SCEPTREPLUS

Final Trial Report

Trial code:	SP19
Title:	Laboratory and greenhouse evaluation of alternative products for control of bacterial canker – *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *mors prunorum*
Crop	Cherry (results also applicable to fruiting and ornamental *Prunus* sp.)
Target	Bacterial canker
Lead researcher:	Angela Berrie / Robert Saville / Matevz Papp-Rupar
Organisation:	NIAB-EMR
Period:	March 2018- September 2018
Report date:	19/09/2018 (report re-issued 25 February 2019)
Report author:	Matevz Papp-Rupar
ORETO Number: (certificate should be attached)	ORETO 321 is NIAB EMR's 2017 certificate of Official Recognition of Efficacy Testing Facilities or Organisations in the United Kingdom. Notification of renewal for 2018 has been received, but the new certificate number has not yet been issued. The certificate and renewal notification letter are given in the Appendix.

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 Authors signature

Trial Summary

Introduction

Bacterial canker of *Prunus* trees in the UK is caused by *Pseudomonas syringae* pv. *syringae (PSS)* and *Pseudomonas syringae* pv. *morsprunorum* (*PSM*). Previously the disease has been kept below economically acceptable levels by applying a copper-based product to knock-down bacterial populations prior to leaf fall. New regulations on use of copper for plant protection means that copper will no longer be available for the control of bacterial canker and new products are required to manage and reduce disease outbreaks.

Methods

Here the current industry standard (Cuprokylt) has been tested alongside two botanical based products (AHDB9884, AHDB9885), two microbial based biocontrol products (Serenade, AmyloX), two common sterilants (sodium hypochlorite, hydrogen peroxide) and a food supplement N-acetyl-L-cysteine (NAC) a small molecule related to L- cysteine which has been shown to be effective in the control of other bacterial diseases. *PSS* and *PSM* were challenged *in vitro* on King's B agar plates, detached cherry leaves and detached fruit and disease severity and incidence scored. Most of the tested products are either approved in the UK for use as plant protection products or approved active substances at the EU level or are basic substances or have potential to be basic substances.

Results

Results are summarised in Table 1. Several products showed promising results with NAC, AHDB9884 and hydrogen peroxide performing better or equivalent to the current industry standard across all three assays reducing bacterial population and disease severity. Serenade ASO and AHDB9885 also showed promising results especially on leaf tests.

Conclusions

All five above mentioned products should be taken forward in future trials and assessed in isolation and in combination in semi-field conditions.

Take home message:

In a laboratory based *in vitro* assay against both *Pseudomonas syringae* pv. *syringae* and pv. *morsprunorum*, NAC, AHDB9884 and hydrogen peroxide, Serenade ASO and AHDB9885 showed promising results. They performed better or equivalent to the current industry standard, Cuprokylt, especially on leaf tests and could offer an alternative to copper based products.

Table 1. A summary table of the direct bactericidal effect of different treatments expressed as reduction of growth or symptom development compared to untreated control (the higher the number the better the treatment performed), NC = inconclusive, / = not tested. No treatments statistically significantly reduced growth/symptom development compared to untreated control.

Treatments	Bacterial strain	Assay		
		Reduction of growth on King B agar (log10))	Detached Leaves (% reduction of lesion count)	Detached fruit (% reduction of lesion severity)
Sodium hypochlorite (10-12% avail. chlorine)	*PSS*	3	/	96%
	PSM	3	56%	/
Cuprokylt (industry standard)	*PSS*	1	/	79%
	PSM	1	79%	/
AHDB9885	*PSS*	NC	/	100%
	PSM	NC	75%	/
Serenade ASO	*PSS*	NC	/	Increase of 43%
	PSM	NC	77%	/
Amylo-X	*PSS*	NC	/	0%
	PSM	NC	39%	/
NAC	*PSS*	6	/	79%
	PSM	7	72%	/
Hydrogen peroxide (30%)	*PSS*	6	/	96%
	PSM	7	70%	/
AHDB9884	*PSS*	6	/	100%
	PSM	7	67%	/

Hydrogen peroxide is a basic substance which is currently only approved for use as a seed treatment or for disinfection of tools. The substance is not approved for use in stone fruit and not approved as a spray treatment.
NAC is not approved for plant protection purposes but AHDB plan to investigate the prospect of getting it approved as a basic substance.
Sodium hypochlorite is not approved for plant protection purposes.

Objective

To identify products with activity against bacterial canker in cherry and benchmark their performance in relation to standard treatments through the following aim:
To identify products with direct bactericidal effects on bacterial canker causing *Pseudomonas spp.* in the UK; *Pseudomonas syringae* pv. *syringae (Pss)* and *Pseudomonas syringae* pv. *morsprunorum* (*Psm*),.

Introduction

Bacterial canker of prunus trees is caused by two pathovars of *Pseudomonas* in the UK; *Pseudomonas syringae* pv. *syringae (Pss)* and *Pseudomonas syringae* pv. *morsprunorum* (*Psm*). Previously the disease has been kept below economically damaging levels by applying a copper-based product up to three times at three week intervals from August to reduce bacterial populations prior to leaf fall. New regulations on use of copper for plant protection means that copper will no longer be available for the control of bacterial canker. SCEPTREplus has identified bacterial canker as a target due to the lack of alternative treatments for the management of this bacterial disease. Products with direct bactericidal effect towards *Pseudomonas spp.* were assessed. These products can reduce populations of bacteria and can be introduced as a direct replacement for copper. SCEPTREplus only tests products (Table 2) which are already approved in the UK for use as plant protection products approved active substances at the EU level or substances which have been listed as approved basic substances (Supp. table 5). The only exception from this rule is for N-acetyl-L-cysteine (NAC) which is a well-known food supplement and have been shown to have an effect on bacterial plant pathogen Xylella fastidiosa (Muranaka et al., 2013). Laboratory assays were conducted and then promising candidate products are discussed. This report covers results from *in-vitro* bioassays for bactericidal effects.

Methods

Experiment 1: Laboratory screen for direct bactericidal effect of tested substances

Aim

To determine the direct bactericidal effect of selected treatments (table 1) on isolates of *Pseudomonas syringae* pv. *syringae* (*PSS*) and *Pseudomonas syringae* pv.*morsprunorum* (*PSM*) when grown on King's B agar plates, an artificial medium which is selective for *pseudomonas* growth.

