Efficacy of *Bacillus subtilis* for the control of *Pseudomonas syringae* pv. *actinidiae* under field conditions

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Abstract

Bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), is a disease included on the EPPO A2 List because it is currently increasing in the Mediterranean region. Climatic conditions determine the multiplication and dispersal behavior of Psa and may influence its biological control (BC). However, information regarding the influence of climatic conditions on growth of the Psa bacterium in Portugal is scarce. The aims of this study were to relate the climatic conditions with the efficacy of *Bacillus subtilis* Serenade Max® in controlling the disease, to determine whether disease incidence increased over the growing season, and to monitor carry-over of disease from one season to the next. Two 'Hayward' orchards of *Actinidia deliciosa* of different ages, located in Valença, in the north of Portugal, were selected for BC assay. Thirty plants were randomly selected from a young orchard (6 years old) and another 30 plants from an old one (30 years old). Psa was identified and characterized by morphological, biochemical and molecular tests. The efficacy of *B. subtilis* was evaluated by disease incidence, and correlated to climatic conditions. A standardized leaf infection index (0-4 scale of symptoms) was used to determine disease severity. Monitoring of Psa symptoms was performed every 30 days, from June 2016 to April 2017. The severity of the disease decreased in the summer season, characterized by unfavorable conditions for Psa such as high temperatures and scarce precipitation. *B. subtilis* was partially effective in controlling Psa in the 6-year-old orchard, and showed no efficacy in the protection of leaves in the 30-year-old orchard. *B. subtilis* applied at flowering could be considered as a suitable solution for the control of Psa biovar 3 only for the new kiwifruit orchard (6 years old).

Keywords: *Actinidia deliciosa*, bacterial canker, *Bacillus subtilis*, climatic conditions

INTRODUCTION

Pseudomonas syringae pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, is a very destructive bacterium, included on the European and Mediterranean Plant Protection (EPPO) A2 List. The first epidemic outbreaks in Europe were recorded in 2008 in Italy (Balestra et al., 2008), followed by France, Germany, Portugal, Spain, Switzerland, and Turkey. Outside the EPPO region, the disease has been reported in New Zealand, Chile, Japan, China and Korea (EPPO, 2014).

At present, five genetically different Psa populations or biovars are known, biovars 1, 2, 3 and 5 (Chapman et al., 2012; Sawada et al., 2014) and biovar 6, present in a small region of Japan (Sawada et al., 2016). Biovar 3 is the most destructive and comprises the pandemic strains isolated in Europe, New Zealand, Chile and China, causing severe economic losses in all major areas of kiwifruit production worldwide (Scortichini et al., 2012), and is the Psa biovar present in the north of Portugal (Moura et al., 2015). Several groups of *P. syringae* with low virulence on kiwifruit, causing foliar spots and not cankers or other symptoms, isolated in New Zealand, Australia (Vanneste et al., 2013), France and Spain (Abelleira et al., 2015; Cunty et al., 2015) and initially named Psa biovar 4, were recently separated from Psa

and assigned as *P. syringae* pv. *actinidifoliorum* (Cunty et al., 2015).

In Portugal, the disease was detected in 2010 (Balestra et al., 2010) in the Entre Douro e Minho Region (REDM), and consequently kiwifruit production decreased (INE, 2015). Nevertheless, the production area is growing: Portugal has a kiwifruit growing area of 2305 ha and a total production of 28,331 t, of which the REDM has the main part of the cultivated area (1721 ha) with fruit production of 23,205 t (INE, 2016).

Climatic conditions determine the multiplication and dispersal behavior of Psa and may influence its biological control (BC). However, information concerning the growth of the Psa bacterium under the climatic conditions of Portugal is scarce. Psa control methods are currently in place and being used to manage the incidence and progression of Psa infection. Chemical control of Psa is based on copper products, most of them authorized for winter treatment and effective against other bacterial pathogens on kiwifruit, such as *P. syringae* pv. *syringae* and *Pseudomonas viridiflava* (Fratarcangeli et al., 2010). Moreover, other chemical products such antibiotics, not authorized in Europe, and elicitors, in addition to orchard management practices, are used to control Psa (Stewart et al., 2011). More recently, antagonistic microorganisms, such as *Bacillus subtilis*, are being used to minimize the damage caused by Psa. However, the efficacy and reliability of such treatments under different environmental conditions is limited and not well studied.

