Effect of *Bacillus subtilis* on fruit yield and quality in *Actinidia deliciosa* orchards infected with bacterial canker in the north of Portugal

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Abstract

Bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidae* (Psa), a disease included in the EPPO A2 List, has been registered in the north of Portugal since 2010. The relationship between the bacterium and cultural practices, namely the application of the biological control agent *Bacillus subtilis* in kiwifruit orchards in Portugal, is poorly understood. The most damaging phase of the disease occurs in winter and involves damage of the main vine structure and overwintering canes, and also in spring, during pollen spread, thus reducing subsequent yield and fruit quality. The main objective of this study was to evaluate the effect of *B. subtilis* Serenade Max® on kiwifruit yield and quality in infected orchards of different ages. Two orchards (*Actinidia deliciosa* 'Hayward') with typical symptoms of Psa were selected for yield and quality assays. Thirty plants were selected from the young orchard (6 years old) and other 30 plants were selected from the old orchard (30 years old), both located in Valença, northwest Portugal. This work showed that the use of *B. subtilis* as a biological control agent against Psa did not affect overall kiwifruit quality, namely the longitudinal and length diameters of fruit, the total soluble solids content, firmness, pH, titratable acidity and dry matter, but contributed significantly to increased yield in the younger orchard compared with the older orchard.

Keywords: *Actinidia deliciosa*, bacterial canker, kiwifruit yield, kiwifruit quality

INTRODUCTION

Pseudomonas syringae pv. *actinidiae* (Psa), the causal agent of bacterial canker in *Actinidia deliciosa* and *Actinidia chinensis*, was first isolated and described in Japan in 1984 (Takikawa et al., 1989). In Europe, the first epidemic outbreaks were reported in Italy (Balestra et al., 2009; Vanneste et al., 2013), and the disease quickly reached pandemic proportions by spreading to France, Spain, Portugal, Switzerland, New Zealand, Chile, Turkey, Korea, Japan and China (EPPO, 2014; Vanneste, 2017).

In Portugal, the disease was detected in 2010 (Balestra et al., 2010) at the Entre Douro e Minho Region (northwest Portugal), causing considerable damage to the national kiwifruit industry (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 region of Entre Douro e Minho makes up the main part of the cultivated area (1721 ha) with fruit production of 23,205 t (INE, 2016).

Psa symptoms are characterized by an oozing of whitish or reddish exudates on the trunks and branches of affected vines, leaf spots that may be surrounded by chlorotic halos, browning of buds and flowers, and the wilting of branches, twigs and of entire plants (Gallelli et al., 2011b; Moura et al., 2015). Affected fruits are misshapen, smaller than healthy fruits, and may collapse as a consequence of the wilting of the branches. Wilted fruits are not marketable (Gallelli et al., 2011b). Moreover, the presence of Psa in an orchard can change the chemical composition and quality of fruits, affecting postharvest quality, shelf life, and susceptibility to postharvest rots of fruit (Prencipe et al., 2016).

Strains of Psa show genetic variability and can be grouped into five biovars (biovars 1, 2, 3, 5, and 6) based on their genetic makeup and biological characteristics (Vanneste et al., 2013; Fujikawa and Sawada, 2016; Sawada et al., 2016). Biovar 3 is the most destructive and is responsible for the most recent outbreaks of Psa, including those in Europe, Chile, and New Zealand and, more recently, those in Japan, Korea and China (Vanneste, 2017), causing severe economic losses (Scortichini et al., 2012; Vanneste, 2017). Several groups of *Pseudomonas syringae* strains 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), initially named Psa biovar 4, were recently separated from Psa and assigned to *P. syringae* pv. *actinidifoliorum* (Cunty et al., 2015).

Because of the virulence of the pathogen, disease management strategies for bacterial canker caused by Psa are important but problematic. Several hundred products, including antibiotics, antimicrobial peptides, heavy metals, disinfectants, elicitors, biological control agents, natural products and bacteriophages, were tested in the glasshouse for their ability to reduce leaf spotting following inoculation of young kiwifruit seedlings with Psa. Few of those products reduced disease incidence significantly and consistently (Vanneste, 2013). Today, the industry relies mostly on copper-based products to limit the impact of the disease (Colombi et al., 2017), 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). Other chemical products such as antibiotics, which are not authorized in Europe, and elicitors are used to control Psa, together with orchard management practices (Stewart et al., 2011). In Portugal, copper-based products, a strain of the antagonistic microorganism *Bacillus subtilis* and the elicitor acibenzolar-*S*-methyl (ASM) have been registered for Psa control.

