Survival of *Pseudomonas syringae* pv. *actinidiae* in non-kiwifruit green compost

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Abstract *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, can survive in the environment outside its host. Determining where Psa can survive in the environment is important to limit the incidence and spread of this disease. Composts are sometimes used in kiwifruit orchards and the goal of this study was to determine the risk associated with such a practice. Three commercially prepared composts made from non-kiwifruit green waste were analysed for presence of Psa and for the ability of Psa to survive. Psa was not found in any of the composts analysed. An antibiotic-resistant derivative of Psa biovar 3 (previously Psa-V) used in this experiment could not multiply in any of the composts and its population declined rapidly. Live Psa could not be detected 7 days after inoculation. In sterile composts, Psa could be detected for at least 8 days after inoculation.

Keywords: bacterial canker of kiwifruit, source of inoculum, epidemiology.

INTRODUCTION

Worldwide production of kiwifruit is being affected by an outbreak of bacterial canker caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) (Vanneste 2012). Until 2008, this pathogen had been identified only in Japan (Takikawa et al. 1989), Korea (Koh et al. 1994), China (Wang et al. 1992) and Italy (Scortichini 1994). The economic impact of those early epidemics on the global kiwifruit production was relatively minor (Vanneste 2012). Since 2010, Psa has been isolated from France (Vanneste et al. 2011b), Portugal (Balestra et al. 2010), Spain (Abelleira et al. 2011), Turkey (Bastas & Karakaya 2011), Chile (EPPO 2011a), Switzerland (EPPO 2011b), Australia (EPPO 2011c) and New Zealand (Everett et al. 2011; Vanneste et al. 2011a). The strains isolated from those recent outbreaks are different

from the strains isolated earlier (Ferrante & Scortichini 2010; Mazzaglia et al. 2011; Chapman et al. 2012; Vanneste et al. 2013). Strains of Psa are now grouped in four categories called biovars (Vanneste et al. 2013). Strains of biovar 3 and biovar 4 have been isolated from New Zealand, but only strains of biovar 3 (previously known as Psa-V) are economically important.

Psa belongs to *Pseudomonas syringae*, a group of plant pathogenic bacteria that can survive in the environment outside their host plants. For example, *P. syringae* has been found surviving in water from rivers and lakes (Vanneste et al. 2008; Morris et al. 2010). Thus the environment could act as a reservoir for the pathogen. Compost is commonly used as a soil conditioner and nutrient source on productive kiwifruit orchards. If Psa

can survive in compost, then the use of compost could increase the inoculum of *Psa* in an orchard or spread this disease to areas that are still *Psa* free. If this is the case then alternative ways to supply nutrients to the vines might need to be found. The goal of this study was to determine whether *Psa* could survive or multiply on different commercial composts available in the Bay of Plenty where *Psa* has been present since 2010.

MATERIALS AND METHODS

Compost samples

Three compost samples (raw compost) were obtained from two commercial compost producers. Composts A and B were from Revital Fertilisers based in Tauranga, compost C was sourced from EnviroFert based at Tuakau in the Waikato district. All composts were made from non-kiwifruit green waste material. Sterile composts were obtained by autoclaving the samples twice at 120°C for 20 min. The samples were left at room temperature overnight between the two autoclavings.

Strains and media

Strains of *Psa* were grown and maintained at 28°C on King's B medium (King et al. 1954). When needed, this medium was supplemented with streptomycin (100 ppm), rifampicin (20 ppm) and/or cycloheximide (50 ppm). When washings from compost A were spread on King's B medium supplemented with streptomycin alone, 2.8×10^6 colony forming units (cfu)/g of compost were recovered. When similar washings were spread on King's medium supplemented with streptomycin and rifampicin, the number of bacteria that could be recovered fell to 1.2×10^4 cfu/g. The presence of a relatively high number of streptomycin-resistant bacteria in the compost precluded using a derivative of *Psa* resistant to only streptomycin to study the survival of *Psa* in compost. Since the natural population of streptomycin-and-rifampicin-resistant bacteria was 100 times lower than that of streptomycin-resistant bacteria, a streptomycin-and-rifampicin-resistant derivative of *Psa* was used to lower the limit of detection of the survival experiments. The strain of *Psa* 10627 Sm^R Rif^R, a spontaneous

streptomycin-and-rifampicin-resistant derivative of *Psa* 10627 (Vanneste et al. 2013), was used for inoculation of the compost samples. *Psa*10627 was isolated from New Zealand and belongs to biovar 3 (previously *Psa* V) (Vanneste et al. 2013).