The aims of this study were to study the impact of climatic conditions and the efficacy of *B. subtilis* in controlling Psa under field conditions at two 'Hayward' orchards of *Actinidia deliciosa* in the north of Portugal, infected with Psa biovar 3, to determine whether disease incidence increased over the growing season, and to monitor carry-over of disease from one season to the next, in order to investigate the use of *B. subtilis* in a program for the control of Psa in this region.

MATERIALS AND METHODS

The 'Hayward' orchards are located in Valença do Minho in the northwest of Portugal (REDM), where Psa was first detected in Portugal (Balestra et al., 2010). One orchard has plants that are 30 years old, and the second, younger, with plants that are 6 years old. These plants are trained in T-bar structures, which have trunks 1.80 m tall and a central leader, which is composed of a horizontal cordon that produces the fruiting canes. The orchards have a total area of 1.15 ha and are irrigated with micro-sprinklers.

Climatic conditions of the orchards

Data for climatic parameters were obtained from a meteorological station of the Regional Services of Agriculture, located in Ganfei (5 km from the orchards). Temperature, precipitation, radiation and air humidity were registered continuously, and used to correlate with the incidence and severity of the disease in 2016 and 2017.

Bacterial isolation

Bacteria were isolated from orchards displaying symptoms of Psa in 2016 and 2017, from leaves, buds, flowers and exudates of *A. deliciosa* 'Hayward' (Table 1). Fragments of infected tissues were removed from the margin of necrotic lesions, dipped into 95% ethanol, washed in sterile water, macerated and incubated in sterile water for 10-20 min at room temperature. Subsequently, 20-µL aliquots of the macerated samples or exudates were plated on modified King B medium (Mohan and Schaad, 1987). Inoculated plates were incubated at 28°C for 72 h. Strains presenting the morphological characteristics described for Psa were selected and purified on King B medium (King et al., 1954), and used for further identification and characterization.

Psa identification and characterization

Psa-like isolates were identified based on morphological, cultural, biochemical, and molecular methods following the protocol proposed by the EPPO for Psa (EPPO, 2014).

Table 1. Strains isolated in 2016 and 2017 from infected plants in new (6 years old) and old (30 years old) kiwifruit 'Hayward' orchards in Valença do Minho. Samples used for Psa isolation are shown.

Year	Old orchard		New orchard	
	Strain	Host/part of plant isolated	Strain	Host/part of plant isolated
2016	VV1	Female/leaf	VN15	Female/leaf
	VV2	Female/leaf	VN16	Female/leaf
	VV3	Female/leaf	VN17	Female/leaf
	VV4	Female/leaf	VN18	Female/leaf
	VV5	Female/leaf	VN19	Female/leaf
	VV6	Female/leaf	VN20	Female/leaf
			VN21	Female/leaf
			VN22	Female/leaf
2017	VV7	Female/ooze	VN23	Female/ooze
	VV8	Male/flower	VN24	Female/leaf
	VV9	Female/leaf	VN25	Female/leaf
	VV10	Male/leaf	VN26	Female/flower
	VV11	Female/leaf		
	VV12	Male/leaf		
	VV13	Female/flower		
	VV14	Female/flower		

Twenty-six Portuguese strains and one reference strain (CFBP 7286) were tested for the presence of cytochrome *c* oxidase activity, production of levan on sucrose-rich medium, ability to hydrolyze arginine, pectolytic activity and tobacco hypersensitivity as described by Lelliott and Stead (1987). Production of a fluorescent pigment from bacterial colonies was observed on King B medium under ultraviolet light (λ=560 nm) (Cunty et al., 2015).

