# Interactions of *Xylella fastidiosa* and Endophytic Bacteria in Citrus: A Review

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## ABSTRACT

Citrus variegated chlorosis (CVC) is a disease of sweet orange [*Citrus sinensis* (L.)] caused by *Xylella fastidiosa* subsp. *pauca*, a phytopathogenic bacterium that has been shown to infect all sweet orange cultivars. *Xylella fastidiosa* is a fastidious Gram-negative, xylem-limited bacterium which was rapidly disseminated by infected nursery trees and by several xylem-feeding sharpshooter insect vectors. In Brazil, CVC is the most economically important of several plant diseases caused by *X. fastidiosa*. One factor that may confer apparent resistance to CVC is the endophytic microbial community colonizing individual *C. sinensis* plants. Endophytes are microorganisms that do not visibly harm the host plant, but can be isolated from the internal tissues of surface-disinfected plants. Furthermore, as they colonize an ecological niche similar to that of certain plant pathogens, they are likely candidates for biocontrol agents. There is evidence that *X. fastidiosa* interacts with endophytic bacteria present in the xylem of sweet orange, and that these interactions, particularly with *Methylobacterium mesophilicum* and *Curtobacterium flaccumfaciens*, may affect disease progress. Studies of endophytic bacterial populations in sweet orange suggest that symptoms of CVC in sweet orange could be influenced by the relative populations of *Methylobacterium* spp., *C. flaccumfaciens* and *X. fastidiosa* subsp. *pauca*. Symbiotic control is a new strategy that uses symbiotic endophytes as biological control agents to antagonize or displace pathogens. Candidate endophytes for use in symbiotic control of CVC must occupy the xylem of host plants and attach to the precibarium of sharpshooter insects in order to have access to the pathogen. In the present review, we focus on interactions between endophytic bacteria from sweet orange plants and *X. fastidiosa* subsp. *pauca*, especially those that could result in some strategy for symbiotic control of CVC.

## CONTENTS

## THE PATHOGEN *XYLELLA FASTIDIOSA*

The first report of symptoms caused by what we now call *Xylella fastidiosa* was in 1884 in the grape-growing region of southern California (US). A disease syndrome, known today as Pierce's disease (PD), was later described in detail (Pierce 1892). Subsequently, similar diseases were reported on many fruit tree and ornamental species, especially in North and South America (Hopkins 1989). *Xylella fastidiosa* is a fastidious Gram-negative xylem-limited bacterium, rod-shaped with distinctive rippled cell walls. It is non-flagellate, does not form spores and measures 0.1-0.5 × 1-5 μm (Nyland *et al.* 1973; Bradbury 1991). This Gram-negative bacterium was formally named only in 1987 (Wells *et al.* 1987), and is characterized by being extremely slow-growing in culture. These traits have made the pathogen difficult to study, and have contributed to its previous obscurity. The taxonomic position of *X. fastidiosa* (Wells *et al.* 1987) is: Bacteria, Gracilicutes, aerobic rods, Category I, Group 4, Subgroup 4 A (Holt 1994). Natural transmission occurs via insects feeding suctorially on xylem sap. Transmission efficiency varies widely among vector species. The bacterium overwinters in the xylem of the host plant as well as in weeds.

**Invited Review**

The bacterium *X. fastidiosa* (Wells *et al.* 1987), as mentioned above, resides in the xylem vessels of a broad range of perennial plants in the New World, and has been shown to cause important diseases in a variety of fruit trees and vines. These include PD in grapevines (Davis *et al.* 1981; Hopkins and Purcell 2002), leaf scorches of pecan (Sanderlin and Heyderich-Alger 2000; Sanderlin and Melanson 2006), pear (Leu and Su 1993), plum (Raju *et al.* 1983), almond (Mircetich *et al.* 1976), mulberry (Kostka *et al.* 1986), elm, sycamore, oak (Hearon *et al.* 1980), maple (Sherald *et al.* 1987), and coffee (de Lima *et al.* 1998), as well as alfalfa dwarf (Goheen *et al.* 1979), phony peach disease (Wells *et al.* 1981), periwinkle wilt (McCoy *et al.* 1978), and citrus variegated chlorosis (Chang *et al.* 1993; Hartung *et al.* 1994). Strains of *X. fastidiosa* have a wide host range in the native flora, where they exist without inducing symptoms of disease, and they are transmitted by common sharpshooter insects (Freitag 1951; Freitag and Frazier 1954). These attributes contribute to the current lack of adequate disease control measures.

## CITRUS VARIEGATED CHLOROSIS (CVC)

Citrus variegated chlorosis (CVC) is a disease of the sweet orange [*Citrus sinensis* (L.)], which is caused by *Xylella fastidiosa* subsp. *pauca* (Chang *et al.* 1993; Hartung *et al.* 1994; Schaad *et al.* 2004) a phytopathogenic bacterium that has been shown to infect all sweet orange cultivars (Li *et al.* 1997a). CVC was first reported in Brazil in 1987 and has rapidly become one of the most economically important diseases affecting sweet orange production in Brazil (Rossetti *et al.* 1990; Lee *et al.* 1991). CVC rapidly became widespread in most major citrus growing areas through unregulated movement of infected nursery stock due to a previous lack of certification programs and high CVC infection rates in Brazil. Currently, CVC is found widespread in citrus orchards in the states of São Paulo, Paraná (Leite and Jacomino 1993), Minas Gerais (Mizubuti *et al.* 1994), Goiás (Tubelis *et al.* 1993), Rio de Janeiro (Lee *et al.* 1991; Rossetti *et al.* 1990), Sergipe, Santa Catarina, Distrito Federal and Rio Grande do Sul (Tubelis *et al.* 1993). Brazil is the largest producer of citrus fruit in the world, supplying most of the international market with concentrated orange juice. More than 80% of the production comes from the state of São Paulo. CVC can be found in at least 90% of the orchards in Brazil (Lambais *et al.* 2000). In Brazil, CVC is responsible for losses of US $100 million per year to the citrus industry (Della-Coletta *et al.* 2001).

