Project title:	Understanding Resilience of Soil Beneficials to Combat Apple Replant Disease
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Project leader:	Louisa Robinson-Boyer, NIAB EMR., Naresh Magan, Cranfield University., Xiangming Xu, NIAB EMR.
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Location of project:	NIAB EMR, Kent and Cranfield University, Bedfordshire
Industry Representative:	N/A
Date project commenced:	October 2018

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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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CONTENTS

GROWER SUMMARY

Headline

Long-term trial established assessing the effect of pre-plant soil amendment with specific microorganisms for Apple Replant Disease (ARD) prevention.

Background

The phenomenon of previously high yielding perennial fruit orchards producing unsatisfactory growth and yield in replanted trees, termed apple replant disease or ARD (Mai & Abawi, 1981), has become an increasing problem as virgin land to establish new plantings becomes increasingly difficult to locate in both nurseries and newly established orchards. Modern, intensive systems of apple production require high yields throughout the orchards life to get a good return on investment however a reduction in yield caused by ARD can limit yield potential. ARD symptoms may decrease profitability by up to 50% during the orchards life (van Schoor *et al.*, 2009). ARD symptoms include stunted growth, discolouration of apple skin, reduced yield, reduced fruit size/weight, altered fruit aroma and tree death (Mazzola & Manici, 2012; Zhu *et al.*, 2014; LIU *et al.*, 2014). The aetiology of ARD is disputed within the scientific community but is likely caused by a consortium of soil pathogenic microorganisms, the main causative agents of which change depending on local conditions.

Current treatments for ARD include pre-plant fumigation of the soils by applying volatile chemical compounds (eg. Chloropicirin & Dazomet) to sterilise the soils (Mazzola & Manici, 2012). These products however are under pressure from government legislation regarding safe chemical use as well as being harmful to the environment. Chemical treatments are therefore not sustainable in the medium to long term. Brassica seed meal (BSM) is a newly developed bio-fumigation treatment that has been extensively studied and used to alleviate ARD symptoms but is yet to be trialled in long term trials in the UK. BSM has been shown to increase apple tree growth over a period of 3 years higher than those observed using conventional fumigation techniques (Mazzola *et al.*, 2015). Specific plant growth promoting microbes are yet to be widely recognised as a treatment for ARD despite the use of arbuscular mychorrizal fungi (AMF) significantly increasing fresh weight of apple seedlings in AMF inoculated soils (Mehta & Bharat, 2013) and improving drought stress tolerance in strawberry (Boyer *et al.*, 2015).

The present research project aims to understand how various soil management practices, including amending soils with specific microbes (Nicola *et al.*, 2017), will impact soil quality in terms of ARD and other apple diseases will be observed such as apple canker development. In addition, we are studying the dynamics of soil microbial communities under climate change scenarios: combinations of elevated CO_2 x temperature x water potential stress.

Summary

In the first year of this study, long term trials were established evaluating beneficial biological soil amendments in ARD predisposed soils. Trials were measured to determine whether treatments have beneficial effects on tree development in the presence of ARD. The growth parameters chosen were height, girth and yield of the tree throughout the first growing season. Further work will include microbiome population analysis of inoculated trees using next generation sequencing and functionality difference tested using carbon utilisation assays. Once population and functionality variation are established, these can be cross-referenced with the long-term growth data to demonstrate a comprehensive assessment of the effectiveness and potentiality of standardising biological soil amendments to mitigate the effects of ARD.

Financial Benefits

It is too early to calculate the financial benefits of this work from the first-year data. As ARD is a prevalent disease in both nurseries and in fruit production and ARD onset can be 1-2 years after planting, significant economic losses can occur for growers from both management and prevention of ARD. Fumigation is an expensive pre-plant option, so a transition to using non-chemical soil amendments applied at planting would save growers both money and time managing ARD. This work aims to identify candidate amendments and optimise their use to reduce ARD in long term field trials, benefiting growers by offering alternatives to chemical treatments.

Action Points

There are no action points for growers as the project is still at an early stage of a 4-year project.

