Recent progress on detecting, understanding and controlling *Pseudomonas syringae* pv. *actinidiae*: a short review

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**Abstract** In the last few years the causal agent of bacterial canker of kiwifruit, *Pseudomonas syringae* pv. *actinidiae* (Psa), has become a global pathogen of economic importance. Since the beginning of this global outbreak, many laboratories in the world have been working on Psa. Today, it is known that Psa is not a homogeneous pathovar and tools that allow the distinction between biovars (sub-pathovar classification) have been developed. The whole genome sequence of several strains of Psa has now been published. Some of the assumptions on the life cycle (ports of entry, epiphytic survival, etc.) made in the early days of the outbreak have now been confirmed. Although few new methods have been found to control Psa, there is now a better understanding of how to reduce the incidence of this disease. This paper reviews the progress made in understanding the pathogen, the disease and how to control it.

**Keywords** aetiology, epidemiology, biovar.

**INTRODUCTION**

Twice as many scientific papers on *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, have been published in the last 5 years (between 2008 and 2013) than in the previous 19 years (between 1989, the year of the first publication on Psa, and 2008). This reflects the huge research effort that has recently taken place in Europe and New Zealand trying to understand and control this pathogen. This research effort was prompted by an exceptionally devastating outbreak of the disease that first appeared in Latina (Italy) in 2008 (Balestra et al. 2009; Ferrante & Scortichini 2009) and which became an economically important global problem in the following 3 years. In New Zealand alone the cost to the industry over the next 5 years has been estimated to be between NZ$ 310 and NZ$ 410 million (Greer & Sanders 2012; Vanneste 2012).

Until 2008, Psa had been reported only in Japan (Takikawa et al. 1989), Korea (Koh et al. 1994), China (Wang et al. 1992) and Italy (Scortichini 1994). But since 2008, it has been isolated from France (Vanneste et al. 2011c), Portugal (Balestra et al. 2010), Spain (Abelleira et al. 2011), Turkey (Bastas & Karakaya 2011), Chile (EPPO 2011c), Switzerland (EPPO 2011b), Australia (EPPO 2011a) and New Zealand (Everett et al. 2011; Vanneste et al. 2011a). Furthermore, prior to 2008, outbreaks of Psa in Italy were of no real economic consequence, few vines had to be removed, and the disease never spread very far from the original focus of infection. In 2008, Psa was found on *Actinidia deliciosa* (green-fleshed...
kiwifruit) and *A. chinensis* (yellow-fleshed kiwifruit) in Latina, which is the most important Italian area for kiwifruit production. By 2010, it had progressed throughout Latina and spread throughout Italy (Vanneste et al. 2011b).

In New Zealand Psa was among a number of pathovars of *P. syringae* that were classified as unwanted organisms. As early as 2007, MAF Biosecurity New Zealand (now the Ministry for Primary Industries) had contracted the development of molecular tools for the identification of 16 pathovars of *P. syringae*, including Psa (Vanneste et al. 2009b). This led to the development of several PCR primers for detection and identification of Psa (Vanneste et al. 2009a; Rees-George et al. 2010). When in early 2009 the economical importance of the Italian outbreak became obvious, and 'Hort16A', a cultivar grown in Italy under contract for Zespri, was one of the cultivars affected by Psa, New Zealand became involved in the early research done overseas on understanding better this pathogen and trying to control it (Vanneste et al. 2011b). However, when the disease was found in 2010 in New Zealand, little information on how to control Psa under New Zealand conditions was available. Consequently, the research effort in New Zealand intensified tremendously. Today the industry is still struggling to contain or control this pathogen, yet important progress has been made. This paper will review some of the most important progress in understanding the pathogen, the disease and how to control it.