CVC affects mostly oranges (*C. sinensis*); it has been observed especially on cultivars 'Pera', 'Hamlin', 'Natal' and 'Valencia'. It occurs on trees propagated on all commonly used rootstocks in Brazil: *C. limonia*, *C. reshni* and *C. volkameriana* (Li *et al.* 1997c). The disease has not been observed on limes (*C. latifolia)* or mandarins (*C. reticulata*), even when the trees were planted in severely affected orange groves (Li *et al.* 1997b). Some weed species are also hosts and act as reservoirs of infection (Smith *et al.* 1997). This disease continues to show an increase in severity, with 35% of the sweet orange trees in São Paulo, Brazil, currently showing yield losses (www.fundecitrus.com.br).

Citrus plants with symptoms of CVC show a brilliant leaf chlorosis, similar to zinc deficiency, as the initial symptom (Laranjeira *et al.* 1998; Anonymous 2000). Later symptoms include wilting, canopy dieback, necrotic leaf lesions, and undersized, hard fruit (Derrick and Timmer 2000; Hopkins and Purcell 2002). The causal agent of CVC has been found to be transmitted in Brazil by sharpshooter leafhoppers (*Cicadellidae*) (Lopes 1996; Almeida and Purcell 2003). CVC has been experimentally transmitted by 11 different sharpshooter species tested in Brazil (Fundecitrus 2005). Also, the pathogen can be transmitted through seeds (Li *et al.* 2003).

Although *X. fastidiosa* subsp. *pauca* was the first plant pathogen to have its genome sequenced (Simpson *et al.* 2000), there is still no effective control for CVC. The pathogen is known to have an extraordinary host range among higher plants in New World ecosystems (Freitag 1951). Interestingly, within the majority of native host plants, *X. fastidiosa* does not damage the host plant and behaves as an endophyte (Purcell and Saunders 1999). In contrast, the horticultural crops that suffer from diseases caused by *X. fastidiosa* are those that have been introduced into New World ecosystems (Chen *et al.* 2002). The observation that a few asymptomatic trees persist in some infected orchards may lead to new approaches to the investigation of the control of CVC. These asymptomatic plants have the same genotype as diseased plants and are located in the same grove under similar climatic and edaphic conditions, suggesting that some other factor is responsible for resistance to CVC. One factor that may influence the resistance to CVC is the nature of the endophytic microbial community colonizing individual *C. sinensis* plants (Araújo *et al.* 2002).

## ENDOPHYTIC MICROORGANISMS AND BIOLOGICAL CONTROL

Endophytes can be isolated from surface-disinfected plant tissue or the inner parts of plants and are defined as bacteria that live within a plant for at least a part of its life cycle, without causing apparent harm to the host (Petrini *et al.* 1989; Hallmann *et al.* 1997; Azevedo *et al.* 2000). The most comprehensive definition was proposed by Azevedo and Araújo (2007), who described endophytes as all microorganisms that may or may not be successfully cultured, that either internally colonize the host plant and do not cause apparent damage and/or visible external structures. Endophytes were reported to contribute to host plant protection and ultimately survival (Sturz and Matheson 1996; Hallmann *et al.* 1998; Azevedo *et al.* 2000; Newman and Reynolds 2005). Since endophytes colonize an ecological niche similar to that of phytopathogens, they are possible biocontrol agents (Hallmann *et al* 1997). The potential for practical applications of endophytes has led to studies addressing the bacterium's ability to control both disease and insect infestations, as well as promoting plant growth (Azevedo *et al.* 2000; Kozdrój *et al.* 2004; Kavino *et al.* 2007).

Indeed, previous work suggested that endophytic microorganisms have the potential to control pathogens (Sturz and Matheson 1996; Duijff *et al.* 1997; Sharma and Nowak 1998; Sturz *et al.* 1998; Lacava *et al.* 2004, 2007a), insects (Petrini *et al.* 1989; Azevedo *et al.* 2000), and nematodes (Hallmann *et al.* 1997). In some cases, endophytes can also accelerate seed emergence, help plant establishment under adverse conditions (Chanway 1997), and increase plant growth and development (Bent and Chanway 1998; Lazarovits and Nowak 1997; Pillay and Nowak 1997; Nassar *et al.* 2005).

Endophytic bacteria may play a significant role in protection against plant pathogens and in the overall productivity of an agricultural ecosystem (Hallman *et al.* 1997; Sturz *et al.* 2000). The mode of action of the endophytic bacterial community may be through induction of disease resistance mediated by the synthesis of structural compounds, such as siderophores and extracellular enzymes (Benhamou and Nicole 1999), and the induction and expression of general molecular-based plant immunity (Benhamou and Nicole 1999). Alternatively, they may act by niche competition, preventing pathogens from becoming established in a host (Sturz *et al.* 2000).

In mature citrus trees, the endophytic environment becomes more stable and uniform over time. This may result from selection of particular genotypes within each local microbial population (Araújo *et al.* 2002). Consequently, bacteria living in an endophytic environment may show a tendency to adapt themselves to this more stable environment, resulting in intense interactions among them (Lacava *et al.* 2004).

