In vitro inhibition of *Pseudomonas syringae* pv. *actinidiae* by wound protectants

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Abstract Preventing *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, from entering pruning wounds would reduce disease incidence and facilitate canopy management. Antibacterial activity against Psa has been determined in the laboratory for active ingredients commercially available in New Zealand. Only tebuconazole, tebuconazole with othililone and a formulation of cyproconazole and iodocarb killed Psa on agar plates or in liquid medium. However, only tebuconazole with othililone killed Psa within 5 min. Psa was detected by polymerase chain reaction following plating on instruments used for applying wound protectants in an infected orchard. This suggests that under some conditions the pathogen could be transferred from vine to vine when applying some wound protectants. Addition of streptomycin sulphate to tebuconazole or a formulation of cyproconazole and iodocarb did not result in a rapid and complete kill of Psa similar to that with a commercial formulation of streptomycin applied alone.

Keywords bacterial canker of kiwifruit, pruning paste, streptomycin.

INTRODUCTION

Pseudomonas syringae pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, has recently spread to most of the major kiwifruit-producing countries around the world (Vanneste 2013). In New Zealand, this pathogen has been isolated from commercial orchards of *Actinidia deliciosa* (green-fleshed kiwifruit), *A. chinensis* (yellow-fleshed kiwifruit) and *A. arguta* (kiwiberry) (Everett et al. 2011; Vanneste et al. 2013; Vanneste et al. 2014). While Psa threatened the economic viability of growing *A. chinensis* and *A. deliciosa* in New Zealand (Vanneste 2012), it had no real economic impact on *A. arguta* (Vanneste et al. 2014). Bacterial pathogens isolated in New Zealand from symptomatic kiwifruit plants

were initially grouped into two biovars: biovar 3 and biovar 4 (Vanneste et al. 2013). However, recently it has been demonstrated that strains of biovar 4 belong to a distinct pathovar now called *Pseudomonas syringae* pv. *actinidifoliorum* (Cunty et al. 2014). Therefore at present, all Psa strains isolated from New Zealand belong to biovar 3 (Vanneste et al. 2013).

Most *Actinidia* species are dioecious, with separate male and female plants. Both male and female kiwifruit plants need to be pruned several times during the year. In winter, pruning is needed to remove dead or unproductive wood. In summer, pruning of female vines is needed to remove unnecessary shoots and to

keep replacement canes to a manageable length. On male vines, the canes that have flowered are removed in early summer once the fruit has set on the female vines. All these pruning wounds could potentially be entry points into the plant for Psa, as is the case for other pathovars of *P. syringae* (Kennelly et al. 2007).

A number of products have been developed over the years to protect pruning wounds of kiwifruit and other plants. These wound-protectant products form a physical barrier that prevents potential pathogens from getting inside the plants. Some of these products also contain one or more active ingredients, which may kill some pathogens. Although most of these products have been developed to prevent fungal infection, the aim of this study was to determine whether any of these products could inhibit or kill Psa *in vitro*. Wound protectants that killed Psa could be used after pruning or grafting when conditions are favourable for infection, reducing disease incidence and facilitating canopy management. The survival of Psa in the products or on the tools used to apply the products in an infected orchard was also examined. This was to determine whether protecting wounds with any of these products could also result in spreading the pathogen in the orchard.

MATERIALS AND METHODS

Strain and media

All the laboratory experiments were conducted in the Physical Containment level 2 (PC2) laboratory of The New Zealand Institute for Plant & Food Research Ltd at the Ruakura Research Centre. The bacterial strain used in these experiments was Psa10627, a strain of biovar 3 isolated in New Zealand in 2010 (Vanneste et al. 2013). Psa was routinely cultivated on King's B medium (KB) (King et al. 1954). Bacterial concentration was determined by plating serial dilutions made in sterile distilled water on KB agar plates after 48 h of incubation at 28°C. When a complete growing medium was required, Luria (Invitrogen Life Technologies Ltd) was used. When necessary, Psa identity was confirmed by polymerase chain reaction (PCR) using the primers Psa F1/Psa R2

(Rees-George et al. 2010) as described previously (Vanneste et al. 2010).

