

Detection of siderophores in endophytic bacteria *Methylobacterium* spp. associated with *Xylella fastidiosa* subsp. *pauca*

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Abstract – The objective of this work was to study the production of siderophores by endophytic bacteria *Methylobacterium* spp., which occupy the same ecological niche as *Xylella fastidiosa* subsp. *pauca* (*Xfp*) in citrus plants. The siderophore production of *Methylobacterium* strains was tested according to chromeazuroil agar assay test (CAS), Csáky test (hydroxamate-type) and Arnow test (catechol-type). In addition, the ability of *Xfp* to use siderophores, in vitro, produced by endophytic bacteria as source of iron, was evaluated. All 37 strains of *Methylobacterium* spp. tested were CAS-positive for siderophore production. *Methylobacterium* spp. produced hydroxamate-type, but not catechol-type siderophores. In vitro growth of *Xfp* was stimulated by the presence of supernatant siderophores of endophytic *Methylobacterium mesophilicum*.

Index terms: citrus variegated chlorosis, endophytes, heterologous siderophores, iron, symbionts.

Detecção de sideróforos nas bactérias endofíticas *Methylobacterium* spp. associadas com *Xylella fastidiosa* subsp. *pauca*

Resumo – O objetivo deste trabalho foi estudar a produção de sideróforos pelas bactérias endofíticas *Methylobacterium* spp., que ocupam o mesmo nicho ecológico que *Xylella fastidiosa* subsp. *pauca* (*Xfp*), em plantas cítricas. A produção de sideróforos, pelas linhagens de *Methylobacterium*, foi testada por meio do ensaio de cromoazuroil-ágar (chromeazuroil agar assay-CAS), teste de Csáky (tipo hidroxamato) e do teste de Arnow (tipo catecol). Além disso, a habilidade de *Xfp* em utilizar sideróforos produzidos por bactérias endofíticas, como fonte de ferro, in vitro, foi avaliada. Todas as 37 linhagens de *Methylobacterium* spp. testadas foram positivas para a produção de sideróforos, pelo teste CAS-ágar. *Methylobacterium* spp. foram capazes de produzir sideróforos do tipo hidroxamato, mas não do tipo catecol. O crescimento in vitro de *Xfp* foi estimulado pela presença de sideróforos no sobrenadante de *Methylobacterium mesophilicum* endofítica.

Termos para indexação: clorose variegada dos citros, endófitos, sideróforos heterólogos, ferro, simbiose.

Introduction

Endophytes are microorganisms which do not visibly harm the host plant, but can be isolated from the internal tissues of surface-disinfected plants (Azevedo et al., 2000; Araújo et al., 2002). Furthermore, as they colonize an ecological niche similar to certain plant pathogens, they are likely candidates for biocontrol agents (Lacava et al., 2007).

Bacterial siderophores are low-molecular-weight compounds with high Fe³⁺ chelating affinity (Sharma &

Johri, 2003) responsible for the solubilization and transport of this element into bacterial cells. Some bacteria produce hydroxamate-type siderophores, while others produce catecholate-types (Neilands & Nakamura, 1991). In a state of iron limitation, the siderophore-producing microorganisms are also able to bind and transport the iron-siderophore complex by the expression of specific proteins (Nachin et al., 2001; Nudel et al., 2001). The production of siderophores by microorganisms is beneficial to plants, because it can inhibit the growth of plant pathogens (Sharma & Johri, 2003). In addition, iron is a

vital element, and its sequestration by specific bacterial siderophores may induce the development of plant disease (Masclaux & Expert, 1995; Nachin et al., 2001; Etchegaray et al., 2004).

Xylella fastidiosa subsp. *pauca* is the causal agent of citrus variegated chlorosis (CVC), which is an important disease of citrus species (Schaad et al. 2004). In Brazil, over 70 million sweet-orange trees (38%) are affected, and CVC is responsible for losses of US\$ 100 million per year by Brazilian citrus industries, besides affecting all commercial sweet-orange varieties (Souza et al., 2005). *X. fastidiosa* subsp. *pauca* (*Xfp*) was the first plant pathogen to have its genome completely sequenced, and putative genes for membrane receptors, including siderophores, were found (Simpson et al., 2000).

