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Research Papers

Bacillus-based products for management of kiwifruit bacterial canker

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Summary. *Pseudomonas syringae* pv. *actinidiae* is an important pathogen of kiwifruit (*Actinidia deliciosa*), and bacterial canker of this host is managed by monitoring and chemical control strategies. The efficacy of the bio-pesticides Amylo-X® (based on *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747) and Serenade Max® (strain QST713 of *B. subtilis*) was evaluated by *in vitro* and *in vivo* experiments. Both antagonists inhibited different biovars of the pathogen in *in vitro* assays; QST713 was more efficient than D747. The two *Bacillus* strains also colonized *A. deliciosa* flowers (*c.* 10^{5-7} cfu per flower) up to 96 h after inoculation. D747 persisted on leaves (*c.* 10^{4-6} cfu cm^{-2}) up to 4 weeks after inoculation, during 2 years in Emilia Romagna and Latium regions of Italy. On flowers, the antagonists reduced pathogen populations, compared to untreated (control) flowers. On *A. deliciosa* and *A. chinensis* plants under controlled conditions, Amylo-X® reduced severity of bacterial canker, providing *ca*. 50% relative protection on *A. deliciosa* and 70% on *A. chinensis*. Serenade Max® was less effective, giving 0% relative protection on *A. deliciosa* and 40% on *A. chinensis*. In a field trial, on *A. deliciosa* plants, Amylo-X® reduced the severity of bacterial canker on leaves, providing *ca*. 40% relative protection. The sensitivity of both antagonistic strains to streptomycin sulphate was confirmed by testing the most used concentration where antibiotics are approved for management of bacterial pathogens.

Keywords. Biocontrol agents, *Pseudomonas syringae* pv. *actinidiae*, *in vitro* assays, antagonist survival, population challenge.

INTRODUCTION

In the last decade, bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), led to extensive economic losses for kiwifruit producers. The pandemic of this bacterial pathogen started in 2008, mainly in the *Actinidia* spp., and the pathogen was especially aggressive on *A. chinensis* cultivars (Abelleira *et al.,* 2011; Mazzaglia *et al*., 2012; EPPO, 2016). At present, five biovars of Psa are recognized (Cunty *et al*., 2015), grouped by biochemical, genetic and pathogenicity characteristics (Renzi *et al*., 2012; Butler *et al*., 2013; Vanneste *et al.,* 2013; Vanneste, 2017; Fujikawa and Sawada, 2019). Biovar 3 is the most virulent, and was responsible for the bacterial canker pandemic. Disease control strategies rely on strict orchard hygiene practices, breeding and deployment of resistant host genotypes, and scheduled use of antibacterial compounds or elicitors activating host immune systems (Cotrut *et al.,* 2013; Cellini *et al*., 2014; Michelotti *et al*., 2018). As well, the use of biological control agents (BCAs) has also been shown to be effective (Cortesi *et al*., 2017; Rossetti *et al*., 2017; Hoyte *et al*., 2018). New and promising strategies have shown the possibility to reduce the use of chemicals by nanotechnological tools (Fortunati *et al*., 2016; Mazzaglia *et al*., 2017; Fortunati and Balestra 2018). Nevertheless, prophylaxis utilizing early diagnostic analyses of asymptomatic plant material remains the most effective method for reducing the primary infection sources (Rees-George *et al*., 2010; Balestra *et al*., 2013; Biondi *et al*., 2013; Gallelli *et al*., 2013).

Chemical control applications against Psa are preventive and/or applied at early stages of the disease development. In open fields, the amount of effective control is dependent on compounds such as streptomycin and/or copper formulations to prevent bacterial blight occurrence (Koh *et al.,* 1996; Nakajima *et al.,* 2002; Lee *et al.,* 2005; Vanneste *et al.,* 2011a). Antibiotics are allowed on most of the continents to control bacterial plant pathogens, but not in Europe, where copper compounds are mostly employed (Balestra and Bovo, 2003; Balestra, 2007; Lee *et al.,* 2005; Vanneste *et al.,* 2011a). Both compounds have different negative properties, including phytotoxicity, pathogen resistance, fruit residues, and accumulation of metal ions in soils (Goto *et al.,* 2004; Marcelletti *et al.,* 2011; Cameron and Sarojini, 2013).Integrated management of kiwifruit bacterial canker is therefore required using multiple strategies for the effective control of the disease. This could include application of resistance inducers, stimulation of host defence responses, and the use of biocontrol agents (Dong *et al*., 1999; Cellini *et al*., 2014).

Several bacterial strains are used as fungicides or bactericides to control different plant diseases; most of these are species of *Bacillus* or *Pseudomonas* (McSpadden Gardener and Driks, 2004; Borriss, 2011), and are used against several plant pathogenic bacteria, including *Erwinia amylovora* (Bazzi *et al*., 2006; Chen *et al*., 2009), *Xanthomonas arboricola* pv. *pruni* (Biondi *et al*., 2009a), and *Pseudomonas siringae* pv. *tomato* (Fousia *et al*., 2016). Some studies have been carried out on control of Psa using biological methods. These have shown the effectiveness of *Pantoea agglomerans* or *Lactobacillus plantarum*, and some bacteriophages and organic substances (Stewart *et a*l., 2011; Frampton *et al*., 2014; Daranas *et al*., 2018; de Jong *et al*., 2019).

In the present study, bacterial strains D747 of *Bacillus amyloliquefaciens* subsp. *plantarum* and QST713 of *B. subtilis*, the principal components, respectively, of the bio-fungicides Amylo-X® and Serenade Max®, were tested *in vitro* for their ability to inhibit the Psa growth, and for their sensitivity to the antibiotic streptomycin sulphate. *In planta*, under controlled conditions and in field trials, the two BCAs were assessed for their capacity to survive on and colonize kiwifruit plants (leaves and flowers), for their efficacy to inhibit Psa epiphytic populations on flowers, and for their effectiveness in reducing the severity of bacterial canker.