Recent results highlighted the relationships among bacterial populations and suggest that CVC symptoms in citrus plants could be influenced by the population balance among

*Methylobacterium* spp., *Curtobacterium flaccumfaciens* and *X. fastidiosa* subsp. *pauca* (Lacava *et al.* 2004, 2006, 2007a). Understanding the relationship among endophytic bacteria within sweet orange trees and *X. fastidiosa* subsp. *pauca* may lead to strategies to control CVC using endophytic bacteria.

## ENDOPHYTIC BACTERIA FROM CITRUS PLANTS AND INTERACTIONS WITH *XYLELLA FASTIDIOSA*

Araújo *et al.* (2001) isolated several endophytic bacteria from citrus trees. The genus *Methylobacterium* was the most frequently isolated endophytic bacterium from CVC-symptomatic citrus plants (*C. sinensis*) (Araújo *et al.* 2002; Lacava *et al.* 2004, 2006a, 2006b). Also, Araújo *et al.* (2002) and Lacava *et al.* (2004) provided data to suggest an interaction between *Methylobacterium* species and *X. fastidiosa* subsp. *pauca* because the genus *Methylobacterium* is frequently isolated from the citrus plants with symptoms of CVC and *M. mesophilicum* could reduce the growth of *X. fastidiosa*, while *M. extorquens* could stimulate the growth of *X. fastidiosa in vitro*. Lacava *et al.* (2004) suggested that CVC symptoms in citrus plants could be influenced by the population balance among the endophytic bacteria *Methylobacterium* spp., *C. flaccumfaciens* and *X. fastidiosa* subsp. *pauca*.

We have focused on the interaction between members of endophytic bacterial community, such as *Methylobacterium* spp. and *C. flaccumfaciens*, which occupy the same ecological niche as *X. fastidiosa* subsp. *pauca* in the xylem vessels of citrus plants (Araújo *et al.* 2002). The genus *Methylobacterium* is classified in the α2 subgroup of the *Proteobacteria* and includes a group of strictly aerobic, Gram-negative, pink-pigmented, facultatively methylotrophic (PPFM) bacteria characterized by their ability to utilize single-carbon compounds like methanol and formaldehyde via the serine pathway, as well as a wide range of multi-carbon growth substrates (Green 1992 Urakami *et al.* 1993; Wood *et al.* 1998; Doronina *et al.* 2000; McDonald *et al.* 2001; Doronina *et al.* 2002; Aken *et al.* 2004; Anesti *et al.* 2004; Van Jourand *et al.* 2004; Gallego *et al.* 2005a, 2005b, 2005c, 2006). The genus *Curtobacterium* had been defined by Yamada and Komagata (1972) as Gram positive and aerobic bacteria and for some so-called motile brevibacteria. *Curtobacterium* strains had been isolated from rice and other plants and *C. flaccumfaciens*, in particular, is a well-established plant pathogen (Collins and Jones 1983). However, *Curtobacterium* have been isolated as endophytes from many crops, including red clover (Sturz *et al.* 1998), rice (Elbeltagy *et al.* 2000), potato (Sturz and Matheson 1996), yam (Tor *et al.* 1992), prairie plants (Zinnier *et al.* 2002), and citrus (Araújo *et al.* 2001). Several reports have indicated that *C. flaccumfaciens* can function as a biological control agent against many pathogens, and may function either by the triggering of induced systemic resistance (Raupach and Kloepper 1998) or by antibiosis (Sturz and Matheson 1996).

The bacterium *M. mesophilicum* seems to reduce the growth of *X. fastidiosa* subsp. *pauca*, while *M. extorquens* could stimulate the growth of *X. fastidiosa* subsp. *pauca in vitro* (Lacava *et al.* 2004). The conclusion is that the symptoms of CVC could be affected by the populations of *Methylobacterium* spp. and also *C. flaccumfaciens* (Araújo *et al.* 2002; Lacava *et al.* 2004).

Lacava *et al.* (2006a) evaluated the co-inoculation of *M. mesophilicum* and *X. fastidiosa* subsp. *pauca* into *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle), an excellent experimental host plant for *X. fastidiosa* subsp. *pauca* and endophytic bacteria (Monteiro *et al.* 2001; Andreote *et al.* 2006). These authors verified that the population of endophytic *Methylobacterium* was lower when inoculated with *X. fastidiosa* subsp. *pauca* than when inoculated alone. Also, the population of *X. fastidiosa* subsp. *pauca* was in turn reduced by *M. mesophilicum*. These initial results are from a single time point only, but are consis-

tent with other data that suggest interactions between *M. mesophilicum* and *X. fastidiosa* subsp. *pauca in vitro* (Lacava *et al.* 2004, 2006a).

The bacterium, *C. flaccumfaciens*, was more frequently isolated from CVC-asymptomatic than from CVC-symptomatic orange (*C. sinensis*) and tangerine (*Citrus reticulata*) plants (Araújo *et al.* 2002; Lacava *et al.* 2004), and it was also suggested, based on *in vitro* interaction experiments, that the growth of *X. fastidiosa* subsp. *pauca* could be inhibited by endophytic *C. flaccumfaciens*. It was also demonstrated that symptoms of *X. fastidiosa* subsp. *pauca* infection in *C. roseus*, such as shortened internodes, reduced flowering and stunting and chlorosis of leaves with occasional scorch symptoms, were reduced or prevented entirely by co-inoculation with *C. flaccumfaciens* (Lacava *et al.* 2007a).

## BIOLOGICAL CONTROL OF CVC

Lacava *et al.* (2004) reported that the growth of *X. fastidiosa* subsp. *pauca* was inhibited by endophytic *C. flaccumfaciens in vitro* and Lacava *et al.* (2007a) demonstrated that *C. flaccumfaciens* reduced the severity of disease symptoms when co-inoculated with *X. fastidiosa* subsp. *pauca* in periwinkle (*C. roseus*) plants (**Fig. 1**).