The genus *Methylobacterium*, which occupies the same ecological niche as *Xfp*, was the most frequently isolated endophytic bacterium from CVC-symptomatic citrus plants (*Citrus sinensis*) (Araújo et al., 2002; Lacava et al., 2004, 2006a, 2006b). Recently, an interaction between *Methylobacterium* species and *Xfp* was strongly indicated (Araújo et al., 2002; Lacava et al., 2004). Lacava et al. (2004) suggested that CVC symptoms in citrus plants could be a result of the population balance between endophytic bacteria *Methylobacterium* spp., *Curtobacterium flaccumfaciens* and *Xfp*.

Acquisition of iron from siderophores produced by other microbial species has already been described for bacteria such as *Escherichia coli*, *Salmonella typhimurium* (Martinez et al., 1990), *Actinobacillus pleuropneumoniae* (Diarra et al., 1996), *Streptomyces* sp. (Imbert et al., 1995), *Arthrobacter flavescens* (Winkelmann, 1991). Certain strains of *Pseudomonas* spp. can also utilize ferric complexes of pyoverdines-siderophores produced by other strains of *Pseudomonas* spp., due to the presence of multiple outer membrane receptors that recognize heterologous pyoverdines (Koster et al., 1995). Furthermore, *Pseudomonas* spp. can utilize iron complexes of different siderophores produced by fungi and bacteria (Meyer, 1992). These include the catechol, enterobactin (Poole et al., 1990) and the hydroxamate, aerobactin, which are produced by members of the Enterobacteriaceae family. In addition, Loper & Henkels (1999) described the ability of *Pseudomonas putida* to use heterologous siderophores produced by rhizosphere microorganisms in its natural habitat.

Methylobacterium is the most frequently found genus associated to *Xfp*, and there is a positive association with the occurrence and intensity of symptoms of CVC (Araújo et al., 2002; Lacava et al., 2004). This interaction may occur by *Methylobacterium* spp. synthesis of pathological factors, such as siderophores, which may be used by *Xfp* (Simionato et al., 2006).

The aim of this study was to evaluate the production of siderophores by *Methylobacterium* spp., isolated as citrus endophytic bacteria (Araújo et al., 2002), and to investigate the *Xfp* capacity of using siderophores produced by *M. mesophilicum* for growth and development.

Materials and Methods

Methylobacterium strains used in this study are part of the collection of the Department of Genetics from Escola Superior de Agricultura Luiz de Queiroz – Universidade de São Paulo, Brazil. The 37 *Methylobacterium* spp. strains tested are listed in Table 1, and *Xfp* strains used in this study were 9a5c (Simpson et al., 2000) and 6570 (Marucci et al., 2003).

The chromeazuroil (CAS) agar assay was described by Schwyn & Neilands (1987), and was modified by Silva-Stenico et al. (2005). Briefly, 60.5 mg of CAS was dissolved in 50 mL of deionised water, and mixed with 10 mL of a Fe³⁺ solution (1 mmol L⁻¹ FeCl₃.6H₂O, 10 mmol L⁻¹ HCl). While stirring, this solution was slowly mixed with 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA) previously dissolved in 40 mL water. The resulting dark-blue solution was autoclaved, cooled to 50/60°C and mixed with 900 mL sterile MM9 (Silva-Stenico et al., 2005) containing 15 g L⁻¹ agar (also kept at 50/60°C). This medium was allowed to gel on Petri dishes, was subsequently inoculated with bacterial strains and incubated in the dark (28°C for 5 days). Positive results were indicated by the formation of a clear halo around the colonies, showing a visual change in color from dark-blue to yellow. Each assay was performed in triplicate.

Catechol-type siderophores were measured in culture supernatants through Arnou assay (Arnou, 1937), while hydroxamate siderophores were measured according to Csáky (1948). In the analyses, 2,3-dihydroxybenzoic acid and hydroxylamine hydrochloride, respectively, were used as standards. Each assay was performed in triplicate.

