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Exploring options for wound protection to prevent *Pseudomonas syringae* pv. *actinidiae* infection of cut stems of *Actinidia deliciosa* 'Bruno' seedlings (VI1486)

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Executive summary

Exploring options for wound protection to prevent *Pseudomonas syringae* pv. *actinidiae* infection of cut stems of *Actinidia deliciosa* 'Bruno' seedlings (VI1486)

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August 2014

Wound protection continues to be a challenge for kiwifruit growers since the incursion of a virulent strain of *Pseudomonas syringae* pv. *actinidiae* (Psa) in New Zealand in November 2010. When several products were tested in the field as wound protectants against natural infection by Psa, there were no significant differences between treatments (Miller et al. 2012). Therefore, there is demand in the kiwifruit sector for a wound protectant suitable for pruning wounds. This project evaluates a range of registered treatments not previously tested for protecting pruning wounds against Psa and explores the use of a penetrant for driving the product into the wound to provide protection in the case of bacteria entering the plant during the cutting process.

Ten replicate 18 month *Actinidia deliciosa* 'Bruno' seedlings were used per treatment, a total of 100 plants. The trial was carried out at the Te Puke Plant & Food Research (PFR Te Puke) field site on 4 February 2014 to enable inoculation on site.

Plants were cut through the main stem at 1 m high with sterilised secateurs. Secateurs were sprayed with 70% ethanol and scrubbed clean with handitowels between each cut. Immediately before cutting, the bark on the outside of the cutting site was sprayed with a 10^8 cfu/ml suspension of Psa to simulate environmental contamination of the bark by rainsplash in a Psa affected zone.

Protectant treatments were applied immediately after cutting. After treatment, the wound was again sprayed with a 10^8 cfu/ml suspension of Psa to simulate environmental deposition of Psa onto the wound following rainsplash.

Treatments were:

1. Greenseal™ Ultra
2. Greenseal Ultra + Kasumin®
3. Greenseall Ultra + Kasumin +penetrant (Engulf®)
4. Greenseal Ultra + penetrant (Engulf)
5. Kocide® Opti (copper) paste
6. Actigard™ paste
7. Kocide Opti (copper) spray
8. No protectant treatment
9. Uninoculated control
10. Uninoculated control + Greenseal Ultra

Liquid treatments were applied according to rates recommended by the manufacturers for field spraying.

Plants were then placed in a shadehouse at PFR Te Puke in a Latin square design.

The length of external lesions was measured at approximately weekly intervals for 14 weeks starting on 26 February 2014 and ending on 13 May. On 14 May, canes were sliced with a knife to expose any brown staining and internal lesions were measured.

None of the treatments used in this experiment were able to protect the wound from infection by *Pseudomonas syringae* pv. *actinidiae* (Psa) at a statistically significant level. This could be due to one of four reasons, or a combination: 1. Psa is such an aggressive pathogen that it is not possible to protect the wound; 2. The inoculum should have been applied at a higher dose to ensure more even infection; 3. The inoculum should have been applied at a lower dose to ensure that the protectants were not overwhelmed by a large inoculum load of Psa; 4. Susceptibility to Psa varied in the seedling 'Bruno' population.

When the treatments were placed in order of lesion size reduction when compared with the inoculated control, the Greenseal Ultra + Kasumin + penetrant treatment was the best treatment following both external and internal measurement. The order of the treatments for this internal lesion length difference was consistent with the hypothesis that the bacterial cells were sucked into the stem following severing of the transpiration stream, the order being: Greenseal Ultra < Greenseal Ultra + penetrant < Greenseal Ultra + Kasumin < Greenseal Ultra + Kasumin + penetrant. This pattern was not repeated for the external measurements.

The protocol of treating wounds and measuring the development of the external lesions during the season, and internal lesions at the end of the trial, worked well. The data were not normally distributed due to very large lesions on one or two plants of several treatments, which made any differences between treatments difficult to ascertain. If this trial is repeated, application of two or three concentrations of Psa to ensure a more even infection rate would be warranted, and/or use of clonal material to ensure there is no plant to plant variability in response to infection by Psa.

Conclusions

- None of the wound protectant treatments significantly reduced Psa lesion development in this experiment.
- The best protectant was Greenseal Ultra + Kasumin + Engulf.