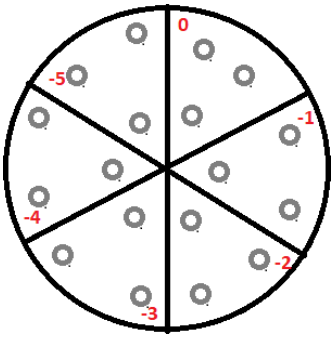
Inoculum preparation

The *PSM* and *PSS* strains were isolated by Michelle Hulin from cherry orchards at NIAB EMR and cultured on King's B agar plates for 2 – 3 days. A single colony was transferred from the plate and then added to the LB-Broth, high salt. Overnight bacterial cultures (50 – 100 ml) were grown in 250 ml conical flasks on an orbital shaker at 180 rpm and 25°C. The next morning multiple 50 ml falcon tubes were centrifuged at 3000rpm for 10 minutes to form a pellet.
Supernatant was removed and cultures re-suspended in 5 ml sterile water. To determine cell number the optical density (OD) at 600nm of suspension was measured on a spectrophotometer and adjusted to 0.2 absorbance units to obtain a suspension of 1×10^8 colony forming units per ml in sterile water.

Experimental procedure

- Double the required concentration of the test product was prepared (Table 2) to allow for optimal concentration after the 1:1 dilution with bacterial suspension.

- Dilution series of OD 0.2 calibrated overnight cultures (see above) of *PSS* and *PSM* was prepared in sterile water (5 steps,10 fold): 1x10^8, 1x10^7, 1x10^6, 1x10^5, 1x10^4 CFU/mL (0, -1, -2, -3, -4, -5).
- 500 µl of each bacterial dilution was mixed with 500 µl of prepared test treatments in a sterile Eppendorf tube.
- 1 minute after mixing 3 x 10 µl drops were plated onto designated position on pre-prepared and pre- labelled King's B agar plates as per diagram below.



- Plates were incubated for 48 h at room temperature. The number of colonies in each drop was counted at 24 and 48h to estimate survival of the *PSS* and *PSM*

Experiment 2: Detached leaf assay

Aim

To estimate the efficacy of treatments to reduce *PSM* symptom development on detached leaves as a simulation of reducing *PSM* populations on leaves.

Inoculum preparation

As per Experiment 1

Plant material

Fresh, blemish-free, about 65 mm long cherry leaves (midrib fold still visible) were collected from susceptible cherry (cv. Sweetheart) 18h before inoculation and stored at 0-4^{o}C until the experiment was setup. Leaves were collected from actively growing shoots on potted trees that had not been treated by any other plant protection substances.

Experimental procedure

- Leaves were cleaned using a standard protocol, briefly leaves were placed in 0.5% sodium hypochlorite for 3 minutes followed by rinsing twice with sterile distilled water (as described in Spotts et al. 2008, Moragrega et al. 2013).
- Ten randomly selected leaves were labelled for each treatment using a permanent marker.
- Lower (abaxial) side of unwounded leaves was sprayed with *PSM* inoculum. 2 presses of atomizer spray were applied to just before runoff and left to dry in a sterile flow hood (as per Mikiciński et al. 2012).
    - The consistency of the atomiser sprayer was measured before use to estimate the volume of inoculum applied. The test indicated that each leaf was sprayed very uniformly with 250 +/- 2 uL of inoculum (appx 2.5 x 10^7 CFU) and treatment solutions.
    - Once the inoculated leaves had dried the inoculum density was determined on control leaves by excising a circular piece of tissue with 0.5 cm diameter using a cork borer, macerating the tissue in an Eppendorf tube with a plastic pestle and plating onto King's B media determine cfu/unit area of leaf.

- Inoculated leaves were sprayed with treatments in random order at single rates (table 2), in the same manner as inoculum (2 presses of atomizer just before run off), and left in the laminar flow hood to dry for 45 min.
- Leaves were placed in high humidity transparent plastic boxes lined with water agar (1%; 5g in 500ml/tray) and wet paper towel to ensure high humidity and incubated in controlled environment cabinets at 22°C and 16h light.
- At 11 days after inoculation, lesions on all leaves were counted. Koch's postulates was satisfied by re-isolation of *PSM* from representative lesions onto King's B agar plates. In all cases *PSM* was re-isolated.

Study design and data analysis

The leaf assay was done with 10 biological replicates per treatment in a randomised block design with 10 blocks and 9 leaves per block (90 leaves in total). Lesion number per leaf was subjected to analysis of variance in Genstat. All products were tested in the same experiment.

Experiment 3: Detached fruit inoculations

Aim

To estimate the efficacy of treatments to reduce the *PSS* symptom development on wounded detached fruit.

Inoculum preparation

As per Experiment 1.

Plant material

Blemish-free immature green cherry fruits sized up to 1 cm in diameter were collected from orchard trees (cv. Sweetheart) 18h before inoculation and stored at 0-4^{o}C until the experiment was setup.

Experimental procedure

- Stalks were removed and fruit surface cleaned using standard plant tissue techniques, briefly, fruit were suspended in 0.5% sodium hypochlorite for three minutes before rinsing three times in sterile distilled water and air dried under the laminar flow hood.
- The fruit was labelled, sprayed with inoculum and test substances, and dried in laminar flow hood.
- Ten randomly selected fruit were labelled for each treatment using a permanent marker.
- Only PSS was used in this assay. Unwounded fruit was sprayed to runoff with inoculum and left to dry in a sterile flow hood. A negative control was mock inoculated with sterile water.
- Treatments at single rate (table 2) were sprayed on the fruit to just before run off in the same manner as inoculum.
- After the products were dry (appx 30 min after spray), one 2 mm deep stab wound was made in each fruit using a sterile needle.
- Inoculated and treated fruit were arranged in clean high humidity plastic boxes lined with water agar (1%; 5g in 500ml/tray) and wet paper to ensure high humidity and incubated in controlled environment cabinets at 22°C and 16h light for 4 days.

- Lesions were assessed at 4 days post inoculation (DPI) using a scale from 0-4: 0 – no necrosis, 1 – lesion diameter 0.1-1 mm, 2 – lesion diameter from 1.1 to 2 mm, 3 – lesion diameter from 2.1 – 4 mm and 4 - lesion diameter >4 mm. PSS was successfully re-isolated from representative lesions at the end of the experiment thus satisfying Koch's postulates.
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Study design and data analysis

Experiments were done with 10 biological replicates per treatment in a randomised block design with 10 random fruit per block. Lesion scores were categorised by a single assessor and subjected to Kruskal-Wallis ANOVA with post hoc Mann-Whitney U test. All products were tested in the same experiment.

