Project title:	**Understanding the impact of phylloplane biocontrol agents on insects**
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Project leader:	Prof Rob Jackson, University of Reading
Report:	Annual report, October 2018
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Location of project:	University of Reading
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Date project commenced:	3rd October 2016
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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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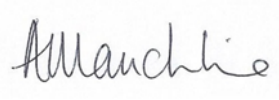
Signature .....  ..... Date 07/01/2018

CONTENTS

GROWER SUMMARY

Headline

Applying experimental evolution to improve naturally aphid-killing bacteria, *Pseudomonas poae*, resulted in a strong biofilm-forming mutant but failed to improve the efficacy of aphid killing.

Background

The control of insect pests in glasshouse systems is a major challenge. Aphids in particular thrive in controlled environmental conditions, causing damage to crops by feeding and by transmission of 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 used against aphids but growers are under increasing pressure from supermarkets and consumers to find alternative, environmentally friendly, non-chemical methods of control. Also, indiscriminate use of chemical pesticides can increase the chance of resistance developing in the aphids and also kills off other 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, particularly against aphids and thrips. 140 bacterial isolates from a variety of plants were tested for virulence against aphids (Hamilton, 2015) and three were found to be most effective: *Pseudomonas fluorescens*, *Citrobacter werkmanii* and *Pseudomonas poae*. Further investigations (Paliwal, 2017) found *Pseudomonas poae* (*P. poae*) to have the highest success rate in killing aphids, with a 70% reduction in aphid populations when treated on plants as well as appearing to deter aphids from going on the plant. Furthermore, application did not have any negative effects on the plants. Not only were they effective at killing a range of aphid species but these bacteria also proved to have no noticeable effect on non-target insects that they may come into contact with, such as species of lepidopterans and ground beetles.

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 to harness 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. Additionally, they have less of an environmental impact and pose little or no threat to biodiversity as they are naturally present in the ecosystem (Lacey *et al*., 2001). 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 that we are investigating for use as a biological control, *Pseudomonas poae* PpR24 (*P. poae*), was originally found on the roots of *Brassica oleracea* and found to be pathogenic to 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*). Previous work investigated its success for a range of application methods and found it to be most effective as a foliar spray or by soil drenching; therefore these are the application methods we intend to use for this project.

So far this project has mainly focused on improving the bacteria to become more efficient as a biological control by experimental evolution and examining the trade-offs between traits that arise. The process of experimental evolution involves identifying beneficial trait of the bacteria we want to enhance or develop, and selecting for it in ‘passaging’ situations over several weeks. At the end of this ‘passaging’ process we examined trade-offs between the evolved strains. This involved comparing whether improving one trait of the bacteria was at a cost to another, for instance improving bacterial toxicity may have caused bacterial growth on a plant to become less efficient. Due to time constraints, we focused on evolving two traits.

Toxicity to aphids

A key outcome of the evolutionary passages was to improve the toxicity of the bacteria. Currently, 70% of aphids are killed by *P. poae* in 42 hours; we hoped to improve this by increasing the overall mortality and reducing the time it takes for the bacteria to be effective.

This would be beneficial to growers as it would significantly reduce the time taken to combat aphid infestations as well as reduce the need for subsequent applications.

Formation of biofilms

We investigated whether the bacteria possess the ability to form biofilms. Biofilms are aggregations of bacteria that are able to adhere to surfaces and form communities. Such an adaptation offers numerous benefits to bacteria which would also be relevant as a biocontrol. Biofilms offer bacteria more protection from the environment, allowing the bacteria to survive longer on the plant, and help create space for the bacteria to grow and move. Not only would this aid in colonisation of plants when it has been applied but it may also remove other, non-desirable microbes from the plant. Furthermore, testing whether *P. poae* can form such a structure may provide insight as to how it kills the aphids, as one theory suggests it coats the insides of the aphids in a biofilm which ultimately may cause the pest to starve to death.

Trade-offs

We explored how the two traits performed in each other's selective environment, as well as investigated how the bacteria performed against the wild-type *Pseudomonas poae* in situations relevant to crop protection, notably how well evolved isolates could survive on a crop plant. An improvement to the colonisation of plant leaves and how long the bacteria can last on the plant would reduce how often it would have to be applied to the crop. This would also provide further insight as to whether the bacteria can sustain itself in the crop environment and the possibility of a single spray solution to aphid infestations

Each property of the bacteria was investigated over 10 passages. We succeeded in evolving biofilm-forming isolates of *P. poae* in a broth environment, with one isolate in particular forming significantly strong biofilms. However, this ability came at the cost of aphid virulence and survival on the crop plant, proving to be significantly poorer than aphid-passaged isolates and the wild-type *Pseudomonas poae.*

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 (Harris and Maramorosch, 1997). 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

Research is still ongoing therefore it is not yet feasible to make well defined action points. However, we would expect to use this microbial based product in an integrated pest management system as a foliar spray alongside other biocontrol agents, such as natural enemies. 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

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).

Previous research discovered the bacteria, *Pseudomonas poae*, to be effective at killing aphids without seeming to harm non-target insects or damage the plant it is applied on (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.

Materials and methods

Media

Bacteria were grown in King's Medium Broth (KB) (King *et al*. 1954) (Proteose peptone (Difco) 20 g, K2HPO4 1.5 g, MgSO4.7H2O 1.5 g, glycerol 10 mL). The aphid diet used in feeding sachets was Mittler diet (Dadd *et al*., 1967).

Plants

All plants used for aphid rearing and bacterial growth passages were sweet pepper Palermo RZ F1-Hybrid *Capsicum annum*, supplied by Rijk Zwaan seeds. The plants were grown at 21°C in a controlled environment room and for 4 weeks before use in experiments or for rearing aphids.

Aphid rearing

The aphids used were *Myzus persicae*. Clones were maintain parthenogenetically in plastic leaf box cages in a rearing room at 21°C on a long day light cycle (16h light/8 h dark) to ensure no sexual reproduction occurred. Large populations were reared on whole plants in cages.