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1 Introduction

Wound protection continues to be a challenge for kiwifruit growers since the incursion of a virulent strain of *Pseudomonas syringae* pv. *actinidiae* (Psa) in New Zealand in November 2010 (Everett et al. 2011; McCann et al. 2013). Several pruning wound protectants have been tested for their activity against Psa, and Greenseal™ Ultra was identified as the product most inhibitory to Psa survival in vitro (Benge & Max 2013). However, when several other products were tested in the field as wound protectants against natural infection by Psa, there were no significant differences between them (Miller et al. 2012). Therefore, there is demand in the kiwifruit sector for a wound protectant suitable for pruning wounds, grafts or girdles, and Greenseal Ultra needs to be tested as a wound protectant on plants to confirm the results of in vitro tests.

An infected orchard is likely to be contaminated with Psa cells on the outside of stems as well as on leaves (Pushparajah et al. 2014) and secateurs (Everett et al. 2012). Penetrants and a bactericide (oxytetracycline) used together reduced lesions caused by *Pseudomonas syringae* pv. *syringae* on wound-inoculated detached cherry fruit when the treatment was applied after inoculation (Carroll et al. 2010) indicating an eradicant activity. Oxytetracycline is not registered for use on kiwifruit in New Zealand so was not tested here. If Psa cells are smeared over the pruning wound at the time of cutting, either from contaminated secateurs or from the outside of the stems, then it is possible that the cells will be sucked into the cut stems by the withdrawal of the lower section of the broken transpiration stream. Therefore, application of a bactericide with eradicant activity induced by concurrent application of a penetrant could improve control. This project evaluates a range of registered compounds not previously tested for protecting pruning wounds against Psa and explores the use of a penetrant for driving the product into the wound to provide protection from bacteria entering the plant during the cutting process.

Figure 1. Containment glasshouse where treatments were applied showing plastic tray and polystyrene screen (arrowed) used around the sprayer when Psa was applied to restrict movement of aerosols (a), labelled plants before treatment (b) and a plant treated with Actigard paste (c).

2 Methods

This trial was conducted at The New Zealand Institute for Plant & Food Research Limited facilities in Te Puke. Treatments were applied to 18-month-old *Actinidia deliciosa* 'Bruno' seedlings purchased from Waimea Nursery, Nelson, delivered to the site on 20 January 2014. There were 10 replicate plants per each of 10 treatments, a total of 100 plants. Treatment plants were laid out in a Latin square design in a shadehouse. Plants were irrigated by natural rainfall.

Plants were cut through the main 'trunk' at 1 m high with sterilised secateurs. Secateurs were sprayed with 70% ethanol and scrubbed clean with handitowels between each cut. Immediately before cutting, the bark on the outside of the cutting site was sprayed with a suspension of Psa adjusted to a concentration of 10^8 cfu/ml in sterile deionised water to simulate environmental contamination of the bark by rainsplash in a Psa affected zone. The concentration of Psa was determined spectrophotometrically (Pushparajah et al. 2014). Plants were sprayed with Psa using two 'puffs' of a Devilbiss atomiser (Somerset, PA) in a containment glasshouse on a plastic tray and a polystyrene screen was used to restrict any aerosol movement of the pathogen (Figure 1 a, see arrow). Treatments were conducted on 4 February 2014.

Treatments were applied immediately after the stem was cut (Table 1). Spray treatments were applied to 'runoff'. Immediately after treatment, the wound was again sprayed with a 10^8 cfu/ml suspension of Psa to simulate environmental deposition of Psa onto treated wounds following rainsplash.

Table 1. Chemicals used to treat wounds of 18-month-old *Actinidia deliciosa* 'Bruno' seedlings.

Trade name	Active ingredient (s)	% a.i.	Application
Greenseal™ Ultra	Tebuconazole + ochthilinone	10 g/L + 17.5 g/L	paint
Kasumin®	Kasugamycin	20 g/L	5 ml/L; spray
Actigard™	Acibenzolar-s-methyl	500 g/kg	2 g/L; paste[1]
Kocide® Opti	Copper hydroxide	275 g/kg	1 g/L; paste[1]and as a spray
Engulf®	Polyether-modified trisiloxane	Not supplied	2 ml/L; spray

[1] Paste was procured by Zespri. For further details, please contact Dr Mary Black. Kocide® is a registered trademark of DuPont or its affiliates. Actigard™ is registerd to Syngenta Crop Protection Limited, Engulf® is a trademark of Etec Crop Solutions Ltd, Kasumin® is a registered trademark of Hokko chemical Industry Co., Ltd.

