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| **Project title:** | Discovery and development of new phylloplane biocontrol agents to control insect pests |
| **Project number:** | CP 82 |
| **Project leader:** | Dr Robert W. Jackson, University of Reading |
| **Report:** | Annual report, October 2012 |
| **Previous report:** | N/A |
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| **Location of project:** | Reading |
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| **Date project commenced:** | 03/10/2011 |
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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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# GROWER SUMMARY

## Headline

Novel aphid-killing biocontrol agents have been discovered that may have use for control of a range of aphids.

## Background

Aphid and thrips pests cause major problems in horticulture, through physical damage of crops, deposition of sticky honeydew and the spread of viruses. With a reduction in available pesticides, predatory wasps and *Bacillus thuringiensis* (Bt) bacteria biocontrol agents represent the few options for control, although Bt resistance can manifest rapidly and broadly within pest populations. There is therefore an urgent need for alternative control measures. The aim of this project is to use bioprospecting to identify novel biocontrol bacteria that can kill aphids and thrips and to characterize their efficacy and mode of action. If possible, it would be useful to understand the population dynamics of the bacteria during plant colonization to determine whether certain plants and/or growing conditions would help to proliferate or maintain the biocontrol bacteria. Ultimately, it is hoped that the novel biocontrol bacteria can be commercially developed.

## Summary

A semi-targeted approach to isolating and identifying candidate biocontrol bacteria was adopted. It was hypothesized that plants that do not suffer from aphid pests, or have an ability to deter them, might not suffer from these pests due to a component of their microbial microflora. For example, bacteria may occupy the plant surfaces or exist as endophytes i.e. living within the plant tissue. Therefore a range of bacteria were isolated (140 colony types) from eleven different plant species and then inoculated them into a novel aphid-feeding assay to identify bacteria that can kill aphids. From 140 strains tested, nine were found to be effective against six different aphid species: peach potato aphid, *Myzus persicae*, black bean aphid, *Aphis fabae*, cabbage aphid, *Brevicoryne brassicae*, lupin aphid, *Macrosiphum albifrons*, carrot lettuce aphid *Nasonovia ribisnigri* and glasshouse potato aphid *Aulacorthum solani*.

DNA sequence analysis was used to identify the nine bacteria isolated. Although some bacteria (eg *Escherichia fergusonii* and *E. albertii*) were undesirable due them being related to opportunistic human pathogens, most of the bacteria were discovered to be related to harmless environmental bacteria. To focus on the most effective bacteria to use in further

experiments, a series of tests were done to discover which strains could be amenable to genetic manipulation and exhibited antibiotic sensitivity – these are key requirements for identification of toxin and virulence factors. The bacteria were also tested for their ability to kill other insects, an important test of host range as we do not wish to work on bacteria that might kill beneficial insects. Finally, bacteria applied to a surface were tested to establish if they would be ingested by an aphid – this test is important in the context of foliar application.

By assessing dose response and timing of killing, the most potent bacteria that killed the aphids were *Pseudomonas poae*, *Pseudomonas fluorescens* and *Citrobacter werkmanii*. All the bacteria were taken up by aphids from surfaces as well as from liquids, indicating they may be useful for foliar application. These bacteria also demonstrated antibiotic resistance, acceptance of plasmids and the ability to be mutated, which means all would be suitable for genetic manipulation to find the mode of action of aphid killing. To facilitate the studies, DNA of each strain has been sent to the University of Exeter sequencing service to have their genomes sequenced *i.e.* to read the entire genetic blueprint of the bacteria. This will help in our mode of action research.

Finally, contact has been made with a research organisation who have kindly agreed to assist us in screening bacteria against thrips. Results from this should be available within the next six months.

## Financial Benefits

Since this project holds more strategic value to gauge the potential for developing novel biocontrol products against aphid and thrips pests, the project remains at an early stage of fundamental science discovery. There is still much work to do in understanding the nature of the aphid-control and thus it is too early at this time to make any recommendations. However, the fact that a range of bacteria that can kill aphids have been discovered suggests it will be possible to develop at least one or more for further characterization and hopefully help in taking them through development and formulation. These would provide growers with a significant financial benefit in reducing losses due to aphids.

## Action Points

None to date.

# SCIENCE SECTION

## Introduction

The global population is predicted to reach 9 billion by the middle of this century, and growth is not set to slow down for at least 40 years (Godfray et al., 2010). Consequently, there is a need to increase the efficiency of food production to realistically feed the future population. In recent years food production has increased (Daily et al., 1998), reducing the proportion of the world's people that are suffering hunger but despite this 800 million people still live without sufficient quantities of food – and this figure is set to increase. Six countries account for half of the annual population increase; India, China, Pakistan, Nigeria, Bangladesh, and Indonesia (Cohen., 2003). The UN predicts that between now and 2050 the major contributors to the population increase will be developing countries – with the developed nations having little or no effect (Carvalho, 2006). There is thus a growing need to improve the productivity and efficiency of agricultural practises in the all countries and particularly developing countries. Available resources must be used efficiently if food production rates are to meet demand.

Whilst effective use of fertilisers and cultivation methods can significantly influence crop growth, as much as a third of global food production is still lost to pathogens (e.g. bacteria, viruses, fungi, chromista), pests (e.g. arthropods, birds, mammals), and weeds (Leake, 2000) . Animal pests cause the second highest amount of damage to crops, being responsible for 18% of all crop losses (Oerke, 2005). The global use of chemical pesticides has been expanding ever since their invention in the 1940s, with a notable, unprecedented increase over the last two decades. However, synthetic pesticides do not offer a sustainable solution to pest management due to noteworthy negative side effects (such as accumulation in the food chain) (Feder, 1979). Many pests that are targeted by pesticides have begun to develop resistance, rendering them ineffective; however the large cost involved in the development and production of new pesticides means that the use of new pesticidal compounds is often not an option for the horticultural industry. The decline in beneficial species such as native Bee species has led to many environmental groups banning the use of newly emerging pesticides such as neo-nicotinoids, along with this there is also increasing pressure from the general public and supermarkets for growers to use less synthetic pesticide.

### *Aphids and thrips*

Aphids and thrips are both very important pests in the horticultural industry. Both cause large scale yield losses which lead to economic losses. Both aphids and thrips cause physical damage to plants, which affects the aesthetic appeal of ornamental crops, and the saleability of fruit from edible crops.

Aphids are an insect pest that causes considerable problems for growers. Their ability to efficiently colonise plants means that they often cause substantial damage to cultivated crops. They are a particular problem for pepper and strawberry growers, where they can cause significant malformation of the fruit. Physical damage is caused when the aphids feed; aphids consume sap via phloem sieve elements of plants. Sap is an almost perfect food source for aphids, with a high sugar content providing an abundant source of carbohydrates and energy; it is also, generally speaking, toxin free (Douglas, 2006). To gain access to the sap, an aphid walks across a leaf until interrupted by a vein, whereupon it inserts its stylus (a syringe-like mouth part) and begins to feed (Pollard, 1973). Not only do they cause physical damage to crops, they are also vectors of several viruses that severely affect many different commercial food crops (such as pepper, tomato and strawberries). Control of aphids has become increasingly difficult due to the restriction of pesticides available for use, and the evolution of resistant aphids to the most commonly approved pesticides has further exacerbated the problem. *Myzus persicae* (one of the most economically important species of aphid) colonies have been shown to have up to 70% resistance to the pesticide Primicarb, a commonly used pesticide used against this species of aphid. The mechanism that accounts for this resistance involves the production of esterases by the aphids, in particular specifically modified acetyl cholinesterase (MACE).