The ability of *X. fastidiosa* to use siderophores produced by endophytic bacteria as source of iron, in vitro, was evaluated. Two strains (AR5.1/5 and AR5.1/6)

of the endophytic bacterium *M. mesophilicum* (Table 1) were individually grown in Fe-free MM9 broth (Silva-Stenico et al., 2005) to the mid-log phase, to stimulate the production of siderophores, before being pelleted by centrifugation at 3,000 *g* for 5 min. Then the supernatant was collected and filtered through a 0.22 μm membrane filter. The supernatant was then added to individual preparations of PW broth medium without a source of iron (hemin chloride). The supernatant was added to a final concentration of 0.2, 2, 20, 100% (v/v) to produce supplemented PW broth medium (Davis et al., 1981) containing *M. mesophilicum* supernatant-siderophore (PWSMm).

Supplemented, unsupplemented (without a source of iron/negative control) and PW broth (positive control) were inoculated with *Xfp* by placing 9 mL of PWSMm in a 30 mL tube, and adding 1 mL of PW broth containing 10^4 viable *Xfp* cells (previously grown at 28°C for 24 hours with agitation) (Lacava et al., 2004). After inoculation, the tubes were incubated at 28°C for 20 days, and the growth of *Xfp* was evaluated at $\lambda = 600$ nm using an Ultrospec 3000 spectrophotometer. Each assay was performed in triplicate.

Analysis of the data was carried out using SAS software package, with a completely randomized analysis of the variances ($p < 0.05$). Tukey test was used for comparison of the means.

Results and Discussion

All strains of *Methylobacterium* spp. were CAS-positive for siderophores production (Table 2), and the CAS-agar assay revealed that 66% CVC-symptomatic, 55% uninfected, 20% asymptomatic and 10% tangerine strains of *Methylobacterium* spp. showed very high siderophore production.

A positive siderophore reaction by the CAS method shows a yellow halo surrounding the bacterial colonies grown under iron-limiting conditions (Schwyn & Neilands, 1987). This is the most universal assay developed so far for siderophores; it only depends on the ability of the compound to bind iron with relatively high affinity, as can see in Figure 1 for AR5.1/5, AR5.1/6, and AR1.6/2 strains of *Methylobacterium*.

All strains of *Methylobacterium* spp. were negative in the Arnou assay (Arnou, 1937), which means that

Table 1. Endophytic *Methylobacterium* strains collected in citrus variegated chlorosis (CVC) symptomatic, asymptomatic and uninfected plants.

Plant host category	<i>Methylobacterium</i> species	Strains	Origin ⁽¹⁾
		CVC-symptomatic	
<i>C. sinensis</i>	<i>M. mesophilicum</i>	AR1.6/1, AR1.6/6	Novais
	<i>M. mesophilicum</i>	AR3/20	Catanduva
	<i>M. mesophilicum</i>	AR4/19	Frutal
	<i>M. mesophilicum</i>	AR5/1, AR5.1/4, AR5.1/5, AR5.1/6	Colina
	<i>M. extorquens</i>	AR1.6/2, AR1.6/3, AR1.6/8, AR1.6/11	Novais
	<i>Methylobacterium</i> sp.	AR1.6/4	Novais
		CVC-asymptomatic	
<i>C. sinensis</i>	<i>M. mesophilicum</i>	ER1/21, ER1.6/1, ER1.6/4, ER1.6/5	Novais
	<i>M. mesophilicum</i>	ER5/2	Colina
		Uninfected	
<i>C. sinensis</i>	<i>M. mesophilicum</i>	SR1.6/6, SR1.6/13	Novais
	<i>M. mesophilicum</i>	SR3/27	Catanduva
	<i>M. extorquens</i>	SR1.6/1, SR1.6/15	Novais
	<i>M. extorquens</i>	SR5/4	Colina
	<i>M. radiotolerans</i>	SR1.4/10, SR1.6/4	Novais
	<i>M. zatmanii</i>	SR1.6/2	Novais
<i>C. reticulata</i>	<i>M. mesophilicum</i>	PR1/3	Novais
	<i>M. mesophilicum</i>	PR1.4/10	Novais
	<i>M. mesophilicum</i>	PR2/2	Elisiário
	<i>M. mesophilicum</i>	PR3/5, PR3/11, PR3/15	Catanduva
	<i>M. fujisawaense</i>	PR5/4, PR5.1/1	Colina
	<i>M. zatmanii</i>	PR3/8, PR3/17	Catanduva

⁽¹⁾All the sites are in São Paulo State, except for Frutal, which is in Minas Gerais State, Brazil.