Table 2: Products with potential bactericidal properties evaluated in laboratory for control of bacterial canker in cherry. Rates for laboratory use are calculated based on 400L/Ha which is standard UK practice.

reat. No.	Product	AHDB Code CODE	Rate of product / ha	Rate of product / L[1]	ate in 25mls (double in brackets)	Active ingredient	Product type	Company	Reasons for inclusion	Probability of route to market
T1	Untreated	/		-	-	-	-			
T2	Sodium hypochlorite (10-12% available chlorine)	No code needed	1%	10 ml	250 ul (500 ul)	Sodium hypochlorite	Disinfectant	Non -proprietary	Effective bactericide for standard for laboratory tests. Already used by growers	
T3	Cuprokylt	No code needed	3 kg	7.5 g	187.5 mg (375 mg)	Copper oxychloride	Fungicide	Certis	Previous industry standard of known efficacy. Copper still used as a foliar feed.	
T4	AHDB9885	AHDB9885	3 L	7.5 ml	187.5 ul (375 ul)	N/D	N/D	N/D	Product with a botanical active substance showed efficacy in work in USA	Manufacturer is committed to having this product registered for use on tree fruit crops in the UK. Good chance
T5	Serenade ASO	No code needed	10 L	25 ml	625 ul (1250 ul)	Bacillus subtilis strain QST 713	BCA	Bayer	Shown to have activity against bacterial diseases in US and Belgium	Already approved on tree fruit crops in UK
T6	Amylo-X	No code needed	2.5 kg	6.25 g	156.3 mg (312.5 mg)	Bacillus amyloliquefaciens subsp. Plantarum strain D747	BCA	Certis	An alternative product to Serenade ASO	Already approved on horticultural crops in UK
T7	NAC	No code needed	0.24%	2.4 g	60 mg (120 mg)	N-acetylcysteine	Bactericide	Non -proprietary	Shown activity against Xylella.spp. requested for inclusion by AHDB	Requested for inclusion by AHDB. Therefore company interest demonstrated
T8	Hydrogen peroxide (30%)	No code needed	0.5%	16.7 ml	416.7 ul (833.3 ul)	H2O2	Disinfectant	Non -proprietary	Requested for inclusion by AHDB	Basic substance
T9	AHDB9884	AHDB9884	0.4 L	1 ml	25 ul (50 ul)	N/D	N/D	N/D	Comparison to other terpene based products being tested	Manufacturer is committed to having this product registered for use on tree fruit crops in the UK. Good chance

Hydrogen peroxide is a basic substance which is currently only approved for use as a seed treatment or for disinfection of tools. The substance is not approved for use in stone fruit and not approved as a spray treatment.
NAC is not approved for plant protection purposes but AHDB plan to investigate the prospect of getting it approved as a basic substance.
Sodium hypochlorite is not approved for plant protection purposes.

Results

Results of *in-vitro* bactericidal tests on agar plates

Bacterial counts obtained on untreated control plated indicated that the initial populations of *PSM* and *PSS* in 10 ul drop were 5x10^6 and 3x10^7, respectively. Several treatments have shown direct bactericidal effect against *PSS* and *PSM*. Industry standard Cuprokylt reduced the bacterial population by one order of magnitude (10x) in both *PSS* and *PSM* in comparison to untreated control (figure 2). Interestingly there was a ring like growth observed on Cuprokylt treated *PSS* (Supp. figure 1) and *PSM*. Ring like growth could indicate that bacteria managed to grow in the zone where concentration of the Cuprokylt was reduced to sub lethal levels due to diffusion in the media. The most evident reduction in bacterial population was observed in treatments NAC, hydrogen peroxide and AHDB9884 (figure 2). No bacterial growth was observed up to 5 days post plating on any of the bacterial dilutions of above mentioned treatments (supp. figure 2) signifying that these treatments reduced bacterial population below levels of detection from appx 5*10^6 CFU or 3*10^7 CFU in10 ul of *PSM* and *PSS* culture, respectively. We concluded that up to 10 CFU/mL could still be present even when no colonies are detected in any of the replicated 10 ul drops, due to the fact that when subsampling 10 uL from 1 mL stock at very low CFU/mL we could have missed the bacterial cells. This assumption is visualised in the case of NAC, hydrogen peroxide and AHDB9884 in figure 2. The exception was treatment AHDB9884 against *PSM* with 0-3 colonies at the highest bacterial concentration.

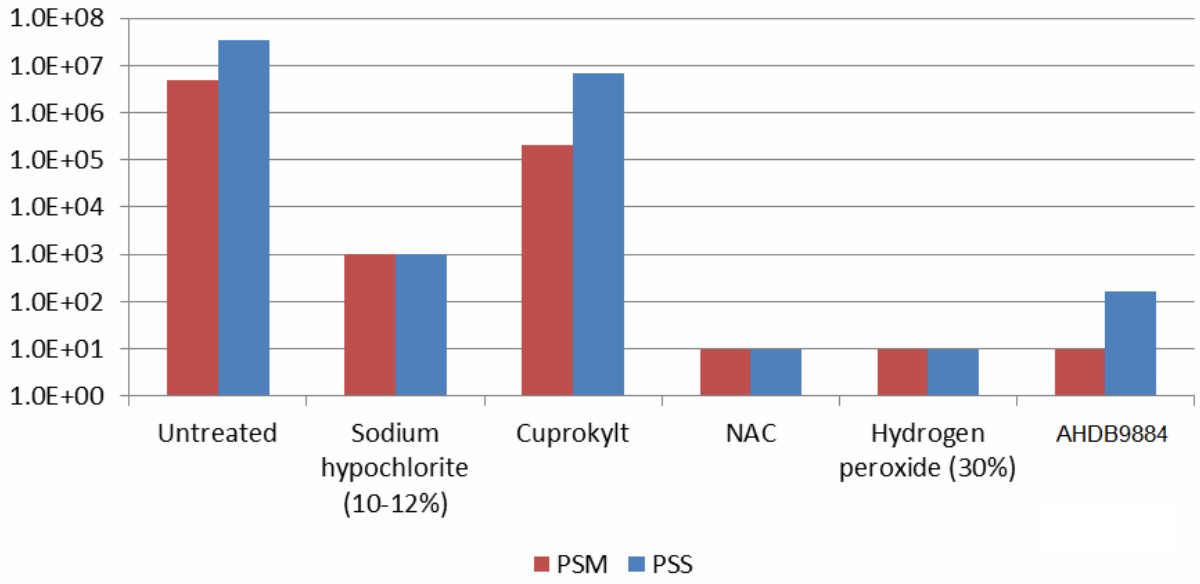


Figure 2: Survival (CFU/ml) of *PSS* and *PSM* after different treatments. Treatments AHDB9885, Serenade ASO and AmyloX were omitted from the figure due to inconclusive results.

