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[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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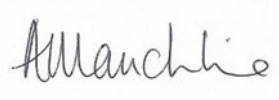
Signature .....  ..... Date 07/01/2018

CONTENTS

GROWER SUMMARY

Headline

Reduce cost of chemical pesticide use in glasshouses by using bacterial biological control alternatives against aphids in an IPM system.

Background

The control of insect pests in glasshouse systems is a major challenge. Aphids in particular thrive in controlled environmental conditions, damaging crops by direct feeding and transmitting plant diseases. Due to their vast range in host plants and rapid reproductive cycle, they are particularly hard to eradicate once they have become established in a glasshouse system.

Chemical insecticides are commonly employed against aphids but growers are under increasing pressure from supermarkets and consumers to find alternative, environmentally friendly, non-chemical methods of control. Intensive and indiscriminate use of chemical pesticides has resulted in certain aphid populations to develop resistance to some chemical-based treatments, limiting effective products available to growers. Additionally, chemical pesticides have significant detrimental effects on beneficial insects used in glasshouses, such as natural enemies and pollinators. The use of microbial agents as biocontrols is a rapidly developing field and work conducted by a previous AHDB-funded student, Dr Amanda Hamilton, investigated the potential for bacteria naturally occurring on plants to act as biocontrol agents against aphids and thrips.

In a process known as 'bioprospecting,' 140 bacterial isolates were taken from the rhizosphere (area around plant roots) and phyllosphere (above ground portion of plants) of a variety of plants and tested for virulence against aphids (Hamilton, 2015). Three isolates were found to be most virulent to aphids when inoculated in artificial set ups: *Pseudomonas fluorescens*, *Citrobacter werkmanii* and *Pseudomonas poae* (*P. poae*). *P. poae* was found to have plant growth promoting properties and no noticeable effect on non-target insects (caterpillars and ground beetles). Further investigations found that of the three isolates, *P. poae* PpR24 had the highest success rate in killing aphids, probably because it reproduced in the insect gut and produced aphicidal toxins. These resulted in a 70% decrease in aphid populations when applied on plants as a foliar spray. Foliar applications also deterred aphids from a plant. *P. poae* PpR24 also proved to be effective at killing aphid clones known to be resistant to chemical pesticide treatments; therefore it has great potential for future use in an IPM system.

This project aims to take the next steps in investigating the potential for using *P. poae* as a biological control in glasshouses.

Summary

Many bacteria and microbial organisms in the natural world play an important role in regulating insects and other microbial populations. Some inadvertently have these beneficial properties and there has been an increase in research in harnessing their abilities as biological controls. Microbial-based biological controls offer many benefits to growers. Compared to chemical pesticides, microbial controls are more cost-effective and safer to use for humans and non-target organisms as they are generally highly specific (only dangerous to a few organisms). They also have less of an environmental impact and pose little or no threat to biodiversity as they are naturally present in the ecosystem. They can also be applied to crops by conventional means, making use of systems in place, such as foliar sprays or soil drenching systems. There is also the potential for bacterial-based treatments to become self-sufficient in the crop, offering protection against target pests without the need to be regularly applied. They may also be a solution to the issue of treatment resistance in pests. As bacteria have a rapid reproduction time, they are quick to evolve and so may be able to evolve alongside the pest species, such as aphids, and prevent them becoming tolerant to the treatment.

The bacteria investigated for use as a biological control, *P. poae* PpR24, was originally found on the roots of *Brassica oleracea* and found to cause disease (pathogenic) in the green peach-potato aphid (*Myzus persicae*), lettuce aphid (*Nasonovia ribisnigri*), glasshouse potato aphid (*Aulacorthum solani*), cabbage aphid (*Brevicoryne brassicae*), lupin aphid (*Macrosiphum albifrons*) and pea aphid (*Aphis fabae*). It worked most effectively as a foliar spray or by soil drenching. For this study, foliar spray application was used and the green peach-potato aphid (*Myzus persicae*) was the target pest on sweet pepper plants (*Capsicum annuum*).

The overall purpose of this project was to investigate whether we can improve the wild version of *P. poae* PpR24 as a potential biocontrol agent and to assess whether it can be used in a glasshouse environment. The project was done in three parts, described below in the next three sections.

Experimental evolution to improve Pseudomonas poae PpR24

Experimental evolution is a well-established method for examining the underlying mechanisms of evolution, such as natural selection. ‘Passaging’ is when bacterial cells are grown in a petri dish, and then some of those cells are transferred to a new petri dish to grow

and multiply again as a different generation (or isolate). This can also be done in insects like aphids. By passaging an organism such as *P. poae* PpR24 in a controlled environment for multiple generations, random mutations and adaptations can appear and the different populations can be tracked. Experimental evolution was used to see whether the ability of *P. poae* PpR24 to kill aphids could be improved. This was done in two ways:

1. *P. poae* PpR24 was passaged through aphids via an artificial diet for ten cycles (ten lineages) to try and evolve the *P. poae* and improve aphid killing.
2. Successive passages of *P. poae* PpR24 were also done in a broth environment in an attempt to evolve biofilm formation. Biofilms are clumps of bacteria that are able to stick to surfaces and form communities, held together by substances produced by the bacteria.

The phylloplane can be a harsh environment for bacteria, with challenges such as UV radiation, nutrient limitations and competition from other microbes, therefore biofilms may improve bacterial survival. Biofilm formation may be beneficial for a biocontrol agent as it may lead to bacteria surviving on the plant for longer, reducing the number of applications and possibly remove other, non-desirable microbes from the plant. Unfortunately, the ability of the bacteria to kill aphids was not significantly improved, but out of the ten lineages, one isolate evolved the ability to form strong biofilms. However, there seems to be a trade-off between being able to form a biofilm (for survival) and killing aphids. There was also no improvement in bacterial colonisation and growth on the host plant.

The effects of Pseudomonas poae PpR24 semiochemicals on Myzus persicae behaviour

The ability to repel pests from crops to minimise damage is especially useful in a biocontrol agent. The wild-type *P. poae* PpR24 has a deterrent effect on aphids when sprayed on a plant. In this project, we identified the volatiles (gases) produced by the *P. poae* PpR24 which may explain the aphid-deterrent properties. In addition, volatiles of the aphid-passaged and biofilm-forming isolates were also studied as, although not as lethal to aphids as the wild-type, they may still have deterrent properties. Mulitple volatile organic compounds (VOCs) emitted by the bacteria were found.

When presented with a choice to settle on either a control plant (no VOCs) or a wild-type spray plant, more aphids settled on the control plant which corroborates what was previously found. No significant difference was seen in aphid host plant choice when aphids were presented with a control sprayed plant and plants either sprayed with the biofilm or aphid-passaged isolates. Different VOC levels were detected between the biofilm isolates to the wild-type which may account for the loss of repellency.

Non-target effects of Pseudomonas poae PpR24 on commercial aphid natural enemies

Natural enemies are often a key component in IPM management systems where they are introduced or encouraged into crop systems as part of augmentative and conservation biocontrol programs. *Aphidius colemani*, *Orius laevigatus* and *Macrolophus pygmaeus* are three commercially produced aphid natural enemies commonly used to control *Myzus persicae* in glasshouse cropping systems. To investigate potential lethal effects the bacteria may have on the insects, three experimental set-ups were devised to simulate likely routes of exposure to *P. poae* if the bacteria is applied as a foliar spray.

Topical application of *P. poae* had no significant effect on *M. pygmaeus* mortality after 72 hours or *A. colemani* mummy emergence. However, an effect was observed for adult *O. laevigatus* and *A. colemani*, implying that *P. poae* does affect their survival. Exposure to spray residues did not have a significant effect on the generalist predators but the bacteria appeared to have a damaging effect on *A. colemani* survival. Finally, when left to feed on *M. persicae* that had ingested the bacteria, no significant lethal effect was observed in *M. pygmaeus* but a significant change in mortality was seen in *O. laevigatus.*

Financial Benefits

The annual cost of crops lost to aphids and the viruses they transmit, including the control methods put in place to fight them, is over £100 million. The annual loss to the UK potato industry alone is estimated at £12 million. In an average protected pepper crop, the focal plant of this study, the cost of everyday aphid control is estimated at £5800 per hectare per season. However, this dramatically increases when serious aphid outbreaks occur due to increased applications of biocontrol and insecticide treatments and cleaning the crop of honeydew.

Action Points

- This microbial-based product could be used in a glasshouse integrated pest management system as a foliar spray alongside other biocontrol agents, such as natural enemies. *Pseudomonas poae* PpR24 may be applicable in both preventative and corrective biocontrol strategies to manage aphid infestations.
- *P. poae*'s aphid deterrent properties may make it suitable to deter aphids onto banker plants. Pre-emptive spraying of crops before serious infestations occur may 'push' pests onto sink banker plants, minimising crop losses. Such a method could be combined with natural enemies established in the banker plant to feed on the displaced aphids.

- There is potential for *P. poae* to be used in a management system alongside aphid parasitoids and predators to ensure maximum aphid control. *Macrolophus pygmaeus* may be the most applicable aphid predator for use together with *P. poae*. Carefully timed spray applications may also mean *P. poae* is applicable for use with other parasitoids and predators. It may also be possible to spray crops when parasitoids are developing as mummies. Juvenile *O. laevigatus* may avoid direct contact with *P. poae* spray as they spend early life-cycle stages in more concealed areas of the plant (e.g. in the flowers) and as such are less likely to directly encounter the bacteria.
- Finally, as this microbial, environmentally friendly form of control is meant to be used instead of chemical based pesticides, a reduction/total loss of chemical based products would also be advised to get the full environmental benefit.

SCIENCE SECTION

Introduction

Aphids are a global problem in horticulture and agriculture (Van Emden and Harrington, 2017), with growers losing up to 10% of crop yields due to direct damage from aphid feeding and aphid-vectored diseases. Since the mid-1940s with the development of commercial synthetic chemical pesticides like DDT, chemical pesticides have been widely applied against insect pests, with over £5 billion spent on pesticides each year worldwide (Pimentel *et al.,* 1980; Alavanja, 2009). Although still effective in many instances, increasing legislation and consumer concerns over the impact of chemical pesticides on the environment and human health has put more pressure on growers to find alternative green, biodiversity-friendly means of crop protection (Lamichhane *et al.*, 2016). Chemical pesticides available to growers are limited further as many are dependent on the season, crop type and pest species. Target pests evolving resistance to treatments has also resulted in pesticides becoming redundant. The intense application of chemical pesticides has resulted in approximately 20 species of aphids evolving resistance to pesticides belonging to the main classes of chemical pesticides (Silva *et* al., 2012; Bass *et al.*, 2014). Integrated pest management (IPM) strategies have become increasingly popular amongst growers in dealing with crop pests. By incorporating more specific natural enemies and biopesticides, combined with favourable crop culturing techniques, IPM offers a holistic approach to combating pests rather than relying on synthetic chemicals that have a wider, and often more detrimental, impact factor (Mann, 2016).

This project, funded by the Agriculture and Horticulture Development Board, explores the potential of using aphicidal bacteria as a biological control agent in sweet pepper glasshouse systems. Two previous PhD research projects discovered that the Gram-negative, rhizosphere-dwelling bacterium, *Pseudomonas poae* PpR24, possessed aphicidal properties when ingested by aphids. PpR24 was found to be able to survive on the leaf surface at no detriment to the plant and was also found to deter aphids from colonising a plant. This study continues the investigations into the potential of PpR24 as a biopesticide, firstly by attempting to improve PpR24's efficacy as a biopesticde. The wild-type PpR24 is capable of killing 70% of aphids within 42 hours in a lab environment and can persist on a plant for three weeks. By employing experimental evolution, this project attempts to improve the virulence of PpR24 to aphids and its growth and survival on the plant. Further investigations will investigate the volatile mechanisms behind the deterrent properties of PpR24 and whether it has undesirable non-target effects on natural enemies commonly applied to control aphids in an IPM system.

Microbial-based biological controls are becoming increasingly popular on the pest control market (Lacey *et al*., 2001; Pandin *et al.,* 2017). They present many advantages over both chemical and arthropod based aphid management strategies. Compared to chemical pesticides, microbial pesticides are more cost-effective and safer to use for humans and non-target organisms as they are generally highly specific. Furthermore, they have less of an environmental impact and pose little or no threat to biodiversity (Lacey *et al*., 2001). With regards to advantages over natural insect enemies, microbial controls can be applied with conventional equipment, produced with artificial media and are easier to store over long periods of time (Lacey *et al*., 2001).

Entomopathogenic pseudomonads

Pseudomonads are Gram-negative bacteria commonly found in the rhizosphere and phyllosphere of plants. They are a particularly promising class of bacteria to exploit as biological controls and extensive research has been conducted into their potential applications (Lacey *et al*., 2001; Couillerot *et al.,* 2009; Hofte and Altier, 2010; Dandurishvili *et* al., 2011). Frequently found in agricultural soils, many Pseudomonads are plant growth-promoting rhizobacteria (PGPR) that enhance plant growth by making nutrients and compounds readily available to the plant as well as protecting their host plant from pathogens and pests (Weller, 2007; Hol *et al*., 2013). Fluorescent Pseudomonads in particular have been known to exhibit plant growth promoting properties. For instance, strains of *Pseudomonas fluorescens* have been found to have antimicrobial properties, suppressing fusarium wilt in tomato (Leeman *et* al., 1995; Duijff *et* al., 1997) and the fungal disease, black root rot, in tobacco plants (Voisard, *et* al., 1989).

Many bacteria in the *Pseudomonas fluorescens* group have also been seen to exhibit insecticidal properties (Rangel *et al*., 2016). In several species, the presence of the *fluorescens* insecticidal toxin (*fit*) gene cluster that regulates the production of FitD, an important insecticidal protein, and Tc gene clusters are known to contribute to insect toxicity (Pechy-Tarr *et al*., 2008). For example, FitD plays a key role in the toxicity of *Pseudomonas protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391 to the diamondback moth *Plutella xylostella*, the African cotton leafworm, *Spodoptera littoralisI,* and the tobacco budworm, *Heliothis virescens*, all important lepidopteran pests (Ruffner *et al*. 2013). *Pseudomonas chlororaphis* strains produce an array of metabolites that have antiomicrobial and insecticidal properties (Anderson and Kim, 2018). *P. chlororaphis* O6 is effective at controlling root knot nematodes, *Meloidoyne* spp., causing juvenile mortality and reducing gall formation. Although *P. chlororaphis* O6 possesses *fit*D, a gene important in regulating Fit D, mutations in this gene did not affect the nematocidal properties of the bacterium. It is believed instead that the

bacterium's virulent properties are due to the production of hydrogen cyanide (Kang *et* al., 2018). *P. syringae* PsyB728a, a bean pathogen, can kill pea aphids in 48 hours when fed in an artificial diet (Stavrinides *et al.,* 2009). This virulence was linked to *fliL* gene, which plays an important role in flagellum assembly and bacterial motility, suggesting motility is key to virulence. However, as *P. syringae* is a plant pathogen, its application in a crop system is questionable.

Pseudomonads and other bacteria can be mass produced relatively easily due to their rapid reproduction cycle and their adaptability to a range of environments means there is scope for their use in a range of agricultural and horticultural settings (Weller, 2007). However, Weller (2007) and Kupferschmied *et al*., (2013) have voiced reservations on producing *Pseudomonas* on a commercial scale as ensuring their survival during long-term storage will be challenging. Before any bacteria are used as treatment in the field they must undergo rigorous trials to ensure there are no deleterious effects on other species or risk to human health and the environment (Kupferschmied *et al.,* 2013). Furthermore, it has been found in some endophytic bacteria that ordinarily mutualistic organisms turn pathogenic under stressful environmental conditions (Sieber, 2007; Slippers and Wingfield, 2007). However, as living organisms bacteria applied as biopesticides have the potential to adapt and evolve along with aphid pests, thus reducing the likelihood of aphids evolving resistance to the management method.

Previous research

Previous research discovered the bacteria, *Pseudomonas poae* PpR24, to be effective at killing aphids with no non-target effects to insects or damage to the host plant (Hamilton, 2015; Paliwal, 2017). This project aims to improve the efficiency of the aphid killing bacteria, via experimental evolution, and prove that they are safe and can be used within an integrated pest management framework.

This project is a continuation of the PhD research conducted in the Jackson Lab by Dr Amanda Hamilton and Dr Deepa Paliwal, investigating the potential phylloplane biocontrol agent *Pseudomonas poae* PpR24. PpR24 is a Gram-negative, rod-shaped bacterium, first isolated from cabbage roots during Dr Hamiltons' research, where bioprospecting was applied in a bid to discover potential biopesticides for use against aphids and thrips (Hamilton, 2015). Out of 140 bacteria isolated from the rhizosphere and phylloplane of a variety of plant species, three were found to be most pathogenic to aphids. *Pseudomonas flourescens*, *Citrobacter werkmani* and *Pseudomonas poae* were effective at killing six species of aphid: *Myzus persicae*, *Brevicoryne brassicae*, *Aphis fabae*, *Macrosiphum albifrons*, *Aulacorthum solani* and *Nasonovia ribsnigri*, with 100% mortality observed after 72 hours when applied at 10^7

CFU ml^{-1}. No non-target effects were observed in the experimental plants or in the five non-target species tested.

The follow-up study conducted by Dr Paliwal (2017), concluded that out of the isolates under scrutiny, PpR24 was the most promising as a potential biocontrol agent. It was hypothesised that *Pseudomonas poae* act by way of gut occlusion – replicating in the insect gut until a maximum population is reached, clogging the aphid gut preventing digestion and producing lethal levels of toxins (Stavrinides *et al.* 2009). Genome analysis discovered three insecticidal toxins, stress response genes and a variety of pathogenicity-related toxins that are likely the cause of PpR24's aphicidal properties, the toxin AprX in particular. Dr Paliwal and Dr Hamilton demonstrated that PpR24 possessed potential plant growth promoting properties and was capable to colonise the phylloplane without triggering a hypersensitive response. Dr Paliwal's research found *P. poae* to be successful at colonising and effective at controlling aphids in pepper, arabidopsis and sugar beet plants when applied by leaf needle-inoculation, soil drenching and as a foliar spray. Olfactometer analysis showed that when applied to the plant as a foliar spray, PpR24 exhibited a deterrent effect to aphids, which was not observed when the aphids were exposed to the bacteria alone. Furthermore, virulence investigations with four aphid clones known to be resistant to numerous pesticides found PpR24 to be effective at killing 50-80% of aphids when applied at bacteria dose of 10^7 CFU ml^{-1}. Overall, PpR24 shows a great deal of promise for future use as a biopesticide product. This project is a continuation of the investigations into PpR24's suitability as a biocontrol.

The model system

This study focuses on the effects of *P. poae* PpR24 against the green peach-potato aphid, *Myzus persicae*, when applied as a foliar spray in sweet pepper crops, *Capsicum annuum*. Over 1.6 million tons of pepper fruit is grown annually across the world. High in calcium and vitamins A and C, it is a popular crop. In the UK pepper is an important protected edible, with 90 hectares of pepper grown in controlled hydroponic glasshouses (as of 2015). Although various pests afflict pepper crops, the primary factor that leads to insecticide use is the need for aphid control. *M. persicae* is a global pest of sweet pepper but also has a wide host range of approximately 400 plant species (Bass *et al.,* 2014) and acts as a vector for over 100 plant viruses. *M. persicae* is renowned for developing resistance to insecticides (Bass *et al.,* 2014) therefore novel, effective forms of control are needed.

The overall purpose of this project is to investigate whether we can improve the wild-type *Pseudomonas poae* PpR24 as a potential biocontrol agent by experimental evolution and to further assess its suitability for use in a glasshouse environment. The aims for each research chapter can be more precisely defined as follows:

- Experimental evolution shall be applied in an attempt to improve the wild-type PpR24's virulence to aphids and improve its' survival on the phylloplane by evolving biofilm formation. Experimental evolution has become a well-established method for examining the underlying mechanisms of evolution, such as natural selection and genetic drift. By passaging an 'ancestor' organism in a controlled selective environment for multiple generations, random mutations and adaptations can arise and the divergent populations can be tracked and observed by experimenters in real time (Lang and Desai, 2014). After cycling ten independent lineages of PpR24 through ten selection passages, either to evolve enhanced virulence or biofilm formation, final derived isolates will be examined as to whether fitness trade-offs or phenotypic changes have occurred between evolved isolates and the wild-type.

- Whole genome Illumina and Nanopore sequencing with variant calling analysis will be conducted in an attempt to observe mutations that may be responsible for the phenotypic and fitness changes that evolved over the course of the experimental passages (Dettman *et* al., 2012; Guidot *et* al., 2014).

- Identification of the volatile organic compounds emitted by PpR24 and the evolved isolates will be conducted to provide deeper understanding of the bacteria's aphid-deterrent properties. Volatile Organic Compounds (VOCs) are compounds of a low molecular mass that are produced by all organisms and play a vital role in intra and inter-specific communication, particularly for plants, bacteria and fungi (Ameye *et* al., 2018). Whether derived isolates of PpR24 are more effective at deterring *Myzus persicae* from crop plants than the wild-type PpR24 shall also be investigated.

- Direct non-target effects of PpR24 on natural enemies commonly used against aphids in glasshouses will be investigated. *Aphidius colemani*, *Orius laevigatus*, and *Macrolophus pygmaeus* will be exposed to PpR24 in three different ways to simulate routes of exposure in the crop system. Insects will be subjected to walking on a sprayed leaf, dorsal application of the bacteria to simulate spray contact and oral ingestion from treated aphids and lethal effects observed.

Materials and methods

General materials and methods

Bacteria

Pseudomonas poae (*P. poae*) PpR24 was originally isolated in the University of Reading Experimental Greenhouse from *Brassica oleracea* roots by Hamilton (2016) and was found to be Ampicillin and Nitrofurantoin resistant (Paliwal, 2017). Frozen stocks were kept at -80°C.

Media

All bacteria, unless otherwise stated, were grown in King's Medium Broth (KB), at 27°C shaken at 200rpm or on a KB agar plate King *et al*. (1954) at 27°C. Proteose peptone 20 g, K_2HPO_4 1.5 g, $MgSO_4.7H_2O$ 1.5 g and glycerol 10ml were added to 1 litre of deionised water. Agar was added to the media to achieve a concentration of 1.5%. Media was autoclaved at 121°C for 20 minutes for sterilisation. Sterile KB agar was melted and 20 ml poured into 9 cm diameter Petri dishes (Thermo Fisher Scientific, Scotland, UK) when cooled to 50°C. For PBS used throughout the project, 1 L of 10xPBS contains 80 g NaCl, 2 g KH_2PO_4, 29 g $Na_2HPO_4.12H_2O$, 2 g KCl; 1xPBS has a pH of 7.4.

Plants

The plants used throughout the project were sweet pepper Palermo RZ F1-Hybrid *Capsicum annuum* L., supplied by Rijk Zwaan seeds. The plants were grown at 21°C at 70% humidity in a controlled environment room on a long day light cycle (16 hr light/8 hr dark) for four weeks before use in experiments or for rearing aphids.

Aphid rearing

All aphids used were *Myzus persicae* Sulzer (Hemiptera: Aphididae) supplied by Rothamsted Research. Clones were maintained parthenogenetically in plastic leaf box cages or on whole plants if large populations were needed. The insects were kept in a rearing room at 21°C on a long day light cycle (16 hr light/8 hr dark) to ensure no sexual reproduction occurred.

Aphid mortality sachets

All aphid mortality assays were conducted in a constant environment, long-day light cycle rearing room at 21°C and 70% humidity. Preparation of the sachets was carried out in a laminar flow hood to mitigate contamination.

The aphid Mittler diet was used in feeding sachets (Dadd, 1967). The solution was then stored at -20°C in 50 ml falcon tubes. Perspex cylinders, 25 mm in diameter by 25 mm in length, were cleaned with 70% ethanol and one end covered with 4 cm^2 of parafilm. Two other sections of parafilm were cut and all were placed under UV light for sterilisation in a safety cabinet for 35 minutes. Room temperature, pre-prepared Mittler diet was sterilised with a 0.22 µm filter syringe. Control sachets were made up of 594 µl diet and 6 µl sterile water, which was pipetted onto the sterile parafilm stretched over the top of the cylinder and carefully covered with a second layer of sterilised parafilm to avoid any spillage.

