Canker Control by the Siderophore Pyochelin from *Pseudomonas fluorescens*

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Abstract

This work identifies a compound able to inhibit *Xanthomonas citri* subsp. *citri* (citrus canker agent) growth in vitro and in vivo. First, we isolated bacteria from citrus leaf surfaces that were able to inhibit *X. citri* subsp. *citri* in vitro. Among the selected isolates, we focused on one with remarkable activity. The strain was characterized as *Pseudomonas fluorescens* after sequencing its 16S rDNA and analyzing the sequence with BLASTn. The purification and chemical analysis of the active compound allowed us to assign the inhibitory activity to enantio-pyochelin (E-Pch). Since this molecule is a siderophore, we wondered if the inhibition observed was a result of iron scavenging. Surprisingly, when we supplemented media with an excess of iron, we observed practically no change in the inhibition activity. In an attempt to identify the action mechanism of E-Pch, we evaluated the ability of E-Pch to generate reactive oxygen species (ROS) within a culture of *X. citri* subsp. *citri* and its correlation with the inhibitory activity. In fact, we observed increased ROS levels when E-Pch was added. In addition, the reducing agent, ascorbic acid, lowered ROS levels and the antibiotic activity, implying that inhibition is probably caused by oxidative stress. Finally, we studied the use of E-Pch in a model of canker disease. E-Pch reduced canker formation on leaves of 'Eureka' and 'Lisbon' lemon cultivars. These results show E-Pch as a promising compound for citrus canker biocontrol.

Keywords: biocontrol, *Xanthomonas*, antagonism, reactive oxygen species, lemon

INTRODUCTION

Citrus canker is a disease caused by *Xanthomonas citri* subsp. *citri*, which is characterized by damaged branches, leaves and fruit that restricts fruit commercialization. This disease was declared endemic in the northeast of Argentina in 1973 and the first cases appeared in the northwest in 2002. Since then, canker disease has greatly affected the Argentinean citrus industry (Canteros, 2001a, b). In canker free citrus areas, canker outbreak management involves eradication campaigns of trees suspected to be infected. However, in endemic areas such an approach is not feasible and the chosen control strategy uses chemicals containing copper (Das, 2003). Nevertheless, *X. citri* subsp. *citri* isolates resistant to copper have been reported, and since the use of copper-containing chemicals represents an environmental hazard, it is of interest to evaluate alternative means for disease control. In that sense, biocontrol represents a promising alternative for canker management. Until now, several bacteria isolated from the citrus phyllosphere (i.e. *Pseudomonas syringae*, *Erwinia herbicola*, *Bacillus subtilis* and *Pseudomonas fluorescens*) showed the ability to inhibit *X. citri* subsp. *citri* in vitro. However, biological control studies are still preliminary probably because the success of a biocontrol depends largely on the ability of the antagonistic bacteria to reside stably on the surface of citrus leaves (Das, 2003). To our knowledge, there is only one report on *X. citri* subsp. *citri*

inhibition in vivo and it involves the use of *Bacillus subtilis* and *Bacillus amyloliquefaciens* on Mexican lime (*Citrus aurantifolia* (Christ.) Swing.) leaves (Huang et al., 2012). Biocontrol is based on the bacterial production of secondary metabolites able to inhibit other microorganisms' growth. Siderophores are among the molecules responsible for the antagonism, since they make iron unavailable by chelation; therefore, affecting the growth of competing microorganisms (Duffy and Défago, 1999). In our laboratory we previously reported the inhibitory activity of the *Pseudomonas aeruginosa* siderophore pyochelin on *X. citri* subsp. *citri* (Adler et al., 2012). Since this siderophore is also produced by other Pseudomonads, we isolated bacteria belonging to the *Pseudomonas* genus from the surface of lemon (*C. limon* (L.) Burn. f.) leaves ('Lisbon'). Next, we studied the isolates ability to inhibit *X. citri* subsp. *citri* in vitro and selected an isolate that was characterized as *P. fluorescens* by 16S rRNA sequence analysis. The active metabolite was purified and identified as enantio-pyochelin. According to the literature, pyochelin from *P. fluorescens* is termed enantio-pyochelin since it is stereochemically different from the siderophore produced by *P.aeruginosa* (Youard and Reimmann, 2010). As with pyochelin from *P. aeruginosa*, enatio-pyochelin exerted an inhibitory mechanism on *X. citri* subsp. *citri* through the generation of reactive oxygen species (ROS). Finally, we evaluated the in vivo effect of enantio-pyochelin and showed that this molecule is capable of preventing canker formation.