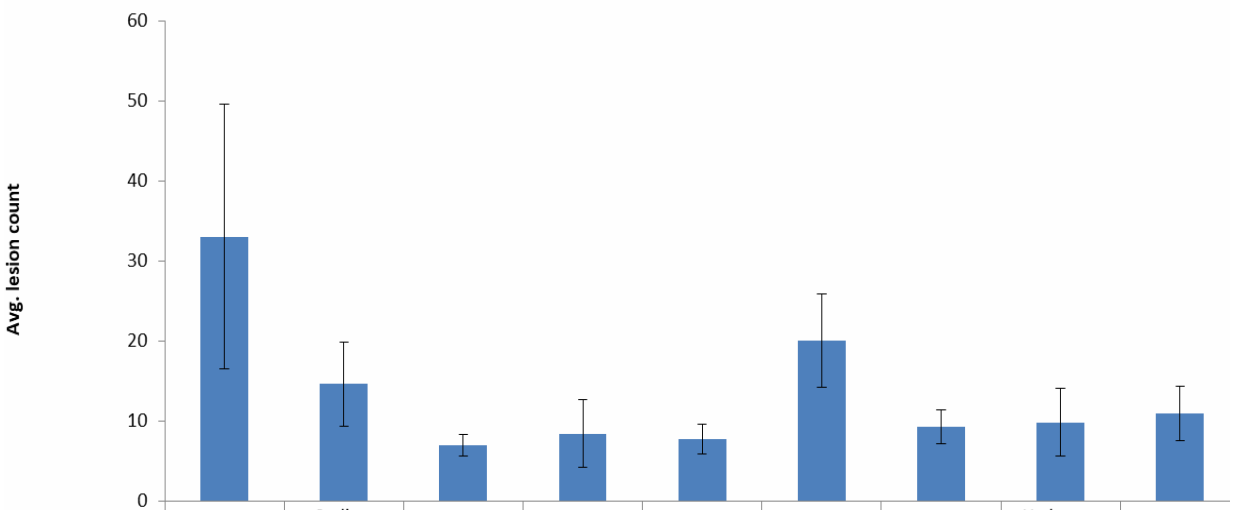
In the case of sodium hypochlorite (SH) we observed abundant growth of *PSS* and *PSM* at the highest bacterial concentration (colonies were indistinguishable and therefore not possible to quantify). At 10x diluted bacterial concentration treated with the same SH concentration we detected no growth indicating that all or almost all bacterial cells were inactivated. The mode of action of SH, i.e. biosynthetic alterations in cellular metabolism and phospholipid destruction, formation of chloramines that interfere in cellular metabolism, oxidative action with irreversible enzymatic inactivation in bacteria, and lipid and fatty acid degradation could explain the observed drop in survival. At high cell concentrations it is conceivable that oxidising potential of SH is being used on cell debris of already inactivated cells and thus depleted before all the cells are inactivated. At lower concentrations this effect is much less pronounced and SH has the capacity to inactivate all the cells.

The treatments with Serenade ASO and AmyloX and AHDB9885 yielded inconclusive results. The bacteria in Serenade ASO and AmyloX overgrew the *PSS and PSM* on King's B media (supp. figure 2) rendering *PSS and PSM* colony count impossible. Although direct bactericidal effect of Serenade ASO and AmyloX on *PSS and PSM* through production of bactericidal secondary metabolites is not excluded we propose that the main mode of action is most likely competition which was better assessed in detached leaf and fruit assays. The problem with AHDB9885 was seemingly random appearance of high bacterial growth across dilution series (Supp. figure 1).

This was observed in both *PSS* (Supp. figure 1) and *PSM* tests (data not shown); however only at the last time point (5 DPI). The most likely explanation is that AHDB9885 has some bactericidal effect on *PSS* and *PSM* indicated by absence of characteristic *PSS* and *PSM* colonies in most droplets with bacterial concentration below 1x10^5, but the product could be contaminated with low concentration of other bacteria resistant to the action of the product that only showed growth at the later time point. The product is not sterile and we make no effort to sterilise the products prior to this assay.

Detached leaf assay results

Successful inoculation of young cherry leaves with *PSM* resulted in formation of lesions in the form of black spots (Supp. figure 2).
All treatments reduced the amount of lesions produced by *PSM* in comparison to untreated control (Figure 3). None of the treatments however, statistically significantly reduced the amount of the lesions observed (Supp. table 2). It is worth noting that the lack of statistically significant differences was most likely due to high variability. In comparison to untreated control most treatments reduced the number of observed lesions by about 70%. Industry standard (Cuprokylt) performed best with 79% reduction in comparison to untreated control (Figure 3) and was also the most consistent, i.e. lowest standard error. Sodium hypochlorite and AmyloX were the only two treatments that performed slightly worse than others. The oxidising potential of sodium hypochlorite could have been depleted by its reaction with plant matter resulting in diminished bactericidal effect and only 59% reduction of bacterial spot. Interestingly, the two microbial based treatments performed markedly different with Serenade ASO achieving second best result with 77% reduction while AmyloX was much less effective with only 39% reduction in bacterial lesions. The results of this assay are in agreement with *in-vitro* agar assay in terms of NAC, hydrogen peroxide and AHDB9884 and sodium hypochlorite performing better than untreated control in both assays. The effects of Cuprokylt were much stronger on leaves compared to *in vitro* agar test probably due to abundance of nutrients and diffusion of its active ingredient favouring the bacteria on *in vitro* agar assay.



	Untreated	Sodium hypochlorite	Cuprokylt	AHDB9885	Serenade	AmyloX	NAC	Hydrogen peroxide	AHDB9884
Avg. lesion count	33.0	14.6	6.9	8.4	7.7	20.0	9.2	9.8	10.9
% reduction	0.0	56%	79%	75%	77%	39%	72%	70%	67%

Figure 3: Mean lesion count per leaf (N=10). Error bars denote +/- 1 S.E.M

Detached fruit results

Only *PSS* was used to inoculate detached fruit upon wounding. At 7 days post inoculation incidence of brown necrotic lesions (Supp. figure 3) on the fruit was counted and lesion size scored (see 4.3). Presence of *PSS* was confirmed by re- isolation from infected lesions. *PSS* as confirmed in all lesions, except for the ones treated with Bacillus spp. based treatments where only Bacillus colonies were observed on King's B agar plates.

