SCEPTREPLUS

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Title:	Polytunnel evaluation of alternative products for control of bacterial canker of cherry
Crop	Cherry
Target	Bacterial canker
Lead researcher:	Matevz Papp-Rupar
Organisation:	NIAB EMR
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Report author:	Matevz Papp-Rupar, Edited by Lucas Shuttleworth.
ORETO Number: (certificate should be attached)	/

I the undersigned, hereby declare that the work was performed according to the procedures herein described and that this report is an accurate and faithful record of the results obtained

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Date

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Authors signature

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Trial Summary

Introduction

Bacterial canker of cherry (*Prunus avium* in the UK) is caused by two pathovars of *Pseudomonas*; *Pseudomonas syringae* pv. *syringae* (Pss) and *Pseudomonas syringae* pv. *mors prunorum* (Psm). Both are found in UK cherry orchards throughout the year and are able to infect all commercial cherry cultivars. In summer, *P. syringae* survives as an asymptomatic leaf epiphyte or causes leaf spot/shot-hole symptoms, depending on cherry cultivar. At leaf fall, the pathogen infects shoots through leaf scars, overwinters in woody tissue and buds causing cankers the following season. Traditionally, bacterial canker of cherry has been kept below economically damaging levels by application of copper-based products at regular intervals starting in late summer to reduce bacterial populations prior to leaf fall. Lower bacterial populations on plant surfaces reduce the probability of infection in the autumn. Regulatory restriction of the use of copper in plant protection and the emergence of copper tolerant *Pseudomonas* strains require new approaches for control of bacterial canker. The objective of this study was to identify alternative products for control of bacterial canker of cherry.

Methods

Potted cherry trees cv. Sweetheart were grown in four randomised blocks in a polytunnel with irrigation. To ensure sufficient disease pressure and uniformity we spray inoculated trees with a mix of *Pseudomonas* strains. Inoculation was done after the first product application and again at leaf fall to increase uniformity of infection. Alternative products ranging from biological control agents, plant extracts, defence inducers and new fungicides were tested (Table1). Based on maximum treatment concentration and dose defined on product labels we applied between four and seven sprays of alternative products (Table 1). Products were applied throughout August and September 2019. Effect of products on the pathogen population size on the leaves was assessed twice. First, after a single preventative spray in summer and secondly after multiple curative sprays at leaf fall. Since defence inducing products were not expected to directly reduce pathogen population on the leaves we also assessed canker incidence and severity in April 2020. Disease incidence was measured as percentage of dead buds and disease severity was measured as the total number canker lesions on the inoculated part of the tree. Phytotoxicity effects of the products such as leaf chlorosis, leaf necrosis and leaf drop were assessed as well.

Results and discussion

Serenade ASO (biocontrol agent), AHDB9957 (defence elicitor) and Amylo-X (biocontrol agent) all showed good control of bacterial canker. Serenade ASO stat. sig. reduced *Pseudomonas* population on leaves after multiple sprays and disease incidence by 89% and 29%, respectively (Table 1). AHDB9957 and Amylo-X did not reduce *Pseudomonas* population on the leaves, but did decrease disease incidence the following spring by ~30%, equivalent to Serenade ASO. Based on this study we recommend using Serenade ASO and AHDB9957 throughout the summer to decrease pathogen population and increase plant defences of the trees before the leaf fall. Amylo-X could be added to the spray program at the beginning of leaf fall for added protection. The effect of these products when combined in a program will need to be investigated further to assess combined efficacy and product compatibility.

Copper-based AHDB9829 was the best product overall reducing *Pseudomonas* populations on the leaves by 93% after a single preventative spray and 91% after multiple sprays. It also reduced disease incidence and severity by more than 60%. The product is not likely to get long-term approval due to its copper-based mode of action. It is however a good candidate for emergency use to protect the most susceptible young trees at the time of high disease risks.

AHDB9831 was another product that showed promising results after a single spray and warrants further investigation into application rate to avoid phytotoxicity.

Table 1. Tested products ordered from most effective (top) to least effective (bottom). Asterisks indicate stat. sig. reduction in comparison to water control. Negative values indicate higher disease incidence and/or severity than water control. No product application resulted in stat. sig. higher disease incidence or severity than in water treated control.

Product	Type	Phyto-toxic	Total number of sprays	Pathogen population reduction on leaves (%)		Disease incidence reduction (%)	Disease severity reduction (%)
				Single preventative spray	Multiple curative sprays		
AHDB9829	**Fungicide**	**no**	**4**	**93***	**91***	**65***	**68***
Serenade ASO	**BCA**	**no**	**7**	65	**89***	**29***	26
AHDB9957	**Defence elicitor**	**no**	**6**	NA	27	**30***	30
Amylo-X	**BCA**	**no**	**6**	14	6	**26***	30
AHDB9831	**Bactericide**	**yes**	**4**	**74***	54	22	-30
AHDB9959	**Defence elicitor**	**no**	**7**	68	65	0	-3
AHDB9885	**Plant extract**	**no**	**7**	45	40	-4	-58

Take home message

- Alternative control products with efficacy against bacterial canker were Serenade ASO, AHDB9957 and Amylo-X.
- Copper-based AHDB9829 can offer very effective emergency protection replacing previous standard Cuprokylt

Objective

The objective was to select plant protection products that are already on the market or close to the market and assess their efficacy against bacterial canker in semi commercial trial.

Introduction

Bacterial canker of cherry (*Prunus avium*) trees in the UK is caused by two pathovars of *Pseudomonas*; *Pseudomonas syringae* pv. *syringae* (Pss) and *Pseudomonas syringae* pv. *mors prunorum* (Psm). Psm was originally divided into two races of *Pseudomonas syringae* pv. *mors prunorum* race 1 (Psm R1) and *Pseudomonas syringae* pv. *mors prunorum* race 2 (Psm R2) based on morphological differences and differential aggressiveness on two cherry cultivars (Freigoun & Crosse, 1975). The race structure currently proposed for Psm is misleading. Phylogenetics revealed that Psm R1 and Psm R2 are distantly related, forming distinct monophyletic clades and have recently been classified as separate species within the *P. syringae* complex (Bull et al., 2010; Hulin et al., 2018). Hulin et at. (20018) could not clearly separate Psm R1 and Psm R2 based on aggressiveness towards different cherry cultivars; however, differences in experimental procedure, strains and climatic conditions prevent a direct comparison with historical results. It seems likely that the field differentiation of Psm R1 and Psm R2 recorded in previous studies is not based solely on single-gene mediated effector triggered immunity, but is dictated by a set of quantitative traits in both host and pathogen. Pss, Psm1 and Psm2 are found in UK cherry orchards throughout the year. They are able to infect all commercial cultivars and cause brown/black lesions on all aerial plant organs, including fruit, leaves and blossom. Over the summer *P. syringae* causes leaf spot/shot-hole symptoms, or survives asymptomatically as an epiphyte on plant surfaces, depending on cherry cultivar. At leaf fall, the populations on plant surfaces infect shoots through leaf scars and overwinter on infected wood.
Bacterial canker of cherry is commonly kept below economically damaging levels by applying copper-based products in late summer to reduce bacterial populations prior to leaf fall. Reduced bacterial populations on plant surfaces prior to leaf fall reduce the probability of leaf scar infection in autumn. However, new regulations on the use of copper for plant protection means in the near future these products will no longer be available. Therefore, the SCEPTREplus consortium have identified bacterial canker of cherry as an important disease to target due to a lack of effective alternative control products.

Aims

To determine efficacy of a range of biocontrol agents, plant defence elicitors and bactericides on reducing bacterial canker.

Two mechanisms of action were investigated.
1) Direct effect of the products on *Pseudomonas* population size on leaves after a single or multiple applications.
2) Plant defence eliciting effect that could result in reduced canker severity with little or no reduction in pathogen popu;ation size on the leaves.

Methods

Trial stages and timing

Protocol development: March to June 2019
Experimental set-up: June to July 2019
Product applications: Aug to Sep 2019
Assessments: Aug to Sept 2019 and again in April 2020
Data analysis and reporting: May 2020

SOPs and guidelines

This study was completed according to ORETO standard. The physical ORETO folder required for full ORETO compliance was not done due to limited funds. The assessment of phytotoxicity was done following "EPPO PP1 - PP1/135(4) - Phytotoxicity assessment" procedure.

