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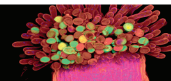

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RESEARCH PAPER

Pseudomonas protegens CS1 from the lemon phyllosphere as a candidate for citrus canker biocontrol agent

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ABSTRACT

- Citrus canker is a worldwide-distributed disease caused by *Xanthomonas citri* subsp. *citri*. One of the most used strategies to control the disease is centred on copper-based compounds that cause environmental problems. Therefore, it is of interest to develop new strategies to manage the disease. Previously, we reported the ability of the siderophore pyochelin, produced by the opportunistic human pathogen *Pseudomonas aeruginosa*, to inhibit *in vitro* several bacterial species, including *X. citri* subsp. *citri*. The action mechanism, addressed with the model bacterium *Escherichia coli*, was connected to the generation of reactive oxygen species (ROS). This work aimed to find a non-pathogenic strain from the lemon phyllosphere that would produce pyochelin and therefore serve in canker biocontrol.
- An isolate that retained its capacity to colonise the lemon phyllosphere and inhibit *X. citri* subsp. *citri* was selected and characterised as *Pseudomonas protegens* CS1. From a liquid culture of this strain, the active compound was purified and identified as the pyochelin enantiomer, enantio-pyochelin.
- Using the producing strain and the pure compound, both *in vitro* and *in vivo*, we determined that the action mechanism of *X. citri* subsp. *citri* inhibition also involved the generation of ROS. Finally, the potential application of *P. protegens* CS1 was evaluated by spraying the bacterium in a model that mimics the natural *X. citri* subsp. *citri* infection.
- The ability of *P. protegens* CS1 to reduce canker formation makes this strain an interesting candidate as a biocontrol agent.

INTRODUCTION

Citrus canker is a worldwide-distributed disease caused by the phytopathogenic bacterium *Xanthomonas citri* subsp. *citri*. In 1973, the disease was officially declared endemic in northeast Argentina, and since 2002 it has affected other citrus-producing areas of the country (northwest). The disease is hard to eradicate and its emergence in Argentina greatly affected the local citrus industry (Canteros 2000, 2004). In canker-free citrus areas, prevention of introduction and establishment of the disease involves rigorous quarantine measures. In the case of outbreaks, trees having symptoms and the surrounding trees within a radius of 500 m are removed (Gottwald *et al.* 2002). In contrast, the chosen strategy in endemic areas is chemical control with preventive sprays of copper-based bactericides (Das 2003). A significant disadvantage of this approach is the selection of naturally copper-resistant *X. citri* subsp. *citri* strains (Rinaldi & Leite 2000; Behlau *et al.* 2011) and the subsequent serious environmental problems due to copper toxicity (Alva *et al.* 1995; Tchounwou *et al.* 2012). Consequently, it is of interest to develop alternative disease management approaches that are more sustainable (Gottwald *et al.* 2002; Graham *et al.* 2004). For example, employing microbiological tools with biocontrol potential represents a promising alternative for canker management (Huang *et al.* 2012). Beneficial bacteria with this potential can be isolated from soil, the rhizosphere, rhizoplane and phyllosphere, and are often considered an alternative to chemical products (Smyth *et al.* 2011). These microorganisms can exert biological control of pathogens by synthesising molecules with antimicrobial activity, by competing for nutrients and by inducing plant systemic defence against a broad spectrum of pests and diseases (Hameeda *et al.* 2008; Martínez-Viveros *et al.* 2010; Vacheron *et al.* 2013). In order to succeed, it is crucial that beneficial microorganisms colonise and establish on plant surfaces (Arevalo-Ferro *et al.* 2005; Amaya-Gomez *et al.* 2015; Gao *et al.* 2015).

We previously reported that the siderophore pyochelin, produced by *Pseudomonas aeruginosa*, has *in vitro* inhibitory activity against bacteria from different genera, including *X. citri* subsp. *citri* (Adler *et al.* 2012). Generally, siderophores are known to antagonise pathogens by making iron unavailable to

them (Duffy & Defago 1999). Interestingly, we observed that the antibacterial activity of pyochelin against *Escherichia coli* was related to the generation of reactive oxygen species (ROS) and was independent of its ability to sequester iron (Adler *et al.* 2012). These results suggested that the action mechanism against *X. citri* subsp. *citri* could also be due to ROS generation. However, to corroborate this assumption, *in vitro* and *in vivo* assays using the plant pathogen were required. Pyochelin and its stereoisomeric variant enantio-pyochelin are produced by several Pseudomonad species (Youard *et al.* 2007; Adler *et al.* 2012; Lim *et al.* 2016). Even though numerous isolates belonging to this genus have been proposed for biocontrol (Walsh *et al.* 2001), many have faced significant difficulties in being registered. That is the case for *P. aeruginosa* that despite displaying interesting biocontrol qualities, field application implies human health risks that are decisive in avoiding its use (Stockwell & Stack 2007). With this in mind, we speculated that non-pathogenic pyochelin-producing Pseudomonads, isolated from lemon leaves, would potentially retain ability to colonise this niche and inhibit *X. citri* subsp. *citri.* Using an isolate with the above-mentioned traits, we aimed to shed light on the action mechanism involved in *X. citri* subsp. *citri* inhibition and pave the way for development of a microbiological alternative to control citrus canker disease.

MATERIAL AND METHODS

Isolation and selection of bacteria active against *X. citri* subsp. *citri*

Pseudomonads were isolated from lemon leaf surfaces (*Citrus limon* cv. Limoneira 8A) using the differential culture medium King B (King *et al.* 1954). For this, healthy leaves were collected and superficially washed with sterile distilled water. Then leaves were put in a 250 ml Erlenmeyer flask containing 20 ml sterile saline solution and agitated for 24 h at 28 °C at 150 rpm. Subsequently, serial dilutions of the bacterial suspension were plated on King B plates and incubated at 28 °C until colony formation. Fluorescent colonies were selected and, to avoid selecting *P. aeruginosa*, we discarded those isolates that grew at 42 °C (Brenner *et al.* 2005). We then tested *in vitro* inhibitory activity against *X. citri* subsp. *citri* of the selected isolates following the protocol described in Adler *et al.* (2012). For each isolate, 5 µl of a cell suspension with an optical density at 600 nm ($OD_{600\ nm}$) of 0.6 (Spectronic 20; Bausch & Lomb, Rochester, NY, USA) were spotted on the surface of M9 medium agar plates supplemented as described below. Plates were incubated for 24 h at 30 °C and then cells were killed by exposure to chloroform vapour for 20 min before overlaying a lawn of *X. citri* subsp. *citri*. After 16 h of incubation at 28 °C, inhibition of the pathogen was evidenced as a zone of clearance around the bacterial spots and measured from the edge of the colony to the edge of the inhibition halo. The isolate showing highest inhibition activity was chosen to continue with the following experiments described here.