Biofilm passage assay

Bacteria and growth conditions

The biofilm passages were carried out over a 10 week period, where each passage lasted 1 week following the methods as devised by Spiers *et al*. (2003). Ten, 10 ml glass universals of King's Broth (KB) media were inoculated with *Pseudomonas poae* PpR24. Five universals were inoculated with 10 µl of bacteria and the other five 100 µl of bacteria. The microcosms were incubated at 27°C for 1 week and kept static to allow biofilms to form at the air-liquid interface. The passages were then continued in fresh KB media. 10 µl of bacterial-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.

Bead test of biofilm strength

After one week, the static microcosms were removed from the incubator and observations on the presence of biofilms were made. 2 mm glass beads were dropped into the centre of the biofilm from a constant height until the biofilm sagged or broke. The more beads it could support, the stronger the biofilm.

Biofilm attachment strength

Bacterial attachment was quantitatively assessed using the crystal violet staining technique as laid out by O'Toole *et al*. (1999). 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 washed out with water. The stain was eluted with 5 ml ethanol, shaken for 15 minutes and the OD_{600} recorded.

<u>Aphid-Killing passage assay</u>

The aphid sachets comprised of Perspex cylinders, washed with 70% ethanol, and cut parafilm sections 4 cm x 4 cm, sterilised under a UV light for 30 – 40 minutes. Overnights were normalised at OD600 to 1 and the volume added to a sterile Eppendorf and spin down for 3 minutes at 5000 rpm. The supernatant was removed and the bacterial cells resuspended and washed twice with 1 ml of sterile PBS, spinning down, removing supernatant and adding sterile PBS each time.

The diet sachets comprised of 6ul bacteria-PBS suspension added to 594 µl of sterile aphid diet to achieve a concentration of 10^7, vortexed for 5 seconds to ensure it was well mixed. The sachets were made by delicately pipetting 600 µl of the bacteria-diet solution onto the centre of the parafilm stretched over the cylinder and carefully stretching a second piece of parafilm over this, ensuring the sterilised side is in contact with the droplet, avoiding spillage.

Ten aphids were added to each cylinder and the bottom sealed with parafilm. The sachet pots were left in long-day light conditions, observing aphid death at 0, 1, 6, 18, 24, 30, 42, 46 and 48 hours. Aphid death was classed as an aphid carcass dead at the bottom of the cylinder.

After 48 hours, the aphids were recovered and surface sterilsed in an Eppendorf of 500 µl 1% sodium hypochlorite for 5 minutes. The sodium hypochlorite was removed and the aphids washed three times in sterile water, spinning down at 5000 rpm for 3 minutes each time.

The aphids were then homogenised using micropestles in 200 µl of sterile PBS, then 100 µl of the homogenised solution was spread on KB and Nitrofurantoin plates. Incubation lasted for 24 hours at 27°C, allowing a lawn of the recovered bacteria to grow. This was washed off the plate using 2 ml of sterile PBD and added to sterile falcon tubes. Frozen stocks were made using 500 µl of solution and 500 µl of 40% glycerol and the process repeated for the new passage sachet.

Assessment of trade-offs

Each of the 20 evolved isolates and the wild type bacteria were tested for trade-offs between traits.

Aphid virulence

The same protocol for aphid-killing passages was followed.

Biofilm formation

The same protocol for biofilm formation passages was followed.

Growth

Changes in growth were assessed using Bioscreen C equipment. Honeycombe plate wells were filled with 180 µl KB media and inoculated with the isolates in triplicate. Sterile PBS was used for the controls. Growth was observed over 24 hours, in continuously shaken and static conditions.

Motility

Swimming assay – Assays were conducted using 30 ml of 0.25% KB agar in standard, 8.5 cm diameter petri dishes. Each plate was inoculated with *P. poae* by stabbing the centre of the plate. Plates were left to grow at 27°C on a flat surface and imaged at 0, 12, 24, 36 and 48 hours. Image J software was used to assess the spread of the bacteria over time.

Swarming assay – Assays were conducted using 30 ml of normal strength KB agar in standard, 8.5 cm diameter petri dishes. Each plate was inoculated with *P. poae* by stabbing

the centre of the plate. Plates were left to grow at 27°C on a flat surface and imaged at 0, 12, 24, 36 and 48 hours. Image J software was used to assess the spread of the bacteria over time.

Survival on plant

Isolates were grown overnight in 30 ml KB, in 50 ml falcon tubes at 27°C and then normalised to OD600 to 1. Only the best aphid-killer isolate and best biofilm former were tested as testing all isolates would have been unfeasible. The bacterial spray is then made. The bacterial overnight solutions are spun down at 400 rpm for 25 mins to pellet the bacteria. This is then re-suspended in sterile PBS and washed twice, before re-suspended in sterile PBS.

The spray solution ins applied to the abaxial and adaxial sides of leaves using hand atomisers, with the control spray as PBS. Treated plants were left to dry in the flow hood.

Leaf sections were taken on days 0, 3, 7, 14 and 21 aseptically with a core borer and add to eppendorfs of 200 µl sterile PBS. The leaf disks were then homogenised using micropestles and samples diluted and plated out as 10 µl droplets in triplicate on KB and Nitrofuratoin agar. Plates were left to grow for 16 hours at 27°C, after which colonies were counted.

Examination of *P. poae* volatiles

The volatile composition of *P. poae* and the best isolates for biofilm formation and aphid killing will be examined using solid-phase microextraction (SPME), with a triple phase fibre. The volatiles from the bacteria in spray form and once sprayed on the plant will be examined.

Aphid colonisation behaviour assay

Plants, aphids and bacterial spray solution were grown, reared and produced as above. Two plants were placed in a sterilised, Perspex box (sterilised as above). The plants were sprayed with different treatments (following the spray preparation above), with sterilised PBS for the control spray. Ten replicates were conducted at a time, with 50 aphids per box. Aphids were place equidistant between the two plants and left for 24 hours. After 24 hours, the number of aphids per plant was counted. A number of choice/no-choice conditions were and are to be investigated.

Control vs control – Aphids were presented with a no-choice situation between two plants both sprayed as controls to ensure the method worked.

Wild type vs control - Aphids were presented with a choice situation between control spray and wild type spray

Wild type vs Aphid killer vs control - Aphids were presented with a choice situation between control spray vs wild type spray, control vs aphid killer.

