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VI1175 Management of Psa short term – Progress Report September 2011

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A report prepared for ZESPRI Group Limited

ZESPRI Group Limited VI1175

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SPTS No. 6163

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Significant effort is being invested to address the problem and there is now a considerable international research effort focused on Psa including a dedicated Plant & Food Research Psa research programme. The suite of projects reported here were established shortly after the Psa incursion occurred and were aimed at addressing immediate research needs. This report provides a summary of the research completed under the cap-stone project VI1175 Management of Psa - Short term. Much of the information presented in this report has previously been reported to ZESPRI and provided to the industry.

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Introduction

The bacterial plant disease *Pseudomonas syringae* pv. *actinidiae* (Psa) is a damaging bacterial pathogen in a number of countries around the world. Psa was provisionally identified in New Zealand on 5 November 2010 from a kiwifruit orchard in Te Puke. Subsequently the identification was confirmed and further infected orchards have been identified across the country, with a more virulent form of the disease being identified in the Bay of Plenty region. Unfortunately, the disease has continued to develop and progress rapidly and is now a very serious threat to the New Zealand kiwifruit industry.

Significant effort is being invested address the problem and there is now a considerable international research effort focused on Psa including a dedicated Plant & Food Research Psa research programme. The suite of projects reported here were established shortly after the Psa incursion occurred and were aimed at addressing immediate research needs. This report provides a summary of the research completed under the cap-stone project VI1175 Management of Psa - Short term. Much of the information presented in this report has previously been reported to ZESPRI and provided to the industry.

Objective 1. Literature review

1.1 and 1.2 Literature Summary and Review

To provide an initial summary of the literature relating specifically to Psa To complete a literature review, incorporating the initial Psa literature summary, relating to Psa and similar bacterial pathogens

Outcome

Final reports have previously been submitted to ZESPRI.

Objective 2. The pathogen and biology within the vine

2.1 Survival of Psa in the environment

This work will be completed as part of a separate project.

2.2 Koch's Postulates

To determine if the bacteria isolated in New Zealand fulfil Koch's postulates

To establish that the bacteria isolated in New Zealand is Psa, it was essential that Koch's postulates were fulfilled. Trials on seedlings were established at MAF Tamaki on 12 November 2010. Bacteria isolated from infected vines were inoculated onto seedlings to test if the bacteria would infect and result in symptoms similar to those observed in the orchards.

Outcome

The trials demonstrated that when a pure culture of the bacterium was inoculated onto kiwifruit vines, symptoms typical of Psa were produced, the pathogen was re-isolated from the diseased tissue and its identity confirmed, thus meeting Koch's postulates.

2.3 Establishment of a specific pathogenicity test for Psa

To establish and utilise a specific pathogenicity test for Psa that will enable the relative pathogenicity of different types of Psa to be established and to determine differences in potential Psa resistance

Internationally it is recognised that there appear to be differences in the pathogenicity of different haplotypes of Psa. It was vital that we establish and then utilise a specific pathogenicity test that will enable different types of Psa found in New Zealand to be compared for their relative pathogenicity and risk. In addition, such assays are essential in identifying variability in Psa resistance in the *Actinidia* germplasm and breeding populations.

Outcome

A pathogenicity test that allows the virulence of different strains of Psa to be determined has been developed. This test forms the basis a number of bioassays and has been used in a range of subsequent studies. The test consists of spraying the underside of young fully developed leaves of *Actinidia deliciosa* 'Bruno' seedlings a suspension of freshly grown Psa. Symptoms are seen within a week of inoculation if plants are kept in a relatively humid environment and at room temperature. Infection seems to be delayed or even stopped if temperatures are too high soon after inoculation. The whole range of symptoms observed in the field was found on the plants inoculated under those conditions, including leaf spots, necrosis, and even production of exudate. This assay can be used to distinguish Psa-V from Psa-LV.

2.4 Diagnostic test for Psa on asymptomatic leaf tissue

Does *P. syringae* pv. *actinidiae* have an endophytic/epiphytic phase on kiwifruit leaves in its life cycle in New Zealand conditions?

Asymptomatic leaves and canes could potentially harbour *Pseudomonas syringae* pv. *actinidiae.* Control or containment procedures rely on knowledge of the pathogen distribution and size of buffer zones required. ZESPRI requested testing of asymptomatic leaves for presence of *P. syringae* pv. *actinidiae*. Kiwifruit leaf tissue was sampled from 10 orchards in the Bay of Plenty. Samples were:

1. Asymptomatic leaf tissue from an isolated uninfected orchard (uninfected control)

2. Asymptomatic leaf tissue immediately adjacent to symptomatic leaf tissue on an infected orchard

3. Asymptomatic leaf tissue 1 m from symptomatic leaf tissue on the same cane on an infected orchard

4. Asymptomatic leaf tissue 2 m from symptomatic leaf tissue on the same vine on an infected orchard

5. Asymptomatic leaf tissue 5 m from symptomatic leaf tissue on a different vine on an infected orchard

6. Leaves with symptoms (infected control).

Outcome

A final report has been submitted to ZESPRI, a summary and recommendations are provided below.