Overall fairly low incidence and severity was observed with 40% successful lesion formation on untreated control (Figure 4). Although the average is not an appropriate representation of scale data recorded on detached fruit we used average for ease of interpretation and illustration of the severity in figure 4. Results of non-parametric ANOVA and box plot representation of the severity data are shown in Supp. table 3. The combined score (product of severity and incidence) for each treatment in shown in Supp. figure 4. Most of the treatments reduced lesion incidence and severity (Figure 4), however only severity could be statistically analysed because incidence data consisted of only one data point per treatment. None of the treatments statistically significantly reduced lesion severity in comparison to the control (Supp. table 3). The only significant difference was observed between Serenade ASO and AHDB9884.The lack of significant differences between control and treatments could be a consequence of low incidence and high variability of severity in untreated control (Supp. table 4). No lesions were observed on fruit treated with botanical products AHDB9884 and AHDB9885 indicating that they performed better than untreated control and industry standard Cuprokylt. Hydrogen peroxide and sodium hypochlorite performed similarly well with less and smaller lesions than industry standard Cuprokylt (figure 4). Treatment with NAC reduced incidence and severity to the same level as the industry standard. AmyloX showed no reduction of lesion size or incidence compared to untreated control and Serenade ASO made lesions marginally more frequent and larger than untreated control. The latter is in contrast with leaf assay in which Serenade ASO performed second best

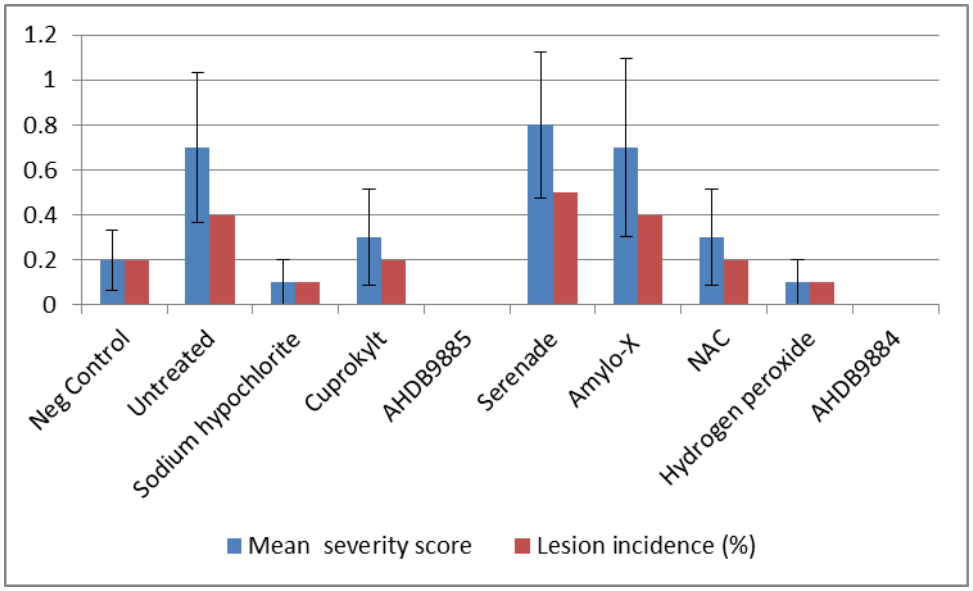


Figure 4: Mean lesion severity score +/- 1 S.E.M. and lesion incidence (%) of *PSS* wound inoculated mature green cherry fruit.

Efficacy

For efficacy of treatments across all 3 assays see **Table 1**. Several products showed promising results with NAC, AHDB9884 and hydrogen peroxide performing better or equivalent to the current industry standard across all three assays reducing bacterial population and disease severity. Serenade ASO and AHDB9885 also showed promising results especially on leaf tests.

Discussion

The selection of the products screened was based on extensive literature searching and consultation with the industry and consultants. There are very few products available for effective control of bacterial diseases and although several products were identified which may have been promising, their route to the UK market was deemed to be unclear or too questionable to consider them for inclusion. The products included represent several classes of product including botanical based products, microbial based products, sterilises and an antioxidant compound.

The use of *in vitro* agar, leaf and fruit assays has enabled the testing of a larger panel of treatments than would have been practical in field/semi-field trials and allowed for comparison in highly controlled conditions. The agar assay allows for a fast and cheap screen of direct bactericidal effect, however it is not an ideal model system due to diffusion of products in the media and its high nutrient content favours bacterial growth. The leaf and fruit assay were therefore used to corroborate the agar results in more natural conditions. These three assay together have provided the evidence on relative efficacy to determine what products should be taken forward in future trials for bacterial canker and also for other bacterial targets within SCEPTREPLUS.

All *in vitro* tests were validated by the fact that the industry standard was reducing bacterial populations compared to untreated control as expected.

The *in vitro* test on agar is an effective test for showing the direct toxic effect of the test products (Supp. figure 1). However the results should be interpreted with caution and in conjunction with the results from the other tests as the situation of mixing full strength test product in solution with the bacterial suspension is far from reality in the field. As expected the testing of microbial-based products in this system is flawed as the biocontrol bacteria grow on the plate and are indistinguishable from *PSS* and *PSM*. The colonies of PSS on King's B media produce fluorescence and yellow coloration of the media which we hoped to observe in spite of the growth of biocontrol bacteria. This however was not the case. The mode of action of the Serenade ASO and AmyloX is likely to include direct ecological competition for resources and effects on plant defence stimulation hence their inclusion in the SAR tests on potted trees.

The detached leaf test provides the most realistic model to simulate the effect of treatments on pseudomonas populations in an orchard system. The results of the leaf assay conducted here was however too variable (best seen in the untreated control, Figure 3, Supp. figure 2) and therefore not conclusive. The trends indicated in this test were that botanical-based products AHDB9885 and AHDB9884, antioxidant NAC, steriliser hydrogen peroxide and microbial-based Serenade ASO reduced *PSM* lesion formation to the similar extend as the current industry standard Cuprokylt. These treatments performed well on direct bactericidal tests on agar plates against *PSS* and *PSM* (Figure 2) suggesting direct bactericidal effect against both strains. They present viable alternative to copper (Cuprokylt) and should be further tested in semi-orchard conditions as single treatments, in combinations of treatments or in combination with SAR inducing treatments. AmyloX and sodium hypochlorite were less efficient than current industry standard.

The detached fruit test, although not realistic for field conditions (the infection route through wounds in fruit is not the major target for this pathogen), does provide a potential proxy for efficacy testing some products against *PSS*. *PSS* is very difficult to use in leaf assays because rapid lesion spread render lesion counting unreliable. Detached fruit test revealed that botanical based AHDB9885 and AHDB9884, antioxidant NAC and steriliser hydrogen peroxide again reduced lesion formation to the same level or better than industry standard. In the fruit assay neither of the *microbial*-based products had any effect on lesion progression and interestingly sodium hypochlorite performed better on fruit than on leaf.

Treatments here identified as potential replacement for copper could be used to develop an integrated approach to control bacterial canker i.e. combining products with direct bactericidal effects with products that elicit plant resistance prior to leaf fall (the main period of infection). The new treatments could provide a viable alternative not just in the light of latest legislation reducing the use of copper, but also in light of copper resistance of *PSS* strains found in UK (Roberts, 2015) and abroad (Scheck et al, 1996; Sundin et al, 1989).

