Project title:	**Understanding the impact of phylloplane biocontrol agents on insects**
Project number:	CP120
Project leader:	Prof Rob Jackson, University of Reading
Report:	Annual report, October 2017
Key staff:	Dr Louise Johnson, Dr Alice Mauchline, Prof Mark Fellowes, Dr Georgina Key
Location of project:	University of Reading
Industry Representative:	Martin Emmett, Tristram Plants, Binstead and Walberton, West Sussex
Date project commenced:	3rd October 2016
Date project completed (or expected completion date):	19th September 2019

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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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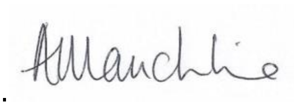
Signature ..  .... Date ....01.11.17.............................

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GROWER SUMMARY

Headline

Initial results indicate that *Pseudomonas poae* is capable of forming biofilms in a broth environment and so may be able to survive for longer on the plant. There are also significant changes to attachment strength over time, indicating we are able to improve this trait over time via experimental evolution.

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 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 employed 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. Of the 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 it 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 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. Furthermore, 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 methods of application we intend to use for this project.

The first year of this project has focussed on improving the bacteria to become more efficient as a biological control. We intend to do this by experimental evolution, where the bacteria's beneficial trait we want to enhance is focused on and selected for over several weeks. At the end of this 'passaging' process, we will compare if there have been any trade-offs between the evolved strains. This involves comparing whether improving one trait of the bacteria will be at a cost to another, for instance improving bacterial toxicity may cause bacterial growth on plant to become less efficient. There are four traits that are the focus of our evolution experiment.

Toxicity to aphids

A key outcome of the evolutionary passages would be to improve the toxicity of the bacteria. Currently, 70% of aphids are killed by *P. poae* in 42 hours, we hope 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.

Growth and persistence on plants

We will attempt to improve the colonisation of bacteria on plant leaves and how long the bacteria can last on the plant, thus reducing 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.

Formation of biofilms

Finally, we intend to investigate 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, thus 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 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.

Each property of the bacteria will be investigated over 10 passages. Only the biofilm passages have been conducted thus far and although the dataset is incomplete, there are promising results. *P. poae* is capable of forming biofilms in a broth environment and there are significant changes in attachment strength (how well the bacteria can adhere to a surface) over the passages, indicating that we are able to improve this over time.

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.

This bacterial biological control has the potential to significantly reduce costs of aphid crop protection as it would remove the need for chemical treatments and the improvements we are working on should increase the efficacy of this approach; therefore decreasing application costs. As the bacteria may be self-sustaining in the crop system, a reduction of applications would be likely. However, it is still very early in the project for definitive figures.

Action Points

As this is the first year of this project, 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

Chemical pesticides have been widely applied in use against aphid pest for decades. However, detrimental, long-lasting side effects on the environment and biodiversity (Hopwood *et al*., 2016), as well as a build-up in resistance to chemical treatments in the aphids (Bass *et al*., 2014), has resulted in increased legislation against their use and a push for alternative forms of aphid control. Using insect natural enemies, such as parasitic wasps, is an increasingly common form of pest management, providing the economic benefit of reducing yield loss without the negative environmental effects (Bianchi et al., 2006; van Lenteren, 2012). However, when used alone, many biocontrol insect species are incapable of totally reducing a pest below their economic threshold (Hall and Norgaard, 1973) and thus they need to be used alongside other control agents in a holistic, integrated management system.

Microbial based biological controls are becoming increasingly popular on the pest control market (Lacey *et al*., 2001; Pandin *et al.,* 2017). Entomopathogens, such as fungi, nematodes and bacteria, naturally play an important role in regulating insect populations and many are now being exploited as current forms of biological controls (Lacey et al., 2001). They present many advantages over both chemical and arthropod aphid management strategies. Compared to chemical pesticides, entomopathogens 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 arthropods, entomopathogenic 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 and prove that they are safe and can be used within an integrated pest management framework.

The first year of this project has focussed on evolving *Pseudomonas poae* to become more efficient as a biological control via experimental evolution. This involves a series of passaging experiments (Lenski *et al.*, 1991) intended to improve bacterial growth and persistence on the crop plant, improve their toxicity against aphids and investigate the potential and encourage the formation of biofilms on the plant surface.