MATERIALS AND METHODS

Strains and Culture Media

P. fluorescens was isolated from lemon leaves ('Lisbon') by swabbing surfaces and streaking on King B plates. Fluorescent colonies were selected and *Xanthomonas* antagonism was evaluated. *P. fluorescens* was routinely cultured on M9 medium containing 2% Casamino acids, 0.2% glucose, 1 mM $MgSO_4$ and 1 mg/ml vitamin B1. *X. citri* subsp. *citri* was obtained from the Estación Experimental Obispo Colombres Culture Collection and cultured on Cadmus medium.

Isolate Characterization

To characterize the isolate, DNA coding for the 16S rRNA was amplified by PCR using primers 27F and 1492R according to the conditions described by Lane (1991), and the sequence obtained was analyzed using the BLASTn program in the GenBank nucleotide database.

Enantio-Pyochelin Purification

Enantio-Pyochelin was obtained from *P. fluorescens* cultures grown for 20 h at 30°C in M9 medium supplemented as described above. The cell free supernatant was loaded into a 1 g C18 cartridge (Phenomenex), which had been equilibrated in 20% MeOH in water, and eluted stepwise 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 to 85% acetonitrile (MeCN) in water containing 0.1% trifluoracetic acid at a flow rate of 1 ml/min. Enantio-pyochelin I and II eluted at 53 and 59% MeCN, respectively.

In Vitro Inhibition Assay

Cell suspension aliquots of 5 µl from the selected isolates (OD_{600} = 0.6) or 10 µl of fractions from the enantio-pyochelin purification process were spotted on the surface of supplemented M9 agar plates. The plates were incubated for 24 h at 30°C and cells were killed afterwards by exposure to chloroform vapors for 20 min. A lawn of *X. citri* subsp. *citri* was overlaid on the surface. After 16 h of incubation at 30°C, zones of clearing were examined around the colony or the purification fraction. Minimal inhibitory concentrations (MIC) were determined in M9 medium supplemented with 0.2% Casamino acids, 0.2% glucose, 1 mM $MgSO_4$ and 1 µg/ml vitamin B1. Aliquots of 10 µl

of double dilutions from a 1 mg/ml enantio-pyochelin solution were spotted on M9 agar plates and a lawn of the corresponding strain was overlaid. The maximum dilution that showed a zone of clearing was recorded as the MIC. $FeCl_3$ and ascorbic acid were used at concentrations of 100 μM and 1 mM, respectively.

Measurement of Reactive Oxygen Species

To determine the level of reactive oxygen species (ROS), exponentially growing cells in supplemented M9 minimal medium, were washed and resuspended in 50 mM sodium phosphate buffer, pH 7, to a final OD_{600} =0.5. The cell suspension was divided into three fractions. Two fractions were added to 2 μl of pure E-Pch (final concentration 15 μM) and one of them was supplemented with 1 mM ascorbic acid. The third fraction with no additives served as the control. Fractions were incubated for 2 h at 30°C and then 2,7-dichlorofluorescein diacetate (H2DCFDA, the oxidation-sensitive probe dissolved in dimethyl sulfoxide) 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). The results are expressed as fluorescence relative to that of the control.

In Vivo Inhibition Assays

Lemon plants ('Eureka' and 'Lisbon') were grown as previously reported (Siciliano et al., 2006). Inoculations of the pathogenic bacteria *X. citri* subsp. *citri* on the lamina of young lemon leaves were performed by pressure infiltration according to published methods (Siciliano et al., 2006). All plant 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 bacterial suspension of *X. citri* subsp. *citri* (OD_{600} = 0.0001) meanwhile only one side was infiltrated with the enantio-pyochelin solution in 20% methanol. The other side (with no E-Pch) was infiltrated with 20% methanol as a control. Inoculated plants were maintained for 21 days in a growth cabinet, with temperatures ranging from 25 to 28°C, high humidity, a photoperiod of 16 h of light, and a light intensity of 150 to 200 $\mu E.sm^{-2}$. The canker lesion count was performed in the area of infiltration after three weeks.

RESULTS

Isolation and Characterization of *Pseudomonas* from Citrus Leaves

Bacteria belonging to the *Pseudomonas* genus were isolated from the surface of lemon leaves ('Lisbon') using a differential culture medium (King B). Antibiotic activity in vitro against *X. citri* subsp *citri* was evaluated for all bacteria isolated (Fig. 1). Based on the antibiotic effect and the colony fluorescence under UV light, we selected an isolate that was characterized as *P. fluorescens* by 16S rRNA sequencing.