"Treated" sachets were prepared in the same way. *Pseudomonas poae* strains were recovered from stocks kept at -80°C and grown for 24 hours on KA plates at 27°C to achieve single colonies. A single colony was picked and the bacteria were then grown overnight, shaken at 27°C in 10 ml KB media for 16 hours. A spectrophotometer was used to analyse the bacterial cell density and normalised to an OD600 to 1, which was equivalent to a bacterial concentration of 10^9 CFU ml^{-1}. After washing in sterile 1xPBS, 6 µl of bacterial suspension was added to 594 µl of Mittler to achieve a concentration of 10^7 CFU ml^{-1} and this formed the sachet filling. Ten aphids were carefully added to each cylinder using a paintbrush and the cylinder sealed with the final piece of parafilm. The cylinder was placed with the sachet at the top so any dead aphids can drop to the bottom and easily recorded. Observations were made at 0, 1, 6, 18, 24, 30, 42, 46 and 48 hours

Bacterial foliar spray

Plants were grown and *P. poae* isolates were recovered and grown from frozen stocks. Bacteria were washed twice in 1xPBS to remove any residual growth media and resuspended in 1xPBS at an OD600 to 1 to form the spray solution. Plants were sprayed with 8 ml of solution (unless otherwise stated) on the abaxial and adaxial leaf surfaces using a hand atomiser spray nozzle at a cellular suspension of 10^{-7} CFU mL^{-1}. Sterile 1xPBS was used as a control spray. Nozzles were cleaned with 70% ethanol and sterilised water before use, with each spray treatment using a different nozzle to prevent contamination. After spraying, plants were left in the laminar flow until completely dry.

DNA extraction

DNA was extracted and purified using a Qiagen Puregene Core A kit following the protocol guidelines for Gram-negative bacteria. A sample was electrophoresed in a 0.8% agarose TBE gel to check for DNA integrity and the concentration was measured using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. Extracted DNA was stored at 20°C. Monarch PCR and DNA clean-up kit was used, following the protocol for dsDNA < 2kb and the samples were sent to Eurofins for Sanger Sequencing.

Polymerase Chain Reaction (PCR)

PCRs were carried out using PCRBIO Taq Mix Red (PCR Biosystems Ltd, London, U.K.), following the protocol of 2x PCRBIO Taq Mix Red, 1 µL of each 10 µM forward and reverse primer, 1-2 µL template and molecular biology grade water to 25 µL. The initial denaturation was at 95°C for two minutes, followed by 30 cycles of denaturation at 95°C for one minute and annealing at 50–65°C for 20 seconds to 1 minute (depending on the primer pair used. Extension was at 72°C for one minute and a final extension step of 72°C for five minutes.

Phusion PCR

Phusion high fidelity DNA polymerase (Thermo Fisher Scientific, Scotland, and U.K.) buffers and enzyme were used for gene sequencing. The reaction was prepared as follows: 10 µL 5x Phusion HF buffer; 1 µL 10 mM dNTPS; 1 µL of each 10 µL forward and reverse primer; 0.5 – 1 µL template; 0.5 µL Phusion polymerase (1 unit/50 µL); molecular biology water to 50 µL. Cycling conditions were as follows: initial denaturation of 98°C for 30 seconds; 30 cycles of 98°C for ten seconds, 58°C for 30 seconds, 72°C at 30 seconds; and a final extension of 72°C for ten minutes. Primers were designed using Primer3Plus and checked for their specificity using NCBI Primer-BLAST.

Statistical analysis

All statistical analysis was conducted in RStudio, version 3.6.1.

Experimental evolution methodologies

The wild-type PpR24 was isolated in a previous study and used as the ancestral strain to seed two evolutionary passages. One passage investigated whether biofilm formation could arise in a population (PpR24b isolates), the other whether improved aphid virulence would arise (PpR24a isolates). In both passage systems, ten independent lineages derived from the same wild-type PpR24 strain was serially passaged and final isolates randomly selected from plates spread with final passage bacteria.

Biofilm passage assay

Biofilm passages were conducted over a ten-week period, where each passage lasted one week following the protocol as devised by Spiers *et al.* (2003). Ten glass universals of 10 ml KB media were inoculated with *Pseudomonas poae* PpR24 from the same ancestral population. Five universals were inoculated with 10 µl of bacteria and the other five 100 µl of bacteria. The microcosms were incubated with loose lids at 27°C for one week and no shaking to allow biofilms to form at the air-liquid interface in aerobic conditions.

After one week, the microcosms were carefully removed from the incubator to ensure no biofilms were dislodged. Visual observations on the presence of biofilms were made. The passages were then continued in fresh KB media. 10 µl of bacteria-broth solution was removed from the biofilm of the old microcosm and added to the new, fresh media. This was also repeated for the 100 µl samples. Frozen stocks were also taken from each isolate, each week to for a 'fossil record' of the bacteria's evolution. At the conclusion of ten passages were conducted for each of the derived ten isolates and PCRs to ensure *P. poae* was recovered were carried out.

Bead test of biofilm strength

To test the strength, or maximum deformation mass (MDM) of the biofilms, 2 mm glass beads were dropped onto the centre of the biofilm from a constant height until the biofilm sagged or broke. The more beads supported was taken to indicate a stronger the biofilm.

Biofilm cellular attachment strength

Biofilm attachment strength was assessed using the crystal violet staining technique as laid out by O'Toole *et al*. (1999). After recording the biofilms' strength, universals containing the bacterial-broth solution were emptied and stained with 1 ml of 0.05% (w/v) Crystal Violet. The vials were agitated for one minute and gently rinsed out with water. The stain was then eluted with 5 ml ethanol, shaken for 15 minutes and the OD600 recorded.

Bacterial virulence assay

Ten aphid mortality sachets were prepared using the ancestor wild-type *P. poae* PpR24 strain. After 48 hours, the aphids were recovered from each sachet and surfaced sterilised with 1% sodium hypochlorite solution for 5 minutes in 1.5 ml eppendorfs. The aphids were then rinsed 3 times with sterile water, centrifuging washes at 5000 rpm for 3 minutes. After removing the sterile water, 200 ul of sterilised PBS was added to the eppendorfs and the aphids were homogenised using sterile micropestles.

Each of the ten samples were aliquoted in 10 µl droplets and spread onto KB and Nitrofurantoin plates with glass spreaders. The plates were incubated at 27°C and left to grow lawns overnight. After incubation, 1 ml of PBS was pipetted onto the plate to loosen the lawns and to enable the bacteria to be collected for the next passage. Frozen stocks of each derived isolate were made from this solution and the next passage sachets made from these stocks. Ten passages were conducted for each of the derived ten isolates and PCRs to ensure *P. poae* was recovered were carried out.

Examination of trade-offs

After undergoing 10 passages for either biofilm formation or aphid-killing, an assessment of whether trade-offs had occurred between traits was carried out with the final evolved isolates. Each isolate was tested in each of the following experimental procedures three times in triplicate.

Aphicidal properties and biofilm formation

Final strain biofilm-passaged bacteria were tested for virulence against aphids following the same sachet methodology describes above. Likewise, aphid-passaged final isolates were tested for biofilm formation as previously mentioned.

Bacterial growth

Final passage isolates were tested for changes in growth when in a broth environment. Isolates from single colonies were grown overnight and resuspended in PBS at an OD to 1. In a 96 well plate, 180 µl of KB broth was added to each well and 20 µl of culture. Growth readings were taken using a Bioscreen C plate reader, at 27°C using a 600 nm filter. Readings were taken every 20 minutes over 24 hours in continuously shaken and a static environment.

Motility assay

Whether the derived isolates differed in motility to the wild-type PpR24 was investigated. Two methods of bacterial motility, swimming and swarming, were examined. Isolates were streaked to single colonies on KB agar plates and grown overnight at 27°C. For swimming, each petri dish contained 30 ml of semi-solid, 0.25% w/v agar with 10% KB. For swarming plates, 0.25% w/v agar with full strength KB was used. Plates were inoculated with strains by stabbing the centre of the plate, taking care not to penetrate through the agar to the bottom. Plates were imaged at 0, 12, 24, 36 and 48 hours and incubated at 27°C between imaging. Images were taken using a G box and analysed using Image J software.

Bacterial colonisation on plant assay

Bacterial spray solutions of the wild-type and passaged isolates were made following the protocol previously describes and applied at a cellular suspension of 10^7 CFU mL^{-1}. Samples were taken on days 0, 1, 3, 7, 14, and 21. Leaf discs, 1 cm^2, were excised from the plants and placed in sterile Eppendorf tubes containing 200 µl sterile PBS. Leaf discs were macerated using micropestles and vortexed for five seconds. Dilution series were made, pipetting 10 µl of each dilution onto KA and nitrofurantoin plates in triplicate. Plates were left to grow for 16 hours at 27°C and colony counts made and averaged to determine CFU per leaf area.

Analysis of isolate volatile organic compounds and aphid host selection methodologies

Solid phase microextraction (SPME) and Gas-chromatography mass spectrometry (GC-MS) analysis of volatile compounds

Volatile compounds were extracted from the three bacterial spray treatments and 1xPBS control by Solid Phase Microextraction (SPME). Using a 50/30 µm DVB/CAR/PDMS Stableflex fiber (Supelco, Poole, UK), 10 ml of bacterial broth suspension were aliquoted into 20 ml glass SPME vials and equilibrated for 30 min at 37°C with agitation (500 rpm). The SPME fibre was then exposed to the suspension headspace for 20 min followed by desorption in the GC injection port (splitless) at 250°C. An Agilent 5975C series GC/MSD coupled to an Agilent 7890A Gas Chromatograph was used, equipped with a Zebron ZB5-MSi column (30 m x 250 µm x 1.0 µm). The oven was held at 40°C for 5 min, increased from 40°C to 220°C at a rate of 4°C /min, increased to 300°C at 8°C /min and then held at 300°C for 5 min. Helium was the carrier gas at a flow rate of 0.9 ml/min. An internal standard was run before and after the samples to indicate any changes to experimental conditions. Mass spectra were recorded in electron impact mode at an ionization voltage of 70 eV and source temperature of 220°C. A scan range of m/z 20-280 with a scan time of 0.69 s was employed and the data were controlled and stored by the ChemStation system. Volatiles were identified by comparison of spectra and linear retention indices (based on C5-C26 alkane series) from authentic compounds.

Dynamic Headspace Extraction (DHE) and GC-MS analysis of volatile compounds

SPME proved unsuitable for extracting volatiles when the bacteria was applied to the plant, possibly due to the larger headspace and more complex volatile blends being produced. A more active extraction approach was required to focus the volatiles onto a trap therefore dynamic headspace extraction was used. Four-week-old sweet pepper plants were sprayed with one of the four treatments, aphid-passaged PpR24a1, biofilm-passaged PpR24b4, wild-type PpR24 and control PBS as a stated above. Individual plants were left in sealed, glass flasks to equilibrate for one hour before volatile extraction occurred. The average ambient temperature was 30.5°C. Controls of soil, no-spray and an empty jar were also recorded. Charcoal-filtered air was pulled through the glass collecting jar containing the sample plant at a flow rate of 120 mL/min and volatiles were collected on a SUPELCO trap. Volatile collection lasted one hour and after each run, the glassware was sterilised in odourless deacon and autoclaved in an oven. A 1 µl of 130.6 mg/µL Dichlorobenzene in ether standard was applied to each trap and charcoal filtered air blown over it for three minutes. Traps were then loaded onto an Analytical Thermal Desorption (ATD) machine and run for 58 minutes. Volatiles were identified for the SPME method, using the Adams library.

Wild-type and passaged isolate choice box assays

Plants were sprayed with their allocated treatment, either wild-type *P. poae* PpR24, aphid-passaged isolate PpR24a1, biofilm-passaged PpR24b4 or 1xPBS as a control. Two sweet pepper plants, one treated with a bacterial treatment and one control, were placed side by side in an aerated perspex box, 11.5 cm x 6 cm x 17.5 cm, ensuring the plants were not touching. One Eppendorf containing 50 final instar aphids was placed equidistantly between the two plants. The lid was open to allow free movement to either the treated or control plant. The number of aphids on each plant was recorded after one week. Ten replicates of each treatment were recorded and the average aphid count per plant calculated. Welch two-sample t-tests were used to assess statistical differences in preference for aphid settling behaviour.

Choice box spray post-settle assay

One untreated sweet pepper plant (Plant 1) was placed in an aerated box as described above. An open Eppendorf containing 50 adult aphids was placed at the base of the plant and left for 24 hours, allowing the aphids to settle on the plant. After 24 hours, the number of aphids settled on the Plant 1 was recorded (Day 0). Plant 1 was then sprayed following the protocol as described above with either wild-type PpR24, 1xPBS or no spray was applied and a second, untreated plant (Plant 2) was introduced to create a 'sink' for the aphids to move to if they desired. The number of aphids on each plant was recorded at 24 hours and 72 hours from the spray application.

Non-target effects on natural enemies

Orius laevigatus, *Aphidius colemani* and *Macrolophus pygmaeus* were produced by Biobest and supplied by Agralan growers. Insects were kept in a controlled environment room at 25°C on a long-day light cycle (16/8 hours). Assays were conducted under the same conditions. Four-week old sweet pepper plants and final instar, adult predators, parasitoids and aphids were used. For each assay, mortality assessment was conducted at 72 hours and insects were considered dead if they failed to move after a light prod with a paint brush. For topical and residual assays, the insects were supplemented with food to ensure they did not starve to death. *O. laevigatus* and *M. pygmaeus* were supplemented with Nutrimac *Ephestia kuehniella* eggs produced by Biobest. *A. colemani* were nourished with 30% honey water.

Topical assay

Insects were chilled at 4°C for 3-5 minutes. This was sufficient time to immobilised them to allow droplet application without causing lasting harm. A 10 µl micropipette was used to administrate 1 µl of wild-type PpR24 at 10^7 concentration onto the dorsal side of the insects. `Non-spray' control insects were not treated with a droplet and PBS controls were treated with

1 µl of 1xPBS as a control for the droplet procedure. Ten insects were used per replicate, with each treatment replicated ten times (100 insects per treatment).

Residual spray assay

A spray solution of the wild-type PpR24 was made up and 1 ml was applied to the abaxial and adaxial surface of an excised leaf. A 1xPBS spray was applied to control leaves. Leaves were left to dry in a laminar flow cabinet until completely dry, then placed in aerated boxes. Five predators or parasitoids were placed in each box and left for 72 hours, after which mortality was observed.

Oral assay

Aphids were allowed to feed on sachets containing PBS control and wild-type PpR24 for 24 hours. Ten final instar aphids were placed in a petri dish with one predator insect and the dish sealed with parafilm. The predators were left to feed on the aphids for 72 hours and their mortality observed.

A. colemani mummy emergence

Ten mummified aphids of *A. colemani*, were placed in a petri dish. As above, 1 µl of wild-type PpR24 at 10^7 concentration was pipetted onto the mummies. `Non-spray' control mummies were not treated with a droplet and PBS controls were treated with 1 µl of 1 x PBS as a control for the droplet procedure. Wasp emergence was recorded over four days.

Results

Experimental Evolution passages

After ten passages were complete for each of the experimental set-ups, to assess whether the two evolution passages were successful, z scores were used to test for statistical differences between final isolates within each lineage. Scores were considered statistically different from the rest if they were outside two standard deviations from the mean. No significant differences in final isolate performance were seen for aphid virulence or biofilm attachment, however one isolate, PpR24b4, formed statistically stronger biofilms than the other isolates (Figure 1).

Virulence passages yielded no consistent improvement in percentage aphid mortality from one passage to the next (Figure 2). This suggests the evolution passages for aphid virulence were unsuccessful. After ten passages, only isolate PpR24b4 evolved strong biofilm formation (Figure 3), consistently forming a biofilm from the seventh passaged, unlike other strains that inconsistently formed biofilms throughout the duration of the study. Cellular attachment of the biofilm-passaged isolates to the glass universal walls appears to increase over the course of the ten passages, however no statistical difference was observed between the final passage isolates (Figure 4).

Microcosms in which biofilms developed showed a loss of pigmentation when compared to non-biofilm forming isolates. In the eight lineages that did not form biofilms, after one week of growth the bacterial-broth suspension was an opaque, yellow-green, whereas the broth of the biofilm-forming isolates was yellow but far more transparent (Figures 5 and 6).

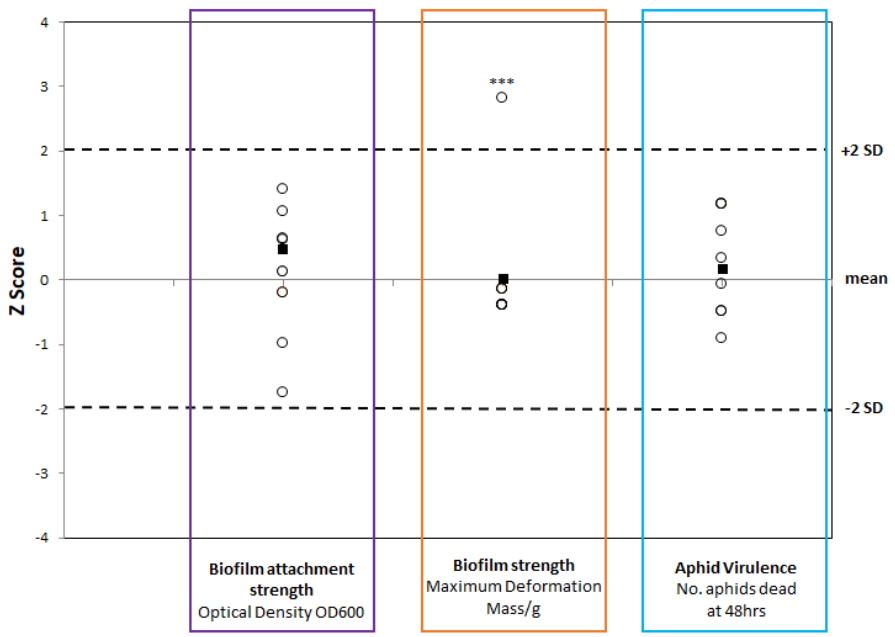


Figure 1 Passage results for the final isolates of the three traits we hoped to evolve after 10 passages. Results were standardised as z scores to significant differences between isolates to be observed. Only one isolate, biofilm former PpR24b4, was significantly different to the other isolates after the passaging process.

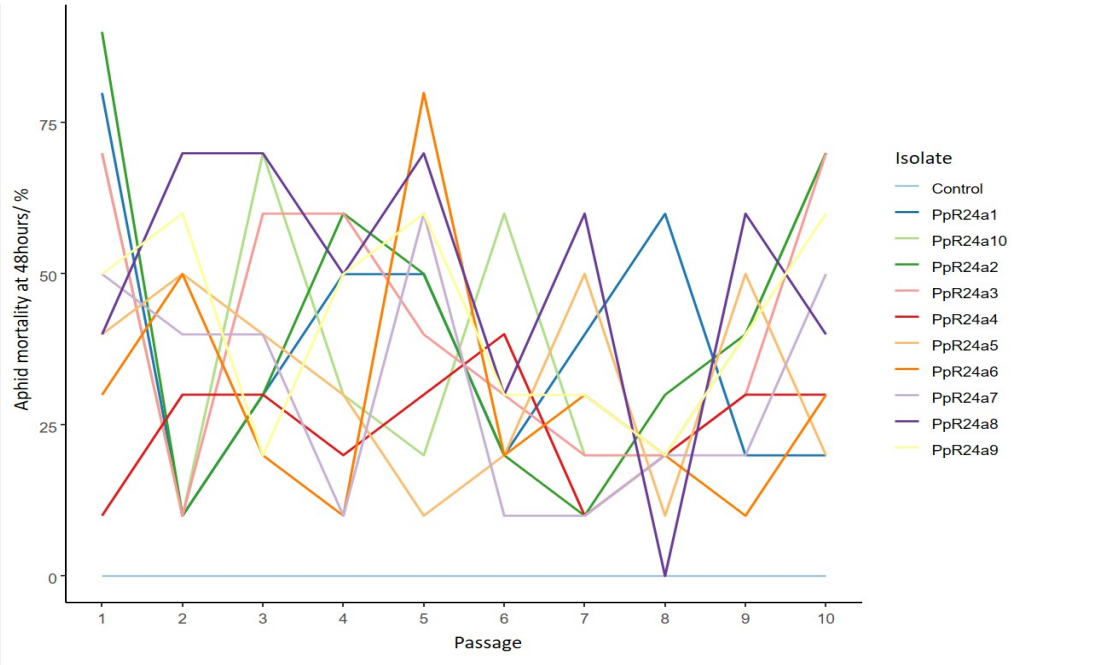


Figure 2 Percentage aphid mortality at 48 hours for each of the 10 passages. Aphid Mittler diet was inoculated with bacteria at a cellular suspension of 10^{-7} CFU $mL^{-1.}$ Ten aphids were left to feed on a sachet for 48 hours, with aphid mortality recordings taken at 0, 1, 6, 18, 24, 30, 42, 46 and 48 hours. Dead aphids were recovered after 48 hours, surface sterilised, homogenised in PBS and the bacterial-aphid slurry plated onto KA and nitrofurantoin plates. After 16 hours of growth, bacteria were recovered from the plate and used to inoculate the next passage.

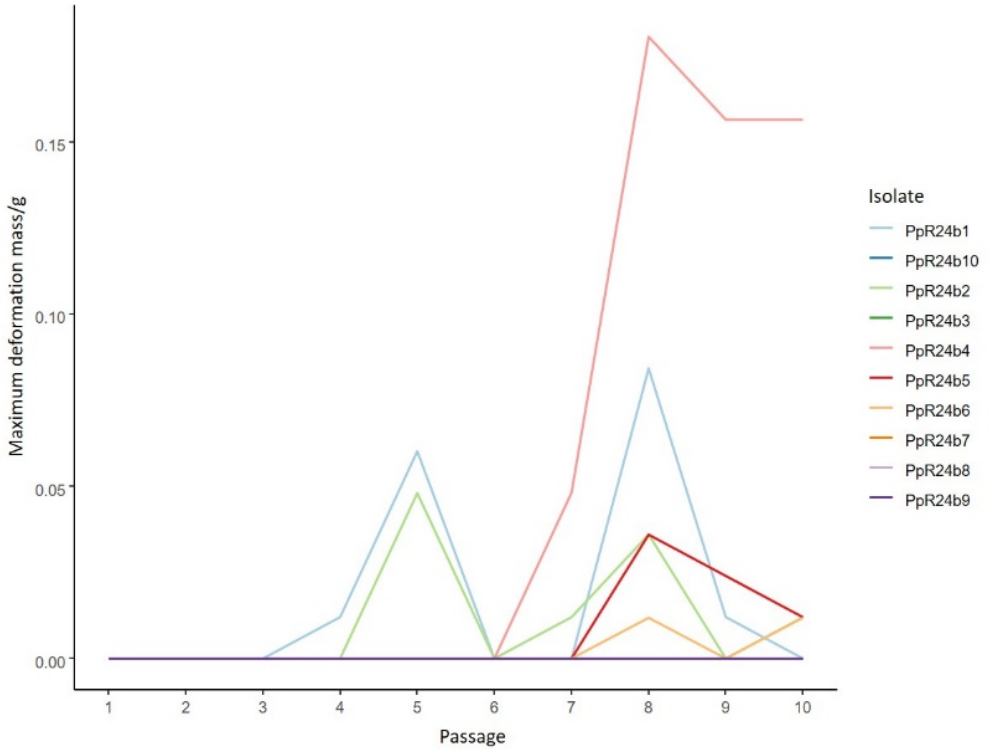


Figure 3 Maximum deformation mass of biofilm passaged isolates. Isolates were left to grow in KB broth for one week at 27ºC without agitation. After a week had elapsed, the maximum deformation mass of potential biofilms was tested by dropping 2 mm diameter glass beads into the centre of the biofilm from a consistent height. The mass supported indicated the strength of the biofilm.