Wild type vs wild type - Aphids will be presented with a no-choice situation between two plants both sprayed as wild type to assess whether aphids are capable of colonising plants in spite of spray.

Wild type spray once aphids established – Allow aphids to establish on a plant for 24 hours and then treat with bacteria to assess whether the aphids are repelled.

Results

Experimental Evolution

Research is still on going and therefore not all results can be presented in this report. We have successfully evolved biofilm-forming isolates over the ten passages (Figures 2 and 3) although attempts to improve bacterial virulence against aphids have been unsuccessful (Figure 1). There are definite trade-offs between the isolates. Aphid-killing isolates proved to be poor biofilm formers (Figures 4 and 5) but remained indifferent to the wild-type in terms of tested trade-offs. Biofilm-formers appear to have a 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). Biofilm-passaged isolates also showed a decrease in swimming (individual cell movement by rotating flagella) and swarming (multicellular movement by rotating flagella) motility (Kearns, 2010) when compared to the wild-type (Figures 8 and 9), as well as showing slower growth in a broth environment (Figure 10). However, statistical analysis still needs to be carried out on this data.

Volatile Analysis

Changes in isolate volatile emissions have been observed, with the biofilm-isolate trace loosing sulphur compounds which are present in the wildtype and aphid-passaged isolates.

Aphid Behaviour Boxes

In a choice environment between a control plant and wild-type sprayed plant, when left for a week, there was a preference for aphids to colonise the control plant. When left to choose for 24 hours, no aphid preference was observed (Figures 11 and 12). Initial aphid colonisation behaviour box tests between evolved isolates indicated that there is no difference in aphid colonisation of plants treated with wild type or aphid-killer bacteria, which may reflect that no significant volatile changes have occurred. As there was no significant difference between control vs control plants we took this to indicate our testing system was effective.

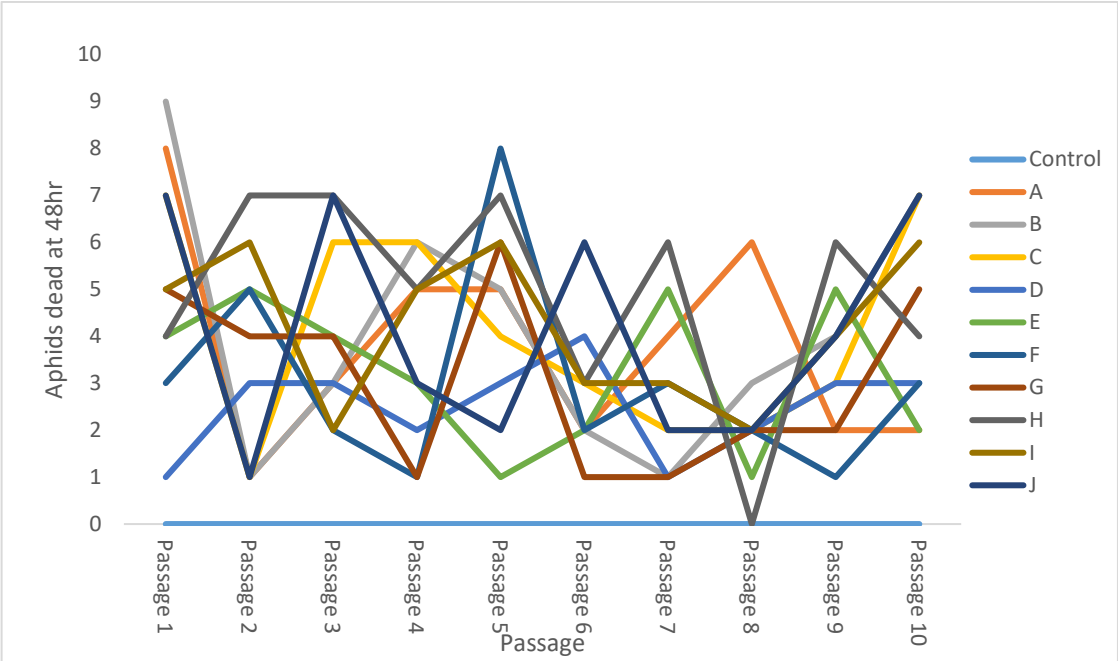


Figure 1 Aphid Virulence passage. Aphid death at 48 hours for each isolate derived from the wild-type *Pseudomonas poae* (A-J) over the course of the ten passages.

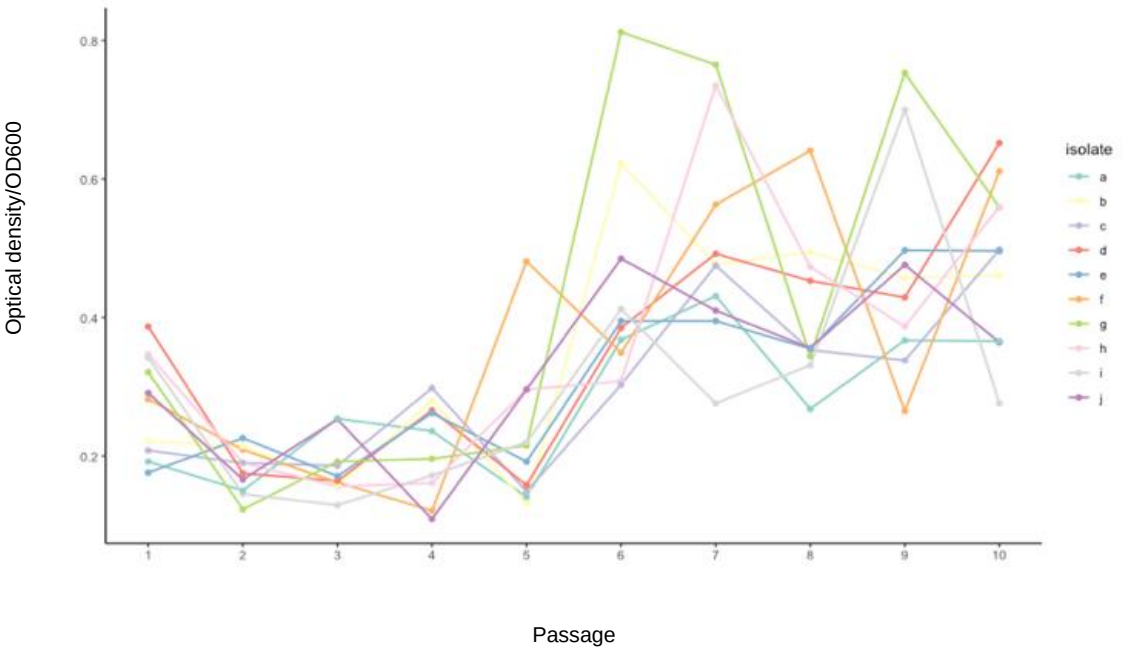


Figure 2 Biofilm attachment strength passage. Bacterial attachment of biofilm passaged isoaltes (A-J) as indicated by optical density at OD600 across the 10 week passage.