Assessment

After 1 week, wounds were inspected for any signs of infection. Ideally a brown-stained lesion will begin progressing down the stem. This was visible (Figure 2), and the length was measured at approximately weekly intervals for 14 weeks. At the end of that time, the bark of the cane was removed with a knife to expose the internal brown staining and the length of the lesions was measured. The first assessments recorded were on 26 February, the last assessment of external lesions on 13 May, and the final destructive assessments on 14 May.

Treatments were:

1. Greenseal™ Ultra
2. Greenseal Ultra + Kasumin®
3. Greenseall Ultra + Kasumin +penetrant (Engulf®)
4. Greenseal Ultra + penetrant (Engulf)
5. Kocide® Opti (copper) paste

6. Actigard™ paste
7. Kocide Opti (copper) spray
8. No protectant treatment
9. Uninoculated control
10. Uninoculated control + Greenseal Ultra

Liquid treatments were applied according to rates recommended by the manufacturers' instructions for field spraying (Table 1).

Figure 2. External lesions on cut stems spray inoculated with a 10^8 cfu/ml suspension of *Pseudomonas syringae* pv. *actinidiae* on 4 February visually assessed on 11 February 2014.

3 Results

3.1 External Psa lesions

Psa lesions starting at the pruning wound and progressing down the stem were visible 7 days after inoculation. Lesions were measured at 1–2-week intervals beginning on 16 February and ending on 13 May 2014 (Figure 3).

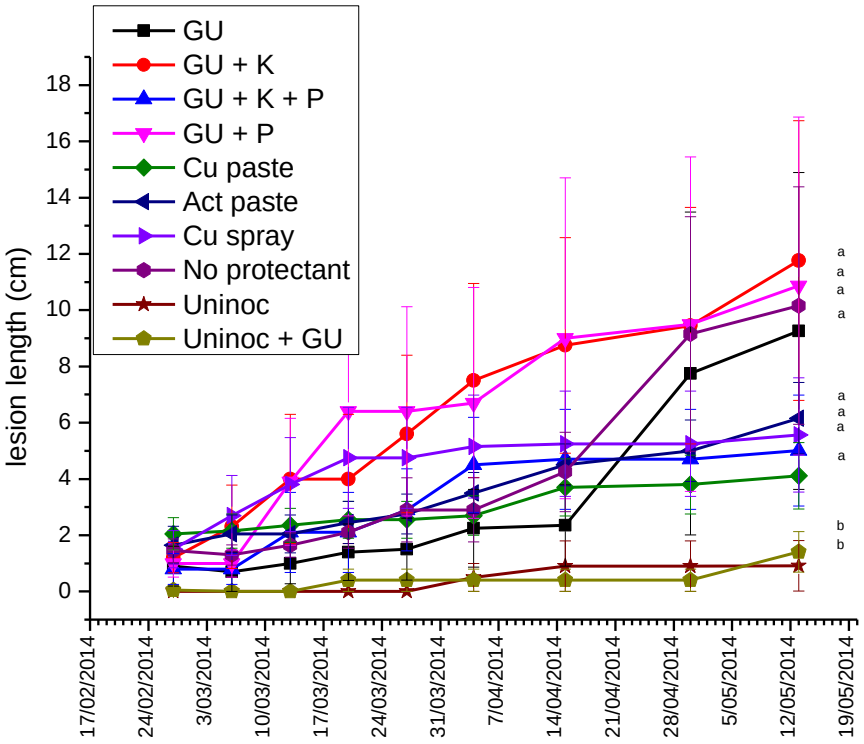

Figure 3. External lesions measured from 26 February until 13 May 2014. Values followed by the same letter were not significantly different according to an analysis of variance of Logten transformed data followed by Dunnett's comparison with the untreated inoculated control (no protectant). Untransformed data are presented. Treatments were 1. GU; Greenseal Ultra 2. GU + K; Greenseal Ultra + Kasumin 3. GU + K + P; Greenseal Ultra + Kasumin +penetrant (Engulf) 4. GU + P; Greenseal Ultra + penetrant (Engulf) 5. Cu paste; Copper paste 6. Act paste; Actigard paste 7. Cu spray; Copper spray 8. No protectant 9. Uninoc; Uninoculated control 10. Uninoc + GU; Uninoculated control + Greenseal Ultra.