Molecular identification and characterization of each Psa-like isolate was performed with DNA extracted from bacterial suspensions, prepared from pure colonies in sterile distilled water and calibrated at an optical density at 600 nm of 0.5, and stored at -20°C before use in PCRs. Identification of the Portuguese strains was performed by duplex PCR with two sets of primers, KN-F/KN-R (Koh and Nou, 2002) and AvrDdpx-F/AvrDdpx-R proposed by Gallelli et al. (2011). A reference Psa biotype 3 strain from Italy (CFBP 7286) was included in all PCR experiments as a positive control. A negative control with no template DNA added was also included. Isolates were characterized by BOX-PCR as described by Louws et al. (1994). Amplification reactions were carried out as described by Gallelli et al. (2011) and Louws et al. (1994) in a Perkin Elmer 2400 thermocycler. Duplex-PCR and Box-PCR amplicons were separated by electrophoresis on 1.5 and 2% agarose gels, respectively, in 0.5× TBE buffer. DNA bands were stained with GelRed™ (Biotium Inc., Hayward, CA, USA) and visualized on a UV transilluminator and the images were recorded for analysis. Band sizes were compared by including a 100-bp molecular marker (BIORON DNA Ladder) in duplex-PCR agarose gels, and 1-kb DNA molecular markers (BIORON DNA Ladder) to estimate the size of the amplicons obtained with the Box1R primer. Psa strains were identified by the presence of amplicons of 492 and 226 bp (Gallelli et al., 2011).

Orchard trials and field application of *B. subtilis*

To test the efficacy of *B. subtilis* Serenade Max®, a 2-year trial was established. A formulation of 15.67% *B. subtilis* QST 713 (Serenade Max®) was applied using a maximum concentration of 1 kg ha^{-1} following the instructions of the manufacturer. In 2016, it was applied at the end of flowering (one application only) and, in 2017, four applications were done during the flowering period. The first application of Serenade Max® started in the third week of May; the second occurred 4 days after, because this biological product acts by contact and has a biological persistence of 7 days and, as it rained, the application was

applied earlier to protect the flowers from Psa infection. The two following applications of Serenade Max® were made 3 days after the previous ones.

Disease severity was determined by regular detailed monitoring of Psa symptoms (10 plants of each replicate treatment) performed every 30 days, from May 2016 to June 2017 in both orchards. A standardized leaf infection index (0-4 scale of symptoms) was used to determine the percentage of infected leaf surface, as follows: 0 = no symptoms; 1 = symptoms in more than 10% of the leaf; 2 = symptoms in more than 25% of the leaf; 3 = symptoms in more than 50% of the leaf; and 4 = symptoms in more than 75% of the leaf. This scale resulted from the adaptation of a standardized Psa disease leaf index described in the testing report for Serenade Max® (New Zealand Institute for Plant and Food Research, 2011).

The experimental design was a randomized block design, including treatments with and without *B. subtilis* with three replicates each of ten plants, in both orchards. Analysis of variance (ANOVA) was performed by the general linear model SPSS procedure using SPSS 17.0 for Windows (SPSS Inc.) and treatments were compared by the least significant difference (LSD) test. A probability level of α=0.05 was applied to determine statistical significance.

RESULTS AND DISCUSSION

Bacterial isolation and phenotypic characterization

The 26 bacterial isolates obtained from symptomatic samples of *A. deliciosa* 'Hayward' (leaves, flower buds, flowers and exudates) formed whitish, translucent, round colonies, 1-2 mm in diameter after 72 h. Some strains produced a light fluorescent pigment on King B medium. Isolated strains were Gram-negative, levan-positive, did not have cytochrome c oxidase or arginine dehydrolase activity, did not cause potato soft rot and produced a hypersensitivity reaction on tobacco leaves.

Molecular identification and characterization of Psa

The 26 Portuguese isolates, and the reference strain CFBP 7286, were characterized by the amplification of two fragments of 492 and 226 bp (Figure 1) and assigned to Psa.

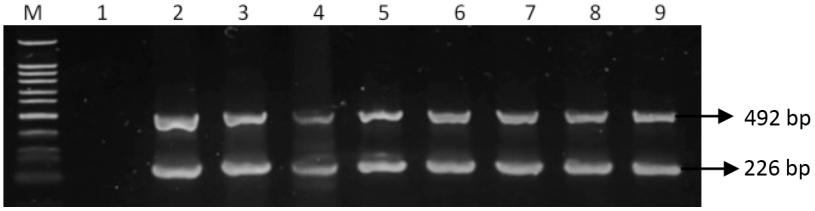

Figure 1. Amplified DNA fragments obtained by duplex-PCR with primers KN-F/R and AvrdDpx-F/R. Lanes: 1, water; 2, CFBP 7286; 3-9, representative Portuguese strains. M, 100-bp DNA ladder marker (BIORON).