Passaging involves inoculating a plant or aphid with bacteria, allowing it to grow and recovering it. The recovered bacteria are then used as inocula for the second passage. This can be repeated several times, where changes in bacterial performance can be observed and each recovered bacterial population frozen and stored as a record for each cycle.

To enhance aphid killing, *P. poae* will be passaged through the aphids by allowing the aphids to ingest the bacteria from inoculated diet, dispensed by a feeding sachet. To improve the growth and persistence of aphids on plants, a similar approach will be conducted on pepper plants, *Capsicum annuum*, where the bacteria will be applied as a foliar spray, recovered and re-passaged.

The majority of work conducted thus far has focussed on the biofilm aspect of the project. When in a broth solution, *P. poae* naturally assumes a planktonic state. However, we wished to investigate whether it has the ability to form biofilms. A biofilm is where cells form aggregations by secreting extracellular substances that are able to attach to each other and surfaces (Popat *et al*., 2012). Biofilms are particularly useful when colonizing a new area as they offer some protection for bacteria from environmental, physical factors as well as provide an advantage to outcompete other microorganisms in their environment (Spiers et al., 2003). Such an attribute may enhance the survival of *P. poae* on plant surfaces and thus be a significant beneficial property to a biocontrol. Furthermore, investigations into whether *P. poae* is capable of forming biofilms in the broth environment may provide insight as to whether it is capable of aggregating in the aphid gut, which may be one contributing factor to aphid mortality.

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 10ul of bacteria and the other five 100ul 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. 10ul 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 100ul 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. 2mm 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 1ml 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 5ml ethanol, shaken for 15 minutes and the OD_{600} recorded.

Results

Biofilm formation and strength

Although the 10 week experiment is not complete and the investigation still ongoing, thus far all microcosms of *P. poae* have been capable of forming at least a thin biofilm at the air-liquid interface of the microcosm (figure 1). The strength of the biofilms were inconsistent from week to week (figure 2), with no biofilms of the 100ul passages able to support any beads at all.

Attachment strength

An analysis of variance (ANOVA) was conducted on the data for OD_{600} using JMP statistical software and Tukey's honest significant difference (HSD) test was applied to test for any significant differences in biofilm attachment strength between passage weeks (figure 3). The results indicate for graph A) that for passages 6 and 7 the bacterial attachment strength is significantly different to that of the other passages. The data for passages 6 and 7 for both of the passaged volumes also indicates a higher OD_{600} reading, suggesting an increase in attachment strength over the passage series.