Thrips are an opportunistic insect pest found on many different crops worldwide. The western flower thrips, *Frankliniella occidentalis,* are economically significant for several food crops including strawberries and peppers, but also on ornamental crops. Thrips feed by piercing a hole in the leaf using their mandibles (mouth parts), and the contents of the cells is then accessed (Kindt, Joosten, Peters, & Tjallingii, 2003). Feeding by thrips on flowers causes them to wither and turn brown prematurely–, and can cause russetting of the fruit receptacle (Vale, 2003). It has been noted that a small population of thrips on strawberry and pepper crops may not be detrimental, but in fact beneficial as they pollinate the crop. However their population needs management in order to maintain a threshold below that which will lead to crop damage (Matos & Obrycki, 2004). The damage caused to ornamental crops includes silver mottling or blotching, streaking and distortion of the leaves

and petals, all of which reduce the marketability of floriculture crops (Raupp , and Davidson et al 2012) As with aphids, the development of resistance to pesticides and the reduced number of pesticides available means that alternative methods of control are becoming more important. Problems with traditional pest management strategies have led to alternatives being sought. We propose that it is possible to use naturally occurring bacteria found in the phylloplane of plants to control both aphids and thrips. Moreover, we suggest that it may be possible to manipulate the phylloplane in order to encourage the predominant growth of these bacteria, providing a natural shield for plants against insect pests.

## Aims and objectives

The aim of this project is to identify naturally occurring phylloplane bacteria and their components that control the pests of pepper and strawberry plants. While there has been success in the search for bacterial controls of strawberry and aphid pests, such as the discovery and use of *Bacillus thuringiensis toxins*, there has been little advancement in the identification of a bacterium (or its product) that can be used as a specific control agent against aphid or thrips pests that cause problems for the strawberry and pepper industry. It is hypothesised that one reason why some plants can resist infestation by aphids and thrips is because they possess a bacterial population that protect the plant from pest colonisation. Plant matter itself, as well as soil and water from surrounding areas, will be analysed for any bacteria that potentially possess pesticidal qualities. The introduction of a biological pesticide with action against aphids and thrips could reduce the reliance upon for conventional chemical pesticides and help to reduce the problems associated with the residues that can be left behind by synthetic pesticides. The identification of such a bacterium or product will help to reduce crop damage by pests and thus increase crop yield. At a time where global food supply is under threat, this research could contribute to improved food security for the future.

## The objectives of this project are to:

**1.** Isolate bacteria from the environment including soil, root, water, leaves and invertebrates, using traditional isolation techniques to obtain bacteria from their sources and isolate single colonies. Store the bacterial isolates for further analysis.

**2.** Identify bacterial isolates that have the potential to cause pathogenicity in insects using toxicity tests.

**3.** Characterise the bacterial isolates using biological, chemical and histological tests including the analysis of antimicrobial and antifungal action.

**4.** Determine the interaction dynamics with aphids (toxicity and pathogenicity), and with other host organisms (to determine host specificity).

**5.** Determine whether toxic bacteria can be transmitted from plant to plant by aphids.

**6.** Apply a genetic screening technique to identify the cause of toxicity. Create random mutations to try and produce reduced toxicity. The mutants that express the reduced toxicity phenotype can then be sent for genetic sequencing in order to identify the genes affected by the mutations.

**7.** Determine the efficacy of bacterial suspension or product in controlling aphid infestations within a crop scenario.

8. Throughout the project relay findings and advancements in the research to the Horticultural Development Company (HDC) and growers.

## Materials and Methods

### *Sample collection*

Bacteria were isolated from 10 different plant species over 7 different locations. Further bacteria were isolated from lake water and from the interior and exterior of a ground beetle, *Broscus cephalotes* both from the same location. All samples were collected using sterile equipment including: scissors, tweezers and spoons, and stored in sealed sterile plastic bags at 4oC until needed. 0.5g of each sample was added to sterile matrix tubes containing a ceramic bead along with 500µl of PBS. These were then homogenised using a tissueruptor set to 40mph for 20s.

### *Isolation of bacteria*

A dilution series was then created using a phosphate buffered saline (PBS) as the diluent. 100µl of each dilution was added on to one plate of Luria agar and one plate of Kings B agar (media routinely used to culture bacteria) and spread to create an even distribution of growth. Each plate was left to grow at room temperature (roughly 20$^{o}$C) for 3 days. When it was possible to select an individual colony (i.e. a colony that was not touching another) a sterile wire loop was used to pick the colony off of the plate, and a 3 point streak method was then used to ensure that the colony was pure. Some of the samples contain high numbers of bacteria, and despite diluting the samples it is not possible to isolate a single colony from a plate. In order to be able to do so a further step must be taken. Using a sterile wire loop drawn across the diameter of the plate, a number of different bacteria were picked up. Samples were then streaked to single colonies (using the 3 point streak method), and subsequently purified.

### *Storage of isolated bacteria*

Once single colonies were isolated from the mixed plates, it was possible to preserve them indefinitely for further use at -80oC. Individual colonies were picked from the plate using a sterile wire loop, and placed into 10ml of sterile Luria broth. These cultures were then incubated, with shaking at 27°C for 12 hours. 900µl of this culture was then added to a sterile Cryotube along with 600µl of 40% glycerol. This was then sealed and placed in to a -80°C freezer until needed.

### *Aphid toxicity assay*

All of the bacterial isolates were screened through 30 *M. persicae* to test for pathogenicity, using a method pioneered by Professor van Emden. Artificial diet was inoculated with 109 bacterial cells per ml of artificial feed within a feeding chamber (Figure 1). Aphids were applied to the diet. Over a period of 72 hours the aphids applied to the bacteria+diet treatment and those applied to a diet control were monitored for signs of illness (turning brown) or death (turning dark brown and dropping from the food source). Once the initial screen of all isolated bacteria was complete, the strains found to be pathogenic were also tested on other aphid species using the same feeding system (see appendix A3). Tests were also performed to ascertain whether the effect of the bacteria was dose dependent, and whether the infection was cell mediated or caused by a secreted toxin.

**Fig.1.** A top view of the feeding chamber used to screen the bacteria through the aphids. Parafilm (a stretchy plastic-similar to cling film) is stretched over a plastic cylinder to form a film over the top. The artificial diet is put on to this film. Another layer of Parafilm is then stretched on top to create a sachet. Aphids are then put inside the cylinder. The aphids move up to the food source and feed as they would from a leaf.