Samples from two orchards were negative by qPCR for Psa, despite severity ratings by symptoms of, respectively, 7 and 8. One of the orchards had previously tested negative for Psa, but the other had tested positive. Blossom blight (*Pseudomonas* sp. or *Pseudomonas viridiflava*) causes leaf symptoms that cannot be distinguished from leaf symptoms caused by

Psa. It is therefore likely that at least one of these two orchards could be infected by blossom blight.

Orchards 4 and 10 were worse infected than all other orchards by both severity ratings, and by number of positives by qPCR. These orchards have been diagnosed by MAF Biosecurity as being infected with the virulent haplotype of Psa (Psa-V).

Of the remaining six orchards, two had strong qPCR positives from one sample each and had been diagnosed as infected with the less virulent haplotype Psa-LV, and four orchards had only weak positives (CT 30-35 cycles) of which two were infected with Psa-V and two with Psa-LV.

On one orchard, two surface-sterilised samples from symptomless leaves on an adjacent vine yielded Psa. This is some evidence that in symptomless leaves Psa can infect inside the tissue without causing symptoms (endophytic infection). It could also have been early stages of infection that were not noticed during sampling. If this was an endophytic infection, it does not appear to be common, as Psa was not isolated from the remaining 198 surface-sterilised samples from symptomless leaves. This result was from testing leaves from an orchard that was infected with the virulent strain of Psa. Before conclusions are made, this result needs to be confirmed by further testing or experimentation.

It was difficult to isolate Psa from leaves with symptoms from orchards infected with Psa-LV. This suggests that the less virulent haplotype of Psa may not have been causing new infections, or only a few new infections, when samples were collected in January. It could also be that the measures used by growers to control Psa-LV were effective.

Psa was isolated from 4/5 leaves with symptoms on the two orchards infected with the Psa-V haplotype. It was also isolated from 3/5 and 2/5 adjacent symptomless leaves, from 5/5 and 3/5 symptomless leaves 1 m away on the same cane, from 2/5 and 3/5 symptomless leaves 2 m away on a different cane, and from 1/5 and 1/5 symptomless leaves on a different vine 5 m away.

Because Psa-V was isolated from symptomless leaves up to 5 m from the epicentres (leaves with symptoms), this suggests that Psa-V was still active, spreading, and possibly causing new infections in January when these samples were taken. It is also possible that Psa-V was able to survive for long periods of time on the outsides of leaves without causing symptoms (epiphytically). Further research is required to determine the length of time that Psa-V can survive on the outsides of leaves in New Zealand kiwifruit orchards.

When orchard severity was plotted against the number of Psa positives (out of 75 possible) for every orchard, there appeared to be three distinct groups of orchards: those that were infected with Psa-V, those infected with Psa-LV, and a group that included the orchard that may be infected with blossom blight. The potential blossom blight group probably had one orchard that really was infected with blossom blight, but the other orchard had no Psa positives when sampled in January, but when sampled earlier (November 2010) it was positive for Psa-LV. This latter orchard had a high severity rating of 8/10, that means there was an abundance of leaves with symptoms, but the Psa could not be isolated from the symptoms. This could be explained by the microclimate of this particular orchard not being suitable for long-term survival of Psa. It could also be explained by the presence of a successful microbial competitor, or by grower applied products suppressing or killing Psa cells. Whatever the reason, further research could help to explain this result.

When the putative blossom blight group was removed from the analysis, there was a strong (r=0.9) and significant (*P*=0.002) relationship between orchard symptom severity and the number of times that Psa was detected by qPCR out of 75 possible samples.

Psa was not easily found on two orchards infected with Psa-V. Both orchards had low severity ratings, and it is possible that these orchards had become infected only a short time before these samples were taken. Although no dates were available for when these samples were tested, the other two Psa-V orchards with more severe symptoms were identified earlier with Psa. These latter orchards are south and inland to the orchard on which Psa was originally diagnosed in New Zealand. These orchards may have been infected earlier because symptoms were more severe and orchards may have been downwind of the prevailing off-sea breezes to the putative epicentre of the current epidemic. However, it could also be because the measures that the growers are using to control Psa are more effective than the measures taken by other growers.

Conclusions

- For effective reduction of Psa-V inoculum in an infected orchard, vines at least 5 m distant from leaves with symptoms need to be removed or treated with bactericides even if they do not show symptoms.
- There was some evidence for an endophytic growth habit of Psa in this study (that is, it resides inside symptomless leaves), but before any conclusions are made these results need to be verified by further experimentation.
- Both Psa-V and Psa A have an epiphytic growth habit, and were thus able to be isolated from the surface of symptomless leaves.
- These results suggest that the Asian strain of Psa is either being managed well by growers, or that it is not very active during January in New Zealand. However, Psa-V on two orchards from this study was very active when samples were taken.
- The methodology used in this study could be used to compare the effectiveness of control measures for Psa in the field.

Recommendations for industry

- These results suggest that symptomless vines at least 5 m away from leaves with symptoms need to be removed, or sprayed with an effective bactericide, for successful elimination of Psa from orchards infected with the Psa-V haplotype.
- The measures used to control Psa by several growers should be scrutinised and compared with those measures used by others.
- The control measures used by growers on those orchards that had tested positive for Psa and for which all samples were negative in this study, or on which Psa was difficult to find, should also be scrutinised.

2.5 Infection pathways

This work will be completed as part of a separate project.