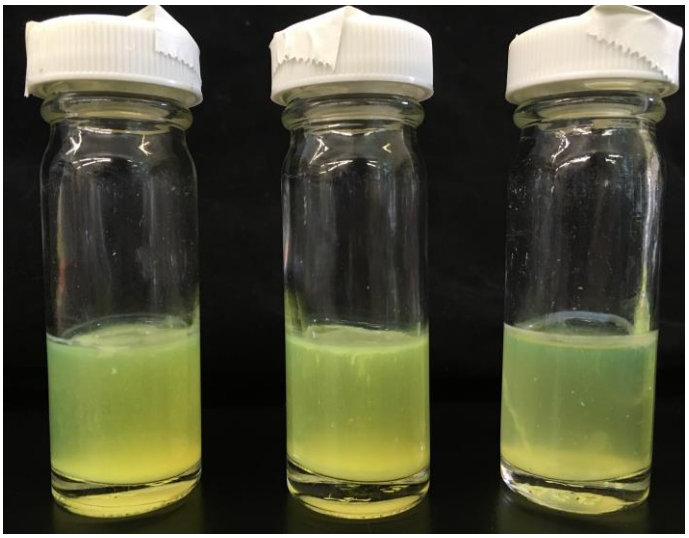
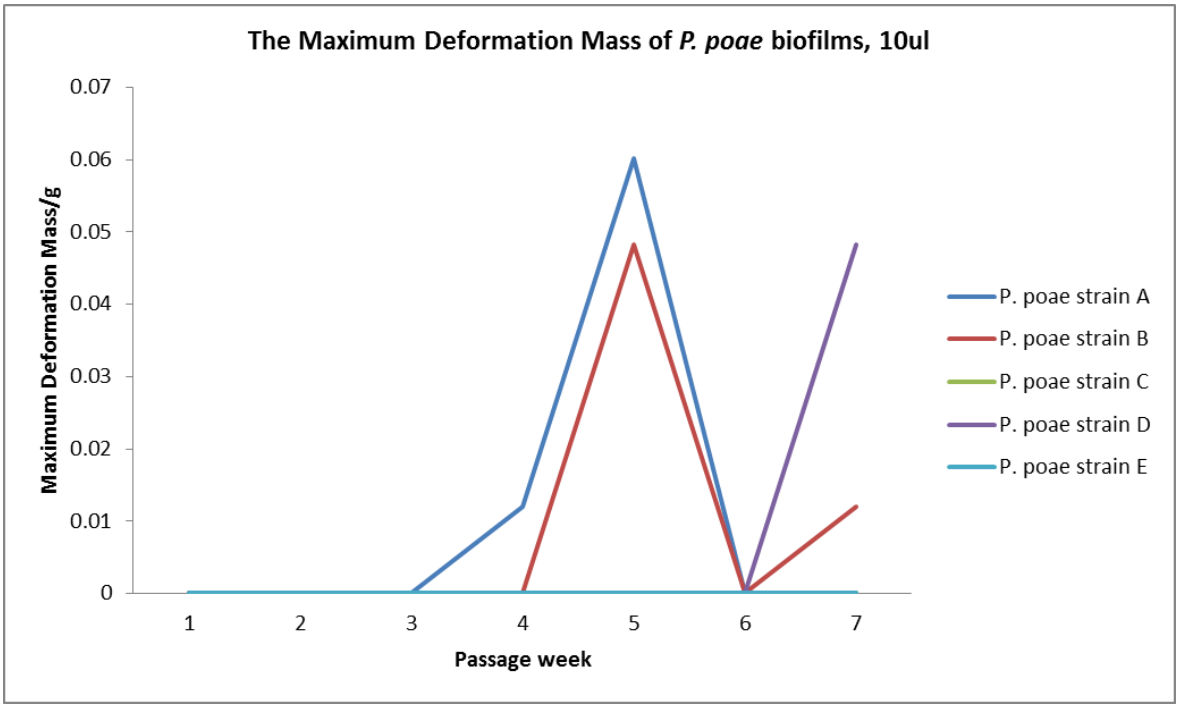


Figure 1. Microcosms of *P. poae* in KB media after 1 week of growth. A defined band at the A-L interface indicates biofilm growth.

A)