SCIENCE SECTION

Introduction

Apple Replant Disease (ARD), previously termed “replant problem” is a disease where previously high yielding perennial fruit orchards show unsatisfactory growth and yield in replanted trees (Mai & Abawi, 1981). ARD has become increasingly difficult to control as finding virgin land to establish new orchards becomes increasingly difficult. Apple (*Malus domestica)* can be severely affected by ARD both in newly planted orchards and particularly in nursery orchards where tree turnover and successive replanting of crops is far more frequent than fruit production orchards where older well-established trees may have a chance to recover from ARD. Modern systems of apple growing require much higher investment to induce higher yields and earlier fruit production (Hoestra, 1968), increasing the number of cases of ARD.

ARD causes a host of negative impacts on the replanted apple trees, such as stunted growth, discolouration of apple skin, reduced yield, reduced fruit size/weight, altered fruit aroma and tree death (Mazzola & Manici, 2012; Zhu *et al.*, 2014; LIU *et al.*, 2014). These changes through ARD symptoms may decrease profitability by 50% during the orchards life (van Schoor *et al.*, 2009). The symptoms of ARD can be easily missed as stunting is often subtle and early stage ARD can only be detected when fumigated and un-fumigated soils are compared (JACKSON, 1979; Jaffee, 1982a). Young apple trees, particularly in nurseries, are of particular concern as the symptoms of ARD can occur as early as 1 year after establishment in the orchard. If death of these young trees does not occur, then characteristic ARD symptoms emerge. Additional to the above ground effects described above, discoloured roots, root tip necrosis and reduction in root biomass are all evident below the surface (Mazzola & Manici, 2012).

There is debate as the cause of ARD being caused by biotic or abiotic factors. It is generally accepted that the cause is biotic due to basic soil properties remaining unaffected in ARD affected tree soils (Simon *et al.*, 2020). The most accepted hypothesis is that changes in the soil microbiome is the basis for the onset of ARD (Mazzola & Manici, 2012). The non-specific interaction of multiple pathogenic microorganisms with each other and the host may be responsible for the onset of ARD. Changes in key components, beneficial or otherwise, in the soil microbiome is also hypothesised due to the absence of speculated ARD pathogens in affected soils (Nicola *et al.*, 2018). It is thus likely that the hypothesis that soil microbiome

composition is pivotal in either the overall health of the plant through rhizosphere microbe interaction with the roots or by the interaction of pathogenic microorganism complexes forming in ARD affected soils.

Various approaches have been made to identify the causal agents of ARD meaning a plethora of different pathogenic microorganisms have been associated with ARD. There is a general agreement that a number of oomycete and fungal genera contribute to the disease globally. These include the oomycetes *Pythium* and *Phytophthora* and the fungi *Cylindrocarpon*, *Rhizoctonia* and *Fusarium* (Mazzola & Manici, 2012). The nematode *Pratylenchus penetrans* has also been associated with ARD and acts to exacerbate the disease further, leading to reduction in vegetative growth of affected apple trees and seedlings (Jaffee, 1982b). It is important to be careful when associating pathogens with ARD as some reported causal agents including *Bacillus subtilis*, *Penicillium* spp., and *Mortierella* spp. are not usually associated with being root pathogens but increased populations in ARD affected soils lead to mis-labelling of them as ARD associated pathogens (Mazzola & Manici, 2012).

The current industry standard treatment for ARD includes pre-plant fumigation of the soils by applying volatile chemical compounds (eg. Chloropicirin, Dazomet & methyl bromide) to sterilise the soils of potential pathogenic microorganisms and pests (Mazzola & Manici, 2012). Broad spectrum chemicals are either currently or soon will be banned due to their negative effect on the environment (Nicola *et al.*, 2017), making these treatments unsustainable. Non-chemical treatments include brassica seed meal (BSM) products that create a fumigation-like effect and provide anti-fungal and anti-nematode action. BSM is effective against fungal pathogens but is not effective against oomycete pathogens such as *Phytophthora*. BSM has been extensively tested as an effective alternative to chemical treatments providing a non-chemical approach amidst the uncertainty over future chemical use (Mazzola & Brown, 2010).