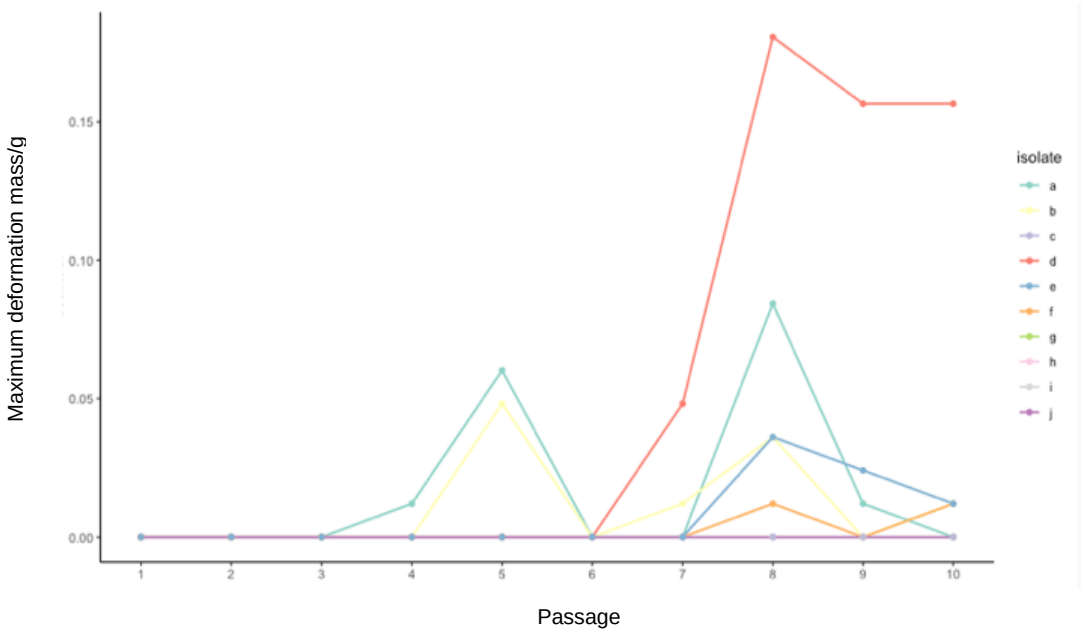


Figure 3 Biofilm strength passage. The maximum deformation mass of biofilm passaged isoaltes (A-J) across the 10 week passage.

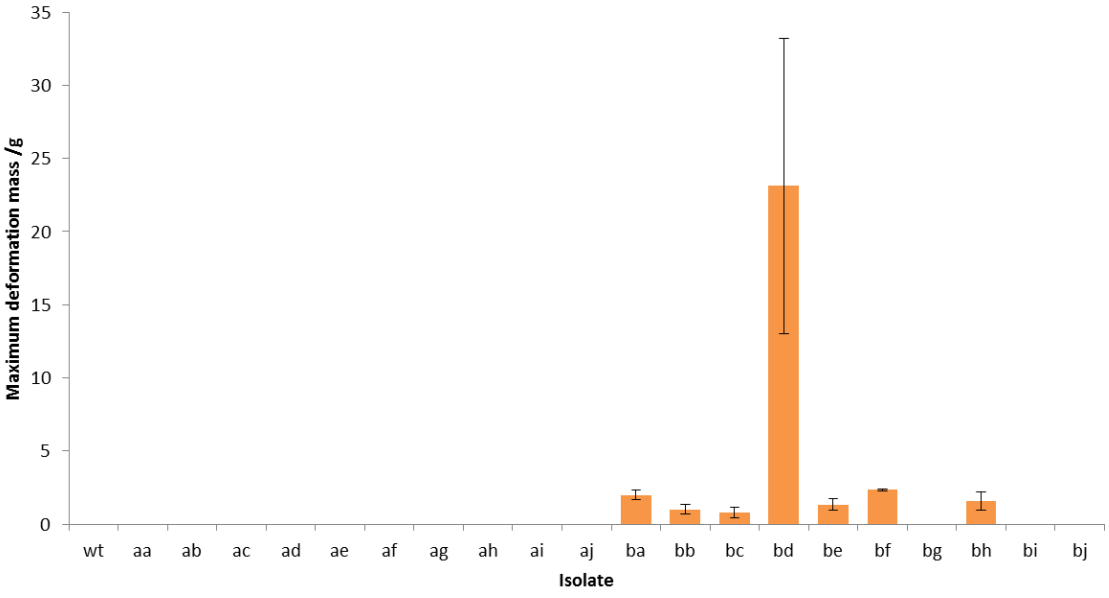


Figure 4 Trade-off of biofilm strength between wild-type (wt), aphid-passaged (a-) and biofilm-passaged isolates (b-).