Wound protectants

The wound protectant formulations used in this project and their characteristics are listed in Table 1. Streptomycin sulphate and KeyStrepto™, a commercial product that contains streptomycin sulphate, are the only two products that are not sold as wound protectants. They were used as positive controls or possible adjuvants to the other products. Of the six other products, five are still commercially available. Garrison NF, which was used in the initial experiments, is no longer available; it has been replaced by Garrison Rapid. Although Garrison NF and Garrison Rapid contain the same active ingredients at the same concentration, they are different colours and gave different results, so they have been treated as two different products. These commercial products are sold to protect pruning wounds. However, Gripset Betta is also widely used in the building industry as a waterproof coating.

Inhibition of Psa on agar plate and in water

Inhibition of Psa on plates was detected by spotting a 10-µl drop of the product to be tested on a soft agar overlay seeded with Psa10627 at ca 5×10^7 cfu/ml. The experiment was conducted on KB and Luria agar plates, with three plates of each medium used per product. All the products except the wax were tested. Presence of a zone of inhibition was detected after 48 h of incubation at 28°C. Water and 10-ppm streptomycin were used on every plate as a negative and a positive control respectively.

To prevent possible interactions between the product and components of the medium in which Psa had grown, inhibition of Psa in liquid was done in water. A bacterial suspension made in water from a fresh plate was mixed with the same volume of the product to be tested. Except when otherwise mentioned, the final solution was a 50/50 (vol/vol) aqueous solution of the product to be tested. After a given incubation time, the population of Psa that survived the treatment was determined by plating 1/10 serial dilutions made in sterile distilled water on KB agar plates and counting the colonies that had

grown 48 h after incubation at 28°C. Determination of the Minimum Inhibitory Concentration (MIC) was carried out in water as described above, using decreasing concentrations of the product to be tested.

Survival and potential transferability of *Psa* in the orchard

The commercial formulations of tebuconazole, tebuconazole with othililone, cyproconazole with iodocarb (formulation 1), wax and bitumen were applied on kiwifruit stumps in August 2012 during the grafting of *A. chinensis* 'Zesy002' (commonly known as Gold3) in an infected orchard in Te Puke, Bay of Plenty, New Zealand. Five samples of each product were used, and for each sample a new application tool was employed. All products except the wax were applied with a small paintbrush. Each brush was used four times; each time, it was dipped into one sample of product and swiped at least twice over the cut area of a grafted stump. The wax was applied with a kitchen butter knife on four stumps. The tools were then individually placed in plastic bags, the product samples were sealed, and all were sent to the PC2 Plant & Food Research laboratory at Ruakura. One sample of each product and one tool were analysed on the day of use, whereas the others were analysed the following day.

Presence of *Psa* on the tools or in the product samples was determined by PCR following plating. For the product samples (except for the wax, which could not be diluted in water), 2.5 ml of products were mixed with an equal volume of water and 500 µl was spread onto KB plates. For the wax, 2 ml of sterile water was deposited on the top of the samples and mixed as thoroughly as possible before spreading 500 µl onto KB plates. The brushes were washed in 5 ml of water and 500 µl was spread onto KB plates. The knives were cleaned with cotton swabs, which were then washed in 2 ml of water; 500 µl of the washing was spread onto a KB plate. After incubation at 28°C for 24 h, a swipe with a pipette tip was made across the plate and used to inoculate a PCR tube containing the primers *PsaF1/R2*. The PCR was carried out as described previously (Vanneste et al. 2010).

RESULTS

Inhibition of *Psa* on agar plates

Cyproconazole with iodocarb (formulation 1) and the bitumen did not inhibit the growth of *Psa* on either KB or Luria agar plates. Tebuconazole, tebuconazole with othililone and cyproconazole with iodocarb (formulation 2) inhibited the growth of *Psa* on KB medium and on Luria plates (Table 2). These three formulations produced a single clear zone of inhibition on both media. In contrast, streptomycin sulphate produced two zones of inhibition – a small clear one and a larger opaque one. On KB, only tebuconazole with othililone produced a zone of inhibition larger than the clear zone of inhibition obtained with streptomycin sulphate at 10 ppm. On Luria, both tebuconazole with othililone and cyproconazole with iodocarb (formulation 2) produced a zone of inhibition larger than the clear zone of inhibition produced by streptomycin sulphate 10 ppm.