The results of BOX-PCR obtained with selected strains from the old and new orchards were similar to those for the reference strain CFBP 7286 (biovar 3) (data not shown).

Field conditions and disease severity

The climatic conditions (Figure 2) show that May 2016 was a very rainy month, with high relative humidity and a mean temperature of 15.3°C. Flowering was late in the season, and the rainfall during this month (160 mm) destroyed many flowers. Therefore, the application of *B. subtilis* Serenade Max® was delayed to the end of May, when climatic conditions were optimal for its application, with temperatures about 19°C and 72% relative humidity.

In the second year, the first application of Serenade Max® started in the third week of May, when 30% of the flowers were open, favoring the efficacy of the application. At this

time of year, in both years of the assay, symptoms of Psa were easily observed in flowers and in young leaves, with larger necrotic spots on the leaves, falling flowers that did not open because of Psa necrosis, and die-back of the young shoots (Figure 3).

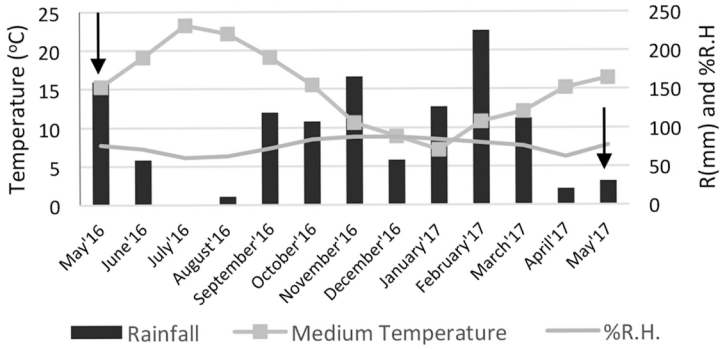

Figure 2. Climatic conditions (May 2016-May 2017) for two kiwifruit 'Hayward' orchards located in Valença do Minho, north Portugal. Arrows indicate application of Serenade Max®. R, Rainfall; R.H., relative humidity.

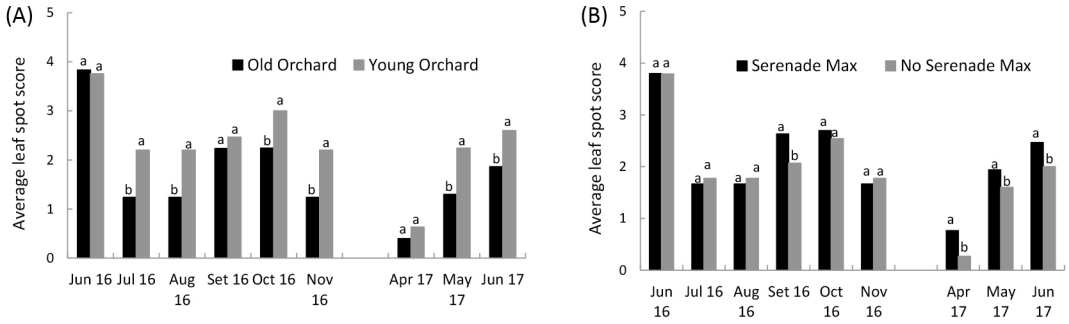

Figure 3. *Pseudomonas syringae* pv. *actinidiae* (Psa) disease severity recorded in two 'Hayward' kiwifruit orchards. (A) Effect of the age of plants (new/old orchard). (B) Effect of *Bacillus subtilis* Serenade Max® application (Serenade Max®/No Serenade Max®) from June 2016 to June 2017. Disease severity was estimated by using a 0-4 disease severity index (0 = no symptoms; 1 = symptoms in more than 10% of the leaf; 2 = symptoms in more than 25% of the leaf; 3 = symptoms in more than 50% of the leaf; 4 = symptoms in more than 75% of the leaf). Statistically different columns are distinguished as a and b.

With the amount of precipitation that occurred in 2017, mostly in February, the dispersal and multiplication of the bacterium were more evident from amounts of exudates in the orchards. In April, the incidence of leaf spotting was similar to the previous season, but the amounts of exudates in 2017 were higher, probably because of the higher rainfall in February.