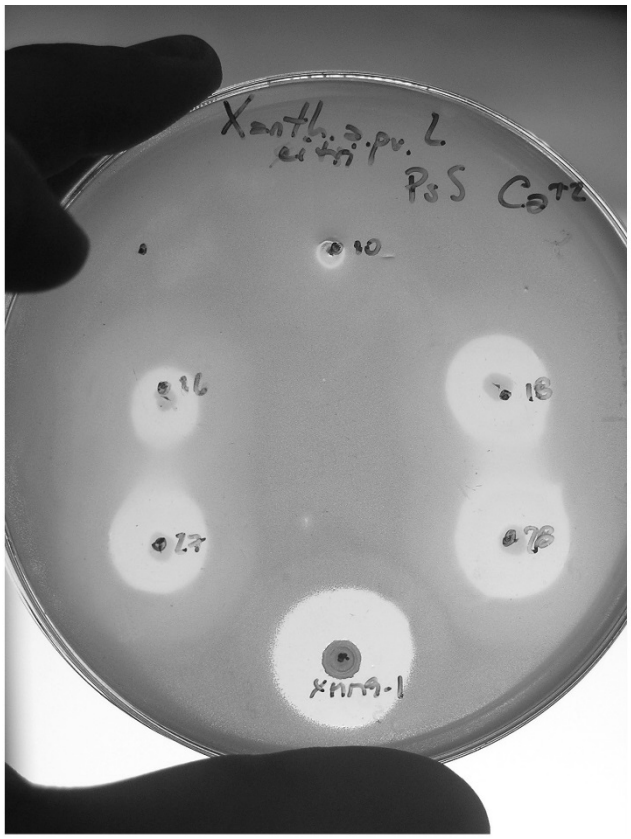

Fig. 1. Bacteria were isolated from lemon leaves using a differential culture medium (King B) and were evaluated for in vitro antibiotic activity against *Xanthomonas citri* subsp. *citri.* Zones of clearing around the bacteria spotted indicate the production of metabolites with antibiotic activity.

Purification and Characterization of Enantio-Pyochelin

Isolation of the metabolite responsible for the anti-*Xanthomonas* activity was performed by culturing *P. fluorescens* in M9 medium and purifying as described in the Material and Methods section. Figure 2A shows the last step of the purification process in which two peaks corresponding to enantio-pyochelin I and II were obtained by HPLC. Of the fractions obtained only these two peaks showed in vitro anti-*Xanthomonas* activity (Fig. 2B). The enantio-pyochelin assignment was based on the equivalent chromatogram (Fig. 2A inset), UV spectrum and inhibition spectrum (data not shown) to pyochelin from *P. aeruginosa* as previously reported (Adler et al., 2012).