Table 2. Siderophore production by endophytic *Methylobacterium* strains⁽¹⁾.

Strain	CAS-Agar universal test	Csáky test (hydroxamate-type)	Arnow test (catechol-type)
AR1.6/1	+	+	-
AR1.6/6	+++	++	-
AR3/20	++	+	-
AR4/19	+	+	-
AR5/1	++	+	-
AR5.1/4	+++	+	-
AR5.1/5	+++	+++	-
AR5.1/6	+++	++	-
AR1.6/2	+	+	-
AR1.6/3	+++	+	-
AR1.6/8	+++	+	-
AR1.6/11	++	+	-
AR1.6/4	+	+	-
ER1/21	+	+	-
ER1.6/1	+	+	-
ER1.6/4	++	++	-
ER1.6/5	+++	+	-
ER5/2	++	+	-
SR1.6/6	+++	+	-
SR1.6/13	++	++	-
SR3/27	++	++	-
SR1.6/1	+++	+	-
SR1.6/15	++	+	-
SR5/4	++	++	-
SR1.4/10	+++	++	-
SR1.6/4	+++	+	-
SR1.6/2	+++	+	-
PR1/3	+	+	-
PR1.4/10	+++	+	-
PR2/2	+	+	-
PR3/5	+	+	-
PR3/11	++	++	-
PR3/15	+	+	-
PR5/4	+	+	-
PR5.1/1	++	+	-
PR3/8	++	++	-
PR3/17	++	+	-

⁽¹⁾The symbols represent the relationship between the halo average diameter and the average diameter of the colony growth (+: small; ++: medium; +++: big) for CAS-Agar universal test, and the intensity of production of siderophores (-: none; +: low; ++: high; +++: very high) for Csáky and Arnow tests.

these strains are negative for catechol-type siderophores (Table 2). However, all strains of *Methylobacterium* spp. were able to produce hydroxamate-type to various degrees, as shown by the Csáky assay (Csáky, 1948) (Table 2).

Growth of *Xfp* strains 9a5c and 6570, in PW broth medium, was stimulated by the presence of *M. mesophilicum* supernatants that contained siderophores (Figure 2); and inhibition of this same strain was observed in the negative control (PW broth medium without a source of iron) (Figure 2).

The competition for iron and the high-affinity iron uptake involved in pathogenesis have been well documented in relation to animal-bacterial pathogen systems; but their role in plant pathogens has received limited attention (Banuett, 1995).

Members of genus *Methylobacterium*, which are frequently isolated as endophytes from citrus plants with CVC symptoms (Araújo et al., 2002; Lacava et al., 2004), are able to produce siderophores. Silva-Stenico et al. (2005) reported that one strain of *M. extorquens*, isolated from *C. sinensis* (Araújo et al., 2002), was able to produce siderophores. Also, the same authors reported that this *M. extorquens* strain was positive for hydroxamate and negative for catechol-type siderophores.

It was detected a high production of siderophores by *M. mesophilicum* and *M. extorquens*. Furthermore, the high levels of siderophore production were grouped in *Methylobacterium* species isolated from both CVC-symptomatic and healthy citrus plants. Simionato et al. (2006) characterized siderophores, produced by *M. mesophilicum* (AR5.1/5 and AR5.1/6 strains) and *M. extorquens* (AR1.6/2 strain), by capillary electrophoresis with mass spectrometry detection (CE-ESI-MS). These authors revealed siderophores with m/z (mass-to-charge ratio)

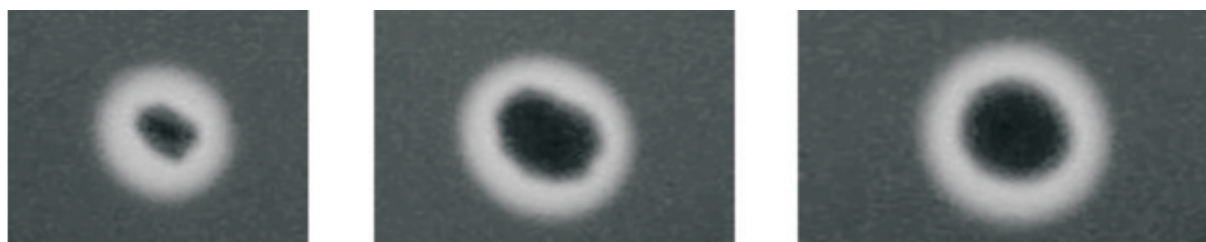


Figure 1. Chromeazuroil S agar test, for siderophore detection in a plate culture of *Methylobacterium* spp. The culture medium with CAS blue dye contains halos indicating the presence of siderophores.

of 1,004.3, for *M. mesophilicum*, and m/z of 798.3 for *M. extorquens*. Mass-to-charge ratio (m/z) is used to denote the dimensionless quantity formed by dividing the mass number of an ion by its charge number.

