential putative antimicrobial metabolites were identified based on untargeted mass spectrometry analysis (rhizoxin, syringomycins and pseudophomin), which could have affected *R. solanacearum* growth suppression in supernatant assays. Additionally, the CLP extracts had suppressive effects against every bacterial strain and nematode species tested. It is unknown whether these extracts contained more than one metabolite. However, as F113 is known not to be able to produce orfamides, this result suggests other cyclic lipopeptides or alternative metabolites were likely present explaining the inhibition mediated by F113 strain. Alternatively, it is possible that the strain F113 is able to produce orfamide-like compounds, but the secondary metabolite cluster has not been yet identified. Together, these findings suggest that much still remain to be discovered in terms of *Pseudomonas* secondary metabolism in the context of pathogen biocontrol.

6.3 The importance of *R. solanacearum* genotypic variation when screening for *Pseudomonas* biocontrol activity

A relatively small effect of *Ralstonia* strain variation was found in comparison to *Pseudomonas* strain variation in *in vitro* biocontrol assays. There are multiple explanations for these results. Six of the seven *R. solanacearum* in this study were isolated from three different locations in the UK and one from Poland. This geographical distribution is much smaller compared to *Pseudomonas* biocontrol strains, which were originally isolated across Europe and North America. This could explain why the *Pseudomonas* variation was more apparent. It might also be that the *R. solanacearum* strains were very similar because the pathogen population in the UK is clonal (Phylotype 2, Race Biovar 3) and may originate from a single introduction (Clarke *et al.*, 2015). Alternatively, they might be similar because they originate from a similar environment (river network) leading to similar adaptations. Previous studies have shown that different *R. solanacearum* strains can respond similarly or differently to certain biocontrol agents. For example, Fujiwara *et al.* (2011) discovered that one bacteriophage could infect 15 *R. solanacearum* strains which belonged to multiple biovars exhibiting a broad infectivity range. In contrast, Yang *et al.* (2016) investigated the antibacterial effects of chitosan polymers on five races of *R. solanacearum*. Results were highly varied with some races being more susceptible to chitosan polymers than others. This showed how *R. solanacearum* genotypic variation could influence biocontrol outcomes. While, it is not yet clear how diverse or homogenic the *R. solanacearum* strains used in this thesis are, ongoing genomic sequencing work in the lab is likely

to reveal this.

To study the role of *R. solanacearum* strain variation in *Pseudomonas* biocontrol potential *in vivo*, we compared the efficacy of CHA0 and Pf-5 strains against two UK strains (#1 and #7) in tomato model. Interestingly, all tested *Pseudomonas* strains (CHA0, Pf-5 and Sbw25 control strain) were associated with reduced levels of bacterial wilting incidence. However, such protective effect was only evident with the *R. solanacearum* strain #7 (no biocontrol effects in the case of strain #1). First, this suggests that pathogen inhibition was likely mediated by DAPG or another similar compound that was present in all *Pseudomonas* strains. Interestingly, while we did not observe significant differences in ancestral *R. solanacearum* strains #1 and #7 susceptibility to DAPG, the strain #7 showed increased susceptibility to DAPG relative to strain #1 in our selection experiment, if they both had been exposed to CHA0 or Pf-5 strains' supernatant. It is thus possible that *R. solanacearum* strain differences were also driven partly by evolutionary changes, which have previously shown to take place within one tomato plant generation in the rhizosphere (Wang *et al.*, 2017, 2019). Alternatively, it is possible that the *R. solanacearum* tolerance to *Pseudomonas* antimicrobials was activated through phenotypic plasticity and that strain #1 was able to do this more efficiently by adjusting its gene regulation accordingly. For example, failures to express genes related multidrug efflux pumps or outer cell membrane could explain why the strain #7 was more susceptible to *Pseudomonas* strains (Brown, Swanson and Allen, 2007; Raman *et al.*, 2019). Efflux pumps are common mechanisms of resistance for bacteria such as *E. coli* and *Pseudomonas aeruginosa* against antibiotics used in human infections such as tetracycline and β-lactam antibiotics (Schweizer, 2003; Poole, 2007). Similarly, strain differences in their ability to grow and reach high densities in the rhizosphere could have affected quorum-sensing-mediated expression of key virulence factors (Kumar *et al.*, 2016). Finally, differences could be explained by responses to host plant defences during the *in vivo* experiments, which have been shown to differ depending on which *R. solanacearum* strain they are exposed to (Milling, Babujee and Allen, 2011). This could have impacted the ability of the pathogen to evade or trigger host recognition and their ability to cause disease symptoms.