### *Identification*

The bacterial strains that were shown to have a pathogenic effect on *M. persicae* were identified. The 16S ribosomal DNA gene sequence was analysed by polymerase chain reaction (PCR) to identify the bacterial strains; DNA was sent off to Source Bioscience UK for sequencing. The sequencing result obtained was compared to an online database (Genbank using BLAST analysis), to find the most similar match, indicating which bacteria have been isolated (see table 2.1 in the appendix for full protocol).

### *Interaction dynamics*

Host specificity tests were performed on all the aphid-pathogens by testing with other insect species. *Oryzaephilus surinamensis* (Grain beetle), *Sitophilus oryzae* (Rice weevil) and *Cryptolestes capillulus* (Grain weevil) were all tested to see if they were affected by the bacteria. 1g of diet was inoculated with 109 bacterial cells, they were then observed every 24 hours for signs of illness and death. After 2 weeks of feeding there was no death or illness observed amongst the insects.

*Galleria mellonella* were also infected with the bacteria, however as the larvae do not feed, the bacteria were injected directly into *G. mellonella* gut and signs of infection and death were observed over 72 hours.

### *Genetic modification trials*

Broth cultures of the pathogenic bacteria (recipients) which are all resistant to the antibiotic nitroforantoin were grown in broth overnight. The donor strains *E.coli* S17-1λpir possessing the plasmid pLAFR3 and *E.coli* DH5αλpir containing the transposon IS-Ω-Km/hah (both resistant to kanamycin) were also cultured overnight in broth. 900µl of each recipient strain was then mixed with 600µl of a donor. This mixture was then spun down at 500rpm for 5 minutes. The majority of the supernatant was aspirated and discarded, leaving between 20 µl and 50µl of supernatant and cell pellet at the bottom of the eppendorf. The cells were then suspended in the remaining supernatant. This ‘sloppy pellet’ was then collected and placed in the centre of an agar plate. This was then incubated overnight at 27°C. The following day the pellet was collected from the plate and spread out on to another agar plate containing the appropriate antibiotics (in this case kanamycin, for the plasmid, and nitrofurantoin for the recipient bacterium.) This prevents the growth of any of the donor cells or any of the pathogenic bacteria that have not taken up the plasmid, thus the only cells that are able to grow are the pathogenic bacteria that have accepted the plasmid or transposon from the donor strain.

## Results

The first task was to identify potential plants that either have no known aphid pests attacking them, or are known to deter aphids. A list of plants was previously obtained from Prof. van Emden and is shown in Table A1.1 in Appendix 1. Tissue from 10 different plants found at 7 different locations on the University of Reading Whiteknights campus was taken and bacteria were re-isolated onto LB agar and plates were incubated at 27$^{o}$C for 48 h. Bacteria were purified to single colonies and 140 bacteria were isolated and used in the aphid in vitro screening test to search for toxic effects. Of these 140 bacteria, nine were found to be pathogenic to *M. persicae (Table 1)*

**Table 1:** Sample origin and name of the bacterium, identified using the 16S rRNA gene (highest similarity match using the BLAST database).

| Plant common name | Latin name | Location | Sample taken | Identification |
|---|---|---|---|---|
| Pepper | *Capsicum annuum* | Private garden | Leaf | *Pseudomonas jessenii (P. jessenii* |
| Tomato | *Solanum lycopersicum* | Private garden | Leaf | *Escherichia fergusonii (E. fergusonii)* |
| Cabbage | *Brassica oleracea* | Experimental green house | Root | *Pseudomonas poae(P. poae)* |
| Hammalias | *Hamamelidae fagale* | Harris gardens | Leaf | *Acinetobacter johnsonii (A. jonsonii)* |
| Viola | *Viola arvensis* | Private garden | Root | *Acinetobacter johnsonii (A. jonsonii)* |
| Viola | *Viola arvensis* | Private garden | Root | *Acinetobacter johnsonii (A. jonsonii)* |
| Pepper | *Capsicum annuum* | Cantelo Nursery | Leaf | *Escherichia albertii (E. albertii)* |
| Strawberry | *Fragaria ananassa* | Experimental green house | Leaf | *Citrobacter werkmanii (C. wekmanii)* |
| Water | | Whiteknights lake | | *Acinetobacter johnsonii (A. jonsonii)* |
| | | | | |

### *Aphid toxicity tests*

The initial tests revealed nine bacterial isolates had a pathogenic effect on *M. persicae.* Five other pathogenic bacteria that were identified in a previous study were included in this project. *Pseudomonas poae* and *Pantoea agglomerans* were found to kill 100% of *M. persicae* after 72 hours (Figure 2). Graphs displaying results for the other aphid species tested can be found in the appendix.

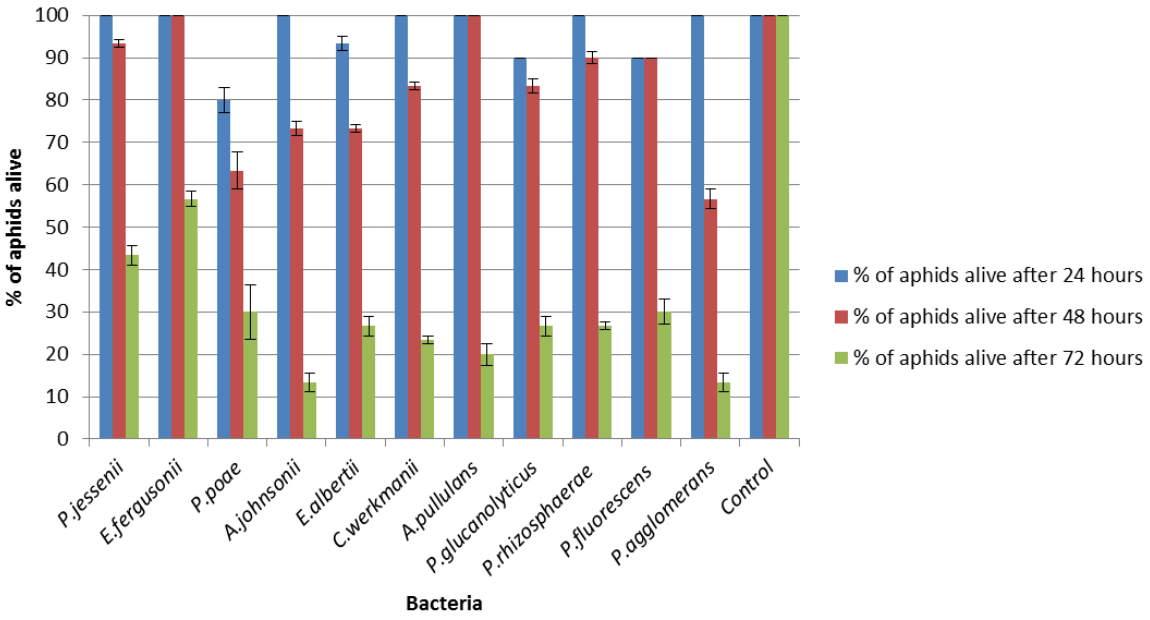


**Figure 2.** Differential killing effects of plant-associated bacteria on *Myzus persicae*. Percentage of aphids alive at 24, 48 and 72 hours after being exposed to pathogenic bacteria. N= average of 3 replicates, each replicate consisting of 10 aphids. The error bars represent the standard error of the mean.