Plant material

The experiment consisted of 48 4-years old and 66 one-year old potted cherry cv. Sweetheart trees (Supplementary Figure 1, Supplementary Figure 2). We had 2 different tree ages because older trees required for sufficient experimental replication were not available at the start of the experiment at any of our suppliers.

Site

The experiment was conducted in polytunnel 6 of Ditton Rough field at NIAB EMR, East Malling.

Products and application

All products were sprayed on whole trees with a motorised knapsack sprayer by NIAB EMR Trials Team at 500 L/Ha spray volume (Table 2). We used the highest recommended application rate and maximum dose per season (Table 3) in line with the product labels.

Table 2. List of alternative control products for bacterial canker of cherry

Product	Active ingredient	Product type	Reasons for inclusion
Untreated, NOT INOCULATED	NA	NA	To monitor background level of *Pseudomonas* spp.
Untreated INOCULATED	Water		Negative control
Cuprokylt (not approved)	Copper oxychloride	Fungicide	Previous industry standard of known efficacy.
AHDB9885	/	Plant extract	Product with a botanical active substance showed efficacy in work in USA
AHDB9829	/	Fungicide	AHDB suggestion
Serenade ASO	*Bacillus subtilis* strain QST 713	BCA	Shown to have activity against bacterial diseases in US and Belgium
Amylo-X	Bacillus amyloliquefaciens subsp. Plantarum strain D747	BCA	Alternative BCA product
AHDB9831	/	Bactericide	Shown activity against *Xylella fastidiosa*. requested for inclusion by AHDB
AHDB9959	/	Elicitor	Evidence of efficacy from USA.
AHDB9957	/	Elicitor	Evidence of efficacy from trials in Belgium. Potential effect shown in the potted tree trial in 2018.

Table 3. Alternative control products for bacterial canker of cherry including product concentration, number of sprays and spray interval.

Product	Concentration based on 500 L/Ha spray volume	No. of sprays	Appx. interval between sprays (days)
Untreated, NOT INOCULATED	/	/	/
Water control	/	6	10
Cuprokylt	3 g / L	4	20
AHDB9885	6 ml / L	7	10
AHDB9829	2.7 g/ L	4	20
Serenade ASO	20 ml /L	7	10
Amylo-X	5 g / L	6	10
AHDB9831	2.4 g /L	4	10
AHDB9959	4.65 ml /L	7	10
AHDB9957	2 ml / L	6	10

Inoculum preparation and tree inoculation procedure

A three strain mix of *P. syringae* was used to reflect natural bacterial populations that can cause bacterial canker. These included *P. syringae* pv. *syringae* strain 9097 (PSS), *P.syringae* pv. *morsprunorum* race 1 strain 5244 (PSM1) and *P. syringae* pv. *morsprunorum* race 2 strain 5255 (PSM2), sourced from the collection of Michelle Hulin stored at NIAB EMR, East Malling, Kent. Isolates were grown overnight in LB high salt broth (Sigma 51208-500G-F) at 25° C., centrifuged at 3000rpm for 10 minutes and re-suspended in sterile water. Suspensions of each isolate were prepared at 1 x 10^6 CFU per ml before mixing the 3 strains together in 1:1:1 ratio.

Due to tree sizes, with the 4 year-old trees, three branches per tree were inoculated. With the one year-old trees the whole trees were inoculated. Leaves and branches on trees were sprayed to run off. Inoculation was completed in the late afternoon/evening to ensure cooler and more humid environment in the first 12 h after inoculation.

Trees were first inoculated at beginning of August, 24 h after the first product application to ensure high disease pressure, and test if a single preventative product application had an effect on *Pseudomonas* populations. The second inoculation was done at leaf fall, 2 weeks after the last application of products. This was done to increase uniformity of disease pressure and the likelihood of symptoms developing the following spring.

Assessments

Phytotoxicity assessment

Phytotoxicity was assessed on 28/9/19 after 2-3 product applications depending on the product. Assessment was done according to EPPO PP 1/135 (4) Phytotoxicity assessment guidelines. Three phytotoxicity categories were assessed:

1) Discolouration of the whole leaf lamina: chlorosis, whitening, other abnormal coloration
2) Necrosis of leaves on current year's shoots: edges along the veins, the whole leaf lamina

3) Deformations of leaves or annual shoots: stunting, dwarfing, curling, etc.

Leaves and shoots on treated trees were compared to the water treated control to distinguish between phytotoxicity, and non-product related blemishes. Photos of all trees and representative leaves were taken.

Bacterial population size

We measured *P. syringae* population size on leaves in summer and autumn. Six leaves were selected at random from each tree. On 4 year-old trees, 3 inoculated shoots were sampled, while on 1 year-old trees the entire trees were sampled. A disc (8 mm diameter) was cut from the lamina of each leaf. Discs were taken from random sites. Six sampled discs per tree were homogenised in 2 ml Eppendorf tubes with 1 ml of sterile water and 2 steel ball bearings using Genogrinder (1 min, 1500 rpm). One hundred µl of homogenate was plated on selective media (KingsB with Cefalexin and Cycloheximide). Undiluted, 10x and 100x diluted homogenates were plated to increase quantification range. Two petri dishes per sample/dilution were prepared and incubated for 48 h at 27°C. Colonies were counted and population size calculated as colony forming units (CFU) per ml.

We measured the size of *Pseudomonas* population on leaves at two time points. The first was to assess whether tested products exhibit any preventative action. The trees were treated with all products except for AHDB9957, which was shipped with more than 4 weeks delay and hence excluded from the first spray application. One day after product application the trees were inoculated and the inoculum load immediately after inoculation measured (6.8.19). Preventative effect of a single spray application on the size of *Pseudomonas* population was measured two days after inoculation.

Second assessment was done before leaf fall (24.9.19). This assessment measured the combined effect of multiple product applications on *Pseudomonas* population size.

Canker symptom assessment in spring 2020

We assessed 1) disease incidence as % infected buds per tree. Buds that failed to develop leaves/flowers by mid-April 2020 or showed small, brown/dry leaves and flowers were considered infected. 2) Disease severity was assessed as the total number of clear canker lesions i.e., sunken lesions, splitting of the bark and/or gumming around the dead bud on assessed shoots/trees. The whole tree was assessed in the case of 1-year old trees. In the case of 4 –year-old trees each inoculated shoot was assessed separately and then an average over the 3 shoots calculated. Percent incidence/severity reduction in comparison to ware control was calculated as:

100 x ((‘Y of control’ – ‘Y of treatment)/ ‘(‘Y of control’)); where Y stands for either mean severity or mean incidence.

Experimental design and data analysis

A randomised block design was selected with 4 blocks each with 9 plots, 3 trees per plot. Blocks 2 and 3 consisted solely of 1-year old trees, block 1 consisted only of 4-years old trees and plots in block 4 consisted of 2 old and 1 young tree each. We had 3 m buffer space between plots. Schematics of experimental design are presented in Supplementary Figure 2. Trees were randomly assigned to treatment groups and treatments were applied in random order. Due to irrigation issues we lost a few trees in blocks 3 and 4 which caused an unbalanced design. Population size data were log10 transformed before analysis. The effect of product application, block and tree age was analysed in Genstat (19th Edition) software with unbalanced ANOVA procedure (y=’block’+’tree age’+’product). ANOVA procedure was followed by Fisher’s LDS test to ascertain which groups were significantly different from water treated control. Significance level of $p<0.05$ was used to declare the product effective.

Study diary

The diary of tasks completed in 2019 is shown in Table 4.

Table 4. Schematic diary of the study. Different numbers/colours represent different tasks: 1) product application, 2) spray inoculation of trees, 3) sampling and bacterial population estimation, 4) phytotoxicity assessment. NA, product was not available for application due to the delay in delivery. Canker disease assessments were completed on 16th and 17th April 2020.