We have shown that *R. solanacearum* strains respond differently to *Pseudomonas* biocontrol *in vivo* which highlights the importance of testing and validating biocontrol efficacy in greenhouse and field experiments. Variability of pathogen genotypes should also be considered when developing biocontrol methods. Generalist biocontrol bacteria — which can impose effects

on multiple pathogens — can be more advantageous when it comes to implementing pathogen biocontrol on a large geographic scale typically containing higher amount of genetic variation (Yang *et al.*, 2016). Moreover, using a combination of *Pseudomonas* could have a greater level of suppression on *R. solanacearum* through an increased number of modes of action as shown in previous studies (Maurhofer *et al.*, 2004; Hu *et al.*, 2016). Not only can combinations of *Pseudomonas* strains aid pathogen suppression by increasing the diversity secondary metabolites produced, they could also have negative effects on pathogen invasiveness by taking up a larger niche breadth via competing for space and nutrients more efficiently with the pathogen (Wei *et al.*, 2015). While no clear benefits were found when combining CHA0 and Pf-5 strains, potentially better outcomes could be observed using if CHA0 would have been combined with relatively more suppressive MVP1-4 strain. Jousset *et al.* (2011) found that using genetically dissimilar *Pseudomonas* biocontrol strains resulted in a more functionally dissimilar community which was able to better exploit complex resources. As CHA0 and Pf-5 have a strong genetic similarity (Ma *et al.*, 2016), it might be combinations with more dissimilar strains could improve their establishment *in vivo* and biocontrol effects on pathogens. More work is thus needed to explore the efficacy of *Pseudomonas* strain combinations and their effects at the community level in the rhizosphere (Maurhofer *et al.*, 1992; Dowling and O'Gara, 1994; Hu *et al.*, 2016).

6.4 Evolution of tolerance to biocontrol agents in *R. solanacearum*

To ensure long-term efficiency of biocontrol, it is important that pathogens do not evolve tolerance or resistance under repeated exposure. This was modelled in a simplified lab experiment *in vitro* and it was found that *R. solanacearum* evolved improved tolerance to a subset of *Pseudomonas* strains within just three weeks of continuous exposure to antimicrobials. By using a combination of mass spectrometry and direct microbiological assays, it was identified that pathogens evolved tolerance specifically to the cyclic lipopeptide orfamides, which has not been observed previously. Also, some evidence of tolerance to DAPG and pyoluteorin was also observed but these effects were much weaker (DAPG) or observed also with the control selection lines (pyoluteorin) which is indicative of selection mediated by growth media instead of *Pseudomonas* supernatants.

Mechanistically, tolerance evolution was likely to be driven by *de novo* mutations as selection experiments using *R. solanacearum* populations were started from single clones. An

example of one potential tolerance mechanism could be increased efflux pump activity, which could help the pathogen to pump out *Pseudomonas* secondary metabolites before they impose negative effects on the pathogen growth. It has been suggested that *R. solanacearum* uses multidrug efflux pumps as a form of protection against host defences (Brown, Swanson and Allen, 2007; Ryan and Adley, 2013; Raman *et al.*, 2019). Polymyxin B is a lipopeptide derived from soil bacteria *Paenibacillus polymyxa* (Hsu *et al.*, 2017) for which *R. solanacearum* is often highly resistant to. The orfamides are also antimicrobial peptides and it is thus possible that slight modifications to existing resistance mechanisms could have resulted in increased orfamide tolerance. Mechanistically, polymyxin B resistance has been linked in modification in cell envelopes and increased smooth lipopolysaccharide production (Hong *et al*., 2014). Interestingly, we saw clear decreases in DAPG tolerance of LB-exposed control and supernatant-exposed populations, which correlated with improved growth in the presence of pyoluteorin. These results suggest that tolerance to these two compounds showed pleiotropic effects as the tolerance to one compound led to increased susceptibility to another (evolutionary trade-off). Evolutionary trade-offs such as these have been observed in other systems for example between phage and antibiotic resistance (Scanlan, Buckling and Hall, 2015; Burmeister *et al.*, 2020), resistance between different antifungal treatments (Hill, O'Meara and Cowen, 2015), or even between insect reproduction or immunity to insecticidal treatments (Schwenke, Lazzaro and Wolfner, 2016) to name a few. Pyoluteorin is a chlorinated polyketide antibiotic and its production is regulated by the DAPG precursor, monoacetylphloroglucinol (Kidarsa *et al.*, 2011). It is thus possible that efficient production of pyoluteorin limits the production of DAPG, and that these compounds are not produced simultaneously.

More mass spectrometry studies are needed to verify this idea, especially in co-cultures with the pathogen, as no DAPG or pyoluteorin production was observed in *Pseudomonas* monocultures. However, it remains difficult to explain why changes to pyoluteorin tolerance and DAPG susceptibility were observed in LB-exposed control populations that were not exposed to *Pseudomonas* supernatants. One potential explanation could be adaptation to the growth media, as reported by Riley *et al.* (2001), which could have affected tolerance evolution indirectly via unknown genetic correlations. This pattern is however, difficult to explain without further detailed transcriptomics studies linking antimicrobial tolerance and primary (growth) metabolism. Genome resequencing could also be used to identify potential mutations and mechanisms underlying orfamide and pyoluteorin tolerance and DAPG susceptibility between LB-exposed control and supernatant-exposed evolved populations. More work is also needed to explore if the

pathogen can evolve tolerance to *Pseudomonas* in the rhizosphere. It is suspected that if that pathogen is able to evolve tolerance within a 21-day time period *in vitro* to *Pseudomonas* antimicrobials, this might also occur *in vivo* when pathogen-biocontrol interactions could continue for a longer time period.