The results obtained in this study are in accordance with those of Mauri et al. (2016), which indicated that climatic conditions over the seasons have a determinant impact on the pathway of dispersal and contamination of kiwifruit plants by Psa, and disease development is related to permissive environmental conditions, such as medium temperature, high humidity, presence of entry points, genetic and physiological features of the host plant.

Progression of Psa in 'Hayward' orchards

Disease severity and progression up to April 2017 in the two 'Hayward' kiwifruit orchards and the effect of the application of *B. subtilis* Serenade Max® are shown in Figure 3.

There was no apparent correlation between the effect of the application of *B. subtilis*

and the age of the orchard. The highest disease severity index was observed in June 2016 in both orchards, without significant differences between them ($p<0.05$). After June 2016, the disease severity was always lower, but increased in the new orchard compared with the old one. Other authors have also reported higher disease severity in young orchards (Scortichini, 1994; Tyson et al., 2015). Likewise, Vanneste et al. (2013) stated that, for the same place, younger plants are more susceptible than older plants.

Increased precipitation was registered in June 2016, conditions that can be favorable for Psa infection (Ferrante et al., 2012); this may explain why the disease severity in June was higher compared with the same period in 2017. Disease severity was lower in the summer (July and August 2016) relative to summer 2017 in both orchards, probably because temperatures were above 20°C.

In 2017, the incidence of the disease increased during the early months (April, May and June) in both orchards (with higher values in the young orchard) and in both treatments (with higher values in the treatment with Serenade Max®). At this time of the year, the temperature was optimal for the growth of the bacterium (12-18°C), which may explain this increase in incidence of the disease during these months (Donati et al., 2014). The lowest values of disease incidence of the two years of the experiment were in April 2017, probably because of the lower precipitation in that month, which can reduce dispersal of the bacterial inoculum (Ferrante et al., 2012).

The effect of Serenade Max® on disease severity was different ($p<0.05$) in September 2016 and April, May and June 2017. The increased disease incidence in plants treated with Serenade Max® in 2017, in comparison with the control plants, might be explained by the need for a wide and stable colonization of the epiphytic population by this biological control agent (BCA) to be effective, which probably did not happen in this assay. These results suggest that this BCA cannot be used alone to control the disease. However, it is possible that this BCA may show a long-term efficacy that was not demonstrated in this assay, as data were collected only from one year.

In 2016, evaluation of disease symptoms in the leaves began only after the *B. subtilis* application (end of May). For the plants not treated with *B. subtilis*, disease severity was higher in July, August and November 2016 as a result of the favorable climatic conditions (Figure 2). Nevertheless, the pathogen spread in September-October at the old orchard, and in October at the new orchard, with a higher severity index (3.0) in this orchard. After this period, during winter (November), the disease index was lower in the old orchard, when compared with the young one.

The application of *B. subtilis* Serenade Max® was not effective in reducing Psa infection (Figure 3B). This was not expected; as reported by Collina et al. (2016), the product should reduce the symptoms and incidence of the disease in plants. In fact, the only statistical difference in leaf spotting indicated a higher incidence in orchards treated with *B. subtilis* than in the control orchard (Figure 3B). It has previously been reported by Donati et al. (2014) that *B. subtilis* can prevent disease infection, whereas other BCAs can reduce leaf spotting only under low disease pressure. Possibly, the disease pressure in these two orchards was too high, so the application of *B. subtilis* was not effective in reducing the Psa population.

CONCLUSIONS

The climatic conditions registered in 2016 and 2017 in the north of Portugal were favorable for Psa growth and infection, which is promoted by temperatures between 10 and 20°C, with an optimal temperature of 15°C. The disease symptoms caused by Psa biovar 3, identified in this study by BOX-PCR, increased with spring and autumn humidity, and led to higher disease severity indexes. In the summer, because of low humidity, and temperatures higher than 20°C, new disease symptoms were not found. Bacterial populations decreased in summer, and disease progression was prevented in the winter.

The antagonistic microorganism tested, *B. subtilis* Serenade Max®, was ineffective in controlling Psa in the 6-year-old 'Hayward' orchard, and also showed no efficacy in the protection of leaves in a 30-year-old orchard. The *B. subtilis* strain tested could be

considered as a suitable treatment for the control of Psa biovar 3 at flowering.

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