2.6 Detection of pathogen in infected canes

This project aims to determine whether Psa can be detected in the cane tissues of vines showing secondary die-back symptoms.

The removal of vines and/or portions of vines will be essential in the control of Psa. This project was to ensure that the recommendations to cut vines 10 cm above the graft union when shoot die-back has been observed were supported by some scientific data. Such a drastic measure is needed only if Psa is present in the cane tissue.

Outcome

A final report has been submitted to ZESPRI and the associated executive summary and recommendations are provided.

Psa was detected on leaf tissue and in cane pieces, indicating that Psa can enter the kiwifruit vine and move rapidly within the canes. How far and how fast it can move was not determined in this trial.

Psa was easily isolated from canes showing blackening, cracking of the bark and necrosis, indicating that those symptoms were probably caused by Psa. In two of the four canes analysed that showed those symptoms, Psa could also be isolated up to 50 cm away from the symptoms. Analysis of six wilting shoots revealed that Psa could not be isolated from the part that was wilted but was found in the samples taken further away from the shoot tip of the cane where the cane wood joined older wood. This suggests that the wilting was not caused directly by Psa, but was a consequence of the vascular system being blocked. In these instances, Psa must have been in the vascular system.

Recommendations for industry

Data indicate that Psa is present in canes of vines where die-back symptoms are observed. This suggests that canes need to be cut and removed when such symptoms exist, to reduce the likely spread of the pathogen throughout the remaining vine.

Objective 3. Bees/pollen transmission

3.1 Contamination of NZ pollen

The aim of this study was to determine whether Psa was associated with kiwifruit pollen collected in New Zealand, or with kiwifruit pollen collected overseas and present in New Zealand.

We have found that at least one sample of pollen collected from Italy in an infected orchard contained some live cells of Psa. Presence of Psa on pollen has some important economic consequences, knowing that most kiwifruit orchards are artificially pollinated and that Psa is a potential epiphytic bacterium. Results from Italy might not be extrapolated to New Zealand because of the differences in kiwifruit pollen collection methods used in Italy (vacuumed from open flowers in the field) and in New Zealand (pollen milled from flowers collected as closed flowers in the field).

Outcome

A final report has been submitted to ZESPRI and the associated executive summary and recommendations are provided.

In several cases, an amplicon of the right size for Psa was obtained after PCR; however, no culture of Psa was isolated from pollen samples collected from New Zealand. Therefore, we cannot conclude that live cells of Psa were present on the samples of pollen analysed in this study.

3.2 and 3.3 Transfer of infested pollen

The aim of this study was to determine whether bees collect or become contaminated by Psa during foraging.

On at least on one occasion, Psa has been found (by PCR?) associated with pollen collected from infected kiwifruit vines. If Psa is associated with pollen, it is reasonable to assume that bees could carry Psa after picking it up from pollen or infected flowers. To determine if honey bees act as vectors for Psa, we sought initially to determine whether Psa could be detected on the bodies of bees or in beehives located in infected orchards.

Outcome

A final report has previously been submitted to ZESPRI.

In this study, no Psa was isolated from any of the samples tested. The limit of detection in this experiment was 40 colony forming units per sample.

However, this is a preliminary result. We do not know whether the bees from that colony were visiting kiwifruit flowers. In addition, few kiwifruit flowers were still present in the orchard at the time we collected the hive, meaning that the bees might have been visiting other flowers such as flowers from the ground cover.

3.4 Presence in beehives of Psa and reuse of hives

The aim of this study was to determine whether Psa collected by bees could contaminate beehives, which could then act as potential reservoirs for inoculum.

As described above, Psa has been found associated with pollen collected from infected kiwifruit vines. If Psa is associated with pollen, it is reasonable to assume that bees could carry Psa after picking it up from pollen or infected flowers. To determine if honey bees act as vectors for Psa, we sought initially to determine whether Psa could be detected on the bodies of bees or in beehives located in infected orchards.

Outcome

No Psa was isolated from any of the honeycomb samples tested or of the bees tested. The limit of detection in this experiment was 40 colony forming units per sample. We do not know whether the bees from that colony were visiting kiwifruit flowers. Few kiwifruit flowers were still present in the orchard at the time we collected the hive. However, the bees might have been visiting other flowers such as flowers from the ground cover.

3.5 Infection of flowers by Psa-contaminated pollen

The aim of this study was to determine whether Psa associated with or carried on pollen could infect flowers.

If Psa is associated with pollen, then it is possible that pollen transferred to another vine by bees, wind or artificial pollination may lead to infection of the vine and in particular the flowers. This study aimed to determine whether Psa-contaminated pollen could infect kiwifruit flowers.

Outcome

Flowers from potted male vines which flowered in the PC2 laboratory and of branches with young flowers transferred from the orchard to the laboratory were inoculated with pollen contaminated with Psa (c. 10^6 cfu/g of pollen). None of the flowers developed any symptoms. Flowers rapidly dried and fell off the vines. Presence of Psa was not detected in any of the flowers analysed. Those experiments are being repeated with female flowers.

3.6 Does pollination lead to vine infection

This work was deferred by the ZESPRI steering group.