All bacterial isolates were routinely cultured on M9 minimal salts (M9) medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.2% (w/v) casamino acids, 0.2% (w/v) glucose, 1 mM $MgSO_4$ and 1 mg·l^{-1} vitamin B1 (Sigma-Aldrich). *X. citri* subsp. *citri* was cultured on Cadmus medium (Cadmus *et al.* 1978).

Characterisation of the selected isolate

Bacterial colonisation on lemon leaves

Bacterial growth and biofilm formation were studied on *C. limon* Limoneira 8A leaves. The selected isolate was grown in a Luria Bertani (LB) shaking culture (Sezonov *et al.* 2007) at 180 rpm and 28 °C until cell density reached 10^6 CFU·ml^{-1}. Leaf disks of approximately 35 mm^2 were cut from healthy leaves, superficially sterilised with 70% ethanol for 1 min and then with 10% sodium hypochlorite for 10 min. Disks were successively washed (three times) with sterile distilled water. For bacterial growth, leaf disks were sprayed with 1 ml of bacterial suspension and incubated at 30 °C. At 0, 8, 15, 24, 48, 72 and 96 h of incubation, the number of cells per leaf disk was evaluated. To detach cells from leaf disks, 1 ml saline solution was added to each disk and then vortexed for 1 min. The number of viable cells, expressed as log CFU·leaf $disk^{-1}$, was determined by plating dilutions in agarised King B medium. For biofilm formation, a protocol adapted from Russo *et al.* (2006) was followed. Briefly, leaf disks were mounted on Petri dishes, adaxial side down, placed into contact with 3 ml of the bacterial suspension and incubated at 30 °C. At 0, 8, 15, 24, 48, 72 and 96 h of incubation, leaf disks were rinsed three times with distilled water and subsequently warmed at 60 °C for 20 min to facilitate dye fixation. Leaves were placed in contact with 3 ml 0.10% (w/v) crystal violet for 45 min at room temperature. The unbound crystal violet was removed and disks gently washed three times with distilled water. As control, leaf disks were placed in contact with 3 ml LB medium with no bacteria and evaluated under the same conditions as described above. At the indicated time points, photographs of the stained surfaces were taken to estimate the amount of surface-attached biofilm.

Genus and species assignment

Sequencing and subsequent analysis of the 16S rDNA and the gyrase subunit B gene (*gyrB*) was used to assign genus and species of the selected isolate. For this, the 16S rDNA was amplified by PCR using primers 27F and 1492R and the *gyrB* gene with primers UP-1 and UP-2r according to conditions described previously (Lane 1991; Yamamoto & Harayama 1995). The BLAST tool of the National Center for Biotechnology Information (NCBI) was then used to compare the sequences obtained with those in the 16S rRNA database and the nucleotide collection (http://www.ncbi.nlm.nih.gov/BLAST). Partial sequence for the 16S rDNA and *gyrB* genes have been deposited in the EMBL/GenBank/DDBJ nucleotide sequence data libraries.

Active compound purification

The active compound was purified from a culture of the selected isolate grown for 20 h at 30 °C in supplemented M9 medium following the antimicrobial activity as described above. The cell-free supernatant was loaded into a 1 g C18 cartridge (Phenomenex, Torrance, CA, USA) equilibrated with a 20% (v/v) methanol (Cicarelli, Argentina)–water dilution, and eluted step-wise with 40, 60, 80 and 100% methanol (in water). The 40% fraction was concentrated *in vacuo* and further purified by HPLC using a C18 Phenomenex Luna column (4.6610 mm, 5 micron) and a gradient of 10–85% (v/v)

acetonitrile (MeCN; Cicarelli) in water containing 0.1% (v/v) trifluoracetic acid (Sigma-Aldrich) at a flow rate of 1 ml·min^{-1}. Elution of the active compound was compared with a standard of pure pyochelin. Mass determination and UV spectra of the peaks showing activity against *X. citri* subsp. *citri* were obtained using an HPLC-MS (Agilent Technologies 6400, Santa Clara, CA, USA).

In vitro inhibition of *X. citri* subsp. *citri*

Effect of additives on antimicrobial activity of the active compound and its producing strain

The minimum inhibitory concentration (MIC) of the pure active compound (enantio-pyochelin) on *X. citri* subsp. *citri* was determined as described in Adler *et al.* (2012). Briefly, 10 μl double dilutions from a 1 mg·ml^{-1} solution of the active compound were spotted on supplemented M9 agar plates and a lawn of *X. citri* subsp. *citri* was overlaid. The maximum dilution that showed a zone of clearing was recorded as the MIC. To test the effect of additives on the MIC, plates were supplemented with either $FeCl_3$ (100 μM) or ascorbic acid (1 mM).

In order to evaluate the effect of additives on the antimicrobial activity of the enantio-pyochelin-producing strain, spots of it were grown overnight in supplemented M9 medium with the addition of either $FeCl_3$ (100 μM) or ascorbic acid (1 mM) and thereafter killed with chloroform vapour. The antimicrobial activity was revealed overlaying a lawn of *X. citri* subsp. *citri* with overnight incubation at 28 °C.

Measurement of ROS

To determine ROS levels, exponentially growing *X. citri* subsp. *citri* cultures in supplemented M9 medium were washed and resuspended in 50 mM sodium phosphate buffer (pH 7) at a final $OD_{600\ nm}$ of 0.5. Cell suspensions were divided into three fractions. Two fractions were supplemented with 2 μl of the pure active compound (final concentration 15 μM) or with 1 mM ascorbic acid. The third fraction with no additives was used as control. Fractions were incubated for 2 h at 28 °C and then 2,7-dichlorofluorescein diacetate (H2DCFDA, the oxidation-sensitive probe dissolved in dimethyl sulphoxide) was added at a final concentration of 10 mM and incubated for 30 min (Davidson *et al.* 1996). After incubation, cells were washed, resuspended and sonicated in the same buffer. Fluorescence intensity was measured using a Perkin Elmer LS55 spectrofluorometer (excitation l, 490 nm; emission l, 519 nm). Results are shown as relative fluorescence to that of the control expressed as a percentage.