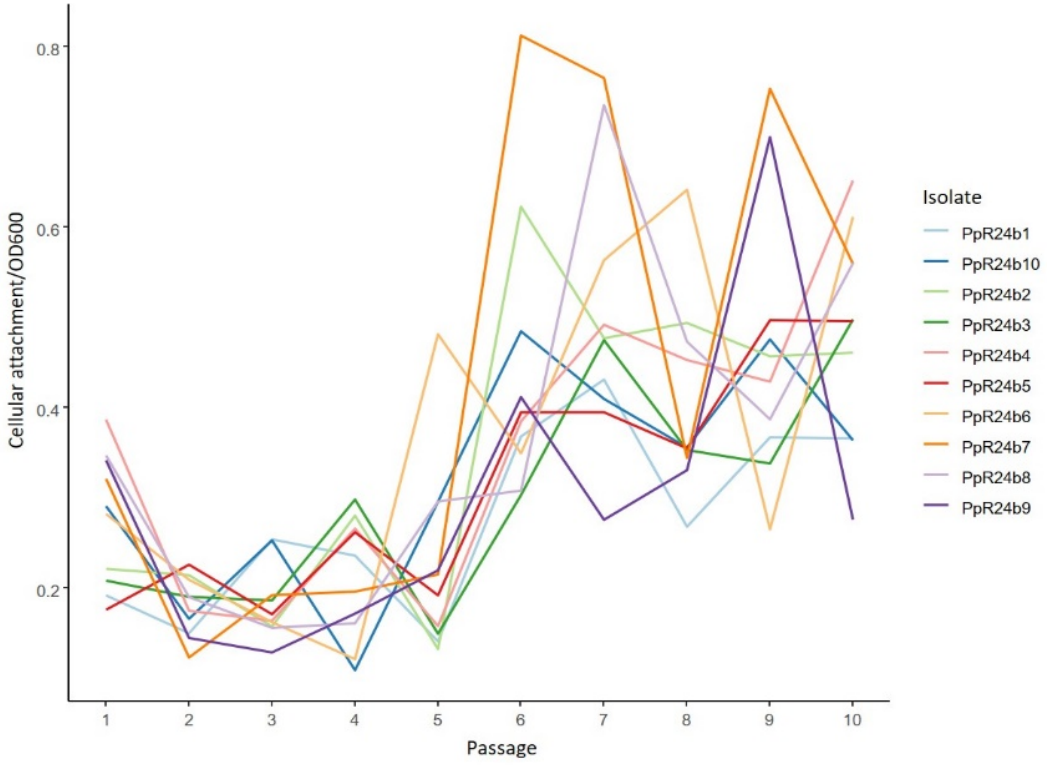


Figure 4 Biofilm attachment strength of biofilm passaged isolates. For each passage, emptied microcosms were stained with 1 ml of 0.05% (w/v) Crystal Violet. The vials were agitated for one minute and gently rinsed out with water. The stain was then eluted with 5 ml ethanol, shaken for 15 minutes and the OD600 recorded.

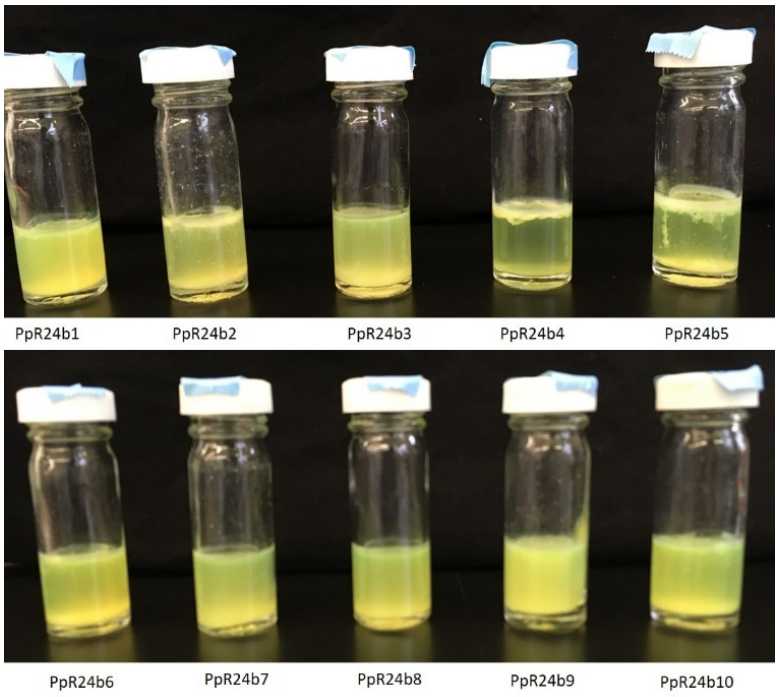


Figure 5 Final evolved biofilm-passaged isolate microcosms after ten passages. Sterile KB broth was innoculated with 10μl of bacteria from the previous passage in isolates PpR24b1-5, and 100μl in PpR24b6-10. Isolates were grown for a week at 27\degree C with no agitation and loose lids.

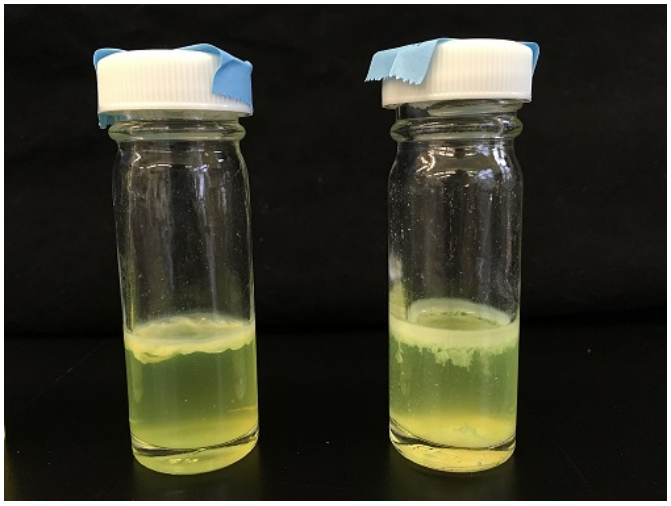


Figure 6 Bioiflm-forming isolates from passage ten, *P. poae* PpR24b4 and PpR24b5.

Analysis of trade-off between the wild-type and evolved isolates

Significant fitness trade-offs were seen between evolved isolate phenotypes and wild-type *Pseudomonas poae* PpR24. No significant difference in aphid killing was observed between the wild-type PpR24 and aphid-passaged isolates, therefore we may conclude no improvement to aphid killing was evolved via the evolution passages.

Kruskal-Wallis rank sum test revealed a significant difference in aphid virulence between final passaged isolate treatments (Kruskal-Wallis chi-squared = 125.86, df = 21, p-value<2.2^{-16}) (Figure 7), with the significant pair-wise differences summarised in Table 1. All aphid-passaged isolates were significantly different to the PBS control and several biofilm-passaged isolates were different to aphid-passaged bacteria, including the strong biofilm forming isolate PpR24b4.

Aphid-killing isolates proved to be poor biofilm formers (Figures 7 and 8) but remained indifferent to the wild-type in terms of tested trade-offs. Biofilm-passaged isolates showed a decrease in swimming and swarming motility when compared to the wild-type (Figures 9 and 10), as well as showing slower growth in a broth environment (Figure 10). Biofilm-formers also had a significantly reduced virulence to aphids (Figure 6) and were unable to persist on the phylloplane of pepper plants for as long as the aphid-passaged isolate and wild-type (Figure 7). No statistically significant improvements in aphid mortality at 48 hours was observed between the standardised aphid-passaged isolates (Figure 6).

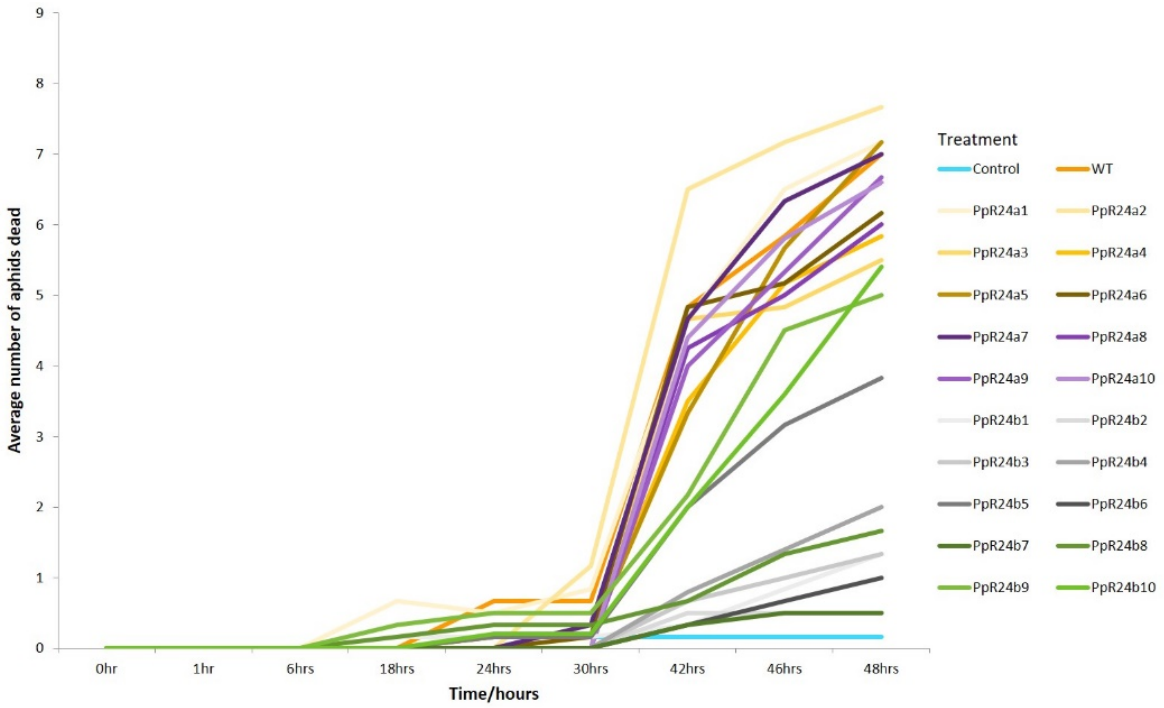


Figure 1 Trade-off in aphid virulence. Aphid mortality was tested for the wild-type PpR24, control PBS and all the final derived isolates from the biofilm and aphid passages. Mittler diet was innoculated with bacteria at a cellular suspension of 10−7 CFU mL−1. Ten aphids were left to feed on a sachet for 48 hours, with aphid mortality recordings taken at 0, 1, 6, 18, 24, 30, 42, 46 and 48 hours.

Tabke 1 Significant pair-waise comparisons from Kruskal-Wallis and Dunn post-hoc test analysis of final evolved isolate aphid virulence, where 'control' is PBS and 'WT' the ancestor wild-type PpR24 strain.

Comparison	Z	P.unadj	P.adj	Sign.
control - PpR24a1	-5.05083084	4.40E-07	0.00010162	***
control - PpR24a10	-3.77333186	1.61E-04	0.03720989	*
control - PpR24a2	-4.79859201	1.60E-06	0.0003691	***
control - PpR24a3	-4.25513732	2.09E-05	0.00482607	**
control - PpR24a4	-4.41170235	1.03E-05	0.00236916	**
control - PpR24a5	-4.75485859	1.99E-06	0.00045873	***
control - PpR24a6	-4.4395838	9.01E-06	0.00208207	**
control - PpR24a7	-4.21009807	2.55E-05	0.0058965	**
control - PpR24a9	-3.77567264	1.60E-04	0.03686216	*
PpR24a1 - PpR24b1	4.4717547	7.76E-06	0.00179211	**
PpR24a2 - PpR24b1	4.23680563	2.27E-05	0.00523728	**
PpR24a4 - PpR24b1	3.83262621	1.27E-04	0.02928676	*
PpR24a5 - PpR24b1	4.17578244	2.97E-05	0.00685986	**
PpR24a6 - PpR24b1	3.86050765	1.13E-04	0.02613804	*
PpR24a1 - PpR24b2	4.70338516	2.56E-06	0.00059109	***
PpR24a2 - PpR24b2	4.46152018	8.14E-06	0.00187988	**
PpR24a3 - PpR24b2	3.90769163	9.32E-05	0.02152507	*
PpR24a4 - PpR24b2	4.06425667	4.82E-05	0.01113092	*
PpR24a5 - PpR24b2	4.4074129	1.05E-05	0.00241655	**
PpR24a6 - PpR24b2	4.09213811	4.27E-05	0.00987326	**
PpR24a7 - PpR24b2	3.86265238	1.12E-04	0.02590955	*
PpR24a1 - PpR24b4	3.89482327	9.83E-05	0.02270045	*
PpR24a1 - PpR24b6	4.0428094	5.28E-05	0.01220016	*
PpR24a2 - PpR24b6	3.82066757	1.33E-04	0.03074401	*
PpR24a5 - PpR24b6	3.74683715	1.79E-04	0.04136708	*
PpR24a1 - PpR24b7	4.09428284	4.23E-05	0.00978232	**
PpR24a2 - PpR24b7	3.87060414	1.09E-04	0.02507874	*
PpR24a5 - PpR24b7	3.79831058	1.46E-04	0.0336534	*
PpR24a1 - PpR24b8	4.03851995	5.38E-05	0.01242538	*
PpR24a5 - PpR24b8	3.7425477	1.82E-04	0.04207987	*
control - PpR24b9	-4.09428284	4.23E-05	0.00978232	**
control - wt	-4.55754375	5.18E-06	0.00119555	**
PpR24b1 - wt	-3.97846761	6.94E-05	0.01602236	*
PpR24b2 - wt	-4.21009807	2.55E-05	0.0058965	**

When tested for biofilm formation and cellular attachment strength, aphid-passaged isolates could not form biofilms and, in general, exhibited weaker cellular attachment than the biofilm passaged isolates (Figures 8 and 9). Kruskal-Wallis rank sum test revealed a significant difference in maximum deformation mass between the final strain isolates (Kruskal-Wallis chi-squared 61 = 119.59, df = 20, p-value = 3.393−16) (Figure 8). Dunn post-hoc tests indicated that isolates PpR24b1 and PpR24b4 had significantly different cellular attachment than the wild-type PpR24, biofilm isolates PpR24b7, PpR24b9, PpR24b10, and all the final strain aphid passaged isolates (PpR24b1 p-value = 0.000379 and PpR24b4 p-value = 0.00106). Kruskal-Wallis rank sum test revealed a significant difference in cellular attachment between the final strain isolates (Kruskal-Wallis chi-squared = 85.838, df = 20, p-value = 3.393−10) (Figure 9).

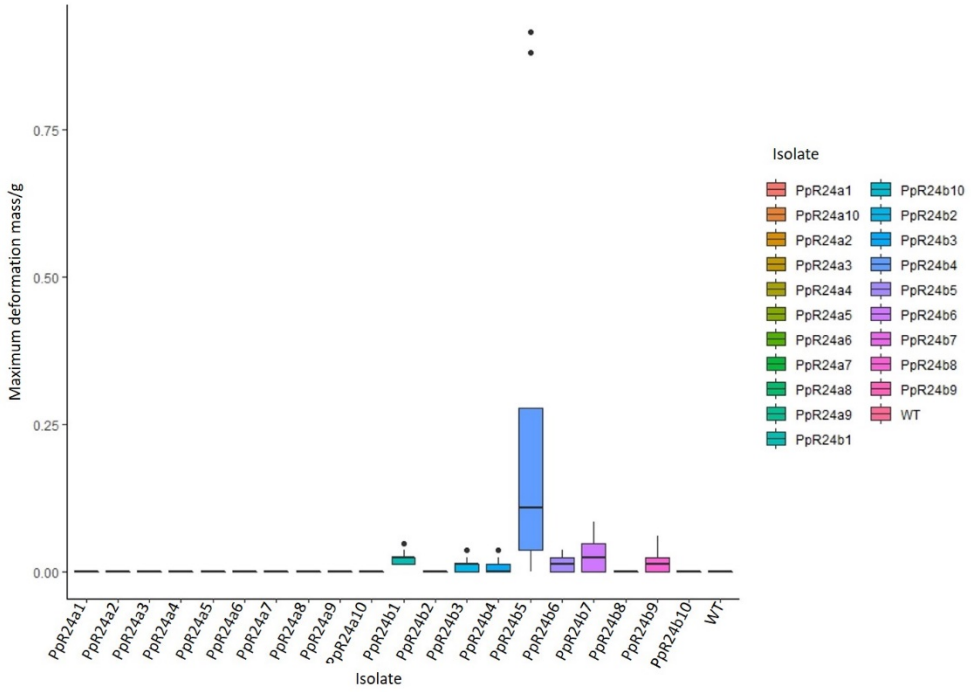


Figure 2 Trade-off biofilm strength. Maximum deformation mass of all final biofilm-passaged and aphid-passaged isolates compared to the wild-type PpR24. Isolates were left to grow in KB broth for one week at 27◦C without agitation. After a week had elapsed, the maximum deformation mass of potential biofilms was tested by dropping 2 mm diameter glass beads into the centre of the biofilm from a consistent height. The mass supported indicated the strength of the biofilm.

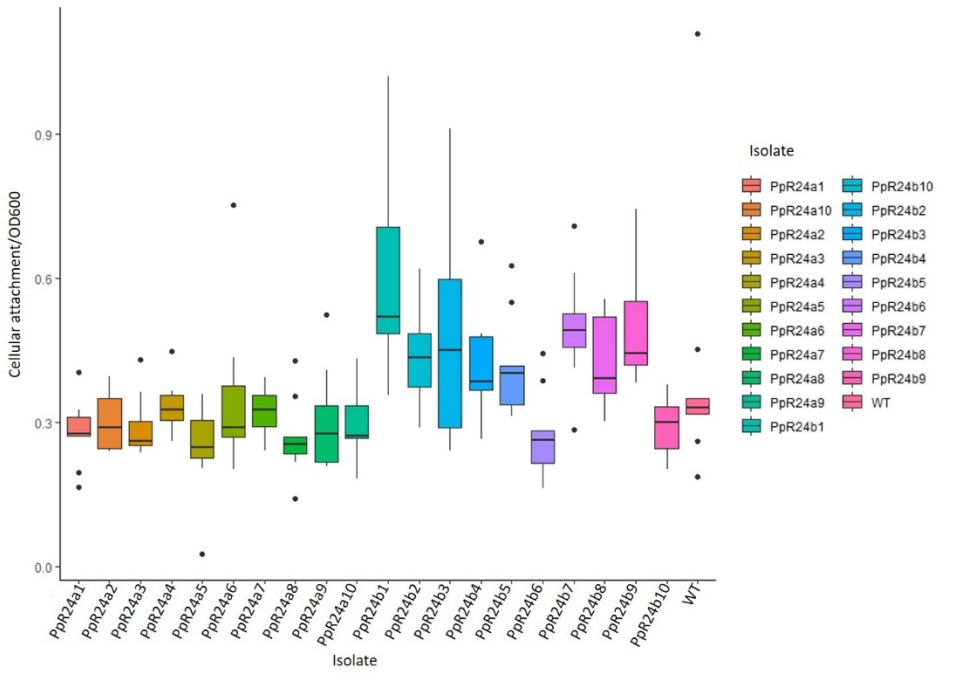


Figure 3 Trade-off biofilm attachment strength. All final biofilm-passaged and aphid-passaged isolates compared to the wild-type PpR24. Isolates were left to grow in KB broth for one week at 27◦C without agitation. After a week had elapsed and maxuimum deformation mass readings taken, emptied microcosms were stained with 1 ml of 0.05% (w\v) Crystal Violet. The vials were agitated for one minute and gently rinsed out with water. The stain was then eluted with 5 ml ethanol, shaken for 15 minutes and the OD600 recorded.

Trade-offs in bacterial growth

In terms of isolate growth and colony morphology, when grown on KA plates for 24 hours, no difference in the amount of growth or colony morphology was observed between the aphid-passaged isolates and the wild-type PpR24. However, the strong biofilm forming isolate PpR24b4 had reduced plate growth when compared to the wild-type as well as smaller, more concise circular colonies than the wild-type (Figure 10).

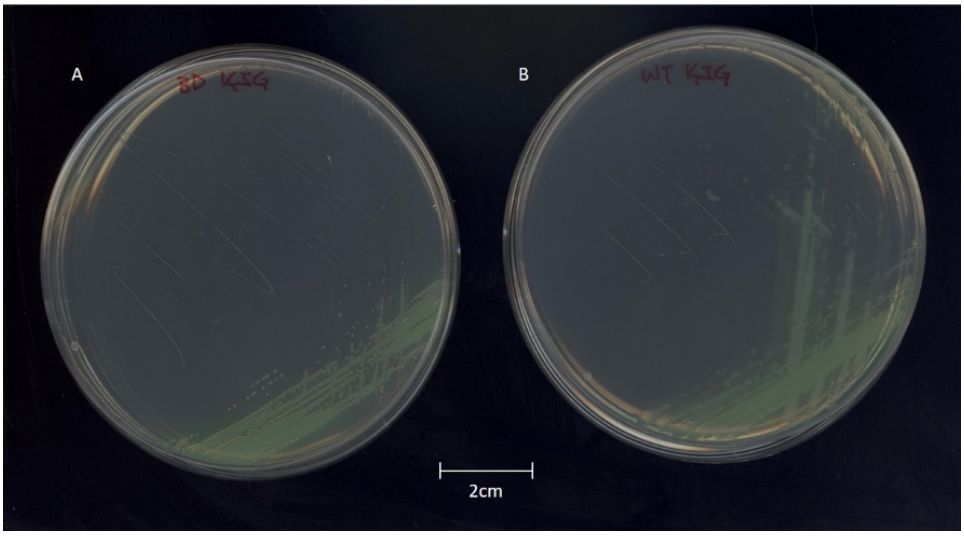


Figure 4 A comparison in colony morphology between A) biofilm forming isolate PpR24b4 and B) wild-type PpR24 after 16 hours.

Little difference was observed in the rate of growth between the wild-type PpR24 and the aphid passaged isolates in both static and shaken conditions. For static growth conditions, at 24 hours Kruskal-Wallis chi-squared indicated significant differences between isolates (p-value = 0.001755). Isolate PpR24b9 in particular took noticeably longer than all other isolates to reach the stationary phase of growth (Figure 11). However, Dunn post-hoc analysis, after Bonferroni adjustment found no significant differences between absorbance levels in the final strain isolates. The same was also found for final absorbance levels after 24 hours of growth of isolates in a shaken environment (Figure 12). Kruskal-Wallis analysis revealed strong statistical significance (p-value = 0.00005445) but post-hoc comparisons with Bonferroni correction found no significant differences between individual isolates. However, by eye it appears the isolates PpR24b4 and PpR24b9 reached the stationary phase at a lower population than the other isolates. Furthermore, nearly all biofilm passaged isolates had less growth in a shaken environment than the wild-type and aphid passaged bacteria.