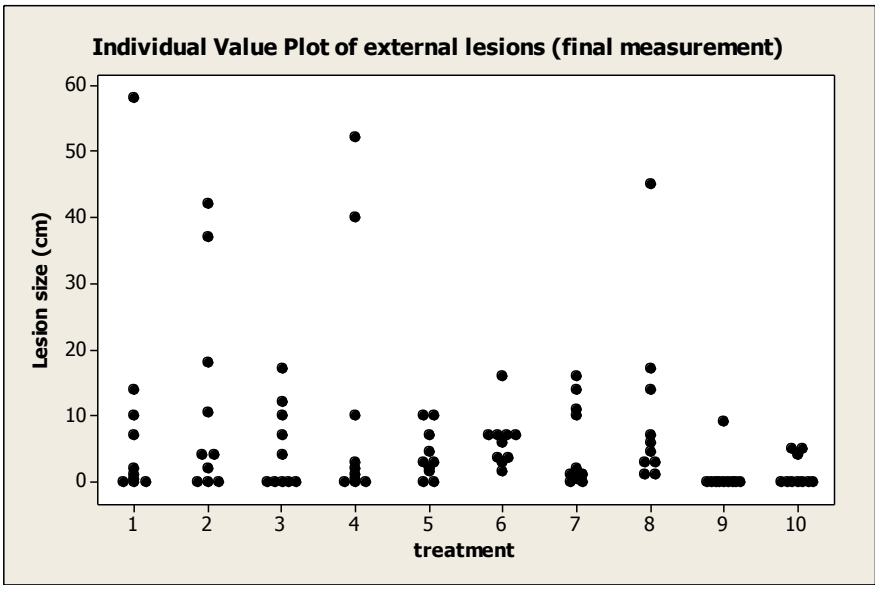

Figure 4. Plot of individual replicate values for each treatment at the final measurement of external Psa lesion length assessed on 13 May 2014. Treatments were: 1. Greenseal Ultra 2. Greenseal Ultra + Kasumin 3. Greenseal Ultra + Kasumin + penetrant (Engulf) 4.Greenseal Ultra + penetrant (Engulf) 5. Copper paste 6. Actigard paste 7. Copper spray 8. No protectant 9. Uninoculated control 10. Uninoculated control + Greenseal Ultra.

When the raw data of external lesions measured on 13 May 2014 (final measurement) was plotted to understand the distribution of the dataset (Figure 4), it was apparent that there were 'escapes' that resulted in one large outlier lesion for treatment 1 (Greenseal Ultra) and treatment 8 (inoculated control) and two large outlier lesions for treatments 2 (Greenseal Ultra and Kasumin) and 4 (Greenseal Ultra and penetrant). There were no statistically significant differences between treatments and the untreated inoculated controls, except for treatment 9 and 10 (uninoculated controls) following a logten transformation prior to analysis using the GLM Procedure of Minitab® version 17 followed by Dunnett's test ($P < 0.05$). Treatment 3 (Greenseal Ultra + Kasumin + penetrant) showed five zero values, the highest number for inoculated stem wounds.

A natural logarithmic transformation of the final measurement data prior to analysis followed by comparison of the treatments with the inoculated control using a mixed model analysis in Statistical Analysis Software (SAS) version 9.4, identified that the untreated and uninoculated controls (Treatment 9 and 10, Table 2) were the only treatments that were significantly ($P < 0.05$) different from the inoculated control (Table 1).

Table 2. Summary of mixed model analysis for external lesions following inoculation with 10^8 cfu/ml Psa after the raw data was natural log transformed prior to ANOVA. All lesion length values were subtracted from the inoculated control and a constant value (0.5) was added to all values before transformation. The adjusted *P* values <0.05 indicate significance.