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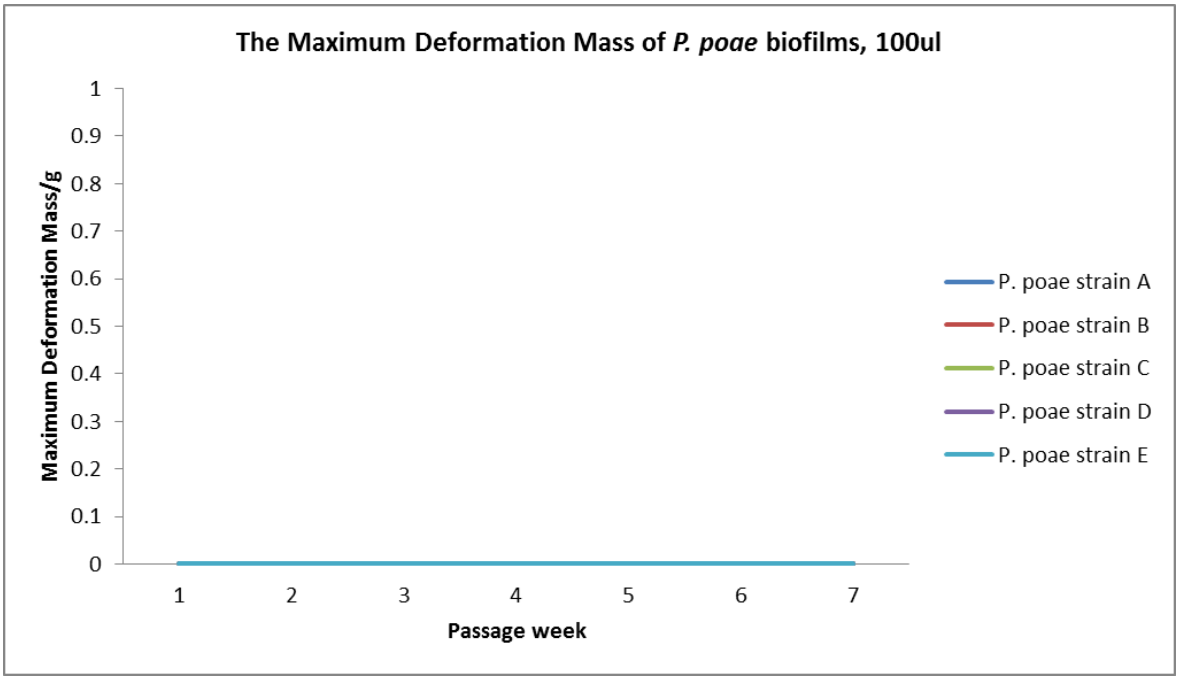
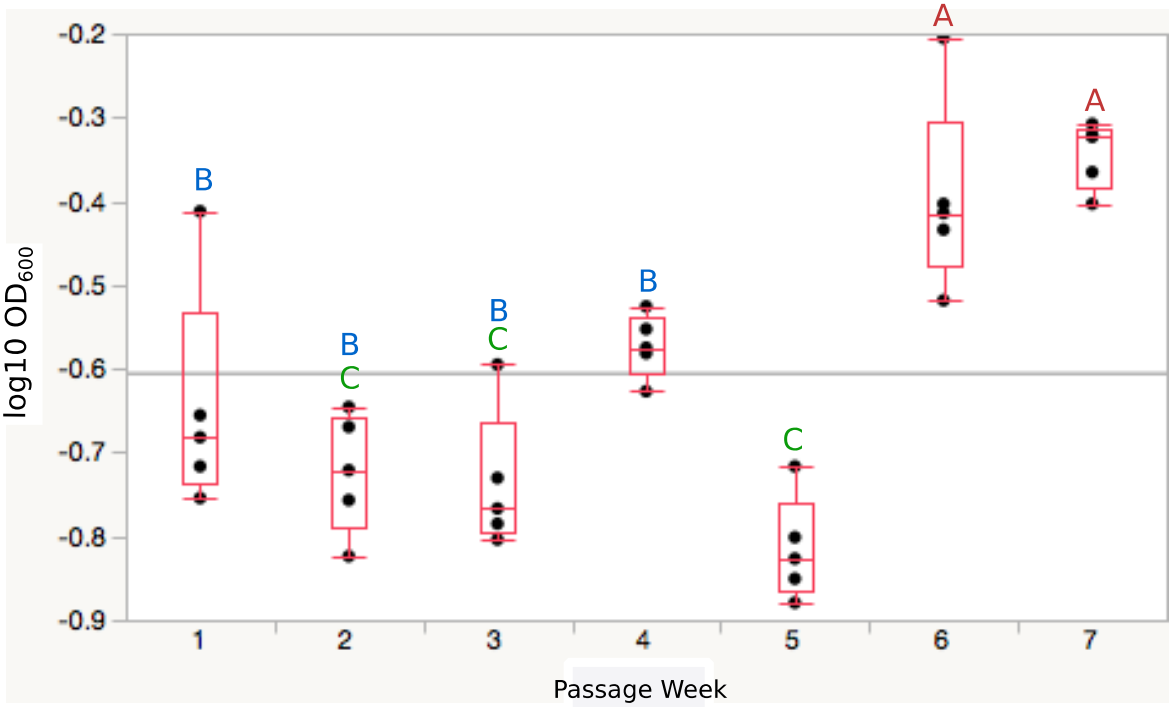


Figure 2. A) The Maximum Deformation Mass of *P. poae* biofilms, 10ul and b)100ul.

A)