Lipid peroxidation

Lipid peroxidation was evaluated by measuring the thiobarbituric acid-reactive substances (TBARS) in *X. citri* subsp. *citri* cell extracts following the method described in Rice-Evans *et al.* (1991). Briefly, *X. citri* subsp. *citri* cultures in Cadmus medium containing (a) 15 μM enantio-pyochelin, (b) 15 μM enantio-pyochelin + 1 mM ascorbic acid, or (c) without additives (control) were lysed using a French press after reaching stationary phase. Lysed cultures were then centrifuged for 15 min at 4000×*g* and the supernatants taken to continue the protocol. An aliquot of each extract was used to determine the amount of protein following the Bradford method (Bradford 1976). Furthermore, 1 ml of each cell extract was precipitated with 1 ml 20% TCA and then centrifuged at 4000×*g*. The supernatants were then mixed with 1 ml 0.65% thiobarbituric acid reagent, vigorously mixed and heated at 100 °C for 60 min. Cooled samples were centrifuged and absorbance of the supernatants at 535 nm measured. TBARS content (expressed in nmol·mg protein^{-1}) was determined using a molar extinction coefficient of 156 mM^{-1}·cm^{-1}.

In vivo inhibition of *X. citri* subsp. *citri*

Lemon plants (*C. limon* cv. Limoneira 8A) were grown as previously reported (Siciliano *et al.* 2006). Inoculations of the pathogenic bacterium *X. citri* subsp. *citri* on the lamina of young lemon leaves were done through pressure infiltration according to published methods (Siciliano *et al.* 2006). For each condition studied, inoculations involved two leaves from each plant and seven plants were used in total. In each leaf, both sides of the rib were infiltrated with a diluted bacterial suspension of *X. citri* subsp. *citri* ($OD_{600\ nm}$ equivalent to 0.0001) while only one side was infiltrated with either the pure compound (0.2 mg·ml^{-1} in 20% methanol) or a combination of the pure compound (0.2 mg·ml^{-1} in 20% methanol) plus 1 mM ascorbic acid. As a control, a 20% methanol solution was used in leaves infiltrated with *X. citri* subsp. *citri*. The same experimental design using a cell suspension of the selected isolate ($OD_{600\ nm}$ equivalent to 0.001) instead of the pure compound was used to study the ability of bacterium to control citrus canker disease. Inoculated plants were maintained for 21 days in a growth cabinet, at temperatures from 25 to 28 °C, high humidity, 16 h light photoperiod, and a light intensity of 150–200 μmol·m^{-2}·s^{-1}. The number of cankers in the area of infiltration was counted after 3 weeks.

Evaluation of the potential application of *P. protegens* CS1 to control canker disease

Inoculation of the pathogenic bacterium (*X. citri* subsp. *citri*) on the lamina of young lemon leaves was done with the spraying method described in Siciliano *et al.* (2006). In this way, as with natural infections, bacteria enter the leaf through openings (*via* stomata) and then colonise the apoplasm.

Experiments were conducted using a randomised design with seven plants for each treatment with three replications. In each plant, both sides of the leaves were sprayed with a bacterial suspension of *X. citri* subsp. *citri* ($OD_{600\ nm}$ 0.1). After 24 h, groups of seven plants were sprayed with a suspension of *P. protegens* CS1 ($OD_{600\ nm}$ 0.1) or supplemented M9 medium (control group). Inoculated plants were maintained for 21 days in a growth cabinet, at temperatures from 25 to 28 °C, high humidity, 16 h light photoperiod, and a light intensity of 150–200 μmol·m^{-2}·s^{-1}. The number of cankers in the area of infiltration was counted after 3 weeks.

Statistical analysis

An ANOVA using a general linear model was applied to analyse log CFU·leaf disk^{-1}, relative fluorescence, TBARS content and number of cankers. Significant differences ($P < 0.05$) between mean values were determined with Tukey's test, using the MINITAB statistical software (version 16 for Windows; Minitab Inc., State College, PA, USA).

RESULTS

Isolation and selection of a non-pathogenic *Pseudomonas* strain active against *X. citri* subsp. *citri*

One goal of this work was selecting a non-pathogenic pyochelin-producing Pseudomonad that would successfully colonise lemon leaves and inhibit *X. citri* subsp. *citri*. For that, we isolated bacteria from the surface of lemon leaves using a differential culture medium for Pseudomonads. After selecting all fluorescent isolates and discarding those that grew at 42 °C (culture criterion to rule out *P. aeruginosa*), six bacterial isolates having different colony morphotypes were obtained. Fig. 1 shows the *in vitro* antimicrobial activity against *X. citri* subsp. *citri* of each isolate. The most active isolate (Fig. 1, Panel C) was selected for further characterisation.

Characterisation of the selected isolate

Considering that effective colonisation of the selected strain on lemon leaves could be necessary to prevent *X. citri* subsp. *citri* growth, we verified the ability of this isolate to grow and form a biofilm on the surface of lemon leaves. Results show that, even though a significant ($P < 0.05$) bacterial growth of 1 log unit (Fig. 2A) was evidenced at 8 h of incubation, no biofilm was observed (Fig. 2B). However, when bacterial growth peaked (15 h of incubation), biofilm formation could be detected. Next, at 24 and 48 h of incubation there was a decrease in both the cell number (3 log units) and the biofilm formed. This trend was further accentuated at 72 and 96 h of incubation (Fig. 2A,B).

The isolate characterisation was followed by genus and species assignment performed through sequencing and posterior analysis of the 16S rDNA and *gyrB* genes with the NCBI algorithm BLAST. A 100% identity with *P. protegens* Pf-5 and a 99% of identity with *P. protegens* CHA0 were obtained for 16S rDNA and *gyrB*, respectively. This level of identity is sufficient to assign the genus and species (Bosshard *et al.* 2003; Mulet *et al.* 2010) and therefore we named the isolate *P. protegens* CS1 (accession numbers, KU755551 and KU836637).