**THE PATHOGEN**

The strains isolated from the recent global outbreak are different from the strains isolated earlier in Japan, Korea and Italy (Ferrante & Scortichini 2010; Mazzaglia et al. 2011; Chapman et al. 2012; Vanneste et al. 2013b). Known strains of *P. syringae* within the pathovar *actinidiae* are now grouped within four sub-pathovar groups called biovars (Vanneste et al. 2013b). Only Psa biovar 1 was present in Italy before 2008. Since 2008, all the strains isolated in Italy and in the rest of Europe belong to biovar 3. Strains of both biovar 3 and biovar 4 have been isolated from New Zealand, but only strains of biovar 3 (previously referred to as Psa-V) are economically important. Strains of biovar 4 (previously referred to as Psa-LV) are found throughout New Zealand, where they cause leaf spotting but do not cause systemic infections or plant death. Initially, tools that allowed the identification of Psa were very useful tools. However, diagnostic tools that allowed characterisation of strains of Psa at the sub-pathovar level became necessary because of the presence in New Zealand of two biovars of very different virulence but both able to cause leaf symptoms. Initially PCR primers were designed to better understand the taxonomic position and the relatedness of Psa with that of other pathovars of *P. syringae* (Sawada et al. 1997; Scortichini et al. 2002). The first set of primers designed for the detection and identification of Psa was developed by Koh & Nou (2002). Those primers, which amplified a 492 bp DNA fragment with all strains of Psa, were identified after a random amplified polymorphic DNA (RAPD) analysis. This amplicon has been shown to contain a sequence similar to part of the DNA sequence of the *ompP1* gene, which in *P. s. pv. tomato* codes for the outer protein P1 (Vanneste et al. 2013b). Another set of primers based on the gene that codes for the outer protein P1 has recently been developed (Biondi et al. 2013). A nested PCR based on presence of the *clf* gene, which codes for synthesis of coronatine, was described shortly after the Koh and Nou primers (Jung et al. 2003). However, of the four biovars of Psa identified today, only biovar 2 produces coronatine (Vanneste et al. 2013b); therefore, this protocol cannot be used for the detection or identification of all biovars of Psa. The Koh and Nou primers were later found to give an amplicon of ca 492 bp with DNA from strains of *P. syringae* pv. *actinidiae* isolated from tomato, *P. syringae* pv. *theae*, and other strains of *P. syringae* (Rees-George et al. 2010). Of those strains that gave a false positive with the Koh and Nou primers, only the strains of *P. syringae* pv. *actinidiae* gave an amplicon of the size expected for Psa using the primers PsaF1/R2 or PsaF3/R4 (Rees-George et al. 2010). However, since 2010 strains of *P. syringae* pv. *avellanea*, pv. *passiflorae,*
and a few other unidentified bacteria isolated from kiwifruit leaves have also been found to give a false positive with the primers PsaF1/R2 (J.L. Vanneste, unpublished data). The primers PsaF1/R2 and PsaF3/R4 amplify a fragment of the ITS region. They are consequently very sensitive, since several copies of this region exist per chromosome. A real-time PCR protocol has also been developed for those primers (Vanneste et al. 2013b). The duplex PCR protocol developed by Gallelli et al. (2011) is based on the primers developed by Koh & Nou (2002), and on primers that target the \textit{avrD} gene. Strains of biovar 3 gave two bands of 492 and 226 bp as expected, but strains of biovar 4 gave only the 492 bp band (Vanneste et al. 2013b). It was found later that the gene \textit{avrD} is indeed present in the strains of biovars 1, 2 and 3 but not in strains of biovar 4 (McCann et al. 2013). This duplex PCR has been one of the tools used in New Zealand to distinguish strains of biovar 3 from those of biovar 4 (Vanneste et al. 2013b).

Before biovars of Psa were defined, strains of Psa were characterised by the DNA sequence of their \textit{cts} gene (Vanneste et al. 2010). Two \textit{cts} haplotypes were initially described, one corresponding to biovars 1 and 2, and one corresponding to strains of biovar 3. Strains of biovar 4 were found to have different \textit{cts} haplotypes from the two described earlier. The differences in the \textit{cts} DNA sequence was the basis for the first tool developed for characterisation of Psa rather than identification of the pathogen (J.F. Mackay, Dnature Diagnostics & Research Ltd, unpublished data). Those were followed by more advanced diagnostic tools based on the whole genome sequence analysis of several strains of biovar 3 and biovar 4 of Psa (Rikkerink et al. 2011; Balestra et al. 2013).