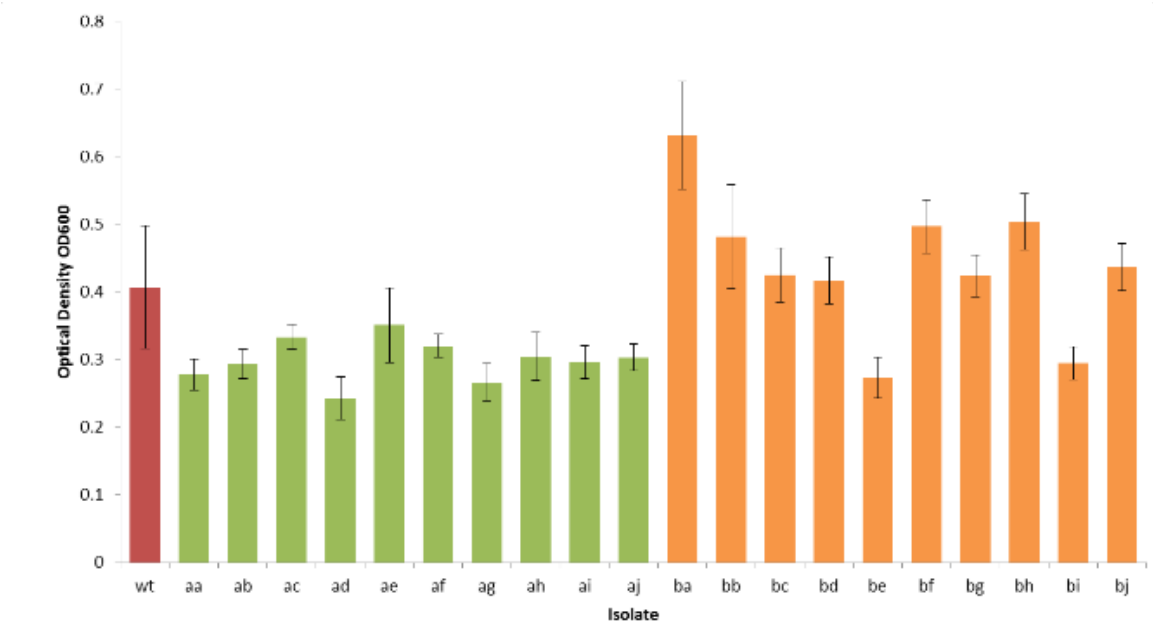


Figure 5 Trade-off of biofilm attachment strength between wild-type (wt), aphid-passaged (a-) and biofilm-passaged isolates (b-).

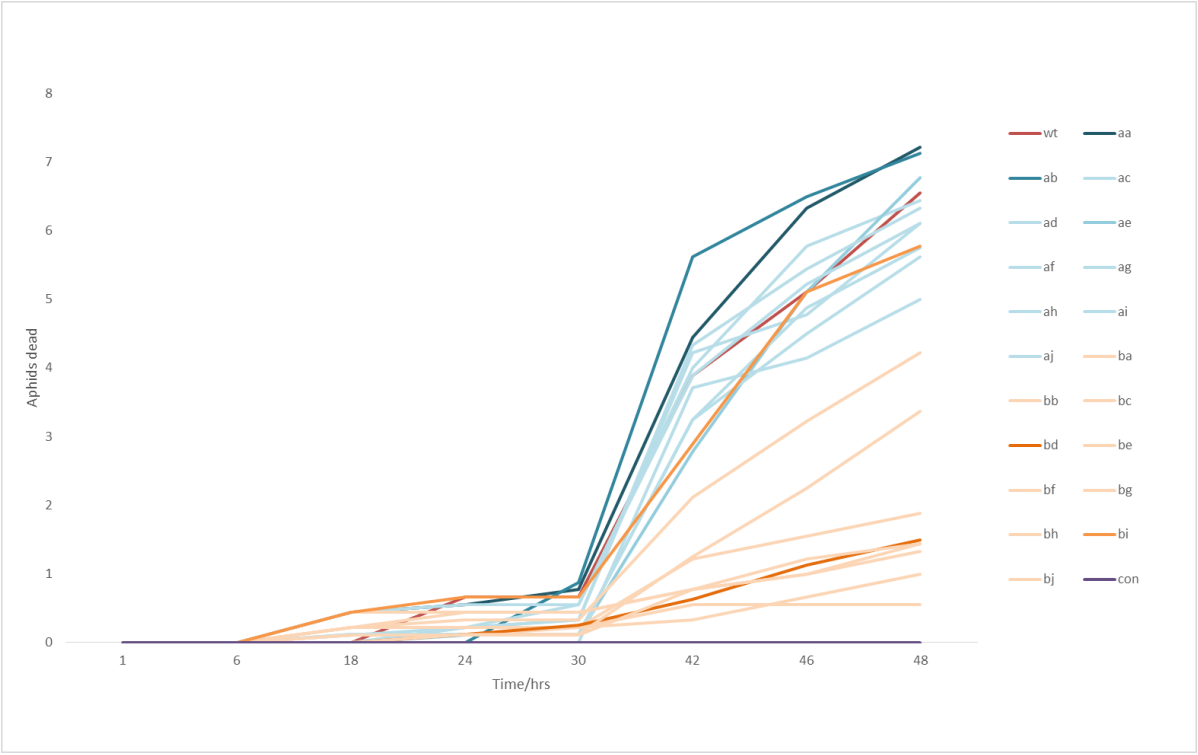


Figure 6 Trade-off of aphid-killing in 48 hours between wild-type (wt), aphid-passaged (A-) and biofilm-passaged (B-) isolates.