Presence of *Psa* in compost samples

Three samples of 1 g of non-autoclaved compost from each batch of compost were diluted in 3 ml of water, and 100 µl aliquots of 1/10 dilutions made in sterile distilled water were plated on King's B medium without antibiotic. After incubation at 28°C for 48 h, any colonies resembling a colony of *Psa* were further characterised by polymerase chain reaction (PCR) using the primers *Psa*F1/R2, as described below.

Inoculation and determination of the bacterial population in compost samples

All the 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. Inoculations were made by mixing a suspension of *Psa* in sterile distilled water to 10 g of compost. The population of *Psa* present in the samples at the different sampling times was determined by washing 1-g samples in 2 ml of water and by depositing 10 µl drops of 1/10 dilutions of the washings made in sterile distilled water onto King's B agar plates supplemented with streptomycin and rifampicin. The numbers of bacterial colonies were recorded after 48 h of incubation at 28°C. Sterile water was used instead of the suspension of *Psa* as a negative control.

To assess the ability of *Psa* to survive in raw composts, 10 g of each compost was inoculated with 1 ml of a 3.2×10^9 cfu/ml suspension of *Psa* 10627 Sm^RRif^R or with sterile water. Every day for 4 days and then 7 days after inoculation *Psa* populations were determined for each of the composts. For each time point a 1-g sample from each compost was analysed.

To compare the ability of *Psa* to survive in raw composts versus sterilised composts, 10 g of raw or sterilised composts samples were inoculated with 1 ml of a 1.1×10^9 cfu/ml suspension of *Psa* 10627 Sm^RRif^R or with sterile water. For each sample, five

1-g samples were analysed for each time point. The standard error of the means for each treatment was calculated for each time point.

Identification of Psa by polymerase chain reaction (PCR)

When confirmation was needed that the bacteria isolated were Psa, it was performed as described earlier (Vanneste et al. 2012). PCRs were carried out using the primers PsaF1 (5'-TTTGTGCTTTGCACACCCGATTTT-3') and PsaR2 (5'-CACGCACCCTTCAATCAGGATG-3'), which yield a 280 bp amplicon with DNA from strains of Psa (Rees-George et al. 2010). Total DNA was isolated with a cell disruptor FastPrep®-24 from MP™ using the ZR fungal/bacterial DNA kit™ from Zymo Research, USA. PCRs were performed on an Eppendorf Mastercycler® Gradient, in 30 µl of the buffer supplied with the DNA polymerase containing 50 to 100 ng of DNA, 10 pmol of each primer, 200 µM of each dNTP, and 1 unit of i-Taq™ from INTRON Biotechnology, Inc. The protocol used was that described by Rees-George et al. (2010) and modified by Vanneste et al. (2010). A negative control, in which the DNA solution was replaced by the same volume of water, and a positive control, in which the DNA was from a strain already identified as Psa, were included in each experiment. The PCR products were separated by horizontal gel electrophoresis on 1% or 2% agarose containing 10 ng/ml of ethidium bromide. Fifteen to 30 µl of each PCR reaction were loaded on the agarose gel and the DNA bands were visualised under UV light. A DNA ladder (ZR 1 kb DNA ladder from Zymo Research, USA) was used on each gel for size comparison.

RESULTS

Presence of Psa in raw composts

None of the bacterial colonies isolated from any of the three compost samples had a colony morphology similar to that of Psa nor gave a 280 bp amplicon after PCR with the primers PsaF1/R2. This indicated that if present in those samples Psa was consistently below the level of detection of this experiment, which was calculated to be 30 bacteria per g of compost.

Survival of Psa in raw composts

No Psa was recovered from compost samples inoculated with sterile water. Over the 4 days after inoculation the Psa population decreased continually from $0.6\text{--}1.2 \times 10^9$ cfu/g to $0.14\text{--}1.5 \times 10^5$ cfu/g (Figure 1). Seven days after inoculation no Psa colonies could be isolated from any of the composts. To check that presence of Psa was not masked by other microorganisms present in the compost, a PCR was done with the DNA obtained from a pipetman tip swiped across the 48 h bacterial growth of the undiluted washing. This bacterial growth was caused by the naturally streptomycin-and-rifampicin-resistant bacteria present in the compost. None of the assays yielded a 280 bp amplicon, indicating that by day 7 Psa populations were below the level of detection. The rate of decline of Psa in compost was similar for the three different composts tested.