To determine whether molecules secreted from the bacteria, rather than the presence of bacterial cells, were causing the toxic effect, the artificial diet was inoculated with a bacterial growth medium where the bacterial cells had been removed. Results showed that aside from one putative *Acinetobacter johnsonii* strain, killing was significantly reduced when the bacterial cells were removed (Figure 3).

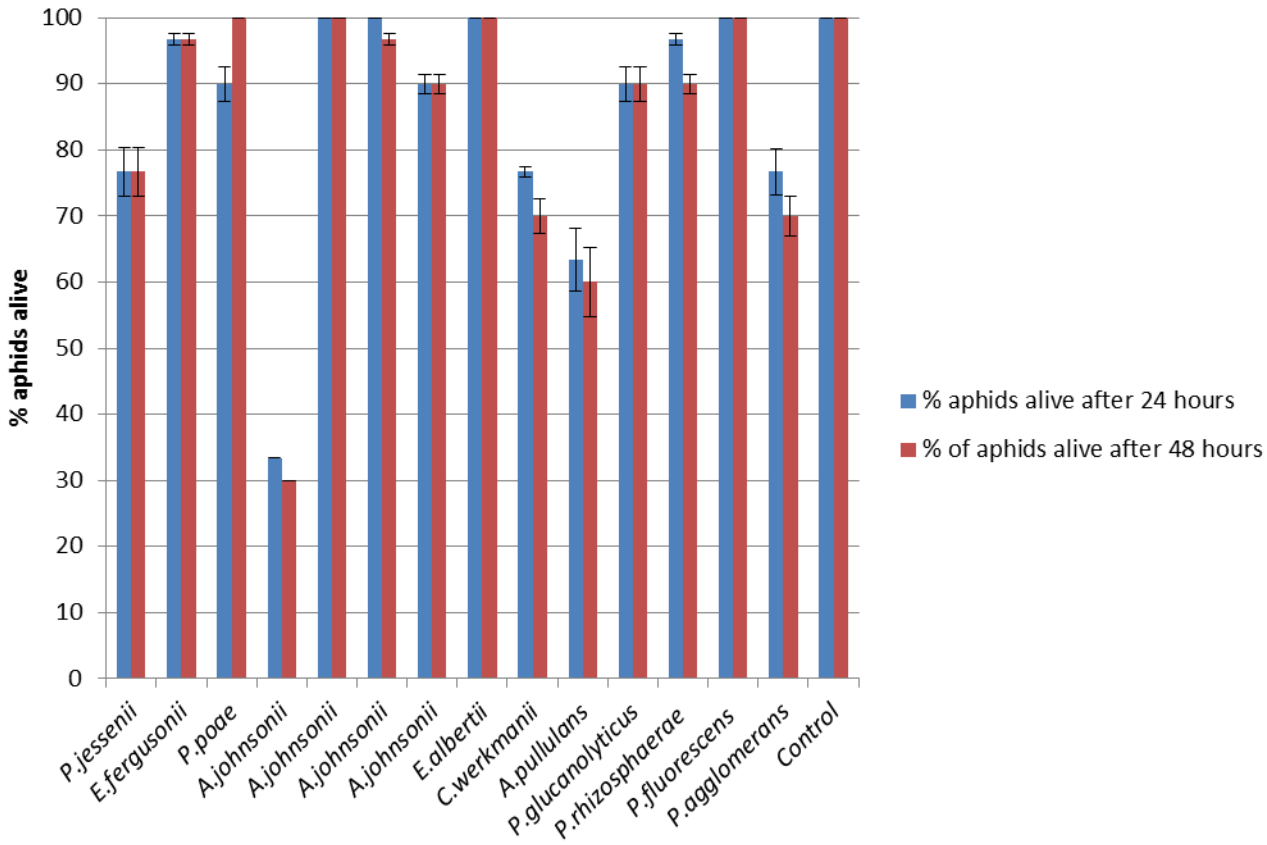


**Figure 3**. Analysis of bacterial broths to determine if putative toxins are secreted. Virulence assay showing the percentage of aphids alive 24 (blue bars) and 48 hours (red bars) after ingestion of artificial diet inoculated with bacterial supernatant. N= average of 3 replicates, each replicate consisting of 10 aphids. Error bars represent the standard error of the mean.

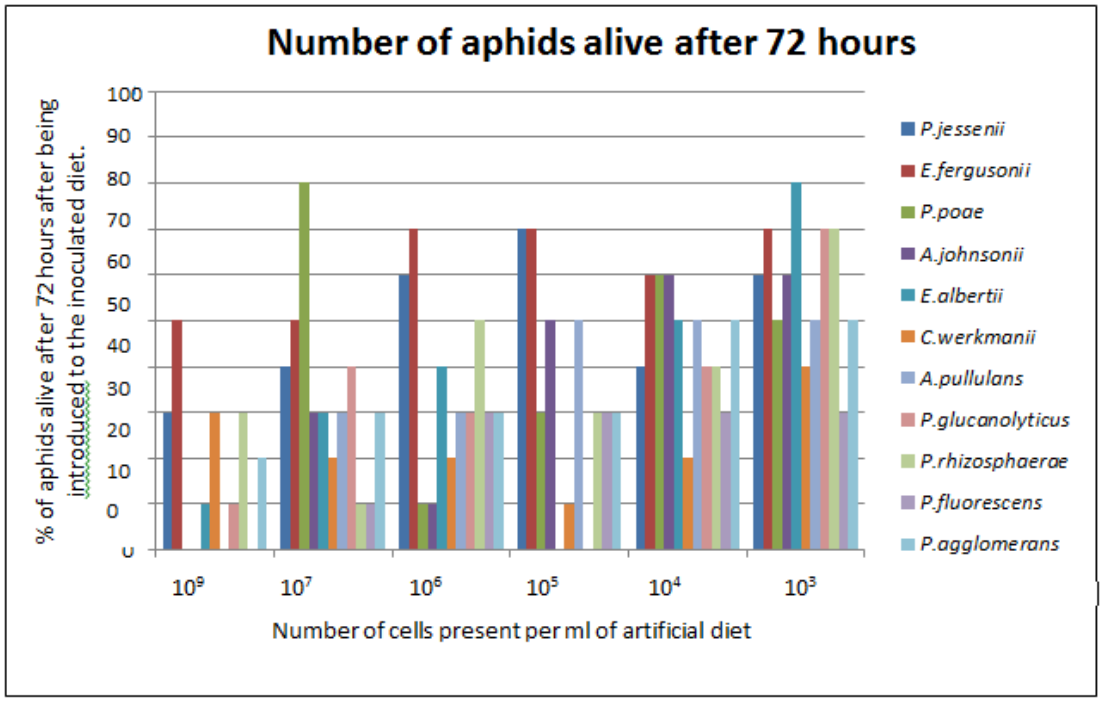


**Figure 4. Efficacy of aphid killing by varying bacterial concentration.** Dose effect of inoculum on aphid killing efficiency. Number of bacterial cells per ml is varied from $10^9$ cells per ml of diet to $10^4$ cells per ml of diet. N= average of 3 replicates, each replicate consisting of 10 aphids. Error bars represent standard error of the mean.