The use of soil biological soil amendments to combat soil borne diseases is becoming more common throughout the literature as increasing numbers of publications report the use and functions of specific soil amendments. There is however a distinct lack of publications describing the effect of biological soil amendments for ARD. Arbuscular mychorrizal fungi (AMF) inoculated soils have been shown to support fewer numbers of the suspected causal agent *P. penetrans* in ARD soils as well as being a well-documented symbiont increasing growth of host plant (Forge *et al.*, 2001; Kyllo *et al.*, 2003). There are also no reports on the correlation between plant-growth promoting Rhizobacteria (PGPRs) such as *Bacillus* spp.,

Streptomyces spp. and *Pseudomonas* spp. on apple tree growth or ARD, making these genera good targets for specific soil amendments to prevent ARD onset at the time of planting (Nicola *et al.*, 2017).

In this research project, we aim to understand whether and, if so, how various soil management practices, including amending soils with specific microbes, will impact soil quality in terms of ARD and canker development. We will be running long term soil amendment trials with specific candidate strains of biological soil amendments to test their efficacy against ARD and also use next generation sequencing techniques to see the impact on soil microbiome populations due to specific biological inoculations and in different climate change scenarios.

The objectives of the project are:

- To determine the effectiveness of specific biological soil amendments on initial growth of the tree to overcome ARD onset.
- To use next generation sequencing techniques to assess the microbiome populations in replanted apple tree soils to assess for potential ARD pathogens, beneficial microorganisms and also access differences in functionality that may correlate with these differences.
- To assess the impact of climate change scenarios (CO_2 increase, drought stress, temperature increase) on soil microbiome and functionality.
- To understand the resilience of biological soil amendments.

Materials and methods

Long Term Growth of Apple Trees inoculated with Biological Soil Amendments

Plant Material

Three different cultivars on M9 rootstocks; Braeburn Mariri Red, Gala (Brookfield) and un-grafted M9 rootstocks, were used (supplied from Frank P Matthews, UK). The trees were planted in an orchard at NIAB EMR, UK, at the original tree stations (where apple trees were removed for this trial).

Soil Amendments

The site was planted in January 2019 and trees were inoculated with three different biological soil amendments, *Bacillus* sp., *Pseudomonas* sp., and a 6 species AMF mix (Plantworks Ltd, UK) as well as pelleted brassica seed meal (Tozer Seeds, UK) at the time of planting (Table 1). The pelleted seed meal was dug in 1 week before planting to allow for the pellets to absorb water, break down and 'de-gas' in order to avoid damage to the roots. *Bacillus* sp. and *Pseudomonas* sp. were applied 1 week after planting to allow time for the tree to establish after the replanting. The microorganisms used for inoculation were selected based on their known plant growth promoting action in apple but lack of investigation against ARD. The site was managed the same as all other conventionally managed apples on the site (not organically).

Table 1: List of inoculants used, time and method of application and the quantity of each added per tree station.

Inoculant	Application Time	Method of Application	Quantity added per tree
Brassica Seed Meal	1 week prior to planting	Pelleted seed dug into ground and soil replaced	300g pelleted seed per tree station
Bacillus sp.	1 week after planting	Evenly poured around the planted tree	500ml per tree station at approximately 10^6 cfu/ml
Pseudomonas sp.	1 week after planting	Evenly poured around the planted tree	500ml per tree station at approximately 10^6 cfu/ml
6 species AMF	At time of planting	AMF mixture sprinkled into bottom of planting	25ml scoop of mixture per tree station

The plot was randomised using a split pot design with the cultivar being the large plot factor and soil amendment the subplot factor. The plot was arranged into 4 blocks of 15 trees (5 treatments per cultivar per block) for a total of 60 trees in the experiment (Figure 1).