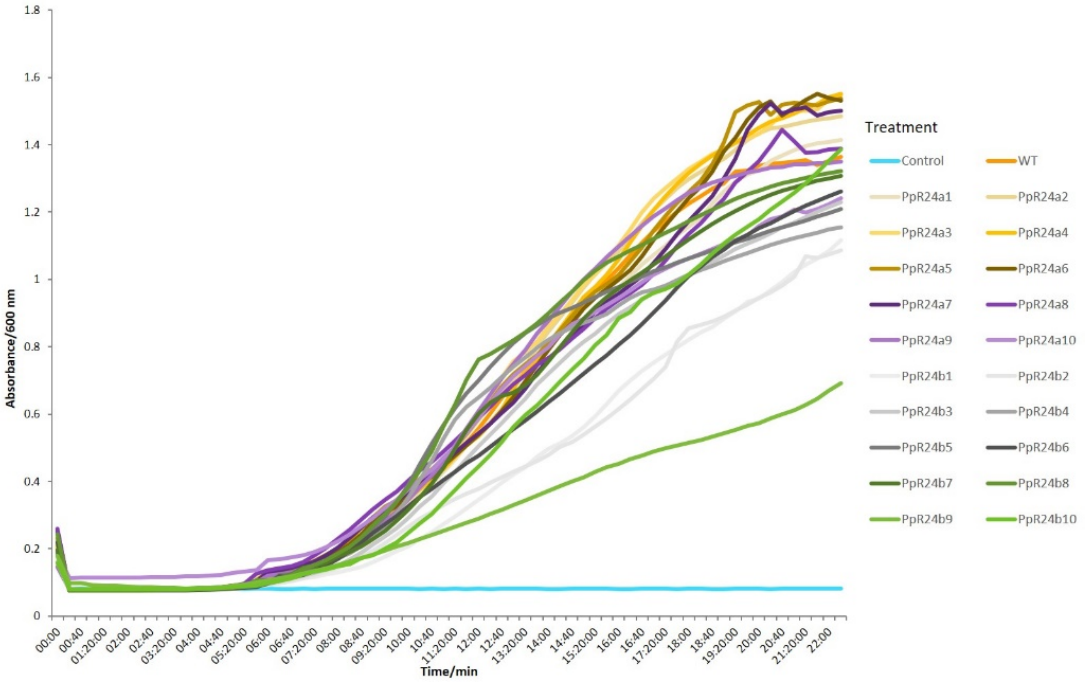


Figure 11 Growth comparison of final evolved isolates, PpR24 wild-type (WT) and PBS control in static conditions. Bacteria were grown in a 100 well honeycomb plate in KB media at 27◦C for 24 hours. Absorbance readings were taken every 20 minutes with a 600nm filter. Kruskal-Wallis chi-squared = 44.957, df = 21, p-value = 0.001755.

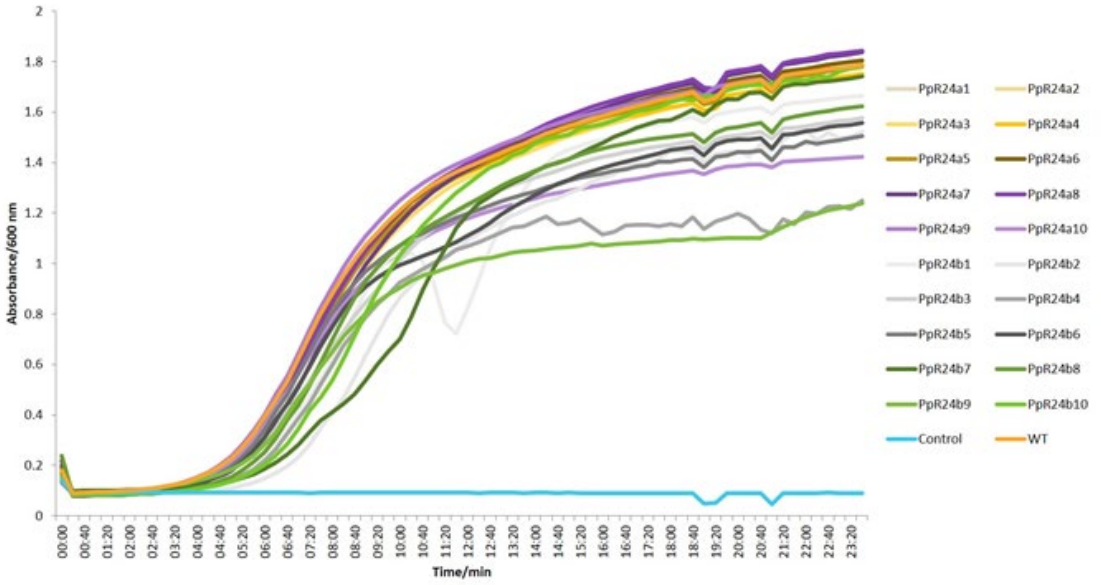


Figure 12 Growth comparison of final evolved isolates, PpR24 wild-type (WT) and PBS control in continuously shaken conditions. Bacteria were grown in a 100 well honeycomb plate in KB media at 27◦C for 24 hours. Absorbance readings were taken every 20 minutes with a 600nm filter. Kruskal-Wallis chi-squared = 55.775, df = 21, p-value = 0.00005445.

Trade-offs in bacterial motility

Isolate motility was investigated as flagella are known to play a role in biofilm surface attachment and are also associated with bacterial virulence. In several bacterial pathogens, flagella-mediated motility has been seen to correlate with pathogen virulence (Josenhans and Suerbaum, 2002). Flagella can play an important role in the early colonisation stages of a host but after colony establishment many species down-regulate flagella production in favour of systems supportive of a sessile lifestyle, such as exopolysaccharide (EPS) production (Josenhans and Suerbaum, 2002; Alsohim *et al.*, 2014). Pathogens can also require maintained motility to thrive in a host. Bacteria may use flagellum for various forms of motility. Swimming motility can be defined as individual cells moving through a medium powered by rotating flagella (Kearns, 2010), whereas swarming describes the flagella-driven, synchronised group movement of bacteria (Verstraeten *et* al., 2008; Morales-Soto *et al*., 2015). Assessing the impact of serial passaging on evolved isolate swimming and swarming behaviour may be of interest in correlation to other phenotypic changes (Ude *et al*. 2006).

There was no significant difference in either mode of motility between the wild-type and aphid-passaged isolates. Analysis of final spread in swimming agar found statistically different differences in the data (Kruskal-Wallis chi-squared = 128.87, df = 21, p-value < $2.2e^{-16}$). No significant differences were observed in the area of spread between aphid-passaged isolates but several of the biofilm-passaged isolates had reduced spread when compared to the aphid-passaged bacteria (Figure 13). Isolates PpR24b2, PpR24b6 and PpR24b8 in particular exhibited statistically different spread when compared to aphid and wild-type isolates (Table 2). Results for area covered by isolates on swarming agar show a stark difference between aphid and biofilm passaged isolates, with biofilm-passaged isolates all having reduced spread compared to aphid-isolate spread (Figure 14). Significant differences were also observed statistically (Kruskal-Wallis chi-squared = 140.84, df = 21, p-value < 2.2e-16). Significant comparisons between isolates, adjusted with the Bonferroni method found in table 3, with isolates PpR24b2, PpR24b6 and PpR24b8 significantly lower than most aphid isolates and the wild-type PpR24.

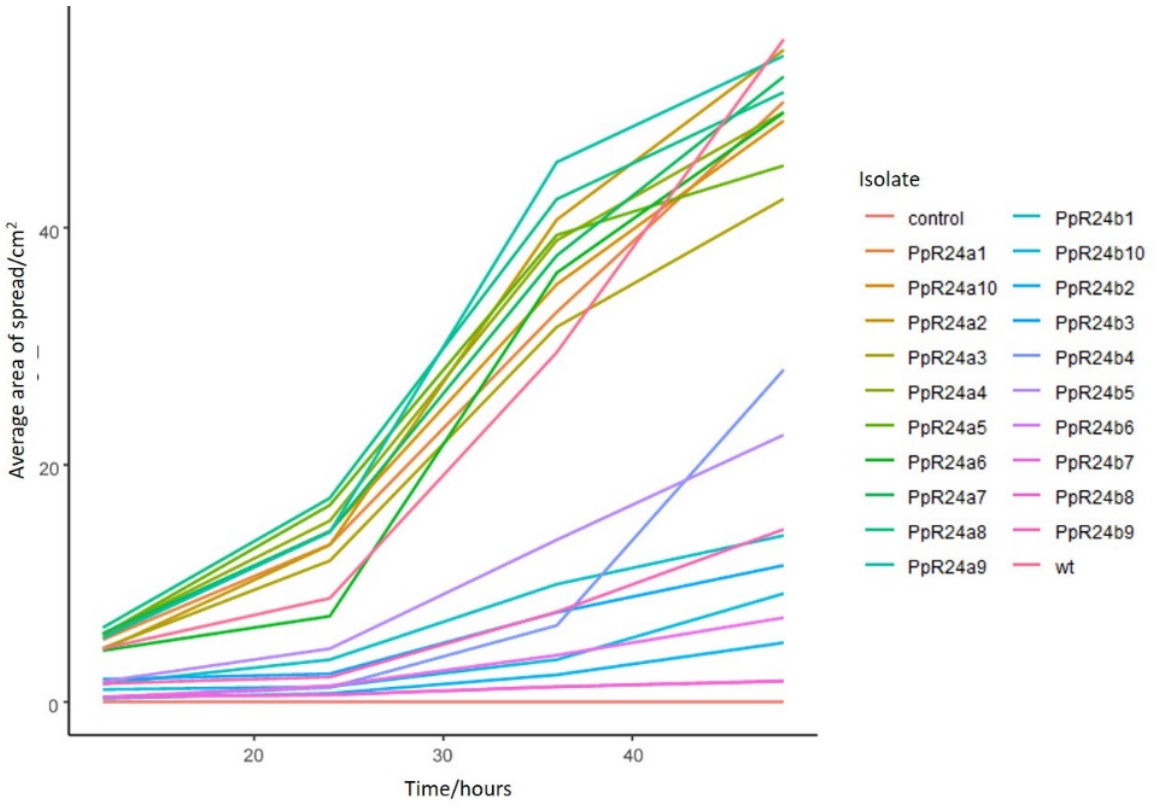


Figure 13 Average bacterial spread in swimming agar between final evolved isolates, the wild-type PpR24 and PBS control. Isolates were inoculated in plates of 30 ml semi-solid, 0.25% w/v agar with 10% KB and images taken at 12, 24, 36 and 48 hours.

Tabke 2 Significant comparisons of isolate swimming spread at 48 hours of growth. Kruskal-Wallis chi-squared = 128.87, df = 21, p-value < 2.2e16, with p-values adjusted using the Bonferroni method. All aphid-passages isolates were strongly significant to the control but have been excluded from this table.

Comparison	Z	P.unadj	P.adj	Sign.
PpR24a9 - PpR24b10	3.8351572	1.25E-04	2.90E-02	*
PpR24a1 - PpR24b2	3.66318092	2.49E-04	5.75E-02	*
PpR24a10 - PpR24b2	3.78453279	1.54E-04	3.56E-02	*
PpR24a2 - PpR24b2	4.40980342	1.03E-05	2.39E-03	*
PpR24a4 - PpR24b2	3.95818442	7.55E-05	1.74E-02	*
PpR24a5 - PpR24b2	3.86886205	1.09E-04	2.53E-02	*
PpR24a6 - PpR24b2	3.93043926	8.48E-05	1.96E-02	*
PpR24a7 - PpR24b2	3.76881185	1.64E-04	3.79E-02	*
PpR24a8 - PpR24b2	4.02723652	5.64E-05	1.30E-02	*
PpR24a9 - PpR24b2	4.85651523	1.19E-06	2.76E-04	***
Control - PpR24b5	-3.683749	2.30E-04	5.31E-02	*
PpR24a1 - PpR24b6	4.05397507	5.04E-05	1.16E-02	*
PpR24a10 - PpR24b6	4.17532694	2.98E-05	6.87E-03	**
PpR24a2 - PpR24b6	4.80059757	1.58E-06	3.65E-04	***
PpR24a3 - PpR24b6	3.858578	1.14E-04	2.63E-02	*
PpR24a4 - PpR24b6	4.35913076	1.31E-05	3.02E-03	**
PpR24a5 - PpR24b6	4.2596562	2.05E-05	4.73E-03	**
PpR24a6 - PpR24b6	4.30956527	1.64E-05	3.78E-03	**
PpR24a7 - PpR24b6	4.14793787	3.35E-05	7.75E-03	**
PpR24a8 - PpR24b6	4.41803067	9.96E-06	2.30E-03	**
PpR24a9 - PpR24b6	5.25746157	1.46E-07	3.37E-05	***
PpR24a2 - PpR24b7	3.79276003	1.49E-04	3.44E-02	*
PpR24a9 - PpR24b7	4.22344207	2.41E-05	5.56E-03	**
PpR24a1 - PpR24b8	4.05397507	5.04E-05	1.16E-02	*
PpR24a10 - PpR24b8	4.17532694	2.98E-05	6.87E-03	**
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PpR24a9 - PpR24b8	5.25746157	1.46E-07	3.37E-05	***
Control - wt	-5.4772885	4.32E-08	9.98E-06	***
PpR24b2 - wt	-4.4427124	8.88E-06	2.05E-03	**
PpR24b6 - wt	-4.8335066	1.34E-06	3.10E-04	***
PpR24b7 - wt	-3.825669	1.30E-04	3.01E-02	*
PpR24b8 - wt	-4.8335066	1.34E-06	3.10E-04	***

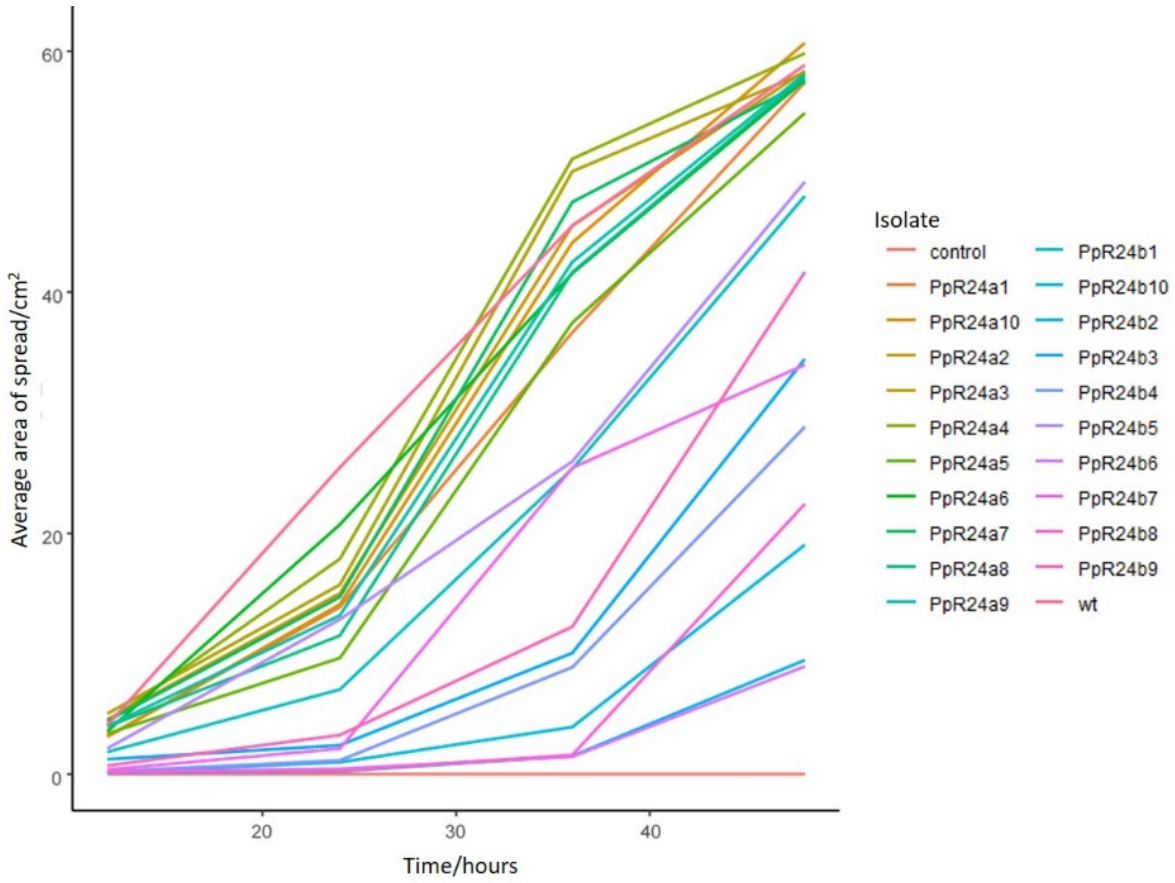

Figure 14 Average bacterial spread in swarming agar between final evolved isolates, the wild-type PpR24 and PBS control. Isolates were inoculated in plates of 30 ml semi-solid, 0.25% w/v agar with full-strength KB and images taken at 12, 24, 36 and 48 hours.

Table 3 Significant comparisons of isolate swarming spread at 48 hours of growth. Kruskal-Wallis chi-squared = 128.87, df = 21, p-value < 2.2e-16, with p-values adjusted using the Bonferroni method. All aphid-passages isolates were strongly significant to the control but have been excluded from this table.

Comparison	Z	P.unadj	P.adj	Sign.
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PpR24a9 - PpR24b2	4.85651523	1.19E-06	2.76E-04	***
Control - PpR24b5	-3.683749	2.30E-04	5.31E-02	*
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PpR24a9 - PpR24b7	4.22344207	2.41E-05	5.56E-03	**
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PpR24a4 - PpR24b8	4.35913076	1.31E-05	3.02E-03	**
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PpR24b7 - wt	-3.825669	1.30E-04	3.01E-02	*
PpR24b8 - wt	-4.8335066	1.34E-06	3.10E-04	***

Trade-offs in bacterial crop persistence

Due to limitations in experimental set up, only one isolate for the biofilm, PpR24b4, and aphid passages, PpR24a1, were investigated. After foliar spray application, the wild-type PpR24, isolate PpR24a1 and PpR24b4 were all present on the plant for the duration of the 21 day experiment (Figure 15). However, Kruskal-Wallis with Dunn post-hoc analysis found no significant difference between the bacterial populations at day 21 (p-value >0.05). Bacterial counts represent the total bacteria recovered from the internal and external leaf surfaces.

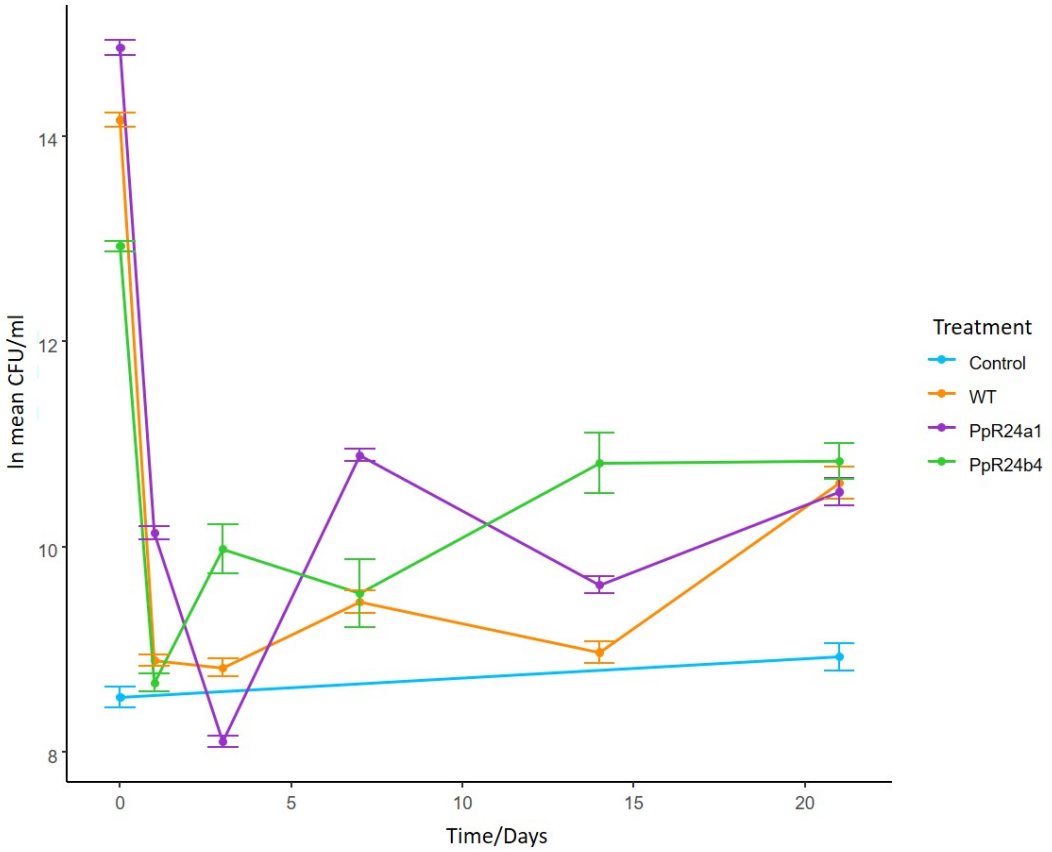


Figure 15 Bacterial isolate persistence on *Capsicuum annum.* Bacteria were applied via a foliar spray at a cellular suspension of 10−7 CFU mL−1. At 0, 1, 3, 7, 14 and 21 days, leaf disc samples were taken in triplicate for each treatment and homongenised in PBS before plating on KB and nitrofurantoin plate for bacterial enumeration.

Genomic changes between PpR24 and the passaged isolates

Whole genome sequencing of the wild-type PpR24 and final passage isolates PpR24a1 and PpR24b4 found significant differences between the genome of biofilm-forming isolate PpR24b4 and the wild-type PpR24. In regions unanimous to all three strains, PpR24, PpR24a1 and PpR24b4, only two single nucleotide variants were identified that differed from the reference wild-type *P. poae* PpR24 genotype. Both SNPs occurred in chromosome 1 of the biofilm passaged isolate, PpR24b4. One at the genomic position 728018 in the gene *cheB* 1 and the other at 3010602 in the gene *barA* 3. In *cheB* 1, a point mutation changed a T to an A, whereas in *barA* 3, a G became T. Both changes resulted in a missense effect.

Sequence alignment of the derived isolate gene sequences against the wildtype corroborated with the variant calling analysis. Consensus alignments of isolate PpR24a1 and the wild-type PpR24 had 100% similarity for both *cheB* and *barA* genes. However, the forward sequence for the wild-type bacteria *barA* gene failed to align, therefore only the reverse sequence was used in comparison the consensus *barA* sequences for PpR24a1 and PpR24b4. In *cheB*, BLAST results found 99% similarity between the gene sequences of PpR24 and the wild-type PpR24. A single point mutation was observed at location 290 on the

gene. Blast results found 99.73% similarity between the *barA* sequences for PpR24b4 and the wild type, with a point mutation at location 110. However, the sequence alignments did not match for the forward and reverse sequences on point mutation in *barA*. Although variant calling found a G on the wild-type became T in PpR24b4, BioEdit alignment found a discrepancy in the nucleotides base pairs at the point mutation site in PpR24b4. The forward sequence read C and the reverse A.

Both *cheB* and *barA* are genes associated with bacterial motility and biofilm formation, therefore it is possible that one, or both of these mutations is responsible for the evolved biofilm properties of PpR24b4. In Pseudomonads, WspF has been found to be homologous to CheB (Hickman *et al*., 2005). Mutations in WspF of *P. fluorescens* SPW25 cause dramatic changes in the bacterial phenotype compared to the wild-type, resulting in a Wrinkly-Spreader phenotype. Wrinkly spreaders form strong bioiflms and have a wrinkled appearance when grown on agar. As well as forming cell aggregations, WS are less motile than the wild-type SPW25 (D'Argenio *et al.*, 2002; Bantinaki *et al.*, 2007; Verstraeten *et al*., 2008). It is thought that the WS's strong biofilm is caused by the malfunctioning WspF methylesterase, which results in the continuous activation of WspR. The active WspR overproduces cyclic-di-GMP (c-di-GMP) and adhesive substances, such as partially acetylated cellulose polymer (Bantinaki *et al.*, 2007). c-di-GMP is a signalling molecule used to coordinate between a motile or sessile lifestyle. At high concentrations c-di-GMP initiates cell aggregation and biofilm formation whereas at low concentrations, cells are motile (Valentini and Filloux, 2016). The gene barA is responsible for the histidine sensor kinase, BarA, in the two-component signal transduction system (TCS) of E. coli, where UvrY acts as its counterpart response regulator (Chavez *et al.*, 2010). In Pseudomonas species, the homolog of this system is the GacS/GacA TCS, where GacS is homologous to BarA (Heeb and Haas, 2001). The GacS/GacA system regulates secondary metabolite and extracellular protein production. In Gram-negative bacteria the GacS/GacA system has been associated with biofilm formation, quorum sensing and bacterial virulence to plants, fungi and animals (Heeb and Haas, 2001; Davies *et al.*, 2007; Workentine *et al*., 2009).