Conclusions

- An initial screen of a list of products purported to have bactericidal properties has been completed with a series of *in vitro* assays.
- All in vitro tests were validated by showing that the industry standard reduced bacterial populations compared to untreated control as expected.
- Several products showed promising results with NAC, AHDB9884 and hydrogen peroxide performing better or equivalent to the current industry standard (Cuprokylt) across all three *in vitro* assays Serenade ASO and AHDB9885 also showed promising results especially on leaf tests.

Acknowledgements

AHDB is acknowledged for funding this work, and also the crop protection companies for their financial contributions as well as providing samples for the trials.

References

-Mikiciński A, Sobiczewski P, Berczyński S (2012) Efficacy of fungicides and essential oils against bacerial diseases of tree fruit. J. Plant Protection Re. 52 (4): 468-471.

-Moragrega, C., Llorente, I., Manceau, C. et al. Susceptibility of European pear cultivars to Pseudomonas syringae pv. syringae using immature fruit and detached leaf assays European Journal of Plant Pathology (2003) 109: 319. doi:10.1023/A:1023574219069

-Muranaka LS, Giorgiano TE, Takita MA, Forim MR, Silva LFC, Coletta-Filho HD, et al. (2013) N-Acetylcysteine in Agriculture, a Novel Use for an Old Molecule: Focus on Controlling the Plant–Pathogen Xylella fastidiosa. PLoS ONE 8(8): e72937. https://doi.org/10.1371/journal.pone.0072937

-Roberts S.J. (2015) Improving the management of bacterial canker in stone fruit. AHDB project TF217 final report.

-Scheck HJ, Pscheidt JW and Moore LW (1996) Copper and streptomycin resistance in strains of Pseudomonas syringae from Pacific Northwest nurseries. Plant disease, 80, 1034-1039

-Spotts and Wallis. (2008) Evaluation of bactericides for control of bacterial canker of sweet cherry using detached leaves.

-Sundin G, Jones A, Fulbright D (1989) Copper resistance in Pseudomonas syringae pv. syringae from cherry orchards and its associated transfer in virto and in planta with a plasmid. Phytopathology, 79, 861-865.