	Transformed data						
Treatment	Transformed data	Standard error	T value	Pr > t[1]	Adj P	Raw data	Back transformed data
1. GU	0.7815	0.6035	1.29	0.0993	0.3823	0.90	1.69
2. GU + K	0.3632	0.6035	0.60	0.2744	0.7055	-1.60	0.94
3. GU + K + P	1.0374	0.6035	1.72	0.0445	0.2115	5.15	2.32
4. GU + P	0.7658	0.6035	1.27	0.1039	0.3942	-0.70	1.65
5. Cu paste	0.7111	0.6035	1.18	0.1209	0.4366	6.05	1.54
6. Act. paste	0.07134	0.6035	0.12	0.4531	0.8719	4.00	0.57
7. Cu spray	0.7555	0.6035	1.25	0.1069	0.4022	4.60	1.63
9. Uninoc.	2.2154	0.6035	3.67	0.0002	0.0016	9.25	8.67
10. Uninoc. + GU	1.8105	0.6035	3.00	0.0017	0.0125	8.75	5.61

[1.] df = 90; Note: Treatments were 1. Greenseal Ultra 2. Greenseal Ultra + Kasumin 3. Greenseal Ultra + Kasumin + penetrant (Engulf) 4.Greenseal Ultra + penetrant (Engulf) 5. Copper paste 6. Actigard paste 7. Copper spray 9. Uninoculated control 10. Uninoculated control + Greenseal Ultra.

When the means of the transformed values were ordered from smallest to largest, the best treatment was Greenseal Ultra + Kasumin + penetrant (Table 3).

Table 3. Natural logarithmic transformed external lesion length data subtracted from inoculated control data, in increasing order.

Treatment		Treatment 8 – Treatment
6	Actigard paste	0.07134
2	Greenseal Ultra + Kasumin	0.3632
5	Copper paste	0.7111
7	Copper spray	0.7555
4	Greenseal Ultra + penetrant	0.7658
1	Greenseal Ultra	0.7815
3	Greenseal Ultra + Kasumin + penetrant	1.0374
10	Uninoculated control + Greenseal Ultra	1.8105
9	Uninoculated control	2.2154

3.2 Internal Psa lesions

Internal lesions were easier to measure than external lesions because there was an easily identified margin between healthy (green) and diseased (brown) tissue (Figure 5). The distribution of the raw data for the internal lesion measurements also showed several 'escapes' (Figure 6). There were two 'escapes' for treatment 2 (Greenseal Ultra plus Kasumin) and one 'escape' each for treatments 4 (Greenseal Ultra plus penetrant) and 8 (inoculated control). The treatment with the most zero values (3) was treatment 2 (Greenseal Ultra plus Kasumin).

Figure 5. Internal Psa lesions in *Actinidia deliciosa* 'Bruno' kiwifruit seedlings 3 months after inoculation of the cut stems with a suspension of *Pseudomonas syringae* pv. *actinidiae* adjusted to 10^8 cfu/ml in sterile deionised water.