Product	05-Aug	06-Aug	08-Aug	14-Aug	26-Aug	29-Aug	03-Sep	07-Sep	12-Sep	23-Sep	24-Sep	03-Oct	15-Oct
Untreated, NOT INOCULATED						4		3			3		
Untreated INOCULATED	1	2 3	3	1	1	4	1	3	1	1	3	1	2
Cuprokylt	1	2	3		1	4			1		3	1	2
AHDB9885	1	2	3	1	1	4	1		1	1	3	1	2
AHDB9829	1	2	3		1	4			1		3	1	2
Serenade ASO	1	2	3	1	1	4	1		1	1	3	1	2
Amylo-X	1	2	3	1	1	4	1		1	1	3		2
AHDB9831	1	2	3	1		4			1		3	1	2
AHDB9959	1	2	3	1	1	4	1		1	1	3	1	2
AHDB9957	NA	2	3	1	1	4	1		1	1	3	1	2

Work responsibilities

- Protocol development: Matevz Papp-Rupar
- Sourcing of trees and trial set up: Matevz Papp-Rupar
- Inoculum prep: Sarah Cohen
- Inoculum application: Tom Passey
- Product application: trials team lead by Alin Borleanu (NIAB-TAG)
- Sampling and bacterial population estimation: Jennifer Kingsnorth, Sarah Cohen, Matevz Papp-Rupar
- Phytotoxicity assessment: Matevz Papp-Rupar
- Canker symptom assessment: Matevz Papp-Rupar
- Reporting: Matevz Papp-Rupar, Lucas Shuttleworth

Results

Phytotoxicity

The majority of the treated trees showed no abnormal leaf discoloration, necrosis or shoot deformations when compared to untreated un-inoculated trees or water treated inoculated trees (Figure 1).

Figure 1: Representative images of blemishes and spots observed on the leaves taken from trees in block 3 (1-year old trees) on 29.8.20. Panel 1 shows Untreated NOT INCULATED leaves. Leaves on all other panels were inoculated with bacterial suspension and treated with water (2), Cuprokylt (3), AHDB9885 (4), AHDB9829 (5), Serenade ASO (6), Amylo-X (7), AHDB9831 (8), AHDB9959 (9) and AHDB9957 (10). Leaf appearance of the product treated trees (3-10) was compared to and water treated control (2).

The leaf appearance was slightly better on 4 year-old trees (data not shown) than on 1-year old trees (Figure 1). We suspect that this was because young trees in smaller pots were more susceptible to heat and accompanying drought stress during the hot days in July and August 2019 (Supplementary Figure 3). Most leaves on most of the trees had minor blemishes and spotting that could be attributed to either *Pseudomonas* infection (discrete spots and shot-holes) or physiological stress and were not distinct from water treated control used for comparison. The only treatment that caused clear phytotoxicity was AHDB9831 (Figure 1, panel 8). Extensive chlorosis and necrosis was observed on the leaves of all trees in all blocks. Moreover, the treatment resulted in 50-70% leaf drop (Figure 2) after 2 product applications. Applications of AHDB9831 were thus reduced from every 10 days to every 3 weeks on average.

Figure 2. Example of leaf drop on trees after 2 applications of AHDB9831 (right panel) compared to water treated control (left panel)

Product effect on *Pseudomonas* population on leaves

Two days after inoculation (3 days after product application) *Pseudomonas* populations on leaves was measured to estimate preventative effect of the products (

Figure 3). Analysis of variance (ANOVA) indicted that 'block' and 'product' applied had significant effect ($p < 0.05$) on *Pseudomonas* population size at this assesmen, while tree age' had no significant effect (Table 5, Supplementary Figure 4). Population size on trees pre-treated with AHDB9829 (fungicide), Cuprokylt and AHDB9831 (bactericide) was significantly lower than on water treated trees (

Figure 3). The most efficacious product was AHDB 9829 which decreased population by ~10 fold (92%) in comparison to water treated control. Cuprokylt and AHDB9831 decreased populations by cca 3 fold (74%). Other products slightly decreased the population size compare to water treated control, the differences however were not significant. The inoculum level on the leaves immediately after inoculation / 1 day after product application was 3,000 CFU/ml (3.5 Log10 CFU/mL).

Table 5. Statistical analysis of *Pseudomonas* population size sampled from cherry leaves on 8th Aug 2019.

Source of variation	***Degrees of freedom***	***Sum of squares***	***Mean squares***	***Variance ratio***	***p-val***
Block	3	5.5003	1.8334	4.3	0.007
Tree age	1	0.5283	0.5283	1.24	0.269
Product	7	11.6847	1.6692	3.91	<.001
Residual	87	37.0952	0.4264		
Total	98	54.8085	0.5593		

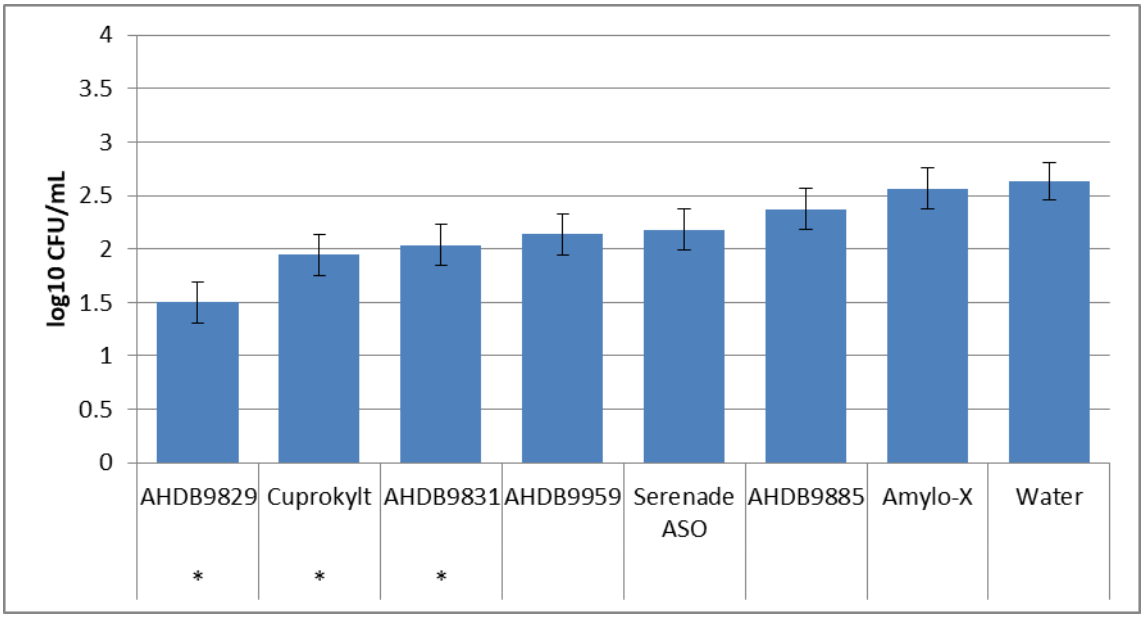

Figure 3. *Pseudomonas* population size on leaves (8 Aug 2019). Colony forming units (CFU) per ml of suspension +/- 1 SEM is shown. Products with *Pseudomonas* population significantly lower than the water control are denoted with an asterisk (*). One ml of suspension equals 1.5 square cm of leaf tissue.

We were concerned that the *Pseudomonas* population on leaves would reduce considerably due to the hot (max daily temp over 30°C) and dry conditions in August 2019 (Supplementary Figure 3) We performed additional assessment of population at the beginning of September (7/9/19), to assess weather disease pressure on inoculated trees was still sufficiently high. Interestingly, *Pseudomonas* population size on water treated control trees was at the same level (~2.5 log10 or ~300 CFU/mL) as at the beginning of August indicating high resilience of the pathogen to adverse weather conditions. Additional summer inoculation was therefore not required.
The last assessment of Pseudomonas population on leaves was done at leaf fall to estimate the combined effect of multiple product applications. ANOVA (Table 6, Supp. figure 5) indicated that the 'product' had a marginal effect (p-val=0.055) on Pseudomonas population size while 'block' and 'tree age' had no statistically significant effect. Fisher's LDS test has identified 2 products that have stat. sig. reduced population below the levels of water treated control, i.e. AHDB9829 and Serenade ASO, both with ~10 fold or 90% decrease in population. Interestingly, Cuprokylt and AHDB9831 have reduced population at the first assessment in summer (

Figure 3) but not at leaf fall (Figure 4). The opposite trend was observed for Serenade ASO that has decreased population in autumn but not in summer. The rest of the products decreased *Pseudomonas* population on leaves at leaf fall by 5 – 40% in comparison to water treated control, which was not stat. significant.