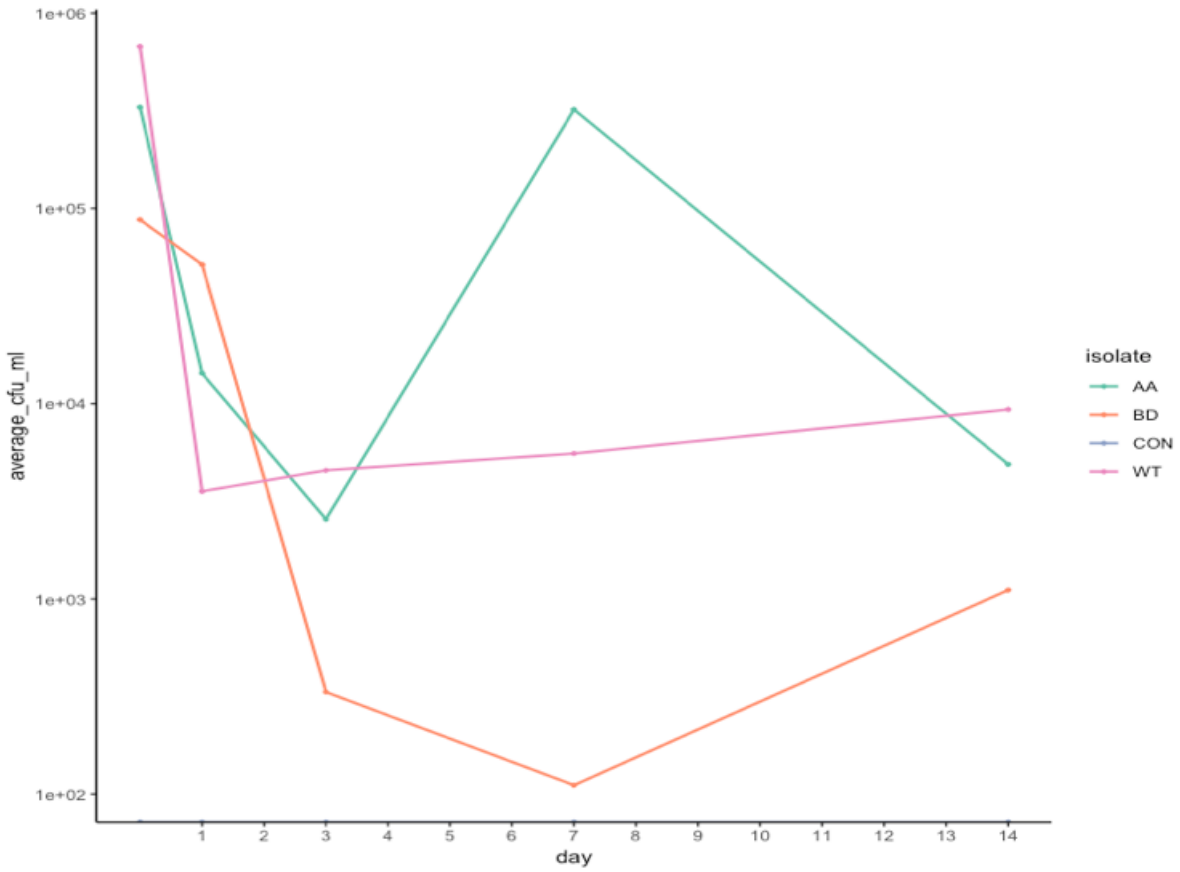


Figure 7 Trade-off of plant survival between wild-type, Isolate AA from the aphid-passages and Isolate BD from the biofilm passages. Log10 of the average colony forming units is presented.

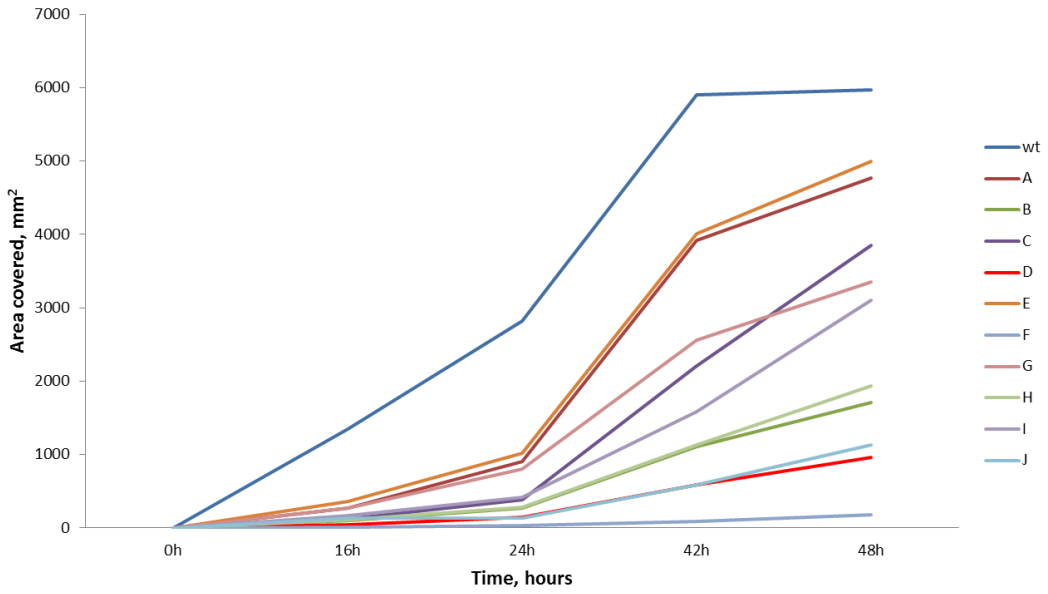


Figure 8 Biofilm isolate motility – Swarming motility of biofilm passaged isolates (A-J) compared to the wild-type *P. poae* (wt) spreading across a soft-agar plate.