3.7 Pollen cleaning

To provide a simple and practical method for cleansing contaminated pollen of viable Psa cells. Can Psa be killed by low temperatures? Will kiwifruit pollen survive temperatures that are lethal to Psa? What other approaches could potentially be used to clean pollen?

Kiwifruit pollen from New Zealand, Italy and Chile has tested positive by PCR for *P.s.* pv. *actinidiae* (Psa). In addition, Psa has been isolated from Italian pollen. Protocols need to be developed to remove viable Psa from contaminated pollen to prevent possible spread.

Outcome

A full briefing on results has been provided to ZESPRI, KVH and the pollen producers.

A wide range of approaches including heat, freezing, sanitisers, UV light and other chemicals were evaluated for their potential to cleanse pollen whilst retaining the pollen viability. Heat was identified as the most promising treatment.

Objective 4. Detection

4.1 Limits of detection

What is the limit of detection for Psa when using the set of primers PsaF1/PsaR2?

All detection methodologies have limits. To fully understand the results of any test, it is important to understand these limits and what they mean.

Outcome

When Psa was used on its own, the level of detection with the primers PsaF1/R2 was 1 colony forming unit (cfu) per reaction tube. When Psa was mixed with *Pseudomonas syringae* pv. *syringae* (Pss):

1. 102 cfu of Psa could be detected in the presence of 107 cfu of Pss

2. 4 cfu of Psa could be detected if there were fewer than 4 106 cfu of Pss

3. 10 cfu of Psa could be detected if there were fewer than 107 cfu of Pss.

This indicates that false negatives could occur when the ratio of Psa/other bacteria was less than $1/10^6$.

4.2 Woody tissue PCR

Is Psa present in hard woody tissue? To develop a tool to directly assay hard woody tissue for Psa

A protocol to assay hard woody tissue directly using PCR needed to be developed to provide a tool to determine if cut-off stumps harbour Psa inoculum. If they do, then stumps will need to be removed from infected orchards to ensure total eradication of Psa should it prove to be aggressive under New Zealand conditions.

Outcome

A protocol has been established and has been used successfully to sample woody tissue in a number of subsequent trials. The protocol involves:

1. The top layer of bark is removed with a pruning knife and the wound site sterilised with 95% ethanol.

2. Drill through the area with a sterilised 3.2-mm wood drill to a depth of 1.5 cm.

3. The shavings adhering to the drill bit are placed in a sterile Eppendorf tube, filled with 1 ml sterile bacteriological saline.

4. Wounds on the vine are then sealed with pruning paste.

5. After agitation, a 0.2-ml aliquot from each sample is removed and streaked across a plate of King's media B.

6. After 48 hours of growth, DNA is extracted from the resultant bacterial colonies and tested for the presence of Psa with qPCR.

4.3 Detection of *Pseudomonas* sp.

This work was deferred by the ZESPRI steering group.

4.4 Development of a method for the detection of Psa in budwood

This work will be completed as part of a separate project.

Objective 5. Orchard hygiene and management practices

5.1 Product testing and baseline sensitivity to copper and streptomycin

What copper products have best efficacy against *P.s.* pv. *actinidiae*?
Are there products other than copper and streptomycin with efficacy against *P.s.* pv. *actinidiae*?

Aim: To carry out *in vitro* tests on products for potential efficacy for control of New Zealand isolates of *P.s.* pv. *actinidiae*

Treatments to limit the spread of Psa in orchards are required. ZESPRI have requested preliminary *in vitro* testing of products for activity against *Pseudomonas syringae* pv. *actinidiae*. In the first trial (Report 1) leaf samples were collected by ZESPRI staff from an orchard on 15 November 2011. The leaves came from blocks that received one of the following treatments: Kocide® 35 g per 100 L; Kocide 70 g per 100 L; Blueshield 50 g per 100 L; Blueshield 100 g per 100 L; or No treatment.

In the second and third trials, *Psa* was isolated from leaf and flower tissue collected from two Bay of Plenty orchards. Single cells from the isolation plates were used to generate the test cultures. Two types of tests were conducted; the first measured the zone of inhibition of a bacterial 'lawn' around filter paper discs infiltrated with various test products (Report 2), and the second measured the growth rate of the bacteria in the presence of these same test products (Report 3). ZESPRI staff sourced and selected those products to be tested.

Outcome

Final reports have previously been submitted to ZESPRI.

Report 1

Although the leaf spot symptoms in this orchard were typical of Psa, we could not detect Psa in this orchard nor could MAF IDF. This result came after we had started this experiment. The results are still interesting, since they indicate the effect of copper on the bacterial load on kiwifruit leaves. Results are presented in the table below.