Volatile Analysis of PpR24 and the effects of volatile organic compounds on aphid behaviour

Identifying the composition of volatile emissions from the bacteria may prove insightful in understanding the mechanisms of the aphid deterrent effect and how PpR24 interacts with the plant when applied as a foliar spray. However, as a result of the experimental passages, biofilm forming isolate PpR24b4 evolved a significantly different phenotype. PpR24b4 was

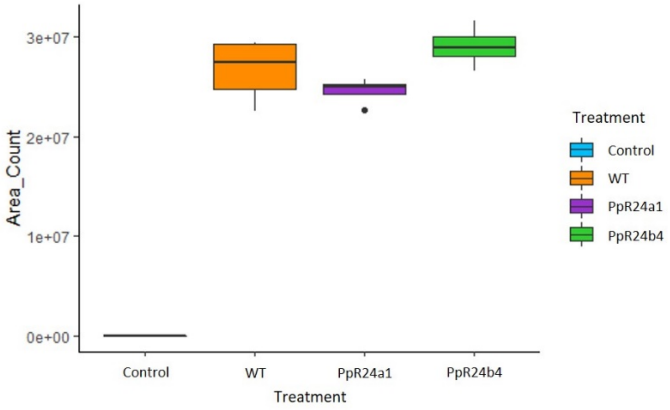
able to form strong biofilms at the air-liquid interface of static microcosms but showed impaired growth on agar and in shaken environments, as well as a reduction in motility. It is possible that as a consequence of these changes, the volatile compounds produced by PpR24b4 have also changed from those produced by the wild-type PpR24 and may affect the bacteria's aphid deterrent properties. The aims of this section were to firstly identify what VOCs are produced by the wild-type PpR24 and whether the volatile composition had changed in the derived isolates PpR24a1 and PpR24b4 as a result of experimental evolution. Secondly, this study aimed to identify the VOCs released when the wild-type and derived isolates were applied to a plant as a foliar spray and finally, whether the deterrent properties of the wild-type PpR24 were retained in the derived passaged isolates was also investigated.

Assessment of volatile organic compounds

The extraction of volatiles from the four spray solutions by SPME identified 11 putative VOCs and one unknown. The identified VOCs were: Acetone, the sulphur compounds Dimethyl sulphide, Dimethyl disulphide and Methanethiol, three alkenes 1-Undecene, 1-Nonene, 1-Decene and four alkanes Undecane, Dodecane, 2-Methylpentane and Tridecane (Table 4). The internal standard did not change more than 10% over the course of the experiment from which we can infer the experimental conditions were constant. The Kruskal-Wallis chi-squared test resulted in significant differences in compound levels between spray types found for dimethyl sulphide, dimethyl disulphide, methanethiol, acetone, 1-undecene, 1-nonene, 1-decene and 2-methylpentane (Table 4). Dunn's post-hoc test was used to assess pair-wise differences between the three isolate treatments and control. Significant changes in compound levels can be seen in Figures 16-22. Isolate PpR24b4 had statistical higher levels of dimethyl disulphide when compared to the control PBS. In all other compounds where a significant difference was observed, pair-wise comparisons indicated the wild-type PpR24 and isolate PpR24a1 were significantly different from the control PBS.

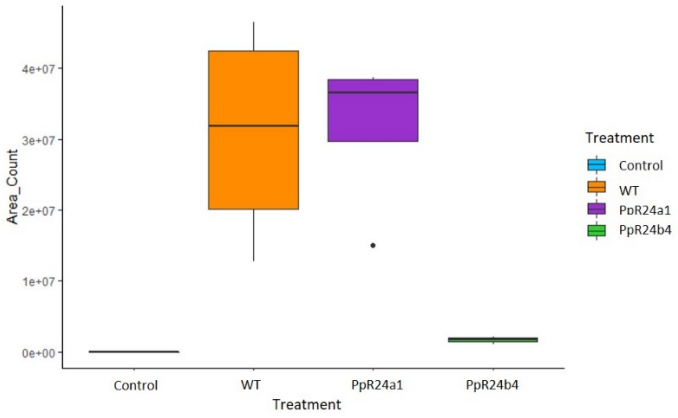
Table 4 Compounds identified in the SPME of volatiles from a control PBS broth, wild-type PpR24 isolate and the two derived isolates PpR24a1 and PpR24b4. Where RT - retention time/mins and p-value indicates whether a significant difference in compound levels between the three treatments and PBS control was observed.

Compound	RT/min	p-value	Sign.
Methanethiol	1.396	0.006187	**
Acetone	2.43	0.03613	*
Dimethyl sulphide	3.057	0.004818	**
2-Methylpentane	4.394	0.01034	**
Dimethyl disulphide	16.207	0.008992	**
1-Nonene	25.019	0.004431	**
1-Decene	29.838	0.005082	**
1-Undecene	34.188	0.005263	**
Undecane	34.556	0.9991	-
Dodecane	38.502	0.9956	-
Unknown 175	40.784	0.835	-
Tridecane	42.17	0.9991	-



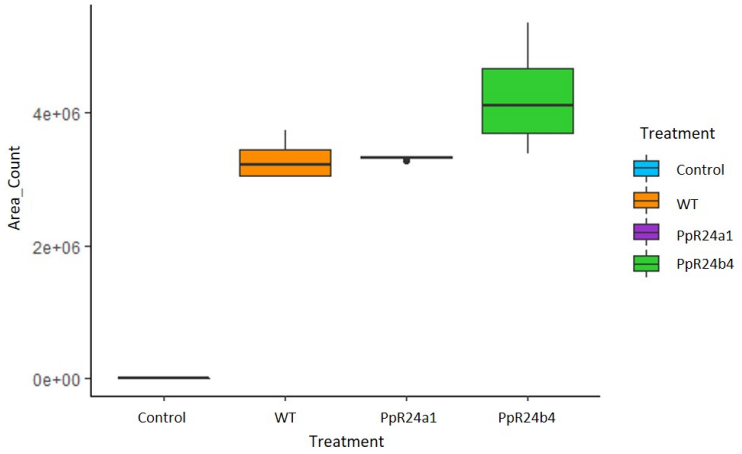
Comparison	Z	P.unadj	P.adj	Sign.
PpR24a1 – PpR24b4	-1.7207036	0.0853046219	0.51182773	-
PpR24a1 – Control	1.5710772	0.1161647168	0.69698830	-
PpR24b4 – Control	3.2917808	0.0009955517	0.00597331	**
PpR24a1 – WT	-0.7481320	0.4543805408	1.00000000	-
PpR24b4 – WT	0.9725716	0.3307662619	1.00000000	-
Control - WT	-2.3192092	0.0203836955	0.12230217	-

Figure 16 Area count of dimethyl disulphide compound levels for each treatment detected by solid phase microextraction and GC-MS. Control is 1xPBS in comparison with the bacterial a spray treatments where WT is the wild-type PpR24, PpR24a1 a final aphid-passaged isolate and PpR24b4 the final passage, strong biofilm-forming isolate. Pairwise comparisons from Kruskal-Wallis chi-squared analysis adjusted using the Bonferroni correction are presented. Kruskal-Wallis chi-squared = 11.575 df = 3 p-value = 0.008992.



Comparison	Z	P.unadj	P.adj	Sign.
PpR24a1 – PpR24b4	1.7207036	0.085304622	0.51182773	-
PpR24a1 – Control	2.9177148	0.003526067	0.02115640	*
PpR24b4 – Control	1.1970112	0.231302188	1.00000000	-
PpR24a1 – WT	-0.1496264	0.881059378	1.00000000	-
PpR24b4 – WT	-1.8703300	0.061438005	0.36862803	-
Control - WT	-3.0673412	0.002159722	0.01295833	**

Figure 17 Area count of dimethyl sulphide compound levels for each treatment detected by solid phase microextraction and GC-MS. Control is 1xPBS in comparison with the bacterial a spray treatments where WT is the wild-type PpR24, PpR24a1 a final aphid-passaged isolate and PpR24b4 the final passage, strong biofilm-forming isolate. Pairwise comparisons from Kruskal-Wallis chi-squared analysis adjusted using the Bonferroni correction are presented. Kruskal-Wallis chi-squared = 12.918 df = 3 p value - 0.004818.



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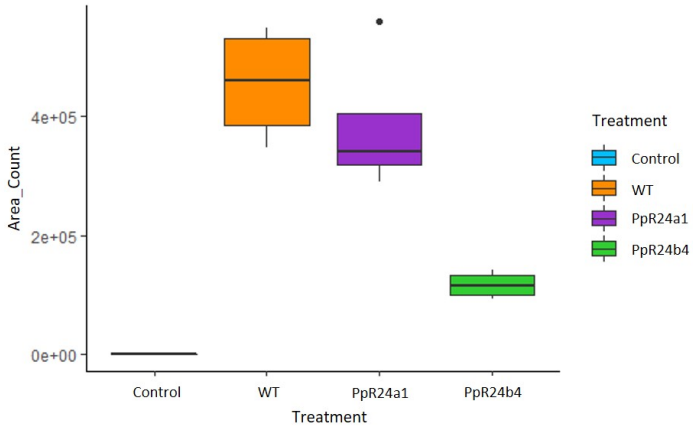
PpR24a1 – WT	-0.1496264	0.881059378	1.00000000	-
PpR24b4 – WT	-1.8703300	0.061438005	0.36862803	-
Control - WT	-3.0673412	0.002159722	0.01295833	**

Figure 18 Area count of methanethiol compound levels for each treatment detected by solid phase microextraction and GC-MS. Control is 1xPBS in comparison with the bacterial a spray treatment where WT is the wild-type PpR24, PpR24a1 a final aphid-passaged isolate and PpR24b4 the final passage, strong biofilm-forming isolate. Pairwise comparisons from Kruskal-Wallis chi-squared analysis adjusted using the Bonferroni correction are presented. Kruskal-Wallis chi-squared = 12.381 df = 3 p-value = 0.006187.



Comparison	Z	P.unadj	P.adj	Sign.
PpR24a1 – PpR24b4	1.5710772	0.116164717	0.696988301	-
PpR24a1 – Control	2.7680884	0.005638616	0.033831695	*
PpR24b4 – Control	1.1970112	0.231302188	1.000000000	-
PpR24a1 – WT	-0.4488792	0.653518801	1.000000000	-
PpR24b4 – WT	-2.0199564	0.043387910	0.260327458	-
Control - WT	-3.2169676	0.001295532	0.007773193	**

Figure 19 Area count of 1-nonene compound levels for each treatment detected by solid phase microextraction and GC-MS. Control is 1xPBS in comparison with the bacterial a spray treatments where WT is the wild-type PpR24, PpR24a1 a final aphid-passaged isolate and PpR24b4 the final passage, strong biofilm-forming isolate. Pairwise comparisons from Kruskal-Wallis chi-squared analysis adjusted using the Bonferroni correction are presented. Kruskal-Wallis chi-squared = 13.097 df = 3 p-value = 0.004431.

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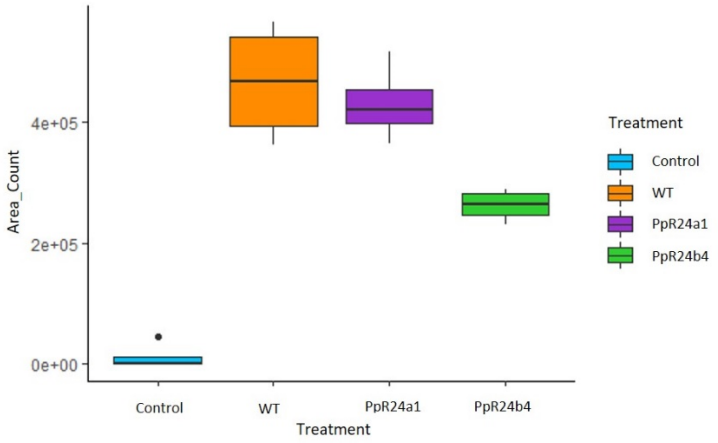
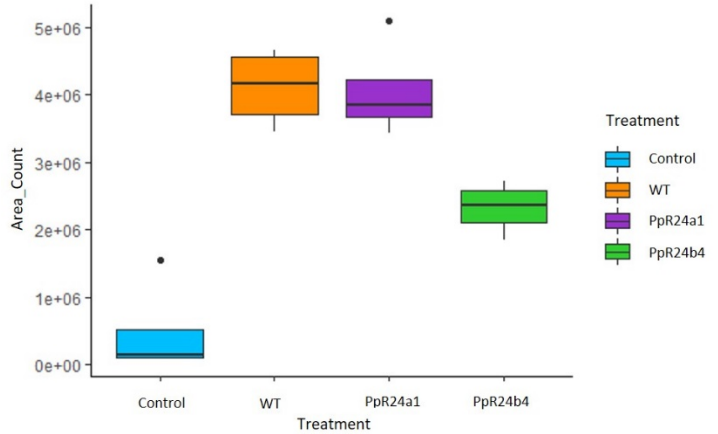


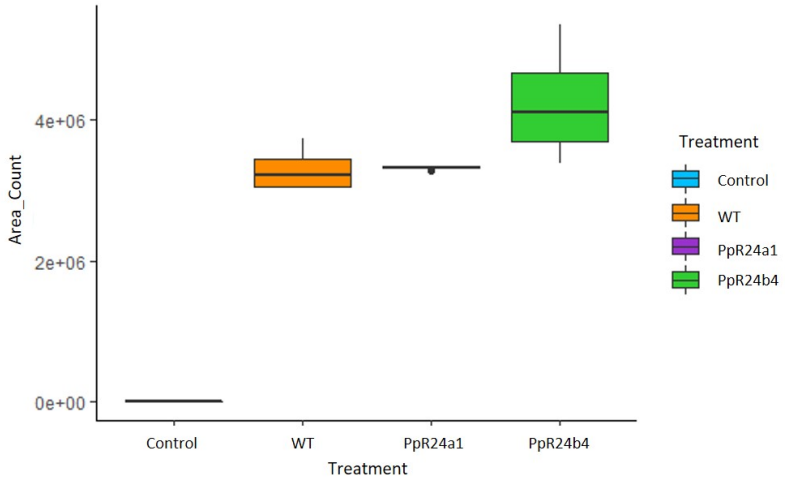
Comparison	Z	P.unadj	P.adj	Sign.
PpR24a1 – PpR24b4	1.7130503	0.086703281	0.52021969	-
PpR24a1 – Control	2.9047375	0.003675612	0.02205367	*
PpR24b4 – Control	1.1916872	0.233383925	1.00000000	-
PpR24a1 – WT	-0.1489609	0.881584488	1.00000000	-
PpR24b4 – WT	-1.8620112	0.062601508	0.37560905	-
Control - WT	-3.0536984	0.002260392	0.01356235	**

Figure 20 Area count of 1-decene compound levels for each treatment detected by solid phase microextraction and GC-MS. Control is 1xPBS in comparison with the bacterial a spray treatments where WT is the wild-type PpR24, PpR24a1 a final aphid-passaged isolate and PpR24b4 the final passage, strong biofilm-forming isolate. Pairwise comparisons from Kruskal-Wallis chi-squared analysis adjusted using the Bonferroni correction are presented. Kruskal-Wallis chi-squared = 12.803 df = 3 p-value = 0.005082.



Comparison	Z	P.unadj	P.adj	Sign.
PpR24a1 – PpR24b4	1.7080045	0.087635505	0.52581303	-
PpR24a1 – Control	2.8961816	0.003777338	0.02266403	*
PpR24b4 – Control	1.1881771	0.234763663	1.00000000	-
PpR24a1 – WT	-0.1485221	0.881930721	1.00000000	-
PpR24b4 – WT	-1.8565266	0.063378529	0.38027117	-
Control - WT	-3.0447037	0.002329097	0.01397458	**

Figure 21 Area count of 1-undecene compound levels for each treatment detected by solid phase microextraction and GC-MS. Control is 1xPBS in comparison with the bacterial a spray treatments where WT is the wild-type PpR24, PpR24a1 a final aphid-passaged isolate and PpR24b4 the final passage, strong biofilm-forming isolate. Pairwise comparisons from Kruskal-Wallis chi-squared analysis adjusted using the Bonferroni correction are presented. Kruskal-Wallis chi-squared = 12.728 df = 3 p-value = 0.005263.



Comparison	Z	P.unadj	P.adj	Sign.
PpR24a1 – PpR24b4	1.7207036	0.085304622	0.51182773	-
PpR24a1 – Control	2.9177148	0.003526067	0.02115640	*
PpR24b4 – Control	1.1970112	0.231302188	1.00000000	-
PpR24a1 – WT	-0.1496264	0.881059378	1.00000000	-
PpR24b4 – WT	-1.8703300	0.061438005	0.36862803	-
Control - WT	-3.0673412	0.002159722	0.01295833	**

Figure 22 Area count of 2-Methylpentane compound levels for each treatment detected by solid phase microextraction and GC-MS. Control is 1xPBS in comparison with the bacterial a spray treatments where WT is the wild-type PpR24, PpR24a1 a final aphid-passaged isolate and PpR24b4 the final passage, strong biofilm-forming isolate. Pairwise comparisons from Kruskal-Wallis chi-squared analysis adjusted using the Bonferroni correction are presented. Kruskal-Wallis chi-squared = 11.272 df = 3 p-value = 0.01034.

The volatiles detected by the DHE and GC-MS analysis differed from those recovered in the SPME analysis. The volatiles mostly comprised of green leaf volatiles with no detectable levels bacterial sulphur compounds which were present in the SPME analysis. Analysis of individual volatiles indicated significant differences in the level of volatile emissions between treatments (Table 5). Dunn post-hoc analysis after adjustment by the Bonferroni method found significant pairwise differences in hexanol, 2-hexanal and 2-hexen-1-ol (Table 6). When comparing the volatile emissions profile of each plant treatment (Figures 23-28), compound emissions appear starkly more reduced for the soil and no-spray treatment in comparison to the plants sprayed with PBS and bacterial treatments. However, significant differences were found between days which meant that it was difficult to accurately establish whether there were statistically significant differences between treatments.

Table 5 Compounds identified from DHE and GC-MS analysis searching by specific ion, where RT - retention time/mins and p-value indicates a significant differences in Kruskal-Wallis chi-squared analysis of compound levels between the five plant treatments.

Compound	Ion	RT/min	p-value	Sign.
3-Hexenal	69	4.206	0.05379	*
2-Hexenal	69	6.17	0.01184	**
Hexanol	69	6.9	0.02144	*
Hexanal	82	4.268	0.1738	-
3-Hexen-1-ol	82	6.288	0.03205	*
2-Hexen-1-ol	82	6.7	0.02998	*
Octanol	82	12.65	0.7339	-
3-Hexen-1-ol acetate	82	12.766	0.01304	**
Toluene	93	3.39	0.05328	*
α-pinene	93	9.362	0.3847	-
β-pinene	93	11.276	0.01043	**
5-Hepten-2-one, 6-methyl	93	11.853	0.7329	-
Limonene	93	13.604	0.08649	-
3-Carene	93	14.471	0.1341	-
g-Terpinene	93	14.876	0.03892	*
Nonanal	93	16.955	0.2394	-
Decanal	112	20.92	0.6524	-

Table 6 Significant pairwise differences in compound levels between plant treatments detected by DHE and GC-MS.

Comparison	Z	P.unadj	P.adj	Sign.
Hexanol				
No-spray - wt	-3.12834477	0.001757938	0.02636908	*
2-Hexenal				
No-spray - wt	-2.8974706	0.003761850	0.05642775	*
Soil - wt	-3.1386575	0.001697237	0.02545856	*
2-Hexen-1-ol				
Soil - wt	-3.0365364	0.002393132	0.03589698	*

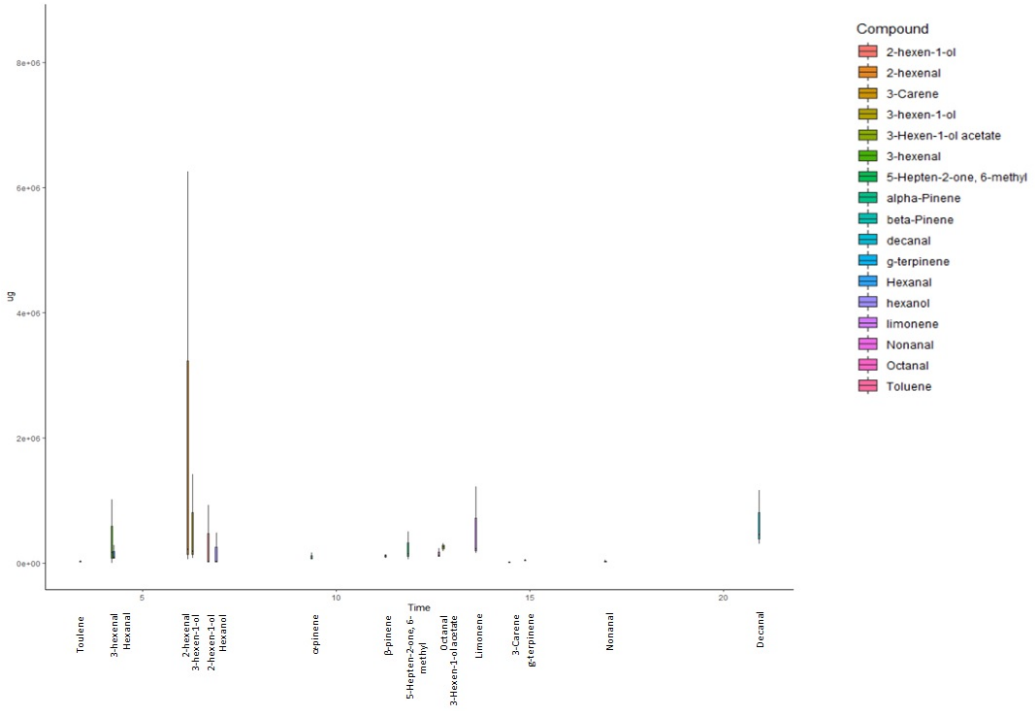


Figure 23 VOC emissions of *C. annuum* plants detected in DHE and GC-MS when treated with wild-type PpR24 foliar spray.

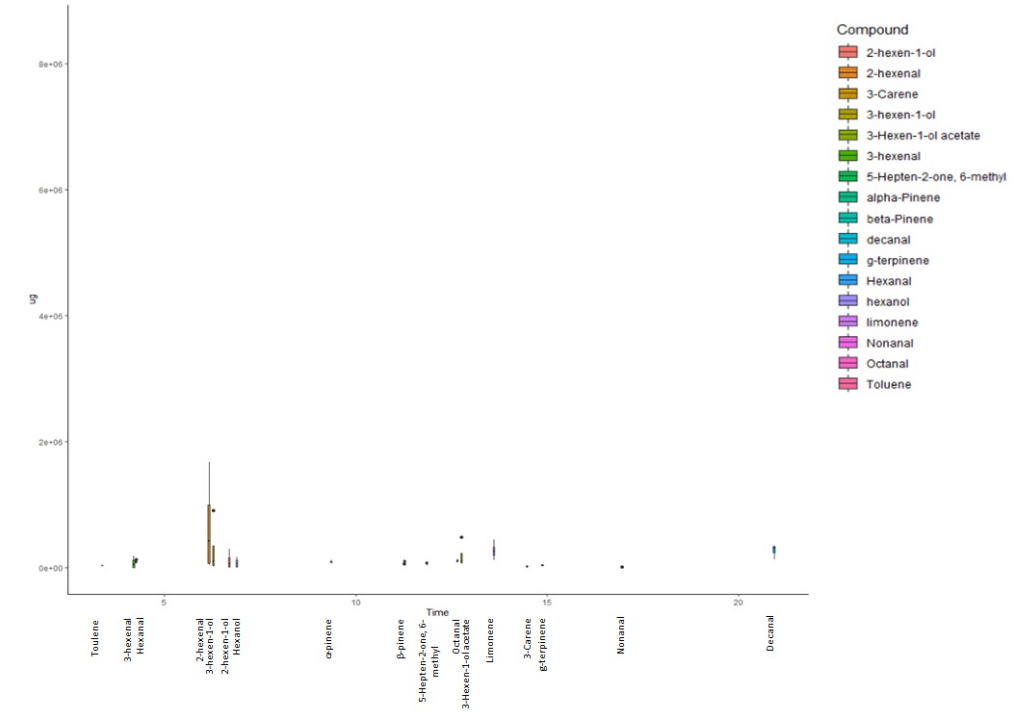


Figure 24 VOC emissions of *C. annuum* plants detected in DHE and GC-MS when treated with isolate PpR24b4 foliar spray.

