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Project leader:	Dr Robert W. Jackson, University of Reading
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Location of project:	Reading
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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

Key message: Novel aphid-killing biocontrol agents have been discovered that may have use for control of a range of aphids and for plant-growth promotion.

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 are becoming increasingly important for control, although Bt resistance manifests 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. We aim 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. We are also aiming to identify the underlying cause of aphid killing. Ultimately, it is hoped that the novel biocontrol bacteria can be commercially developed.

Summary

We have adopted a semi-targeted approach to isolating and identifying candidate biocontrol bacteria. We 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 living within the plant tissue. We therefore isolated a range of bacteria (140 colony types) from eleven different plant species and then inoculated them into our 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: *Myzus persicae* (peach-potato aphid), *Aphis fabae* (pea aphid), *Brevicoryne brassicae* (brassica aphid), *Macrosiphum albifrons* (lupin aphid), *Nasonovia ribsnigri* (lettuice aphid) and *Aulacorthum solani* (glasshouse-potato aphid).

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

related to other apparently 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 genetically manipulated 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, we also tested whether bacteria applied to a plant surface 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 are *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 demonstrate 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. This technique is intended to determine the mechanism behind the bacterial virulence, and is not intended to modify the bacterium for further use. To facilitate our studies, we have had the genomes sequenced *i.e.* to read the entire genetic blueprint of the bacteria of these three bacteria. We visited an insect control group in Spain led by Prof. Primitivo Caballero and in collaboration with them we discovered a range of insecticidal toxin genes in each of the genome sequences. The three strains were also tested in feeding assays with Lepidoptera caterpillars, but none of the strains were able to kill the insects, providing some hope that the toxicity is highly specific.

We had intended to carry out testing in thrips last year, but we were unable to do this due to logistical issues with ADAS. However, at the recent AAB meeting at EMR in November 2013, Dr Jean Fitzgerald informed us that she has a thrips colony and will happily collaborate with us – this is particularly important because of our dual interests in strawberry control. We have also contacted Prof. Ameur Cherif in Tunisia who has kindly agreed to collaborate with us such that we can gain access to their bee larvae pathogenicity test. This test allows us to culture our aphid-killing bacteria in the presence of bee larvae to determine if they can kill the larvae – this is a key test for determining safety and host specificity.

We have made a very exciting discovery in a recent experiment. In a previous small scale experiment, we had observed that plants treated with *P. poae* may deter aphids from colonizing. This has been upscaled to 3 plants each treated with water or the bacterial suspension and then placing the plants into an insect tent containing 10 aphids in growth sachet. The plants were monitored over 4 weeks and there appears to have been a preferential colonization, and subsequent replication, of aphids on plants treated with water. This effect lasted for three weeks. This suggests the aphids are deterred from colonizing the

plants treated with the bacterium when given a choice of an untreated control. We clearly need to repeat this experiment again with more plants, but if this proves to be a realistic result, then the bacterium may be useful as a deterrent agent, allowing growers to apply the bacterium at an early stage of plant growth and then reapplying.

In the last few weeks, we have also discovered that there may be a plant growth promotion effect provided by *P. poae*. Cells of this bacterium were inoculated into potting mix (containing peat) and pepper seeds sown into the soil. Over a period of 6 weeks, the plants were monitored for total plant weight, root length, and shoot length. Compared to a water control, the plants exhibited a 20-35% increase in each parameter, suggesting the bacterium may provide some beneficial property to the plant. This might show promise for using the bacterium as a biofertiliser.

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 fairly early stage of fundamental science discovery. There is still much work to do in understanding the nature of the aphid-control, but several bacteria show great promise for development. We have recently been contacted by an SME who are interested in acquiring the IP on *P. poae* and developing it commercially. They have the equipment to allow them to grow the bacterium in bulk and produce different formulations. The University of Reading research and knowledge exchange office are involved for examining all the legal ramifications. With our industry contacts we hope to make connections that would allow us to test the aphid-killing bacteria within an industry setting. If the trials prove successful, then the bacteria could provide growers with a significant financial benefit in reducing losses due to aphids.

Action Points

In the last 8 months of her PhD project Amanda Livermore will carry out the following experiments:

1. Travel to East Malling Research to carry out thrips tests
2. Travel to Tunisia to carry out bee tests
3. Repeat plant growth promotion experiment on a larger scale
4. Repeat aphid deterrent experiment on a larger scale
5. Test the application of *P. poae* on aphids established on leaves for killing effects
6. Knockout the toxin genes in *P. poae* to elucidate their effect on killing

SCIENCE SECTION

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 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 synthetic 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 leading to serious environmental and human health impacts(Feder, 1979). Many pests that are targeted by pesticides have begun to develop resistance, rendering the pesticide useless; 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 bees has led to a temporary ban on some of the newer pesticides such as the neo-nicotinoids. Along with this there is also increasing pressure from the general public and supermarkets for growers to use fewer types of, and 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 from the 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 inserts its stylus (a syringe-like mouth part) into the leaf and begins to feed (Pollard, 1973). Not only do they cause physical damage to crops through feeding, 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. In *Myzus persicae* (one of the most economically important species of aphid) colonies it has been shown that up to 70% of the individuals have developed resistance to the pesticide Pirimicarb a once favoured pesticide. The mechanism that accounts for this resistance involves the production of esterases by the aphids, in particular modified acetyl cholinesterase (MACE). Thrips are an opportunistic insect pest found on many different crops worldwide. The western flower thrips 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, *et al. 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 be 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 global food security for the future. Moreover, the tightening of legislation around pesticides with the review of endocrine disrupting properties, and the issues around Neonicotinoids that are a serious threat to horticulture, highlights the need to find alternative controls that could be of real benefit to the UK growers.

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 DNA 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.

Methods

Sample collection

Bacteria were isolated from 10 different plant species from 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 4^{o}C until needed. 0.5g of each sample was added to sterile matrix tubes containing a ceramic bead along with 500µl of phosphate buffered saline (PBS). These were then homogenised using a tissueruptor set to 4.0 m/s 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 (LA) 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 contained high numbers of bacteria, and despite diluting the samples it was not possible to isolate a single colony from a plate. In order to be able to do so a further step was 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 -80^{o}C. Individual colonies were picked from the plate using a sterile wire loop, and placed in to 10ml of sterile Luria broth (LB). These cultures were then incubated, with shaking, at 27°C for 12 hours. Nine hundred microlitres (900µl) of this culture

was then added to a sterile Cryotube along with 600µl of 40% glycerol. This was then sealed and placed into 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 10^9 bacterial cells per ml of artificial feed within a feeding chamber (Figure 1). Aphids were placed in the feeding chamber and moved onto the parafilm sachet containing the feed. 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 (more information on this is available in last years annual report).

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.

Separation of Bacterial cells and supernatant.

Overnight bacterial cultures were set up by inoculating 10ml of LB with an isolated bacterial colony. This was incubated at 27^{o}C for between 12 and 15 hours, shaking constantly to prevent the development of a biofilm. 1.5ml of the overnight-culture was then aliquoted into a sterile Eppendorf, and spun at 5000rpm for 5 minutes to render a pellet of bacterial cells at the bottom of the tube. The supernatant was then removed for use, being careful not to disturb the cell pellet at the bottom of the Eppendorf.

Identification

The bacterial strains that were shown to have a pathogenic effect *on M. persicae* were identified. The 16S ribosomal DNA gene sequence was amplified by polymerase chain reaction (PCR) and DNA sequencing 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 were 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 *Sitophilus granaries* (Grain weevil) were all tested to see if they were affected by the bacteria. 1g of diet (oats for the grain weevils and red lentils for the rice weevil.) was inoculated with 10^9 bacterial cells, the insects were added directly to the inoculated food and left to feed. 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.

A range of Lepidoptera were tested in Prof. Primitivo Caballero's laboratory in Spain. An insect diet consisting of **(g/L)** Wheat germ 72g; Soya protein 25g; Sugar 29.25g; Yeast 14.25g; Western salt mix 9.4g; Cholesterol 0.94g; Nipagina 0.94; Agar 18.75g; Sorbic acid 1.5g; Benzoic acid 1.5g; Chloric acid 0.94g was made up by combining all ingredients except sorbic acid, benzoic acid and chloric acid in water up to 1L and autoclaved. Once the mixture had cooled to 50^{o}C the remaining ingredients were added. This was mixed well and blended to give a smooth texture. The diet was then distributed between sterile Petri dishes and allowed to cool. The diet was stored at 4^{o}C until needed.

Florella Blue mg/ml; Sucrose 100mg; Florella blue 10mg.

In 1ml of sterile ultra pure water 100mg of sucrose and 10mg florella blue were combined. The ingredients were mixed until dissolved solubilised.

Several species of Lepidoptera as well as other caterpillar species were tested using two different techniques.

Blue dropassay

Larvae were reared in an insectory at 28^{o}C and with 16 hour days. Larvae were used at 2nd instar and they had been starved for 12 hours prior to the assay. Liquid cultures of bacteria were incubated overnight at 28^{o}C shaking. The cultures were normalised to an OD600 of 1. The cells were washed and re-suspended in 90µl phosphate buffered saline (PBS) and 10µl

florella blue. 50µl of the bacterial suspension was dotted evenly in a sterile Petri dish (Roughly 10 5µl drops). 20 Larvae were then added to the petri dish. These were left for 10 minutes to feed on the bacterial suspension. If the larvae had fed on the bacterial suspension their gut turns blue, this colouration is visible on their backs. This indicates that a sufficient amount of the diet was consumed. These larvae were then moved using a paintbrush to a 24 well microtitre plate. Each well of the microtitre plate contained a piece of insect diet 0.5cm^2. The larvae are left to feed on the diet for 4-5 days at 23^{o}C. Observations for sickness and death were made every 24 hours. The larvae were classed as sick if they moved only when stimulated. They were classed as dead if they were shrivelled and dry and did not move when stimulated. Water and *E. coli* OP50 were used as negative controls and *B. thuringiensis* was used as a positive control.

Surface inoculation assay

Larvae were at 1st instar and had not eaten prior to the assay. 20 larvae were added to a sterile Petri dish. A piece of insect diet 0.25cm^2 in size was placed in to the centre of the petri dish. Working rapidly to prevent larvae feeding on the diet before inoculation, 10µl of OD 1 bacterial suspension was added directly to the insect diet. The larvae were then allowed to feed on the inoculated diet for 24 hours. The larvae were moved to individual pieces of non-inoculated diet. They were allowed to feed on the non-inoculated diet for 4-5 days at 23^{o}C. The larvae were observed for sickness and death every 24 hours. The larvae were classed as sick if they moved only when stimulated. They were classed as dead if they were shrivelled and dry and did not move when stimulated. Water and *E. coli* OP50 were used as negative controls and *B. thuringiensis* was used as a positive control.

Bacterial growth in artificial aphid diet

It was observed that over the time course of the aphid toxicity experiment the inoculated diet turned from a clear golden liquid to a cloudy colour, suggesting that the bacteria were replicating in the diet over 72 hours. To determine the rate at which they were growing a growth assay was conducted. A Flat bottomed 96 well plate was filled with artificial aphid diet (180µl of diet in each well). Each well was then inoculated with 5µl of bacterial culture to give a final OD of 0.1. The plate was then put into an automatic OD reader that maintained a temperature of 27^{O}C, and shook the plate at 200rpm for 10 seconds every 10 minutes before each OD reading to prevent a biofilm forming, and OD 600 reading was taken every 30 minutes.

Surface inoculation

Bacterial pre-cultures were made as previously described. The OD600 of the culture was normalised to an OD of 1, and the cells were washed to remove any remaining growth medium. 500µl of this bacterial culture was then added to the inner surface of Parafilm that the aphids would pierce with their stylets to access the artificial diet. The bacterial culture was spread evenly over the Parafilm and allowed to dry for 30 minutes with occasional agitation to distribute the bacteria evenly .