The recent use of *B. subtilis* to minimize damage caused by Psa has gained particular interest because of its safety, widespread distribution in very diverse habitats, remarkable ability to survive under adverse conditions by development of endospores, and the production of compounds that are beneficial for agronomic purposes (McSpadden Gardener, 2004; Earl et al., 2008).

The aim of this work was to evaluate the effect of *B. subtilis* treatment on kiwifruit (*A. deliciosa* 'Hayward') yield and fruit quality in two commercial orchards infected with Psa biovar 3.

MATERIAL AND METHODS

Characterization of the orchards

The experiment was performed in two commercial orchards of *A. deliciosa* 'Hayward' located in Valença, northwest Portugal, where Psa was first detected in the country (Balestra et al., 2010). One orchard has plants that are 30 years old and the other has 6-year-old plants. These plants were trained in T-bar structures, with a trunk height of 1.80 m and a central leader, with a horizontal cordon that produces the fruiting canes. The orchards have a total area of 1.15 ha and are irrigated with micro-sprinklers. Both orchards were infected with Psa biovar 3, confirmed by duplex-PCR (Gallelli et al., 2011a) and Box-PCR (Louws et al., 1994).

Orchard trials and field application of *B. subtilis*

A randomized block design with three replications was used. Treatments included two different ages of kiwifruit orchard, with plants that were 6 (young) and 30 (old) years old, with (S Max), and without (No S Max) the application of *B. subtilis* Serenade Max®. The numbers of plants used to determine disease severity and to collect plant measurements were five for each replicate (three) in each of the four experimental treatments, giving a total of 60 plants.

To test the effect of *B. subtilis* on disease severity, a formulation of 15.67% *B. subtilis* QST 713 was applied at flowering (end of May) in 2016, using a maximum concentration of

1 kg ha^{-1}, following the instructions of the manufacturer. Disease severity was determined by regular detailed monitoring of Psa symptoms, performed every 30 days, from May to November, 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 on more than 10% of the leaf; 2 = symptoms on more than 25% of the leaf; 3 = symptoms on more than 50% of the leaf; and 4 = symptoms on 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).

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.

Kiwifruit productivity parameters, yield and quality

Kiwifruit productivity parameters and total yield were evaluated on five plants for each of three replicates in each of four experimental treatments (n=60). The numbers of canes per plant, lateral branches per plant, fruits per plant, and fruits per lateral branch, as well as the fresh weight per plant, were recorded.

Quality parameters gauge (longitudinal and length diameters of fruits), total soluble solids (TSS), firmness, pH, titratable acidity (TA) and dry matter (DM) were evaluated at harvest (16 November 2016) on 10 fruits for each replicate of the four experimental treatments, giving a total of 120 fruits. Fruit firmness, expressed in kg mm^{-2}, was determined with a TR-Turoni® penetrometer with a 6-mm plunger tip after removal of the skin to a vertical depth of 1 mm on two sides of the fruit. TSS were quantified with a hand-held digital refractometer (Atago®) by squeezing one drop of kiwifruit juice. TA and pH were determined using an automated titrimeter CRISON®-pH Burette 24. Six milliliters clarified kiwifruit juice was mixed with 30 mL distilled water, placed into a sample cup and titrated to the endpoint of pH 8.0 using 0.1 M NaOH. The results were expressed as percentages of citric acid.

Fruit DM content was determined after drying the fruits in a ventilator oven at 60°C for 48 h. The longitudinal and length diameters of the fruits were measured with a Vernier caliper.

RESULTS AND DISCUSSION

Disease severity

There was no apparent correlation between the effect of the application of *B. subtilis* and the age of the orchard. The higher disease severity index was observed in June 2016 in both orchards. After June, the disease severity was always lower, but it was higher in the younger orchard compared with the older one (Figure 1). Other authors have also reported higher disease severity in young orchards (Scortichini, 1994; Tyson et al., 2015). Likewise, Vanneste et al. (2011) indicated that, for the same location, younger plants are more susceptible than older plants.