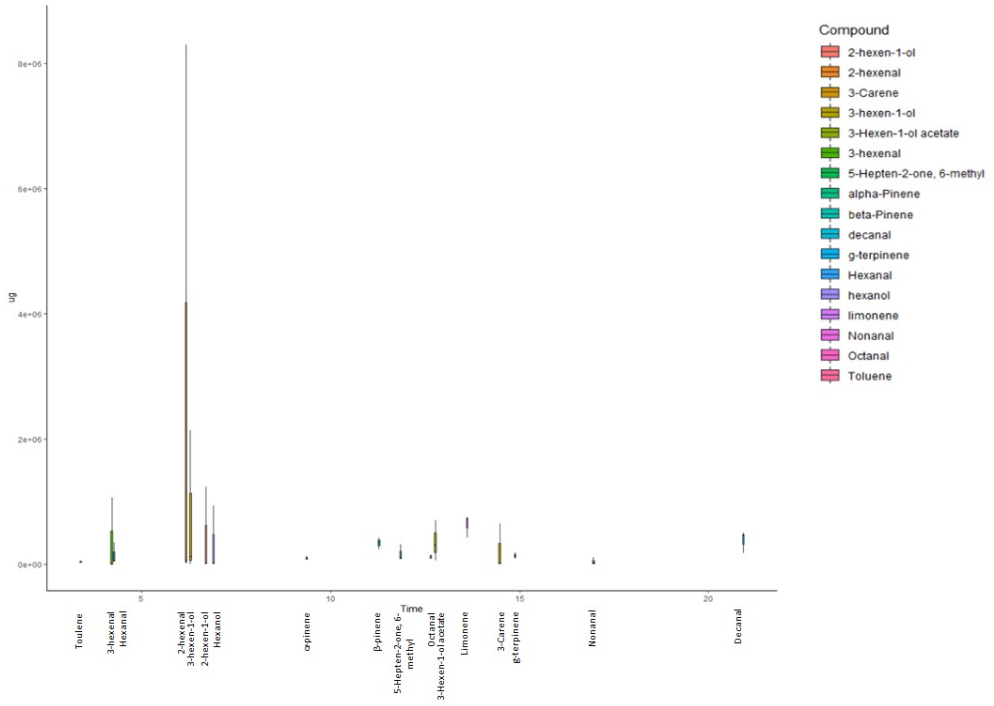


Figure 25 VOC emissions of *C. annuum* plants detected in DHE and GC-MS when treated with isolate PpR24a1 foliar spray.

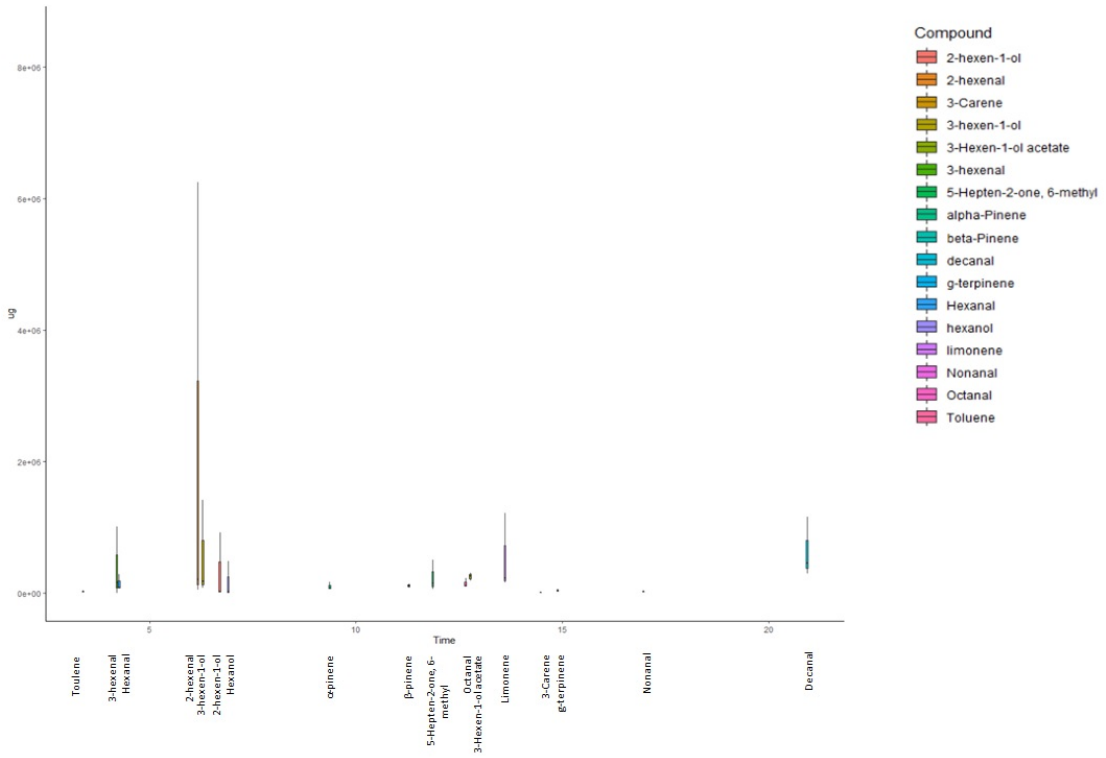


Figure 26 VOC emissions of *C. annuum* plants detected in DHE and GC-MS when treated with 1xPBS foliar spray.

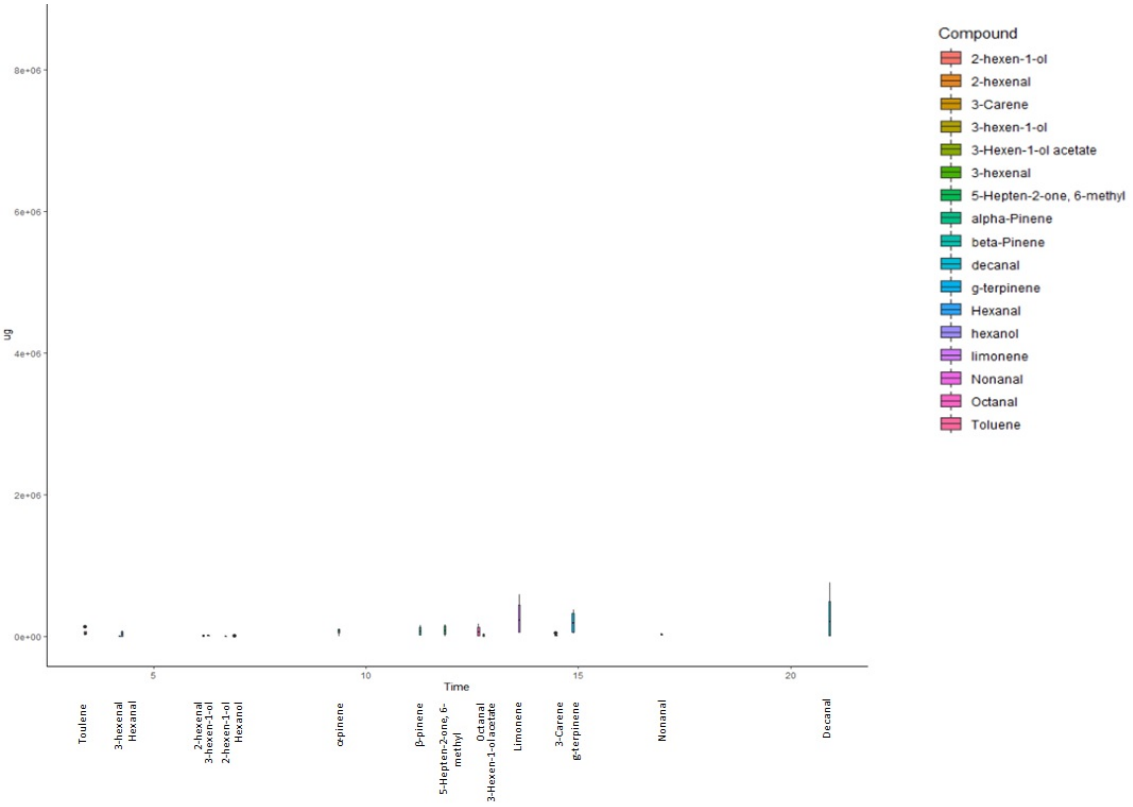


Figure 27 VOC emissions of *C. annuum* plants detected in DHE and GC-MS when no spray is applied.

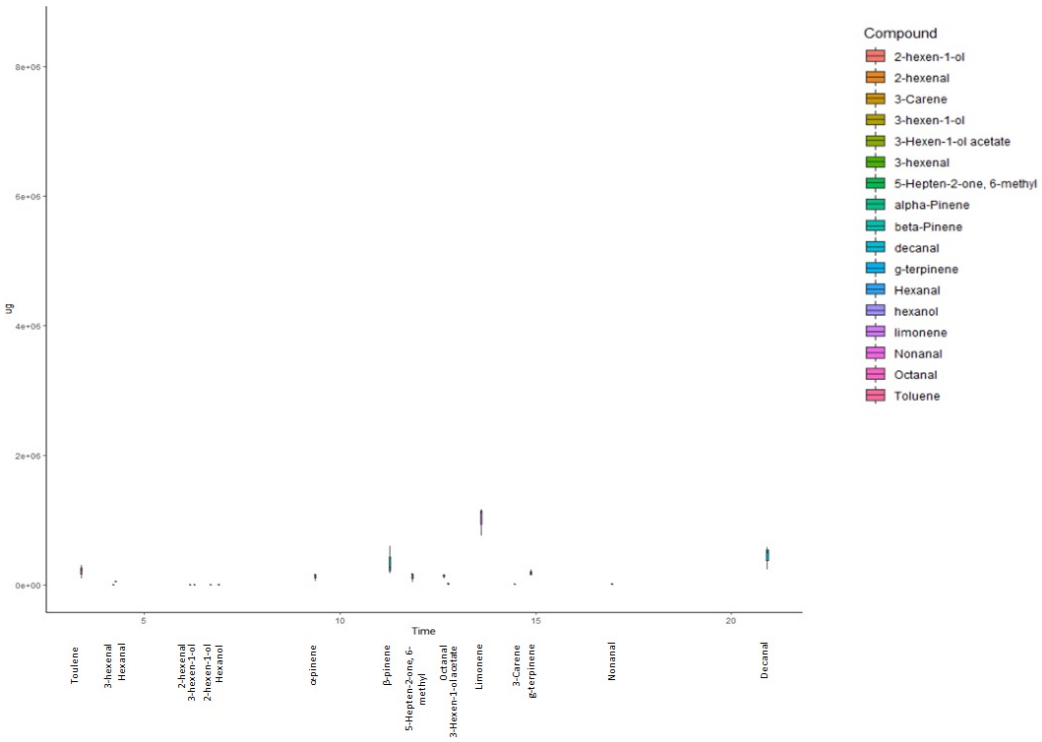


Figure 28 VOC emissions of potting soil used for *C. annuum* plants detected in DHE and GC-MS.

Aphid Behaviour Boxes

To investigate how wild-type and evolved isolates affected aphid host-plant choice, aphids were allowed to choose between plants sprayed with PBS or a bacteria-sprayed plant treated with wither the wild-type, PpR24a1 or PpR24b4 isolate. When presented with a choice between a plant sprayed with the wildtype PpR24 or a PBS control spray, significantly more aphids were present on the control plant (t = 2.2251, df = 13.824, p-value = 0.04325, Figure 29), which corroborated Dr Paliwal's findings of a potential deterrent effect of PpR24. When presented with a choice to settle on plants spray with PBS control or either isolate PpR24b4 or PpR24a1, no significant difference was observed between the numbers of aphids present on control or plants treated with PpR24b4 (p-value = 0.2754) or PpR24a1 (p-value = 0.278) (Figures 30 and 31).

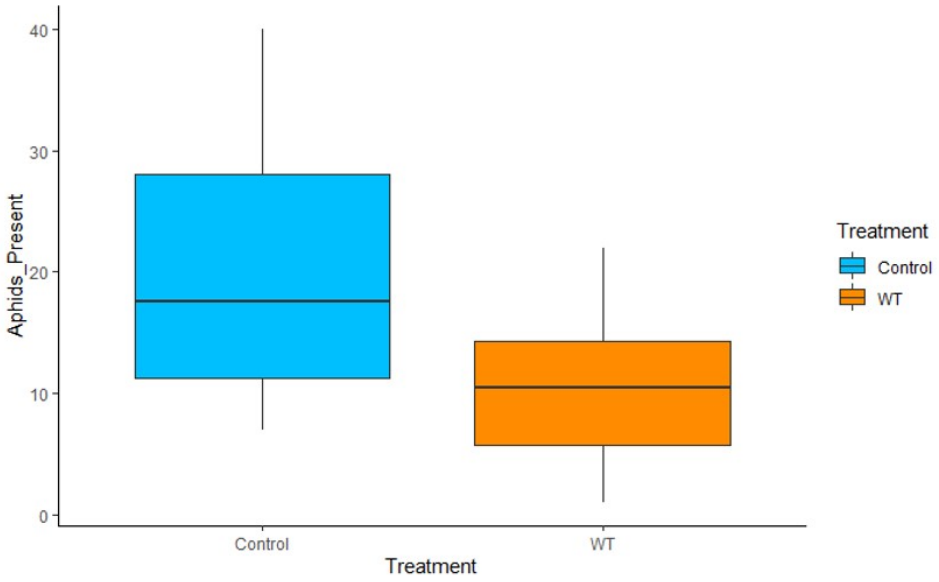


Figure 29 Choice box wild-type *P. poae* and 1xPBS sprayed control plant. Results of choice box assays. 50 *M. persicae* were given a choice between control and treated plants. After 1 week counts of aphid presence were made. t = 2.2251, df = 13.824, p-value = 0.04325.

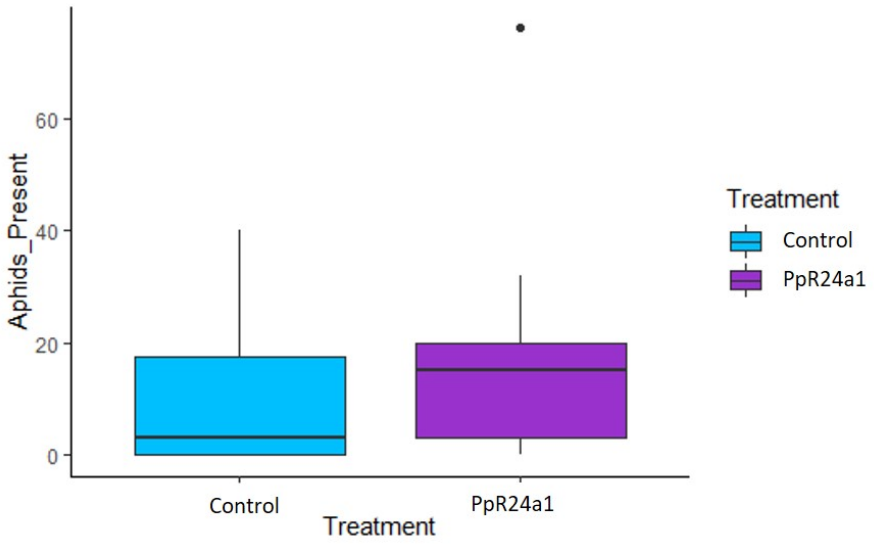


Figure 30 Choice box isolate PpR24a1 vs 1xPBS sprayed control plant. Results of choice box assays. 50 M. persicae were given a choice between control and treated plants. After 1 week counts of aphid presence were made. t = 1.1262, df = 14.804, p-value = 0.278.

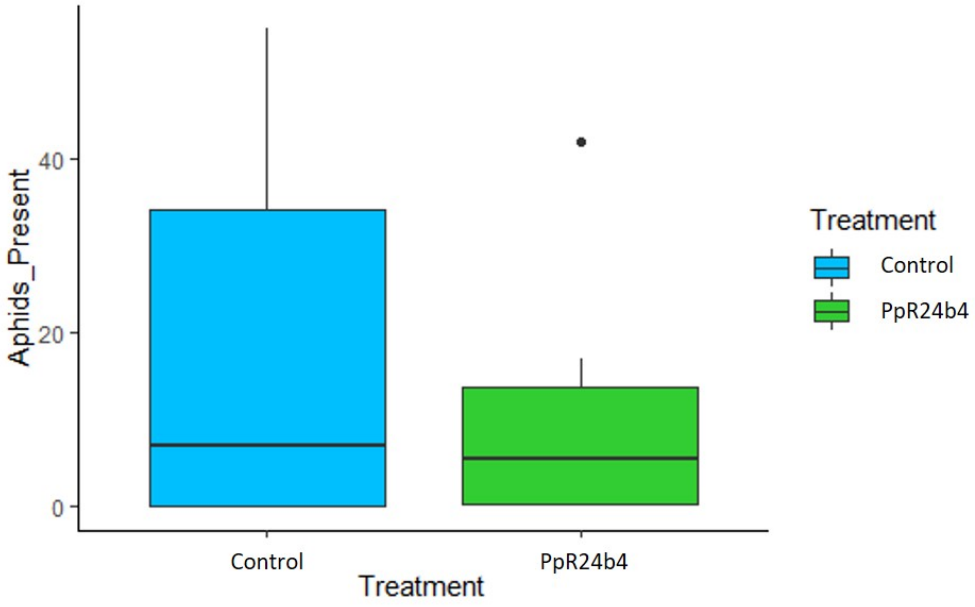


Figure 31 Choice box isolate PpR24b4 vs 1xPBS sprayed control plant. Results of choice box assays. 50 M. persicae were given a choice between control and treated plants. After 1 week counts of aphid presence were made. t = 1.1341, df = 14.325, p-value = 0.2754.

Observations were made into how the bacteria affected aphids once they were already established on the crop. All treatments saw aphids dispersing from the original host plant (plant 1) to the introduced plant (plant 2). Kruskal-Wallis rank sum test with Bonferroni correction found statistically fewer aphids were present on the original plant by day 3 for both un-sprayed control (p-value = 0.01695) and PBS sprayed plants (p-value = 0.004289).

However, no statistical difference was observed in the number of aphids on plant 1 for plants sprayed with PpR24 (p-value = 0.3679) (Full post-hoc analysis can be found in the appendix). That being said, by day 3, more aphids were present on the introduced, un-sprayed plants than the original plant for both spray treatments (Figure 32).

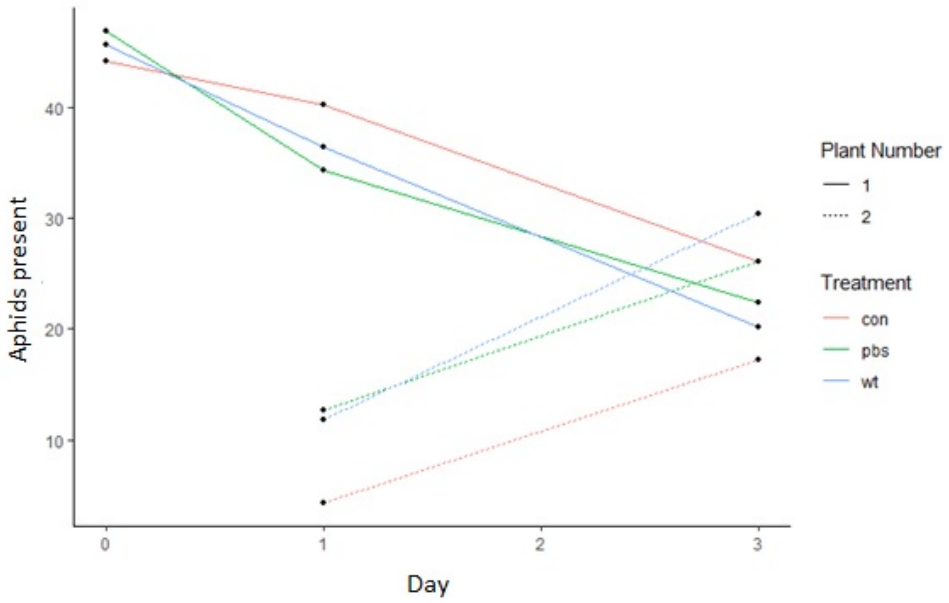


Figure 32 Results of spraying aphids after settling on the host pepper plant and how it affects dispersal to an untreated plant. Fifty aphids were allowed to establish on an un-sprayed sweet pepper plant (plant 1) for 24 hours. On day 0, plants are sprayed with either the wild-type PpR24, 1xPBS or not sprayed at all and a second, un-sprayed plant is introduced (plant 2) (where 'con' is no-spray, 'pbs' is 1xPBS spray and 'wt' is the wild-type PpR24 spray). The number of aphids on each plant are recorded 24 and 72 hours after plant 2's introduction. A statistical difference was seen in aphids present on plant one for both the no-spray (Kruskal-Wallis chi-squared = 8.1551, df = 2, p-value = 0.01695) and PBS treatments (Kruskal-Wallis chi-squared = 10.904, df = 2, p-value = 0.004289) but this was not the case for the PpR24 sprayed aphids (Kruskal-Wallis chi-squared = 2, df = 2, p-value = 0.3679).

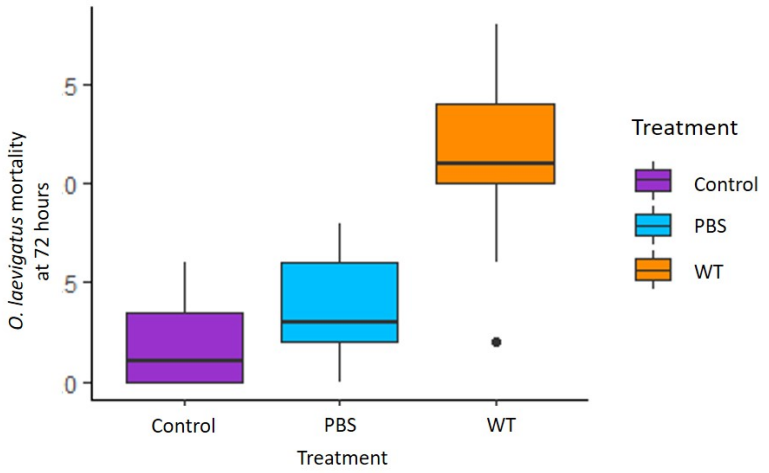
Lethal effects of Pseudomonas poae on non-target natural enemies

In previous investigations, *Pseudomonas poae* PpR24 has been shown to have a high specificity to aphid species, proving non-harmful to non-target insects it may encounter in a crop environment (Hamilton, 2015). This section takes this investigation further by focussing on the potential direct, lethal effects of the wild-type *Pseudomonas poae* PpR24 on three commercially available beneficial insects by simulating likely routes of exposure the insects may encounter in the glasshouse crop environment. *Orius laevigatus*, *Macrolophus pygmaeus* and *Aphidius colemani* are three natural enemies produced for aphid control that are commonly applied in glasshouse sweet pepper systems (Messelink *et al*., 2011; De Backer *et al*., 2015). Experiments included topical application of the bacteria directly onto the

insect cuticle, walking on excised leaves sprayed with bacteria and finally, in the case of *O. laevigatus* and *M. pygmaeus*, feeding on *M. persicae* that have been allowed to feed on artificial diet treated with PpR24.