Determining the minimum exposure time needed for optimum aphid mortality

10 Adult Aphids were applied to inoculated sachets and left for varying amounts of time. They were then removed from the inoculated sachets and placed on to a non-inoculated sachet. The aphids were observed over a period of 72 hours after being removed from the diet for signs of death

Targeted mutagenesis of pathogenic bacteria

The genomes of the 3 most promising pathogenic bacteria were sequenced by the sequencing service at the University of Exeter. With the sequences it was possible to interrogate them using a set of known insecticidal toxin genes stored in a custom database (Leopoldo Dovis, personal communication). We identified several genes in all 3 of our bacteria that were similar to genes within the data base. This allows us to create targeted knockout mutants. By silencing the homologous genes we will be able to see if toxicity is reduced. We hope to work with all 3 bacteria, but are currently focusing on *P.poae*

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.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, Kings B agar and minimal media 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 identified in a previous study were included in this project. *Pseudomonas poae* and *Pantoea agglomerans* were found to kill between 70-88% of *M. persicae* after 72 hours (Figure 2). Several other aphid species were tested using the same method. Determining whether the bacteria we found to be pathogenic to *M. persicae* also caused death in other species indicates whether they could be used as a broad or narrow spectrum bio-pesticide. Graphs displaying results for the other aphid species tested can be found in the appendix.

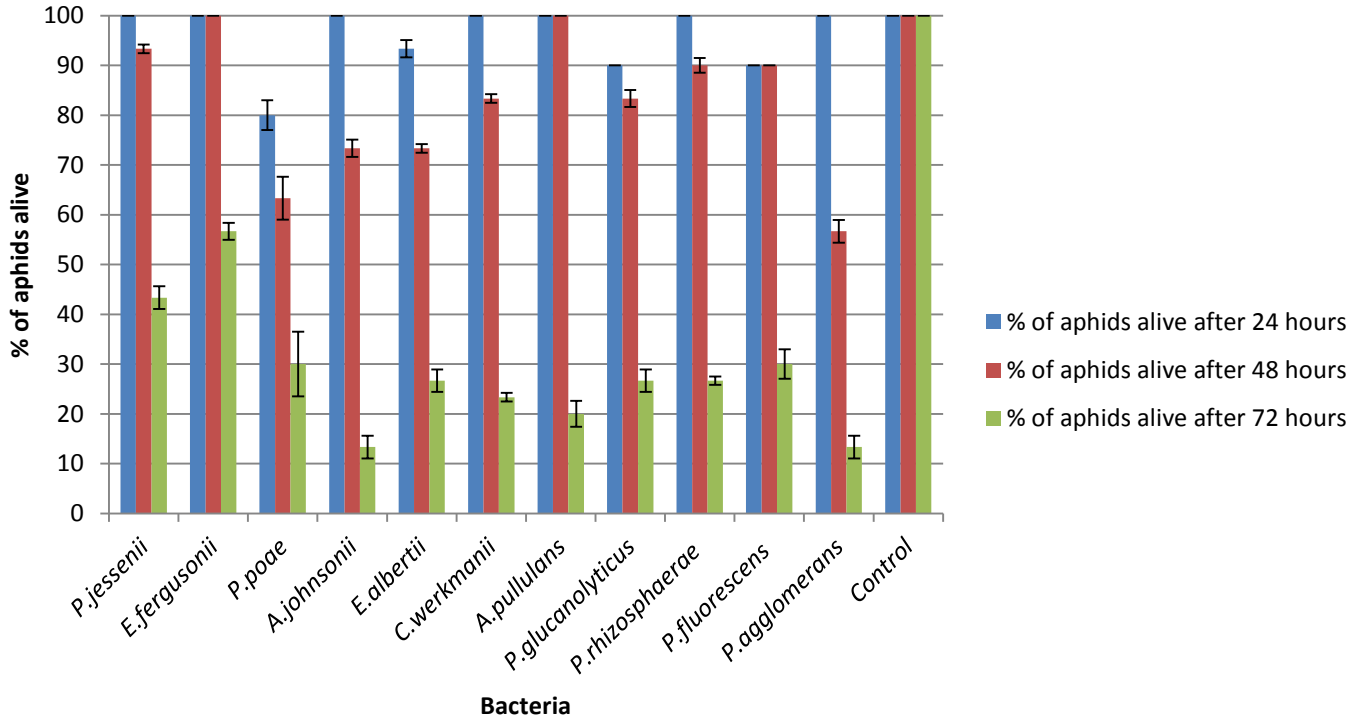


Figure 2. Differential killing effects of plant-associated bacteria on *Myzus persicae*. Percentage of aphids (N = 10) alive at 24, 48 and 72 hours after being exposed to pathogenic bacteria.

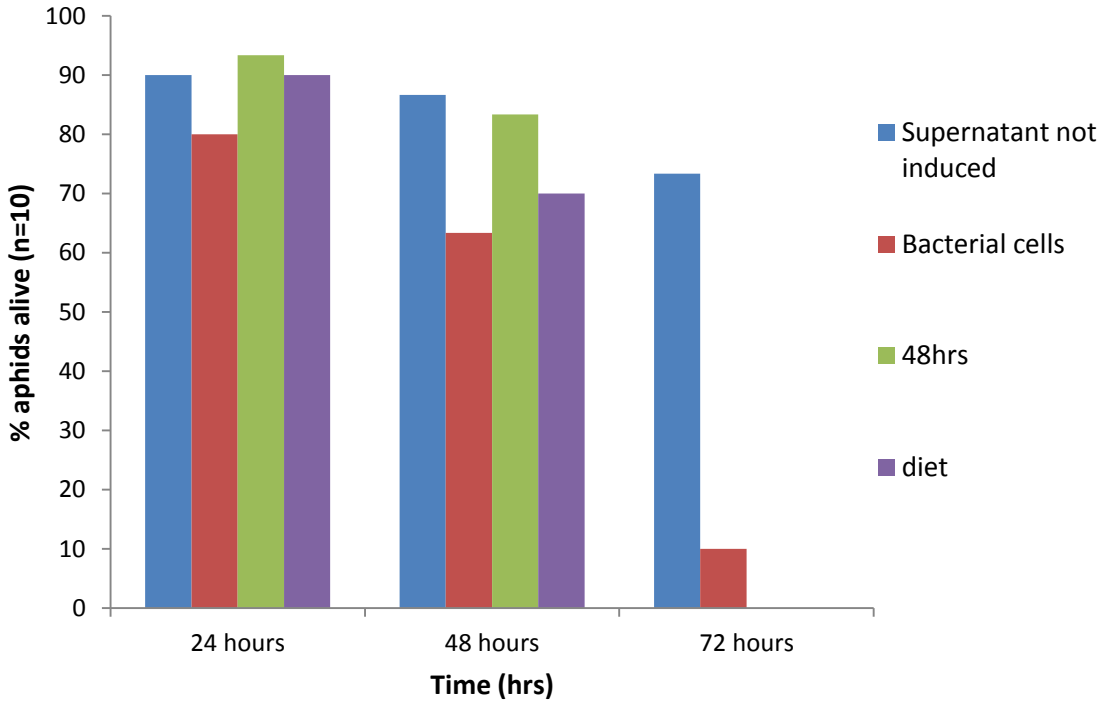


Figure 3. **Analysis of bacterial broths to determine if putative toxins are secreted.** Virulence assay using *Pseudomonas poae* showing the percentage of aphids alive 24 (blue bars) and 48 hours (red bars) after ingestion of artificial diet inoculated with bacterial supernatant. Supernatant not induced indicates where bacteria were grown in a broth shaking at 27^{O}C for 12 hours. The cells were then separated from the growth media, and the growth media was used to inoculate the aphid diet. After 12 hours of growth in broth we assume that the bacteria are not under environmental stress, and therefore secretion of potential toxins not induced.

Bacteria were grown for varying amounts of time and in different media (LB and aphid diet) to deduce whether the toxic effect being observed was due to toxic substances being secreted by the bacterium, or due to the bacteria replicating in the aphid gut (for example, some bacteria can aggregate in the gut and occlude food passage, Stavrinides *et al*., 2009). The bacterial cells were removed from the growth media and used to inoculate the artificial diet. Figure 3 shows that when the bacteria were grown for 16 hours in broth, the cells removed and the remaining supernatant used to inoculate the aphid diet, death rates compared to those seen when the bacterial cells were present were reduced. However, if the bacteria were grown for either a prolonged time in broth, or 16 hours in aphid diet, death rates were restored to a similar level seen when bacterial cells were present (fig. 3). This may suggest that the bacteria are being induced to secrete toxic products which are causing death.

To determine whether there was a dose response effect, all of the aphid killing 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. The graph below depicts the effect of altering the numbers of *P. poae* used to inoculate the aphid diet on death rates in *M. persicae* exposed to the diet. The other bacterial strains were also tested and the results from these can be found in the appendix section.

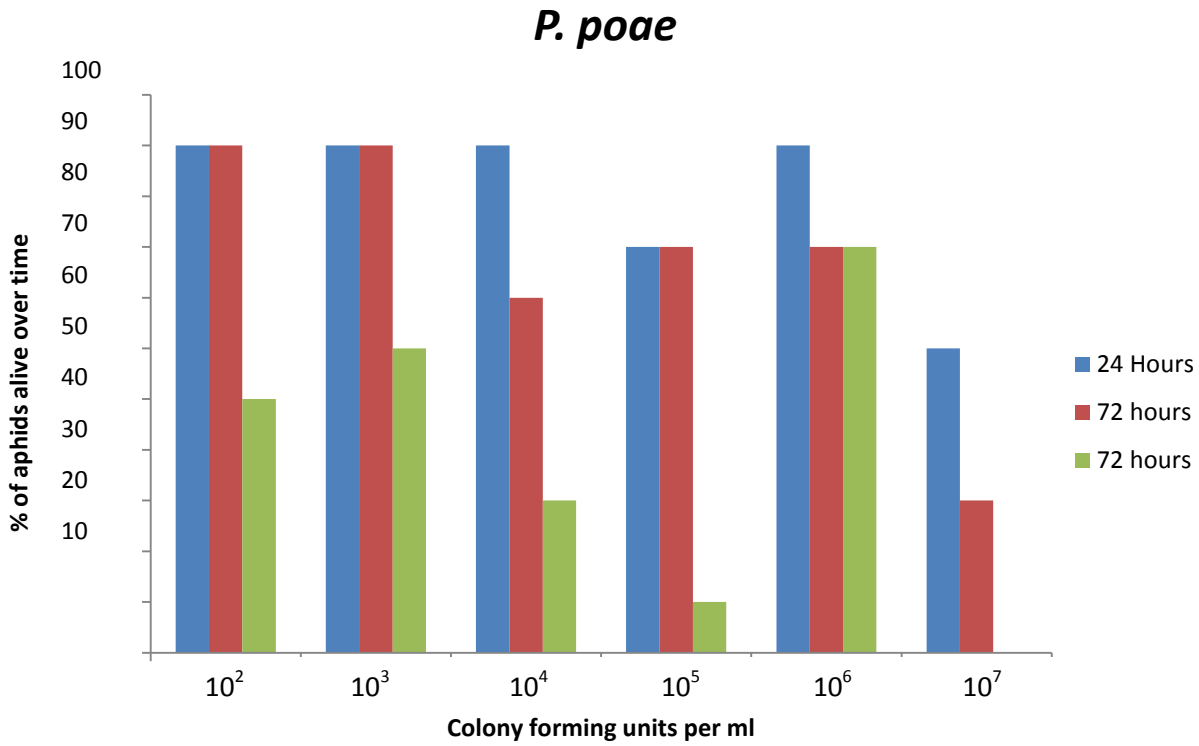


Figure 4. Efficacy of aphid killing by varying bacterial concentration. Dose effect of inoculum on aphid killing efficiency. Number of bacterial cells per ml is varied from 10^7 cells per ml of diet to 10^2 cells per ml of diet.