The use of isolation and denaturing gradient gel electrophoresis (DGGE) techniques revealed several genera of bacteria as colonizers of glassy-winged sharpshooter (GWSS) heads and as identified by 16S sequencing, these included *M. extorquens* and *C. flaccumfaciens*. The GWSS, *Homalodisca vitripennis* Germar (Hemiptera: Cicadellidae) [formerly *H. coagulata* (Takiya *et al.* 2006)] is the most widespread sharpshooter insect vector of *X. fastidiosa* in the United States. In addition, Kirkpatrick and Wilhelm (2007) have also isolated strains of *C. flaccumfaciens* as part of the endophytic bacterial community of grapevine in California. In Brazil, *C. flaccumfaciens* is consistently isolated as an endophytic bacterium from citrus plants (Araújo *et al.* 2002; Lacava *et al.* 2004).

It is likely that endophytic bacteria are introduced into sweet orange trees by sharpshooter insects in the same manner as *X. fastidiosa* subsp. *pauca*. In the study of Gai (2006), *Curtobacterium* sp. was the most important bacterium colonizing the heads of the insect vectors of *X. fastidiosa* subsp. *pauca* in Brazil. To isolate the bacterial community associated to head of insect vectors of *X. fastidiosa* subsp. *pauca*, Gai (2006) started with surface sterilization which involved placing sharpshooter body in 75% ethanol for two minutes, transferring them to a container with sodium hypochlorite solution (2% available Cl⁻) for two minutes, and rising them twice in sterile double-distilled water. After the surface sterilization step, each sharpshooter was transferred into a sterile Petri dish and its head and eyes were removed using a sterile scalpel. The head, which contained the foregut, was removed, the eyes excised and the remainder of the head was used in the isolation, then placed into a sterile 1.5 ml microcentrifuge tube containing 150 μl of sterile phosphate buffered saline (PBS). The mixture was macerated using an electric mortar and sterile plastic pestles. An
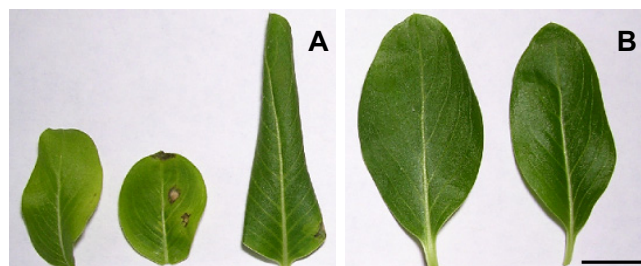


**Fig. 1** Leaf stunting and chlorosis induced in *Catharanthus roseus* leaves 2 months after inoculation with (**A**) *X. fastidiosa* subsp. *pauca* (left). (**B**) Symptom free leaves from a plant doubly-inoculated with *X. fastidiosa* subsp. *pauca* and *C. flaccumfaciens* (right). Scale bar: 1 cm.

aliquot of 100 µl of this solution was plated on 5% TSB media (Tryptone Soy Broth, DIFCO). Plates were incubated at 28°C for 5 days and colonies were counted, classified according to morphological groups and the number of colony forming units per insect head (CFU/insect head) was determined.

*Curtobacterium flaccumfaciens* was implicated in playing an important role in the prevention of CVC symptoms in citrus trees (Araújo *et al.* 2002; Lacava *et al.* 2004, 2007a). The citrus endophyte, *Curtobacterium* sp., colonizing vector insect heads could explain why the transmission efficiency of *X. fastidiosa* subsp. *pauca* by vectors is low (5 to 10%), when compared to the transmission of *X. fastidiosa* subsp. *fastidiosa* by GWSS, which transmit PD (45%) (Krügner *et al.* 2000; Redak *et al.* 2004).

Endophytic bacteria could influence disease development by reducing the efficiency of transmission by insects due to competition with pathogens in host plants and also in insect foreguts (Gai 2006). In addition, the bacterial communities in the foregut of insect vectors of *X. fastidiosa* subsp. *pauca* changed with time, environmental conditions and in different insect species. However, since members of the genus *Curtobacterium* were consistently detected in the insect vectors of *X. fastidiosa* subsp. *pauca* (Gai 2006), they may be candidates for biological control of *X. fastidiosa* subsp. *pauca*, which requires endophytic bacteria (Lacava *et al.* 2007a) that can colonize both the insect vectors of CVC and citrus plants.

## SYMBIOTIC CONTROL (SC)

The technique of paratransgenesis was developed as a novel method to create conditions that render insect vectors incompetent. The strategy of symbiotic control (SC) employs both paratransgenic (defined below) and non-recombinant methods to control disease or health problems. In some cases, these solutions may result in competitive displacement of the pathogen with a more benign microbe.

The strategy, paratransgenesis, was developed in order to prevent the transmission of pathogens by insect vectors to humans (Beard *et al.* 1998, 2001, 2002; Rio *et al.* 2004). The key concept in paratransgenesis is the genetic alteration of symbiotic microbes that are carried by insects (therefore, they are paratransgenic insects). The genetic alterations of the symbiotic microbes are designed to increase their competitiveness within the insect vector at the expense of the pathogen. This overall strategy of disease prevention is an example of SC and is a variation on the theme of symbiotic therapy (Ahmed 2003). Genetic manipulation has fitness costs that must be factored in to the application (Durvasula *et al.* 1997; Miller 2007).