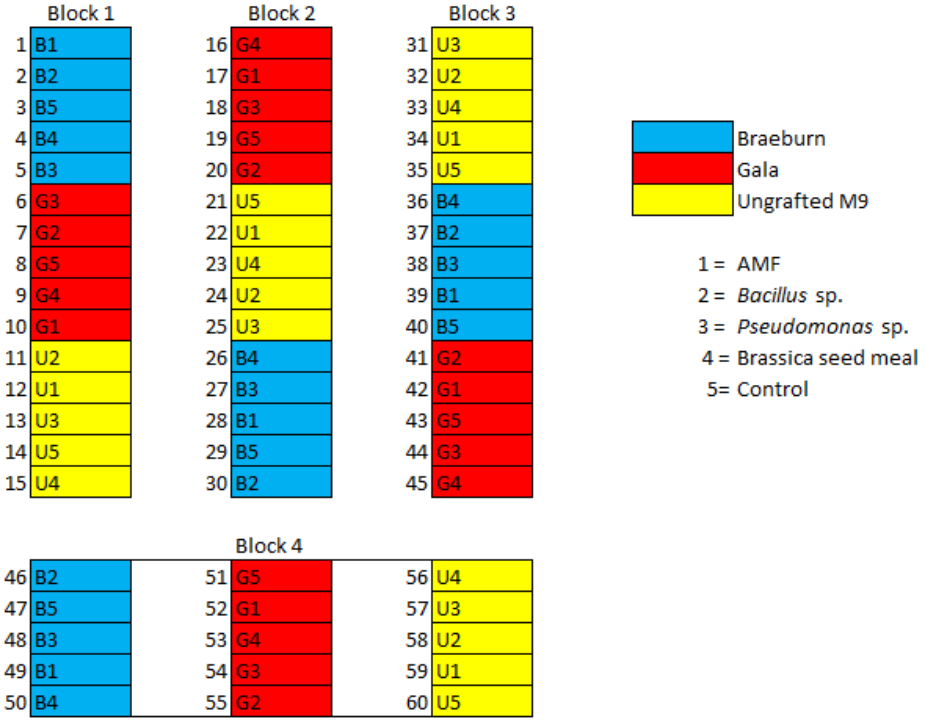

Figure 1: Split-Plot design of the plot. Code is the first letter of the cultivar (U – Ungrafted M9, B- Braeburn, G- Gala) followed by the number code of the treatment shown in the legend.

Growth Measurements

Growth measurements were taken every 3 months from the beginning of the growing season to late autumn, just before dormancy. Growth measurements taken were the height of the tree (from ground level to end of the highest shoot, not including leaf height from this shoot), girth of the tree 5 cm above the graft union on grafted trees and 15cm from ground on un-grafted trees (circumference of the tree measured rather than width using callipers), and number of fruit per tree at harvest.

Statistical Analysis

All statistical analysis was performed in R Studio (R Version 3.5.1).All statistical analysis was conducted from raw growth data from the field. Change in height or girth were calculated in R by the final measurement for height or girth minus the initial value at the time of planting. ANOVAs were performed on the data using the agricolae package to see whether any

treatments showed any significant differences. ANOVAs were also conducted for the differences between cultivars and blocks to check any differences observed were due to treatment, cultivar or both rather than the environment due to tree position. A generalised linear model (quasipoisson distribution) was used to model yield and a pairwise comparison of grouped treatments was compared to the full model using a deviance test (chi-squared).

Results

The un-grafted M9 trees in this orchard did not survive the replanting process and hence were excluded from the statistical analysis. Mean girth and height change was calculated as the difference between the initial measurement at the time of planting in March 2019 to the measurement at the end of the apple season in October 2019. Yield was calculated as the number of fruit per tree rather than mass measurements due to the low number of apples in the first year of growth of both cultivars. All the data presented are after one year of growth of a replanted tree in a 3-year long term trial. The data are therefore preliminary.

In the first year of growth there was no statistical differences between height change ($P = 0.235$) or girth change ($P = 0.81$) between cultivars. Treatment did not show any statistical differences for height change ($P = 0.201$) or girth change ($P = 0.499$). The yield of Gala had a higher mean of 11.2 fruit per tree compared to Braeburn cultivar with a mean of 7.55 ($P = 0.049$). Treatment effect on yield was also statistically significant ($P = 0.024$) from the ANOVA. Braeburn had a lower mean yield in the control trees compared to all other amendments used whereas gala yield appeared similar between the different treatments (Figure 2). Summary of ANOVA results are shown in Table 2.