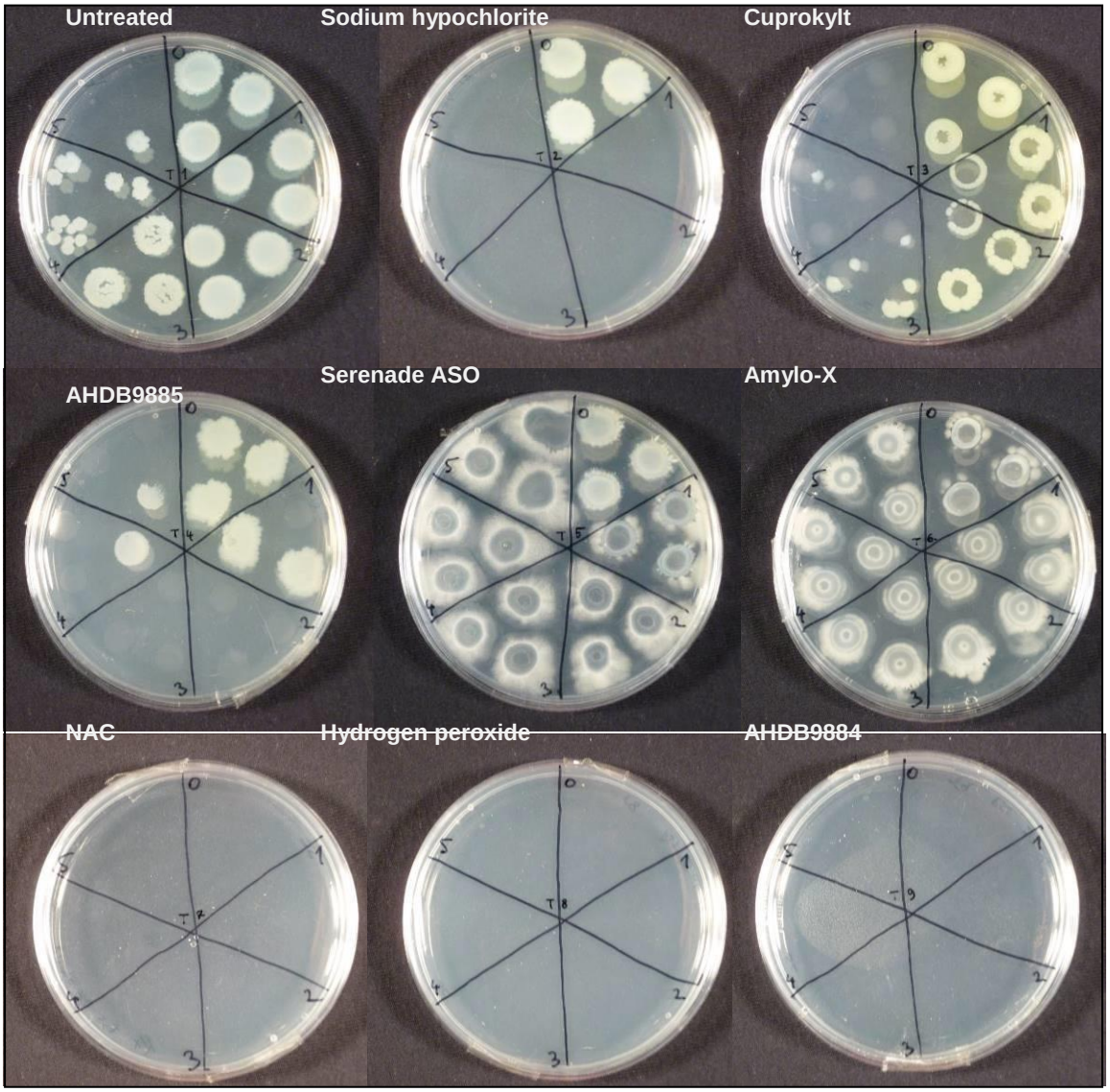
Appendix

Trial diary

Date and name	Record of work done
15.03.18 SC	Kings B autoclaved, made in preparation.
04.04.18 SC	Made up LB broth and sent to autoclave.
09.04.18 SC	Kings B poured into plates.
13.04.18 SC	Transferred *PSS* and *PSM* R1 from glycerol stock to Kings B plates.
23.04.18 SC	In vitro Assay Prep: Single colonies of *PSS* and *PSM* R1 transferred from Kings B to LB in falcon tubes.
24.04.18 SC/Mat/RS	In vitro Assay: Treatments made up, concentration adjustment of *PSS* and *PSM* R1 to $1x10^8$ plus a serial dilution. Droplet plated onto Kings B
25.04.18 SC/Mat	In vitro Assay Assessments: Looked at plates late in the afternoon. The two *Baciillus* only plates had grown a lot already – they grow faster than the *Pseudomonas* on Kings B, meaning the T5 and T6 *Pseudomonas* plates won't be quantifiable by a colony count. The untreated *PSS* and *PSM* (T1) plates were beginning to grow. Plates dropped in the floor…assay will be repeated.
25.04.18 SC	In vitro Assay #2 Prep: Single colonies of *PSS* and *PSM* R1 transferred (from the same Kings B plates as on 23.04.18)
25.04.18 Mat	In vitro Assay #2 Prep: Poured fresh Kings B plates.
26.04.18 SC/Mat	In vitro Assay #2: Treatments made up, concentration adjustment of *PSS* and *PSM* R1 to $1x10^8$ plus a serial dilution. Droplet plated onto Kings B.
27.04.18 SC/Mat	In vitro Assay #2 Assessments: Took a look at the plates and took photos.
28.04.18 SC	In vitro Assay #2 Assessments: Took a look at the plates and took photos.
01.05.18 SC	In vitro Assay #2 Assessments: Took a look at the plates, counted the quantifiable plates and described them all.
01.05.18 SC	Discussed leaf assay protocol and booked required incubators for the following week.
04.05.18 SC	Leaf Assay Prep: Transferred *PSM* R1 from glycerol stock to Kings B plates
08.05.18 SC	Leaf Assay Prep: Cleaned and put sheets on 10x trays – later, made 1% water agar and poured
09.05.18 SC	Leaf Assay Prep: Single colonies of *PSM* R1 transferred from Kings B to LB in falcon tubes.
09.05.18 SC/RS	Leaf Assay Prep: Collected over Sweetheart cherry leaves from P11 and stored in the fridge overnight ready to use the next day for the assay.
10.05.18 SC/RS	Leaf Assay: Treatments made up, concentration adjustment of *PSM* R1 to $1x10^8$. Leaves were surface- sterilised, rinsed with sterile distilled water and left to dry in a flow hood. Leaves were sprayed with the bacterial inoculum and left to dry in the flow hood. Inoculated leaves were used for the 9 treatments and laid randomly in the trays.
11.05.18 SC/RS	Leaf Assay Check: Checked leaves in the incubator. Leaves are being kept moist in the bagged trays.
14.05.18 SC	Leaf Assay Check: Checked leaves in the incubator. No change. Leaf Assay Plate Check: Plates removed from fridge. No growth. Back at room temperature now.
15.05.18 SC	Leaf Assay Check: Checked leaves in the incubator. No change. Leaf Assay Plate Check: No growth.
16.05.18 SC	Leaf Assay Check: Checked leaves in the incubator. No change. Leaf Assay Plate Check: No growth.

17.05.18 SC	Leaf Assay Check: Checked leaves in the incubator. No change. Leaf Assay Plate Check: Growth on some of the inoculated
18.05.18 SC	Leaf Assay Check: Checked leaves in the incubator. No change. Leaf Assay Plate Check: Growth on some of the inoculated.
21.05.18 SC	Leaf Assay Check: Checked leaves in the incubator. Lesions on closer inspection. Leaf Assay Plate Check: Growth on some of the inoculated.
21.05.18 SC	Leaf Assay Assessment: Counted lesions on each leaf and recorded. Took photos.
22.05.18 SC	Leaf Assay Photos: Took photos of leaves.
23.05.18 SC	Leaf Assay Re-isolation: Re-isolating *PSM* R1 from lesions on untreated leaves.
25.05.18 SC	Leaf Assay Re-isolation: Koch's Postulates confirmed by growth of *PSM* R1 on Kings B plates from 23.05.18.
30.05.18 SC	Collected over Sweetheart green cherry fruits from Rookery Fields and stored in the fridge ready to use next week for the assay.
01.06.18 SC	Fruit Assay Prep: Transferred *PSS* from glycerol stock to Kings B plates.
04.06.18 SC	Fruit Assay Prep: Made water agar and poured into 4x trays, leaving to set. Single colonies of *PSS* transferred from Kings B to LB in falcon tubes.
05.06.18 SC	Fruit Assay: Treatments made up, concentration adjustment of *PSS* to $1x10^8$ Fruits were surface- sterilised with sodium hypochlorite, rinsed in sterile distilled water and left to dry in a flow hood. 90x fruits were submerged in bacterial inoculum.
08.06.18 SC	Fruit Assay Check: AM (2.5 days post inoculation). No lesions on any fruits yet (treated or untreated or negative controls).
12.06.18 SC	Fruit Assay Assessment. Fruit assay re-isolation for Koch's Postulates test.
14.06.18 SC	Fruit Assay Re-isolation: Koch's Postulates satisfied.

Trial photographs



Supp. figure 1: In vitro bactericidal assay

Supp. figure 2: Black lesions caused by *PSM* on young cherry leaves (untreated control).

Supp. figure 3: *PSS* induced lesion on detached fruit (untreated control)

Raw data from assessments and statistical tests

Supp. table 1: Raw data (colony counts) from in vitro bactericidal assays on King's B agar

PSS	**Invitro Assay: 26.04.18**	**Counts taken: 01.05.18**

x = not quantifiable due to excessive colony growth - either the single colony had grown too much to be distinguishable or too many colonies

No.	**Treatment**	**Colony number in a 10µl drop**																		**Ave. Cfu/ml**	**Log10 reduction vs control**	**Description/Comments**
		0			-1			-2			-3			-4			-5					
T1	Untreated	x	x	x	x	x	x	x	x	x	x	x	x	**3**	**3**	**9**	0	0	0	5.0E+06		Obvious increase in growth from -5 to 0.
T2	Sodium hypochlorite (10-12%)	x	x	x	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.00E+03	3.70	Obvious growth on 0. No visible growth on -1 to -5.
T3	Cuprokylt	x	x	x	x	x	x	x	x	x	**3**	**1**	**2**	x	x	x	x	x	x	2.0E+05	1.40	Obvious increase in growth from -5 to 0. Ring-like growth could signify development of resistance along the concentration gradien.
T4	AHDB9885	x	x	x	x	x	x	0	0	0	0	0	0	0	x	0	0	0	x	NC	/	Nothing quantifiable. Lack of consistency between dilutions points to contamination of tratment solution with another bacteria.
T5	Serenade ASO	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	NC	/	Overtaken by *Bacillus* .
T6	Amylo-X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	NC	/	Overtaken by *Bacillus* .
T7	NAC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	6.70	No growth at al.
T8	Hydrogen peroxide (30%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	6.70	No growth at al.
T9	AHDB9884	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	6.70	No growth at al.