Next Generation Sequencing allowed several laboratories to sequence the whole genome of a number of strains of Psa (Baltrus et al. 2011; Marcelletti et al. 2011; Mazzaglia et al. 2012; Butler et al. 2013; McCann et al. 2013). A detailed analysis of the information obtained from comparing those sequences among themselves and with those of other strains of \textit{P. syringae} is beyond the scope of this limited review. However, the classification in four biovars, which was based on multi-locus sequence typing (MLST) analysis and some biological characteristics, has been validated in several of those studies (Marcelletti et al. 2011; Mazzaglia et al. 2012; Butler et al. 2013; McCann et al. 2013). An in-depth analysis of the DNA sequence also revealed that strains of biovar 3 isolated from Italy were different from those isolated from New Zealand (Mazzaglia et al. 2012; Butler et al. 2013; McCann et al. 2013). On the other hand, some strains isolated from China were similar to strains isolated from Italy and from New Zealand. This suggests that Italy is not the origin of the New Zealand outbreak but that Italian and New Zealand strains might have had independent origins in China. A large number of effector proteins (bacterial proteins that are injected into the host plant cells and dictate the outcome of the plant-bacteria interaction) has been identified. Further study of those effectors will help understanding of how Psa causes disease and hopefully will help in the selection of kiwifruit plants resistant to Psa.

**THE DISEASE**

The rapid progression of the disease in Italy prompted a series of hypotheses on how the bacteria was surviving, moving and infecting its plant host (Vanneste et al. 2011b). Based on the Japanese studies available at the time (Serizawa et al. 1989; Serizawa & Ichikawa 1993a, b, c, d; Serizawa et al. 1994), on the knowledge of how other \textit{Pseudomonas syringae} infect their host plant, e.g. Rudolph (1995), and on the observations of symptom development in the field, a model was presented in August 2010 (Vanneste et al. 2011b). One of the main features of the cycle was that the two most important infection periods were spring and autumn. Observations in Italy were in agreement with reports in the literature that found that Psa populations decrease when temperatures increase (Serizawa & Ichikawa 1993b) and that above 25°C Psa inoculations did not lead to exudate or infection (Serizawa & Ichikawa 1993a). Few new symptoms could be found when the average temperature over a 10-day period was...
above 20°C (Serizawa & Ichikawa 1993a). Such temperatures are easily reached in Latina during summer. Although frost probably encourages infection creating entry points for the pathogen, during winter in Latina temperatures are most probably too cold for infection to progress.

The situation in New Zealand is quite different from the situation in Italy. In summer average temperatures are never hot enough, and in winter they are never cold enough to prevent progression of Psa infections. Since Psa is also an epiphytic bacterium, which can survive outside its host plant, infection can occur almost all year round. The ability to identify potential periods of infection is therefore quite important. A model to predict potential periods of infection has been developed (Beresford 2012). Initially the model was based on prediction models developed for another devastating bacterial disease: fire blight, caused by *Erwinia amylovora*, but the model has been progressively updated as more information on Psa response to temperature and wet period has become available. This model has been made available to New Zealand kiwifruit growers (http://www.kvh.org.nz/Psa_risk_model).

As for most pathogenic bacteria, once Psa is inside the plant there is very little, besides cutting out the affected parts, that can be done to prevent the bacteria spreading throughout the plant. Understanding where and when the bacteria enter the plant might offer clues on how to better protect the plants. This explains why a lot of attention has been given to finding the port of entry of Psa and the conditions under which it causes symptoms. The development of Psa marked with a *gfp* gene indicated that Psa can infect the plant from stomata, leaf abscission scars and broken trichomes (Spinelli et al. 2011). Colonisation of the major lower parts was also demonstrated using those marked strains of Psa (Spinelli et al. 2011). Psa has been found associated with pollen (Vanneste et al. 2011d) and this might help its dissemination (Stefani & Giovanardi 2011).

The ability of Psa to survive in the field as an epiphyte on asymptomatic kiwifruit plants was reported early on (Vanneste et al. 2011a). Psa was later found to multiply on kiwifruit leaves in the laboratory (Horner et al. 2011) and to survive for at least 15 weeks on leaf litter and on cane prunings in the laboratory and in the field (Tyson et al. 2012). However, it survived for only a very limited time on the few non-host plants that have been examined (Vanneste et al. 2012a) and in compost (Vanneste et al. 2013a).