Inhibition of *Psa* in liquid medium

When a suspension of *Psa* containing 7.7×10^9 cfu/ml was mixed in a 50% (vol/vol) aqueous solution of the different formulations, tebuconazole with othililone killed *Psa* within 5 min (data not shown) and tebuconazole killed *Psa* within 30 min (Table 3). However, tebuconazole did not always kill *Psa* within 30 min; a minimum 3-h incubation time was necessary to kill *Psa* consistently (data not shown). Cyproconazole with iodocarb (formulation 2) killed *Psa* within 24 h (Table 4). The other formulations: cyproconazole with iodocarb (formulation 1), the bitumen and the wax, did not kill *Psa* even after 24 h of incubation. However, the bitumen reduced the population of *Psa* from 7.7×10^9 to 6.0×10^6 cfu/ml (Table 4), and in at least one experiment, cyproconazole with iodocarb (formulation 1) reduced the *Psa* population from 10^9 to 10^8 cfu/ml after 24 h.

The MIC when using ca 2×10^7 cfu/ml was 1% for the formulation of tebuconazole with othililone after 1 h exposure and was 40% after 3 h exposure for the formulation of tebuconazole (Table 5). The MIC for cyproconazole with iodocarb (formulation 2)

Table 1 Wound protectant and other products used in this study against *Pseudomonas syringae* pv. *actinidiae*.

Name in this study	Active ingredient	Commercial name	Company	Comments and recommended use
Tebuconazole	10 g/litre tebuconazole	Bacseal Super®	Bayer New Zealand Ltd	Tebuconazole is a triazole fungicide used to control plant pathogenic fungi.
Tebuconazole with octhilinone	10 g/litre tebuconazole and 17.5 g/litre octhilinone in the form of a paint	Greenseal ULTRA™	Omnia Primaxa Ltd, New Zealand	Octhilinone is a fungicide used for treating canker and tree wounds. It has some bactericide activity.
Cyproconazole with iodocarb (formulation 1)	2.5 g/litre cyproconazole and 1.0 g/litre iodocarb in the form of a paint; proprietary blend of resins and fungistatic additives	Garrison NF	AgriNova (New Zealand) Ltd	Cyproconazole is a systemic fungicide with protective, curative, and eradicant actions.
Cyproconazole with iodocarb (formulation 2)	Same active ingredients as Garrison NF	Garrison Rapid	AgriNova (New Zealand) Ltd	See comments above
Wax	Wax	Smith Grafting Wax	Skelton Ivory Ltd, New Zealand	Used to seal graft unions
Bitumen	Bitumen rubber made of a latex polymer (30–40%) and bitumen emulsion (40–50%)	Gripset Betta	Gripset Industries Pty Ltd, Australia	Waterproof membrane used after tree surgery, pruning and grafting
Streptomycin commercial formulation	15–20% w/w streptomycin sulphate	KeyStrepto™	Key Industries Ltd, New Zealand	Used for control of bacterial pathogens such as <i>P. syringae</i> pv. <i>syringae</i> and <i>Erwinia amylovora</i>
		Streptomycin sulphate	Sigma-Aldrich	Used in the laboratory to test for susceptibility or resistance to streptomycin

Table 2 Average diameter of the zone of inhibition in mm (+/- SE) of *Pseudomonas syringae* pv. *actinidiae* on two types of agar plates, King's B and Luria (n=3).

Product	King's B	Luria
Tebuconazole	11.3 +/- 0.3	10.7 +/- 0.7
Tebuconazole with octhilinone	22.3 +/- 0.3	18.3 +/- 0.3
Cyproconazole with iodocarb (formulation 2)	13.7 +/- 1.5	13.7 +/- 0.9
Cyproconazole with iodocarb (formulation 1)	0	0
Bitumen	0	0
Streptomycin sulphate 10 ppm (clear zone)	18.0 +/- 1.0	11.7 +/- 0.3
Streptomycin sulphate 10 ppm (opaque zone)	23.7 +/- 0.7	25.7 +/- 1.5