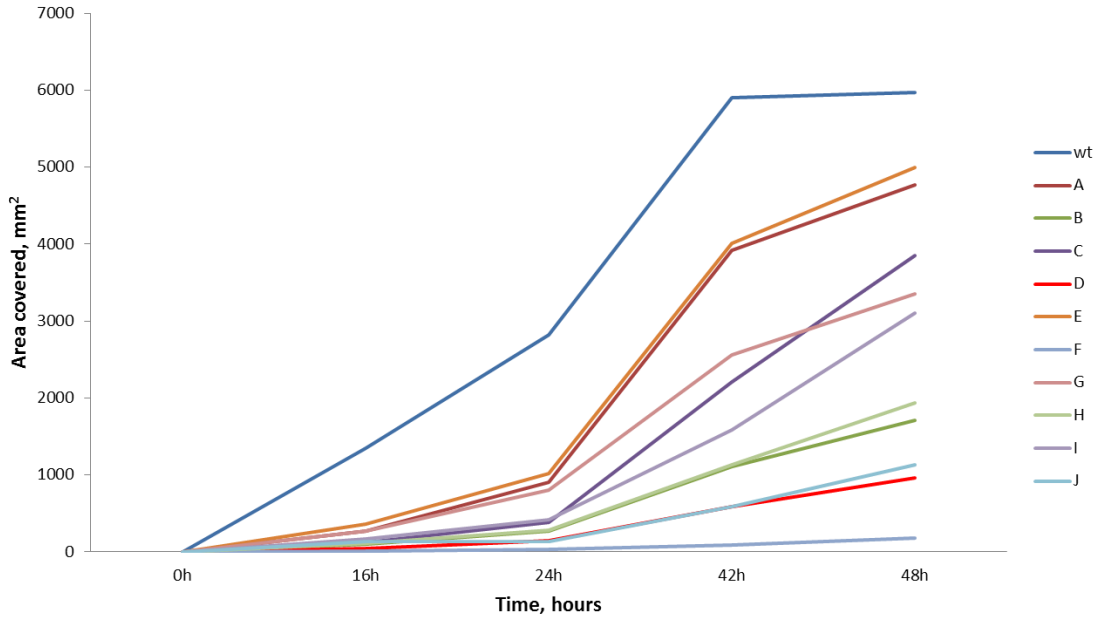


Figure 9 Biofilm isolate motility – Swimming motility of biofilm passaged isolates (A-J) compared to the wild-type *P. poae* (wt) spreading across a soft-agar plate.

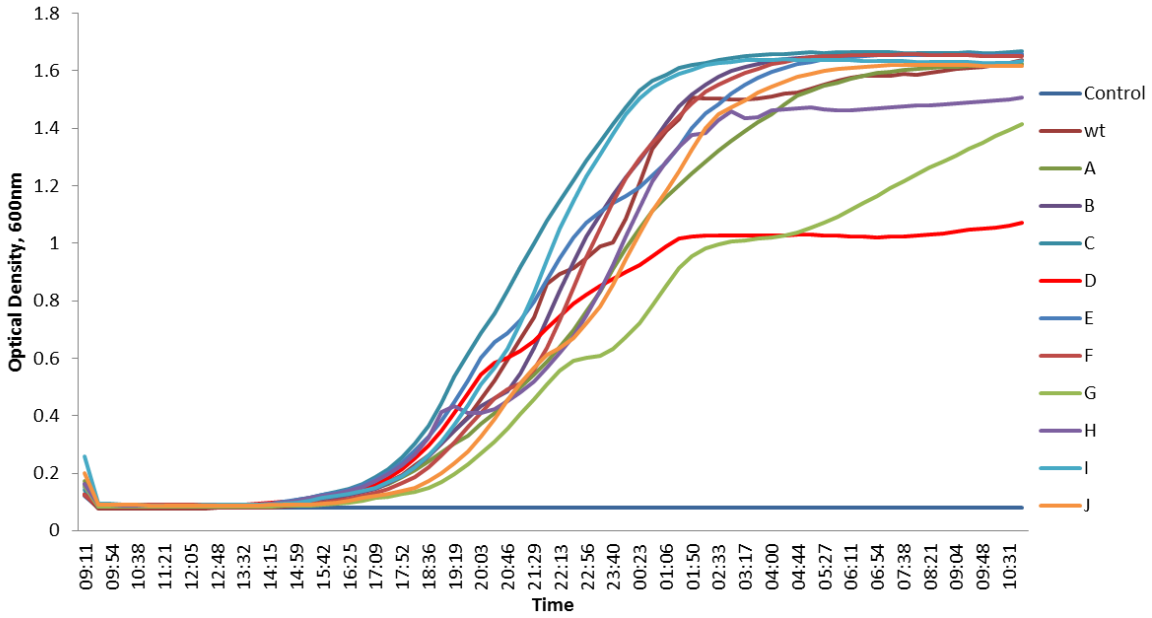


Figure 10 Biofilm isolate growth in KB broth at 27ºC.

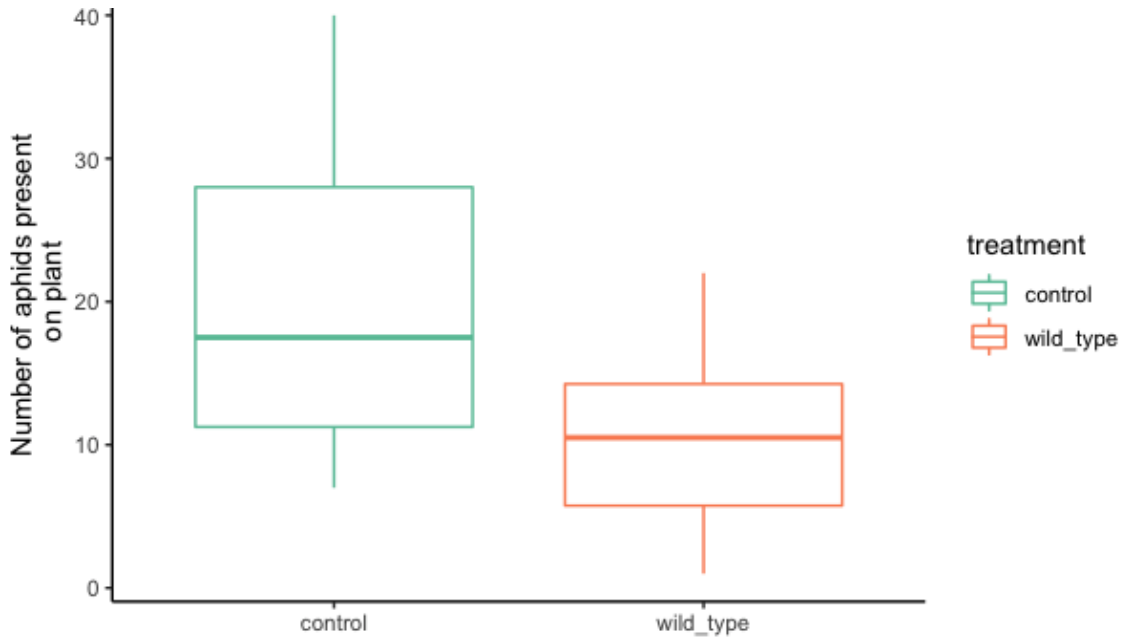


Figure 11 Aphid choice box test, wild-type *P. poae* vs control spray. Aphids left to colonise plants for one week showed a statistical difference, Welch Two sample t-test p-value = 0.04325.