Host specificity

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 University. The weevil and beetle species tested were exposed to the bacteria by inoculating their food. 1g of the insects diet (either red lenitls or porridge oats) was inoculated with 1ml of bacterial culture (10^9 cfu per ml). 10

insects were then exposed to the inolcuated diet for 1 week and kept at room temperature. Control groups were given food that had been soaked in water

G. mellonella were exposed to the diet via injection. 10µl of bacterial culture (10^9, 10^8 and 10^7 cfu per ml) was injected directly into the the larval gut using a sterile hypodermic needle and 6 larvae per concentraion were injected. Once injected the larvae were kept at room temperature and observed every 24 hours for a total period of 3 days. Controls were injected with sterile water.

The results showed 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 resist the infection.

Lepidoptera species

No effect was seen when the different catterpillar, *Spodoptera exigua, Spodoptera litoralis, Mamestra brassicae, Spodoptera frugiperda and Helicoverpa armigera*, were exposed to the pathogenic bacteria. The blue drop assay and the feeding assay both exposed the larvae to the bacteria via an oral route. Both methods described were used to ensure replication and rigour in the experimental approach, and the caterpillars were observed over 1 week for signs of illness and death. 10 larvae were used per bacterial concentration, the concentraions used were as follows 10^9, 10^8, 10^7 and 10^6 cfu per ml. Controls of *Bacillus thuringensis* were used, and as expected the caterpillars exposed to it died.

Bacterial growth in aphid diet.

During the toxicity trials, the bacteria were suspended in the artificial diet. This is a high sugar nutrient solution, and therefore has the potential to be an environment that supports bacterial growth. If the bacteria are able to readily reproduce and increase their numbers in this solution it may have an effect on the pathogenic potential of the bacteria. The performance of the bacteria were assessed by measuring growth curves of the bacteria in the aphid diet (Fig.5). The bacteria were intitially grown in LB with shaking at 27^OC, they were then normalised to an OD of 1 and 20 µl of this suspension was added to 180µl of aphid diet in a 96 well plate.

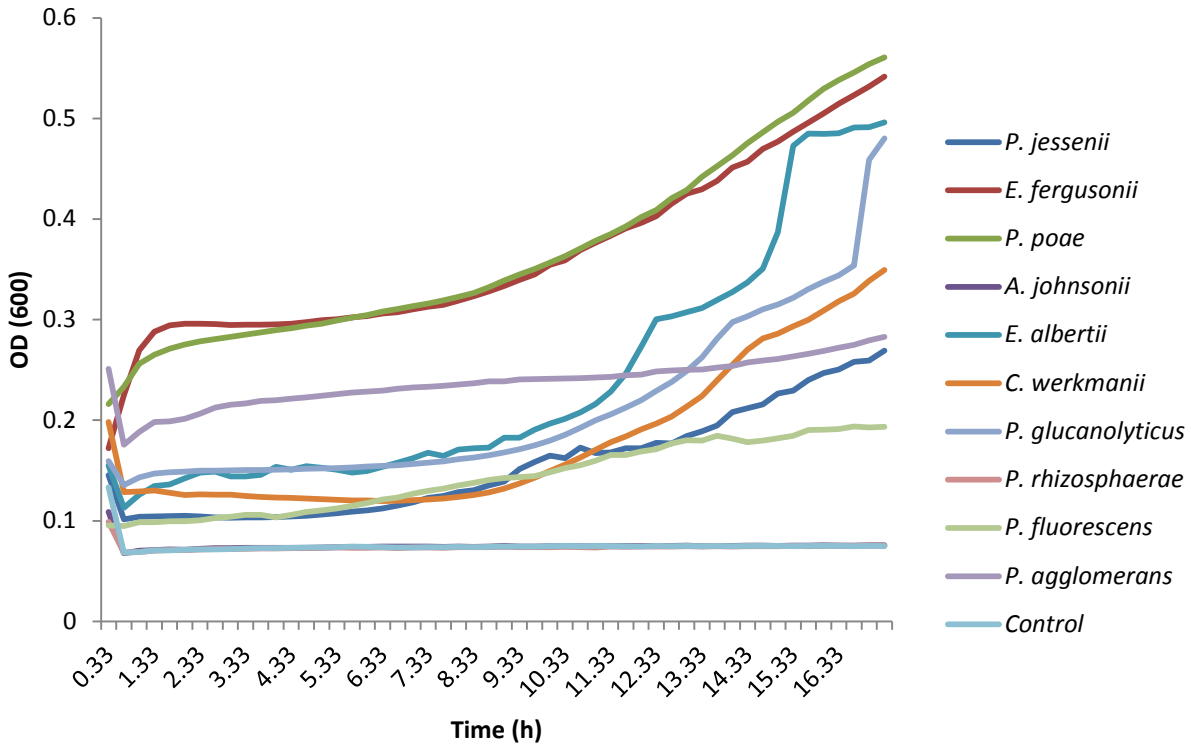


Figure 5. Growth curve showing increase in bacterial cell density over time when grown in artificial diet.

This was then incubated at 27^{O}C with shaking, and OD readings were taken every 10 minutes. In some cases, the bacterial population dropped in numbers most likely as they adjusted to the new conditons of the growth medium; thus their metabolic rates drop, and this accounts for why initially we observed a drop in the OD. *P. poae* and *E. fergusonii* grew particularly well with *P. poae* reaching the highest cell density. Since this particular bacterium performed well in all of the toxicity tests against all of the aphid species, *P. poae's* ability to thrive in the diet, which is similar to the aphids regular diet and thus the contents of the aphids gut, may be correlated to its high pathogenicity

Surface inoculation

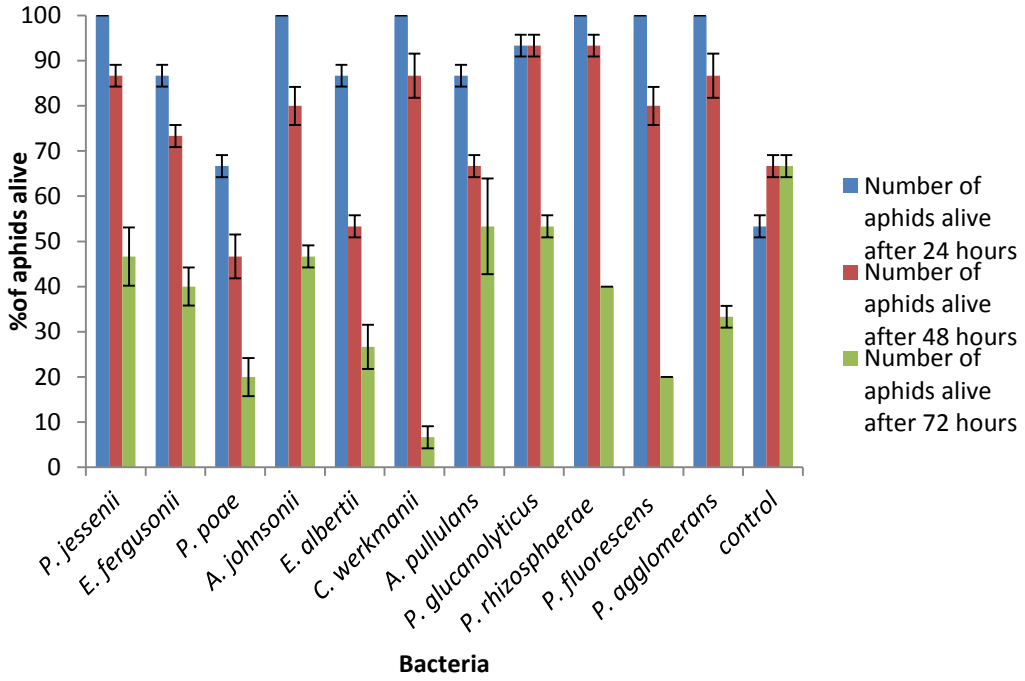


Figure 6 Efficacy of aphid killing by bacterial uptake from the surface of parafilm. Effect of inoculum being applied to surface of parafilm on aphid killing efficiency. Number of bacterial cells on surface of parafilm is $5x10^8$. Values represent 3 replicates and error bars represent the standard error of the mean.

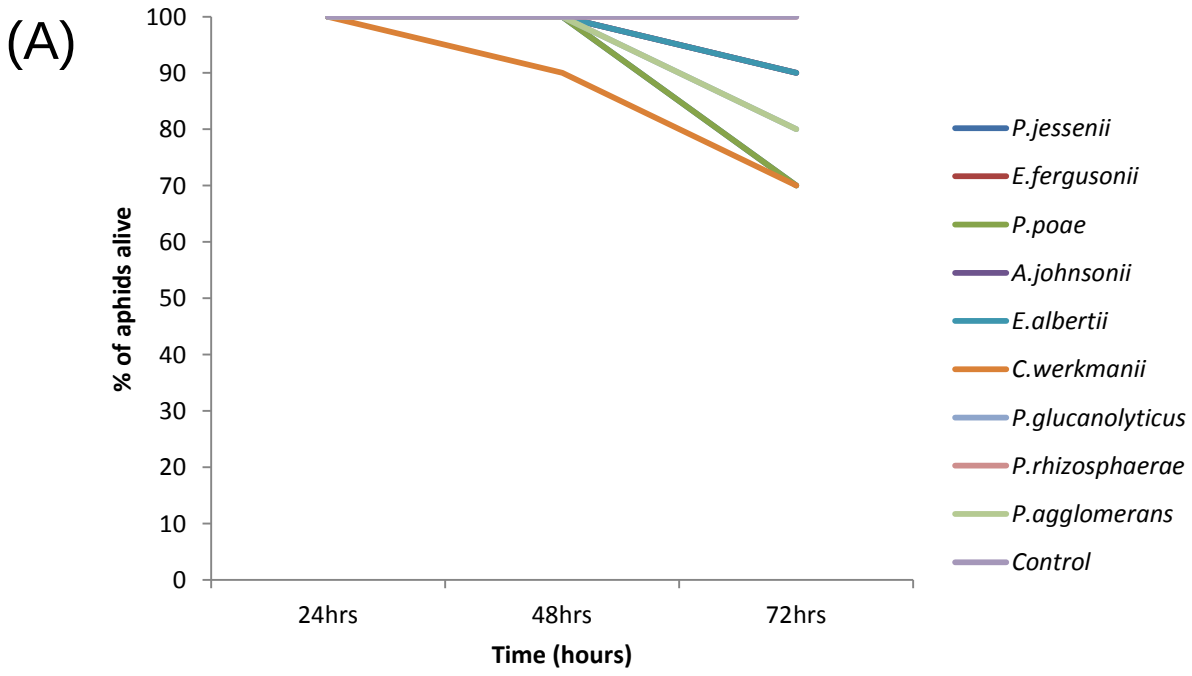
A useful aphicidal control agent would potentially be sprayed onto a leaf surface. To understand whether aphids can ingest bacteria from a surface, an experiment was performed to investigate whether the aphids are able to ingest bacterial cells from the surface of the parafilm. $5x10^8$ bacterial cells in suspension of PBS were applied to the surface of the parafilm and the liquid was allowed to evaporate; a control of PBS was also used. The feed sachet was then made as previously described, without inoculating any of the diet. Aphids were placed in the tube and observation

s for illness and death made at 24, 48 and 72 hours (figure 5). Varying levels of death were observed, but in most cases the bacteria cause more than 50% of aphid death after 72 h. *P. poae*, *P. fluorescens and C. werkmanii* all caused the highest levels of aphid death. These levels of death were comparable to the toxicity data obtained when bacteria were within the feed. This clearly demonstrated that the aphids were able to ingest enough cells to cause death. It was also observed that the diet in the sachet changed over the 72 hours from a clear golden colour to cloudy. This suggested that the aphids may be transferring bacterial cells across the Parafilm and into the diet. This may have implications if it is reflected *in*