MATERIALS AND METHODS

Bacterial strains

The Psa strains NCPPB 3739 (biovar 1), CRA-FRU 3.1 (biovar 3), CFBP 7286 (biovar 3) and DISTAL (*ex*-IPV-BO) 9312 (biovar 3; Biondi *et al*., 2018) were routinely grown at 27°C for 48–72 h, on NSA (Crosse, 1959) or KB (King *et al*., 1954) media. The mutant strain CRA-FRU 3.1rif^r, resistant to rifampicin, was grown at 27°C for 72–96 h on KB medium supplemented with 20 ppm rifampicin.

Bacillus amyloliquefaciens strain D747, the active ingredient of Serenade Max®, and *B. subtilis* strain QST713, in Amylo-X®, were routinely grown on LPGA (Ridè *et al*., 1983) at 27° or 36°C for 24 h.

Release of antibacterial compounds by Bacillus *strains against different Psa strains*

The production of antimicrobial compounds by strains D747 and QST713 was assessed *in vitro* using the method of Vanneste *et al.* (1992). Axenic 24-h-old colonies of each strain were transferred with a loop to the centres of (*c.* 1 cm spot diameter.) Petri dishes containing minimal medium (MM: K_2HPO_4, 7.02 g L^{-1}; KH_2PO_4, 3.02 g L^{-1}; L-asparagine 3.0 g L^{-1}, $(NH_4)_2SO_4$, 2.0 g L^{-1}; nicotinic acid 0.5 g L^{-1}, D-glucose 4.0 g L^{-1}, $C_6H_5Na_3O_7$ $2H_2O$ 0.5 g L^{-1}, $MgSO_4.7H_2O$ 0.01 g L^{-1}; Bacto agar, 18.0 g L^{-1}). The dishes were then incubated at 27°C for 48 h. Two diameters of each resulting bacterial macro-colony were then measured, and the colony was then scraped off the plate with a lancet. The plates were then exposed to chloroform vapours for 45 min. Each Petri dish was then homogeneously covered with 5 mL of MM soft-agar (MM medium containing 0.7% agar) inoculated with Psa strains NCPPB 3739, DISTAL 9312 or CRA-FRU 3.1 (*ca.* 10^6 cfu mL^{-1}). After 48-96 h at 27°C, inhibition haloes were each assessed by subtracting the mean of the diameters of the antagonist macrocolony from the mean of the inhibition halo diameter. Psa strains were used as negative controls, and the assay was repeated three times.

Activity of streptomycin sulphate against Bacillus strains

In vitro experiments using the diffusion plate method were carried out on NA medium (nutrient broth, 8 gL^{-1}; agar 18 gL^{-1}). A water suspension of 24-h-old culture of D747 (approx. 10^6 cfu ml^{-1}) was used for the Petri dish inoculations (100 µL per dish). Three paper disks (6 mm diam.) were placed on the inoculated agar medium in each test dish, and 30 µL of streptomycin at 25 or 50 µg mL^{-1} were pipetted on two of the discs; 30 µL of sterile distilled water (SDW) were applied to the third disc as the experimental control. After incubation at 27°C for 48 h, the inhibition halo diameters in the test plates were determined by subtracting the antibiogram disk diameters (6 mm) from the halo diameters. This test was repeated five times with three replicates each, and the standard deviations were calculated.

The *in vitro* experiments using macro-dilution were carried out in 50 mL Falcon tubes each containing 15 mL of LB broth (Bacto Peptone 10.0 gL^{-1}, Yeast Extract 5.0 g L^{-1}, NaCl 10.0 g L^{-1}, pH 7.0). The tubes were each inoculated with 150 µL aqueous suspensions containing approx. 10^7 cfu mL^{-1} of spores of Amylo-X® (2.0 g L^{-1}) or Serenade Max® (3.0 g L^{-1}). The inoculated tubes were then amended with streptomycin sulphate (100 ppm) or SDW as negative controls. The tubes were then incubated at 27°C at 80 rpm for 24 h. The bacterial population in each tube was evaluated after 1 and 24 h by collecting 1 mL of inoculated broth. Each sample was tenfold diluted and, 10 µL from each dilution were added to LPGA, and the inoculated plates were incubated at 27°C for 24 h. The bacterial populations were then quantified by counting the colonies. The assay was repeated three times, and the standard deviations were calculated.

In planta experiments

Amylo-X® and Serenade Max® against Psa

The efficacy of Amylo-X® and Serenade Max® against Psa were assayed under greenhouse conditions on kiwifruit plants of *A. deliciosa* (cv. Hayward) and *A. chinensis* (cv. Hort16A). The plants were grown in 7.0 L capacity pots in randomized replicates (three plants in four replicates per treatment). Amylo-X® (2.0 g L^{-1}; *c.* 10^7 cfu mL^{-1}) and Serenade Max® (3.0 g L^{-1}; *c.* 10^7 cfu mL^{-1}) were applied to the leaves (*c.* 100 mL per plant) using a sprayer 48 h before inoculation with the pathogen (BPI). After treatment application, the plants were inoculated by spraying a water suspension (OD_{600} = 0.01; *c.* 10^7 cfu mL^{-1}) of the virulent Psa strain DISTAL 9312. The plants were then sealed in polyethylene (PE) bags for 2 d to favour pathogen penetration in the leaves. The greenhouse conditions were set at 16 h light, 23°C and 8 h dark, 17°C, and maintaining the RH% at greater values than 70% (Biondi *et al.*, 2018; Perez *et al.*, 2019) until disease assessments. Streptomycin sulphate (100 ppm) and SDW were used as, respectively, positive and negative experimental controls. Disease severity was evaluated 21 d after Psa inoculations, by counting the number of leaf spots on ten leaves per plant (*c.* 120 leaves per treatment). The data collected were analysed using ANOVA and Duncan's test at $P \leq 0.05$) with SPSS software Windows v15.0 (SPSS Inc.), and the proportions (%) of protection provided by each treatment relative to the negative controls (SDW-treated plants) were calculated.

Selected symptomatic leaf samples were used for Psa isolation and identification. The leaves were surface sterilized by washing with 2% sodium hypochlorite. Necrotic lesions were aseptically collected and crushed with pestel and mortar with 2 mL of SDW. The resulting plant extract and three ten-fold SDW dilutions were plated (30 mL) on NSA. The plates were the incubated for up to 72 h. Psa-like colonies were subcultured on KB plates and identified with PCR assays (Biondi *et al.*, 2013).