PSM	**Invitro Assay: 26.04.18**	**Counts taken: 01.05.18**

No.	**Treatment**	**Colony number in a 10µl drop**																		**Ave. Cfu/ml**		**Description/Comments**
		0			-1			-2			-3			-4			-5					
T1	Untreated	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**2**	**5**	**3**	3.3E+07		Obvious increase in growth from -5 to 0.
T2	Sodium hypochlorite (10-12%)	x	x	x	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1.00E+03		Obvious growth on 0. No visible growth on -2 to -5.
T3	Cuprokylt	x	x	x	x	x	x	x	x	x	x	x	x	**9**	**6**	**5**	0	0	0	6.7E+06		Obvious increase in growth from -5 to 0. Ring-like growth could signify development of resistance along the concentration gradien.
T4	AHDB9885	x	0	0	0	x	x	x	0	x	0	0	0	0	0	x	0	0	0	NC		Nothing quantifiable. Lack of consistency between dilutions points to contamination of tratment solution with another bacteria.
T5	Serenade ASO	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	NC		Overtaken by *Bacillus* .
T6	Amylo-X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	NC		Overtaken by *Bacillus* .
T7	NAC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1		No growth at al.
T8	Hydrogen peroxide (30%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1		No growth at al.
T9	AHDB9884	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1		No growth at al.

Supp. table 2: Means and statistical analysis of data from detached leaf test

Treatment	Avg. lesion count	N	SEM	SD	% reduction
Untreated	33.0	10	16.5	52.3	/
Sodium hypochlorite	14.6	10	5.2	16.5	56%
Cuprokylt	6.9	10	1.3	4.2	79%
AHDB9885	8.4	10	4.3	13.5	75%
Serenade	7.7	10	1.9	6.0	77%
AmyloX	20.0	10	5.8	18.4	39%
NAC	9.2	10	2.1	6.7	72%
Hydrogen peroxide	9.8	10	4.3	13.5	70%
AHDB9884	10.9	10	3.4	10.6	67%

Analysis of variance
Variate: Lesion_count

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	8	5657.5	707.2	1.62	0.131
Residual	81	35273.9	435.5		
Total 89 40931.4					

Supp. table 3: Independent samples Kruskal-Wallis test (non-parametric ANOVA) on severity scores and a boxplot

Total N	100
Test Statistic	18.273
Degrees of Freedom	9
Asymptotic Sig. (2-sided test)	.032

1. The test statistic is adjusted for ties.

Supp. table 4: Frequency table for severity of lesions on fruit (0= no lesion; 1= lesion from 0.1 to 1mm; 2=1.1-2.0 mm, 3= 2.1-4 mm and 4= more than 4 mm)

	Severity score frequency					
Treatments	**0**	**1**	**2**	**3**	**4**	Total
Neg Control	8	2				10
Untreated	6	2	1	1		10
Sodium hypochlorite	9	1				10
Cuprokylt	8	1	1			10
AHDB9885	10					10
Serenade	5	3	1	1		10
Amylo-X	6	3			1	10
NAC	8	1	1			10
Hydrogen peroxide	9	1				10
AHDB9884	10					10
Grand Total	79	14	4	2	1	100

Additional material

Supp. table 5: EU list of approved Basic substances 11 August 2017

Name	Status under Reg. (EC) No 1107/2009	Date of approval
Calcium hydroxide	Approved	01/07/2015
Chitosan hydrochloride	Approved	01/07/2014
Clayed charcoal	Approved	31/03/2017
Diammonium phosphate	Approved	29/04/2016
Equisetum arvense L.	Approved	01/07/2014
Fructose	Approved	01/10/2015
Hydrogen peroxide	Approved	29/03/2017
Lecithins	Approved	01/07/2015
Salix spp. cortex	Approved	01/07/2015
Sodium hydrogen carbonate	Approved	08/12/2015
Sucrose	Approved	01/01/2015
Sunflower oil	Approved	02/12/2016
Urtica spp.	Approved	30/03/2017
Vinegar	Approved	01/07/2015
Whey	Approved	02/05/2016

Certificate of

Official Recognition of Efficacy Testing Facilities or Organisations in the United Kingdom

This certifies that

East Malling Research
(and East Malling Services Ltd)

complies with the minimum standards laid down in Regulation (EC) 1107/2009 for efficacy testing.

The above Facility/Organisation has been officially recognised as being competent to carry out efficacy trials/tests in the United Kingdom in the following categories:

Agriculture/Horticulture
Biologicals and Semiochemicals
Stored Crops

Date of issue: 7 January 2013
Effective date: 1 January 2013
Expiry date: 31 December 2017

Signature _____
Authorised signatory

Certification Number
ORETO 321

HSE
Chemicals Regulation Directorate

Department of Agriculture and Rural Development

HSE

Health and Safety Executive

Chemicals Regulation Directorate

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Mr A Harris
NIAB EMR
New Road
EAST MALLING
Kent
ME19 6BJ

Date 7 March 2018

Reference TOR 144

Dear Mr Harris,

Re: 'Official Recognition of Efficacy Testing Facilities or Organisations' - Inspection Report

Please find attached a copy of the report prepared following the inspection of your organisation's Official Recognition facilities on 14 February 2018.

As stated at the de-brief, East Malling Research satisfied the requirements of Regulation EC No 1107/2009 and Commission Regulation (EU) No 284/2013), and therefore passed the inspection.

Your facility/organisation will therefore continue to appear in the list of 'Officially Recognised Efficacy Testing Facilities or Organisations in the UK', which is published on CRD's website.

I would like to draw you attention to points detailed in the De-brief section of the report, most of which were raised during the inspection. Points1 to 22 identify deficiencies in the current procedures which should be addressed as soon as possible. Many of these will need to be reflected in the relevant SOPs. Points 23 to 24 are of a more advisory nature and identify possible improvements to procedures or areas where procedures may need changing under certain circumstances.

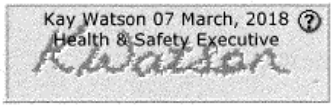
CRD does not normally require a formal response on these issues at this time, however it is noted that you have submitted an application for the renewal of your ORETO status. Part of the documentation in support of that application requires an explanation of the steps taken to address any issues raised in the last inspection report. In this particular instance, considering the timing of the inspection in relation to the expiry of your certificate, we request from you an explanation of the steps you intend to take to address the issues raised in this inspection report. This must be submitted as soon as possible, but by the end of March at the latest, so it can be considered with your renewal application.

We will be happy to provide further advice if required.

If you find anything factually incorrect in the enclosed report or if you have any questions, please contact me at the above address, by telephone, or e-mail to tony.fisher@hse.gov.uk

Please confirm receipt of this letter and report.

Yours sincerely



HSE Digital Signature

Tony Fisher
Official Recognition Inspection Team

For more information on Plant Protection Products visit: www.hse.gov.uk/pesticides

Official recognition information can be accessed via: http://www.hse.gov.uk/pesticides/topics/pesticide-approvals/pesticides-registration/efficacy-guides/official-recognition-introd.htm