The key to SC, and therefore paratransgenesis, is to find a local candidate microbe having an existing association with the pathosystem that includes the problem or condition at hand. The local candidate microbe should occupy the same niche as, or have access to, the target pathogen or condition (Durvasula *et al.* 1997). The local origin of the biocontrol microbe in SC differs from classical biological control, where microbes, herbivores, parasites or predators are sought from outside of the local ecosystem for establishment in the local ecosystem to control a pest, such as a plant or invertebrate (Miller 2007). In SC, all elements originate at the local site and are already co-evolved with and established in the pathosystem; foreign exploration is not only unnecessary, but also most likely counter-productive. Because of these strict requirements, a suitable symbiotic candidate may not always be found or may not be amenable to practical manipulation (Miller 2007).

Microbes chosen for symbiotic control must be able to pass subsequent regulatory scrutiny (Miller 2007). Once a candidate symbiont is identified as a control agent for paratransgenesis, all genetic or other manipulations can be local. Indeed, a symbiotic control or paratransgenic solution developed for a specific location may not be suitable for another site or condition elsewhere (Durvasula *et al.* 1999, 2003;

Miller 2007).

Once a microbe is identified as having potential for symbiotic or paratransgenic control, it is studied to define requirements for culture and reintroduction into the pathosystem, and suitability for genetic alteration, if necessary. The methods selected have to be adaptable to ordinary practices in the target area. In the case of paratransgenic control, a gene or genes to be introduced into the endosymbiont to influence its interaction with the pathogen must be identified. Beard *et al.* (2001) have isolated and characterised symbiont bacteria from various triatomine species, vector of Chagas disease, and developed a method for genetically transforming them. These authors have reintroduced them into triatomine species, thereby producing stable paratransgenic insects that are able to express heterologous gene products.

## SYMBIOTIC CONTROL OF PIERCE'S DISEASE AS A MODEL FOR CVC CONTROL

PD was first detected in Southern California in 1884, where it destroyed approximately 40,000 acres of grapes in Anaheim, CA, during a 5-year outbreak (Pierce 1892; Goodwin and Purcell 1992). After this devastating experience, PD became only an occasional concern to West Coast viticulture for decades until the mid-1990s, when the GWSS became established in California. The GWSS is a major concern for horticultural industries beyond viticulture due to its ability to transmit *X. fastidiosa* strains causing scorch diseases in a number of host plants, including *X. fastidiosa* subsp. *fastidiosa* that causes PD in grapevines (Purcell 2005). As with other sharpshooter insects, *H. vitripennis* is a xylophagous insect that feeds on hundreds of plant species (Purcell and Hopkins 1996; Purcell and Saunders 1999); citrus is one of its preferred hosts (Blua *et al.* 2001). Perring *et al.* (2001) demonstrated a relationship between PD incidence in grapes and the proximity of vineyards to citrus orchards. This leafhopper, which can serve as a vector of *X. fastidiosa*, has the capacity to feed on more than 70 different plant species and can survive winter temperatures as low as -6°C (Park *et al.* 2006). Moreover, compared with other *X. fastidiosa*-carrying insects associated with PD and native in California, GWSS has a longer flight range (up to a quarter mile). These traits make the GWSS a very serious threat to the wine industry of Southern and Central California (Castle *et al.* 2005). Indeed, since the first identification of GWSS in the California vineyards, programs aimed at controlling the dissemination of this insect as a strategy to prevent PD outbreaks have involved more than US $160 million of direct investments (http://www.cdfa.ca.gov/ phpps/pdcp/). Control of any of the GWSS transmitted diseases of horticultural crops in California by a SC or paratransgenic approach would be of immediate interest to other industries as well. The objective or rationale for developing a method of SC for PD is to disrupt vector transmission with the least effect on other crops. SC would be available to local vineyards for local control instead of area-wide treatments of alternative host plants such as is done now. Treatment of citrus with systemic insecticides for GWSS to reduce the chance of acquiring and spreading pathogens in adjacent vineyards cannot be seen as a long-term solution. SC would be more selective and have less side-effects on other biological control practices. The SC organisms inhabit the xylem fluid of the target plants yet do not contaminate the berries of the grapevines. It remains to be seen if one treatment would be effective for an entire season (Miller 2007).

Three potential bacterial candidates, *Alcaligenes* sp., *Chryseomonas* sp., and *Ralstonia* sp., for SC of PD were collected from GWSS in southern California (Bextine *et al.* 2004). All were endophytes transmitted to different host plants by GWSS in a manner analogous to the pathogen; thus, the candidates had access to the pathogen in host plants or in the insect vector, providing the needed access property. *Alcaligenes denitrificans* var. *xylosoxidans* (Axd)

was selected for further development because the endophytic bacterium should have most of the requirements for a successful paratransgenesis strategy such as: a) a population of microbes that is amenable to culture and genetic manipulation *in vitro* must exist within a disease-transmitting vector; b) facile methods for isolating and transforming the endophytic bacteria must be present; c) transformation of the symbiotic/endophytic bacteria must result in stable mutants; d) genetic manipulation of the bacteria should not affect their symbiotic functions in the host vector; e) genetic manipulation of symbiotic bacteria should not render them virulent, either to the target vector or other organisms in the environment. Furthermore, bacteria chosen as gene-delivery vehicles must not be pathogens themselves.

Successful delivery to and colonization of Axd in the foregut regions of GWSS suggest that a paratransgenic approach to manage, prevent, and/or control Pierce's disease is possible (Bextine *et al.* 2004).