Table 3 Population of *Pseudomonas syringae* pv. *actinidiae* in cfu/ml after 30 min of incubation in a 50% (vol/vol) aqueous solutions of tebuconazole and tebuconazole with othililnone formulations

Product	Experiment				
	1	2	3	4	5
Nil	3.3×10^7	3.7×10^7	3.2×10^8	1.0×10^9	7.7×10^9
Tebuconazole	0	6.7×10^6	1.6×10^8	0	0
Tebuconazole with othililnone	0	0	0	0	0

Table 4 Population of *Pseudomonas syringae* pv. *actinidiae* (cfu/ml) after 24 h of incubation in a 50% (vol/vol) aqueous solution of different wound protectant formulations.

Product	Experiment 1	Experiment 2	Experiment 3
Nil	1.5×10^9	1.3×10^9	3.3×10^8
Cyproconazole with iodocarb (formulation 1)	1.3×10^9	3.0×10^8	1.8×10^8
Cyproconazole with iodocarb (formulation 2)	0	0	0
Wax	4.6×10^9	ND ¹	ND
Bitumen	6.0×10^6	ND	ND

¹ND: Not done.**Table 5** Populations of *Pseudomonas syringae* pv. *actinidiae* (Psa) in cfu/ml when grown with different concentrations of tebuconazole, tebuconazole with othililnone, and cyproconazole with iodocarb (formulation 2). The minimum inhibitory concentration is defined as the lowest concentration that completely inhibited the growth of Psa.

Product	50%	40%	30%	20%	10%	1%	0.1%	0.01%	0%
Tebuconazole with othililnone (1 h incubation)	0	0	0	0	0	0	2.3×10^7	2.0×10^7	1.3×10^7
Tebuconazole (3 h incubation)	0	0	1.6×10^6	1.1×10^7	1.4×10^7	2.0×10^7	4.0×10^7	2.0×10^7	1.1×10^7
Cyproconazole with iodocarb (formulation 2) (24 h incubation)	0	0	0	0	0	0	1.1×10^7	1.2×10^7	2.7×10^6

was determined after 24 h incubation and was 1%. The time of exposure was different for each of the formulations since the products required different times to kill Psa.

Survival and potential transferability of Psa in the orchard

Psa was found in two of the five samples of wax, but was not found in any of the other product samples used in the orchard (Table 6). Psa was

found on brushes used to spread all the products except tebuconazole with othililnone (Table 6). Of the eight samples in which Psa was detected, six had been plated the day after use in the orchard.

Effect of streptomycin as an adjuvant to wound protectants

While streptomycin at 100 ppm, provided as streptomycin sulphate or as a commercial formulation of streptomycin, killed Psa in a liquid

containing ca 10^7 cfu/ml within 15 min, addition of streptomycin at the same concentration to a 50% (vol/vol) solution containing tebuconazole or cyproconazole with iodocarb formulation 1 or formulation 2 did not result in a complete kill of *Psa* within 30 min for tebuconazole, 1 h for cyproconazole with iodocarb (formulation 2), or even within 24 h for cyproconazole with iodocarb (formulation 1) (Table 7).

DISCUSSION

Very few studies have looked at the impact of pruning on incidence of bacterial diseases and even fewer have looked at how to reduce that impact using commercially available wound protectants. One exception is a study on sweet cherry, which concluded that copper or phosphite had a very limited or no effect on incidence of bacterial canker caused by *P. syringae* pv. *syringae* (Carroll et al. 2010). One of the reasons for this very limited number of studies might be that most wound protectants were developed for protection against fungal pathogens.

Of the products commercially available in New Zealand, three formulations – tebuconazole, tebuconazole with othilinone and cyproconazole with iodocarb (formulation 2) – inhibited *Psa* on agar plates. The inhibition as measured by the diameter of the zone of inhibition was in some cases stronger than that given by streptomycin sulphate at 10 ppm. However, while the wound protectants were deposited undiluted on the bacterial overlay, streptomycin sulphate at 10 ppm is only 1/10 of the recommended rate for streptomycin.