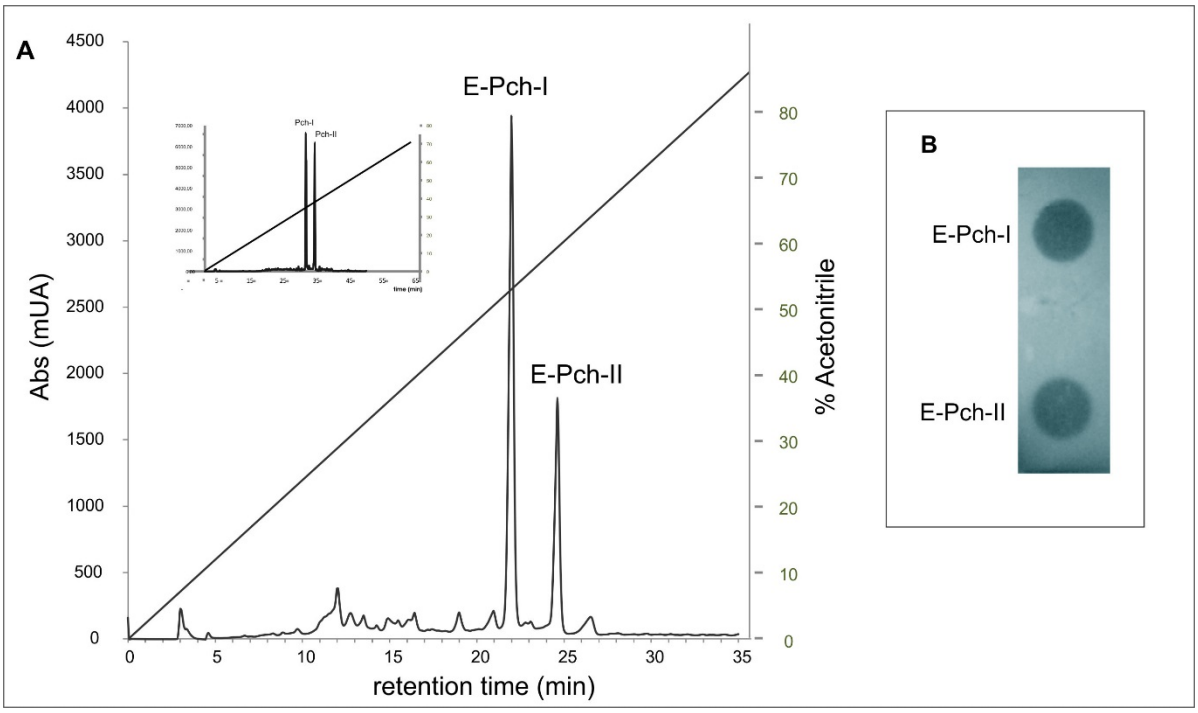

Fig. 2. A: HPLC chromatogram obtained in the last step of the Enantio-pyochelin (E-Pch) purification from *Pseudomonas fluorescens*. The two major peaks correspond to E-Pch I and E-Pch II, which are equivalent to Pch I and II from *P. aeruginosa* (see inset). B: In vitro antibiotic activity against *Xanthomonas citri*. subsp. *citri* of the two major peaks (E-Pch I and II).

Mechanism of Action

As previously determined for pyochelin from *P. aeruginosa* (Adler et al., 2012), we determined the MIC of enantio-pyochelin on *X. citri* subsp. *citri* and evaluated the effect of iron and ascorbic acid addition (Table 1). Interestingly, iron supplementation did not rescue *X. citri* subsp. *citri* from pyochelin toxicity, indicating that inhibition is not caused by iron deprivation. On the contrary, the addition of the reducing agent ascorbic acid lessened *X. citri* subsp. *citri* sensitivity to E-Pch. In concordance, we showed that E-Pch is also able to generate ROS on *X. citri* subsp. *citri* cultures and that ascorbic acid addition prevents ROS generation (Fig. 3).

Table 1. Minimal inhibitory concentrations (MIC) of Enantio-pyochelin against *Xanthomonas citri* subsp. *citri*.

	Conditions of determination
M9 medium[1]	48
M9 medium + $FeCl_3$[2]	96
M9 medium + ASC[3]	768

[1]MIC determination in M9 medium without additives.
[2]MIC determination in M9 medium with the addition of 100 µM iron chloride.
[3]MIC determination in M9 medium with the addition of 1 mM ascorbic acid.