6.5 Considering *Pseudomonas* biocontrol efficacy against *C. elegans* nematode model and PPNs

To explore if *Pseudomonas* had the potential to act as a biocontrol against more than one type of plant pathogen, we tested the same strains against *C. elegans* and plant parasitic nematodes. By using feeding assays of *C. elegans* and *Pseudomonas* strains, it was found that some strains were chosen more often over the others indicative of chemoattractance and avoidance behaviour. The feeding (quantified by pharyngeal pumping rates) itself was associated with negative impacts on the nematode. Strains CHA0 and Pf-5 stood out clearly as highly toxic bacteria in *C. elegans* studies, as did the metabolite DAPG and CLP extracts isolated from CHA0. Pedersen *et al.* (2009) reported that *C. elegans* did not feed on *Pseudomonas* strain CHA0 reporting it as inedible which supports our results (Pedersen *et al.*, 2009). The strain CHA0 was thus highly effective against both *R. solanacearum* (Ramesh, Joshi and Ghanekar, 2009; Hu *et al.*, 2016) and *C. elegans* (Neidig *et al.*, 2011) as reported previously. Another way to investigate the mechanism of nematode killing this would be to tag *Pseudomonas* strains with green fluorescent protein prior to *C. elegans* feeding assays which may give more insight into detecting gut-related toxicity (Pedersen *et al.*, 2009). By using *C. elegans* as a first nematode model in this study, we were able to narrow the *Pseudomonas* strains of interest and identify potential secondary metabolites for direct *in vitro* metabolite experiments. This resulted in furthering the understanding of the underlying mechanisms of *Pseudomonas* biocontrol by investigating the effects of orfamides, pyoluteorin and DAPG on PPNs which helped strengthen explanations for observations made. To conclude this, *C. elegans* was a useful first nematode model in this research and results from these experiments led to novel findings of *Pseudomonas* metabolite effects on both *C. elegans* and PPNs.

Pseudomonas strain effects were less variable in PPN studies; all *Pseudomonas* strains suppressed the PPNs in culture, whereas only CHA0 and Pf-5 showed such a strong suppression in the case of *C. elegans*. Variation became more apparent between the two PPN species during individual secondary metabolite *in vitro* studies, with *Globodera pallida* being more susceptible

than *Meloidogyne incognita* to all four tested *Pseudomonas* metabolites. *G. pallida* mobility decreased over time in the presence of all metabolites (DAPG and orfamides A and B), but the addition of NaOH showed that the nematodes were still alive. A knock-out study hindering DAPG production in *P. fluorescens* strain F113 abolished *Pseudomonas* biocontrol effects on *Globodera rostochiensis* (yellow potato cyst nematode) which further supports DAPG involvement in the suppression of PPNs (Cronin *et al.*, 1997). This study also showed that *Pseudomonas* soil applications suppressed PPN juvenile mobility, but not infection numbers. Together, these previous findings support our observations of suppressed mobility of juveniles during our *in vitro* studies. It is unknown if this was the case in our *in vivo* results as the mobility of recovered juveniles was not tested. Of the metabolites tested, pyoluteorin was the most effective which might have been what deterred *C. elegans* in earlier experiments as described by Neidig *et al.* (2011). However, pyoluteorin was predicted to be produced by only two of the eight *Pseudomonas* strains used in this study. *Pseudomonas* PGPB have the potential to produce numerous secondary metabolites and this study tested only four of them. It is thus possible that also other metabolic compounds played a role in our studies. When comparing *C. elegans* and PPN studies, clear mortality was observed only with *C. elegans* and not with PPNs. This is not yet fully understood and as we have shown it is unlikely to be due to feeding effects, another physical difference between the nematode species must be responsible for this.

Pseudomonas strains CHA0 and Pf-5, which displayed biocontrol inhibition effects against *R. solanacearum* during *in vivo* experiments, were also tested for their biocontrol efficacy with the two PPNs, *G. pallida* and *M. incognita*. There was a non-significant trend that *Pseudomonas* strains reduced *G. pallida* PPN infection of tomato plants, but this was not observed with *M. incognita*. *G. pallida* J2s were more sensitive to *Pseudomonas* during *in vitro* studies, which does suggest some success in the translation of results from *in vitro* to *in vivo* studies. The key challenges faced for *in vivo* studies are successful colonisation of the plant roots by the biocontrol bacteria and then interaction with the pathogens to suppress their activity. It might be that the nematode evades the *Pseudomonas* either by detecting it before choosing to select which plant root it infects, or by travelling to parts of the plant which the *Pseudomonas* has not necessarily colonized (Neidig *et al.*, 2011). This was more difficult to see positive effects compared to *Pseudomonas-R. solanacearum* interactions which have been extensively studied and reported in the literature (Hayward, 1991; Vanitha *et al.*, 2009; Hu *et al.*, 2016) over the years compared to *Pseudomonas*-PPN interactions. However, we saw that *Pseudomonas in vitro* effects were strong against PPNs so there is clearly something hindering biocontrol effects when the experiments are