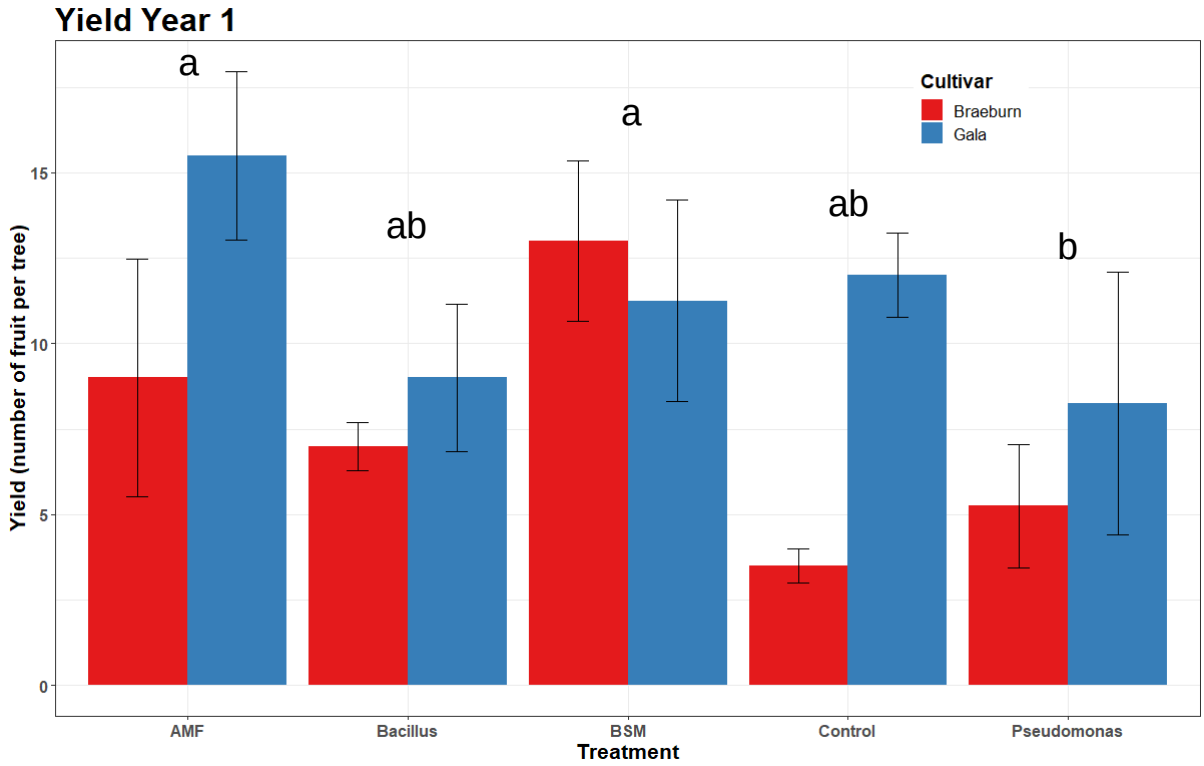

Figure 2: Year 1 yield of conventionally managed orchard. Significant lettering compares linear models of treatments. Same letter indicates no statistical difference.

Table 2: Split-Plot ANOVA analysis for Height change, Girth change and Yield (no. fruit per tree). * denotes significant difference (P < 0.05).

Height Change		Mean Sq	DF	F	P	
Between Groups	Block	61.25	3	4.021	0.206	
	Cultivar	42.99	1	2.822	0.235	
Within Groups	Treatment	25.39	4	1.628	0.201	
	Residuals	15.59	23			

Girth Change		Mean Sq	DF	F	P	
Between Groups	Block	0.157	3	2.701	0.218	
	Cultivar	0.004	1	0.069	0.81	
Within Groups	Treatment	0.033	4	0.866	0.499	
	Residuals	0.038	24			

Yield Difference		Mean Sq	DF	F	P	
Between Groups	Block	89.42	3	6.937	0.073	
	Cultivar	133.23	1	10.33	0.049	*
Within Groups	Treatment	54.5	4	3.43	0.024	*
	Residuals	15.89	24			

The pairwise comparison of Treatment impact on Yield (Table 3) confirmed significant impact of cultivar on Yield (P = 0.029). The difference between Pseudomonas and AMF Treatment (P = 0.038) and Pseudomonas and BSM Treatment (P = 0.042) were statistically different. None of the Treatment impacts on Yield were statistically different to the Control trees in the first year.

Table 3: Pairwise comparison using deviance test of Treatment impact on Yield. Paired treatments were compared with full model. AMF – Arbuscular Mychorrizal Fungi, Bacillus – Bacillus sp., BSM – Brassica Seed Meal, Pseudomonas – Pseudomonas sp., Control – Untreated trees. * denotes significant difference ($P < 0.05$).