Bacillus strain colonization of kiwifruit flowers and their effects on Psa populations

Experiments were carried out on detached flowers of kiwifruit plants of cv. Hort 16A (very susceptible to Psa).

The flowers were kept in Eppendorf tubes containing sterile distilled water. Freshly opened flowers were sprayed with an aqueous spore suspension of Serenade Max® (3.0 g L^{-1}, *c.* 10^7 cfu mL^{-1}) or Amylo-X® (2.0 g L^{-1}, *c.* 10^7 cfu mL^{-1}). The mutant strain CRA-FRU 3.1 Rif^r (*c.* 10^6 cfu mL^{-1}) was sprayed on the flowers 24 h after application of the biocontrol treatments. After incubation at 25°C in humid chamber, five flowers per time point (1, 24, 48, 72 or 96 h from antagonist and pathogen application) were individually washed in 3 mL 10 mM $MgSO_4$. Antagonist and Psa populations present on each flower were assessed by plating tenfold dilutions in 10 mM $MgSO_4$ on LPGA or KB plates (amended with 20 ppm of rifampicin) (Biondi *et al.*, 2006), and incubating these at 36°C for 20 h (for LPGA) or 27°C for 72–96 h (for KB). Numbers of bacterial colonies recovered from treated flowers were counted, and the populations of both the antagonists and the pathogen were calculated for each flower. SDW and untreated, non-inoculated flowers were used as experimental controls.

Survival of *Bacillus* D747 on kiwifruit leaves

The ability of *Bacillus* strain D747 to survive on leaf surfaces of *A. deliciosa* cv. Hayward trees was evaluated during 2017 and 2018, in the Emilia Romagna and Latium regions of Italy. Kiwifruit plants (two trees per replicate and four replicates), located in open fields in Faenza (Emilia Romagna) and Viterbo (Latium) provinces, were sprayed with Amylo-X® (2.0 g L^{-1}; *c.* 10^7 cfu mL^{-1}) or SDW (negative controls). The treatments were carried out after blooming: in Emilia Romagna on 15/05/2017 and 08/05/2018, and in Latium on 14/06/2017 and 04/06/2018. The bacterium population survival was monitored up to four weeks: at each time point six leaves were randomly collected from each tree, washed in 250 mL of 100 mM $MgSO_4$ in a rotating incubator at 120 rpm for 45 min at 25°C. The resulting washing fluids were each filtered through sterile gauze and then centrifuged at 10,000 *g* for 20 min at 4°C, and the resulting pellet was resuspended in 1.0 mL SDW. Bacterial antagonist populations present in the resuspended pellets were determined by plating tenfold dilutions in 10 mM $MgSO_4$ on LPGA plates, and then incubating these at 36°C for 24 h. The bacterial colonies recovered from treated leaves were counted, and the populations of the antagonist per cm^2 of leaf was calculated ((bacterial concentration per mL × 250 mL/six leaves) × 1 / mean leaf area). SDW was used as the negative control. DNA was extracted from selected axenic colonies recovered from the field assessments, using the Plant DNeasy Minikit (Qiagen). A BOX-PCR was carried out on DNA templates diluted at 50 ng μL^{-1}.

PCR assays were performed in 50 µL reaction mixture containing 1× PCR Go Taq Flexi buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 4 U Go-*Taq* Flexi DNA polymerase (Promega), 2.0 µM BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'), and 4 µL template DNA. The BOX-PCR thermal profile consisted of an initial denaturation step (95°C for 7 min), followed by 30 cycles each at 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min, and a final extension step of 16 min at 65°C (Versalovic *et al.*, 1994). All the amplification products were analyzed on 2.0% agarose gel in TAE buffer (0.04 M Tris, 0.001 M NaEDTA and 0.02 M glacial acetic acid), after staining in 0.03% ethidium bromide and visualization under UV light (312 nm). SDW and the D747 strain were used as, respectively, negative and positive controls.

Field activity of Amylo-X® against Psa

In Viterbo (Latium region) during 2018, a kiwifruit orchard (*Actinidia deliciosa*, cv. Hayward) with 7-year-old plants severely affected by Psa (*c.* 30% of plants symptomatic), was used to evaluate activity of Amylo-X® against Psa. The trial included two groups of plants divided into four plots (ten plants each) per treatment. One group was treated three times with 1.5 kg ha^{-1} of Amylo-X®, before bud opening (on 10/05/2018), then 1 week later at the blooming initiation (on 17/05/2018), and then at 04/06/2018. The second group of plants did not receive any phytosanitary treatment, as experimental controls. A disease severity scale was used to evaluate the treatment results. The scale took account of the number of spots (necrotic areas surrounded by yellow haloes) on leaves of 1- and 2-year-old branches (ten leaves from four branches per plant), for four plants in two replicates. Disease assessments were carried out in the second week of May, June or September, 2018.

Four leaf spot severity classes were defined as: class 1 = 0 (no symptoms), class 2 = 25% leaf surface area affected, class 3 = 50% and class 4 = 75% leaf surface are affected. Disease severity was then calculated using the following formula: N° leaves in class 1 × 0 + N° leaves in class 2 × 0.25 + N° leaves in class 3 × 0.50 + N° leaves in class 4 × 0.75 / total N° leaves assessed. As well, the percentage of branches per plant with healthy (asymptomatic) leaves was also determined. The data obtained were statistically analysed using GraphPad Prism v5.0 software for (ANOVA), and differences among mean values for treatments were determined using Tukey's HSD test ($P \leq 0.05$).

RESULTS

In vitro *experiments*

Results from the *in vitro* experiments indicated that both antagonist strains, D747 and QST713, produced compounds that inhibited the growth of the three Psa strains of different biovars (Figure 1). *Bacillus* strain QST713 was significantly more effective for Psa inhibition than D747. Strain QST713 gave mean inhibition haloes ranging from 29 to 31 mm, which were larger than those induced by D747 (22 to 24 mm) (Figure 1). The inhibition haloes produced by antibacterial compounds of each *Bacillus* strain were similar ($P > 0.05$) for the three Psa strains tested, belonging to the biovars 1 and 3 of the pathogen.