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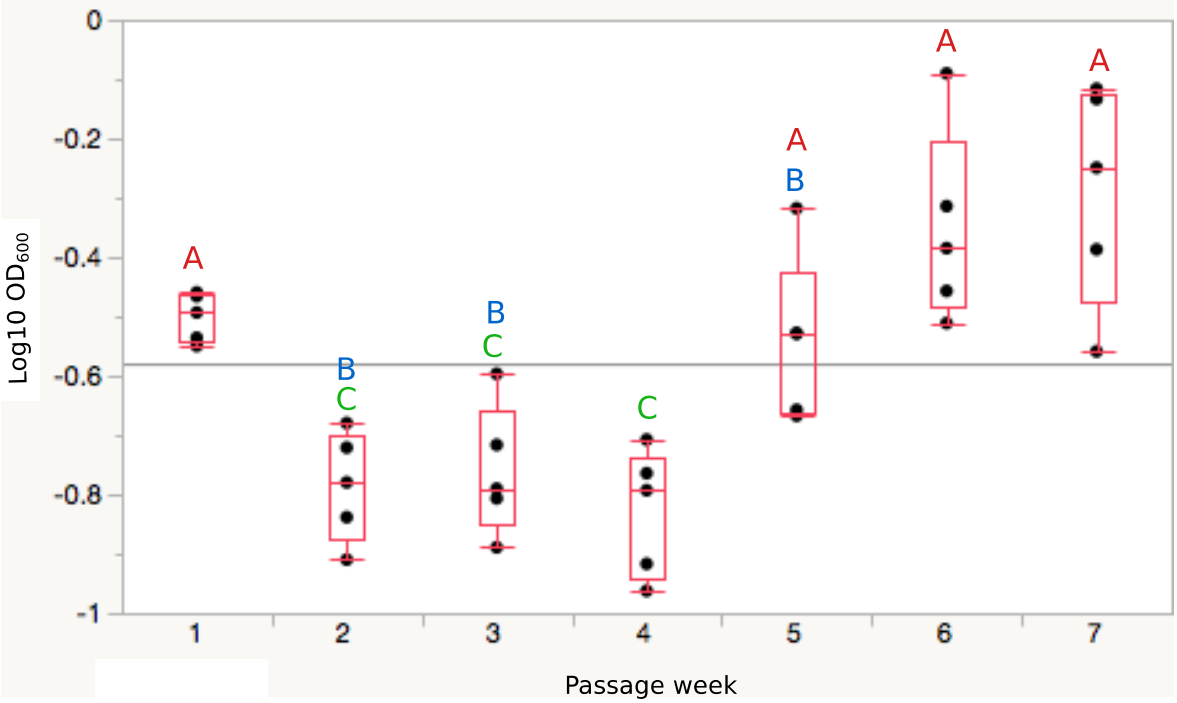

Figure 3. A) log10 OD_{600} over time for 10ul *P. poae* passages and B) log100 OD_{600} over time for 100ul *P. poae* passages. Letters indicate statistically significant groups.

Discussion

The results for the biofilm passages so far indicate that, although biofilms form at the air-liquid interface, biofilm strength does not increase consistently over time. It appears to be a fluctuating property which is possibly due to the stochastic nature of the mutations responsible for biofilm formation. However, there does appear to be a positive trend in biofilm attachment strength, with the data for weeks six and seven indicating they are significantly different to previous weeks.

It is possible that over the week long period the biofilms are left to grow, they become too thick and dense and sink to the bottom of the microcosm as their attachment strength is not enough to support themselves. Another explanation for the weak biofilm strength may be there are cheats arising in the biofilm. Biofilms are populations of cells that perform many role, such as acquiring nutrients and protection from the environment, working as a community to survival. However, this allows cheats to arise in the population as they may take advantage of the products produced by other cells in the biofilm without the cost of producing them themselves (Popat *et al.*, 2012). By benefitting from the products of other cells in the biofilm while avoiding the cost of producing them themselves, such 'cheats' are able to spend their energy in more selfish ways, such as reproduction (Dionisio and Gordo, 2006). This enables them to spread through the system, reducing the thickness and density of the biofilm to the detriment to the population as a whole (Popat *et al*., 2012). An additional explanation that must be consider is that there may be a limit to biofilm adhesion (Dunne, 2002), implying that there will be a maximum attachment strength we will not be able to improve beyond. Investigations are ongoing to understand the processes and potential applications of *P. poae* biofilms.

Conclusions

In conclusion, *P. poae* is capable of forming biofilms, which may bring many promising qualities for use as a potential biocontrol, such as persistence on crop plants therefore reducing the frequency of applications. However, the dataset is not yet complete and research is still ongoing to understand how these biofilms work.

Passages to attempt to improve aphid-killing properties and bacterial survival on plants will be conducted over the coming weeks. At the end of each passage series, it will be investigated whether changes in bacterial genotype has resulted in any trade-offs between the traits we have attempted to enhance and whether bacterial performance is still effective. Once a superior strain is identified, the next stage in the project where we investigate the effects of the bacteria on aphid biological control insects, can commence.

Knowledge and Technology Transfer

- AHDB annual student conference November 2016
- The BSPP conference 2017
- Visit to Walberton nursery for a week long work experience. Included visits to local nurseries and growers, such as Tangmere Airfield Nurseries LTd
- AHDB studentship industry visit 2017

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