The presence of putative open-reading frames (ORFs), encoding iron uptake in the genome of *Xfp* (Simpson et al., 2000), suggests a potential role for chelating agents in the development of the disease

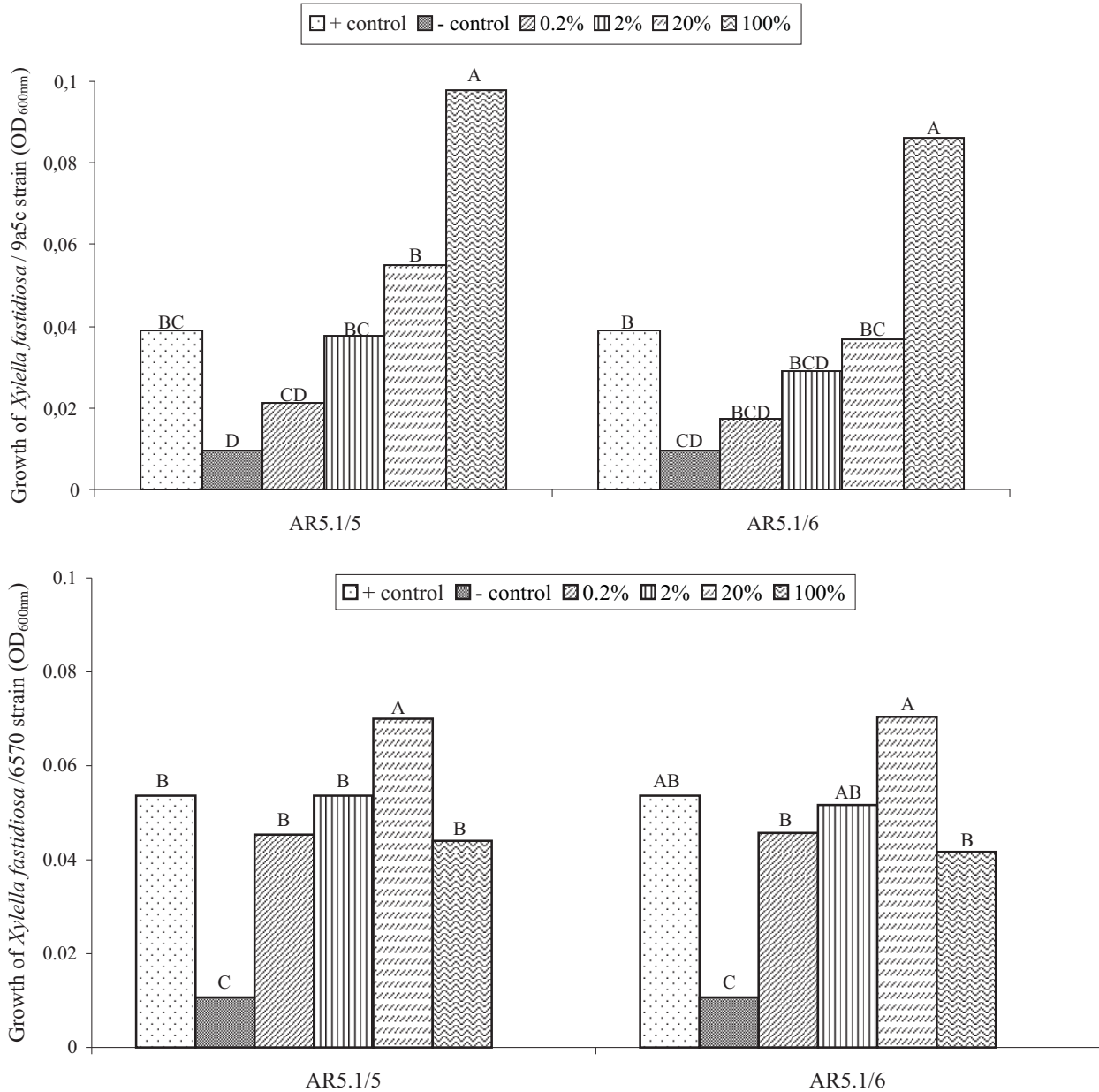


Figure 2. Effect of cell-free supernatants of the endophytic bacteria *Methylobacterium mesophilicum* (AR5.1/5 and AR5.1/6 strains) with siderophore production on the growth of *Xylella fastidiosa* subsp. *pauca* (9a5c and 6570 strains), in PW broth medium. The supernatants were individually tested by adding them to PW broth at a final concentration of 0.2, 2, 20, 100% (v/v), inoculating the supplement PM broth with *X. fastidiosa* subsp. *pauca*, and evaluating growth spectrophotometrically at $\lambda = 600$ nm, after 20 days. Negative control is represented by PW broth medium without a source of iron, and positive control by standard PW broth medium. Different letters on bars for the same treatment means statistic difference by Tukey's test, at 5% probability.

symptoms. In this case, if *Xfp* is able to use siderophores produced by some strains of *Methylobacterium* in environmental conditions, there might be a strain-specific relationship between *Xfp* and some *Methylobacterium* strains isolated from citrus plants with CVC symptoms.

It was observed that *Methylobacterium* strains with high siderophore production were isolated from citrus plants in the same areas of São Paulo State (Table 1). This suggests that there is a correlation between where these strains were isolated and their production of siderophores.

The present data corroborates the hypotheses that there is a relationship between *X. fastidiosa*, the causal agent of CVC, and the endophytic bacteria *Methylobacterium* (Araújo et al., 2002; Lacava et al., 2004). In addition, our results indicated that *Xfp* was able to use *Methylobacterium* siderophores in vitro, as source of iron (Figure 2), and suggested that in some instances *Methylobacterium* could help the growth of *Xfp*, particularly under environmental conditions, where iron sources are limited. Siderophore complexes are taken up by specific transport systems, but some microorganisms have also developed transport systems for heterologous siderophores produced by other species (Raaijmakers et al., 1995).