Lacava *et al.* (2007b) used isolation and denaturing gradient gel electrophoresis (DGGE) techniques to identify several genera of bacteria as colonizers of heads of GWSS collected in orange groves. As identified by 16S rRNA sequencing, these included *Bacillus*, *Cryocola*, *Microbacterium*, *Micrococcus* and *Pedobacter*. In addition, *Methylobacterium extorquens*, *Curtobacterium flaccumfaciens*, *Baumannia cicadellinicola* and various *Pseudomonas* and *Wolbachia* species were found. Of these genera, *Bacillus*, *Pseudomonas*, *Methylobacterium* and *Curtobacterium* were previously described as endophytes that are able to colonize citrus plants. The work of Araújo *et al.* (2002) strongly indicated interactions among *Methylobacterium* spp., *C. flaccumfaciens* and *X. fastidiosa* subsp. *pauca*. These results reinforced the idea that all of these bacteria could interact in the vector insect as well as in the host plant.

Furthermore, Lacava *et al.* (2004) suggested CVC symptoms in citrus plants could be influenced by the interactions among these three species. In a study of the diversity of bacterial communities associated with GWSS foreguts, they used culture-dependent methods as well as procedures based on sequence polymorphisms (DGGE) of the 16S rRNA gene present in total DNA extracted from GWSS foreguts. Lacava *et al.* (2007b) suggested that the diversity profiles obtained with culture dependent (isolation in culture) techniques indicated a low bacterial diversity. However, the same authors described higher bacterial diversity when using PCR-DGGE, a culture-independent method. These initial results from Lacava *et al.* (2007b) showed that PCR-DGGE is suitable for the analysis of bacterial diversity in GWSS heads. In the future, species such as *C. flaccumfaciens* and *Methylobacterium* spp., found as part of the bacterial community in GWSS, could be investigated as potential candidates for use in an SC or SC-paratransgenic based strategy to control the spread of *X. fastidiosa*.

Using methods perfected in previous studies (Lampe *et al.* 1999, 2000), Axd was genetically altered to contain a *Ds*Red fluorescent marker gene in the chromosome (Bextine *et al.* 2004) to demonstrate the ability of *Ds*Axd to colonize the cibarial region of the GWSS foregut for up to 5 weeks post-exposure. The conclusion was that Axd occupies the same region in the foregut as the pathogen, *X. fastidiosa* (Bextine *et al.* 2004). *Ds*Red Axd was found to be transmitted by GWSS and to colonize various plants (Bextine *et al.* 2004, 2005). *Ds*Red Axd could be introduced into grapevines by misting the leaves or by soil drenching or by direct injection of the stem of the grapevine. Interestingly, Axd appeared to be better adapted to citrus than to grapevine (Bextine *et al.* 2005). Indeed, the original samples of GWSS from southern California were obtained from citrus groves in the Agricultural Operations plots at the University of California, Riverside, so it is likely that the endophytes in the GWSS sampled originally came from citrus. Bextine *et al.* (2004) describe the successful delivery of Axd to, and colonization of, the foregut of GWSS by Axd. These results suggest that a paratransgenic approach to manage, prevent, and/or control PD by SC may be possible. We proposed



**Step 1**: Looking for a symbiotic and endophytic bacterium that can survive in the vector insect and in the host plant.

**Step 2**: Identification of the pathogen in the host plant and in the vector insect.

**Step 3**: Confirmation that a symbiotic bacterium (SB), candidate for symbiotic control (SC), is able to colonize the host plant and the vector insect; that is, occupy the same niche as the pathogen.

**Step 4**: Genetic modification of the SB to control the pathogen and confirmation that after transformation the SB retains the ability to colonize the insect vector and the host plant.

**Step 5**: Control of the disease, or blocking pathogen transmission using a SB genetically modified bacterium with ability to colonize the vector insect and the host plant.

● Symbiotic bacterium (SB)  ● *Xylella fastidiosa*  ● Genetically-altered SB

Plant without symptoms  Plant with symptoms  Vector insect (GWSS)
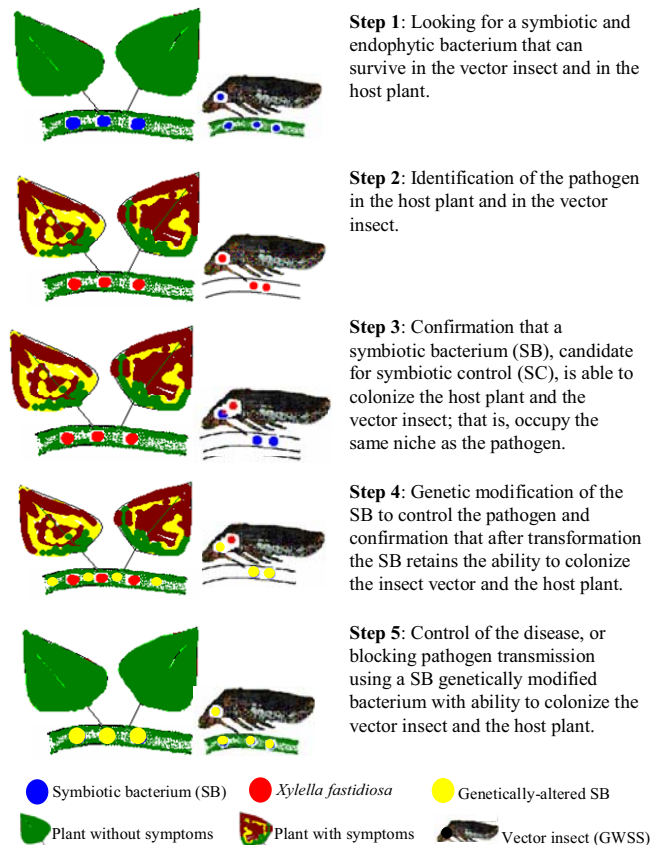
**Fig. 2 Chronological steps to develop a strategy of paratransgenic-symbiotic control, using endophytic bacteria, in cycle disease where the causal agent is *Xylella fastidiosa*.** Source: adapted and developed from Bextime *et al*. (2004, 2005).