In the *in vitro* experiments using the diffusion method, streptomycin sulphate reduced the growth of D747 24-h-old living cells. At 25 and 50 ppm, mean inhibition haloes were, respectively, 7.2 and 8.5 mm, while in the control (SDW), the bacterial growth was not reduced, and no inhibition haloes were observed (Table 1).

In the macro-dilution experiments, streptomycin sulphate treatments of spore suspensions of both bioproducts did not affect the bacterial populations, which were *c.* 10^4 cfu mL^{-1}, statistically similar to the populations in the control tubes (*c.* 10^5 cfu mL^{-1}). In contrast, at 24 h, streptomycin sulphate reduced the concentrations of D747 (*c.* 10^4 cfu mL^{-1}) and QST713 (*c.* 10^4 cfu mL^{-1}) strains, in comparison with the control (*c.* 10^7 cfu mL^{-1}). After 24 h the sensitivity to streptomycin sulphate (100 ppm) was statistically similar for D747 and QST713 strains (Table 1).

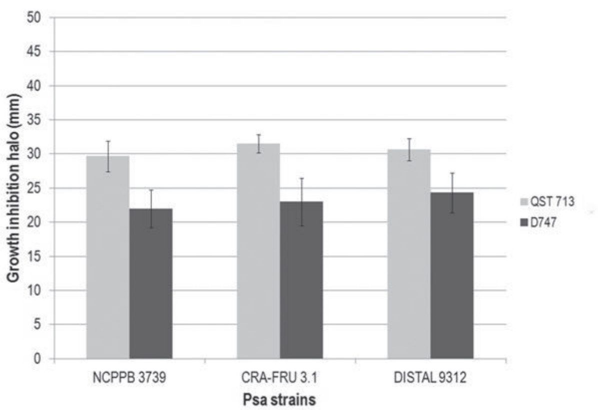

Figure 1. Mean diffusion plate proportions (%) of growth inhibition of three Psa strains (biovar 1, strain NCPPB 3739, biovar 3, strains CRA-FRU 3.1 and DISTAL 9312), caused by two strains of antagonistic *Bacillus* strains (QST 713, light histograms; D747, dark histograms). Bars indicate standard deviations ($P \leq 0.05$).

In planta *experiments*

Controlled conditions

Strain D747, inoculated at high concentration, colonized the kiwifruit flowers up to 96 h after inoculation. After 1 h the concentration of bacteria was *c.* 10^6 cfu per flower, but from 24 to 72 h, the population rapidly increased, to *c.* 10^8 cfu per flower. At 96 h the population decreased to *c.* 10^7 cfu per flower. The strain QST713 was not able to colonize the flowers, but it could survive on them. The mean bacterial concentration was *c.* 10^6 cfu per flower from 1 to 48 h after application, while at 72 and 96 h, the population decreased to *c.* 10^5 cfu per flower. The concentrations of both antagonist strains were similar after 1 h. From 24 to 96 h, the populations of D747 were larger than those of QST713 (Figure 2). Flowers treated with SDW were free of *Bacillus* sp. No phytotoxicity was observed on flowers treated with Amylo-X® or Serenade Max®.

On the same batch of flowers, the mutant Psa strain CRA-FRU 3.1rif^r colonized the flowers treated with SDW (controls) up to 72 h from inoculation. At 1 h, the recorded Psa population was *c.* 10^6 cfu per flower, and this increased to *c.* 10^7 and 10^8 cfu per flower at, respectively, 24 and 48 h. At 72 h, the population had decreased but remained high (*c.* 10^7 cfu per flower). On flowers treated with the antagonist strains, the Psa populations were significantly less than those on control flowers at most of the time points. After 1 h, the Psa populations on QST713-treated flowers was similar to that on those treated with SDW, but up to 96 h, the Psa populations were up to two orders of magnitude less than in the control. Similarly, the Psa populations on D747-treated flowers were less by more than one order of magnitude than those for the controls, at each time point. In general, QST713 more effectively reduced the Psa populations than D747, although QST713 had less ability to colonize flower surfaces than D747. After 1 h, the D747 treated flowers had less Psa (*c.* 10^4 cfu per flower) compared to flowers treated with QST713 or SDW (*c.* 10^6 cfu per flower). At 24 h, the Psa populations on D747- or QST713-treated flowers were less and with similar concentration (*c.* 10^6 cfu per flower) of those of the controls (*c.* 10^7 cfu per flower). From 48 to 72 h, the populations of Psa on flowers treated with both antagonists were reduced (*c.* $10^{5\text{-}6}$ cfu per flower) compared to those on control flowers (*c.* $10^{7\text{-}8}$ cfu per flower); in particular, in QST713-treated flowers, the Psa populations were less than those detected on flowers treated with D747 (Figure 2).

Table 1. Streptomycin sulphate concentrations (ppm) used against strains D747 and QST713 of *Bacillus* sp. for *in vitro* experiments using diffusion and macro-dilution methods.

Diffusion Method		
Strains (inoculated at time 0 h as living cells)	Streptomycin concentration	Growth inhibition halo (standard deviations)
Sterile Distilled Water (negative control)	/	0.00 mm (± 0.00)
D747 living cells	25 ppm (0.75 μg*)	7.2 mm (± 0.3)
D747 living cells	50 ppm (1.50 μg*)	8.5 mm (± 0.4)

Macro-dilution Method			
Strains (inoculated at time 0 h as spores)	Streptomycin concentration	Bacterial concentrations (standard deviations)	
		After 1 h	After 24 h
Sterile Distilled Water (negative control)	/	$3.7{\cdot}10^5$ cfu mL^{-1} (±$1.3{\cdot}10^5$ cfu/mL)	$1.9{\cdot}10^7$ cfu/mL (±$2.2{\cdot}10^6$ cfu/mL)
D747 spores (Amylo-X®)	100 ppm	$7.2{\cdot}10^4$ cfu/mL (± $6.9{\cdot}10^4$ cfu/mL)	$8.2{\cdot}10^3$ cfu/mL (± $9.7{\cdot}10^3$ cfu/mL)
QST713 spores (Serenade Max®)	100 ppm	$8.8{\cdot}10^4$ cfu/mL (± $4.6{\cdot}10^4$ cfu/mL)	$4.2{\cdot}10^3$ cfu/mL (± $4.0{\cdot}10^3$ cfu/mL)

*Streptomycin sulphate quantity in the antibiogram disk (μg).