Many of the important processes of *X. fastidiosa*, related to the diseases that it causes, probably rely on proteins such as hemolysins, adhesins, xanthan gum-producing enzymes, virulence factors, and detoxification enzymes involved in one way or another with transport. This calls attention to transport proteins (Meidanis et al., 2002). Membrane receptors were reported from the *Xfp* genome sequence, including siderophores, ferrichrome-iron and hemin receptors that are all associated with iron transport. The energizing complexes, TonB-ExbB-ExbD and the paralogous TolA-TolR-TolQ, essential for the functioning of the outer membrane receptors, were also present in *Xfp* genome. In all, 67 genes encoded proteins involved in the iron metabolism of *Xfp* (Simpson et al., 2000) and in iron sequestration might play an important role in leaf chlorosis, since this is the initial symptom of the disease (Silva-Stenico et al., 2005) caused by *Xfp*.

Endophytes must compete with plant cells for iron supply, therefore, siderophore production may be highly important for endophytic growth (Idris et al., 2004). Additionally, the production of siderophores has been reported to be one of the mechanisms to outcompete

pathogens (O'Sullivan & O'Gara, 1992), and may have the same function in endophytes. The present study suggested, as preliminary results, that *Xfp* can use molecules produced by endophytic bacteria as siderophores. In this context, as a factor influencing the symptom of CVC (Silva-Stenico et al., 2005; Pacheco et al., 2006), the genus *Methylobacterium* could help *Xfp* to survive inside the xylem vessels. However, additional studies are needed to provide a better understanding on the effect of iron availability in the production of siderophores by *Methylobacterium* spp., which could be involved in the iron-related virulence of the phytopathogen *Xfp*.

Conclusions

1. *Methylobacterium* spp. have no ability of producing catechol-type siderophores, but are capable to produce hydroxamate-type siderophores.
2. In vitro growth of *Xylella fastidiosa* subsp. *pauca* is stimulated by the presence of a supernatant-siderophore of endophytic *Methylobacterium mesophilicum*.

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