Bacterial populations on kiwifruit leaves in colony forming units per ml with copper treatments.				
Treatment	*Maximum Population*	*Minimum Population*	*Median*	*Average population*
Kocide® 35 g per 100 L	3.3×10^6	4.3×10^4	2×10^5	6.2×10^5
Kocide 70 g per 100 L	2.6×10^7	2.3×10^4	3.6×10^5	2.9×10^6
Blueshield 50 g per 100 L	5.7×10^5	7.3×10^3	1.6×10^5	6.2×10^5
Blueshield 100 g per 100 L	3×10^6	4×10^3	5.3×10^4	3.1×10^5
No treatment	3.6×10^7	5.3×10^4	4×10^6	1.1×10^7

Report 2

Key Strepto® was most effective against both KEP1 and KEP3. These isolates were from an orchard reported to be infected by, respectively, Psa-LV and Psa-V haplotypes. It was interesting that KEP3 appeared to be more sensitive to streptomycin than was KEP1, as measured by the clear zone of inhibition. There were no differences between concentrations of Serenade® Max, suggesting that the establishment of the bacterium on King's medium B was not affected by the initial inoculum concentration. Zonix® was slightly inhibitory against KEP1, but not effective against KEP3, and PNS was slightly inhibitory against KEP3 but not effective against KEP1. Neither of these last two products could be recommended as controls, based on these results. Of the biological products, only Serenade® Max was effective in this test. Elicit® and dodine showed some potential as products that could inhibit Psa in these tests.

Report 3

All chemical products tested here had some efficacy against Psa. Ideally these tests should be repeated, but because of time constraints, these results have been released. The best products by EC_{50}/EC_{95} ratios were, in order of effectiveness, copper, streptomycin, dodine, Teracep, Citrolife, PNS, Citrox, and Elicit. The product that was most effective at the lowest rate was dodine, followed by Teracep. The biological products were difficult to test using this method, but results showed that Serenade Max and Blossom Bless were the best products, with Serenade Max showing efficacy over a slightly wider range of concentrations than Blossom Bless. In order to validate the results of these tests, field testing is required, as environmental conditions and the presence of plant material can affect the efficacy of these products. However, results of overseas trials have shown that in field conditions both copper and streptomycin reduce disease caused by Psa on kiwifruit. For disinfection of tools, products such as dodine, Teracep, Citrolife, PNS, Citrox and Elicit show promise and should be further tested to ensure there is no reduction of efficacy in field conditions.

Recommendations

Report 1

At this stage, no recommendations are made, since we do not know the effect of copper on Psa on kiwifruit leaves.

Report 2

Key Strepto® was the most effective product in these tests and should be further tested, including field trials, if marketing considerations allow its use on New Zealand orchards. Dodine, Serenade® Max and Elicit® should also be further tested, but field testing needs to be considered only if these products also perform well in the growth rate inhibition test.

Report 3

Of the products tested, copper and streptomycin were the most effective chemical products by EC_{50}/EC_{95} ratio, and these products have known field efficacy, so can be recommended for field control of Psa. The chemical product that was most effective at the lowest rate was dodine, followed by Teracep, but these products have not been field tested against Psa so cannot be recommended at this stage for that purpose. Of the biological products, Serenade Max was the most effective, but has not been field tested against Psa so cannot yet be recommended for field application.

5.2 Assessment of effectiveness of sanitisers for sterilising equipment

The aim of this project was to determine whether those sanitisers do indeed kill Psa.

It is suspected that Psa can be transferred via pruning equipment. One of the recommendations is for all pruning equipment to be dipped in a solution of a cheap and easily available sanitiser.

Outcome

A final report has previously been submitted to ZESPRI.

Results of the trial are presented in the table below.

Efficacy of common sanitisers in killing or inhibiting the growth of Psa.		
Treatment	*Population of viable Psa after a two minutes treatment*	*Inhibition of Psa on plate* [a]
Water	5 X 108 cfu/ml	No
Virkon® 1% (recommended strength)	Below 102 cfu/ml	Yes
Virkon 0.5%	Below 102 cfu/ml	Yes
Sodium hypochlorite 3%	Below 102 cfu/ml	Yes
Sodium hypochlorite 1%	Below 102 cfu/ml	Yes
Sodium hypochlorite 0.5%	Below 102 cfu/ml	Yes
Janola® (full strength)	Below 102 cfu/ml	Yes
Janola (1:8 dilution recommended strength)	Below 102 cfu/ml	Yes
Sporicidin disinfectant solution (Neat)	Below 102 cfu/ml	Yes
Path-Away™ (Neat)	Below 102 cfu/ml	Yes
Sporicidin lotion soap (1:10 dilution)	Below 102 cfu/ml	No

[a]This was determined by the presence of a zone of inhibition. 'Yes' means a zone of inhibition was present where the product tested had been deposited. 'No' means no zone of inhibition could be detected.

The discrepancy in the results for the Sporicidin lotion soap diluted 1:10 could be explained by the soap being absorbed or diluted in the agar.

Recommendations

The products mentioned here at the concentrations studied can be used for "sterilisation" of the pruning tools as long as the tools are in contact with those solutions for a minimum of two minutes.

5.3 Orchard spray trial

This work was deferred by the ZESPRI steering group.

5.4 Testing of Psa transmission

Does Psa inoculum lodge on clothing, tools and vehicles? To identify key human/material vectors, to aid minimisation of orchard to orchard, and within-orchard, spread of Psa

Orchard workers, tools and vehicles are possible carriers of Psa inoculum. The source of potential spread from orchard to orchard needs to be determined.

5.5 Assessment of the efficacy of wound treatments

This work will be completed as part of a separate project.

5.6 Remediation of budwood

Can systemic infections by *P.s.* pv. *actinidiae* be remediated? To kill all Psa bacterial cells on or in propagating material

There is evidence that canes of vines infected with *P.s.* pv. *actinidiae* become systemically infected. Canes are used for propagating new varieties of kiwifruit, and existing varieties. It would be useful to find a method to ensure that propagating material is free of Psa, especially if a valuable irreplaceable new variety becomes infected by Psa.