Purification of the active compound

The active compound produced by *P. protegens* CS1 was purified from a liquid culture as described above. Given that the goal of this work was to find a non-pathogenic strain that produces pyochelin, we used the pure siderophore as a standard in the last step of the purification process (HPLC fractionation). Figure 3A shows the HPLC chromatogram obtained for both the active fraction of *P. protegens* CS1 (40% fraction) and that of pyochelin. Both chromatograms

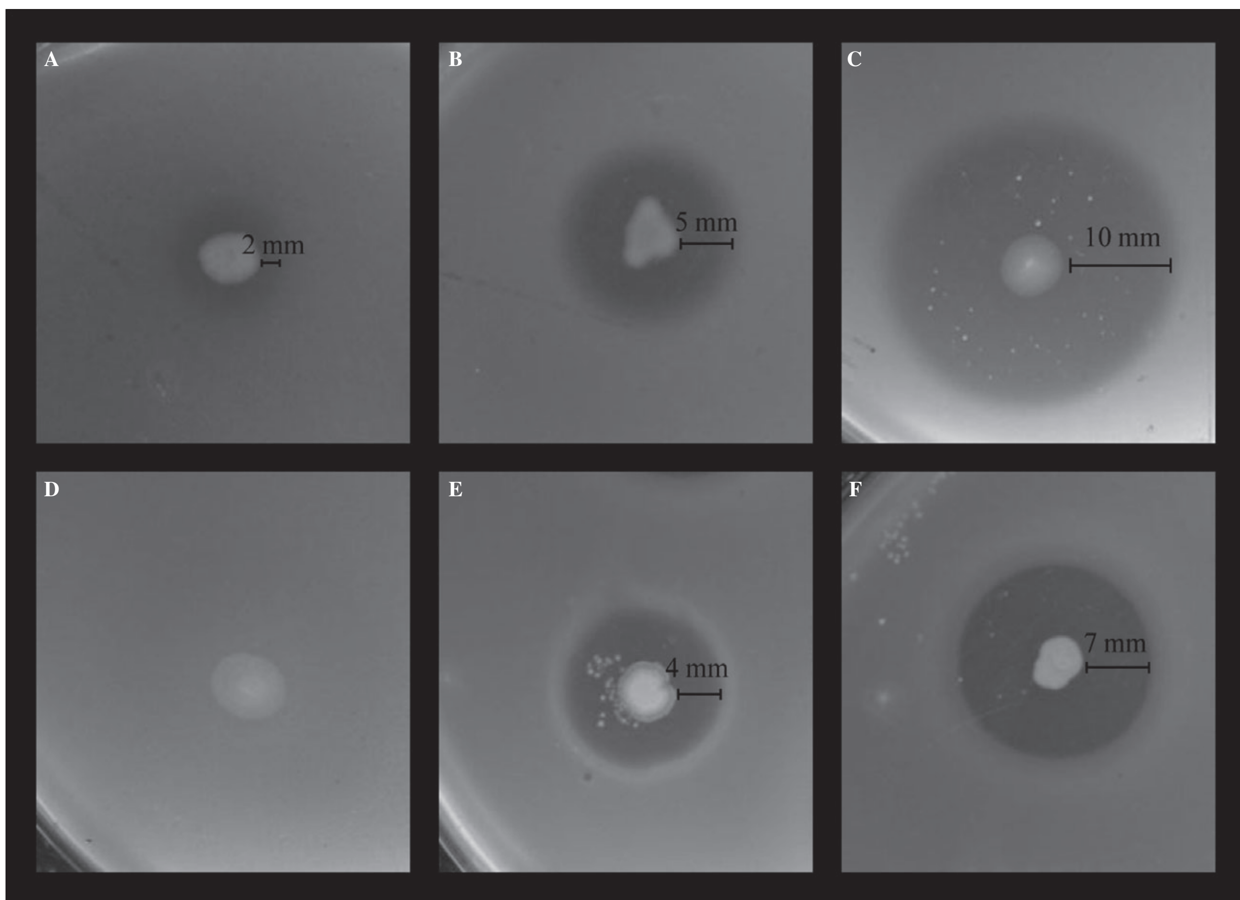

Fig. 1. Antimicrobial activity of bacteria isolated from *Citrus limon* cv. Limoneira 8A leaves against *X. citri* subsp. *citri.* Area of clearance around spots of each of the six isolated morphotypes, reveal the inhibitory activity against *X. citri* subsp. *citri* (Panels A–F). Inhibition was recorded as millimeters from the edge of the colony to the edge of the inhibition halo. Panel C corresponds to the isolate with highest antimicrobial activity (10 mm).

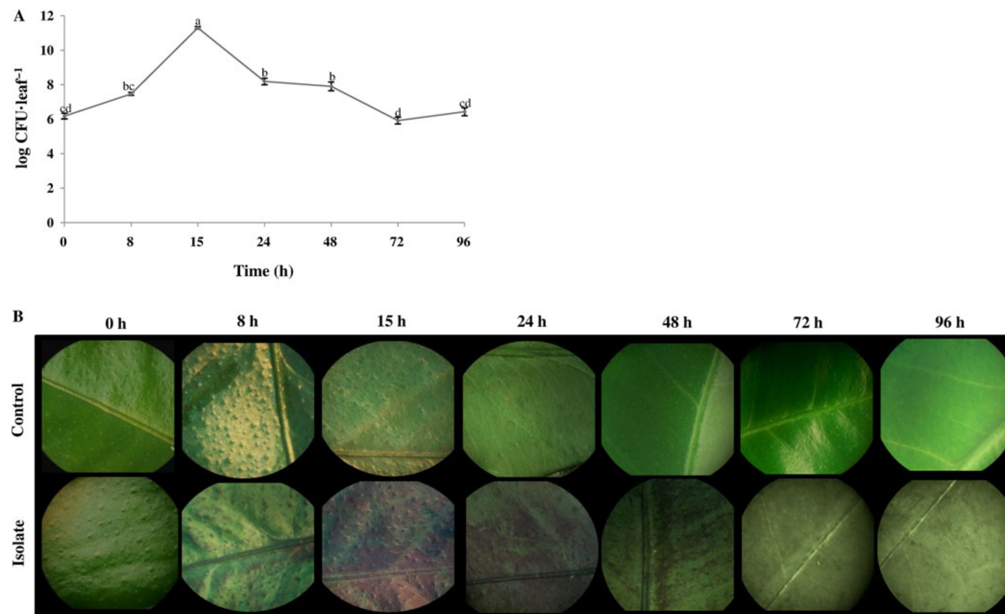

Fig. 2. Bacterial growth and biofilm formation on lemon leaves. A: Viable cell number of the selected isolate on lemon leaves after 0, 8, 15, 24, 48, 72 and 96 h of incubation. Data are plotted as mean values of viable cell numbers (log CFU·leaf·disk^{-1} ± SE). Different letters indicate differences between experimental groups ($P < 0.05$). B: Ability of the selected isolate to form biofilm on lemon leaves over time (0, 8, 15, 24, 48, 72 and 96 h). Crystal violet stain indicates bacterial biofilm on leaf surface. Control indicates leaf disks in contact with LB medium without bacteria.