Only tebuconazole with othilinone killed *Psa* in liquid medium in less than 5 min. In some cases, tebuconazole killed *Psa* in 30 min, but

this was not consistent and the reason for this variability is not known. However, by increasing the time of contact between the pathogen and the product to 3 h, the bacteria were consistently killed. Although the products had to be diluted to determine their ability to kill or inhibit *Psa*, the MIC of the three products that killed *Psa* was well below the 50% vol/vol rate at which the experiments were conducted.

Because the products that killed *Psa* required a long period of contact with the bacteria to kill them, the possibility that using those products in the orchard might help to spread the pathogen was investigated. *Psa* was found surviving in two of the five samples of wax used to treat infected stumps, but was not found in any of the other product samples used in the same orchard (Table 6). It might be that by analysing a larger number of product samples, or using each sample for more than four stumps, *Psa* would have been found in the products that have shown to be unable to kill or inhibit *Psa in vitro*. *Psa* was found on the brushes used to spread all the products except the formulation of tebuconazole with othilinone (Table 6). This is not surprising, given that this product was shown to kill *Psa* at very low concentrations and in a very short time. *Psa* was found most often on the bitumen, which did not kill *Psa* in the laboratory.

To determine whether addition of streptomycin to products that did not kill *Psa*, or needed a long contact time to kill it, could prevent the spread of the pathogen via orchard tools, the survival of *Psa* in water supplemented with a wound protectant and streptomycin (100 ppm) was determined. The addition of streptomycin

Table 6 Presence of *Pseudomonas syringae* pv. *actinidiae* (No. with *Psa*/Total No.) in product samples used in the orchard and on tools used to apply those wound protectants in the orchard.

Product	Container	Brush or knife
Tebuconazole	0/5	1/5
Wax	2/5	1/5
Cyproconazole with iodocarb (formulation 1)	0/5	1/5
Tebuconazole with othilinone	0/4	0/4
Bitumen	0/5	3/5

Table 7 Population of *Pseudomonas syringae* pv. *actinidiae* (cfu/ml) in a 50% (vol/vol) aqueous solution of different wound protectant formulations supplemented with a commercial formulation of streptomycin sulphate (100 ppm).

Experiment No.	Time of incubation	Product	Psa population
1	15 min	Nil	4.3×10^7
		Streptomycin sulphate	0
		Tebuconazole	1.7×10^7
		Tebuconazole + Streptomycin sulphate	6.7×10^6
2	30 min	Nil	3.7×10^7
		Streptomycin sulphate	0
		Tebuconazole	6.7×10^6
		Tebuconazole + Streptomycin sulphate	4.3×10^4
3	1 h	Nil	2.3×10^7
		Streptomycin sulphate	0
		Cyproconazole with iodocarb (formulation 2)	1.3×10^7
		Cyproconazole with iodocarb (formulation 2) + Streptomycin sulphate	8.7×10^5
4	24 h	Nil	3.3×10^8
		Streptomycin sulphate	0
		Cyproconazole with iodocarb (formulation 1)	1.8×10^8
		Cyproconazole with iodocarb (formulation 1) + Streptomycin sulphate	3.3×10^4

sulphate or a commercial formulation of streptomycin sulphate to cyproconazole with iodocarb (formulation 1) did not result in a complete kill of Psa within 24 h. Addition of a commercial formulation of streptomycin sulphate to tebuconazole or cyproconazole with iodocarb (formulation 2) reduced the Psa population after 30 min and 1 h respectively, while the commercial formulation of streptomycin sulphate by itself killed Psa with 15 min (Table 7). These results indicate that these three products somehow interfere with the ability of streptomycin sulphate to kill Psa. Similar results have been reported for tebuconazole using lower concentrations of streptomycin (10 ppm) (Dowlut et al. 2013).

In conclusion, of the wound protectant formulations commercially available in New Zealand only tebuconazole with octhilinone

showed some strong antibacterial activity. The main advantage of the other compounds might be in physically preventing the pathogen from entering the plant, rather than killing the pathogen. However, since Psa can survive in some of those compounds or on the tools used to apply them, there might be a risk of distributing the pathogen onto non-infected plants, when using them in an infected orchard. Addition of a strong bactericidal compound, such as streptomycin, did not result in a rapid and complete kill of Psa and therefore would not prevent the spread of the pathogen in an infected orchard when using those products.

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