Outcome

A final report has previously been submitted to ZESPRI.

1. A three-way analysis of variance with isolate (replicate), time and temperature as factors showed that isolate was not significant, and that time and temperature were significant ($P<0.0001$).

4. Raw data showed that 50°C was a lethal temperature for Psa, even after only 5 min of exposure (Figure 1).

5. Any time:temperature combination greater than 45°C for 5 minutes killed Psa (Figure 2).

6. Modelling the thermal response suggested that 42.5°C for longer than 150 minutes was the lowest time:temperature combination that could be used to kill the bacterial cells.

Recommendations

For elimination of bacteria in infected tissue, temperatures of 50°C for 1 hour at the centre of infected wood, fruit or leaves should result in 100% mortality of Psa.

5.7 Killing Psa at relatively low temperatures

The aim was to determine if a 10-minute treatment at 65°C is enough to kill or to inactivate Psa?

The disposal of plant tissues affected or presumed affected by Psa from a kiwifruit orchard represents a major problem, as the material is usually difficult and expensive to burn or to bury. ZESPRI suggested an alternative, the use of a BioVapor heat system, which is a fully mobile and self-contained heat-treatment system. A 20-ft mobile unit could be brought onto an orchard for treatment on site of the pruning wastes.

Outcome

A final report has previously been submitted to ZESPRI.

The population of Psa in the tube that was not subjected to heat treatment was 5 x 108 cfu/ml. In the tube that was subjected to heat treatment, no colonies grew after 48 hours. The limit of detection of this assay is 33 colony forming units per ml.

Recommendations

We demonstrated in the laboratory that a 10-minute BioVapor treatment at 65°C would kill Psa. The recommendation might be for the industry to set up some field experiments with the BioVapor equipment.

Objective 6. Progression within orchards, and effectiveness of aggressive containments

6.1, 6.2, and 6.3 Progression within orchards and effectiveness of aggressive containment

This project aims to map and follow progress of Psa disease development in kiwifruit orchards carefully, and to provide answers to the following questions: Does Psa spread within the canopy during the season, and if so, when? What is the pattern of spread? Do leaf symptoms progress to cane/trunk symptoms? How will next season's disease relate to the current season? Does aggressive containment work? Are widespread Psa symptoms in some orchards a result of Psa or other agents?

Little is known of the spread of Psa within orchards during the season, or of the potential progression of symptoms from mild to severe within vines in New Zealand.

Selected orchards will be used to provide a benchmark for disease progression within orchards and the potential for restricted leaf spots to progress to systemic infections via the petioles. The same orchards could potentially be used as study sites for a number of years and be used to develop the relationship between weather events/environmental conditions and disease incidence and severity. This ongoing data collection will also help to guide chemical control strategies.

It is anticipated that this work will relate to wider industry monitoring carried out in conjunction with MAF, ZESPRI and growers. Advice for growers wishing to carry out their own disease progress surveys will also be provided.

Outcome

A full report has been presented to ZESPRI with results on bay level assessments, cane and shoot level assessments, assessments on individual leaves and spray diaries.

Bay level assessments

There were some clear trends in disease severity recorded within bays during the summer months. In all six 'Hayward' orchards with Psa-V, there was an obvious change during the season, with progressively more bays moving into the higher disease severity classes as the season progressed. The progression to higher disease classes appeared similar in all six orchards, with numbers of vines in the '0' category declining and numbers in the '<1%' and '1-5%' or '5-15%' categories increasing.

- In both 'Hayward' orchards with the Psa-LV strain, there was, in general, little change in average disease severity ratings during the summer (Orchards 13 &14).
- In 'Hort16A' orchards with the low-V strain of Psa (Orchards 7 to 11), there was either little change or a decline in the average disease severity ratings during the season.
- Orchard 8, diagnosed with Psa-LV early in the season, but subsequently diagnosed with Psa-V, showed a slight increase in the number of bays where Psa symptoms were detected. Some vines in this orchard developed secondary symptoms (cane die-back) in February 2011, which is when the V-strain was first diagnosed, and by April secondary symptoms were recorded in six of the monitored bays.
- In Orchard 12, there was a large increase in leaf spot symptom severity during the season. This orchard is in Motueka and presumed to be Psa-LV, but the Psa strain in this orchard has not yet been determined. Recent isolation attempts in samples from this orchard have failed to confirm Psa.

Cane and shoot level assessments

- In the six 'Hayward' orchards with Psa-V, there was an increase in the number of shoots with one or more infected leaves (cane analysis) and number of infected leaves on shoots as the season progressed (Orchard 1 to 6).This increase was substantial and highly significant for the four orchards where statistical analyses could be carried out.
- Data from two orchards could not be validly analysed: on Orchard 2, many of the tagged canes were pruned out during canopy thinning, leaving insufficient numbers for valid analysis, and on Orchard 3, all vines were cut back to the main stump in early February.
- In the two 'Hayward' orchards with the low-V strain of Psa, on tagged canes and shoots there was a small but statistically significant ($P<0.05$) increase in the number of leaves with Psa symptoms during the summer (Orchards 13 &14).
- In 'Hort16A' orchards with the low-V strain of Psa (Orchards 7 to 11), numbers of leaves with Psa symptoms on tagged canes and shoots increased significantly during the season, although in most cases this increase was small.
- The exception was Orchard 12 (Psa strain unconfirmed, but presumed Psa-LV), where there was a large increase in number of leaves with spot symptoms recorded during the season, as noted earlier in analysis of the bay data.