To determine whether there was a dose response effect, the bacteria were inoculated into aphid feed at 10-fold dilutions: the results (Fig. 4) showed a dose response effect, *i.e*. the less bacterial cells per ml in the feed, the slower and less effective the bacteria are at killing.

In some initial tests we have looked at whether the aphids are able to ingest bacterial cells from the surface of the parafilm. This is analagous to bacteria being present on a leaf surface. $5x10^8$ bacterial cells in suspension were applied to the surface of the parafilm and the liquid was allowed to evaporate. The feed sachet was then made as previosly described, without inoculating any of the diet. Aphids were placed in the tube and observations for illness and death made at 24, 48 and 72 hours (figure 5).

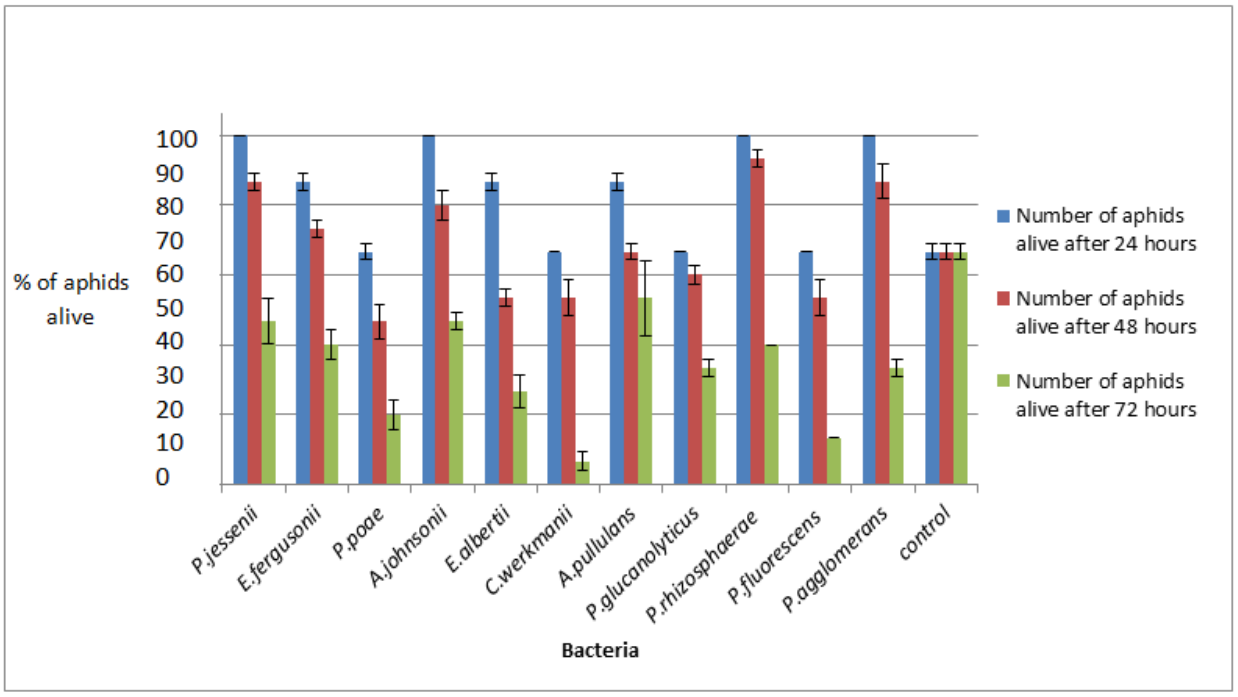


**Figure 5.** Efficacy of aphid killing by bacterial uptake from the surface of parafilm. Effect of inoculum being applied to surface of parafilm on aphid killing efficiency. Number of bacterial cells on surface of parafilm is $5x10^8$. N= average of 3 replicates, each replicate consisting of 10 aphids. Error bars represent standard error of the mean.

An important consideration for use of these bacteria in controlling insect pests is to ensure they do not harm beneficial insects. To examine host range, the bacteria were tested against a range of available insects at Reading. The results show that, with the exception of some weak effects against *G.mellonella*, there was no pathogenic effect on the other species that were exposed to the bacteria. The *G.mellonella* showed some signs of infection, and signs that their immune system was activated (i.e. melanisation), but they did not die, and thus were able to overcome the infection.

### *Genetic modification*

For further experimental analysis, it will be necessary to genetically modify the host bacteria to identify toxins and virulence factors. Thus, an important aim was to determine which bacteria could be genetically modified. Tests were performed to see if the pathogenic bacteria isolated were able to be genetically modified using a transposon or a plasmid..The bacteria were tested to determine if they are receptive to receiving plasmids from other bacteria via direct conjugation. All of the isolated bacteria appear to have accepted the plasmids pLAFR3 and pBBR1MCS-2 from a donor strain apart from *P. rhizosphaerae*. All isolated pathogenic bacteria were able to accept the transposon IS-Ω-Km/hah apart from *A. johnsonii.*

## Discussion

In this study 140 bacteria were isolated from the phylloplane and rhizosphere of a range of plants; nine of these bacteria were observed to be pathogenic to aphids. This raised the total of aphid killing bacteria to 14 when including five other bacteria from a previous study (Figure 1). The pathogenic bacteria after initially being tested using *M. persicae*, were tested to see if they had an effect on other aphid species. Lettuce aphid (*Nasonovia ribisnigri*), glasshouse potato aphid (*Aulacorthum solani*), cabbage aphid (*Brevicoryne brassicae)*, lupin aphid (*Macrosiphum albifrons*) and black bean aphid (*Aphis fabae*) were all exposed to the 14 bacterial strains. All 14 pathogens were able to cause some degree of death within a 3 day period and in all of the aphid species (see appendix for full reults).

When the artificial feed was innoculated with the media supplemented with cell-free supernatant from overnight cultures, there was much less death than when the feed was inoculated with bacteria cells. This could be due to the bacterial cells needing to be present in the aphids gut to cause death. We also looked to see if the pathogenic effect was dose dependant. Using cell concentrations ranging between $10^9$ and $10^3$ cells per ml of artificial diet we are able to discern the most effective concentration. The results show that the cell concentration $10^9$ was the most effective for all of the bacterial strains. Notably *P. poae* was still 50% effective at $10^5$ cells. These results will allow us to determine the numbers of cells that need to be present *in planta* for the bacteria to be effective against aphids. We have also shown that the aphids are able to aquire enough bacterial cells from the surface of the parafilm to cause death, thus enough bacterial cells are ingested when the surface of the parafilm is punctured by the aphids stylets.