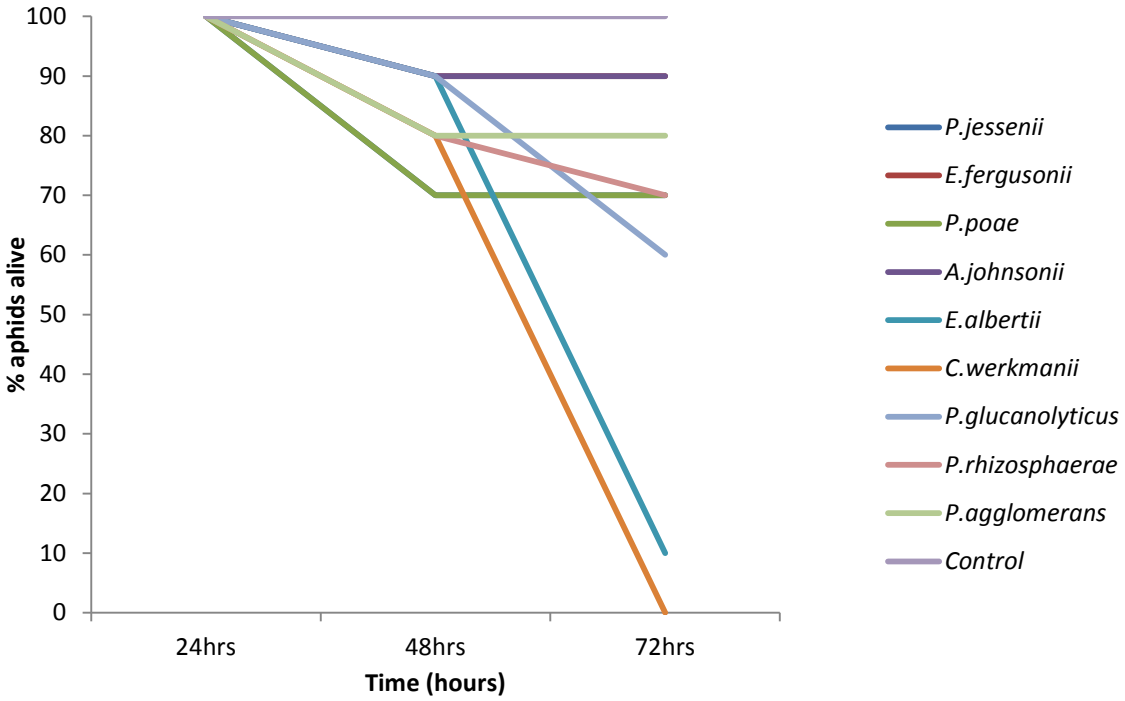
planta and the aphids are able to transfer the bacteria from the surface of the plant to its vascular system making it systemic. Potential outcomes could be detrimental, if the bacteria cause problems for the plant, or beneficial, if the aphids are disseminating the bacterial cells and thus promoting spread of the control agent.

Determining the minimum exposure time needed for optimum aphid mortality

A key question is to understand how rapid the application of the bacteria are to helping in control of the aphids. To determine how long the aphids need to be exposed to the pathogenic bacteria a series of experiments were undertaken. The aphid diet was inoculated with bacterial cells as previously described, 10 adult aphids were introduced to the inoculated feed for varying periods of time before being removed and placed in a feeding sachet with non-inoculated diet. They were observed for signs of death and illness over a period of 72 hours (Fig. 7. To achieve high levels of death in M. persicae the aphids needed to be exposed to the inoculated diet for at least 12 hours. Two of the bacteria, *E. albertii* and *C. werkmanii* were able to cause high levels of death after only 8 hours of exposure. On the other hand the aphids needed to be exposed to P.jessenii for 48 hours before acceptable levels of death were observed.



(B)



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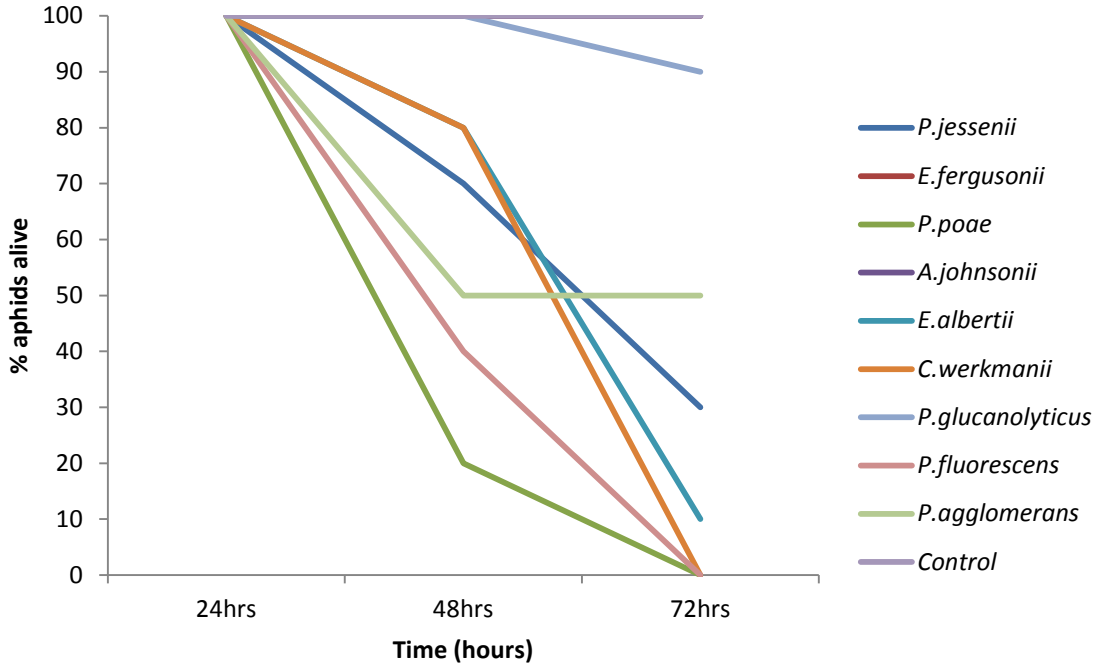

(D)

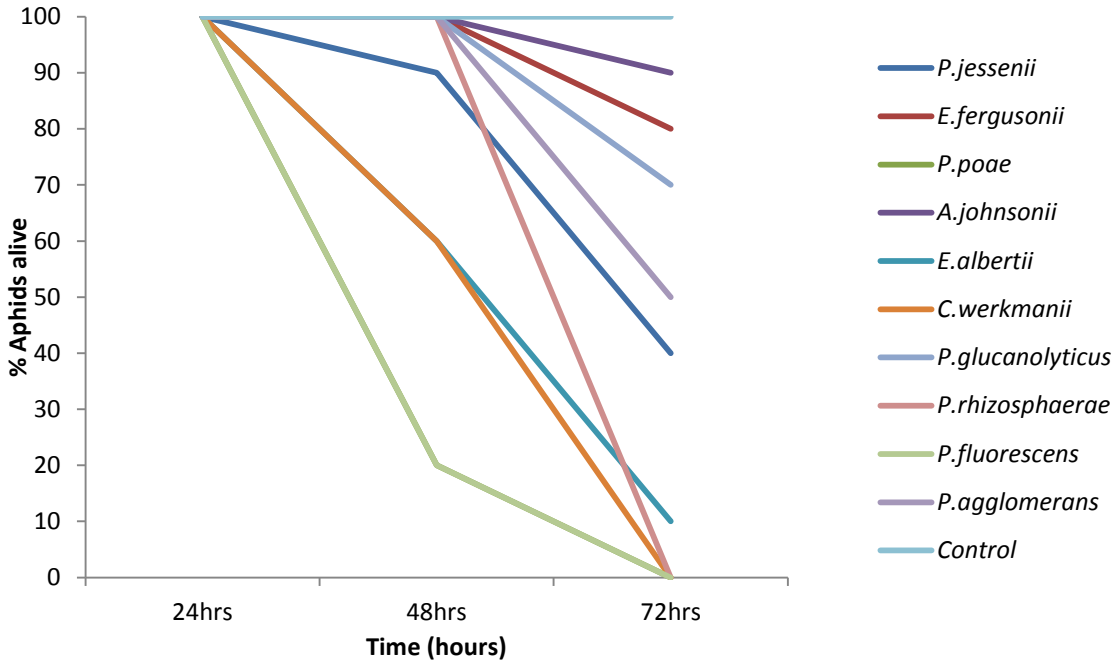

Figure 7. (A-D) Effect of differing exposure times to pathogenic bacteria on the mortalilty of aphids. Aphid feed was supplemented with 10^7 cells per ml of each bacterium and aphids were exposed to the inoculated feed for varying times (A) 1 hour, (B) 8 hours, (C) 24 hours (D) 48 hours. Aphids were removed after being exposed and placed on non-inoculated feed. They were then assessed for death

Genome sequencing and identification of putative insecticidal toxins

From the experiments carried out so far, the three strains that provided the best aphid killing effects, from both in-feed and from surfaces, were *P. poae*, *P. fluorescens* and *C. werkmanii*. These three strains also showed the best susceptibility patterns for antibiotic resistance, an important trait for any future genetic manipulation. Additionally, all three strains could accept the broad host range cloning plasmid pBBR1MCS-5. Therefore, these three strains hold the most promise for development. To aid future work, total DNA of the three strains was extracted and sent to the University of Exeter for sequencing. A draft genome for each strain was developed and supplied as a set of contigs accompanied by a RAST analysis to indicate putative open reading frames. There are 49 supercontigs for *P. fluorescens* genome with a predicted size of 5.95 Mbp with 60% GC content encoding 5266 predicted genes. There are 44 supercontigs for *P. poae* genome with a predicted size of 6.18 Mbp with 60% GC content encoding 5465 predicted genes.

To identify putative aphicidal toxin genes, a custom search analysis of each genome was carried out by Leopoldo Palma Dovis at the Universidad Publica de Navarra. In total, 20 genes were identified in the three genome sequences.

Targeted mutagenesis of *Psedomonas poae*

To test the hypothesis that *P. poae* insecticidal toxins are important for aphid killing effect of the bacterium, some of the genes will be inactivated by insertional knockout. To do this, the internal sequences of three target genes were amplified by PCR and cloned into vector PCR 2.1. The current aim is to now electroprate these constructs into *P. poae*, where it will recombine with the target gene and inactivate it. Once this is done, the mutant strains will be tested for reduced toxicity.

Aphid deterrent effect

Two sets of treated pepper plants, three plants sprayed with *P. poae* and three sprayed with water, were exposed to 10 aphids (removed from any food source) inside an insect tent. The aphids were left to migrate to any of the 6 plants over several days. Over several weeks the number of aphids on each plant was monitored. White paper was placed around the base of each plant in an attempt to visualise any aphids that had died and fallen from the plants. After a 3 week period there was an average of 42 aphids visible on the water treated plant versus 11 aphids visible on the *P. poae* treated plant. No dead aphids were seen on the white paper surrounding any of the plants, suggesting that the aphids were repelled from the *P. poae* treated plant, choosing instead to migrate to the plants sprayed with water.

Plant growth promotion

Very recent results indicate that as well as being pathogenic to aphids *P. poae* may also possess plant growth promotion attributes. Four treatments were used over six weeks to water pepper plants. For each treatment group there were 4 plants used. The plants were kept in conditions as follows 16 hour days at $20^{O}C$, they were sewn in to potting compost containing peat. The control group of pepper plants were watered every other day with 10ml of water. The treated peppers were either watered with 1ml of *P. poae* culture (10^{7} cfu per ml) and 9ml water, 3ml of *P. poae* culture and 7ml water or 5ml culture and 5ml water. Four plants for each treatment were grown and treated from seed until six weeks old. At this six week point several measurements were taken: average measurements for shoot, root and whole plant length as well as whole plant weight were taken. The treated plants were on average at least 5 cm longer than the non-treated plants. In all treated plants there was an increase in root, shoot and whole plant length as well as an increase in the whole plant

weight. The increase for all of the measurements when compared with the plants that were treated with only water can be seen in table 2.

Table 2. Amount of increase in plant length and weight of plants treated every other day with *P. poae* compared to plants watered with 10ml of water every other day. Percentage increase was calculated from the average measurements where n=3.