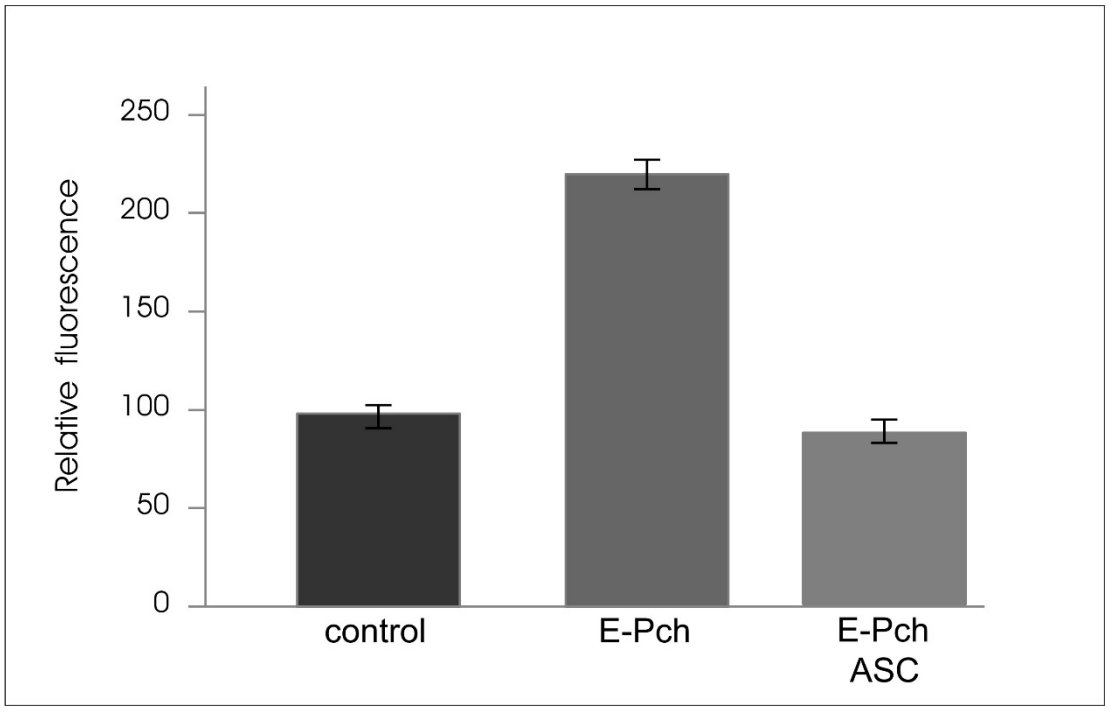

Fig. 3. Reactive Oxygen Species (ROS) determination using the 2,7-dichlorofluorescein diacetate (H2DCFDA) fluorescent probe. Results are expressed as fluorescence relative to that of the control (without additives). Enantio-pyochelin (E-Pch) was added to a final concentration of 15 μM and ascorbic acid (ASC) to a final concentration of 1 mM.

Canker Control by Enantio-Pyochelin

Using two lemon cultivars ('Lisbon' and 'Eureka') we tested E-Pch ability to inhibit *X. citri* subsp. *citri* infection in a model of canker disease. Figure 4A shows the reduction in canker lesion numbers after E-Pch treatment of infected leaves compared with the control (vehicle). Figure 4B shows a photography of a representative assay, where the difference in the number of cankers along both sides of the leaf mid rib (treated with E-Pch and control) is noteworthy.