Table 6. Analysis of an unbalanced design using Genstat regression, variable: *Pseudomonas* population size on 24th Sep 2019

Source of variation	*Degrees of freedom*	*Sum of squares*	*Mean squares*	*Variance ratio*	*p-val*
Block	3	3.8508	1.2836	1.39	0.25
Tree age	1	0.9191	0.9191	1	0.321
Product	9	16.1319	1.7924	1.94	0.055
Residual	92	84.8004	0.9217		
Total	105	105.7021	1.0067		

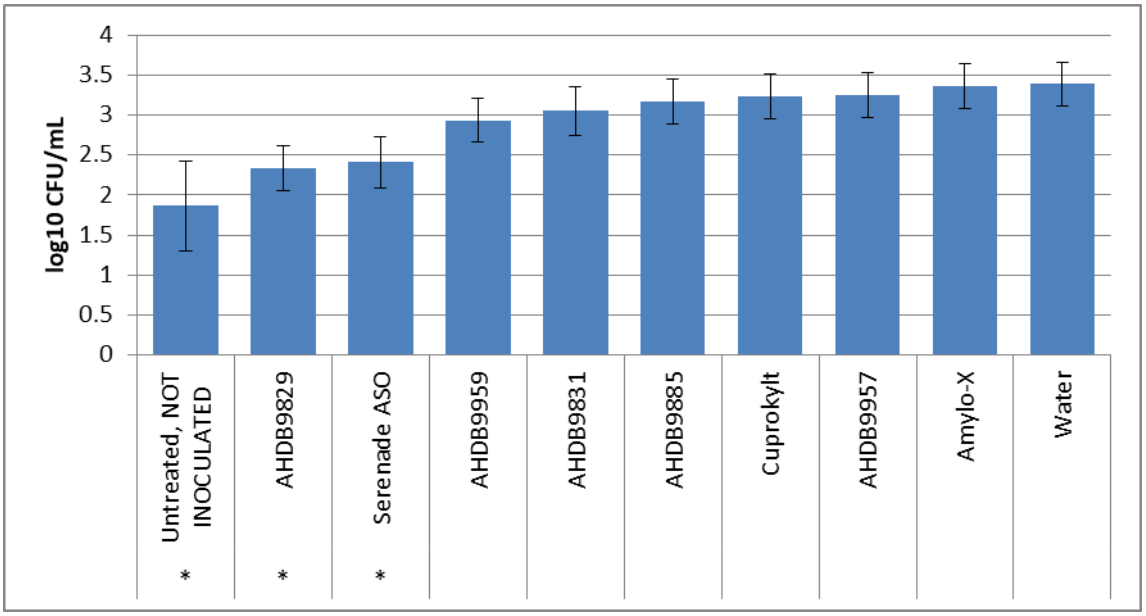

Figure 4. Pseudomonas population size on leaves (24 Sep 2019). Colony forming units (CFU) per mL of suspension +/- 1 SEM is shown. Products with Pseudomonas population stat sig. smaller than the water control are denoted with an asterisk (*). One mL of suspension equals to 1.5 square cm of leaf tissue.

Product effect on bacterial canker symptom expression

Elicitor products (AHDB9959, AHDB9957) increase plant defence rather than decrease population of *Pseudomonas* directly therefore we conducted additional symptom assessment at the end of flowering period in April 2020.If elicitors do increase plant defence then we would expect to observe less bacterial canker symptoms in the following spring despite bacterial populations at leaf fall not being significantly different from water treated trees (Figure 4). To ensure that pathogen population was sufficiently high and uniform to detect small differences in symptom expression we re-inoculated all treated trees again at cca 50-75% leaf fall.
ANOVA analysis indicated that 'block', 'tree age' and 'product' had significant effect on disease incidence (Table 7) and severity (Table 8). Since 'tree age' was uniform in 3 out of 4 blocks the 'block' effect could mostly be attributed to the 'tree age' effect. Smaller, younger trees had considerably higher % infected buds and much higher no. of cankers than older trees. This indicates that young trees in the first 1-2 years after planting may be more susceptible.

Table 7. Analysis of canker incidence (% of infected buds)

Source of variation	Degrees of freedom	Sum of squares	Mean squares	Variance ratio	p-val
Block	3	36133.5	12044.5	34.94	<.001
Tree age	1	8051.4	8051.4	23.36	<.001
Product	9	22516.2	2501.8	7.26	<.001
Residual	89	30676.5	344.7		
Total	102	97377.7	954.7		

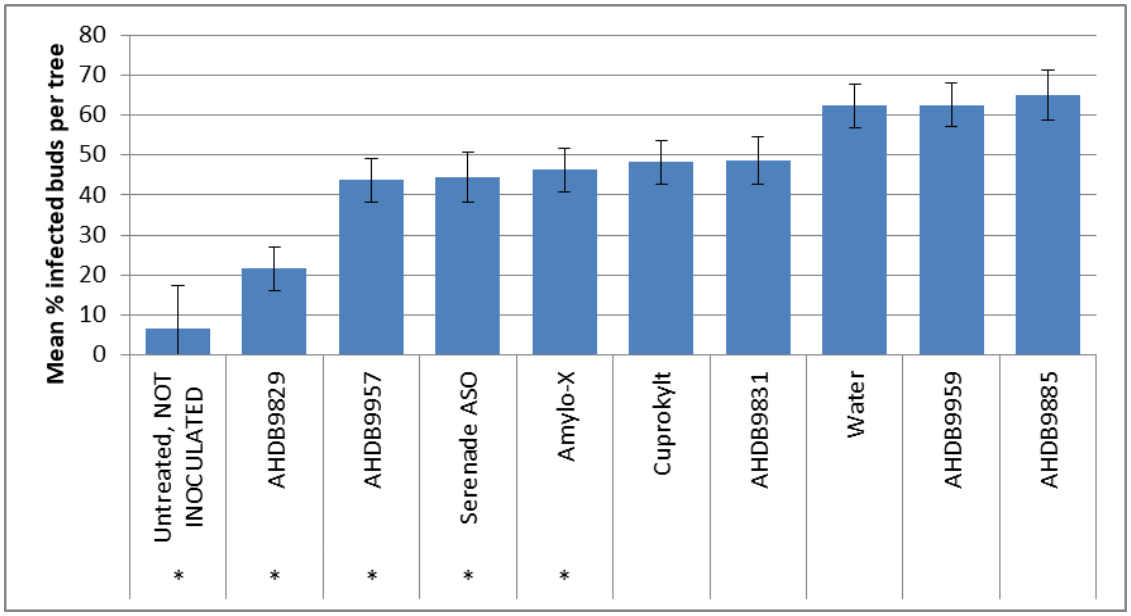

Figure 5. Mean % infected buds +/- 1 SEM. Brown/dry buds together with buds showing gumming and splitting were considered infected. Products with stat. sig. lower mean % of infected buds in comparison to water control are denoted with an asterisk (*)

Four products showed significantly lower incidence (% dead buds) than the water control (Figure 5). Interestingly, AHDB9829 (fungicide) and Serenade ASO (biocontrol agent) that had reduced bacterial populations in summer (

Figure 3) and autumn (Figure 4) had also decreased disease incidence in spring (Figure 5)

despite the fact that all products were applied at least 2 weeks before inoculation at leaf fall. Furthermore, AHDB9829 treated trees were the observed to be the healthiest (Figure 7, left panel) and were the only trees with significantly lower number of cankers compared to the water control (Figure 6). Interestingly, AHDB9957 (elicitor) and Amylo-X (biocontrol agents) also significantly decreased disease incidence to a similar level as Serenade ASO (Figure 5), but did not significantly reduce bacterial populations in summer or autumn (

Figure 3, Figure 4) nor canker severity (Figure 6). No product application resulted in significantly higher disease incidence or severity than in water treated control. Examples of different canker incidence and severity are presented in Figure 7 and 8.