The experiment performed on *A. deliciosa* plants under greenhouse conditions demonstrated the ability of Amylo-X® to reduce the disease severity (bacterial leaf spots) caused by the inoculated Psa DISTAL 9312 strain. The disease severity was low in all treatments. In particular, the severity on plants treated with Amylo-X® (mean = *c.* 2 spots per leaf) was statistically similar to that recorded on plants treated with streptomycin sulphate (positive control, *c.* 0.3 spots per leaf), and significantly lower than that on the negative controls (*c.* 4 spots per leaf). The disease severity on plants treated with Serenade Max®, in contrast, was similar to the one of the negative control plants (*c.* 4 spots per leaf) (Figure 3).

In the experiment carried out on *A. chinensis* plants, the disease severity was higher than observed on *A. deliciosa* plants. These results confirmed the ability of Amylo-X® to reduce bacterial leaf spot severity caused by the virulent Psa. The disease severity on the plants treated with Amylo-X® (mean = *c.* 14 spots per leaf), was significantly lower than that on control plants (*c.* 62 spots per leaf). The severity was also reduced in the plants treated with Serenade Max® (*c.* 32 spots per leaf), but this was higher than that in Amylo-X® treated plants (Figure 3).

Field trials

On *A. deliciosa* plants in Emilia Romagna, the D747 strain survived on leaf surfaces for up to almost 4 weeks after Amylo-X® application in both field experiments (2017 and 2018). The D747 strain, employed in both trials at the same concentration, produced larger antagonist populations at 1 h after application in 2017(*c.* 8×10^6 cfu cm^{-2}), in comparison with 2018 (*c.* 2×10^6 cfu cm^{-2}). This difference in population remained stable until 6 d, while from 9 to 27 d from application, the populations of D747 were similar in both experiments (10^5 cfu cm^{-2}) (Figure 4).

In Latium region, the strain D747 survived on leaf surfaces with high populations for 28 d (from *c.* 10^4 to *c.* 10^5 cfu cm^{-2}). In both experiments, the population dynamics of D747 were similar for the first assessments, up to 14 d from Amylo-X® application (*c.* $10^{4\text{-}5}$ cfu cm^{-2}). During 2017, populations of the antagonist at 21 and 28 days were larger (*c.* 10^5 cfu cm^{-2}) than those evaluated in the second year (approx. 10^4 cfu cm^{-2}) (Figure 4).

Although the antagonist populations on the kiwifruit trees in Latium were smaller than those recorded in Emilia Romagna from the first to the last assessments, the populations remained high for the whole assayed period.

The plants in the negative control treatments (untreated or SDW) were, in most cases, free of *Bacillus* species. In the other cases, some *Bacillus*-like colonies were found in the re-isolations (*c.* 10–10^2 cfu mL^{-1}). At each assessment time point, selected re-isolated colonies were identified as strain D747, using BOX-PCR (Figure 1S).

In the assayed kiwifruit orchards, the use of Amylo-X® led to a general reduction of bacterial wilt on diseased branches. In May, the mean disease severity index (DI) of 1-year-old branches in the D747-treated plot was *c.*

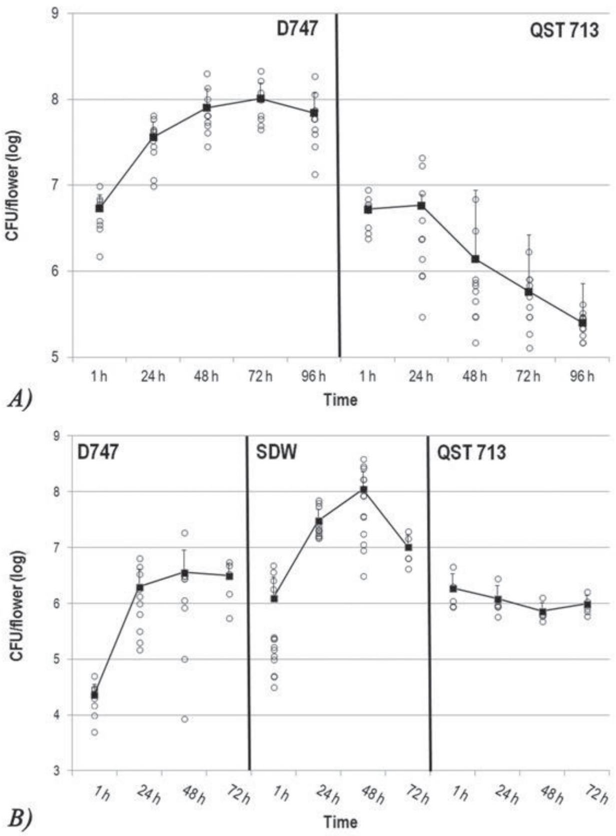

Figure 2. A) Colonization of *Bacillus* strain D747 on flowers of *A. chinensis* 'Hort16A' up to 96 h from its application. Empty circles indicate populations on each flower, and black squares and line indicate the mean populations on ten flowers (five flowers per experiment, two replicates). Bars indicate standard deviations ($P \leq 0.05$). B) Colonization of *Pseudomonas syringae* pv. *actinidiae* mutant strain CRA-FRU 3.1rif^r applied to flowers of *A. chinensis* 'Hort16A' flowers pre-treated with *Bacillus* spp. strains D747 or QST713, or sterile distilled water (SDW, control). The pathogen population was monitored for 72 h. Empty circles indicate CRA-FRU 3.1rif^r strain populations on each flower, and black squares and line indicate the mean populations present in ten flowers. Bars indicate standard deviations ($P \leq 0.05$).