conducted in the rhizosphere. Duijff, Alabouvette and Lemanceau (1996) found *P. fluorescens* treatment significantly increased tomato plant biomass - a trend we also observed in this study. This increase in biomass may be partly due to the bacteria colonizing the hypodermal cells inside the cortical cell walls of the plant contributing to the biomass (Duijff, Alabouvette and Lemanceau, 1996). Translating the virulent effects of *Pseudomonas* interactions *in vitro* to *in vivo* has proved to be a challenge, but this work has revealed a deeper understanding of the mechanisms involved and more work is needed to develop a successful rhizospheric biocontrol.

6.6 Considering the *Pseudomonas* strains with the strongest biocontrol potential against both bacterial plant pathogen *R. solanacearum* and plant parasitic nematodes *G. pallida* and *M. incognita*

One of the aims of this thesis was to screen for effective *Pseudomonas* biocontrol strains which could suppress both *R. solanacearum* as well as both types of PPNs. *Pseudomonas* strain CHA0 displayed the strongest antagonism against *R. solanacearum* strains as shown in Chapter 3. This was evident in liquid culture and supernatant assays and is likely linked to its relatively diverse secondary metabolism as suggested by our comparative genome analysis. However, MVP1-4 strain was also highly suppressive despite possessing fewer secondary metabolite clusters. This shows that having a higher number of clusters, does not always translate into the greatest biocontrol potential. In Chapter 4, CHA0 supernatant displayed the most suppressive effects against *R. solanacearum* populations in the selection experiment, whereas MVP1-4 remained the most 'tolerance-proof' strain during fitness assays. Mass spectrometry confirmed the production of orfamide A in the supernatant of CHA0 which may have contributed to it being one of the most effective *Pseudomonas* strains in this study. Lastly, in Chapter 5 considering *Pseudomonas* biocontrol of nematodes, *C. elegans* nematodes were strongly suppressed by CHA0 and Pf-5 in survival assays. Mass spectrometry also confirmed DAPG and orfamide A production in the supernatant of CHA0 in these studies depending on which growth medium it was cultured in. *Pseudomonas* strains CHA0 and Pf-5 could thus be the best strains for controlling multiple pathogens.

It is interesting that *Pseudomonas* can suppress species across multiple kingdoms. However, this thesis is not the first time this has been reported, and previous studies have

reported *Pseudomonas* biocontrol effects against bacteria, nematodes and fungal pathogens (Pierson *et al.*, 1998; Ramesh, Joshi and Ghanekar, 2009; Hu *et al.*, 2016). The underlying mechanisms of *Pseudomonas* strains' inhibitory activity gives further insight on how *Pseudomonas* can suppress pathogens from different taxonomies. The common antimicrobial DAPG has been shown to affect bacteria, nematode and fungal pathogens (Cronin *et al.*, 1997; Maurhofer *et al.*, 2004), cyclic lipopeptide orfamides produced by *Pseudomonas* strains have been reported to cause insecticidal, fungal, algal and viral toxicity (De Bruijn *et al.*, 2007; Olorunleke *et al.*, 2015; Loper *et al.*, 2016; Aiyar *et al.*, 2017; Flury *et al.*, 2017) and volatile organic compounds produced by *Pseudomonas* can display bacterial and fungal toxicity (Zhai *et al.*, 2018). Understanding the genetics of this vast metabolic diversity, and the production of different secondary metabolites *in vitro* and *in vivo* across different environments, could thus play a key role in engineering the production of highly suppressive biocontrol bacteria.