(**Fig. 2**) a sequence of steps needed to develop a strategy of SC, using endophytic bacteria, to disrupt the disease cycle where the causal agent is *X. fastidiosa* (adapted from Bextine *et al.* 2004, 2005).

A number of candidate antimicrobial peptides were screened against *X. fastidiosa* (Kuzina *et al.* 2006). In this study the authors show that antibiotics and antimicrobial peptides have some activity against the pathogen, *X. fastidiosa* and may have application in protecting plants from developing PD. The potential use of these antimicrobial peptides in the protection of grapevines will depend on the development of a delivery system, such as SC (Kuzina *et al.* 2006). Also, Lampe *et al.* (1999, 2000) further screened single chain antibodies from a phage antibody library for ability to bind the coat protein of the pathogen, *X. fastidiosa*. These authors selected an antibody fragment, designated S1, that was specific for the strain of *X. fastidiosa* causing PD and which did not recognize closely related *X. fastidiosa* strains.

## STRATEGY OF SYMBIOTIC CONTROL FOR CVC

The key to symbiotic control is finding a candidate microbe having an existing association with the ecosystem that includes the problem or condition at hand and that occupies the same niche as or has access to the target pathogen (Miller 2007). Bacteria of the genus *Methylobacterium* are known to occupy the same niche as *X. fastidiosa* subsp. *pauca* inside citrus plants (Araújo *et al.* 2002; Lacava *et al.* 2004). During feeding, insects could acquire not only the pathogen, but also endophytes from host plants. Gai (2006) and Gai *et al.* (2007) reported the localization of the endophytic bacterium, *M. mesophilicum*, in the *C. roseus* model plant system and the transmission of this endophyte by *Bucephalogonia xanthophis*, a sharpshooter insect vector of *X. fastidiosa* subsp. *pauca*.

*Methylobacterium mesophilicum*, originally isolated as an endophytic bacterium from citrus plants (Araújo *et al.* 2002), was genetically transformed to express GFP (Green Fluorescent Protein) (Gai *et al.* 2007). The GFP-labeled strain of *M. mesophilicum* was inoculated into *C. roseus* (model plant) seedlings and was observed colonizing its xylem vessels. The transmission of *M. mesophilicum* by *B. xanthophis* was verified with insects feeding on fluids containing the GFP-labeled bacterium. Forty-five days after inoculation, the plants exhibited endophytic colonization by *M. mesophilicum*, confirming this bacterium as a nonpathogenic, xylem-associated endophyte (Gai 2006). These data demonstrate that *M. mesophilicum* not only occupies the same niche as *X. fastidiosa* subsp. *pauca* inside plants, but also that it may be transmitted by *B. xanthophis*. The transmission, colonization and genetic manipulation of *M. mesophilicum* is a prerequisite to examine the potential use of paratransgenic-SC to interrupt transmission of *X. fastidiosa* subsp. *pauca*, the bacterial pathogen causing CVC, by insect vectors. We propose *M. mesophilicum* as a candidate for a paratransgenic-SC strategy to reduce the spread of *X. fastidiosa* subsp. *pauca*. It is known that *X. fastidiosa* subsp. *pauca* produces a fastidian gum (da Silva *et al.* 2001) which may be responsible for the obstruction of xylem in affected plants (Lambais *et al.* 2000), so the production of endoglucanase by genetically modified endophytic bacteria may transform the endophytes into symbiotic control agents for CVC. Azevedo and Araújo (2003) have used the replicative vector pEGLA160 to produce genetically modified *Methylobacterium* expressing antibiotic resistance and endoglucanase genes. Furthermore, other strategies can be evaluated such as the production of genetically modified *Methylobacterium* to secrete soluble anti-*Xylella* protein effecting citrus, such as Lampe *et al.* (2006) suggested in the *Escherichia coli* α-hemolysin system for use in Axd to secrete soluble anti-*Xylella* protein effectors in grapevine and GWSS. Also, Lampe *et al.* (2007) suggested the evaluation of proteins secreted from the grapevine bacterial symbiont *Pantoea agglomerans* for use as secretion partners of anti-*Xylella* protein effectors. One strategy that can be adopted as the next step for SC control of CVC is producing a genetically modified endophytic bacterium, like *Methylobacterium*, to secrete anti-*Xylella* protein effectors.

## CONCLUSION

Our strategy is similar to that developed by Bextine *et al.* (2004) for a paratransgenic strategy for SC of PD in grapevine. Bextine *et al.* (2004) suggested that the genus *Alcaligenes*, an endophytic bacterium that can colonize the GWSS vector of *X. fastidiosa* subsp. *fastidiosa*, would be a candidate for paratransgenic SC of PD in the USA. We believe that the endophytic bacterium *M. mesophilicum* from citrus plants is likewise a candidate for paratransgenic-SC of CVC. Our results indicate that this endophyte colonizes the same niche as *X. fastidiosa* subsp. *pauca* in citrus plants (Araújo *et al.* 2002; Lacava *et al.* 2004; Andreote *et al.* 2006; Lacava *et al.* 2006a). *M. mesophilicum* is also transmited by an insect vector of *X. fastidiosa* subsp. *pauca* (Gai 2006).