Survival of Psa in sterilised composts versus raw composts

This experiment aimed to determine whether sterilising the composts would influence the ability of Psa to survive in those composts. The population of Psa over time was determined in

Figure 1 Population of *Pseudomonas syringae* pv. *actinidiae* (cfu/g) recovered from three different composts inoculated with a bacterial suspension of Psa 10627 Sm^RRif^R. The limit of detection is indicated by the horizontal dashed line.

Figure 2 Population of *Pseudomonas syringae* pv. *actinidiae* (cfu/g) recovered from raw or sterilised compost after inoculation with Psa 10627 Sm^RRif^R. Results for composts A, B and C are presented in panels (a), (b) and (c), respectively. Populations from raw compost are represented by a solid line and populations from sterile compost are represented by a dotted line. Vertical bars represent standard errors of the means. The limit of detection per sample is indicated by the horizontal dashed line.

each of the three composts after being sterilised and inoculated or kept raw and inoculated. The results for composts A, B and C are presented in Figure 2. No Psa was isolated from any of the samples inoculated with sterile water. In the non-sterile compost samples, Psa populations were below the level of detection by day 6 for composts A and C and by day 8 for compost B. Those results are consistent with those obtained in the first experiment, in which Psa populations dropped below the level of detection by day 7 (Figure 1). For all the three composts, Psa survived longer in sterile than in non-sterile composts. In sterile composts, Psa could be detected until 8 days after inoculation, which is several days longer than when it could be detected in non-sterilised composts.

DISCUSSION

The composts analysed in this study were sourced from suppliers in the Bay of Plenty, where Psa is well established, and the Waikato region. Psa was not detected or recovered from any of the compost samples tested, which might simply reflect the fact that those composts being derived from non-kiwifruit green waste were not carrying Psa. This is consistent with the fact that, although only a few plants used as shelterbelt and a few common weeds have been analysed so far, Psa has not yet been found on plants other than kiwifruit, (Vanneste et al. 2012; J.L. Vanneste, unpublished data). Alternatively, if Psa had been present on the green waste it might have been killed during the composting process. In experiments using plant material infected with *P. syringae* pv. *syringae*, all plant pathogenic bacteria were killed during the composting process (Elorrieta et al. 2003; Suarez-Estrella et al. 2007). However, whether the heating phase of the composting process would have been enough to kill Psa if it had been present on the green waste (for example kiwifruit material) has yet to be demonstrated.

In both experiments, Psa populations in non-sterile composts declined rapidly and were below the level of detection within 7 days. A number of biotic and abiotic factors could explain this relatively rapid decline. Psa is not a soil bacterium and it might find it difficult to compete with better adapted organisms. To determine whether

the inability of *Psa* to multiply in compost and its limited ability to survive was due to the presence of microorganisms naturally growing in the compost, a comparison of the ability of *Psa* to survive in those three composts before and after sterilisation was carried out. *Psa* can survive on sterile compost slightly longer than on non-sterile composts. This suggests that the elimination of naturally occurring microbial populations in the compost by autoclaving allowed the *Psa* to survive longer. However, even in sterile compost, the population of *Psa* decreased relatively rapidly indicating that this bacterium is not adapted for survival in compost. A similar result has been obtained when autoclaved soil was inoculated with *Psa* (J.L. Vanneste, unpublished data). While the reduction of plant bacterial diseases using compost has been documented (Noble & Coventry 2005), the ability of plant pathogenic bacteria to survive in mature compost has been only rarely studied and only for soil-inhabiting bacteria, such as *Ralstonia solanacearum* (Tomlinson et al. 2011). This appears to be the first report on survival of an aerial plant pathogenic bacterium in mature compost.

These experiments showed that green waste composts made from non-kiwifruit material do not constitute a reservoir of inoculum for *Psa*. *Psa* could not multiply and did not survive for more than a few days in the composts that have been tested.

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