- Orchard 8, diagnosed with Psa-LV early season but subsequently diagnosed with Psa-V, showed only a slight increase in the number of leaves with Psa symptoms.

Assessments on individual leaves

Ten leaves were photographed on each orchard at each visit. In most cases, the pattern of spots and area covered at the first visit (December or January) changed little in the following months. This was true for both 'Hayward' and 'Hort16A', and where either the Psa-V or Psa-LV strain had been diagnosed. Occasionally, on individual leaves, the area of leaf covered by spots increased significantly. In almost all cases where this occurred, the leaves were substantially colonised at the first visit, suggesting that defences had been overwhelmed. In the late season visits (April and May), brown lesions appeared around the margins of some leaves, coinciding with the guttation points. While these lesions were not specifically tested for Psa, it is likely that they relate to late-season infection in the dew droplets that form around the leaf margin in cool late summer nights, with entry into leaves through the hydathodes.

Orchard spray diaries

Orchard spray diaries for the 14 monitored orchards were supplied by ZESPRI. Copper sprays and other potentially antibacterial sprays were summarised. There was a wide variation in the number of copper sprays applied. Most orchards did not apply copper in winter 2010. During summer, after Psa diagnosis, the number of copper sprays ranged from none to five, with some orchards also applying Serenade™ Max. It is difficult to draw any conclusions about relationships between copper use and progression of disease in orchards. Variations in timing of copper application, timing of infections, varietal and Psa strain differences confound such analysis.

6.4 Collection of plant material for testing

MAF-funded work and plant material collection completed between Mike Manning and MAF directly.

Objective 7. Sequencing and genomics

7.1 Molecular characterisation

Are the isolates of Psa isolated from New Zealand similar or different from those identified overseas; in particular, are they as virulent as those isolated overseas?

A comparison of the New Zealand isolates with those from overseas is required to ensure that appropriate management strategies are implemented to control this outbreak. If the virulence of the New Zealand isolates is different from that of overseas isolates, then the control methods might need to be modified to be appropriate to the New Zealand situation. Isolates of Psa obtained from Te Puke and Motueka, from 'Hort16A' and 'Hayward' orchards and isolates obtained from overseas (Italy and Japan), were characterised using genetic fingerprinting techniques (such as BOX PCR). These techniques have previously been used to study and characterise strains of *Pseudomonas syringae* at the sub-species and sub-pathovar levels. The comparison was extended by sequencing of the cts gene to determine which haplotypes of the cts gene of Psa were present in New Zealand and to determine whether the haplotypes present in New Zealand were similar or different from the haplotypes identified from overseas isolates. The work also looked at presence of genes that code for effectors (bacterial molecules responsible for either host range or virulence of pathogenic strains) in the isolates from New Zealand.

Outcome and conclusions

A final research note was completed in December 2010 with the following results:

1. Isolates collected from leaf washings from three Te Puke orchards (two 'Hort16A' and one 'Hayward') had a BOX PCR pattern similar to those of strains isolated in Italy from 2008 to 2010.

7. None of the strains isolated to date from New Zealand has a BOX PCR pattern similar to that of strains isolated from Japan.

8. Strains of Psa from Motueka and Hawke's Bay have a PCR BOX pattern that has not previously been identified from strains of Psa.

9. The haplotype of the *cts* gene from isolates collected from two Te Puke 'Hort16A' orchards was similar to those of strains isolated from Italy in 2010, whereas the haplotype of the cts gene from the strains from Motueka was different from any haplotypes previously identified. There were two base pairs different between the Japanese and the Italian cts haplotypes, and there were five base pairs different between the New Zealand and the Japanese or Italian cts haplotypes.

10. All strains, including those from New Zealand, Italy and Japan, were found to carry DNA coding for the effectors hopA1 and hrpK1, but only strains from Motueka carried DNA coding for hopAF1.

11. Several isolates from the two Te Puke 'Hort16A' orchards were analysed; they always gave similar results, suggesting that the Psa populations present in those orchards are homogeneous.

Recommendations

Based on the above research, no recommendations are made, because we still do not know:

1. Whether strains from New Zealand are as virulent or less virulent than strains from overseas

12. What is the relationship between virulence and cts haplotypes or BOX-PCR patterns.

If they are all equally virulent, then the determination of which cts haplotype is present in an orchard carries little practical significance.

7.2 and 7.3 Psa Genome sequencing

This work will be completed as part of a separate project.

7.4 API

This has been merged with objective 7.1.

7.5 Testing Kumeu isolates

Is Psa present in an historic collection of bacteria isolated from macerated kiwifruit buds, flowers and fruitlets from the Kumeu Research Orchard during spring 1991?

The origin and the length of time that Psa has been in New Zealand is currently unknown. However, there have been comments made by growers that some of the symptoms observed have been seen in previous seasons. It is important to establish how long Psa has been present in New Zealand, to help to determine the appropriate management approach.