6.7 Future development and limitations of biocontrol

Results from this thesis highlight the need for more investigations to successfully translate *in vitro* biocontrol effects into an *in vivo* setting. One option could be to research and confirm secondary metabolite expression in the soil. Results in this thesis have shown *Pseudomonas* genomes possess numerous metabolic clusters, but this does not necessarily mean they are functionally active as proven by the mass spectrometry results. Of the four metabolites known and tested to be produced by *Pseudomonas*, DAPG production was only recognised in one strain when grown in LB broth, and Orfamide A in CPG broth. It is known that the culture conditions bacteria are grown in affect secondary metabolite expression (Köhl, Kolnaar and Ravensberg, 2019). Furthermore, if these metabolites can only be detected in low concentrations from bacterial culture supernatant, detecting them in more complex soil samples would be even more challenging. It might be that although the bacteria possess the ability to inhibit pathogens, these secondary metabolites may not be produced to a high enough quantity to suppress the pathogens when interacting in the soil. Metabolomics could be conducted to identify bacteria and quantify their secondary metabolites produced from soil samples using mass spectrometry analysis. This has been proven to work on *P. fluorescens* (Alhasawi *et al.*, 2015; Swenson *et al.*, 2015). Transcriptomic studies could also be used to explore the activation of these secondary metabolite clusters by directly focussing on antimicrobial gene regulation and expression in the rhizosphere (Perazzolli *et al.*, 2016; Hennessy *et al.*, 2017). Another factor to consider is the persistence and potential inactivation of secondary metabolites in the soil (Arseneault and Filion, 2017). Our *in vitro* studies

in Chapter 3 showed DAPG stocks prepared from a range of 72- to 0 hours before an *in vitro* study had no effect on the level of suppression. However, it has been reported that DAPG has a half-life of 0.25 days in the soil which would put into question the long-lasting potential of this as an antimicrobial. However, as long as *Pseudomonas* biocontrol strains survive and remain active, DAPG can be produced at required levels (Arseneault and Filion, 2017).

Confirming the physical interaction of *Pseudomonas* and pathogens *in vivo* is another aspect which could support studies. Chen *et al.* (2016a) described how silver nanoparticle alternatives to agrochemicals could not control pathogenic infections as the particles themselves would bind to the soil and not to the pathogen. The *Pseudomonas* secondary metabolites might have been lost and decomposed in the soil and therefore unable to interact with the pathogen. *R. solanacearum* are known to invade and multiply mainly in the stem of the plant resulting in wilting effects, so this might be the key region in the plant for the biocontrol bacteria to reside for effective suppression (Yang *et al.*, 2012). When discussing *Pseudomonas* biocontrol colonization, it is generally focused on *Pseudomonas* accumulating on the surface of the root systems (Rainey, 1999; Weller, 2007). Yet, it is not widely reported if *Pseudomonas* remain on the roots or move within the plant. Microscopy could be used to confirm this, and for example, immunofluorescence microscopy has been used in the past to observe the location of *R. solanacearum* inside the plant (Mcgarvey, Denny and Schell, 1999).

Lastly, the timing, regularity and set up of the biocontrol system can significantly affect the biocontrol success. Metabolite production of DAPG and HCN could require the presence of high zinc and iron availability in the soil, which might have been affected by the autoclaving of the soil (Jamali *et al.*, 2009). It has also been shown that autoclaving of the soil may result in a flush of ammonia and nitrogen which could potentially affect the microbial growth dynamics in the soil (Serrasolsas and Khanna, 1995). Instead of autoclaving, soil could be sterilised using irradiation. The nutrition of the host plant can also affect the virulence of *R. solanacearum* as described by Yamazaki (2001). This study revealed tomato hosts with greater calcium uptake exhibited plant resistance to bacterial wilting, which might also be affected by autoclaving the soil (Yamazaki, 2001). It might also be that multiple inoculations over a field season would be required for an effective biocontrol, as frequent applications are a common practice in UK agriculture e.g. mandipropamid agrochemical spray programmes to prevent potato blight caused by *Phytophthora infestans* is recommended a minimum of four applications per season (Syngenta, 2019). This is also the case for newly developed biocontrol agents such as the insecticide Madex-

Top which is recommended to be applied up to ten times a year to control *Cydia pomonella* (Codling Moth) (AndermattBiocontrol, 2017). Disease incidence can also vary with different host plants due to the effects of plant root exudates and natural systemic resistance which could influence if biocontrol effects are observed (Jamali *et al.*, 2009). The type of *Pseudomonas* application can range from soil drenching — such as we used — to seed treatment or root injections. Soil drenching could be considered the most appropriate for large scale studies (Xue *et al.*, 2009); but other studies have found *Pseudomonas* seed treatment to be more effective than soil applications, particularly in PPN studies (Timper *et al.*, 2009). Lastly, in *R. solanacearum in vivo* experiments, the tomato roots were wounded prior to inoculating with *R. solanacearum* to encourage entry sites for visible plant invasion. This however brings into question whether this made the infection levels representative of a natural environment.

6.8 Concluding remarks

This thesis has given an in-depth investigation into different aspects of biocontrol. It has considered species, strain and genotype interactions of *Pseudomonas* biocontrol bacteria with several plant pathogen models, considered short- and long-term effects, both *in vitro* and *in vivo* and revealed potential activity of underlying mechanisms which may have contributed to biocontrol effects.