Treatment	**% increase in root length**	**% increase in shoot length**	**% increase in whole plant length**	**% increase in whole plant weight**
1ml Culture 9ml water	49.4	35	49.5	42.4
3ml culture 7ml water	37.8	23	18.1	30.7
5ml water 5ml water	38.5	28.1	28.0	37.5

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 were tested against *M. persicae*, and five other aphid species: lettuce aphid (*Nasonovia ribisnigri*), glasshouse potato aphid (*Aulacorthum solani*), cabbage aphid (*Brevicoryne brassicae*), lupin aphid (*Macrosiphum albifrons*) and pea 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. Other bacteria used as pesticides reflect this ability to infect different species within the same family. For example, *Bacillus thuringiensis* produces toxin proteins that are specific to a particular family of insect, and their effect spans all insects within that species (Höfte & Whiteley, 1989).

When the artificial feed was inoculated with the 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. Using cell concentrations ranging between 10^7 and 10^2 cfu per ml of artificial diet it was possible to

discern the most effective concentration. The results show that the cell concentration 10^7 cfu per ml was the most effective for all of the bacterial strains. Notably *P. poae* was still 50% effective at 10^5 cfu per ml. The number of bacterial cells needed to cause 50% death is reflected in work done by Grenier *et al*. using *P. fluorescens* (Grenier *et al*., 2006). 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. It was also observed that the aphids are able to acquire enough bacterial cells from the surface of parafilm to cause death, thus enough bacterial cells are ingested when the surface of the parafilm is punctured by the aphids stylets. This result, combined with work by Stavrinides *et al.* (2009) that showed aphids were able to secrete bacterial cells in the aphid honeydew, suggests that bacteria may be a self-sustaining pesticide.

My results showed that the aphids do not need to be exposed to the bacteria for the full 72 hours, for death to occur - they are able to ingest enough bacterial cells to cause an infection and subsequently death within a 24 hour period. Further tests showed that 8 hours of exposure to the inoculated food is not enough to render the same results as 24 hours exposure. Stavrinides *et al.* (2010) showed that aphids need to exposed to inoculated feed for 17-18 hours for death to occur (Stavrinides, No, & Ochman, 2010). This may be important, because if the bacteria are able to survive on the surface of a plant for any time period longer than 24 hours this could be sufficient for causing mortality within an aphid infestation. However, it should be noted that different species of *Pseudomonas* were used in the two studies and this could reflect differences in bacterial performance and survival.

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 (data not shown); these tests showed that the majority of bacterial strains isolated were resistant to both ampicillin and Nitrofurantoin. The genomes of the 3 most promising bacteria were sequenced to aid further investigation. Based on analysis of these genomes, and comparing them to a database of known insecticidal toxins we can attempt to determine the genetic mechanism behind toxicity.The 14 bacteria were also used to challenge other insect species to determine if the pathogenic effect was confined to aphids. Host specificity tests were performed on all of the aphid-pathogens by testing with insect species: *Oryzaephilus surinamensis*, *Sitophilus oryzae* , *Galleria mellonella*, *Sitophilus granaries*and several Lepidoptera species. 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 capable of causing death to aphid species. *Bacillus thuringiensis cry* proteins are known to have a restricted host range, specific pathogenic mechanisms relating to their hosts (de Maagd *et al.,* 2003),

however *Photorhabdus luminescens* is able to kill a wide range of insects, suggesting a broader acting pathogenic mechanism (Brillard *et al*. 2002). 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. It is also important to determine the mechanism behind the pathogenicity as this may help to further determine insect hosts that may be affected.

The newest results have prompted us to look *P. poae* from two new angles. Not only can the bacterium kill aphids, it may also have the potential to promote the growth of plants, as well as act as an aphid repellent, , acting as a bacterial shield on the plants leaf surface to prevent aphid colonisation from becoming established. This gives scope within formulation development to use *P. poae*,(or potentially a combination of *P. poae* and several other aphid killing bacteria) as a treatment during initial growing stages of plants, a treatment throughout the plants life providing protection from aphids, and as a top-up treatment if colonisation occurs. In other words, it could potentially be used in varying ways to enhance growth and protect the plant during the growing process. However, more work needs to be done to confirm these results, and to further our understanding of the full potential of the pathogenic bacteria.

Conclusions

We have made good progress in developing the experimental system to hone the host specificity, learning that the bacteria seem to be aphid specific, to defining a potential plant growth promoting effect as well as an aphid deterrent effect. These latter observations need to be reproduiced with better replication to provide convincing proof of these effects. There is good promise that has attracted an interaction with a potential SME partner.

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 liaison staff 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).

Knowledge transfer in 2nd year 2012-2013

In the past year Amanda Livermore has attended and given presentations on her work at several meetings and conferences including the HTA technical committee meeting, HDC studentship conference, The pepper technology group meeting (September 2013), Association of Applied Biologists (AAB) 'IPM: Pushing back the frontiers' conference. Amanda has also presented a poster at the AAB 'Fruits and Roots celebrating a centenary at EMR'. Amanda has won the David Miller travel bursary from the SCI horticultural group,she will have £250 expenses to claim when she travels to work in Tunisia. Amanda has also carried out some public engagement activities attending the Big Bang fair 2013 and The One show Road show, representing the Society for General Microbiology and The British Society for Plant Pathology respectively. Amanda has become a committee member for the SCI horticultural group. Recently, we have entered into discussions with an SME who are interested in obtaining the IP on the P. poae to culture it and formulate it for use by horticulturists.

Glossary

PCR-polymerase chain reaction

PBS- phosphate buffered saline

DNA- deoxyribosenucleic acid

µ- 100µl in 1ml

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Appendix

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

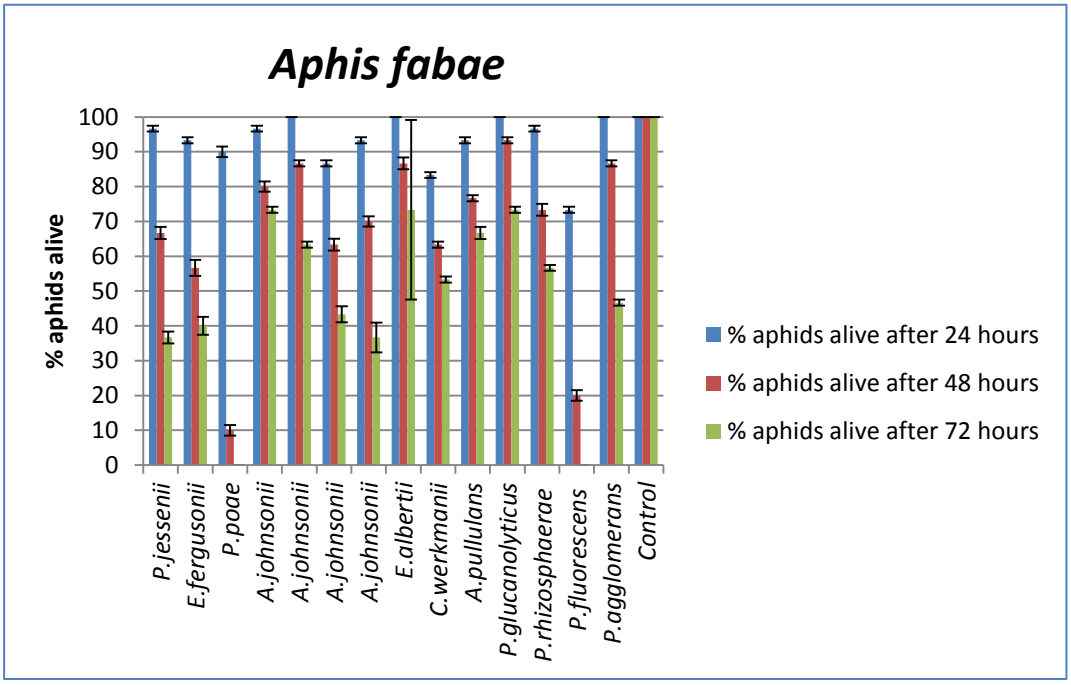
Once the reaction was complete 8µl of the PCR product was mixed with 2µl of 5X loading dye. The sample was loaded into a 1% agarose gel. The DNA was run through the gel in an electrophoresis tank at 120 volts for 40 minutes. The gel was then viewed under a UV wavelength to visualise DNA fragments.

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.

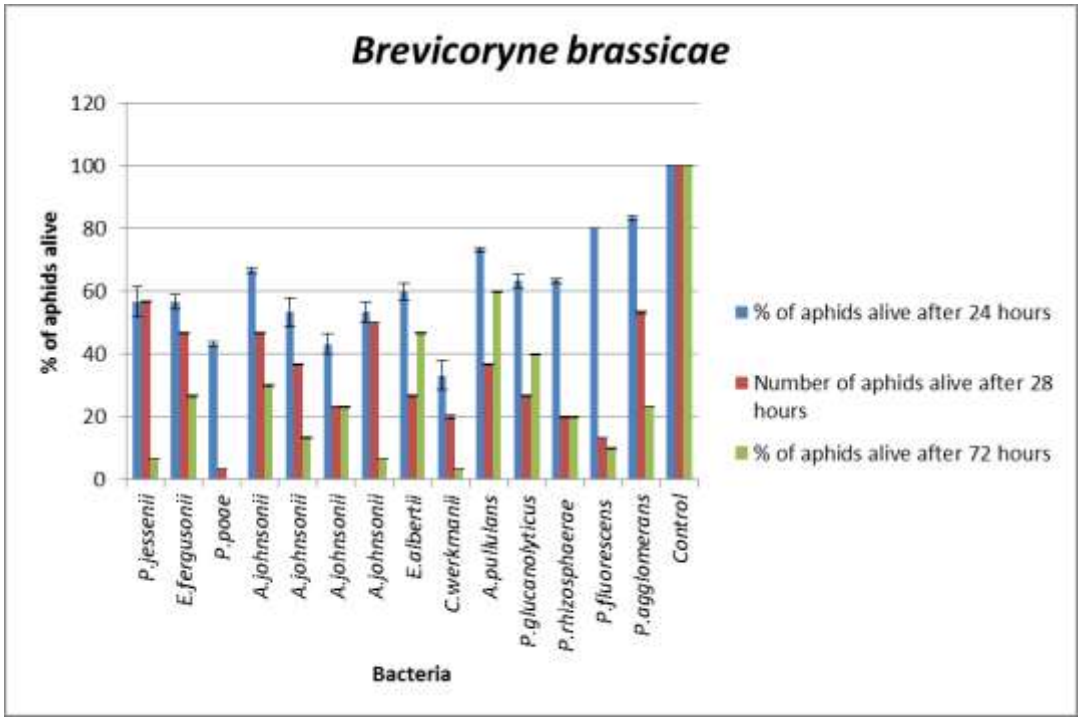
A3. Graphs of all of the aphid species tested with the pathogenic bacteria.

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).