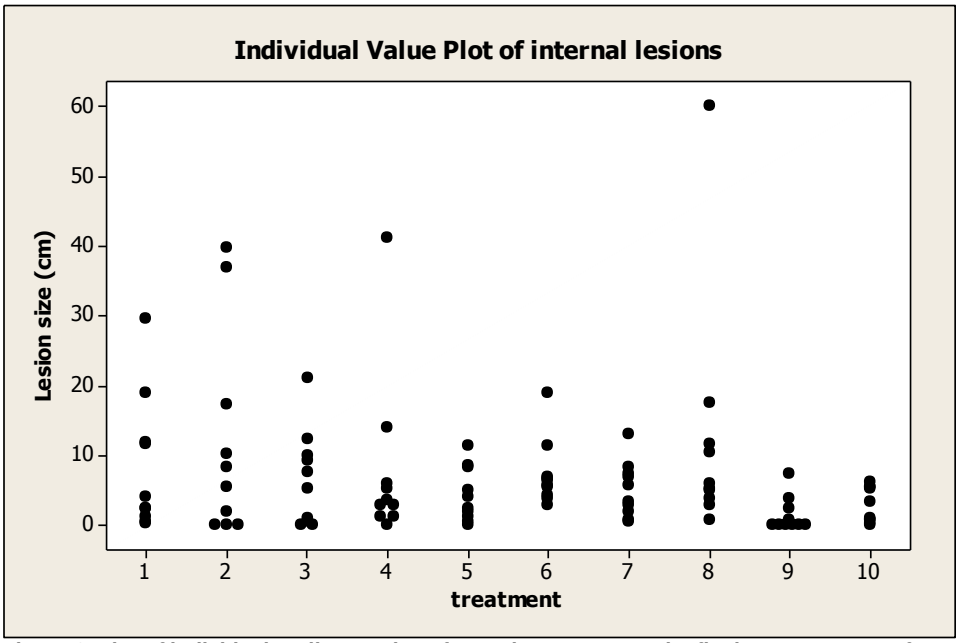

Figure 6. Plot of individual replicate values for each treatment at the final measurement of internal Psa lesion length following inoculation with 10^8 cfu/ml Psa assessed on 14 May 2014. Treatments were: 1. Greenseal Ultra 2. Greenseal Ultra + Kasumin 3. Greenseal Ultra + Kasumin +penetrant 4.Greenseal Ultra + penetrant 5. Copper paste 6. Actigard paste 7. Copper spray 8. No protectant 9. Uninoculated control 10. Uninoculated control + Greenseal Ultra.

The mixed model analysis for internal lesions was similar to that of the analysis of the external lesions except only the uninoculated control (Treatment 9, Table 4) was significantly different ($P < 0.05$) from the other treatments.

Table 4. Summary of mixed model analysis for internal lesions following inoculation with 10^8 cfu/ml Psa after the raw data was natural log transformed prior to ANOVA. All lesion length values were subtracted from the inoculated control and a constant value (0.5) was added to all values before transformation. The adjusted *P* values <0.05 indicate significance.

	Transformed data						
Treatment	Transformed data	Standard error	T value	Pr > t[1]	Adj P	Raw data	Back transformed data
1. GU	0.7003	0.8006	0.87	0.1920	0.5808	4.11	1.0143
2. GU + K	1.0948	0.8006	1.37	0.0874	0.3487	0.30	1.9886
3. GU + K + P	1.2086	0.8006	1.51	0.0673	0.2886	5.62	2.3489
4. GU + P	0.8573	0.8006	1.07	0.1436	0.4867	4.56	1.3568
5. Cu paste	1.1284	0.8006	1.41	0.0811	0.3305	8.00	2.0909
6. Act. paste	0.06615	0.8006	0.08	0.4672	0.8799	5.12	0.0684
7. Cu spray	0.6794	0.8006	0.85	0.1992	0.5931	7.29	0.9727
9. Uninoc.	3.3185	0.8006	4.14	<.0001	0.0003	10.89	26.6181
10. Uninoc. + GU	1.8797	0.7822	2.40	0.0091	0.0562	9.50	3.8495

[1.] df = 91; Note: Treatments were 1. Greenseal Ultra 2. Greenseal Ultra + Kasumin 3. Greenseal Ultra + Kasumin + penetrant (Engulf) 4.Greenseal Ultra + penetrant (Engulf) 5. Copper paste 6. Actigard paste 7. Copper spray 9. Uninoculated control 10. Uninoculated control + Greenseal Ultra.

When the means of the transformed values were ordered from smallest to largest, the best treatment was Greenseal Ultra + Kasumin + penetrant (Table 5).

Table 5. Natural logarithmic transformed internal lesion length data subtracted from inoculated control data , in increasing order.

Treatment		Treatment 8 – Treatment
6	Actigard paste	0.06615
7	Copper spray	0.6794
1	Greenseal Ultra	0.7003
4	Greenseal Ultra + penetrant	0.8573
2	Greenseal Ultra + Kasumin	1.0948
5	Copper paste	1.1284
3	Greenseal Ultra + Kasumin + penetrant	1.2086
10	Uninoculated control + Greenseal Ultra	1.8797
9	Uninoculated control	3.3185

4 Discussion

None of the products evaluated in this experiment were able to protect stem wounds from artificial infection by Psa to a statistically significant level. These findings agree with the earlier study that could not identify a wound protectant with efficacy against Psa in the field (Miller et al. 2012). This could be due to one of four reasons, or a combination: 1. Psa is such an aggressive pathogen that it is not possible to protect the wound; 2. The inoculum should have been applied at a higher dose to ensure more even infection; 3. The inoculum should have been applied at a lower dose to ensure that the protectants were not overwhelmed by a large inoculum load of Psa; 4. Susceptibility to Psa varied in the seedling 'Bruno' population.