Pseudomonas strains have a broad specificity due to their ability to produce a wide variety of secondary metabolites, which we have described and shown to affect a variety of plant pathogens. This can be considered an advantageous attribute for a biocontrol in terms of limiting resistance evolution, because a pathogen would have to overcome resistance to multiple mechanisms at once. In Chapter 4 however, we did see evidence of tolerance evolution occurring to the *Pseudomonas* strains, specifically to orfamide metabolites. One way to minimise this risk further, would be to use combinations of *Pseudomonas* strains which is something to consider in the future. It has been described that combining *Pseudomonas* strains in co-culture can enable strains to upregulate the secondary metabolites of each other including DAPG (Maurhofer *et al.*, 2004). Combinations of orfamides with other *Pseudomonas* metabolites such as phenazine have shown to be more effective against fungal pathogens and bacterial pathogens (Olorunleke *et al.*, 2015; Hu *et al.*, 2016). Liu *et al.* (2012) showed using combinations can also increase the biocontrol of PPNs using *Bacillus spp.* and arbuscular mycorrhizal fungus *Glomus mosseae* on

tomato soil drenching application (Liu *et al.*, 2012). Similar results were observed upon combining *P. fluorescens* and the fungus *Purpureocillium lilacinum* in seed treatment and soil drenching applications (Seenivasan, 2018). The concomitant application of both agents in carrot studies reduced RKN *Meloidogyne hapla* disease incidence significantly (Seenivasan, 2018). It might be that combinations of different types of biocontrol might be a feasible approach for future studies. However, combining species by random could also have unexpected side effects. For example, Becker *et al.* (2012) showed that some selected combinations of biocontrol bacteria had "negative complementary effects" on each other.

Bridging the gap between *in vitro* and *in vivo* experiments to discover biocontrol agents that effectively work in the field requires more attention. *In vitro* studies of *Pseudomonas* bacterial interactions with a pathogen, followed by individual mechanistic metabolite studies and mass spectrometry, can extend our understanding of biocontrol interactions with the pathogen. However, *in vivo* interactions are much harder to interpret. More natural experimental systems are required to bridge the gap, but these come with a vast set of limitations such as space, time and cost. All of these factors can limit a study and how many biocontrol strains can be screened *in vivo*. One way to screen a greater number of potential biocontrol strains rather than in a full glasshouse study could be using microplate soil microcosms which is more complex than an *in vitro* system and can give high throughput results from more *Pseudomonas* strains in a short period of time (Martel *et al.*, 2020). Moreover, developing semi-natural model systems, such as rhizoboxes (Wei *et al.*, 2019), could offer a middle way for the development of biocontrol applications in relatively controlled but more natural settings. Both approaches would require fewer resources rather, and the most successful strains from these screenings could then be expanded to glass house level studies which can increase the complexity of the interactions, then perhaps pave the way for field trials. Alternatively, we discovered pyoluteorin from the secondary metabolite T1PKS cluster had the clearest biocontrol activity of the metabolites tested. Screening potential biocontrol strains for the presence of this metabolic cluster in early biocontrol research might speed up the process of identifying effective biocontrol strains. Moreover, in order to discover biocontrol agents with the ability to suppress multiple pathogens, it is vital to bring together researcher from different specialist fields. The expertise at the University of York with *R. solanacearum* bacteria and at the University of Leeds with PPNs is a good example of this. In the future, this line of research could pave the way for the development of novel alternatives to pesticides and lead to a greener agriculture.

Appendices

Appendix A: Chapter 3

Appendix Table A.1 AntiSMASH5.0 cluster types recognised in *Pseudomonas* CHA0 genome including the types of cluster, the % hit to the database and which bacterial strain it is most related to.

Cluster	Type	Most similar known cluster	Homologous gene cluster
1.1	Bacteriocin		100% to *P. CHA0*
1.2	CDPS		27% to *P. CCOS*
1.3	NRPS	Putisolvin (100%) Orfamide (94%)	96% to *P.CHA0*
2.1	other	Pyrrolnitrin (100%)	91% to *P. CHA0*
2.2	NRPS	Pyochelin salicyclate (100%)	100% to *P. CHA0*
2.3	T1PKS	Pyoluteorin (100%)	97% to *P. CHA0*
3.1	NRPS	Lipopeptide (6%)	100% to *P.CHA0*
3.2	NAGGN		20% to *P. veronii*
3.3	NRPS	Pyoverdine (16%)	100% to *P.CHA0*
4.1	T3PKS	DAPG (100%)	100% to *P. CHA0*
4.2	Bacteriocin		100% to *P. CHA0*
5.1	Arylpolyene	APE Vf (40%)	97% to *P. CHA0*
6.1	NRPS	Viscosin (31%) Pyoverdine (14%)	45% to *P.CHA0* 100% to *P. NFPP17*
6.2	Betalactone	Mycosubtilin (20%)	37% to *P. resinovorans*
8.1	NRPS	Pyoverdine (19%)	62% to *P. CHA0*
10.1	NRPS	Pyoverdine (3%)	17% to *P. extremaustralis*
13.1	NRPS	Syringopeptin (100%) Cyanopeptin (50%)	18% to *P. aeruginosa*

Appendix Table A.2 AntiSMASH5.0 cluster types recognised in *Pseudomonas* Pf-5 genome including the types of cluster, the % hit to the database and which bacterial strain it is most related to.