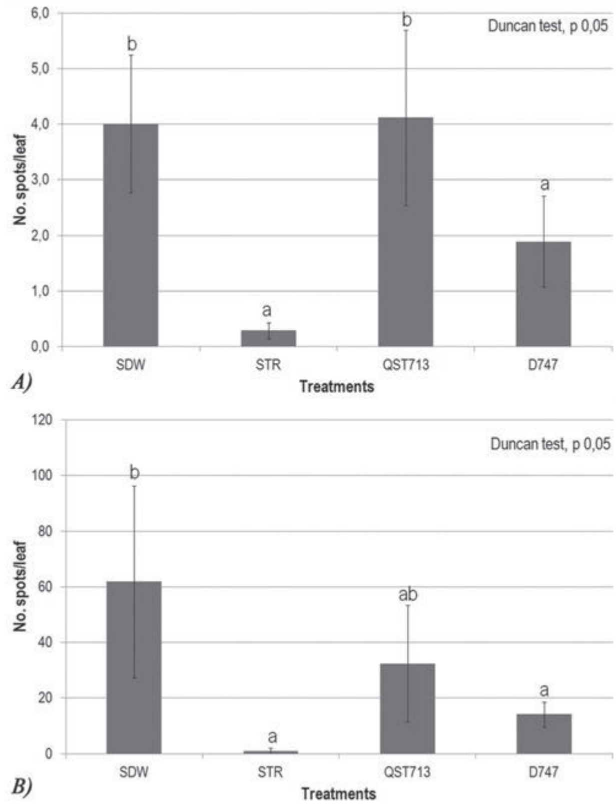

Figure 3. Mean bacterial leafspot severity after treatments with *Bacillus* strains applied to leaves of *A. deliciosa* (A) or *A. chinensis* (B) plants following inoculations with *Pseudomonas syringae* pv. *actinidiae* (strain DISTAL 9312). Treatments applied were: SDW (sterile distilled water, negative control); STR (streptomycin sulphate, positive control); QST713: *B. amyloliquefaciens* strain QST713 (active ingredient in Serenade Max®); D747: *B. amyloliquefaciens* strain D747 (in Amylo-X®). Bars indicate standard deviations. Histograms accompanied by different letters are different (Duncan's test, $P \leq 0.05$).

0.16, while in the control (untreated plot) was *c.* 0.26. In June, the DIs were *c.* 0.17 for untreated and 0.32 from the D747-treated plants. At the last assessment (in September) the mean DI for Amylo-X® treated plants (0.26) was less than that in the experimental controls (0.43) (Figure 5).

For the 2-year-old branches, the DIs in both plots were higher than those recorded for 1-year-old branches. In May, the mean DI in Amylo-X®-treated plants was *c.* 0.30, significantly lower than that of the control plants (*c.* 0.42); in June, the mean DIs were 0.38 for the treated plot and 0.71 for the untreated plot. In September, the mean DI for treated plants was *c.* 0.40, and less than that in untreated plants (*c.* 0.79) (Figure 5). The proportions of healthy leaves on 1- and 2-year-old branches in May was *c.* 70% in the control plot, less than in the treated plot (*c.* 80%). In June and in September, the proportions of healthy branches in the Amylo-X®-treated plot was similar (*c.* 78% in June and 75% in September) to that recorded in May; while in the untreated plot, these proportions were significantly decreased at the June (*c.* 35%) and September (*c.* 28%) assessments (Figure 6).

DISCUSSION

Bacillus bacteria have been described as microbial factories capable of producing many biologically active

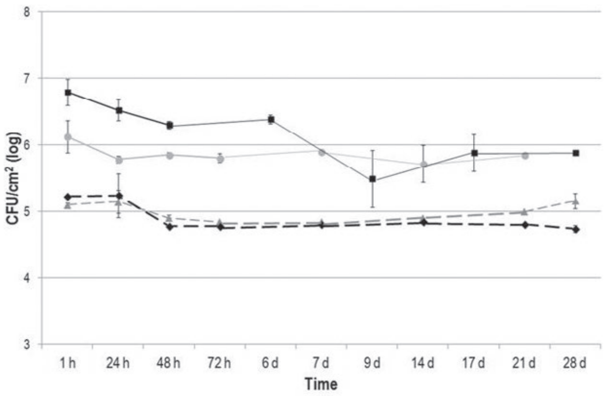

Figure 4. Mean numbers of *Bacillus amyloliquefaciens* strain D747 (active ingredient in Amylo-X®) on leaves of field-grown *Actinidia deliciosa* cv. Hayward plants, in Emilia Romagna (continuous line) or Lazio regions (dotted line) during the 2017 (light grey circles and triangles) or 2018 growing seasons (black squares and diamonds). Mean antagonist populations on leaf surfaces (cfu cm^2) were monitored for 3 to 4 weeks. Bars indicate standard deviations ($P \leq 0.05$) for each time point.

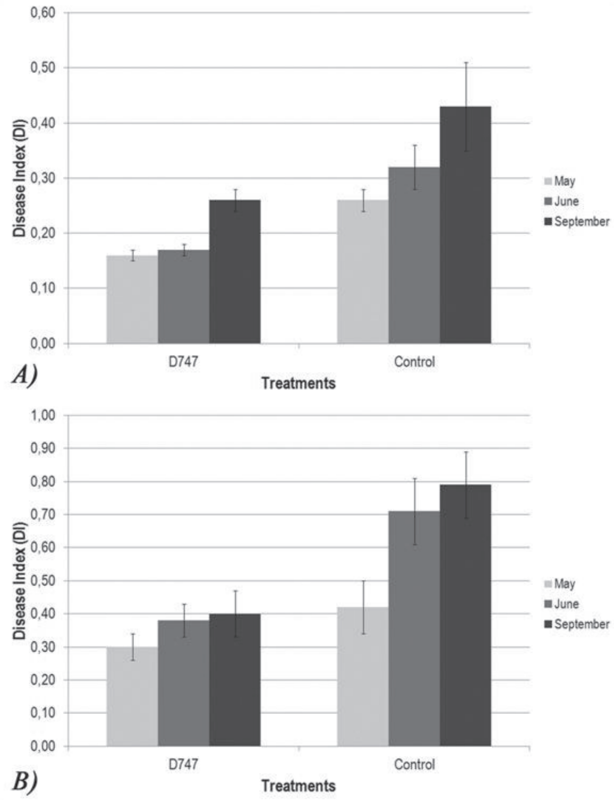

Figure 5. Mean bacterial leafspot indices (DI) caused by natural *Pseudomonas syringae* pv. *actinidiae* infections on kiwifruit plants after treatments with *Bacillus* strain D747 (active ingredient in Amylo-X®) or treated trees (control). The DIs were determined from phytopathometric assessments performed in May, June or September 2018, of leaves of 1-year-old (A) or 2-year-old (B) branches. Bars indicated standard deviations ($P \leq 0.05$).