Table 8. Analysis of canker severity (number of canker lesions)

Source of variation	***Degrees of freedom***	***Sum of squares***	***Mean squares***	***Variance ratio***	***p-val***
Block	3	357.547	119.182	20.81	<.001
Tree age	1	30.083	30.083	5.25	0.024
Product	9	156.085	17.343	3.03	0.003
Residual	89	509.624	5.726		
Total	102	1053.34	10.327		

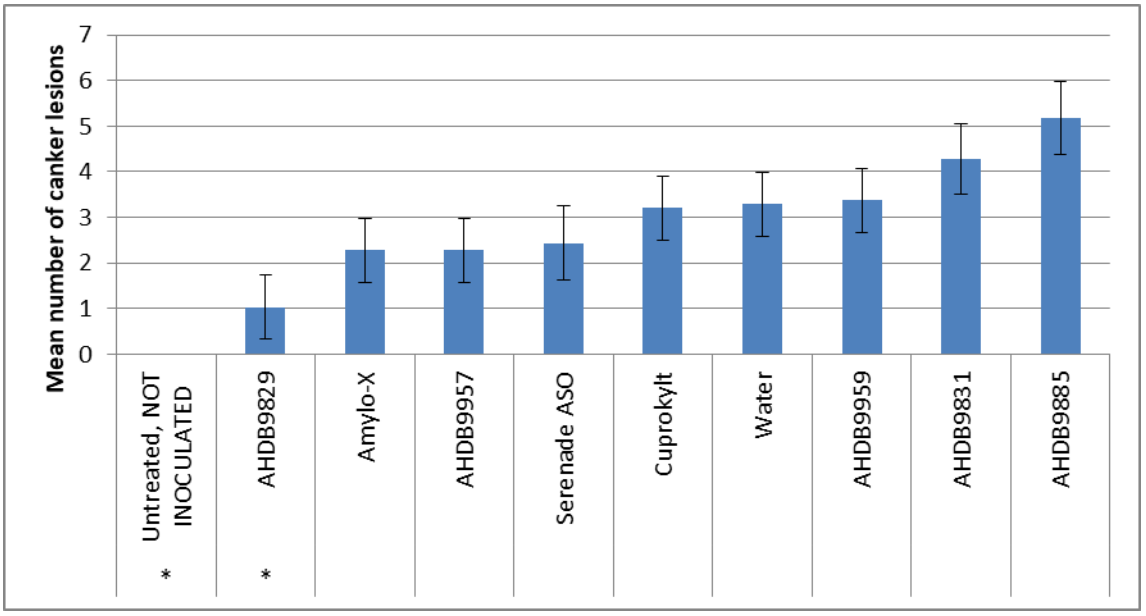

Figure 6. Mean number of canker lesions per tree +/- 1 SEM. Products with stat. sig. lower number of canker lesions in comparison to water control are denoted with an asterisk (*).

Figure 7. Examples of one year-old trees with different amounts of dead buds. On the left is an AHDB9829 treated tree with very low number on dead buds (appx 5%, arrows). On the right is an ADHB9885 treated tree with majority of the buds dead (80%)

Figure 8. Examples of bacterial canker symptoms observed on the trees: splitting of the bark (left) and gumming/oozing observed around and between dead buds (right).

Discussion

We assessed phytotoxicity of products after 2-3 applications. The only product with conclusive signs of phytotoxicity was AHDB9831, which caused leaf yellowing/necrosis and leaf drop. We assume that the application rate of 2.4 g/L was too high for cherry leaves. The same application rate was successfully used to control Citrus Variegated Chlorosis caused by *Xylella fastidiosa* on sweet orange (Muranaka et al., 2013). The study reported no phytotoxicity of the product when sprayed, after being supplied through hydroponic solution or applied with organic fertiliser. Higher sensitivity of cherry trees to this product in comparison to sweet orange is probably the reason for the observed phytotoxicity. Optimisation of spray rate and also assessment of alternative application methods such as application as granules in soil at panting would need to done to properly assess the efficacy of this product. Additional investigation of this product is recommended because a) this product is relatively safe, i.e. it is used in medicine (Sansone & Sansone, 2011) and as a food supplement, and b) it stat. sig. decreased *Pseudomonas* population after a single preventative spray. This preventative scenario would be applicable in the field when a warm and wet weather spell conducive for bacterial proliferation is forecasted. In this circumstances preventative spray might be one of the options for effective control.

Two other products decreased *Pseudomonas* populations after a single preventative spray, i.e. AHDB9829 and Cuprokylt, which are both copper based. However, Cuprokylt is no longer approved, and was used for efficacy comparison only. Product AHDB9829 was overall the most effective. It decreased *Pseudomonas* populations on both assessments by cca 90% (10 fold) and was the only product that significantly decreased both incidence and severity of the symptoms in spring 2020 (both by cca 60%). Reduced disease after second inoculation was most likely due to copper build up on the trees that kept the second inoculum dose and subsequent bacterial populations from causing disease. It was more effective than Cuprokylt on all assessments and is the best product for its direct, although short-term substitution. Due to copper-based mode of action of AHDB9829 it is unlikely to obtain approval for use long term. The latest pesticide legislation is severely restricting the use of copper based products due to the detrimental effects on human health and environment. Moreover, copper-resistant *Pseudomonas* strains have been found in the UK (Roberts, 2015) and abroad (Scheck et al, 1996; Sundin et al, 1989). Our results on population size on Cuprokylt treated trees also hint to potential resistance to copper oxychloride. The *Pseudomonas* population on leaves was reduced after a single use in summer, but not after repeated use in autumn. To minimise resistance, AHDB9829 should be limited to emergency use, or for uses such as protecting young trees in the first few seasons after planting. For best results, spray timing should follow the timing used in this study.

The second most effective product overall was Serenade ASO. This product did not significantly decrease *Pseudomonas* populations after a single preventative spray but the decrease was still sizable (~65%) and it significantly reduces *Pseudomonas* populations after multiple curative sprays. The lack of immediate effect after a single application was expected since biocontrol bacteria (*Bacillus subtilis* strain QST 713) require time to establish. Moreover, Serenade ASO significantly reduced disease incidence in April 2020 and slightly reduced severity as well, indicating that it either remains active as a biocontrol for some time after the last application or primes plant defences. It would be interesting to measuree the survival of *Bacillus subtilis* strain QST 713 on the trees in autumn and spring to investigate this further. Serenade ASO is environmentally friendly, user and consumer safe and is probably the most obvious alternative option for control of bacterial canker. Its drawbacks are associated with higher labour and product cost associated with high frequency of spray applications, and dependence of its efficacy on environmental conditions.

The last two products with some efficacy were AHDB9957 and Amylo-X. As expected, defence elicitor AHDB9957 did not directly decrease *Pseudomonas* population size on leaves in summer or autumn. It did however stat. sig decrease disease incidence and slightly reduced severity as well. The effects were not large, ~30% hence we would recommend to use this product combined in a program with Serenade ASO as additional protection.

We would expect a biocontrol product like Amylo-X, to either compete with the pathogen or actively control it by secreting antibacterial compounds resulting in reduced populations on leaves. Surprisingly, we only observed small (6-14%) reductions of *Pseudomonas* populations on Amylo-X treated trees in summer and autumn assessments. This indicated that the active bacterial component of Amylo-X probably occupied a different environmental niche than *Pseudomonas* spp in summer and autumn and hence did not effectively control population size. Amylo-X did however stat. sig. decrease disease incidence (29%), and also slightly decreased severity. It is possible that the ecological niche of the active organism in Amylo-X is limited to leaf scars and/or other wounds where it successfully competes with or controls *Pseudomonas* spp. to reduce disease incidence. Therefore, we would recommend using Amylo-X in combination with either Serenade ASO and/or AHDB9957.
We observed no stat. sig. control using AHDB9959 and AHDB9885 products. However, AHDB9959 did slightly decrease summer and autumn populations of *Pseudomona*s. Therefore, it might provide some additional control when used in combination with the other effective products identified in this study.

The study was not without challenges. The delivery of AHDB9957 was delayed for several weeks which resulted in delayed study commencement and also in exclusion of AHDB9957 from the first spray application. Hence, we couldn't assess the efficacy of single preventative spray for this product.
A knock on effect of delayed trial start was that it pushed back the last spray application and population assessment closer to full leaf fall period. To make sure we had sufficient amount on leaves for randomised sampling on all trees we decided to assess the autumn *Pseudomonas* populations earlier than planned, i.e. before the last product application. Thus, the measurement of *Pseudomonas* populations at leaf fall did not capture the effect of the last product application. The compound effect of all product applications was only captured in the spring symptom assessment.
High inoculation success and symptom expression allowed reliable estimation of product efficacy in terms of reducing disease incidence and severity which has been difficult in the past. Inoculation success was in line with reports from Crosse & Garrett, (1970) that showed almost 100% infection rate when inoculating fresh leaf scars on field grown trees with a high inoculum concentration of Psm ($1x10^7$ CFU / ml).

Conclusions

Serenade ASO, AHDB9957 and Amylo-X exhibited the most effective control of bacterial canker of cherry. They could become a key part of an integrated disease management program before and during leaf fall.

Copper-based AHDB9829 was the most effective and could offer direct replacement for Cuprokylt to protect high risk orchards in the short term. Bactericide AHDB9831 showed some encouraging results, however further investigation in application method and rate is required to fully assess its potential in combating bacterial canker of cherry.