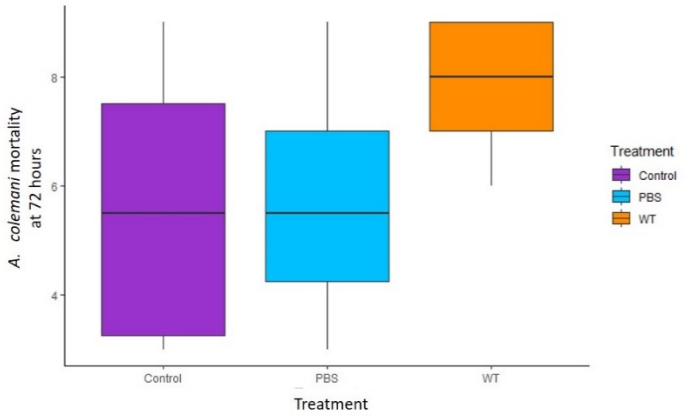
Topical application

The response to the topical application of PpR24 varied with species. After 72 hours, a strong statistical significance was observed for *O. laevigatus* treated with PpR24 droplets (Kruskal-Wallis chi-squared = 15.671, df = 2, p-value = 0.0003954) (Figure 33) and a statistically significant difference was observed between the control and PpR24 treated *A. colemani* when exposed to sprayed pepper leaves (Kruskal-Wallis chi-squared = 7.1972, df = 2, p-value = 0.02716) (Figure 34). However, there was no significant difference between control and PpR24 for *M. pygmaeus* (Kruskal-Wallis chi-squared = 5.2394, df = 2, p-value = 0.07282) (Figure 35).



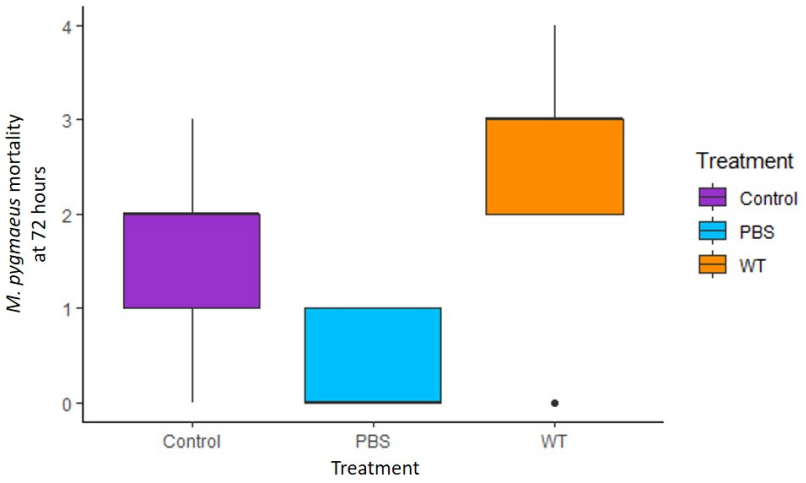
Comparison	Z	P.unadj	P.adj	Sign.
Control - PBS	-1.068215	0.2854233733	0.8562701200	-
Control - WT	-3.835279	0.0001254217	0.0003762651	***
PBS - WT	-2.767064	0.0056563665	0.0169690996	**

Figure 33 Topical assay results for *O. laevigatus*. Insect mortality was recoded at 72 hours (p-value = 0.0003954).



Comparison	Z	P.unadj	P.adj	Sign.
Control - PBS	-0.2569614	0.79720860	1.00000000	-
Control - WT	-2.4411330	0.01464126	0.04392379	*
PBS - WT	-2.1841716	0.02894964	0.08684892	-

Figure 34 Topical assay results for *A. colemani*. Insect mortality was recoded at 72 hours (p-value = 0.02716).



Comparison	Z	P.unadj	P.adj	Sign.
Control - PBS	1.456438	0.14527153	0.43581460	-
Control - WT	-0.801041	0.42310792	1.00000000	-
PBS - WT	-2.257479	0.02397815	0.07193445	-

Figure 35 Topical assay results for *M. pygmaeus*. Insect mortality was recoded at 72 hours (p-value = 0.07282).

Exposure to spray residues

Significant lethal effects to PpR24 spray residue exposure also varied with species. A statistically significant difference in insect mortality was observed between the control and PpR24 treatments for *A. colemani* (p-value = 0.04021) (Figure 36). However, no significant affect was observed for *O. laevigatus* (p-value = 0.2111) (Figure 37) or *M. pygmaeus* (p-value = 0.8137) (Figure 38).

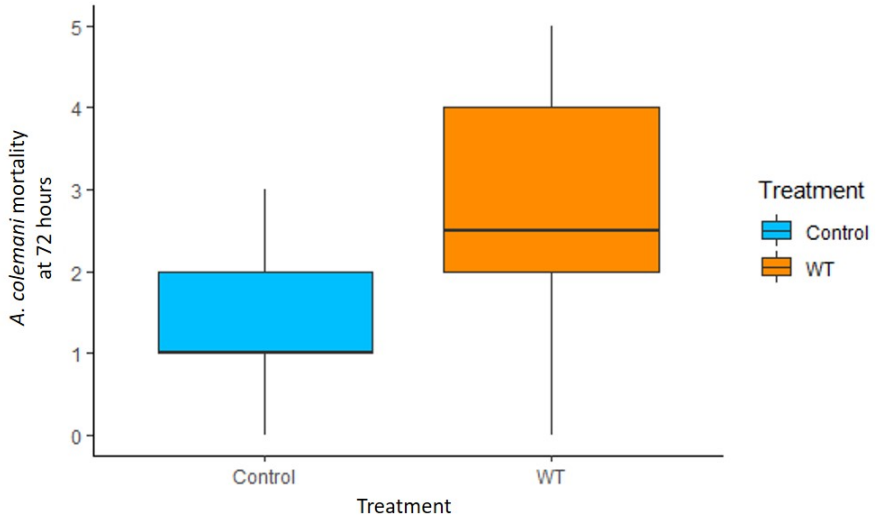


Figure 36 Mortality results for *Aphidius colemani* exposure to PpR24 spray residuals. After exposure to sprayed excised pepper leaves for 72 hours, no significant difference between the wild-type PpR24 and PBS spray treatment was observed (p-value = 0.04021).

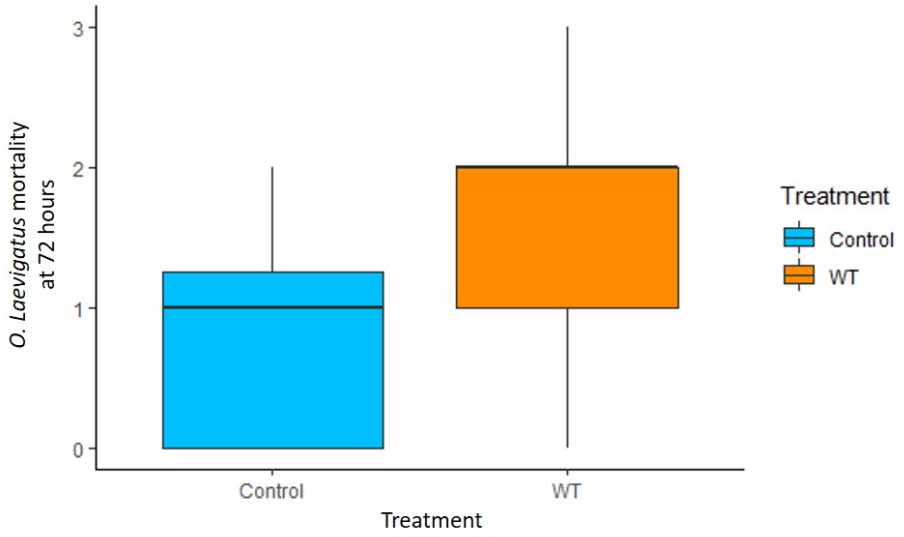


Figure 37 Mortality results for *Orius laevigatus* exposure to PpR24 spray residuals. After exposure to sprayed excised pepper leaves for 72 hours, no significant difference between the wild-type PpR24 and PBS spray treatment was observed (p-value = 0.2111).

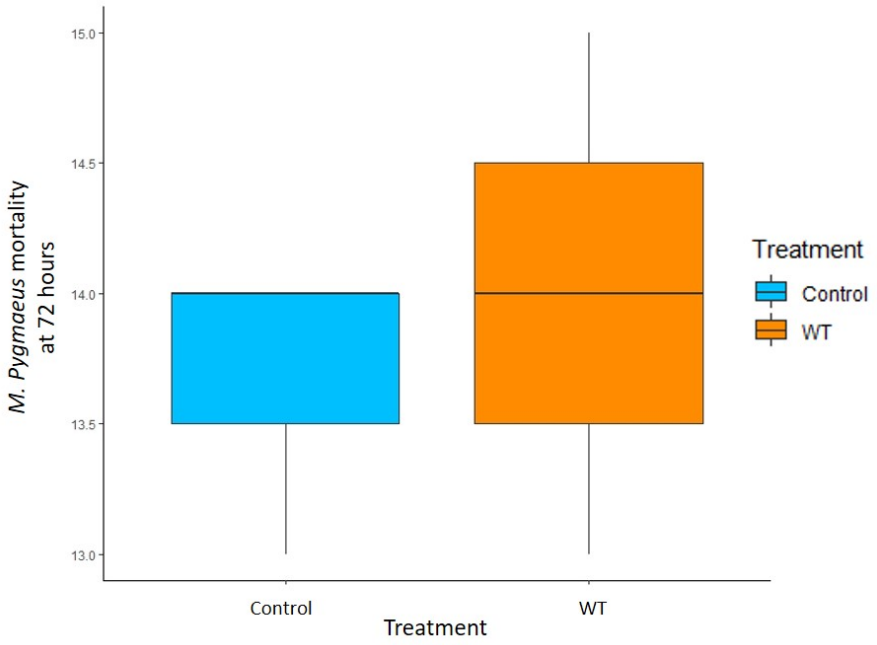


Figure 38 Mortality results for *Macrolophus pygmaeus* exposure to PpR24 spray residuals. After exposure to sprayed excised pepper leaves for 72 hours, no significant difference between the wild-type PpR24 and PBS spray treatment was observed (p-value = 0.8137).

Ingestion of PpR24-fed aphids

To investigate whether aphid predators were lethally affected by eating aphids exposed to PpR24, *O. laevigatus* and *M. pygmaeus* were left to feed on aphids reared on artificial diet inoculated with PpR24. No statistical difference was seen in *M. pygmaeus* survival when fed on treated or untreated aphids (p-value = 1) (Table 7). However, a statistical difference was seen for *O. laevigatus*, where insects fed on aphids treated with PpR24 had a higher mortality (p-value = 0.03251) (Table 8).

Table 7 *Macrolophus pygmaeus* ingestion of *M. persicae* fed on aphids. Fisher's exact test outputs, p-value = 1.

	Insects dead	Insects alive	**Marginal Row Totals**
Control	3	7	10
P. poae	3	7	10
Marginal Column Totals	6	14	20 (Grand Total)

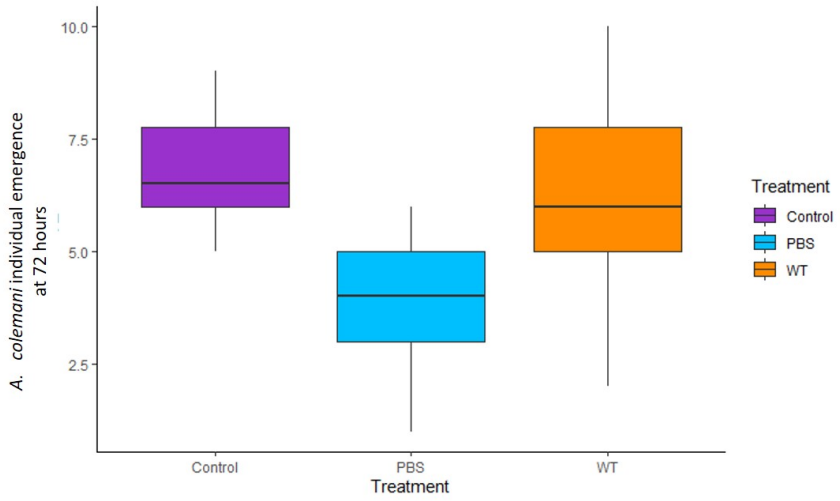
Table 8 *Orius laevigatus* ingestion of *M. persicae* fed on aphids. Fisher's exact test outputs, p-value = 0.03251.

	Insects dead	Insects alive	**Marginal Row Totals**
Control	5	5	10
P. poae	10	0	10
Marginal Column Totals	15	5	20 (Grand Total)

Aphidius colemani mummy emergence

A similar protocol to the topical application experiment was applied to assess whether spray contact of PpR24 affected parasitized aphid mummy emergence. The Kruskal-Wallis

test indicated that there was a significant difference between the treatments on mummy emergence (Kruskal-Wallis chi-squared = 10.598, df = 2, p-value = 0.004998). However, the post-hoc test revealed there was a significant difference between the Control and PBS treatment and the WT and PBS treatment. There was no significant difference between the Control and WT PpR24 treatments (Figure 39).



Comparison	Z	P.unadj	P.adj	Sign.
Control - PBS	3.1810870	0.001467235	0.004401706	**
Control - WT	0.9916749	0.321356134	0.964068403	-
PBS - WT	-2.1894121	0.028566898	0.085700695	-

Figure 39 *A. colemani* emergence from mummies after 72 hours, where 'control' treatment is no droplet, 'PBS' a control PBS droplet and 'WT' the wild-type PpR24.

Discussion

The overall aim of this research was to investigate whether experimental evolution could be applied to improve the efficacy of *Pseudomonas poae* PpR24 as an aphid biocontrol agent and to better understand how PpR24 directly and indirectly affects aphids and non-target insects. Wild-type isolates of PpR24 were serially passaged in environments intended to select for either aphid-virulence or biofilm formation, after which final passaged isolates were observed for different phenotypes and trade-offs between traits. Aphid virulence was not improved but a significantly strong biofilm-forming isolate evolved at a cost to aphid virulence. Whole genome sequencing and variant calling analysis identified two point mutations plausibly responsible for the biofilm phenotype and reduction in aphid virulence. Identification of volatile organic compounds emitted by the wild-type *P. poae* PpR24 was carried out to elucidate the deterrent effect seen in the Paliwal study, as well as further examine phenotypic differences between the wild-type and biofilm-forming isolate. Finally, further assessment of PpR24's interactions with non-target insects was carried out, investigating the lethal effects of PpR24 on commercially used aphid natural enemies.

Experimental Evolution of Pseudomonas poae PpR24 and phenotypic trade-offs

Novel aphicidal biopesticides are in high demand and experimental evolution may offer a means of evolving more efficient microbial aphid control without the stigma of genetically modified organisms. An experimental evolution approach was applied in a bid to improve PpR24's virulence to aphids and to investigate whether biofilm formation can evolve as a means to improve bacterial persistence on a plant, thus reducing the number of applications needed to provide efficient control. Ten independent lineages of PpR24 were passaged in either aphid diet sachets to improve aphid virulence or King's broth microcosms to evolve biofilm growth in vitro.

After the ten passage cycles through aphids, no improvement to virulence was seen in any of the ten isolates derived from the wild-type PpR24. It is likely that ten passages were not sufficient to allow any virulence mutations to evolve and by alternating the bacteria through the aphids and on a plate, there was not a strong enough selection pressure on the isolates to improve virulence over the passages. Previous studies following a similar methodology to enhance the virulence of entomopathogenic fungi against target pest insects proved unsuccessful in improving virulence (Vandenberg and Cantone, 2004; Scully and Bidochka, 2005). For example, no significant change was observed in *B. bassiana* virulence to malarial mosquitoes after ten passage cycles (Valero-Jiménez *et al.*, 2017). Another factor that may affect the evolution of virulence in PpR24 is the life-cycle of the bacterium and how it is transmitted from the aphid host (Bell, 2008). The trade-off hypothesis between virulence

and transmission assumes that an organism of high growth and virulence may kill its host before it has time to spread to other hosts, whereas a species with slow growth may have a high transmission to another host. An equilibrium between virulence and transmission is needed for the parasite to maximise its fitness. It is possible that the wild-type PpR24 is already at this equilibrium and a higher virulence would adversely affect the bacterium's fitness (de Roode *et* al., 2008; Alizon and Michalakis, 2015; Valero-Jiménez *et al*., 2017).

Attempts to evolve biofilm formation were more successful that the virulence passages. After ten passages, one isolate out of the ten lineages, PpR24b4, evolved the ability to form consistently strong biofilms at the air-liquid interface. As seen in similar studies conducted with SPW25 Wrinkly Spreader, forming a biofilm may provide mutants with a fitness advantage by accessing the oxygen-rich surface of a microcosm, so more resources are available for growth, allowing the mutants to proliferate through the system (Ude *et al*., 2006; Udall *et al.*, 2015). PpR24b4's biofilm formation appears to have established in the system at passage 7, whereas other isolates under the same selection exhibited more stochastic and inconsistent biofilm growth over the ten passages. However, it is possible that over the weeklong period that each passage was left to grow, biofilm structures become too thick and dense to be self-supported and cellular matter may have sunk to the bottom of the microcosm, thus avoiding any forms of quantitative measurement. However, isolate PpR24b4's attachment strength to the glass microcosm walls was not significantly stronger than that of the other biofilm isolates. By the final passage, isolate PpR24b2 and PpR24b5 also appeared to form a biofilm at the air-liquid interface. However, these biofilms were unable to support any significant mass before breaking or sinking. It is possible that in these biofilms, 'cheats' had established in the system. Such biofilm cheats are cells that take advantage of the communal goods and services supplied by cells in the biofilm aggregation, without contributing to the formation of the biofilm themselves (Popat *et al*., 2012). This enables the cheats to spend their energy in more selfish ways, such as reproduction, enabling these undesirable mutants to proliferate through the system.

The evolution of PpR24 from planktonic to biofilm-former appears to be a two-stage process, with passages first forming an opaque, yellow broth solution with a weak biofilm at the air-liquid interface that cannot support much/any weight. When a strong biofilm is formed, as in PpR24b4, the yellow colouration is lost which may indicate a loss or reduction of siderophore production. After peaking at approximately the sixth passage, the attachment strength of the PpR24b4 isolate appears to plateau. This may be due to a variety of limiting factors, such as nutrient availability in the microcosm, which mean it is unable to develop any further (Dunne, 2002). No significant differences were observed between the aphid passaged bacteria and wild-type PpR24. However, several trade-offs were observed to be associated with the biofilm passaged isolates. Although well adapted to growth in static broth conditions,

biofilm isolates performed less well than the wildtype and aphid-passaged isolates when grown on agar plates and in a shaken environment. When grown on an agar plate, the wild-type and aphid-passaged colonies were round, approximately 2 mm in diameter and yellow in colour with a blurred boundary at the edge of the colony. In contrast, PpR24b4 formed smaller, circular, dot-like colonies that were milky in colour strikingly different conformation to colonies formed by the SPW25 WS mutant, therefore it is possible the biofilm mutation evolved in different pathways. Other studies have observed similar trade-offs in growth, such as the *Pseudomonas fluorescens* SPW25 WS mutant which had significantly poorer growth in shaken broth conditions and when grown on a plate when compared to static microcosms (Udall *et al*., 2015).

The most important trade-off observed between the two selection lineages was that biofilm-passaged isolates were less virulent than those of the aphid passaged isolates. The statistically strong biofilm forming isolate PpR24 was significantly less virulent to aphids in 48 hours than the wild-type and aphid-passaged strains. This loss in virulence may in part be due to the biofilm-passage isolates reduced growth in non-static condition. Evidence of reduced growth on agar plates and in shaken broth conditions may be extrapolated to imply poor adaptation and fitness in the aphid gut environment. Biofilm isolate populations may not have reached the lethal concentrations require to cause aphid mortality within the 48 hour duration of the experiment, therefore occlusion in the aphid gut may not have occurred (Stavrinides *et al*., 2009).

Furthermore, poorer growth may entail the biofilm isolates to be less competitive in the aphid gut environment. The aphid gut microbiome plays host to a variety of endosymbionts, many with anti-pathogenic properties. For instance, *Regiella insecticola* provides its aphid host with protection against pathogens and parasitoids (Guo *et al*., 2017). It is possible that the reduced growth of the derived biofilm isolates inhibits their ability to compete with the aphids' endosymbionts, thus reducing aphid virulence.

Another contributing factor to the loss of biofilm isolate virulence may be that the genomic changes responsible for biofilm formation have also inhibited aphicidal toxin production. The loss of virulence in biofilm forming isolates can be associated with their reduced motility in swimming and swarming agar. In many pathogens, motility is considered an important factor contributing towards virulence (Josenhans and Suerbaum, 2002). Flagellum-driven motility enables pathogens to move to more favourable environments and find resources and loss of flagella has been correlated with a loss of virulence (Davey and O'Toole, 2000), for instance *Pseudomonas aeruginosa* M-2 mutants with no polar flagellum lost their virulence (Montie *et al*., 1982). However, although biofilm mutants had reduced motility in swarming and swimming soft agars, regardless of whether a biofilm was present in at the air-liquid interface of the microcosm, no isolates exhibited a total loss of motility. Indeed,

motility can be key in the initial attachment stages of biofilm formation (Davey and O'Toole, 2000). Therefore, rather than due to a loss of flagellum, the reduction in cellular movement may be due to the reduced growth of the isolates on agar. Furthermore, upon establishing as a mature biofilm colony, cells adjust to their sedentary lifestyle and lose their energy demanding flagella, instead increasing EPS production (Davey and O'Toole, 2000; Josenhans and Suerbaum, 2002). As the final isolates taken as stocks for PpR24b4 were from a mature biofilm colony, this may also account for its reduced motility.

Biofilm formation did not improve bacterial persistence of sweet pepper crops as hypothesised. This study found that after three weeks of growth on pepper plants, no significant difference in bacterial fitness from populations recovered from the plants was found between the wild-type and either aphid-passaged or biofilm-passaged final evolved isolates. Although PpR24b4 was capable of forming a strong biofilm in static microcosms, it cannot be concluded from this study that the PpR24b4 isolates recovered from the phylloplane were growing in biofilm aggregations. However, having too successful a biofilm on the phyllosphere may prove adverse to the plant survival, potentially impeding vital leaf functions by blocking stomata which would prove detrimental to the plant and possibly result in higher yield loss if applied to a crop.

Genome Sequencing

Whole genome sequencing of the wild-type PpR24 and final passage isolates PpR24a1 and PpR24b4 found significant differences between the genome of biofilm-forming isolate PpR24b4 and the wild-type PpR24. Two single nucleotide polymorphisms were found on the PpR24b4 genome, one in the gene *cheB* and the other in *barA*, which was confirmed by Sanger sequencing. No genomic differences were observed between the wild-type and aphid passaged isolate PpR24a1. Both *cheB* and *barA* are genes associated with bacterial motility and biofilm formation, therefore it is possible that one, or both of these mutations is responsible for the evolved biofilm properties of PpR24b4.

In E. coli, CheB is a methylesterase protein in the chemotaxis signalling pathway CheIV, a key pathway in swarming behaviour (Verstraeten *et al*., 2008). Along with the proteins CheA and CheY, CheB controls direction of flagellum rotation, switching the rotation from clockwise to the default state of counter-clockwise movement. This allows the bacterium to move towards or away from a stimulus (Hickman *et al*., 2005; Verstraeten *et al*., 2008). In chemotaxis, directional movement in response to a chemical stimulus, chemical stimuli bind to methyl-accepting chemotaxis proteins (MCPs) in the bacterial membrane which triggers a change in the MCP that modulates the autophosphorylation of an associated histidine kinase (CheA). CheA-P phosphorylates the response regulator CheY, and CheY-P binds to the flagellar motor, causing the flagella to rotate clockwise. Concurrently, CheA also modulates

the phosphorylation state of CheB through phosphotransfer. CheB-P is active as a methylesterase and acts in conjunction with constitutively active methyltransferase (CheR) to adjust the methylation state of glutamate residues on MCPs. By shifting the methylation state, the flagella rotation is restored to the counter-clockwise direction (Hickman *et al*., 2005; Bantinaki *et al.,* 2007).