When the treatments were placed in order of degree of lesion size reduction compared with the inoculated control, the Greenseal Ultra + Kasumin + penetrant treatment was the best treatment following both external and internal measurement. The order of the treatments for this internal lesion length difference was consistent with the hypothesis that the bacterial cells were sucked into the stem following severing of the transpiration stream, the order being: Greenseal Ultra < Greenseal Ultra + penetrant < Greenseal Ultra + Kasumin < Greenseal Ultra + Kasumin + penetrant. This pattern was not repeated for the external measurements.

The protocol for treating wounds and measuring the development of external lesions over the time course of this experiment, and destructively sampling and measuring the length of the internal Psa lesions at the end of the experiment, worked well.

The data were not normally distributed because of very large lesions on one or two replicate plants of several treatments, which made any differences between treatments difficult to ascertain. If this trial is repeated, application of two or three concentrations of Psa to ensure a more even infection rate would be warranted, and/or use of clonal material to ensure there is no plant to plant variability in response to infection by Psa.

5 Conclusions

- None of the wound protectant treatments significantly reduced Psa lesion development in this experiment.
- The best protectant was Greenseal Ultra + Kasumin + Engulf.

6 Acknowledgements

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7 References

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Appendices

Appendix 1. Summary of mixed model analysis for external lesions following inoculation with 10^8 cfu/ml Psa after the raw data was natural log transformed prior to ANOVA. The adjusted *P* values <0.05 indicate significance.

Differences of least squares means									
Effect	TMT	_TMT	transformed data	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
TMT	8	1	0.7815	0.6035	90	1.29	0.0993	Dunnett	0.3823
TMT	8	2	0.3632	0.6035	90	0.60	0.2744	Dunnett	0.7055
TMT	8	3	1.0374	0.6035	90	1.72	0.0445	Dunnett	0.2115
TMT	8	4	0.7658	0.6035	90	1.27	0.1039	Dunnett	0.3942
TMT	8	5	0.7111	0.6035	90	1.18	0.1209	Dunnett	0.4366
TMT	8	6	0.07134	0.6035	90	0.12	0.4531	Dunnett	0.8719
TMT	8	7	0.7555	0.6035	90	1.25	0.1069	Dunnett	0.4022
TMT	8	9	2.2154	0.6035	90	3.67	0.0002	Dunnett	0.0016
TMT	8	10	1.8105	0.6035	90	3.00	0.0017	Dunnett	0.0125

Note: All treatment means were subtracted from the inoculated controls (8) before analysis. TMT = Treatments, and these were: 1. Greenseal Ultra 2. Greenseal Ultra + Kasumin 3. Greenseal Ultra + Kasumin + penetrant (Engulf) 4.Greenseal Ultra + penetrant (Engulf) 5. Copper paste 6. Actigard paste 7. Copper spray 9. Uninoculated control 10. Uninoculated control + Greenseal Ultra.

Appendix 2. Summary of mixed model analysis for internal lesions following inoculation with 10^8 cfu/ml Psa after the raw data was natural log transformed prior to ANOVA. The adjusted *P* values <0.05 indicate significance.

Differences of least squares means									
Effect	TMT	_TMT	Estimate	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
TMT	8	1	0.7003	0.8006	91	0.87	0.1920	Dunnett	0.5808
TMT	8	2	1.0948	0.8006	91	1.37	0.0874	Dunnett	0.3487
TMT	8	3	1.2086	0.8006	91	1.51	0.0673	Dunnett	0.2886
TMT	8	4	0.8573	0.8006	91	1.07	0.1436	Dunnett	0.4867
TMT	8	5	1.1284	0.8006	91	1.41	0.0811	Dunnett	0.3305
TMT	8	6	0.06615	0.8006	91	0.08	0.4672	Dunnett	0.8799
TMT	8	7	0.6794	0.8006	91	0.85	0.1992	Dunnett	0.5931
TMT	8	9	3.3185	0.8006	91	4.14	<.0001	Dunnett	0.0003
TMT	8	10	1.8797	0.7822	91	2.40	0.0091	Dunnett	0.0562

Note: All treatment means were subtracted from the inoculated controls (8) before analysis. TMT = Treatments, and these were: 1. Greenseal Ultra 2. Greenseal Ultra + Kasumin 3. Greenseal Ultra + Kasumin + penetrant (Engulf) 4.Greenseal Ultra + penetrant (Engulf) 5. Copper paste 6. Actigard paste 7. Copper spray 9. Uninoculated control 10. Uninoculated control + Greenseal Ultra.

DISCOVER. INNOVATE. GROW.