Cluster	Type	Most similar known cluster	Homologous gene cluster
1.1	NRPS	Pyoverdine (14%)	100% to *P. NFPP12*
1.2	Betalactone	Mycosubtilin (20%) Plipastatin (15%) Fengicynin (13%)	37% to *P. resinovorans*
1.3	Other	Pyrrolnitrin (100%)	91% to *P. NFPP12* 88% to *P. CHA0*
1.4	NRPS	Pyochelin salicyclate (100%)	91% to *P. NFPP12* 72% to *P. CHA0*
1.5	NRPS-like	Rhizoxin (100%)	90% to *P. protegens*
1.6	T1PKS	Pyoluteorin (100%)	97% to *P. protegens FDAARGOS* 92% to *P. CHA0*
2.1	Bacteriocin		100% to *P. protegens* 100% to *P. fluorescens*
2.2	CDPS		27% to *P. CCOS*

2.3	NRPS	Orfamide (94%), 100% Putisolvin (100%) Bananamides (100%) Syringomycin (47%)	90% to *P. protegens FDAARGOS* 90% to *P. NFPP12* 86% to *P. CHA0*
3.1	NRPS	Pyoverdine (21%)	100% to *P. protegens*
3.2	NAGGN		20% to *P. resinovorans*
3.3	NRPS	Lipopeptide (6%)	97% to *P. protegens*
4.1	T3PKS	DAPG (100%)	100% to *P. protegens*
4.2	Bacteriocin	APE Vf (40%)	100% to *P. protegens*
5.1	Arylpolyene		97% to *P. protegens*
7.1	NRPS	Pyoverdine (19%)	100% to *P. NFPP19*
9.1	NRPS	Rhizomide (100%)	5% to *P. protegens*

Appendix Table A.3 AntiSMASH5.0 cluster types recognised in *Pseudomonas* Q2-87 genome including the types of cluster, the % hit to the database and which bacterial strain it is most related to.

Cluster	Type	Most similar known cluster	Homologous gene cluster
1.1	NAGGN		22% to unknown *Pseudomonas*
1.2	NRPS	Serobactins (23%) Pyoverdine (10%)	100% to *P. Q287*
1.3	Betalactone	Mycosubtilin (20%)	37% to unknown *Pseudomonas*
2.1	Butyrolactone		100% to *P. Q287*
4.1	T3pks	DAPG (100%)	100% to *P. Q287*
4.2	Bacteriocin		100% to *P. Q287*
5.1	Bacteriocin		100% to *P. Q287*
6.1	NRPS	Cupriachelin (35%)	90% to *P. Q287*
7.1	Arylpolyene	APE vf (40%)	97% to *P. Q287*
8.1	NRPS	Sulfazcin (11%) Ishigamide (11%)	91% to *P. Q287*
9.1	NRPS	Delfibactin (42%) Pyoverdine (19%)	96% to *P. Q287*
9.2	Ectoine		84% to *P. Q287*
15.1	Bacteriocin		100% to *P. Q287*
19.1	Lanthipeptide		78% to *P. Q287*

Appendix Table A.4 AntiSMASH5.0 cluster types recognised in *Pseudomonas* Q8R196 genome including the types of cluster, the % hit to the database and which bacterial strain it is most related to.

Cluster	Type	Most similar known cluster	Homologous gene cluster
1.1	NRPS	Pyoverdine (10%)	100% to *P. Q287*
1.2	Betalactone	Fengycin (13%)	37% to *P. resinovorans*
1.3	T3PKS	DAPG (100%)	97% to *P. brassicacearum*

1.4	NRPS	Cupriachelin (11%)	82% to *P. brassicacearum*
1.5	Butyrolactone		100% to all *Pseudomonas*
4.1	Arylpolyene	APE Vf (40%)	97% to *P. brassicacearum*
6.1	NAGGN	Taiwachelin (11%)	22% to *P. veronii*
9.1	NRPS	Viscosin (31%) Pyoverdine (10%)	69% to *P. brassicacearum*
12.1	Bacteriocin		100% to all *Pseudomonas*
20.1	NRPS-Like	Mangotoxin (71%)	96% to *P. brassicacearum*
23.1	NRPS	Delftibactin (28%) Pyoverdine (3%)	94% to *P. fluorescens*
24.1	NRPS	Taiwachelin (11%) Pyoverdine (3%)	11% to *P. rhodesiae*
25.1	NRPS		100% to all *Pseudomonas*

Appendix Table A.5 AntiSMASH5.0 cluster types recognised in *Pseudomonas* 1M1-96 genome including the types of cluster, the % hit to the database and which bacterial strain it is most related to.