compounds, which are potential inhibitors of phytopathogen growth. Examples are kanosamine and zwittermycin A from *B. cereus* (Emmert and Handelsman, 1999). The spore-forming capacity of *Bacillus* spp. makes these bacteria good candidates for development of efficient bio-pesticide products. *Bacillus* spp. are frequently used in biocontrol of plant pathogens, and includes a heterogeneous group of Gram-positive, aerobic or facultative anaerobic bacteria (Dworkin, 2006). These bacteria have been utilized for control of several plant diseases, including fire blight of pomaceous plants (caused by *Erwinia amylovora*) (Laux *et al.,* 2003; Broggini *et al.,* 2005; Bazzi *et al.*, 2006;), crown gall of grapevine (*Agrobacterium vitis*) (Biondi *et al*, 2009b) and bacterial speck of tomato (caused by *P. syringae* pv. *tomato*) (Fousia *et al.*, 2016). *Bacillus* spp. act through a variety of mechanisms, including competition, induction of systemic host resistance, and production of antibacterial compounds, the last of these being commonly recognized as the most important (Zuber *et al.* 1993; Thomashow and Weller, 1996; Koumoutsi *et al.*, 2004; Reva *et al.*, 2004; Lahlali *et al.*, 2013; Chowdhury *et al.*, 2015).

In the present study, results obtained *in vitro* confirmed the capacity of both *Bacillus* strains in each of the biocontrol formulations tested to produce antibiotic compounds. The cell-free diffusion procedure was carried out with a minimal medium that contained low concentrations of nutrients and salts, as would be the case in host phyllospheres (Vanneste *et al.*, 1992). This activity highlighted the antibacterial abilities of both

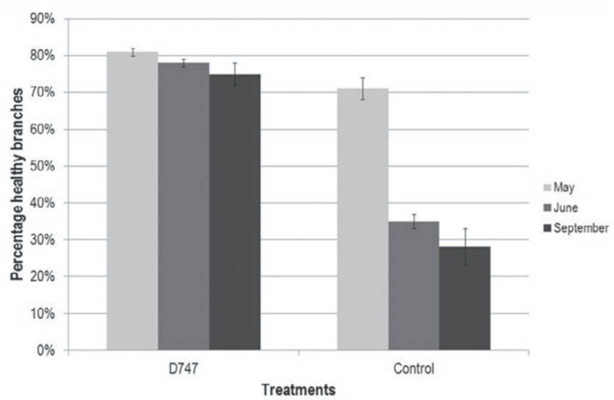

Figure 6. Mean percentage of healthy (disease free) 1-year and 2-year-old kiwifruit branches in May, June or September, 2018, in plots treated with *Bacillus* strain D747 (active ingredient of Amylo-X®) or controls (untreated plots) in a kiwifruit orchard in Latium region. Bars indicate standard deviations ($P \leq 0.05$).

antagonists under the unfavourable conditions. The effectiveness, isolation and identification of the antibacterial compounds produced by both antagonist strains have been assessed in previous studies. In particular, the strain QST713 was found to produce three families of non-ribosomal lipopeptides (LPs), the iturins, fengycins and surfactins (Mora *et al.*, 2011; Cawoy *et al.*, 2014). Strain D747 can produce surfactin, iturin and the serine proteinase subtilisin (Caulier *et al.*, 2019; EFSA, 2014). The *in vitro* activity of strains D747 and QST713 was also evaluated against *Xylella fastidiosa* (Zicca *et al.*, 2020), and these strains showed similar effectiveness against three Psa strains, one of biovar 1 and two of biovar 3. The QST713 strain resulted more antibacterial than strain D747.

The *in vitro* experiments assessing the activity of streptomycin sulphate against the two *Bacillus* strains QST713 and D747, carried out in solid and liquid media, showed the sensitivity of both strains to streptomycin sulphate. This antibiotic is commonly used against bacterial plant diseases. In the liquid medium experiment, the concentration of streptomycin sulphate was similar to that used in the field against bacterial diseases (Sundin *et al.*, 2009; Vanneste *et al.*, 2011a). From 0 h, the populations of both *Bacillus* strains were reduced by the antibiotic. After 24 h, the populations of QST713 and D747 were not completely eliminated, but were reduced by 3 to 4 orders of magnitude, thus confirming the need of significant reductions of antibiotic-based treatments applied in orchards, when these compounds are used in combination with biopesticides based on bacterial antagonists.

Flower colonization by strain D747 was higher than that of strain QST713, but strain QST713 survived at constant levels from application to 96 h. Strain D747 showed the highest population level after 72 h, which was more than one order of magnitude higher than that reported immediately after application. The BCA ability to colonize flowers indicated competition for nutrients and physical occupation of infection sites, as has been observed in previous studies. Psa can penetrate kiwifruit plants through stigmata and nectarii (Donati *et al.*, 2018), and the pathogen could be transmitted by contaminated pollen through natural and artificial pollination (Stefani *et al.*, 2011; Balestra *et al.*, 2018). Natural Psa populations can also predominate on *A. chinensis* compared with *A. deliciosa* flowers, indicating that the flowers of *A. deliciosa* may be less susceptible to penetration by the pathogen (Purahong *et al.*, 2018). Treatment of *A. chinensis* flowers with bacterial antagonists may protect against Psa infections, which rapidly become systemic, leading to the death of host plants (Renzi *et al.* 2012). In the present study, the high populations of both antagonists affected populations of the inoculated Psa mutant strain, which were reduced by more than one order of magnitude compared to the controls, from 48 to 72 h after Psa inoculation.