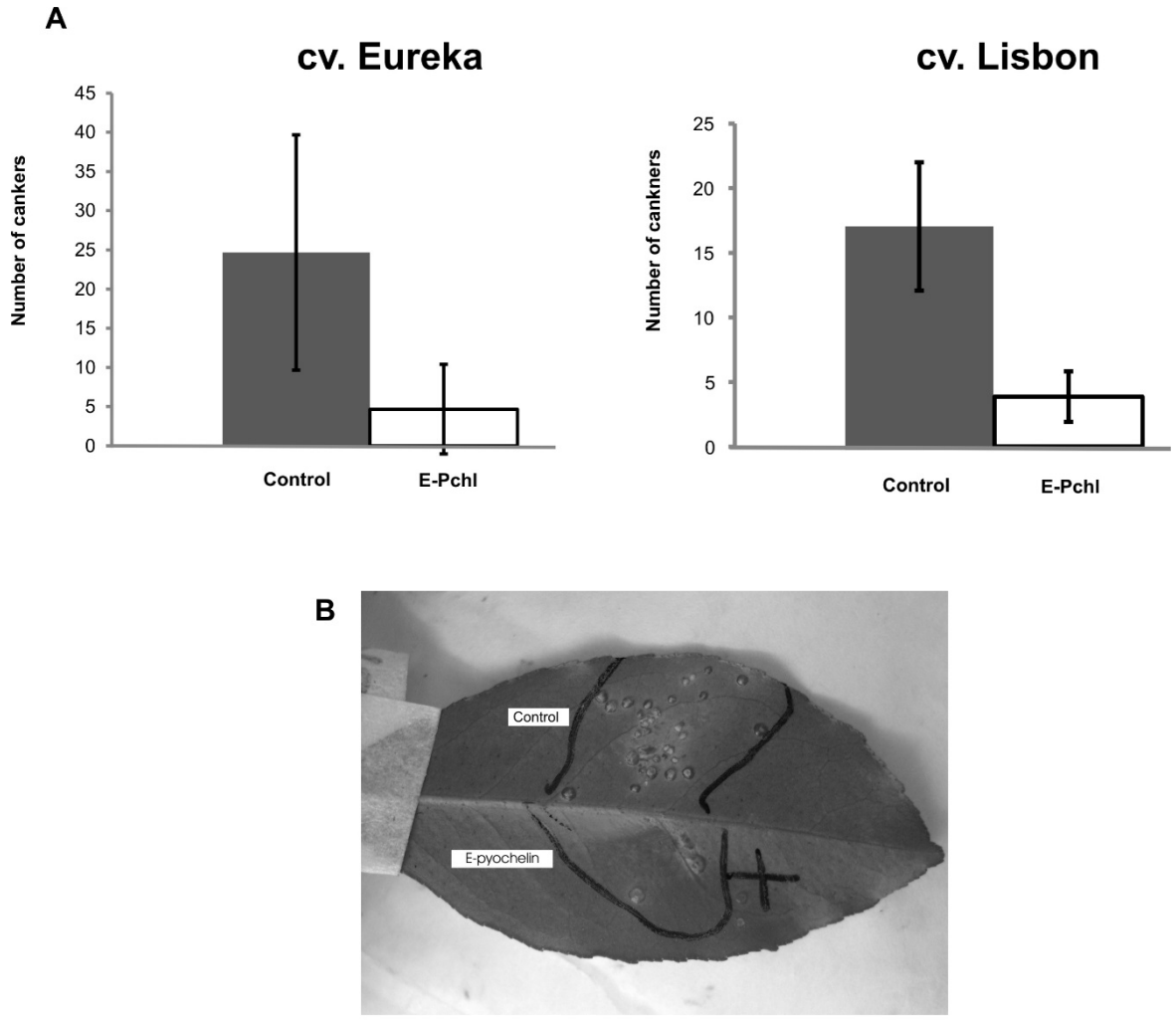

Fig. 4. In vivo inhibition of *Xanthomonas citri* subsp. *citri*. A) Figures show average canker counts for the control and enantio-pyochelin treated leafs of two lemon cultivars. B) Leaf photography of a representative assay. Both sides of the rib were infiltrated with a bacterial suspension of *X. citri* subsp. *citri* meanwhile only one side was infiltrated with the enantio-pyochelin solution.

DISCUSSION

In this work we report the isolation of *P. fluorescens* from the surface of lemon leaves and the purification of enantio-pyochelin produced by this isolate. This siderophore displayed antibiotic activity in vitro and in vivo against the citrus canker agent *X. citri* subsp. *citri.* The phyllosphere represents the aboveground parts of plants inhabited by a complex microbial community. These microorganisms could be involved in fixing atmospheric nitrogen, producing plant-growth regulators, and in controlling plant pathogens either by stimulating plants to synthesize phytoalexins or by producing antimicrobial compounds (Whipps et al., 2007). *P. fluorescens* has been described as a microorganism that colonizes citrus leaf surfaces and is reported to have antagonist properties over several bacterial strains, including the *Xanthomonas* species (Unnamalai and Gnanamanickam, 1984; De la Cruz-Quiroz et al., 2011). However, the action mechanisms exerted by *P. fluorescens* in bacterial antagonism are diverse and still confusing (De la Cruz- Quiroz et al., 2011). In this work we associated the production of the siderophore enantio-pyochelin with the antibiotic effect of a *P. fluorescens* isolate against *X. citri* subsp. *citri*. Moreover, we define a possible action mechanism that is

independent of iron chelation and is related to ROS generation. In addition, we showed that E-Pch is also able to inhibit *X. citri* subsp. *citri* in a citrus canker model suggesting that this metabolite has potential for its use in canker management. It is interesting that canker control based on copper-containing compounds is probably also inhibiting the growth of *X. citri* subsp. *citri* by ROS generation (del Campo et al., 2009), indicating that *X. citri* subsp. *citri* is a bacterium with a low tolerance to oxidative stress.

P. fluorescens produces a pyochelin molecule (pyochelin enantiomer) that is an optical antipode of the pyochelin synthesized by *P. aeruginosa* (Youard and Reimmann, 2010). Since enantio-pyochelin is not recognized by the *P. aeruginosa* pyochelin receptor, uptake of pyochelin is highly selective within *Pseudomonas* species. However, in this work we demonstrate that enantio-pyochelin has the same antagonistic effect as pyochelin on *X. citri* subsp. *citri*. This observation indicates that the pyochelin transport into *X. citri* subsp. *citri* is unspecific.

In conclusion, we report the in vitro and in vivo antimicrobial effects of enantio-pyochelin produced by a bacteria isolated from the phyllosphere of lemon plants. These results highlight the relevance of the microbiome present on plant surfaces and its potential in the control of phytopathogens. Future evaluation of the direct control of *X. citri* subsp. *citri* by *P. fluorescens* may allow the design of canker control strategies based on bioproducts.

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