Bacteria chosen as gene-delivery vehicles for paratransgenesis-SC must not be pathogens themselves. *M. mesophilicum* is not a pathogen and several requirements for successful paratransgenesis-SC strategy as described by Durvasula *et al.* (2003) have been demonstrated a) *M. mesophilicum* is amenable to culture and genetic manipulation *in vitro*; b) facile methods for isolating and transforming the endophytic bacteria have been developed; c) transformation of the symbiotic/endophytic bacteria has resulted in mutants that were stable in planta. Future genetic manipulation of *M. mesophilicum* to achieve paratransgenic-SC should not affect its symbiotic functions in the plant host and insect vector, and of course genetic manipulation of symbiotic bacteria should not render them virulent, either to the host plant or target.

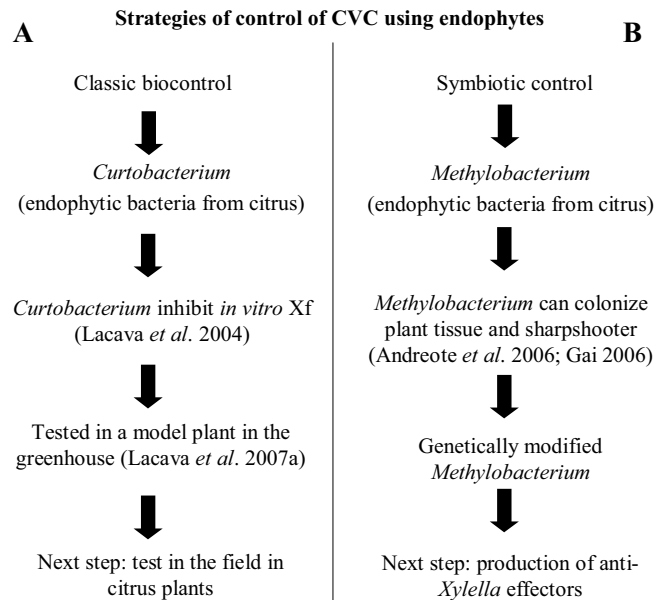*C. flaccumfaciens* is also a candidate for biological con-

**Strategies of control of CVC using endophytes**



**Fig. 3 Hypotheses and strategies to control Citrus variegated chlorosis (CVC) using endophytic bacteria form citrus plants.** (**A**) We suggest the endophytic bacterium *Curtobacterium flaccumfaciens* as a classical biological control agent. *C. flaccumfaciens* has the ability to colonize plant tissues in the presence or absence of *Xylella fastidiosa* subsp. *pauca*. This is a prerequisite for the use of this bacterium as a biocontrol agent. The data indicate that *C. flaccumfaciens* interacted with *X. fastidiosa* subsp. *pauca* in *Catharanthus roseus*, and reduced the severity of the disease symptoms induced by *X. fastidiosa* subsp. *pauca* (Lacava *et al.* 2007a). (**B**) Also, it is suggests the endophytic bacterium *Methylobacterium mesophilicum* as a qualified candidate for a paratransgenic-symbiotic control (SC) strategy because the transmission, colonization and genetic manipulation of *M. mesophilicum* is a prerequisite to examining the potential use of SC to interrupt transmission of *X. fastidiosa* subsp. *pauca*, the bacterial pathogen causing CVC, by insect vectors.

trol of CVC. Interaction and antagonism between *C. flaccumfaciens* and *X. fastidiosa* subsp. *pauca* was strongly indicated on the basis of the frequency of isolation from sweet orange of *C. flaccumfaciens* (Araújo *et al.* 2002). In addition, *in vitro* interactions between *X. fastidiosa* and *C. flaccumfaciens* have been described, including the inhibition of the growth of *X. fastidiosa* subsp. *pauca* by cell-free supernatants of nutrient medium in which *C. flaccumfaciens* had been grown (Lacava *et al.* 2004). Also, Lacava *et al.* (2007a) demonstrated that *C. flaccumfaciens* interacted with *X. fastidiosa* subsp. *pauca* in *C. roseus*, and reduced the severity of the disease symptoms induced by *X. fastidiosa* subsp. *pauca* (**Fig. 1**). The ability demonstrated by *C. flaccumfaciens* to colonize plant tissues in the presence of *X. fastidiosa* subsp. *pauca* and the reduction of disease symptoms caused by *X. fastidiosa* subsp. *pauca* (Lacava *et al.* 2007a) are prerequisites for the use of this endophytic bacterium as a biocontrol agent. Since members of the genus *Curtobacterium* were consistently detected in the insect vectors of *X. fastidiosa* subsp. *pauca* (Gai 2006), they fulfill another requirement of candidates for biological control of *X. fastidiosa* subsp. *pauca* (Lacava *et al.* 2007a), i.e. they can colonize both the insect vectors of *X. fastidiosa* subsp. *pauca* and citrus plants. In the case of biocontrol of *X. fastidiosa* subsp. *pauca* and CVC disease, it would be desirable if *C. flaccumfaciens* could be transmitted by budwood, but this has yet to be demonstrated. The reduction of disease symptoms caused by *X. fastidiosa* subsp. *pauca* in the presence of *C. flaccumfaciens* may be attributable to direct killing of *X. fastidiosa* subsp. *pauca* by *C. flaccumfaciens*. Consistent with this hypothesis, three bacteriocins showing activity against *X. fastidiosa* subsp. *pauca* have been recently described from *C. flaccumfaciens* (Cursino 2005).

We propose two complementary strategies for control of

CVC using endophytic bacteria from citrus plants. We suggest the endophytic bacterium *C. flaccumfaciens* as a classical biological control agent and the endophytic bacterium *M. mesophilicum* as a qualified candidate for a paratransgenic-SC strategy. The details of these strategies are summarized in **Fig. 3**.

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