The 14 bacteria found to have pathogenic qualities were identified using 16S rRNA sequence analysis. The bacteria were also characterised using biochemical and histochemical tests such as antibiotic resistance testing, testing for anti-fungal activity, and

Gram-staining The 3 most promising strains were sent for full genome sequencing, which will aid molecular analyses of the bacteria. We are expecting the results from this shortly.

The 14 bacteria were also exposed to other insect species in order to determine if the pathogenic effect was confined to aphids, or if there was a chance other insects that may come in to contact with the bacteria would be affected. Host specificity tests were performed on all of the aphid-pathogens by testing with insect species: *Oryzaephilus surinamensis*, *Sitophilus oryzae* , *Galleria mellonella* and *Cryptolestes capillulus*. The results from these tests are promising as no death was observed when the insects were exposed to the bacteria. This suggests that the bacteria may only be capapble of causing death to aphid species. These interaction dynamics need to be investigated further using insects often used for biological pest control, as it is important that the bacteria do not negatively impact IPM (integrated pest management) strategies already in place in glasshouses and in outdoor crops.

### *Future Work*

We have begun to investigate how the isolated bacteria interacts with the aphids and potential host plants. We plan to inoculate pepper and strawberry plants with a known number of bacterial cells per $cm^2$ of leaf surface, and over time recover the bacteria, to see whether their populations have increased, decreased or remained the same. We also plan to investigate whether aphids are able to transmit the bacteria from one plant to another, either by secreting honey dew that has the bacteria in it, or by the bacteria adhering to the aphids body. We also want to observe the effect of the bacteria on aphids if they are exposed for a set amount of time, before being removed from inoculated feed and placed on to non inoculated feed. This will allow us to determine how long aphids need to be exposed to the bacteria for death to be induced. While these investigations are ongoing we will also begin work on the genetic screening technique to identify the genetic causes of toxicity.

All agreed year 1 project milestones have been achieved using aphids, apart from fully investigating whether the aphids are able to transmit the bacteria between plants, but these tests are ongoing. No experiments have yet been conducted using thrips, however an experimental culture on which to carry out pathogenicity testing will be available in early 2013 via Dr Jude Bennison at ADAS.

## Conclusions

- Several bacteria have been isolated that kill aphids.
- Dose response data indicates *Pseudomonas poae* is particularly effective in killing aphid.

- *Citrobacter werkmanii and Pseudomonas fluorescens* strains are viable alternatives for assessment.
- All three of the above bacteria can be ingested by aphids from surfaces as well as from liquid.
- All three bacteria can be genetically modified for further analysis of mode of action.
- Efficacy against thrips is now planned.

## Knowledge and Technology Transfer

No technology transfer has been carried out to date. However, Amanda Livermore has been communicating results from the project to the industrial liasions via regular monthly updates and two meetings in 2012. Results have also been communicated to stakeholders via presentation of posters at HDC conference ($4^{th}$-$5^{th}$ of July Norton park hotel Winchester), SCI conference ($3^{rd}$ July at the University of Reading) and CropWorld global conference ($6^{th}$-$7^{th}$ November at the Queen Elizabeth the $2^{nd}$ conference centre).

## Glossary

PCR-polymerase chain reaction

PBS- phosphate buffered saline

DNA- deoxyribosenucleic acid

µ- 100µ in 1ml

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# Appendices

## A1. Isolation of bacteria with pathogenic potential

| | Table 1. | | |
|---|---|---|---|
| **Plant** | **Latin name** | **Location** | **Sample taken** |
| Maize | *Zea mays* | Experimental gardens | Leaf, root, soil. |
| Cabbage | *Brassica oleracea* | Experimental gardens | Leaf, root soil |
| Chilli pepper | *Capsicum annuum* | Experimental gardens | Leaf, root soil |
| Pepper | *Capsicum annuum* | Private garden | Leaf, root, soil |
| Tomato | *Solanum lycopersicum* | Private garden | Leaf, root, soil |
| Hammalias | Hamamelidae, Urticales | Harris gardens | Leaf, soil. |
| Magnolia | *Magnolia virginiana* | Harris gardens | Leaf, root, soil |
| Viola | *Viola arvensis* | Private garden | Leaf, root, soil |
| Invertebrate | *Broscus cephalotes* | Whiteknights lake | Whole organism |
| Strawberries | Fragaria ananassa | Experimental glass houses | Leaf, root, soil |
| Peppers (Cantelo) | *Capsicum annuum* | Cantelo nursery | Leaf, root, soil |
| Water | N/A | Whiteknights lake | Vial of water from edge of lake |

**Table A1.1** of plant species and locations bacteria were isolated from

## A2. 16S rRNA polymerase chain reaction (PCR) full protocol

Colony-PCR was performed on the frozen bacterial samples previously collected. The quantities of each component needed for the PCR are shown in table 2.1. All of the constituents were combined in one Eppendorf and then aliquoted into individual Eppendorfs if more than one sample was being processed. The total volume of reaction solution needed per sample is 50µl.

| **Table 2.1. Components and quantities used for a rRNA PCR.** | | |
|---|---|---|
| Component | Quantity (µl) | Concentration |
| Optibuffer (X10) | 5µl | |
| Magnesium chloride (MgCl) | 2.5µl | 50mM |
| DNTPs | 1µl | 100mM |
| Forward primer (GCAAACAGGATTAGATAG) | 1µl | 100pmol/µl |
| Reverse primer (CGTTTGTCCTAATCTATC) | 1µl | 100pmol/µl |
| Sterile water | 38µl | n/a |
| Enzyme (bioxact short) | 0.5µl | 4u/µl |
| Bacterial DNA | 1µl | n/a |
| Total | 50µ | |

Once the mix was aliquoted into the 200µl Eppendorfs it was essential to work quickly with the samples in ice. The DNA sample was provided from a single bacterial colony from a LB plate. A small amount of a bacterial colony was picked up using a sterile pipette tip, the tip was then placed into the Eppendorf. The tip was then moved around while in the PCR reaction mix to ensure that the bacterial cells were dislodged from the pipette tip and were suspended in the PCR reaction, the samples were then placed into the PCR machine and the thermal cycles were as follows:

**1st cycle, perform 1 cycle**
95$^{o}$C for 5 minutes
58$^{o}$C for 1 minute
**2nd cycle, 30 repeats**
95$^{o}$C for 30 seconds
58$^{o}$C for 30 seconds
70$^{o}$C for 1 minute
**3rd cycle, perform 1 cycle**
70$^{o}$C for 10 minutes
4$^{o}$C infinity