**CONTROL**

Bacterial diseases are notoriously difficult to control. Few products are available to control bacteria and even fewer for the control of plant pathogenic bacteria. This is because the majority of the products that kill bacteria are either heavy metals, which are toxic and have adverse environmental consequences, or antibiotics, which are reserved for the control of human and animal bacterial pathogens.

An extensive screening for compounds that could be used for control of Psa was started in New Zealand in early 2011, to answer the immediate needs of the New Zealand kiwifruit industry. Products were assessed on kiwifruit seedlings in a PC1+ physical containment glasshouse on their ability to reduce infection as assessed by the percentage of the treated leaves that showed necrosis after inoculation with a virulent strain of Psa. The products were grouped according to their potential mode of action: biological control, sterilants or elicitors. Elicitors were applied 7 to 10 days before inoculation, while biological control agents were applied 48 h before inoculation. Sterilants, which include all the products that act directly on Psa, were applied just before inoculation. In over 40 experiments, more than 450 treatments representing over 150 products have been assessed. About 25 products showed some efficacy in those glasshouse trials. All of those 25 products that have been advanced for field assessment were also found to give some control in the field.

Although copper-containing products did not reduce significantly the incidence of Psa in glasshouse experiments, there have been consistent observations from commercial orchards indicating that copper does reduce Psa incidence.
The discrepancy between the glasshouse experiments and the field might be related to the fact that, to be active, copper-containing products need environmental conditions that lead to the release of cupric ions. It is those cupric ions that inhibit or kill Psa. The conditions necessary for the release of the cupric ions are not present in the glasshouse.

In parallel to this untargeted approach, in which products were assessed without any a priori except for their potential mode of action, a project was initiated based on the fact that strains of Psa biovar 2 produce a toxin called coronatine, which mimics jasmonic acid (JA). JA is a plant hormone involved in a signalling pathway for resistance to necrotrophic pathogens. Elicitation of the JA pathway suppresses elicitation of the salicylic acid (SA) pathway, which is commonly associated with resistance to biotrophic pathogens. This led to the hypothesis that not only was Psa not affected by elicitation of the JA pathway, but that it might be eliciting this pathway to suppress the SA pathway to which it is susceptible. Work carried out in Italy and New Zealand showed that treatment with JA or ethylene did not reduce the incidence of the disease on young kiwifruit seedlings in the glasshouse, but that salicylic acid or derivatives, such as the commercial compound acibenzolar S methyl (ASM), significantly decreased disease incidence (Vanneste et al. 2012b).

From those research programmes, two new products have been made available to New Zealand growers: streptomycin and Actigard™, which contains a functional analogue to salicylic acid. However, there are still very few products available to growers and the best way of using the compounds available is not always known. The screening project has now been expanded to answer both these concerns.

CONCLUSIONS
In the last few years a massive amount of work has been carried out in response to a highly devastating global outbreak of Psa. Understanding of the pathogen has consequently increased considerably. It is now known that strains of the pathovar actinidiae can be classified in sub-pathovar groups called biovars. One of these biovars, biovar 3, is the one responsible for the recent worldwide outbreak. Tools have been developed that allow the detection and identification of Psa at the biovar level. The whole genome DNA sequence of several strains of Psa, including strains of biovar 3, is now publicly available, and will greatly facilitate further in-depth studies on how Psa causes disease.

The life cycle of the disease is also much better understood. Unfortunately the climatic conditions that limit the infections during periods of the year in Italy and in Asia are not present in New Zealand. In particular, it would seem that summers are never hot and dry enough nor are the winters cold enough to stop the disease cycle. The development of a prediction model might help considerably the ability to target the spray of the few products identified so far to periods of greatest infection risk.

In addition to the progress reported in this paper, breeding programmes are now including resistance to Psa as a major criterion of selection. Some species of Actinidia are much less susceptible than A. delicosa and A. chinensis, and some cultivars of those two species also show signs of resistance to Psa. The new cultivars that will emerge from those breeding programmes will also benefit from an understanding on how to reduce Psa incidence.

So while significant progress has been made in the understanding and management of Psa, considerably more is necessary for kiwifruit production to regain its previous degree of commercial success in New Zealand.

ACKNOWLEDGEMENTS
Work in the author’s laboratory is supported by Zespri Group Limited, Kiwi Vine Health Ltd and Plant & Food Research.

REFERENCES


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