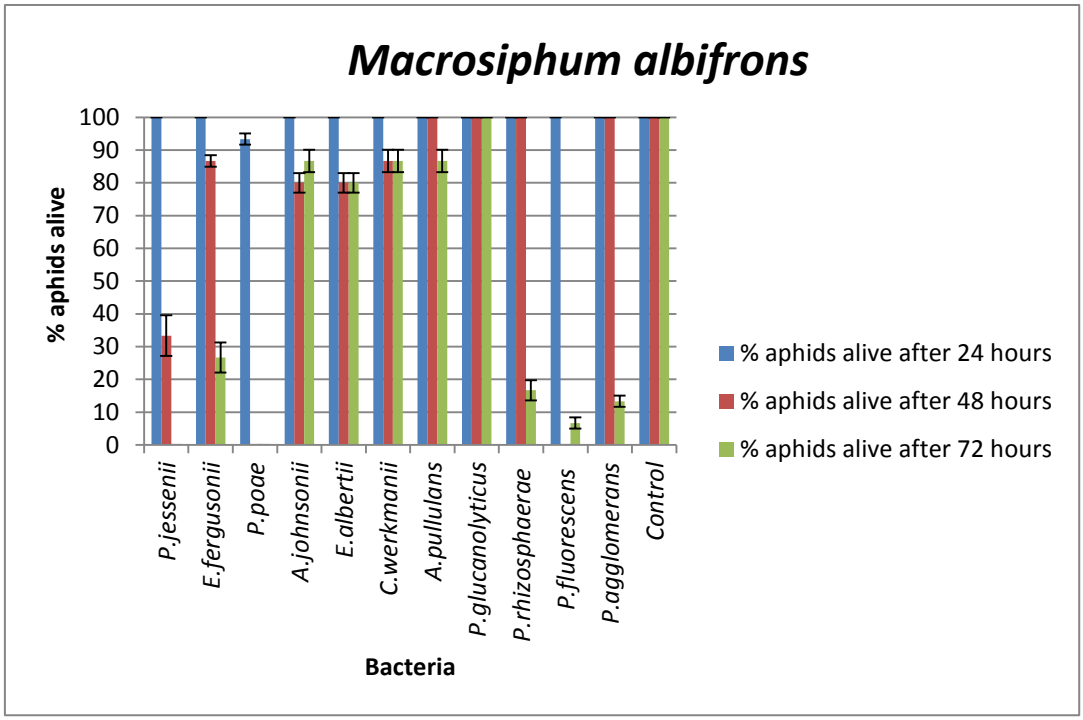
(A)



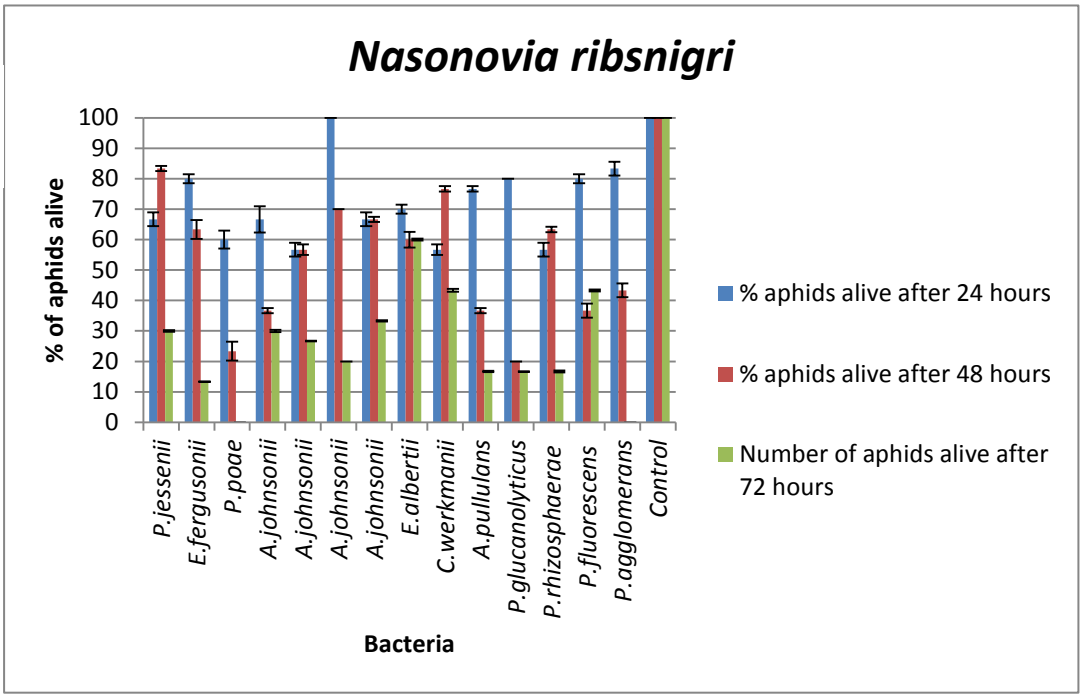
(B)



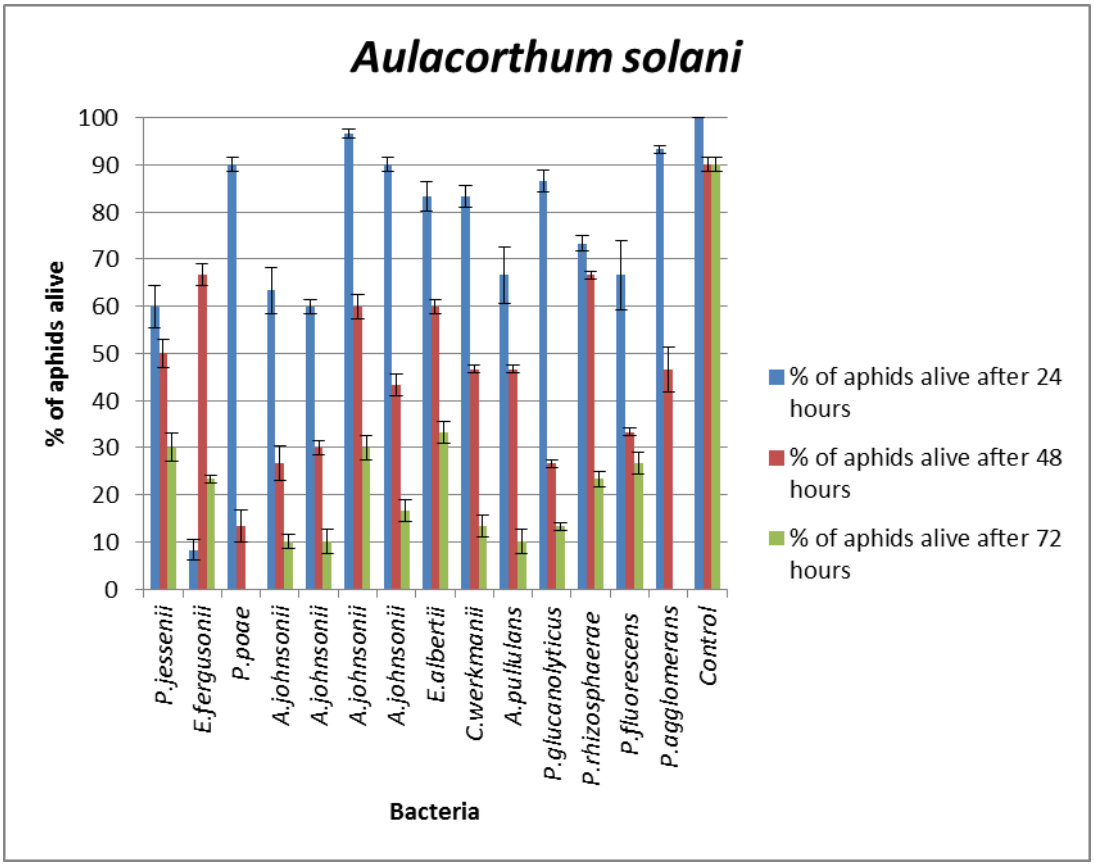
(C)



(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.

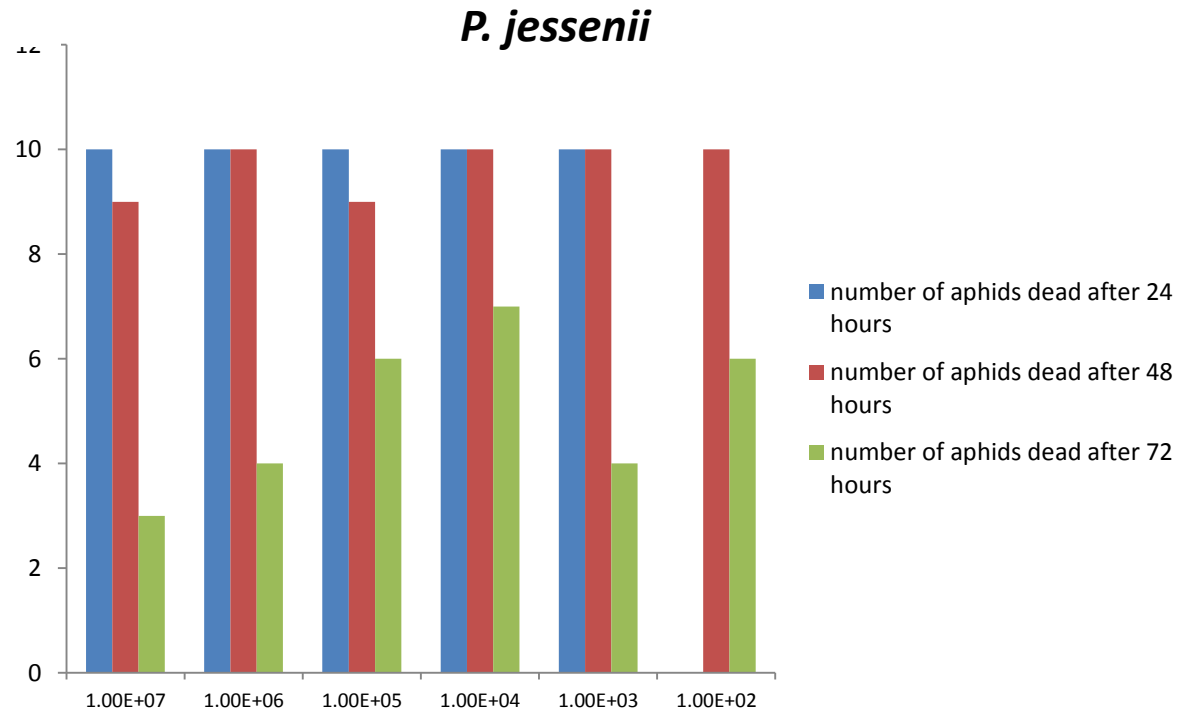
A4. Dose related effect of all pathogenic bacteria on *Myzus persicae*.

Figure 4. Efficacy of aphid killing by varying bacterial concentration.

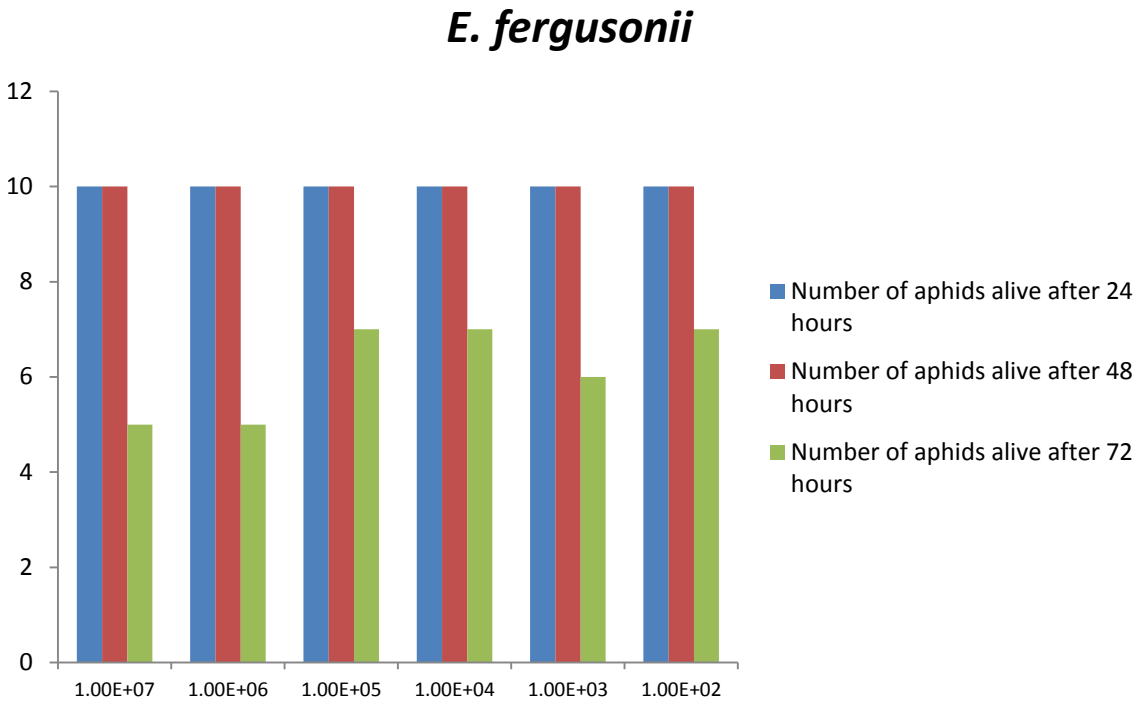
Dose effect of inoculum on aphid killing efficiency. Number of bacterial cells per ml is varied from 10^7 cells per ml of diet to 10^2 cells per ml of diet.