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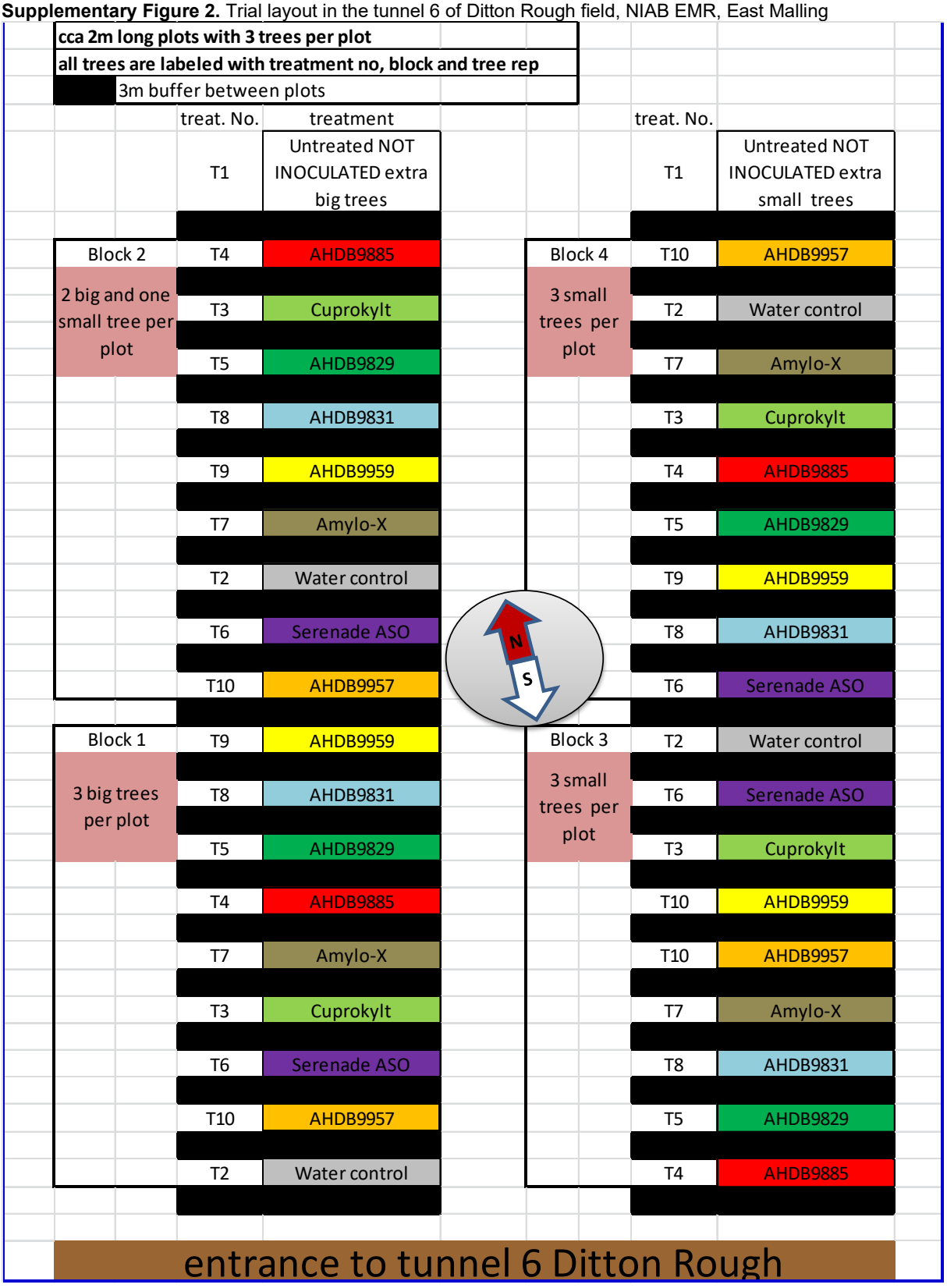
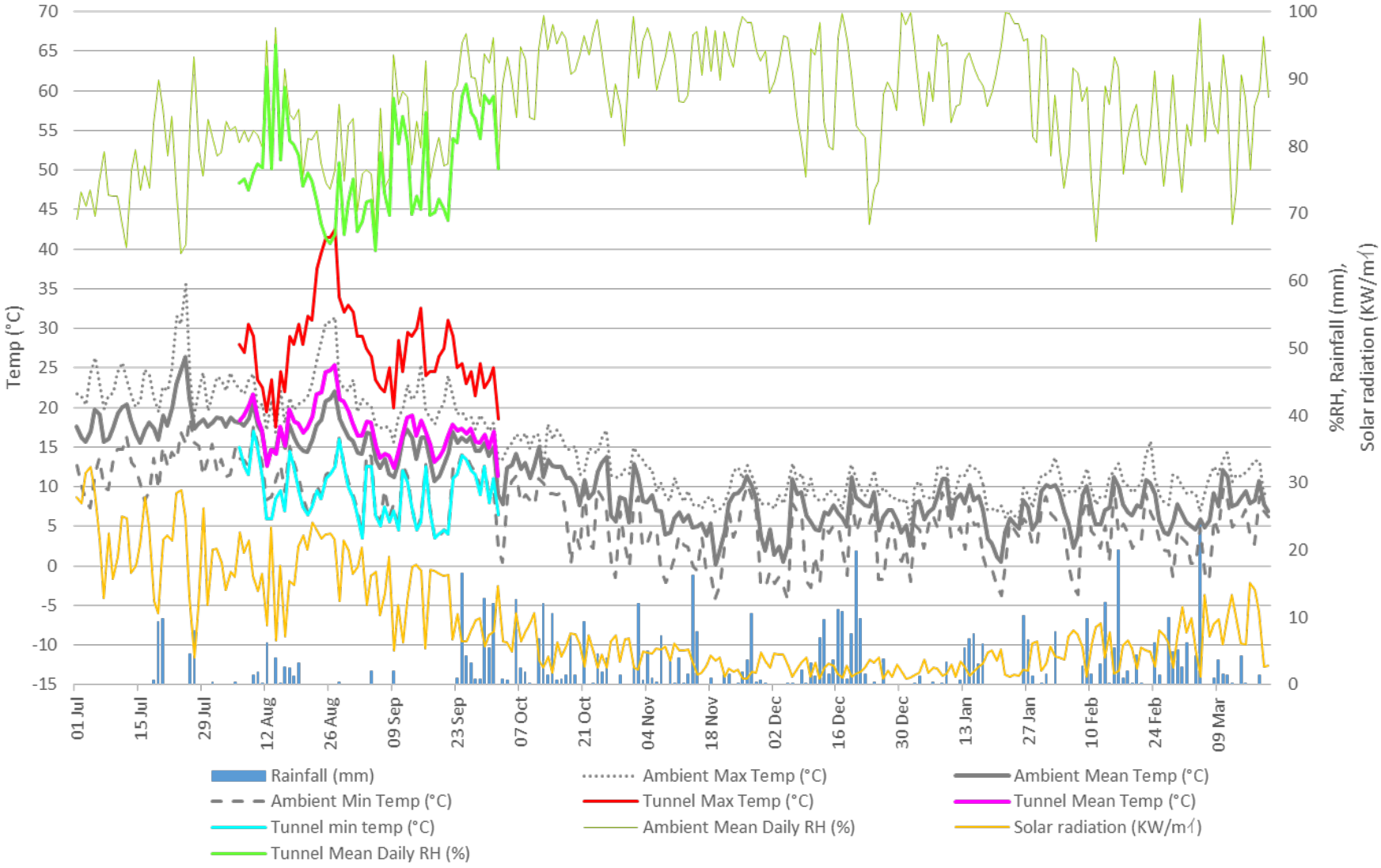
Supplementary data:

Supplementary Figure 1. The polytunnel at NIAB EMR showing the bacterial canker of cherry experiment. Two different tree ages used. Note the plot size of three trees per plot separated by 3 m buffer.

Supplementary Figure 2. Trial layout in the tunnel 6 of Ditton Rough field, NIAB EMR, East Malling



Supplementary Figure 3. Weather parameters recorded in the experimental tunnel and at the NIAB EMR weather station (ambient) during the study.