Model comparison		P	
Between Groups	Treatment	0.105	
	Cultivar	0.029	*
Within Groups	Control - AMF	0.099	
	Control – Bacillus	0.918	
	Control – BSM	0.108	
	Control - Pseudomonas	0.668	
	AMF – Bacillus	0.122	
	AMF – BSM	0.967	
	AMF – Pseudomonas	0.038	*
	Bacillus – BSM	0.132	
	Bacillus – Pseudomonas	0.595	
	Pseudomonas - BSM	0.042	*

Discussion

This report presents preliminary data of a long-term experiment. ARD symptoms may not manifest until the next growing seasons due to the 1- to 2-year gap between planting and onset previously observed. This work highlights the nature of tree fruit to grow slower in early years after planting and the differing responses a Braeburn and Gala cultivar has to the different inoculations. This provides the framework to observe the impact of the pre-plant inoculations on growth parameters of newly planted trees and after continuation in future years will provide a detailed model of the efficacy of using these specific microbial inoculations to improve the health of the orchard soils. This report has focused on just one aspect of the research project whilst I concurrently conduct the following experiments:

- Soil microbial community analysis of amended orchards compared to see interaction of treatments with native population and presence absence of ARD causal pathogens.
- Functionality of inoculated soils compared to see differences in carbon source usage between treatments.
- Climate change stress exposure on soil (increased atmospheric CO_2, increased temperature and drought tolerance) conducted to see response of microbiome populations and functionality.

Conclusions

- There were no statistical difference between Treatments for Height or Girth change in either cultivar.
- AMF and BSM Treatments showed a statistical difference to the Pseudomonas Treatment.
- No Treatment was statistically different to the Control when analysing Yield differences.
- Future work will include:
    - Next generation sequencing of soil microbiomes to observe population differences due to soil amendments
    - Differences in carbon utilisation of amended soils to test for functional differences.

Knowledge and Technology Transfer

AHDB Student Industry Visit – Dundee - July 2019

Presented Poster at Fruit Focus 2019 – July 2019

Thatchers Cider Orchard Visit – August 2019

Glossary

ARD – Apple Replant Disease

BSM – Brassica Seed Meal

AMF – Arbuscular Mychorrizal Fungi

PGPR – Plant Growth Promoting Rhizobacteria

ANOVA – Analysis of Variance

NGS – Next Generation Sequencing

sp. – Species

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Appendices

Table S1: Girth Change data

Treatment	Cultivar	Mean girth change (year 1) (cm)	Standard Deviation	Standard Error of the Mean
AMF	Braeburn	0.275	0.050	0.025
	Gala	0.300	0.081	0.041
Bacillus sp.	Braeburn	0.425	0.150	0.075
	Gala	0.400	0.230	0.115
Brassica Seed Meal	Braeburn	0.400	0.182	0.091
	Gala	0.475	0.434	0.217
Pseudomonas sp.	Braeburn	0.475	0.125	0.063
	Gala	0.375	0.298	0.149
Control	Braeburn	0.375	0.287	0.144
	Gala	0.500	0.141	0.071

Table S2: Height Change data

Treatment	Cultivar	Mean height change (year 1) (cm)	Standard Deviation	Standard Error of the Mean
AMF	Braeburn	9.00	9.309	4.655
	Gala	15.75	3.593	1.797
Bacillus sp.	Braeburn	13.75	2.362	1.181
	Gala	15.75	4.573	2.287
Brassica Seed Meal	Braeburn	17.00	1.825	0.913
	Gala	14.75	7.182	3.591
Pseudomonas sp.	Braeburn	14.00	3.829	1.915
	Gala	19.67	4.932	2.466
Control	Braeburn	14.25	4.349	2.175
	Gala	13.50	3.109	1.555

Table S3: Yield data

Treatment	Cultivar	Mean Yield (year 1) (fruit no.)	Standard Deviation	Standard Error of the Mean
AMF	Braeburn	9.00	6.976	3.488
	Gala	15.50	4.933	2.466
Bacillus sp.	Braeburn	7.00	1.414	0.707
	Gala	9.00	4.320	2.160
Brassica Seed Meal	Braeburn	13.00	4.690	2.345
	Gala	11.25	5.909	2.955
Pseudomonas sp.	Braeburn	3.50	1.000	0.500
	Gala	12.00	2.449	1.225
Control	Braeburn	5.25	3.594	1.797
	Gala	8.25	7.676	3.838