Productivity parameters and yield

The number of canes per plant was similar in the two orchards. The number of fruits per plant, and the number of fruits per lateral branch in the young orchard, were significantly lower ($p<0.05$) than those in the old orchard (Table 1). The results of the application of *B. subtilis* to control Psa showed that, with the exception of the number of canes, all the parameters analyzed were significantly higher ($p<0.05$) when *B. subtilis* was used (Table 1).

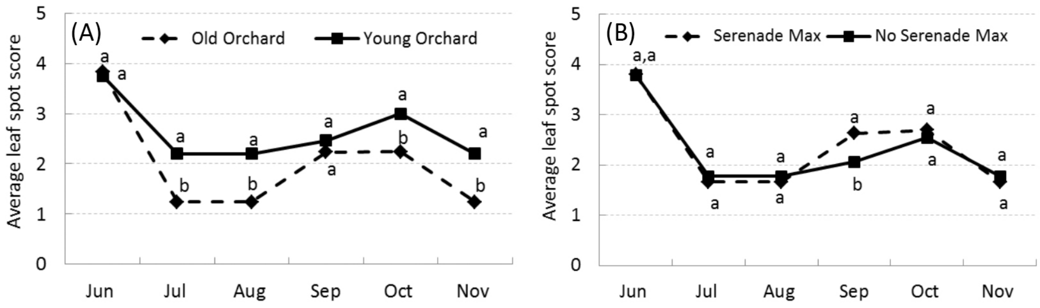

Figure 1. *Pseudomonas syringae* pv. *actinidiae* (Psa) disease severity recorded in two 'Hayward' kiwifruit orchards in Valença, Portugal. (A) Effect of the age of plants. (B) Effect of *Bacillus subtilis* Serenade Max® application from June to November 2016. Disease severity was estimated by using 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; and 4 = symptoms in more than 75% of the leaf. Different letters indicate significant differences by the LSD test ($p<0.05$).

Table 1. Total number of canes plant^{-1}, total fruits plant^{-1} and total fruits lateral branch^{-1} in two kiwifruit 'Hayward' orchards at Valença, Portugal, of different ages, with and without application of *B. subtilis* Serenade Max®, during the fruit growing season of 2016. Different letters in each row for orchards (young/old) or Serenade Max® (SMax/no SMax) indicate significant differences by the LSD test ($p<0.05$)

Yield parameter	Orchard		Serenade Max®	
	Young	Old	SMax	No SMax
Canes plant^{-1}	23.6±3.1a	24.6±2.9a	25.8±3.0a	22.4±2.0a
Fruits plant^{-1}	223.7±87.0b	310.3±66.4a	318.7±53.8a	215.2±86.5b
Fruits lateral branch^{-1}	2.1±2.0b	2.6±2.0a	2.5±0.2a	2.2±0.3b

The interaction between the effect of orchard age and the application of *B. subtilis* was significant ($p<0.05$) for the number of lateral branches plant^{-1} and for the total yield (kg plant^{-1}), indicating a Serenade Max® effect dependent on the age of the orchard. It was observed that both the total yield and the number of lateral branches were lower in the younger orchard without Serenade Max® application (Figure 2).

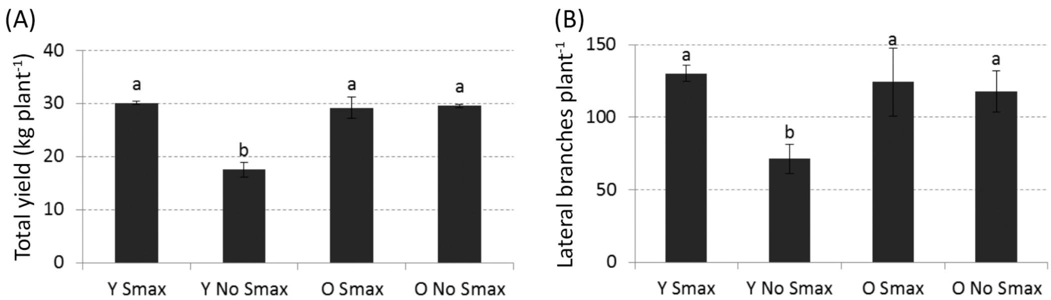

Figure 2. Effect of the application of Serenade Max® (SMax, with application of Serenade Max®; No SMax, without application of Serenade Max®) in young (Y) and old (O) orchards at Valença, Portugal, during the fruit growing season of 2016. (A) Total number of lateral branches plant^{-1}; (B) total yield (kg plant^{-1}). Different letters indicate significant differences between treatments by the LSD test ($p<0.05$).