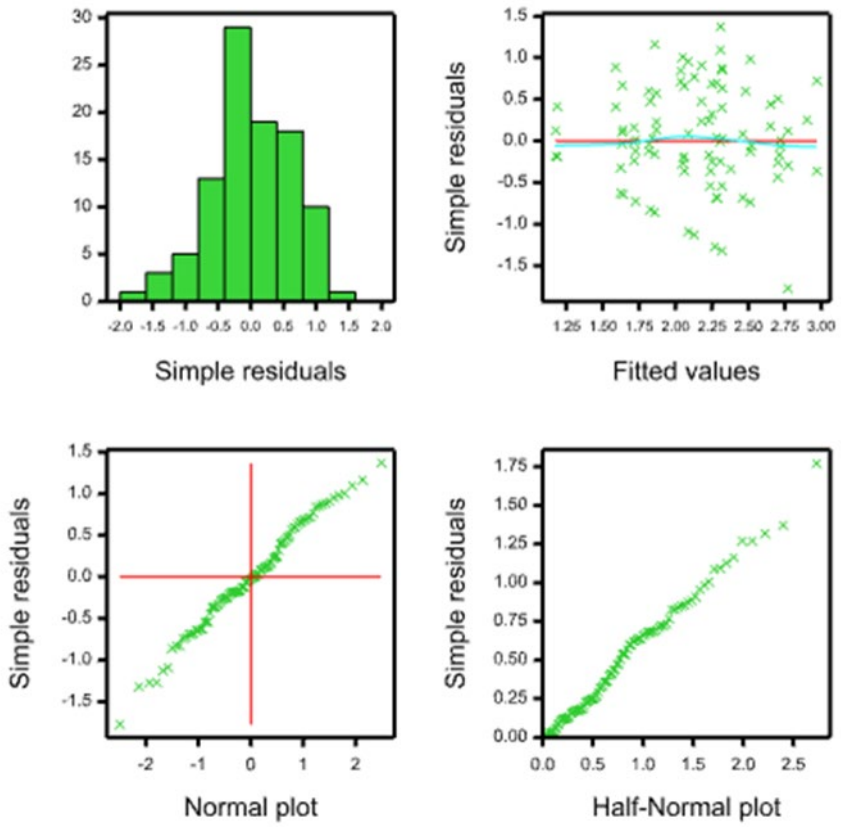


Supplementary Table 1. Data obtained in the study as means per tree.

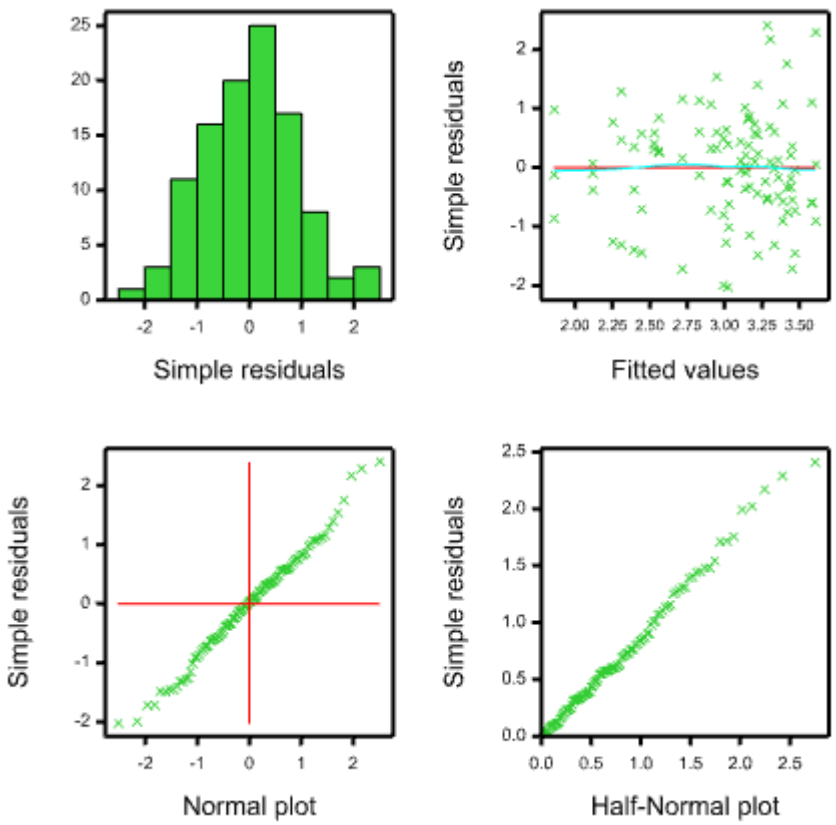
Block	Tree	Tree age (years)	Treatment no.	Product code	*Pseudom.* population on leaves (log10 cfu/ml)		% infected buds per tree, April 2020	Canker symptoms per tree, April 2020
					8/8/19	24/9/19		
2	1	1	1	Untreated, NOT INOCULATED	/	1.00	5.00	0
2	2	1	1	Untreated, NOT INOCULATED	/	1.74	10.00	0
2	3	1	1	Untreated, NOT INOCULATED	/	2.85	5.00	0
1	1	4	2	Water	3.40	3.19	23.33	0
1	2	4	2	Water	3.68	2.88	16.67	0
1	3	4	2	Water	2.30	3.48	30.00	0
2	1	1	2	Water	2.89	5.90	85.00	7
2	2	1	2	Water	1.00	3.66	90.00	4
2	3	1	2	Water	2.48	2.70	70.00	3
3	1	1	2	Water	3.19	2.85	95.00	8
3	2	1	2	Water	1.00	4.04	80.00	6
3	3	1	2	Water	2.72	2.02	80.00	3
4	1	1	2	Water	3.70	3.65	60.00	0
4	2	4	2	Water	2.66	3.26	58.33	5
4	3	4	2	Water	2.73	3.11	21.67	0
1	1	4	3	Cuprokylt	1.00	3.94	16.67	0
1	2	4	3	Cuprokylt	1.00	3.30	36.67	0
1	3	4	3	Cuprokylt	1.30	3.78	23.33	0
2	1	1	3	Cuprokylt	1.00	1.74	60.00	2
2	2	1	3	Cuprokylt	2.18	3.81	50.00	6
2	3	1	3	Cuprokylt	3.04	2.90	50.00	1
3	1	1	3	Cuprokylt	1.60	3.65	65.00	4
3	2	1	3	Cuprokylt	2.30	2.78	90.00	7
3	3	1	3	Cuprokylt	1.74	1.74	80.00	4
4	1	1	3	Cuprokylt	1.60	2.74	60.00	7
4	2	4	3	Cuprokylt	2.88	5.70	5.00	4
4	3	4	3	Cuprokylt	2.75	2.78	5.00	0
1	1	4	4	AHDB9885	1.78	3.15	31.67	0
1	2	4	4	AHDB9885	3.05	2.78	8.33	0
1	3	4	4	AHDB9885	1.85	4.16	40.00	0
2	1	1	4	AHDB9885	2.39	2.48	100.00	12
2	2	1	4	AHDB9885	1.78	2.81	80.00	13
2	3	1	4	AHDB9885	3.50	4.47	60.00	5
3	1	1	4	AHDB9885	1.88	3.54	80.00	3
3	2	1	4	AHDB9885	2.72	4.49	70.00	2
3	3	1	4	AHDB9885	1.70	3.28	90.00	9
1	1	4	5	AHDB9829	1.00	3.60	16.67	0
1	2	4	5	AHDB9829	1.30	1.00	6.67	0
1	3	4	5	AHDB9829	1.00	2.78	13.33	0
2	1	1	5	AHDB9829	1.00	3.41	10.00	0
2	2	1	5	AHDB9829	1.78	2.85	10.00	0
2	3	1	5	AHDB9829	1.00	2.81	30.00	2
3	1	1	5	AHDB9829	1.00	2.19	65.00	2
3	2	1	5	AHDB9829	1.60	1.74	10.00	2
3	3	1	5	AHDB9829	1.00	2.02	40.00	0
4	1	1	5	AHDB9829	1.88	2.74	10.00	3
4	2	4	5	AHDB9829	2.00	2.02	5.00	0
4	3	4	5	AHDB9829	2.48	1.00	5.00	0
1	1	4	6	Serenade ASO	1.70	1.74	15.00	0
1	2	4	6	Serenade ASO	3.02	3.02	13.33	0
1	3	4	6	Serenade ASO	1.00	1.00	13.33	0
3	1	1	6	Serenade ASO	2.10	3.02	75.00	7
3	2	1	6	Serenade ASO	2.44	3.02	80.00	3
3	3	1	6	Serenade ASO	2.00	1.00	35.00	2
4	1	1	6	Serenade ASO	2.46	3.11	80.00	4
4	2	4	6	Serenade ASO	1.00	2.93	6.67	2
4	3	4	6	Serenade ASO	1.00	2.85	40.00	0

Supplentary Table 2. Data obtained in the study as means per tree (cont').