show two major peaks that elute with a 2.5 min difference between each other. Retention times were 18.6 and 21.1 min for pyochelin and 21.5 and 24.0 min for the active fraction of *P. protegens* CS1. Only HPLC fractions that corresponded to the above-mentioned major peaks (from *P. protegens* CS1 and the pyochelin standard) exhibited *in vitro* activity against *X. citri* subsp. *citri* (data not shown). When analysed with HPLC-MS, all major peaks gave a molecular mass of 325.1 and exact same mass and UV spectra (Fig. 3B,C). Both pyochelin and its optical antipode enantio-pyochelin have a characteristic chromatogram pattern with two peaks corresponding to two interchangeable stereoisomeric forms of the same molecule (Youard *et al.* 2007). The observed differences in retention time for peaks of the active compound of *P. protegens* CS1 and those of the pyochelin standard are in concordance with the differences reported for pyochelin and enantio-pyochelin (Youard *et al.* 2007). *In toto*, the typical chromatogram pattern obtained for the active compound of *P. protegens* CS1, having two active peaks with higher retention times than the pyochelin standard, along with identical molecular mass and mass and UV spectra to those of pyochelin, indicates that the active compound produced by *P. protegens* CS1 is enantio-pyochelin (Youard *et al.* 2007; Adler *et al.* 2012). Considering this and that *P. protegens* strains are reported to produce only enantio-pyochelin (Youard *et al.* 2007; Lim *et al.* 2016), in this work we continue naming the active compound of *P. protegens* CS1 as enantio-pyochelin.

In vitro inhibition of *X. citri* subsp. *citri* by enantio-pyochelin-induced ROS

To evaluate if pure enantio-pyochelin inhibits *X. citri* subsp. *citri* by generating ROS, as previously reported for pyochelin and *E. coli* (Adler *et al.* 2012), we assessed the effect of medium supplementation with either iron or ascorbic acid (reducing agent) on the antimicrobial activity of enantio-pyochelin. The MIC of enantio-pyochelin for *X. citri* subsp. *citri* with no additives was 48 μM. Remarkably, ascorbic acid addition substantially increased the MIC (nine-fold), while $FeCl_3$ addition only slightly increased the MIC (one-fold; Table 1). A similar pattern was observed when the effect of the same additives was evaluated on the antimicrobial activity of the enantio-pyochelin -producing strain (*P. protegens* CS1) against *X. citri* subsp. *citri*. While medium supplementation with iron did not affect the inhibitory activity of *P. protegens* CS1, ascorbic acid addition prevented it (Fig. 4). These results suggest that the antimicrobial activity of *P. protegens* CS1 against *X. citri* subsp. *citri* is mediated by enantio-pyochelin and that the inhibition mechanism involves oxidative stress and is independent of iron deprivation. To confirm this, we measured ROS and lipid peroxidation in *X. citri* subsp. *citri* cultures supplemented with enantio-pyochelin. Figure 5 shows that enantio-pyochelin addition significantly ($P < 0.05$) increased ROS (Panel A) and lipid peroxidation (Panel B) levels when compared to the control. Finally, as expected, ascorbic acid supplementation reduced the level of both evaluated parameters (Fig. 5A,B). Thus, the counteracting effect of ascorbic acid on ROS and lipid peroxidation levels generated by enantio-pyochelin in *X. citri* subsp. *citri*, supports that the inhibition mechanism implies ROS at least targeting lipids.

Inhibition of canker formation by either enantio-pyochelin or *P. protegens* CS1

In order to further characterise the ability of enantio-pyochelin or its producing strain (*P. protegens* CS1) to inhibit *X. citri* subsp. *citri*, we performed *in vivo* experiments. A model of canker disease involving lemon leaf infiltration with the pathogen

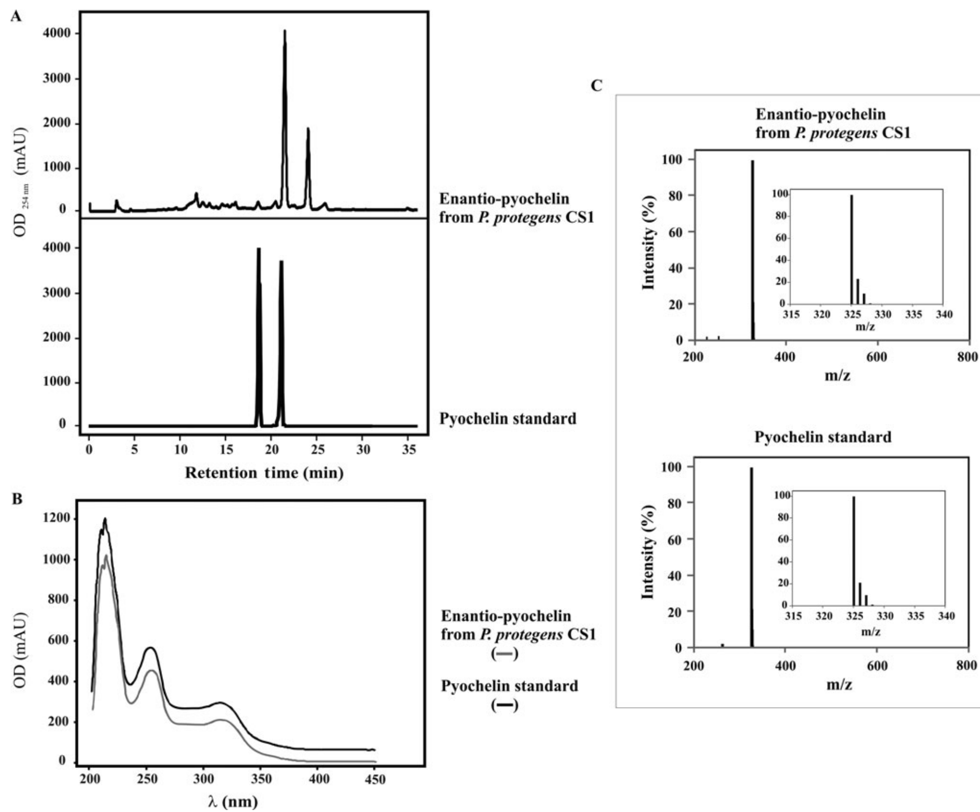

Fig. 3. *Pseudomonas protegens* CS1 active compound purification. A: Comparison of HPLC chromatograms obtained for the active fraction of *P. protegens* CS1 and a pyochelin standard. B: UV spectra for the active major peaks obtained by HPLC fractionation from *P. protegens* CS1 and with the pyochelin standard. C: Positive-ion mode mass spectra for pyochelin and enantio-pyochelin. In each case, the inset corresponds to a magnified view of the major mass ion. In all cases [M + H]+ observed = 325.1.