The lower kiwifruit yield obtained in the younger orchard without Serenade Max® application (Figure 2A) can be explained to some extent by the higher Psa disease severity observed in these plants during the growing season (Figure 1).

This higher disease severity probably had a negative effect on yield, as already described by other authors. Froud et al. (2014) reported a yield reduction in orchards infected with the most virulent Psa population, Psa-V. We also concede that the different yield results obtained between the young and old orchards could be a consequence of differences in soil fertility, water availability during the production season and/or kiwifruit orchard age.

Fruit quality

Regarding quality analysis of kiwifruit, the results obtained show that the interaction between the effect of orchard age and *B. subtilis* application was not significant ($p<0.05$) for any of the studied parameters. The results of the main effects of orchard age and the application of the biocontrol agent on fruit quality are presented in Table 2. The results for pH, DM and gauge did not show significant differences ($p<0.05$) between the older and younger orchards. However, the results obtained for firmness, TSS and TA were different ($p<0.05$). TSS was higher in fruits produced in the young orchard (6.7±0.6), and fruits from the old orchard had greater firmness (6.0±0.1) and TA (1.4±0.1%) (Table 1). The application of *B. subtilis* did not affect overall kiwifruit quality (Table 2).

Table 2. Kiwifruit quality parameters [gauge (longitudinal and length diameters of fruits, cm), total soluble solids content (TSS; %), firmness (kg mm^{-2}), pH, titratable acidity (TA; %) and dry matter (DM; %)] measured in young (6 years old) and old (30 years old) orchards at Valença, Portugal, with and without application of Serenade Max®, during the fruit growing season of 2016. Different letters in each row for orchards (young, old) or Serenade Max® (SMax, No SMax) indicate significant differences by the LSD test ($p<0.05$).

Quality parameter	Orchard		Serenade Max®	
	Young	Old	SMax	No SMax
Gauge longitudinal (cm)	5.4±0.5a	5.8±0.5a	5.6±0.5a	5.6±0.6a
Gauge length (cm)	5.6±0.2a	5.9±0.2a	5.8±0.1a	5.7±0.4a
TSS (%)	6.7±0.6a	6.1±0.1b	6.7±0.7a	6.2±0.1a
DM (%)	16.4±8.7a	14.8±0.6a	14.8±0.6a	16.4±8.7a
TA (%)	1.2±0.1b	1.4±0.1a	1.3±0.1a	1.3±0.1a
Firmness (kg mm^{-2})	5.3±0.2b	6.0±0.1a	5.6±0.5a	5.7±0.3a
pH	3.2±0.1a	3.2±0.1a	3.2±0.1a	3.2±0.0a

The fruit quality characteristics obtained in this study can be compared in some way to those presented by Prencipe et al. (2016), who also concluded that Psa can affect fruit quality. According to these authors, DM content and TSS were higher in fruits from plants infected with Psa, while TA was lower. In our study, fruit DM was apparently higher (16.4±8.7%) in the younger orchard, with higher disease severity, compared with the older orchard (Figure 1).

CONCLUSIONS

This work provides evidence that agricultural practices, such as the use of biological control agents against Psa, may significantly contribute to increased yields of kiwifruit. The results of the application of *B. subtilis* Serenade Max® during flowering of *A. deliciosa* 'Hayward' infected with Psa showed that, under the production conditions of the studied orchards, the total yield depends on the age of the orchard. It was observed that the number of lateral branches and the total yield were lower in the young orchard without the application of *B. subtilis*, whereas the disease severity was higher. The use of *B. subtilis* did not affect the overall fruit quality, namely the longitudinal and length diameters of fruits, TSS, firmness, pH, TA and DM, but significantly contributed to increased yield in the younger orchard compared with the older orchard.

As these data are for one growing season only, further experiments are needed to better understand the influence of biological control agents on kiwifruit yield and quality, under field conditions.

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