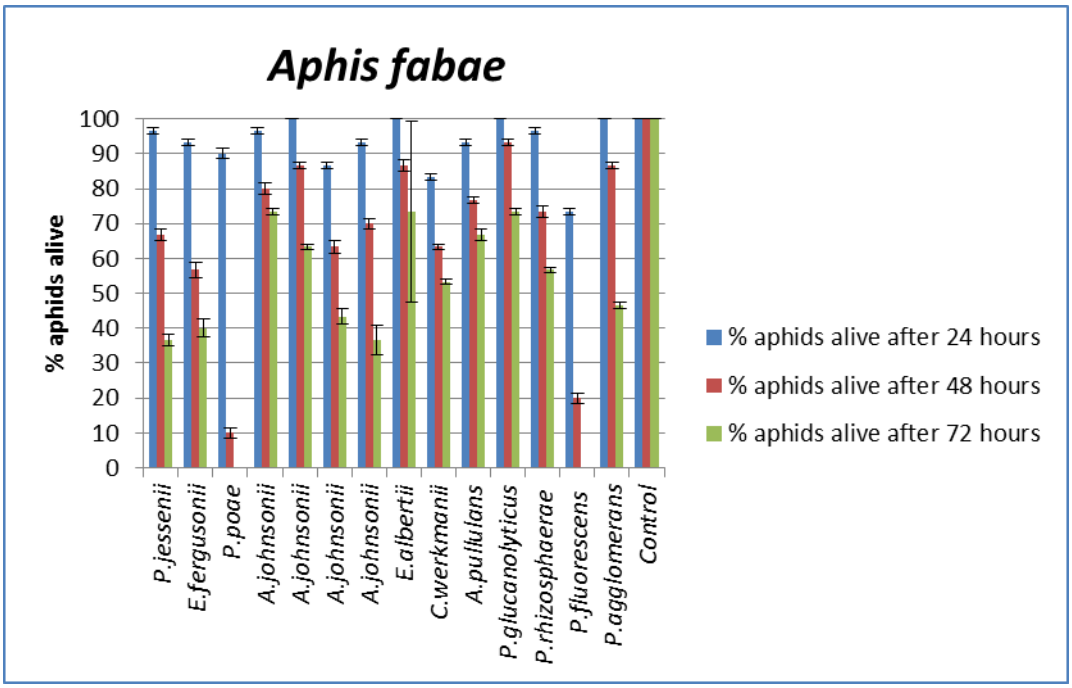
Once the reaction was complete 8µl of the PCR product was mixed with 2µl of 5X loading dye. The sample was loaded into a 1% agarose gel. The DNA was run through the gel in an electrophoresis tank at 120 volts for 40 minutes. The gel was then viewed under a UV wavelength to visualise DNA fragments.

DNA samples were purified using the QIAquick PCR Purification Kit (5). The amount of DNA product yielded from the PCR reaction and purification was measured using a Nanodrop spectrophotometer. If the sample attains a value of less than 10 nanograms of DNA per microliter of elutant it was probable that the sequencing process would not be successful, therefore samples with less than 10 nanograms of DNA per microliter were discarded. Samples were sent for sequencing to Source BioScience UK limited, Oxford. Once the results were received they were compared to an online database of DNA sequences using BLAST. Similarity values are obtained and this allows us to identify the bacteria.

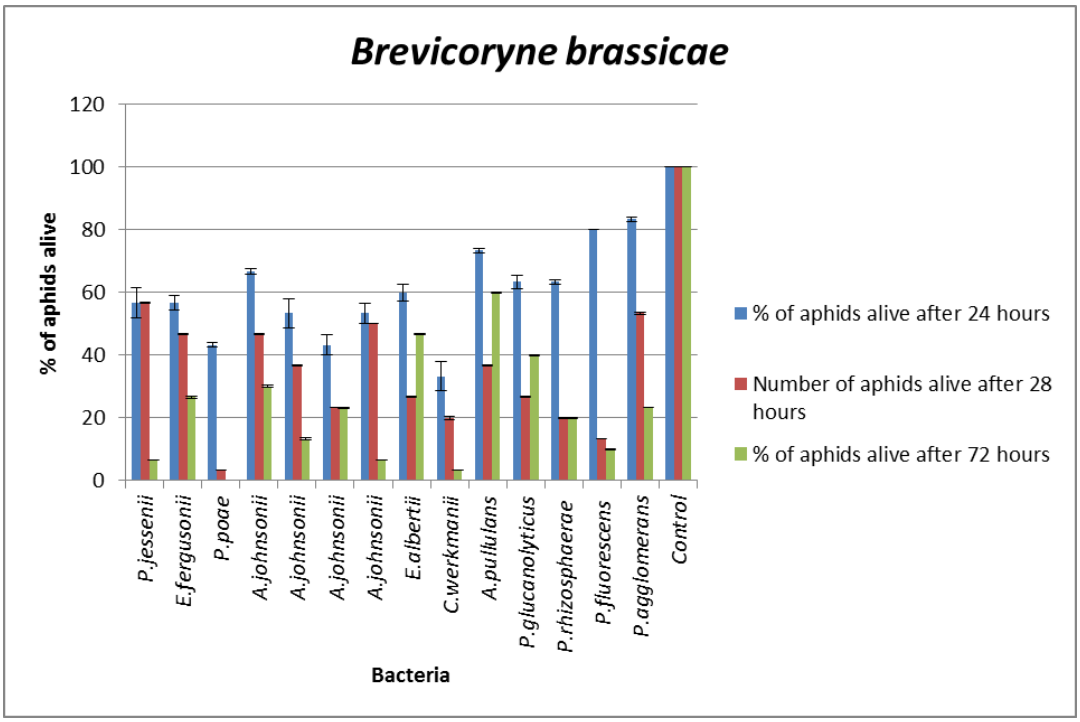
**A3. Graphs of all of the aphid species tested with the pathogenic bacteria.**

The graphs show an average number (3 replicates) of aphids alive after being exposed to the bacteria for 24, 48 and 72 hours (Figures A3.1a-e).

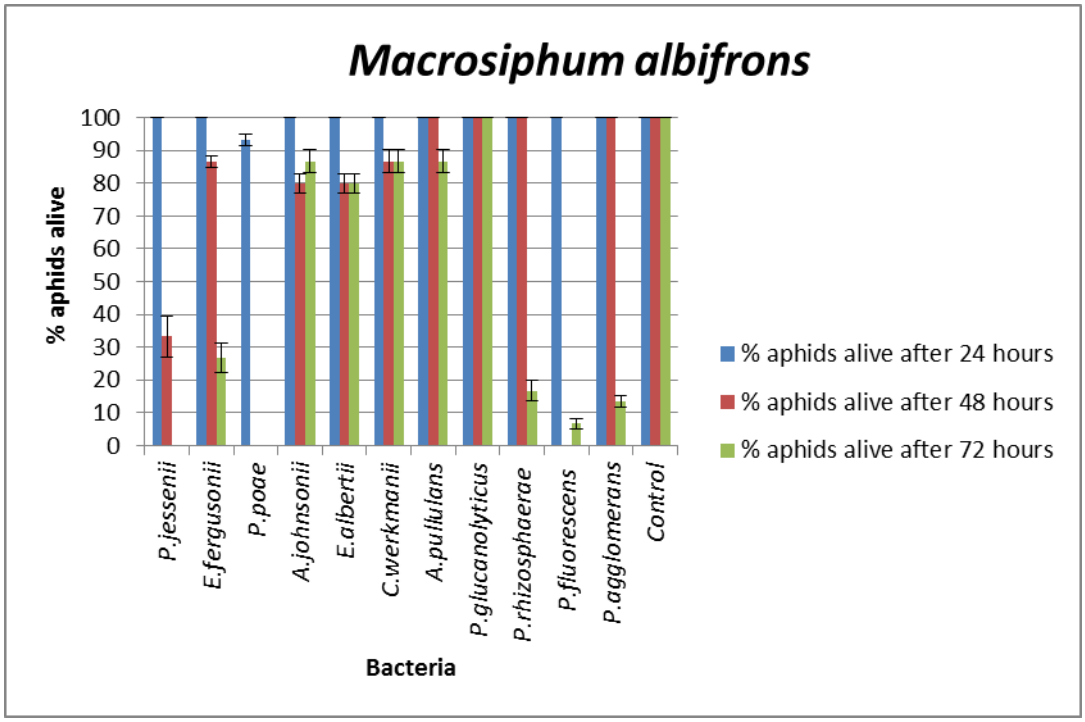
(A)