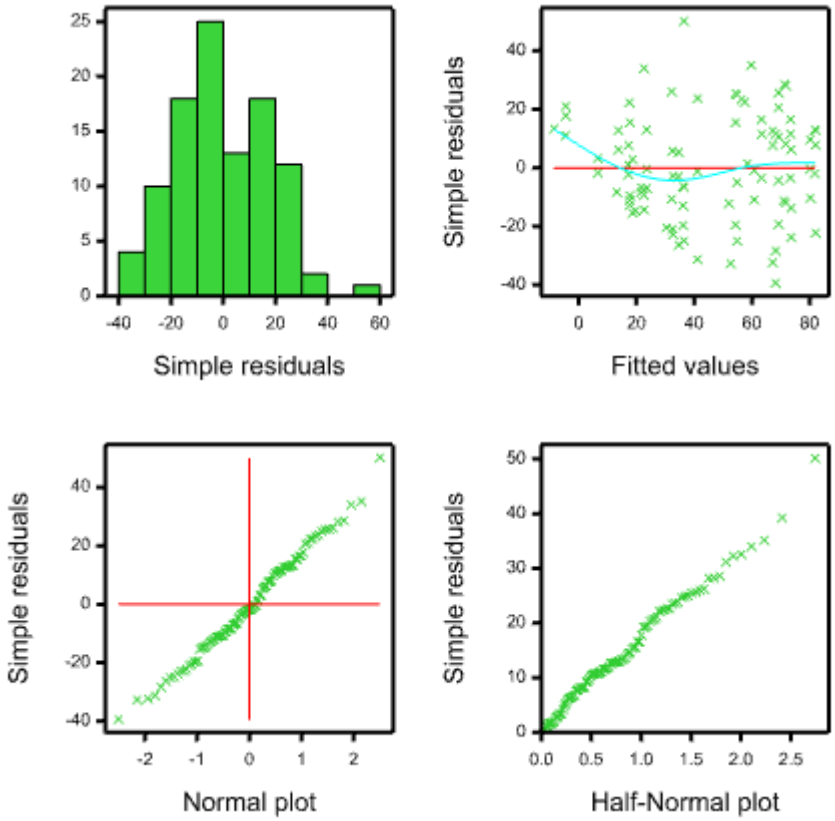
Block	Tree	Tree age (years)	Treatment no.	Product code	Bact population on leaves (log10 cfu/ml)		% infected buds per tree, April 2020	Canker symptoms per tree, April 2020
					8/8/19	24/9/19		
1	1	4	7	Amylo-X	1.88	3.31	18.33	0
1	2	4	7	Amylo-X	1.70	3.94	15.00	0
1	3	4	7	Amylo-X	1.70	2.02	25.00	0
2	1	1	7	Amylo-X	2.27	4.69	70.00	2
2	2	1	7	Amylo-X	3.21	2.98	35.00	2
2	3	1	7	Amylo-X	2.44	3.00	80.00	6
3	1	1	7	Amylo-X	2.00	3.18	50.00	5
3	2	1	7	Amylo-X	2.94	3.35	95.00	5
3	3	1	7	Amylo-X	2.57	3.06	80.00	2
4	1	1	7	Amylo-X	3.16	5.18	40.00	2
4	2	4	7	Amylo-X	2.83	2.66	5.00	0
4	3	4	7	Amylo-X	3.09	3.08	5.00	0
1	1	4	8	AHDB9831	1.70	1.00	8.33	0
1	2	4	8	AHDB9831	1.48	2.78	13.33	0
1	3	4	8	AHDB9831	1.88	3.41	56.67	0
2	1	1	8	AHDB9831	3.09	3.04	100.00	0
3	1	1	8	AHDB9831	1.74	3.44	29.00	2
3	2	1	8	AHDB9831	1.60	2.70	40.00	12
3	3	1	8	AHDB9831	1.00	3.97	80.00	7
4	1	1	8	AHDB9831	2.04	3.22	80.00	8
4	2	4	8	AHDB9831	2.89	3.54	21.67	0
4	3	4	8	AHDB9831	1.00	3.06	3.33	0
1	1	4	9	AHDB9959	2.41	3.23	30.00	0
1	2	4	9	AHDB9959	1.70	3.98	86.67	0
1	3	4	9	AHDB9959	1.78	2.19	11.67	0
2	1	1	9	AHDB9959	1.60	4.00	60.00	5
2	2	1	9	AHDB9959	2.32	3.54	50.00	4
2	3	1	9	AHDB9959	2.98	3.97	80.00	9
3	1	1	9	AHDB9959	1.00	2.88	95.00	8
3	2	1	9	AHDB9959	2.30	3.88	60.00	4
3	3	1	9	AHDB9959	1.00	1.00	90.00	7
4	1	1	9	AHDB9959	3.08	2.19	100.00	0
4	2	4	9	AHDB9959	2.48	1.00	11.67	0
4	3	4	9	AHDB9959	2.06	3.48	38.33	0
1	1	4	10	AHDB9957	/	3.78	8.33	0
1	2	4	10	AHDB9957	/	2.78	5.00	0
1	3	4	10	AHDB9957	/	4.62	33.33	0
2	1	1	10	AHDB9957	/	2.02	30.00	0
2	2	1	10	AHDB9957	/	2.74	80.00	0
2	3	1	10	AHDB9957	/	3.43	50.00	0
3	1	1	10	AHDB9957	/	2.41	80.00	4
3	2	1	10	AHDB9957	/	2.02	75.00	6
3	3	1	10	AHDB9957	/	2.41	60.00	6
4	1	1	10	AHDB9957	/	5.48	20.00	3
4	2	4	10	AHDB9957	/	3.40	20.00	5
4	3	4	10	AHDB9957	/	4.00	26.67	0



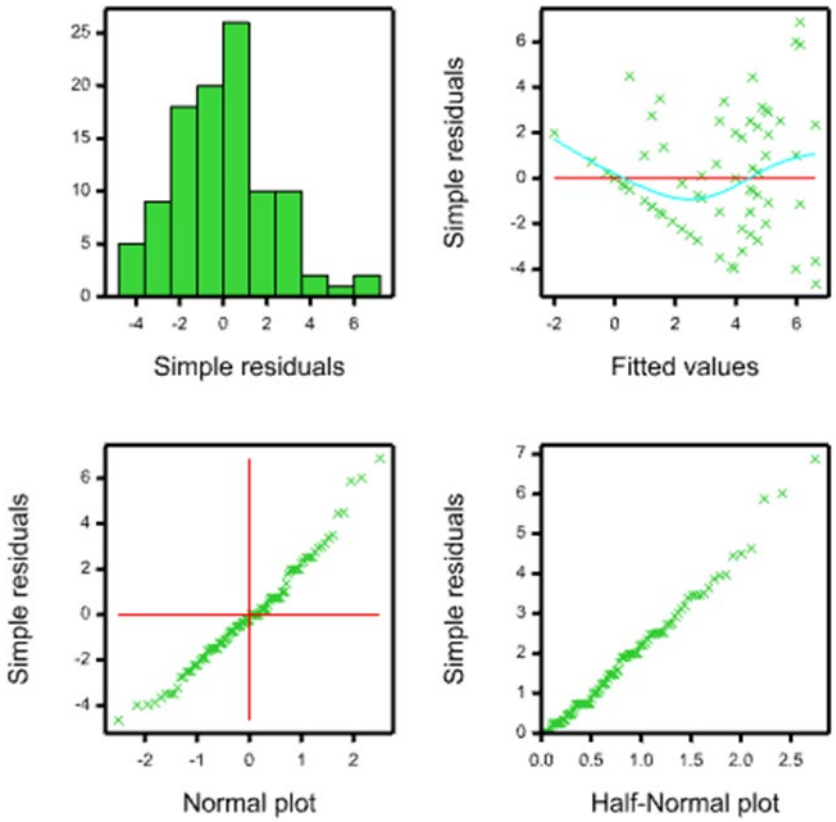
Supplementary Figure 4. Residual plots for Pseudomonas population size on leaves measured on 8th Aug 2019.



Supp. figure 5: Residual plots of Pseudomonas population size on leaves measured on 24th Sep 2019.



Supplementary Figure 6. Residual plots of % infected buds per tree as assessed in April 2020



Supplementary Figure 7. Residual plots of total no. of clear bacterial canker symptoms per tree