In Pseudomonads, WspF has been found to be homologous to CheB (Hickman *et al*., 2005). Mutations in WspF of *P. fluorescens* SPW25 cause dramatic changes in the bacterial phenotype compared to the wild-type, resulting in a Wrinkly-Spreader (WS) phenotype. Wrinkly spreaders form strong biofilms and have a wrinkled appearance when grown on agar. As well as forming cell aggregations, WS are less motile than the wild-type SPW25 (D'Argenio *et al.,* 2002; Bantinaki *et al.,* 2007; Verstraeten *et al*., 2008). It is thought that the WS's strong biofilm is caused by the malfunctioning WspF methylesterase, which results in the continuous activation of WspR. The active WspR overproduces cyclic-di-GMP (c-di-GMP) and adhesive substances, such as partially acetylated cellulose polymer (Bantinaki *et al.,* 2007). c-di-GMP is a signalling molecule used to coordinate between a motile or sessile life-style. At high concentrations c-di-GMP initiates cell aggregation and biofilm formation whereas at low concentrations, cells are motile (Valentini and Filloux, 2016).

The gene *barA* is responsible for the histidine sensor kinase, BarA, in the two-component signal transduction system (TCS) of E. coli, where UvrY acts as its counterpart response regulator (Chavez *et al*., 2010). In *Pseudomonas* species, the homolog of this system is the GacS/GacA TCS, where GacS is homologous to BarA (Heeb and Haas, 2001). The GacS/GacA system regulates secondary metabolite and extra cellular protein production. In Gram-negative bacteria the GacS/GacA system has been associated with biofilm formation, quorum sensing and bacterial virulence to plants, fungi and animals (Heeb and Haas, 2001; Davies *et al*., 2007; Workentine *et al*., 2009).

Gac is an acronym for global activator of antibiotic and cyanide synthesis (Haas and Keel, 2003). Many antibiotic and virulent exoproducts in fluorescent pseudomonads are dependent on the GacS/GacA regulatory system, such as hydrogen cyanide, pyocyanine, phenazines, lipopeptides and exoproteases (Heeb and Haas, 2001; Haas and Keel, 2003; Ruffner *et al*., 2013), as well as the *P. fluorescens* insecticidal toxin (Fit) (Pechy-Tarr *et al*., 2008; Ruffner *et al*., 2013). Mutations in *gacS/gacA* have been seen to result in a reduction or total loss of biocontrol abilities in plant growth promoting bacteria, and a loss of virulence in plant and animal pathogens (Blumer *et al*., 1999; Willis *et al*., 2001). Mutants have also been seen to have altered colony structure and changes in motility (Kyung *et al*., 2007). Variants of *P. fluorescens* F113 with *gacS/gacA* mutations had significantly increased swimming motility than the wild-type (Navazo *et al*., 2009). Inactivation of the *gacS* gene in *Pseudomonas aeuriginosa* PA14 resulted in hypermotility and a reduction in biofilm formation.

However, biofilms of these sensor kinase deficient mutants gave rise to small colony variants when cultured, which were less motile and showed a hyper-biofilm-forming phenotype (Davies *et al*., 2007).

It is possible that one, or both, of the point mutations discovered are responsible for the phenotypic changes in PpR24b4. However, although these missense mutations are in genes previously associated with biofilm formation and virulence, their presence is not definitive evidence that they are the cause of biofilm formation in PpR24b4. Inserting the point mutations seen in *barA* and *cheB* into the wild-type genome may provide more robust evidence as to their effects on the phenotype. The presence of only two missense mutations is in accordance with the average number of SNPs for similar experimental evolution studies (Guidot *et al.*, 2014). However, by only sequencing the end-point isolates of the final passage, we are presented with a snap-shot of the fixed mutations established in the genome, not the evolutionary process as a whole. Previous studies have seen the most significant mutations that affect population fitness occur early on in evolutionary passages (Dettman *et al*., 2012; Lang and Desai, 2014), therefore it would be interesting in further research to sequence the entirety of each lineage to pin-point where both mutations occurred and whether it corresponds with the emergence of the biofilm phenotype.

Volatile organic compounds and their effect on aphid behaviour

Volatile organic compounds play an important role in the interactions between bacteria, plants and insects and there is growing interest to incorporate pest-preventing VOCs in IPM systems. Previous research found PpR24 was deterrent to aphids when sprayed on a plant (Paliwal, 2017). This study aimed to identify the VOCs emitted by PpR24 in a broth and on a plant environment in an attempt to find volatiles that may account for this deterrent action. Experimentally evolved isolate PpR24b4 showed significant changes in phenotype from the wild-type PpR24, exhibiting reduced motility, growth rate and aphid motility but strong biofilm formation, likely due to point mutations in *cheB* and *barA*. It is possible that changes to PpR24b4's volatile emissions also occurred, which may affect the bacterium's ability to deter aphids from a host plant as seen in the wild-type. Therefore, a comparison of the volatiles emitted by the wild-type and derived isolates, and whether changes to the deterrent properties of the isolates was also investigated.

Identification of volatiles

Solid-phase microextraction and GC-MS detected 11 putative volatiles from the bacteria when suspended in a spray solution. Several of the VOCs detected have previously been associated with plant growth promotion and potential biocontrol properties (Lee *et al*., 2012). As such, it is possible that the volatiles detected by SPME may contribute to PpR24's

plant growth promoting properties (Hamilton, 2015). For example, 1-nonene has been identified as a VOC that may benefit plant growth emitted by the PGPR *Pseudomonas fluorescens* SS101 (Park *et al*., 2015). Potential anti-oomycete activity has also been associated with 1-decene and dodecane in *Pseudomonas* strains (Hunziker *et al*., 2015). The presence of dimethyl disulphide (DMDS) and 1-undecene was of particular interest. DMDS has been recognised in the volatile spectra of a variety of bacteria, including many Pseudomonads (Labows *et al*., 1980; Filipak *et al*., 2012), and is associated with antifungal and anti-microbial properties (Avalos *et al*., 2018). For instance, DMDS effectively suppresses gall growth caused by *Agrobacterium sp*. in tomato plants (Dandurishvili *et al*., 2011). There is also evidence to suggest that DMDS can elicit induced systemic resistance in plants. DMDS was the dominant volatile product of *Bacillus cereus* C1L in disease control assays and when applied as a soil drench, where corn and tobacco plants were protected from southern leaf blight and grey mould disease (Huang *et al*., 2012). DMDS is considered to play a defensive role when plants are damaged in *Allium* and *Brassica* species, providing protection from plant pathogens. Due to this, DMDS is under investigation as a potential biocontrol agent for fumigating soil to control plant-fungal pathogens and nematodes (Kyung and Lee, 2001; Dandurishvili *et al*., 2011). As well as providing control against microbial attack, DMDS has been seen as an important influence of insect behaviour. Ferry et al. (2009) found that artificially increasing the presence of DMDS around a broccoli crop significantly deterred egg laying by the cabbage fly pest, *Delia radicum*, and functioned as a kairomone, increasing the presence of the pests' natural predators.

1-Undecene is another potential anti-fungal volatile associated with rhizosphere Pseudomonads that may limit plant pathogen growth (Labows *et al*., 1980; Hunziker *et al*., 2015; Anderson and Kim, 2018). Exposure to 1-undecene inhibited mycelial growth of the potato blight, *Phytophthora infestans* (Hunziker *et al*., 2015). However, the antimicrobial efficacy of 1-undecene does appear to be case dependent (Dandurishvili *et al*., 2011). Popova *et al*. (2014) found 1-undecene to have minor antimicrobial capabilities but instead found exposure to 1-undecene to be significantly effective at limiting the development of the nematode *Caenorhabditis elegans* and caused significant mortality in *Drosophila melanogaster*. Studies into the potential application of 1-undecene as a semiochemical in attractant traps are being carried out. For example, 1-undecene is particularly attractive to the broad bean weevil, *Bruchus rufimanus*, but traps were only effective when the compound was applied with a blend of other semiochemicals (Bruce *et al*., 2011). Many insects rely on specific blends and ratios of VOCs for prey and host location and are extremely sensitive to slight changes in the balance of volatiles (Bruce *et al*., 2005). Therefore, although individual compounds may play important roles in interactions between organisms, it is vital to recognise the importance of the volatile bouquet as a whole.

Dynamic headspace extraction and GC-MS detected 17 volatiles, several of which have been associated with GLVs and HIPVs produced under stress as well as compounds known to act as kairomones. Aldehydes and alcohols detected, such as hexanal, 2-hexenal, 3-hexen-1-ol and 3-hexen-1-ol acetate are important GLVs in insect host plant location, such as in searching for suitable oviposition sites in stem-boring moths, *Chilo partellus* and *Busseola fusca* (Chamberlain *et al*. 2006). 2-Hexenal inhibits the growth of the fungal pathogen *Botrytis cinera*, as well as several species of bacteria, and has been thought to be emitted as part of a plant-wound response to prevent microbial infection (Scala *et al*., 2013). β-Pinene has also been found to possess anti-bacterial and anti-fungal properties (Avalos *et al*., 2018). α-pinene and limonene have previously been associated with volatiles induced by aphid herbivory that act as kairomones to attract the parasitoid *A. ervi* (Sasso *et al.,* 2007).

Although SPME analysis detected significant differences in compound levels between the bacterial isolates and the PBS control, no statistical differences were observed in pairwise comparisons between isolates. As there were no significant phenotypic or genetic changes between PpR24 and PpR24a1, this was not unexpected. However, although not statistically significant, lower levels of 1-decene, 1-nonene and DMDS were seen in the biofilm isolate PpR24b4. Similarly, DHE and GC-MS analysis showed reduced levels of green leaf volatiles produced by PpR24b4 compared to the wild-type PpR24 but these differences were not statistically important. It is likely that the differences in volatile emissions in the evolved isolate PpR24b4 are due to the mutations in *cheB* and *barA* genes.

In Pseudomonads, the GacS/GacA regulatory system (homologous to the two-part component BarA/UvrY of *E. coli*) has been associated with VOC and secondary metabolite production. *Pseudomonas fluorescens* SBW25 GacS mutants showed reduced levels of volatile compound production, including 1-undecene and dimethyl sulphide, indicating volatile products are at least in part regulated by the GacS sensor kinase (Cheng *et al*., 2016). However, not all volatile production is dependent on the GacS/GacA regulatory system. In *Pseudomonas donghuensis* P482, dimethyl sulphide was found to be regulated by the GacS/GacA system whereas dimethyl disulphide was not (Ossowicki *et al*., 2017). This may explain why dimethyl sulphide was found at lower levels in the PpR24b4 isolate compared to the wild-type.

None of the volatiles detected in the SPME analysis of the spray treatments were present in the DHE of treated plants. It is possible that the sulphur compounds produced by the bacteria are in such low quantities in comparison to the green leaf volatiles emitted by the plant that they are undetected by the set-up. Large amounts of noise in the volatile spectra may also mask the presence of small, highly volatile bacterial compounds with a low retention time. Although significant differences between bacterial treatments and the PBS control were seen in SPME analysis, no statistical differences were detected in the DHE GC-MS of pepper

plants sprayed with bacteria or PBS control spray. It is possible that the act of spraying alone, regardless of the treatment, is enough to trigger the emission of the GLVs.

Plants have been seen to emit GLVs in response to physical disturbance. Most plants emit low-levels of GLVs in an undisturbed state, which may account for their presence in the no-spray control plants. However, plants are extremely sensitive to abiotic stresses such as physical disturbance (Ameye *et al*., 2018). Therefore, it is more likely the volatiles are emitted as a result of moving the sample plants into the experimental conditions, which should be reduced as much as possible in future replications of this method.

Isolate effects on aphid behaviour

When aphids were presented with a choice to settle on either a control or a wild-type spray plant, statistically more insects settled on the control plant. This corroborated with the previous study where in an olfactometer setting the combined extracted volatiles from plants sprayed with PpR24 deterred winged *M. persicae* (Paliwal, 2017). No significant difference was seen in aphid host plant choice when aphids were presented with a control PBS sprayed plant and plants either sprayed with PpR24b4 or PpR24a1. It is possible that the loss of deterrency is due to differences in the levels of compounds emitted by PpR24 and PpR24b4 that were detected by SPME and DHE, such as a reduction in dimethyl sulphide.

However, no significant genetic, phenotypic or volatile difference was seen between the wild-type PpR24 and aphid-passaged PpR24a1 but there was no statistical difference between aphids colonising control or PpR24a1 sprayed plants, implying a loss of deterrency. It may be that the method of volatile detection is not sensitive enough to discern subtle differences in the emissions and that volatiles are lost in the noise of the spectra. Olfactometer assays may be used to confirm whether it is solely due to volatiles, coupled with electroantennography to identify specific compounds or blends that aphids respond to. On the other hand, visual factors may also influence aphid host-plant choice. It is possible that aphids are able to detect the presence of the bacteria as they probe for a suitable feeding site and are repelled by the bacteria's presence (Powell *et al.*, 2006; Wamonje *et al.,* 2020).

There is evidence to suggest that the act of spraying a plant is enough to induce the emission of GLVs to instigate the movement of *Myzus persicae* off a host plant. Although there are serious issues with the experiment preventing definitive conclusions, DHE-GC-MS analysis found applying a PBS spray caused similar blends of VOCs to be emitted from the plants to that of the wild-type. Indeed, the soil and plants with no-spray treatment produced significantly less GLVs than all the spray treatments, including the PBS spray. In addition, when plants with established aphid colonies were sprayed with either a control PBS or wild-type treatment, both treatments resulted in the dispersal of aphids to a new, un-sprayed plant, with more aphids settled on the fresh plant that the original after three days. It may be that

the movement of aphids is due to the disturbance caused by the droplets, rather than the volatiles induced in the plant by the treatments. For instance, when disturbed on a plant some aphid species drop off the host plant as an anti-threat response (Harrison and Preisser, 2016). It may be possible that a similar behaviour is being exhibited here.

Non-target effects on beneficials

It is important to consider non-target effects in any form of crop protection. In an IPM system, biopesticides are often used alongside beneficial natural enemies and pollinators. In glasshouses in particular, natural enemies are commonly applied to control aphid infestations therefore it is prudent to assess the effects of PpR24 on common commercial aphid natural enemies. This project explored the direct effects of PpR24 on *Orius laevigatus*, *Aphidius colemani* and *Macrolophus pygmaeus* by simulating likely routes of exposure in a crop environment. The results showed that natural enemies varied in response to PpR24 dependent on the route of exposure.

Topical applications of PpR24 at aphid-lethal concentrations (Paliwal, 2017) simulated droplet contact if sprayed in a crop environment. Significantly higher natural-enemy mortality was observed in *O. laevigatus* and *A. colemani* than PBS or control treatments, but no significant effect was observed in *M. pygmaeus*. It is possible that this effect is related to the insect's size. Adult *M. pygmaeus* are about 3-6 mm in length whereas *O. laevigatus* and *A. colemani* are much smaller, at 1.4-2.4 mm and 2-3 mm respectively. As such, *O. laevigatus* and *A. colemani* may be more susceptible to lower doses of the bacteria. It is possible that the bacteria enter the insects through their spiracles, holes in the cuticle used for respiration, and the toxins perforate throughout their body from there. The Hamilton study (Hamilton, 2015) hypothesised that the wild-type PpR24's aphicidal ability is in part due to gut occlusion (Stavrinides *et al*., 2009). It may be that PpR24 is forming occlusions in the spiracles of the insects, preventing respiration. Spiracle-blocking is seen in several commercial insecticides and is also the mode of action for biopesticides *Beavaria bassiana* and *Lecanicillium muscarium* which proved harmful to *Orius sp*. after dipping in insecticide solutions (Nakaishi and Arakawa, 2011).

Exposure to PpR24 spray residues on excised pepper leaves resulted in a statistically significant difference in *A. colemani* mortality when comparing PBS and wild-type PpR24 treatments. No significant differences in mortality were seen for *O. laevigatus* and *M. pygmaeus*. Similar protocols assessing the residual activity of pesticides on natural enemies have also found *A. colemani* to be sensitive to residual contact (Stara *et* al., 2011; Roubos *et al.,* 2014). However, topically applying aphid mummies with PpR24 had no significant effect on wasp emergence. It is possible the hardened cuticle of the mummy prevents the bacteria

affecting the developing wasp. Whether there are any long-term effects on the emergent wasps remains to be seen.

Both *M. pygmaeus* and *O. laevigatus* are taxonomically related to aphids as Hemipterans (Johnson *et al*., 2018), therefore it is possible that toxins produced by PpR24 may affect them in a similar way to aphids. However, oral ingestion of aphids fed on PpR24 showed no significant effect for *M. pygmaeus* but a statistically significant effect was observed in *O. laevigatus*. Again, this may be due to the smaller size of *O. laevigatus* as the larger *M. pygmaeus* may be able to withstand the ingested dose of the bacteria.

As *Macrolophus pygmaeus* showed no significant differences in survival over 72 hours in any of the topical, residual or oral exposure assays to PpR24, it may indicate that *M. pygmaeus* would be suitable for use alongside *P. poae* PpR24 in an IPM system. Although detrimental effects were observed in *O. laevigatus* and *A. colemani*, it does not definitively rule out the application of the bacteria when the insects are present in the system. *Orius* nymphs tend to be more concealed on a plant, such as in flowers, and so may avoid contact with the bacterial spray. Furthermore, PpR24 had no significant effect on *A. colemani* mummy emergence. It may be possible to apply the bacteria as a spray treatment when the insects are in juvenile stages with minimal beneficial casualties. As only adults were tested in this study, may be directed to examine the effects of PpR24 on juvenile beneficials, as well as examine potential sublethal effects PpR24 may induce. For instance, many chemical treatments have been known to affect insect fecundity and predator egg development, which may prove problematic for growers if they wish to establish the natural enemies in the system. Timing of natural enemy and biopesticide application would be key to an effective combination of control (Otieno *et al*., 2017).

Experimental design can have pronounced effects on a study and the limitations to the experiments in this section must be discussed. Unanimous to all experimental procedures in this set up, control insects untreated with PpR24 died which suggests improvement to the set-up is required. However, as the insects used in this study were ordered in for each experiment, it is possible that these deaths are due to study insects being at the end of their life. Culturing natural enemies in future to confidently age insects used in the assays may provide a solution to this issue. Furthermore, mortality observed in these lab-based experiments may not accurately reflect the mortality in field environments. For instance, in lab bioassays investigating the effects of spinosad on *Orius insidiosus*, a significant mortality effect was seen suggesting it would be unsuitable for use in an IPM system, whereas in glasshouse and field trials, no significant effects were observed (Studebaker and Kring, 2003). Lab-based experiments are unable to take into account field conditions that may affect

biopesticide action, such as changes to humidity, light and temperature, but also the movement of the insects in such large areas.

Going forward, it would be interesting to assess any sub-lethal, indirect effects of the bacterium on natural enemy efficacy, such as fecundity and prey location. As mentioned earlier, herbivore induced plant volatiles can play an important role in natural enemy prey location and pest repellency. For instance, *Orius* bugs have been seen to induce heightened emission of plant defence volatiles in sweet pepper which repelled the whitefly *Bemisia tabaci* and thrip *Frankliniella occidentalis*, as well as proving attractive to the whitefly parasitoid *Encarsia formosa* (Granell *et al*., 2018). Bacterial induced plant volatiles have also been seen to act as kairomones and it would be interesting to explore whether the volatiles induced by PpR24 on the plant are synergistic with natural enemy prey location or antagonistic. Another interesting avenue to peruse would be to see where the predators and parasitoids act as vectors for PpR24, directly transferring the bacterium from aphid to aphid, as well as from plant to plant. *O. laevigatus* has previously been seen to effectively disseminate the entomopathogenic fungus *Lecanicillium longisporum* or *L. muscarium* when doused with the fungal conidia, where 98% of *M. persicae* that came in to contact with leaf discs exposed to treated *O. laevigatus* became infected (Down *et al*., 2009).

Conclusions

The work presented in this study further supports evidence that *Pseudomonas poae* PpR24 has potential for commercialisation as an aphid biocontrol agent in an IPM system.

The experimental evolution passages were successful at evolving a biofilm-forming isolate, PpR24b4, however biofilm formation came at a cost to aphid virulence and bacterial growth, likely due to point mutations in the genes *cheB* and *barA*. Due to this, the wild-type *Pseudomonas poae* PpR24 still remains the best candidate for an aphid biocontrol agent.

Derived biofilm-forming isolates may still have value as plant growth promoters. This study successfully identified several volatile organic compounds emitted by the bacteria in a broth microcosm and from the plant sprayed with bacteria headspace. Several volatiles detected in all bacterial isolates have been associated with antimicrobial and plant growth promoting properties. A significant difference in isolate PpR24b4's volatile spectra from the wild-type was also noted in the broth environment, potentially another consequence of biofilm formation. Although no differences were observed in volatile emissions from isolates sprayed on the plant, both the passaged isolates did not exhibit the wild-type *Pseudomonas poae* PpR24's aphid-deterrent properties.

This study found the presence of PpR24 on a sweet pepper plant was able to deter aphids to a control plant where the bacteria was not present. Furthermore, sprayed plants emitted green leaf volatiles used in plant defence that may act to prime nearby plants to potential aphid threats. PpR24 could be used effectively as a foliar spray in both curative and preventative strategies, directly reducing aphid populations, but also deterring target pests from crop plants. However, to minimise the likelihood of aphids developing resistance to PpR24 treatment, it may be best if used in rotation with other pest control methods. If used in a system with banker-plant buffer zones that remain unsprayed by the bacteria, it may be that any pests are deterred from the crop to the banker plants. Pre-emptive spraying of crops before serious infestations occur may 'push' pests onto banker plants acting as sink-zones, minimising crop losses. Such a method may be combined with natural enemies established in the banker plant to feed on the displaced aphids.

P. poae may be suitable for use alongside aphid natural enemies to ensure maximum aphid control. Of the insects used in this study, *Macrolophus pygmaeus* may be the most applicable aphid predator for use in conjunction with *P. poae* as no significant lethal effects were observed in lab-based experiments. Carefully timed spray applications may also mean *P. poae* is suitable for use with other parasitoids and predators, although more research must be conducted into this. *Aphidius colemani* adults were significantly affected by topical application of PpR24 and exposure to spray residues, however as developing wasp emergence was not affected by topical applications of the bacteria, it may possible to spray crops when parasitoids are developing as mummies. Furthermore, juvenile *O. laevigatus* may avoid direct contact with *P. poae* spray as early life cycle stages are spent in more concealed areas of the plant and thus are less likely to directly encounter the bacteria. Timing bacterial application with the life-cycle of introduced natural enemies may reduce losses of other, non-target beneficials present in the crop as pollinators and to control other pest species.

In all avenues of agricultural research, lab-based trials can only take research so far. Up to this point, all investigations involving PpR24 have taken place in lab-based environments. Rigorous field trials would need to be conducted to fully understand the potential of *P. poae* PpR24 as a biocontrol agent as well as further steps taken to improve *P. poae's* shelf-life as a product.

Knowledge and Technology Transfer

- Week placement at Walberton nurseries, West Sussex, working and visiting local growers
- Two-day AHDB industry visit 2017, Lincolnshire
- Oral presentations at AHDB Conferences 2016 and 2017, AHDB Vine Conference 2019, University of Reading SBS Symposium 2018 and 2019
- Conference poster presentations at BES 2018, PopGroup 2019, International Symposium Ecology of Aphidophaga 2019
- 2017 AHDB Crops PhD Conference Poster Winner
- Author on collaborative review paper, *Endophytes vs tree pathogens and pests: an insight into an underutilised source of biological control agents*
- Presentations on research at Biobest and Syngenta

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