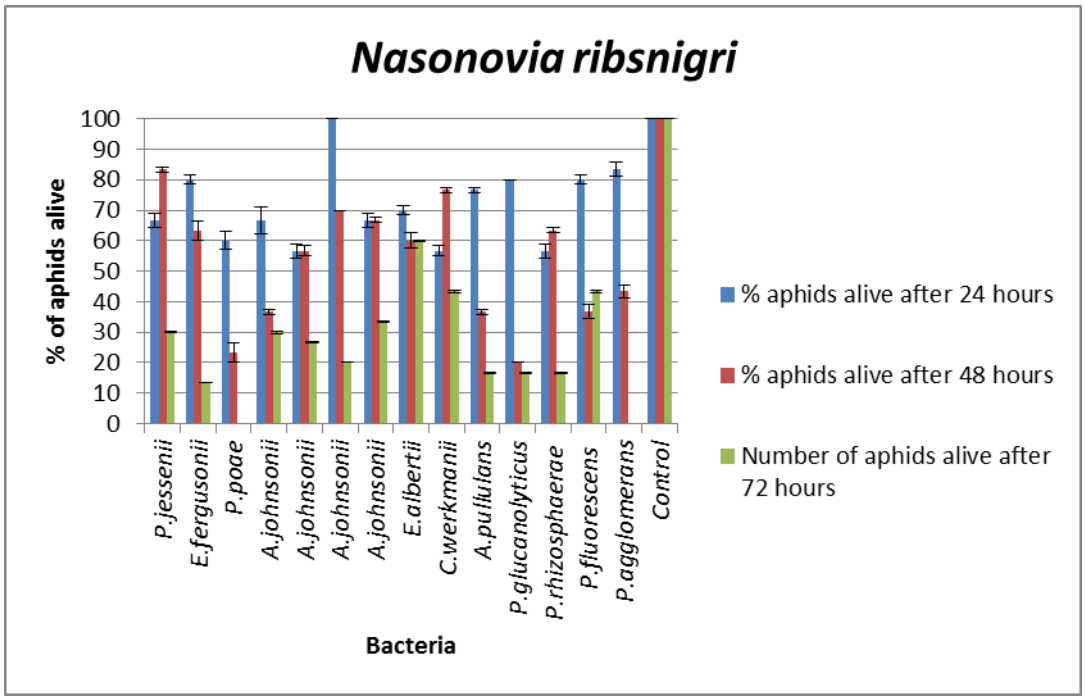

(B)



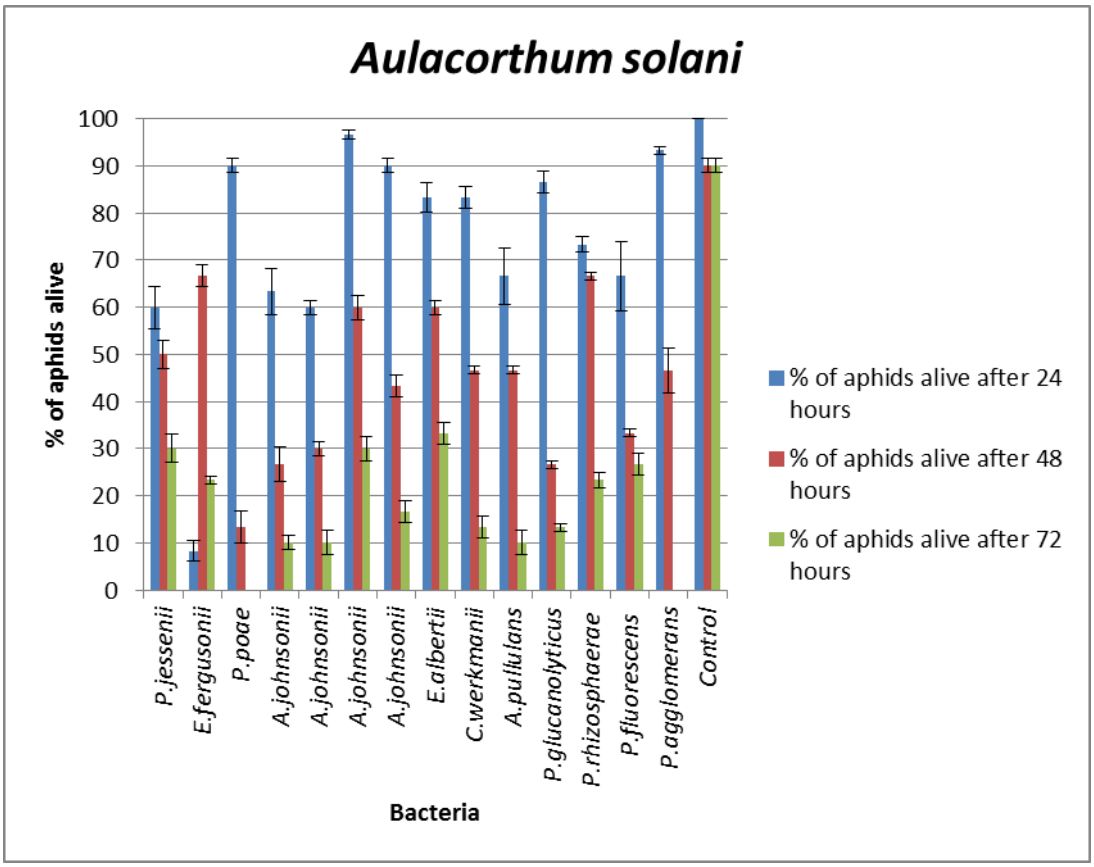
(C)




(D)



(E)




**Figures A3.1 Differential killing effects of plant-associated bacteria on different aphid species.** Aphid feed was supplemented with $10^9$ cells per ml of each bacterium and aphids (A) *Aphis fabae*, (B) *Brevicoryne brassicae*, (C) *Macrosiphum albifrons* (D) *Nasonovia ribsnigri*, (E) *Aulacorthum solani* were assessed for death over 72 h. N= average of 3 replicates, each replicate consisting of 10 aphids. Error bars represent standard error of the mean.