Outcome

A final research note was delivered to ZESPRI with the following results:

1. There were two faint positive bands from isolate 848 and isolate 872 from the Kumeu Research Orchard using the protocol of Rees-George et al. (2010) with the modification of 35 cycles. All other isolates from Kumeu Research Orchard (HortResearch), New Zealand were negative, as was the water control. The expected band was produced from DNA amplified from the two known Psa isolates used as controls.

13. When the PCR protocol and primers of Rees-George et al. (2010), using the 30 recommended cycles, was used then no product was amplified from DNA extracted from isolates 848 and 872.

14. No product was amplified from DNA extracted from isolates 848 and 872 when the primers and protocol of Koh & Nou (2002) were used.

Conclusions

Because the bacterial samples from isolates 848 and 872 were derived from single cell cultures, it is unlikely that the faint positive bands obtained using 35 cycles were from positive Psa isolates. When they were retested using the protocol of Rees-George et al. (2010) and 30 cycles, no bands were produced. However, the Rees-George et al. (2010) primers are likely to be more sensitive than the Koh & Nou (2002) primers, because the region that these primers amplify has five copies, whereas the region that the Koh and Nou (2002) primers amplify has only one copy. Both the higher copy number (5), and the higher number of cycles (35) mean that the Rees-George et al. (2010) primers are more sensitive than the low copy number (1) and lower number of cycles (30) of the primers designed by Koh and Nou (2002). Consequently, it is possible that Psa was present but only in small quantities as a contaminant. However, this is considered to be unlikely. On this basis, it is concluded that Psa was not present on *Actinidia deliciosa* buds, flowers and fruitlets on Kumeu Research Orchard in 1991.

Future Research steps

1. To be certain that Psa was not present on tissue from *Actinidia deliciosa* on Kumeu Research Orchard in 1991, DNA extracted from isolates 848 and 872 should be re-tested using 40 cycles and the primers of both Koh & Nou (2002) and Rees-George et al. (2010), and any products should be sequenced. This will establish if the faint positive bands are Psa, or not.

15. The rpoD gene region also needs to be sequenced to compare *P.s.* pv. *actinidiae* with other pathovars of *Pseudomonas syringae* that cannot be distinguished on the basis of the ITS sequence (*P.s.* pv. *passiflorae*), or are very similar (*P.s.* pv. *morsprunorum*). Downloading rpoD sequence of a New Zealand isolate of *P.s.* pv. *passiflorae* and overseas isolates of *P.s.* pv. *morsprunorum* from GenBank showed that in this region there are 9/472 differences in DNA sequence to *P.s*. pv. *passiflorae*, and 7/472 differences to *P.s*. pv. *morsprunorum*. If the rpoD gene region of isolates 848 and 872 are sequenced and compared with these pathovars, we expect that it will provide further evidence that these isolates are not Psa.

Recommendations

Our results suggest that it is unlikely that Psa is present in this historic culture collection from the Kumeu Research Orchard. However, DNA sequencing confirmation is required. Isolates from this study (Everett & Henshall 1994) that were deposited in ICMP were also negative for Psa when tested with the Rees-George et al. (2010) primers, confirming the results of our study.

Objective 8. Quick response breeding action plan

8.1 Propagation of top elite material from breeding lines

Rescue and propagation of up to 600 vines from germplasm and top elite material from breeding lines in order to produce available material for planting elsewhere and for producing rooted cuttings that will allow testing resistance to Psa in a glasshouse under a replicated experiment.

Psa is widespread in New Zealand orchards and eradication may no longer be feasible. Therefore management practices and precautions need to be devised and put into practice. As part of protecting the unique germplasm and elite material on the Te Puke site, a programme for the replication of the material has been established. The resultant plants will be used not only for the preservation of the material but also in Psa screening.

This work is funded by Plant & Food Research.

Outcome

Rooted cuttings from 250 genotypes were propagated in late November 2010 and comprised of a wide range of material (mainly *A. chinensis*). These cuttings were transported to Palmerston North, where they are being grown in a protected environment and have been used to establish a tissue culture repository. Some of cuttings have also been used to screen for Psa susceptibility. A wide variation in susceptibility has been shown, with the different genotypes ranging from 0% to 60% of leaf infection.

A second batch of approximately 400 rooted cuttings, mainly *A. deliciosa*, has also been sent to Palmerston North.

8.2 Crosses with *Actinidia deliciosa* towards a gold flesh

Vines will be selected, pollen collected and crosses made in the spring. Germination of this material can happen in the autumn and plants established in the ground in December 2011. With the right treatment, most of this vines will be flowering and assessed by 2014, with the possible outcome of a resistant gold *A. deliciosa*.

There are some very early indications that *Actinidia deliciosa* may be more tolerant to Psa than *A. chinensis*. Enough variability exists within the selected lines *A. deliciosa* in terms of Hue Angle to mean that it is possible guarantee selections with gold flesh. A number of crosses will be made to

This work is funded by Plant & Food Research.

Outcome

During flowering in 2010, 11 crosses were made between five selected female parents and two unrelated male parents. The resultant seeds have been germinated in the Palmerston North facility, with a target of 400 seedlings per family. It is anticipated that the seedlings will shortly be transported to Te Puke where they will be screened for their Psa susceptibility.

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