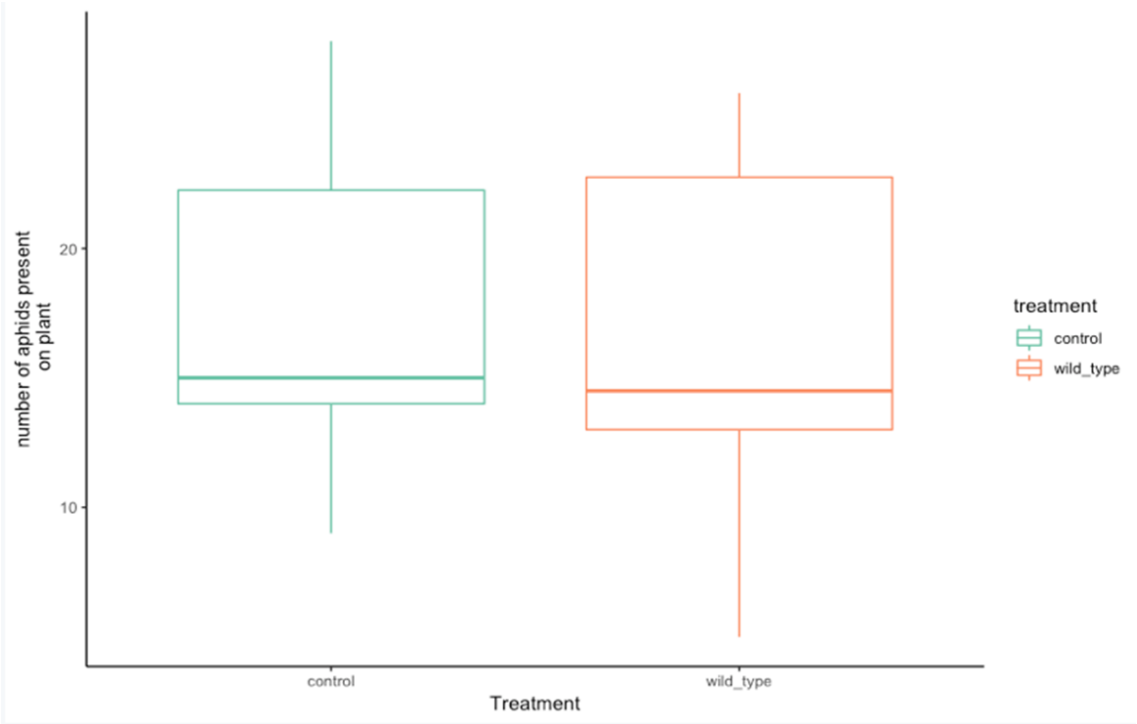


Figure 12 Aphid choice box test, wild-type *P. poae* vs control spray. Aphids left to colonise plants for 24 hours. No statistical diference observed, Welch Two sample t-test, p-value = 0.7071

Discussion

Experimental Evolution

The results for the biofilm evolution passages indicate that we have been successful in evolving *Pseudomas poae* to form biofilms. Isolate D proved to be significantly stronger than the other isolates although biofilm attachment strength did not improve over the wild type. However, there was no improvement to aphid-killing ability over time, with no significant changes from the wild-type. This may be due to selection pressure on the bacteria to select for aphid-killing not being specific enough. Furthermore, if the passages were to be continued for longer, it is possible that improved virulence may occur in the population over time but it is unfeasible to continue it throughout the remainder of this project.

Numerous trade-offs were observed between the biofilm isolates and the wild-type and aphid-passaged isolates. Aphid-passaged isolates were poor at forming biofilms, having low attachment strength and no ability to support beads when compared to biofilm-passaged isolates. However, this was to be expected as they were not under selection for the trait in a broth environment. More interestingly, there was a trade-off between biofilm formation and aphid-virulence, with biofilm-passaged isolates killing far fewer aphids over 48 hours. Biofilm isolates also had poorer swimming and swarming motility than the wild-type *P. poae* as well as slower growth rates. Genome sequencing may shed light on the changes that have occurred and may reveal other changes that we have not tested for.

Investigations are ongoing into the causes of these trade-offs, but we have achieved the original aim of the project by successfully evolving *Pseudomonas poae* by experimental evolution to develop traits that may be desirable to growers. However, it is unfortunate that improved biofilm formation decreased the bacteria's fitness on the crop plant and caused a loss of virulence to aphids, thus making it unsuitable for use as a biocontrol.

Behaviour Assays

Although no difference in choice has initially been observed between aphids presented with wild-type bacteria vs aphid-passaged bacteria, this is likely as there have been no observable significant changes between them. However, it will be interesting to see if the improved biofilm-forming isolate has a significantly different effect as initial results from the volatile analysis of the evolved biofilm isolate D indicate there is a change in the volatile composition given off by the bacteria. This may have an effect on the host detection of the aphids or deter aphids from the plant more effectively than the wild-type.

Conclusions

We have successfully employed experimental evolution to evolve isolates of *Pseudomonas poae*. Although we failed to evolve an improved aphid killing isolate, we succeeded in forming an effective biofilm-forming isolate. However, significant trade-offs have arisen at the cost of this biofilm formation, making it unsuitable as an aphid control. For instance, lack of aphid virulence and poor survival on the crop.

Research into the effects of *P. poae* on the behaviour of aphids and their ability to colonise plants is underway. Initial studies indicate that there is no difference in aphid behaviour between treatments of wild type and aphid-killer. Although initial tests have found differences between the volatile traces of biofilm-forming isolates and wildtype/aphid-passaged isolates,

further examination of the bacteria's volatile emissions remains to be done, which may prove further insight into any deterrence behaviour.

As well as completing behaviour tests on the bacteria's effects on aphids, our next step focuses on examining the non-target effects of *P. poae* on aphid natural enemies. *Orius laegivatus*, *Macrolophus pygmaeus* and *Aphidius colemani* will be the focus of the study, initially examining short-distance effects on prey location, insect mortality and whether the bacteria is transferred to the predator insect. Time permitting, we hope to also investigate long-distance effects on prey detection using olfactometers, as well as potential impacts of *P. poae* on adult bee health.

Knowledge and Technology Transfer

- AHDB annual student conferences November 2016, 2017, 2018
- The BSPP conference 2017
- BES annual meeting 2018 – presented poster
- Pop Group 52 – presented poster

References

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