Cluster	Type	Most similar known cluster	Homologous gene cluster
2.1	T3PKS	DAPG (100%)	72% to *P. fluorescens*
2.2	NRPS	Cupriachelin (11%)	94% to *P. brassicacearum*
2.3	Butyrolactone		100% to all *Pseudomonas*
3.1	NAGGN		17% to *P. veronii*
4.1	NRPS	Serobactin (23%) Pyoverdine (10%)	97% to *Ps. F113*
8.1	Betalactone	Mycosubtilin (20%) Fengicynin (13%)	37% to *P. resinovorans*
9.1	Bacteriocin		100% to all *Pseudomonas*
17.1	Arylpolene	APE Vf (40%)	82% *P. Q287*
32.1	Lanthipeptide	Putative class II	36% *P. syringae*
44.1	NRPS-like	Mangotoxin (71%)	86% *P. corrugata*
49.1	Lanthipeptide	Putative class II	89% *P. fluorescens*
62.1	NRPS	Viscosin (25%) Pyoverdine (6%)	100% to *P. brassicacearum*

Appendix Table A.6 AntiSMASH5.0 cluster types recognised in *Pseudomonas* MVP1-4 genome including the types of cluster, the % hit to the database and which bacterial strain it is most related to.

Cluster	Type	Most similar known cluster	Homologous gene cluster
2.1	Betalactone	Mycosubtilin (20%) Fengicynin (13%)	37% to *P. resinovorans*
2.2	LAP		17% to *P. NBRC*
4.1	NRPS	Cupriachelin (11%)	80% to *P. fluorescens*

4.2	T3PKS	DAPG (100%)	83% to *P. brassicacearum*
6.1	Arylpolyene	APE Vf (40%)	97% to *P. NFACC51*
9.1	Nrps-ike	Mangotoxin (71%)	88% to *P. kilonesis*
10.1	Bacteriocin		100% to all *Pseudomonas*
13.1	NAGGN		22% to *P. veronii*
24.1	Lanthipeptide	Putative class II	100% to all *Pseudomonas*
30.1	Butyrolactone		100% to all *Pseudomonas*
32.1	NRPS	Viscosin (31%) Pyoverdine (10%)	76% to *P. fluorescens*
35.1	NRPS	Pyoverdine (10%)	94% to *P. fluorescens*
36.1	NRPS	Pyoverdine (8%) Serobactin (15%) Taiwachelin (11%)	64% to *P. NFACC563*

Appendix Table A.7 AntiSMASH5.0 cluster types recognised in *Pseudomonas* F113 genome including the types of cluster, the % hit to the database and which bacterial strain it is most related to.

Cluster	Type	Most similar known cluster	Homologous gene cluster
2.1	Arylpolyene	APE Vf (40%)	93% to *P. F113*
4.1	NAGGN		22% to *P. veronii*
4.2	NRPS	Pyoverdine (10%) Serobactins (23%)	97% to *P. F113*
5.1	NRPS-like	Lankacidin (26%)	100% to *P. F113*
6.1	Bacteriocin		100% to all *Pseudomonas*
11.1	Betalactone	Mycosbutilin (20%) Fengicyin (13%)	37% to *P. resinovorans*
12.1	T3PKS	DAPG (100%)	96% to *P. F113*
16.1	NRPS	Delftibactin (42%) Viscosin (31%) Pyoverdine (11%)	64% to *P. F113*
19.1	Butyrolactone		100% to *P. F113*
20.1	Lantipeptide		83% to *P. F113*

Appendix Table A.8 AntiSMASH5.0 cluster types recognised in *Pseudomonas* Ph11C2 genome including the types of cluster, the % hit to the database and which bacterial strain it is most related to.

Cluster	Type	Most similar known cluster	Homologous gene cluster

1.1	NAGGN		17% to *P. veronii*
1.2	NRPS	Serobactins(23%) Pyoverdine (10%)	100% to *P. fluorescens*
1.3	Betalactone	Mycosbutilin (20%) Fengicyin (13%)	37% to *P. resinovorans*
2.1	NRPS	Mycobactin (20%) Coelibactin (18%)	100% to *P. thivervalensis*
2.2	Bacteriocin		100% to *P. thivervalensis*
2.3	Butyrolactone		100% to *P. thivervalensis*
4.1	Bacteriocin		100% to all *Pseudomonas*
5.1	NRPS-like	Mangotoxin (71%)	92% to *P. 9738*
5.2	Bacteriocin	Pyoverdine (1%)	100% to *P. thivervalensis*
5.3	Arylpolyene	APE Vf (40%)	100% to *P. thivervalensis*
6.1	NRPS	WLIP (28%) Cupriachelin (11%) Pyoverdine (2%)	97% to *P. thivervalensis*
6.2	T3PKS	DAPG (100%)	100% to *P. thivervalensis*
7.1	NRPS	Viscosin (31%) Taiwachelin (27%) Pyoverdine (19%)	93% to *P. NFACC37*
12.1	Lanthipeptide	Putative class II	100% to *P. thivervalensis*

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