Table 1. MIC of enantio-pyochelin against *X. citri* subsp. *citri*.

conditions of determination	MIC (μM)
M9 medium[a]	48
M9 medium + $FeCl_3$[b]	96
M9 medium + ASC[c]	768

[a]MIC determination in M9 medium without additions.
[b]MIC determination in M9 medium with addition of 100 μM iron chloride.
[c]MIC determination in M9 medium with addition of 1 mM ascorbic acid.

was used. Figure 6 shows that treatment with either enantio-pyochelin (Panels A and B) or *P. protegens* CS1 (Panels C and D) significantly reduced ($P < 0.05$) the number of cankers per centimeter squared in infected leaves when compared with the control. These observations confirm that *in vivo,* enantio-pyochelin is the metabolite responsible for the inhibition observed. Moreover, ascorbic acid addition abrogated the inhibitory effect of both enantio-pyochelin and *P. protegens* CS1, indicating that ROS generation is also the most likely inhibition mechanism *in vivo.*

Evaluation of the potential application of *P. protegens* CS1 to control canker disease

The prospective application of *P. protegens* CS1 for canker disease biocontrol was evaluated using a method that mimics the natural *X. citri* subsp. *citri* infection process. The method involved spraying lemon plants with *X. citri* subsp. *citri* and *P. protegens* CS1 under temperature, humidity and light conditions that favour plant infection (see Material and Methods). Figure 7 shows that treatment with *P. protegens* CS1 significantly reduced ($P < 0.05$) the number of cankers per centimeter squared in infected leaves when compared with the control.

DISCUSSION

Nowadays, citrus canker management involves the use of copper-based bactericides (Rinaldi & Leite 2000; Behlau *et al.* 2011). This approach encompasses significant problems, such as environmental concerns due to the copper toxicity (Alva *et al.* 1995; Tchounwou *et al.* 2012), and the treatment effectiveness reduction from increasing resistance of *X. citri* subsp. *citri* strains. Hence, it is necessary to develop sustainability-oriented strategies to control this disease, and one valuable alternative is disease biocontrol. When applied as a biocontrol agent, efficiency of bacterial colonisation on the plant tissue is a prerequisite for effectiveness (Lugtenberg *et al.* 2001; Mercado-Blanco & Bakker 2007). In the unfavourable environment of the phyllosphere, colonisation and survival requires the formation of bacterial aggregates (Lindow & Brandl 2003). Therefore, in an attempt to find a non-pathogenic bacterium that successfully colonises the lemon phyllosphere and inhibits

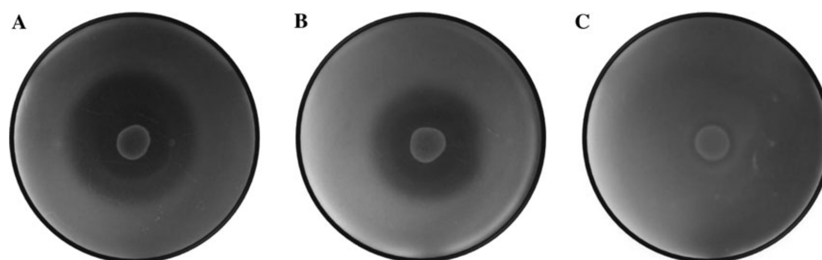

Fig. 4. Effect of iron or ascorbic acid addition on antimicrobial activity**.** *In vitro* antibiotic activity of the selected isolate (*Pseudomonas protegens* CS1) against *X. citri* subsp. *citri* determined in A: M9 medium without additives, B: M9 medium with 100 μM $FeCl_3$ and C: M9 medium with addition of 1 mM ascorbic acid. Zones of clearing around the bacterial spots indicate antimicrobial activity.

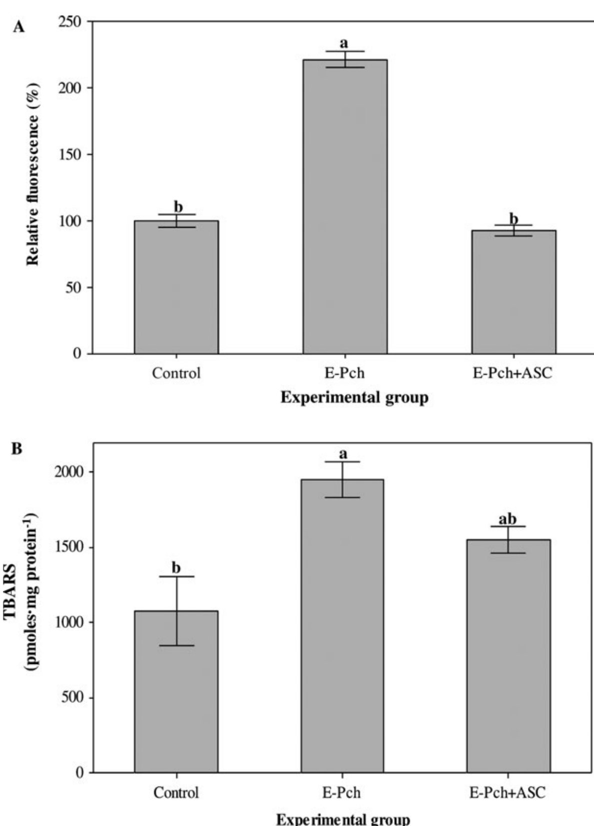

Fig. 5. ROS and lipid peroxidation induced by enantio-pyochelin. A: Quantification of ROS and B: lipid peroxidation levels in *X. citri* subsp. *citri* cultures, expressed as relative fluorescence percentage and thiobarbituric acid-reactive substance (TBARS) content, respectively. *X. citri* subsp. *citri* cultures were supplemented with 15 μM enantio-pyochelin (E-Pch), 15 μM enantio-pyochelin + 1 mM ascorbic acid (E-Pch+ASC) or without any additions (control). Data are plotted as mean values of relative fluorescence percentage or TBARS content ± SE. Different letters indicate differences between experimental groups ($P < 0.05$).

X. citri subsp. *citri,* we selected lemon plant leaves as the source of such bacteria. The isolation process implied a cultural bias that selected Pseudomonads but excluded *P. aeruginosa.* This bias was imposed to increase the chance of selecting a generally regarded as safe (GRAS) microorganism.