The graphs show an average number (3 replicates) of aphids (out of 10) alive after being exposed to the bacteria for 24, 48 and 72 hours (Figures A4.1a-K).

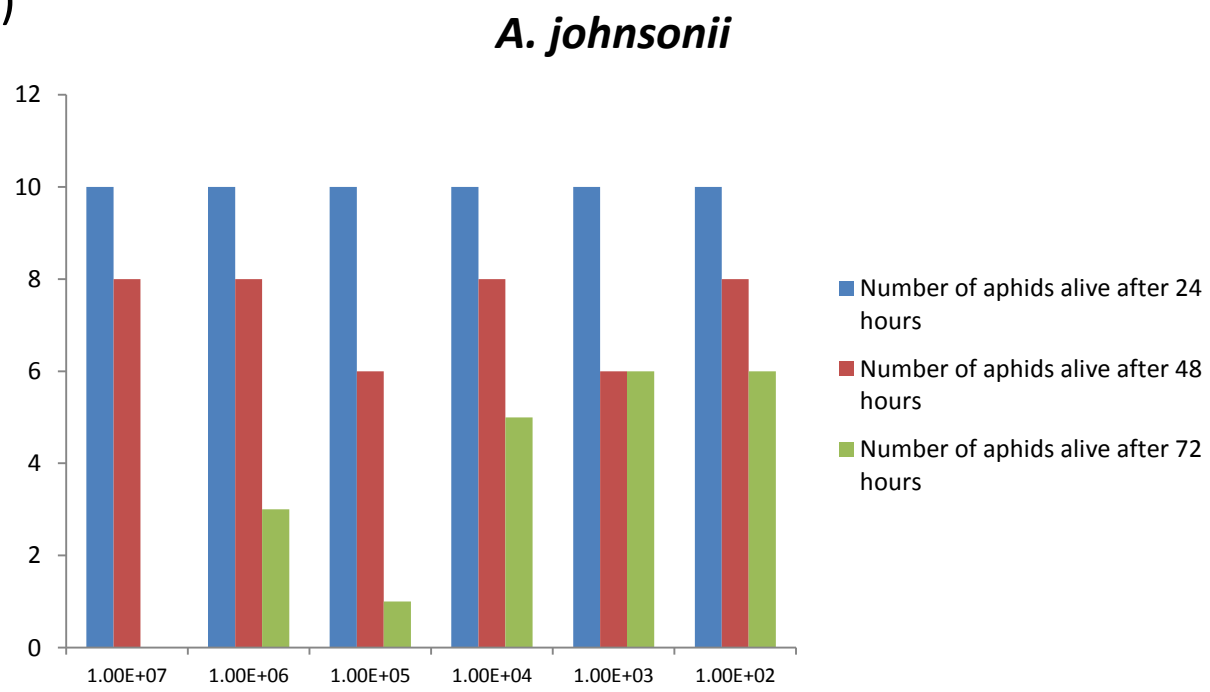
(A)



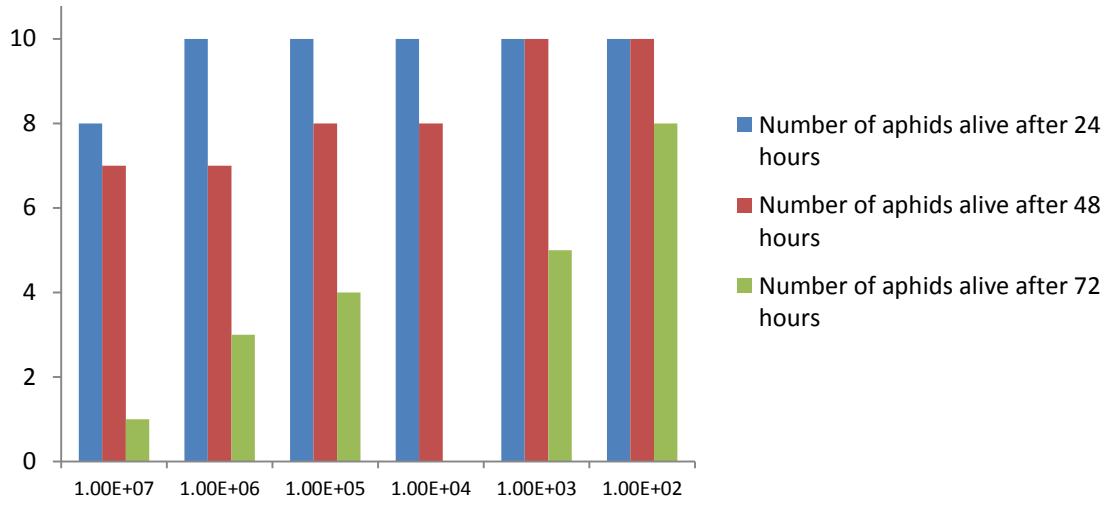
(B)



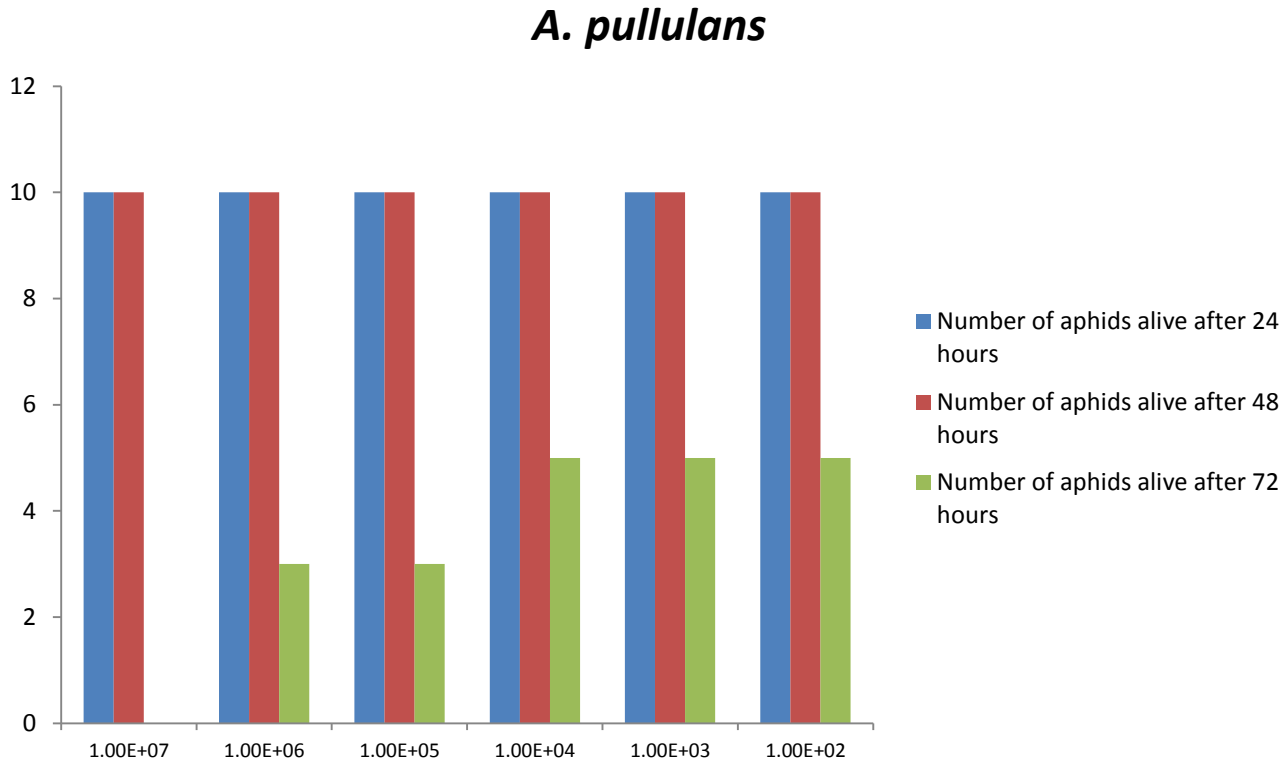
(C)



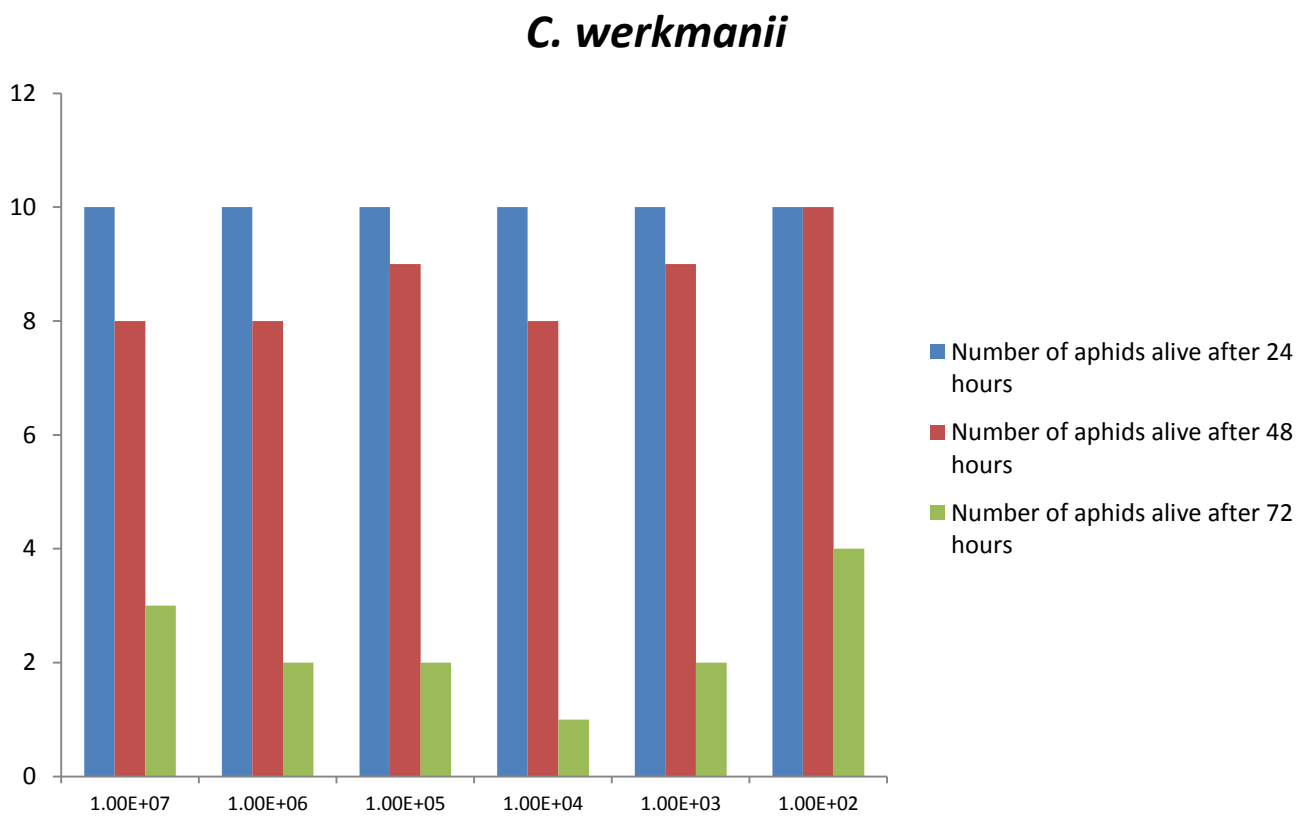
(D)