The ability to colonize flowers made the strain D747 a good candidate for orchard trials to monitor its survival under field conditions. This strain survived on leaves of *A. deliciosa* for almost 1 month at high population levels (*c.* 10^{4-5} cfu cm^{-2}), during 2 years in Emilia Romagna and Latium, located, respectively, in Northern and Central Italy. In Emilia Romagna, strain D747 was more abundant in both years than in Latium from the first assessment (1 h post inoculation). This may have been due to the type and/or time of treatment. The capacity of D747 to survive on kiwifruit leaves for long periods also indicates a reliable efficacy of this biocontrol agent.

Purahong *et al.* (2018) correlated the epiphytic bacterial populations present on leaves of kiwifruit plants to the ability of Psa to shape diversity of epiphytic bacterial populations, thus making the plants more susceptible to bacterial canker. Several bacterial genera were identified, particularly, *Bacillus* spp. were not significant compared to other genera. *Pseudomonas* species on leaves, including the pathogens *P. syringae* pv. *syringae* and *P. viridiflava*, were not predominant compared to the other genera, while on *A. deliciosa* these *Pseudomonas* species were predominant, ranging from 30 to 90%. In combination with the lower genetic susceptibility of kiwifruit plants with green fleshed fruit (EPPO, 2016; Perez *et al.*, 2019), treatments of leaves with D747 may protect from Psa penetration, and may reduce the subsequent secondary inoculum sources.

Under controlled conditions, the *A. deliciosa* plants were less susceptible to Psa than those of *A. chinensis*, which confirms previous results of intermediate susceptibility of *A. deliciosa* genotypes compared to several accessions of *A. chinensis* (Cotrut *et al.*, 2013; EPPO, 2016; Perez *et al.*, 2019). Results from the present study showed that Serenade Max® was ineffective against bacterial leaf spots in *A. deliciosa*, while Amylo-X® reduced the disease severity and provided relative protection of more than 52%. These results were partially confirmed by those on *A. chinensis* against the same pathogen strain; on plants producing yellow-fleshed fruit, Amylo-X® gave 77% relative protection, and Serenade Max® reduced the disease severity (relative protection *c.* 47%. The streptomycin sulphate treatment provided 93% relative protection on the green fruit variety and 98% relative protection on the yellow fruit variety.

The results on *A. deliciosa* plants, obtained under controlled conditions, partially confirmed those obtained in similar environmental conditions by Collina *et al.* (2016). They showed that Serenade Max® applied 48 h before the pathogen inoculation reduced the disease severity and provided more than 60% relative protection. Amylo-X® was more efficient and provided approx. 80% relative protection. In the same study, the ability of both antagonists to reduce the disease severity was similar when they were applied 24 h before Psa inoculation, and both provided *c.* 40% relative protection.

In open field conditions (in Latium region) and with natural pathogen inoculation pressure, repeated applications of Amylo-X® reduced the bacterial canker severity and an increased the number of healthy branches during the entire host vegetative season, in comparison to the control plants. Up to September, where the disease severity was higher in untreated plants, Amylo-X® provided 40-50% relative protection on all leaves. On the leaves of 2-years-old branches, the disease severity was reduced by more than 50% compared to control plants. The orchard results confirmed those obtained under controlled conditions. The strain D747-based product consistently reduced bacterial canker severity and achieved protective action against new and re-infections by Psa. This was confirmed by the higher proportions of healthy leaves on all branches (1-year and 2-year old branches) in plants treated with Amylo-X® compared to control plants, in all the assessments. On control plants, the disease incidence was higher at each assessment. On plants treated with the antagonist, although the disease increased at each assessment, the incidence was always lower. Daranas *et al.* (2018) have also demonstrated the effectiveness of Amylo-X® against bacterial canker of kiwifruit, in semi-field and field conditions. The ability of Serenade Max® to reduce kiwifruit bacterial canker was not optimal as against different bacterial pathogens, including those causing bacterial spot of stone fruits, angular leaf spot of strawberry and fire blight of pomaceous plants, although the product reduced the disease incidence and severity (Biondi *et al.*, 2006; Daranas *et al.*, 2018). The reduction of secondary inoculum sources, provided by the first treatments assessed during the optimal environmental conditions for the pathogen (spring, early summer), and the persistence of the antagonist strain D747 on the leaves surfaces throughout the growth season, emphasised the ability of this *Bacillus* strain to survive efficiently in the phyllospheres, and its capacity to persist under the microclimatic conditions of kiwifruit orchards. The Psa infections were detectable until September, but on Amylo-X®-treated plants the disease severity was reduced, confirming the ability of strain D747 to survive in different environmental conditions, including the high temperatures during summer. *Bacillus* species can form heat-, aridity-, and radiation-tolerant endospores allowing survival under non-optimal conditions (Dworkin, 2006).

In addition, when evaluated on flowers, those treated with Serenade Max® and Amylo-X®, in the climatic chamber and in the field, did not show phytotoxicity.

Purahong *et al.* (2018) showed that *Bacillus* species are not predominant on leaves, with respect of the other bacterial genera. Further research is required to evaluate the influence of *Bacillus* based products on the phyllosphere microbiomes during and after the crop growing season. Risks to biodiversity on kiwifruit organs should be prevented, both by reducing the use of agrochemicals, and by avoiding the persistence of bacterial antagonists (EFSA, 2014; Montesinos *et al.*, 2009).

CONCLUSIONS

This study has demonstrated that the bio-product Amylo-X® could be an effective tool for biological control of kiwifruit bacterial canker, because of efficacy against Psa and survival of the D747 *Bacillus* strain, the principal component of Amylo-X®, on kiwifruit flowers and leaves. The study has also demonstrated that this biocontrol agent is not compatible with antibiotic-based treatments against Psa.

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