The selected isolate, which showed the highest antimicrobial activity *in vitro* (Fig. 1) and retained its ability to colonise the lemon phyllosphere (Fig. 2), was characterised as *P. protegens* after sequencing the 16S rDNA and *gyrB* genes. *P. protegens*, formerly classified as *P. fluorescens,* is the archetype of fluorescent Pseudomonads used in biocontrol (Nowak-Thompson *et al.* 1994). In general, fluorescent Pseudomonads are ubiquitous inhabitants of plant surfaces (Redondo-Nieto *et al.* 2013) and many strains have antagonistic properties over phytopathogens (Unnamalai & Gnanamanickam 1984; Das 2003; Stockwell *et al.* 2009; Cruz-Quiroz *et al.* 2011; Maldonado-Gonzalez *et al.* 2015). However, the action mechanisms exerted by *P. fluorescens* in bacterial antagonism are varied and still require further elucidation (Unnamalai & Gnanamanickam 1984).

In order to characterise the chemical nature behind the antimicrobial activity of *P. protegens* CS1 on *X. citri* subsp. *citri,* we purified the active compound. HPLC fractionation showed two peaks (Fig. 3A) having activity against *X. citri* subp. *Citri*, while no other fraction displayed antimicrobial activity. When the two active peaks were loaded into an HPLC-MS, both had an identical mass of 325.1, which corresponds to the mass of pyochelin. In addition, both the mass and UV spectra of the purified compound were identical to those of the pyochelin standard (Fig. 3B,C). The chromatogram obtained for the active fraction derived from *P. protegens* CS1 is similar to that of the pyochelin standard in that both have two major peaks. However, the retention times for the active peaks from *P. protegens* CS1 are higher than those for pyochelin (Fig. 3A). This is consistent with the reported retention time differences for pyochelin and its optical antipode enantio-pyochelin (Youard *et al.* 2007). The two peaks obtained in the HPLC fractionation correspond to the two interchangeable stereoisomeric forms of the same molecule. In the case of the pyochelin standard, these forms are pyochelin-I and pyochelin-II with a chiral centre configuration of 4′R, 2″R, 4″R and 4′R, 2″S, 4″R, respectively (Schlegel *et al.* 2004). For enantio-pyochelin, the stereosimeric forms are enantio-pyochelin-I (4′S, 2″S, 4″S) and enantio-pyochelin-II (4′S, 2″R, 4″S) (Youard *et al.* 2007). To our knowledge, only *P. aeruginosa* (Castignetti 1997; Braud *et al.* 2009), *Burkholderia cepacia* (Darling *et al.* 1998; Thomas 2007) and *Streptomyces scabies* (Seipke *et al.* 2011) produce pyochelin. On the other hand, enantio-pyochelin is exclusively synthesised by *P. fluorescens* and *P. protegens* (Youard *et al.* 2007; Lim *et al.* 2016). The classification of the producing strain as *P. protegens* CS1, along with chemical characterisation of the active compound, indicate that the purified metabolite is enantio-pyochelin.

Previously, using an *E. coli* mutant strain deficient in the production of the siderophore enterobactin (a siderophore

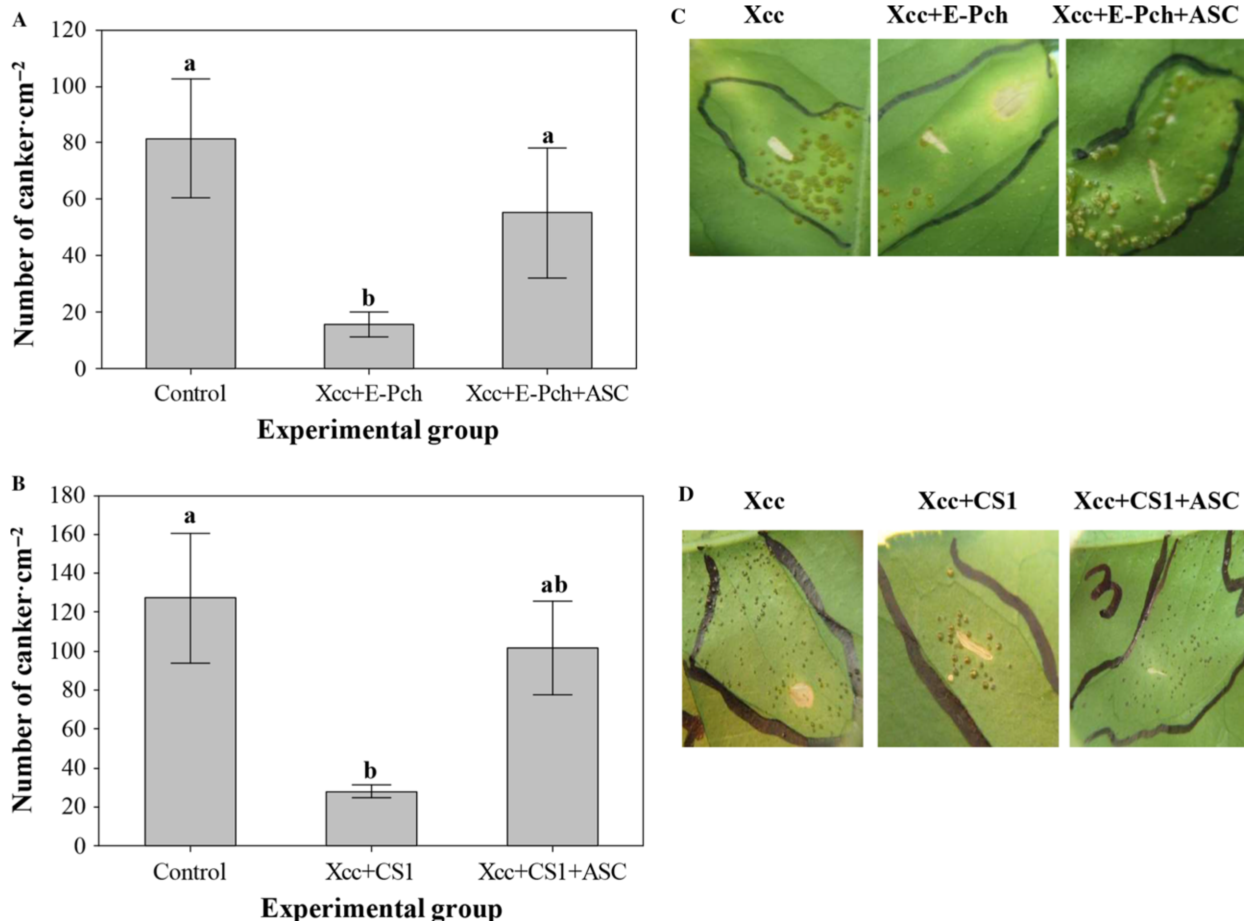

Fig. 6. Canker control by enantio-pyochelin and *Pseudomonas protegens* CS1 in a canker disease model. Leaves of *C. limon* cv. Limoneira 8A were infiltrated with *X. citri* subsp. *citri* (Xcc). Panels A and B show canker control mediated by enantio-pyochelin and *P. protegens* CS1, respectively. Both panels also show the effect of ascorbic acid addition. Data are plotted as mean values for the number of cankers per centimetre squared ± SE. E-Pch indicates infiltration with 0.2 mg·ml^{-1} enantio-pyochelin, CS1 denotes leaf infiltration with a cell suspension of *P. protegens* CS1 ($OD_{600\ nm}$ equivalent to 0.0001), ASC indicates infiltration with 1 mM ascorbic acid and Control refers to infiltration only with Xcc. Different letters indicate significant differences between experimental groups ($P < 0.05$). Panels C and D show representative photographs (from at least three independent experiments) of canker control by enantio-pyochelin and *P. protegens* CS1, respectively.