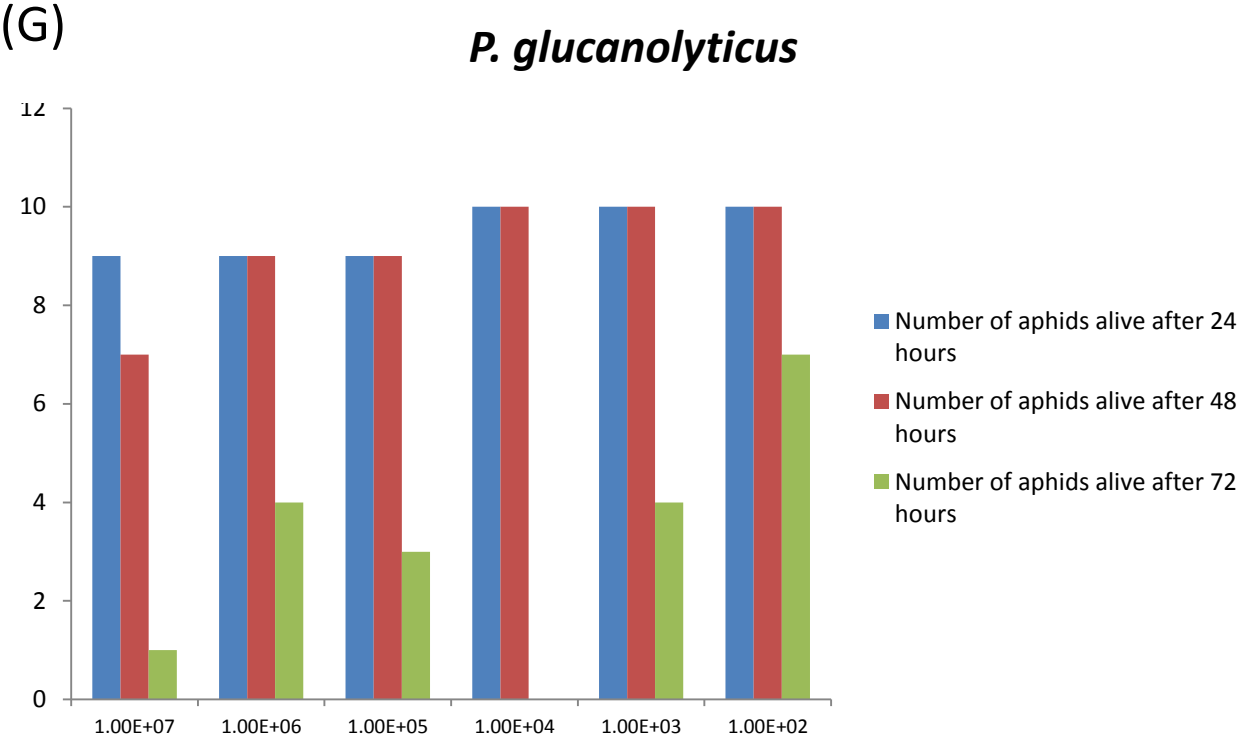
(E)



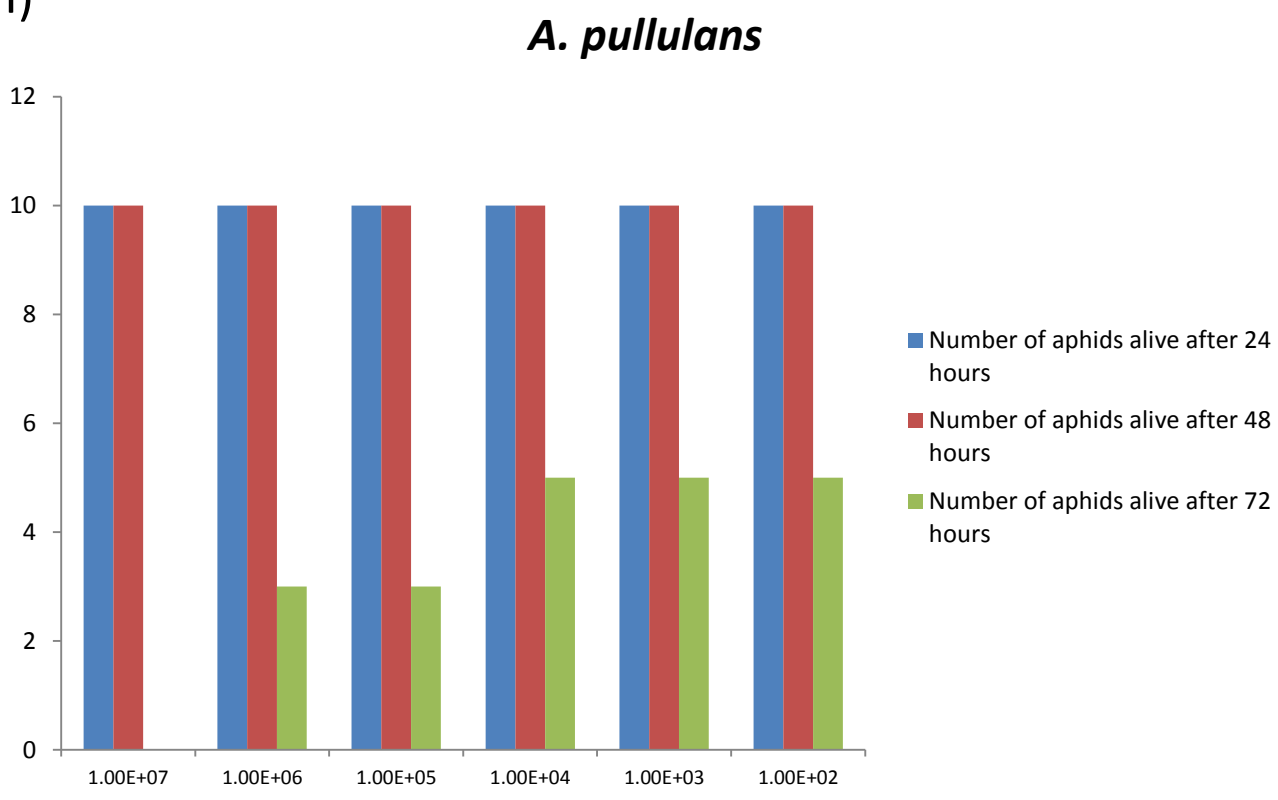
(F)



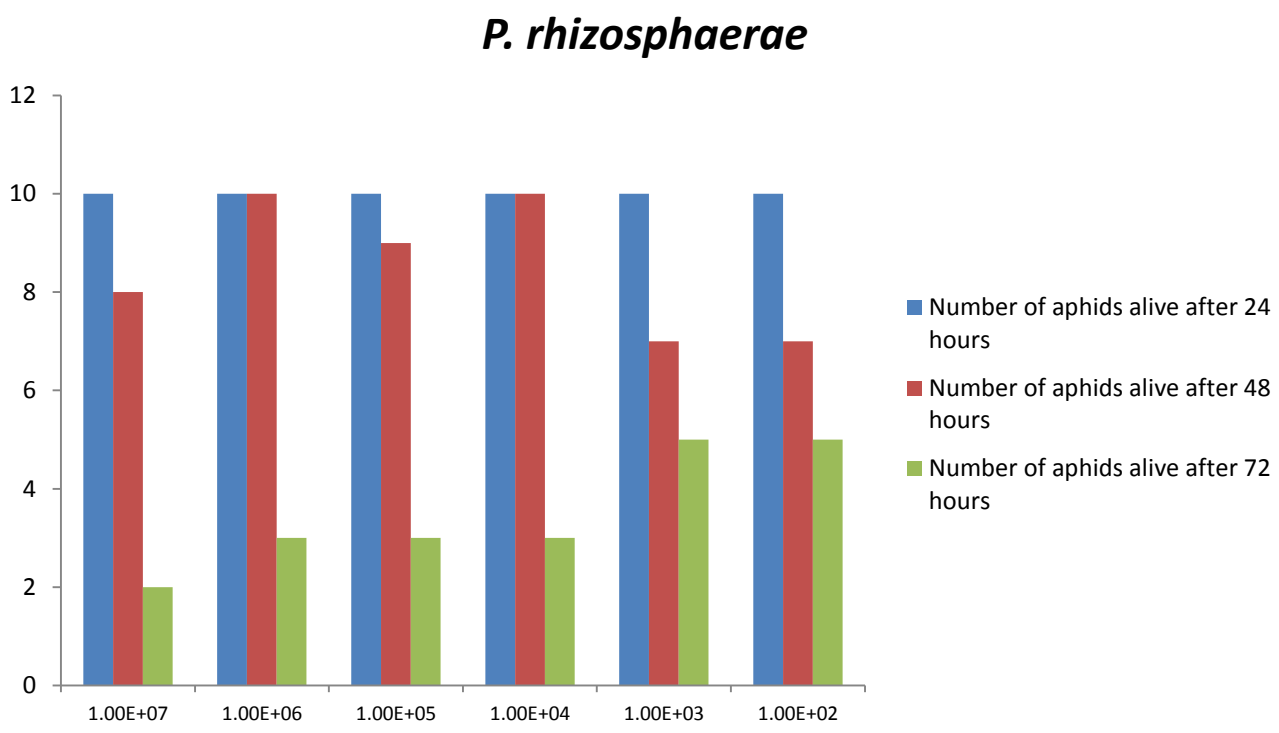
(G)



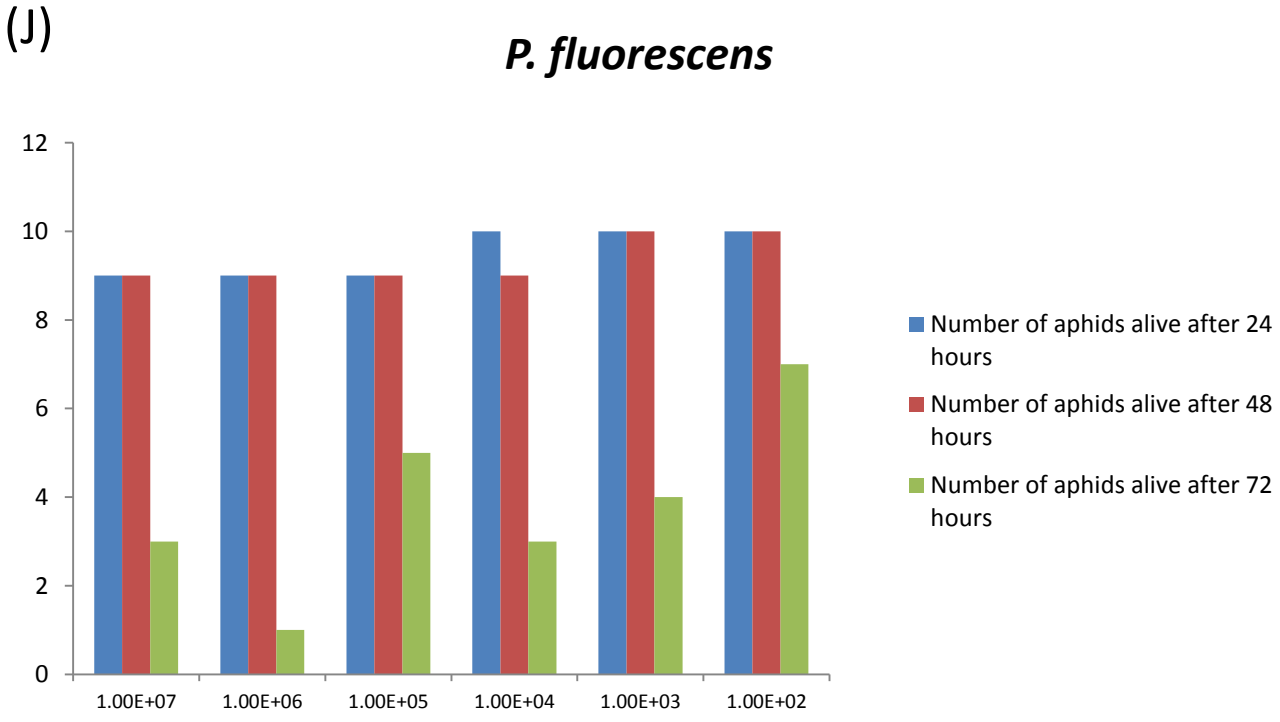
(H)



(I)



(J)



(K)