with antioxidant properties), we were able to link the antimicrobial activity of pyochelin to ROS generation. In that work, the action mechanism of pyochelin on wild-type sensitive bacteria (including *X. citri* subp. *citri*) was not addressed (Adler *et al.* 2012). Given the remarkable structural similarity between pyochelin and enantio-pyochelin, we considered it likely that enantio-pyochelin would also inhibit *X. citri* subp. *citri* through ROS generation. Therefore, we evaluated the effect of iron and ascorbic acid addition on the antimicrobial activity. As expected, whereas iron supplementation barely affected *X. citri* subsp. *citri* sensitivity to pure enantio-pyochelin, ascorbic acid considerably reduced it (Table 1). A similar pattern was observed with the enantio-pyochelin-producing strain, *P. protegens* CS1 (Fig. 4). These observations are highly suggestive of the involvement of oxidative stress in enantio-pyochelin bacterial inhibition. This was confirmed when *X. citri* subsp. *citri* cultures exposed to enantio-pyochelin showed higher ROS levels that were reduced upon ascorbic acid addition (Fig. 5A). Moreover, we further characterised the action mechanism on *X. citri* subsp. *citri* by studying the ROS-mediated cellular damage using a lipid peroxidation assay. Even though our results *in vitro* show increased peroxidation of lipids due to the enantio-pyochelin-generated ROS (Fig 5B), cellular distress of bacteria exposed to enantio-pyochelin and the resulting antimicrobial activity can potentially involve other cellular targets for ROS and perhaps other action mechanisms. In concordance with *in vitro* observations, we found that enantio-pyochelin and the producing strain (*P. protegens* CS1) effectively inhibited *X. citri* subsp. *citri in vivo* and that this effect is also mediated by ROS generation (Fig. 6). In contrast, a report by Ruiz *et al.* (2015) indicates that enantio-pyochelin produced by *P. protegens* Pf-5 controls the fungus *Fusarium* by limiting iron availability. This is an example in which the same species and its secreted metabolite can exert biocontrol in different settings with different action mechanisms involved. This is consistent with multiple chemical traits of the same molecule (*i.e.*, iron chelation and ROS generation) and the diverse biological nature of the microorganisms subjected to biocontrol (*i.e.*, variable iron scavenging resources and different ability to cope with oxidative stress). In addition, it also highlights the importance of the microenvironment where biocontrol occurs and the nature of the interaction between the pathogen, the

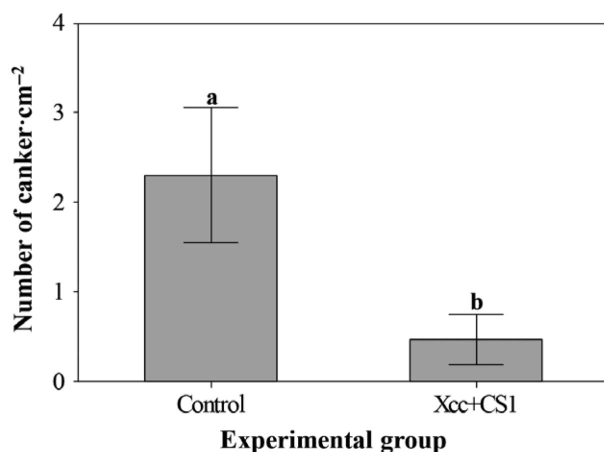

Fig. 7. Evaluation of *Pseudomonas protegens* CS1 as a potential biocontrol agent for citrus canker disease. Plants of *C. limon* cv. Limoneira 8A were sprayed with *X. citri* subsp. *citri* (Xcc) and 24 h later with a *P. protegens* CS1 (CS1) suspension at $OD_{600\ nm}$ of 0.1 (Xcc + CS1) or with M9 medium (Control). Data are plotted as mean values for the number of cankers per centimetre squared ± SE. Different letters indicate significant differences between experimental groups ($P < 0.05$).

residing microflora and the biocontrol microbes used. In fact, the inconsistency of biological control in the phyllosphere was attributed to poor understanding of the ecology of this type of interaction (Stromberg *et al.* 2000). With the advent of next-generation sequencing techniques, the study of plant-associated microbiomes and the impact of biocontrol agents (Massart *et al.* 2015) is providing the foundation for a holistic view that will most likely shape the design of biocontrol strategies.

To our knowledge, there are few reports that demonstrate an *in vivo* antagonistic effect of *Bacillus* and *Pseudomonas* species against *X. citri* subsp. *citri* (Kalita *et al.* 1996; Khodakaraminan *et al.* 2008; de Oliveira *et al.* 2011; Huang *et al.* 2012). These reports do not examine the active metabolites and the possible action mechanisms involved in biocontrol. In addition, the vast majority of the literature suggests biocontrol candidates based solely on their ability to inhibit *X. citri* subsp. *citri in vitro* (Das 2003; Tan *et al.* 2006; Ahmad *et al.* 2014).

Finally, in order to gain some insight into the potential application of *P. protegens* CS1 to control canker disease, we tested the ability of the above-mentioned strain to reduce the number of cankers in a model that mimics the natural infection process. Experimental evidence showing the ability of *P. protegens* CS1 to reduce canker development (Fig. 7) makes this strain a promising candidate as a biocontrol agent. Currently, citrus canker control makes use of copper-based compounds. Interestingly, these compounds most likely inhibit *X. citri* subsp. *citri* through ROS generation (del Campo *et al.* 2009). Thus, it is possible that *X. citri* subsp. *citri* is a bacterium with low tolerance to oxidative stress, and therefore combined strategies based on ROS generation might be promising. However, before considering the development of technology based on *P. protegens* CS1, further studies including bacterial formulation and field application should be carried out.

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