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Transmission of *Methylobacterium mesophilicum* by *Bucephalogonia xanthophis* for Paratransgenic Control Strategy of Citrus Variegated Chlorosis

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***Methylobacterium mesophilicum*, originally isolated as an endophytic bacterium from citrus plants, was genetically transformed to express green fluorescent protein (GFP). The GFP-labeled strain of *M. mesophilicum* was inoculated into *Catharanthus roseus* (model plant) seedlings and further observed colonizing its xylem vessels. The transmission of this endophyte by *Bucephalogonia xanthophis*, one of the insect vectors that transmit *Xylella fastidiosa* subsp. *pauca*, was verified by insects feeding from fluids containing the GFP bacterium followed by transmission to plants and isolating the endophyte from *C. roseus* plants. Forty-five days after inoculation, the plants exhibited endophytic colonization by *M. mesophilicum*, confirming this bacterium as a nonpathogenic, xylem-associated endophyte. Our data demonstrate that *M. mesophilicum* not only occupy the same niche of *X. fastidiosa* subsp. *pauca* inside plants but also may be transmitted by *B. xanthophis*. The transmission, colonization, and genetic manipulation of *M. mesophilicum* is a prerequisite to examining the potential use of symbiotic control to interrupt the transmission of *X. fastidiosa* subsp. *pauca*, the bacterial pathogen causing Citrus variegated chlorosis by insect vectors.**

***Keywords*:** endophytic symbionts, *Citrus sinensis*, *Methylobacterium mesophilicum*, sharpshooters, symbiotic control, *Xylella fastidiosa* subsp. *pauca*

Citrus variegated chlorosis (CVC) is a disease of sweet orange (*Citrus sinensis* (L.)) trees caused by *Xylella fastidiosa* subsp. *pauca* (Hartung *et al.*, 1994; Schaad *et al.*, 2004). In Brazil, CVC is responsible for losses of US $ 100 million per year to the citrus industry (Della-Coletta *et al.*, 2001). The disease continues to increase in severity, with 35% of the sweet orange trees in São Paulo, Brazil, currently showing loss of yield (www.fundecitrus.com.br).

Endophytes are microorganisms that do not visibly harm the host plant but can be isolated from surface-disinfected plant tissue or the inner parts of plants. They colonize an ecological niche similar to that of phytopathogens, and this fact might favor them as candidates for biocontrol agents (Hallmann *et al.*, 1997) because they have access to and could interact with phytopathogens (Azevedo *et al.*, 2000; Azevedo and Araújo, 2007). Many endophytic bacteria have been isolated from sweet orange (Araújo *et al.*, 2001, 2002), but our research has focused on the genus *Methylobacterium*, which occupies the same ecological niche as *X. fastidiosa* subsp. *pauca* in the xylem vessels of plants (Araújo *et al.*, 2002; Newman *et al.*, 2003; Lacava *et al.* 2004; Andreote *et al.*, 2006; Lacava *et al.*, 2006). The genus *Methylobacterium* is described as a main player in the interaction between the endophytic community and the pathogen *X. fastidiosa* subsp. *pauca* (Araújo *et al.*, 2002; Lacava *et al.*, 2004).

Catharanthus roseus (L.) G. Don has been shown to be an excellent experimental host for *X. fastidiosa* subsp. *pauca* (Monteiro *et al.*, 2001). Symptoms of *X. fastidiosa* subsp. *pauca* infection in *C. roseus* include shortened internodes, reduced flowering, stunting, and chlorosis of leaves with occasional scorch symptoms and wilting (Monteiro *et al.*, 2001). In comparison with sweet orange, *C. roseus* is much easier to maintain in a greenhouse, and the induction of symptoms following inoculation with *X. fastidiosa* subsp. *pauca* is both more rapid and more reliable. *Catharanthus roseus* has also been used to study the interactions between *X. fastidiosa* subsp. *pauca* and endophytic bacteria (Andreote *et al.*, 2006; Lacava *et al.*, 2006; Lacava *et al.*, 2007a, 2007b).

Xylem-feeding leafhoppers (Homoptera: Cicadellidae, tribes Cicadellini and Proconiini) are unique organisms in terms of their nutritional ecology; they are able to feed from xylem fluid, which is difficult to access and a nutritionally dilute food (Young, 1968; Raven, 1984). A clear association has been observed between Cicadellinae leafhoppers xylem-feeding habit and ability to transmit *X. fastidiosa* (Costa *et al.*, 2000; Almeida and Purcell, 2003). In Brazilian citrus groves, *Dilobopterus costalimai* Young, *Oncometopia facialis* (Signoret), and *Acrogonia citrina* Marucci & Cavichioli are the most common sharpshooters found, whereas *Bucephalogonia xanthophis* (Berg) is the most commonly trapped in citrus nurse-

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ries and young groves (Redak *et al.*, 2004).

A new genetic transformation tool, called paratransgenesis, has been used to prevent the transmission of pathogens by insect vectors to humans (Beard *et al.*, 1998, 2001; Rio *et al.*, 2004). Paratransgenesis means genetic alteration of symbiotic microbes that are carried by insects. The overall strategy of disease prevention is called symbiotic control and is a variation on the theme of symbiotic therapy (Ahmed, 2003). 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), so during feeding, insects could acquire not only the pathogen but also endophytes from host plants. In this paper, we report the localization of the endophytic bacterium, *M. mesophilicum*, in *C. roseus* model plant and the transmission of this endophyte by *B. xanthophis*. Also, we propose *M. mesophilicum* as a candidate for a symbiotic control strategy to reduce the spread of *X. fastidiosa* subsp. *pauca*.

Materials and Methods

Growth media, plasmids, and strains

A strain of *M. mesophilicum* SR1.6/6 was grown in all experiment on CHOI3 medium (Toyama *et al.*, 1998). For selection of the SR1.6/6 strain expressing the green fluorescence protein (GFP), called SRGFP strain, tetracycline (tc) was added to CHOI3 agar plates at final concentrations of 50 μg/ml. The following plasmid was used in this study: pCM88 broad-host-range plasmid that contains the gene of GFP, tetracycline resistance, both controlled by *LacZ* and *mxaF* promoters and the oriV and ColE1 origin of replication (Marx and Lidstrom, 2001). The plasmid was propagated and isolated from *Escherichia coli* and purified with the Plasmid Miniprep kit (Mo Bio, USA) according to the manufacturer's recommendations. The strain SR1.6/6 was previously isolated from healthy *C. sinensis* (Araújo *et al.*, 2002), and cultures of this strain were routinely grown at 28°C.

Preparation of *M. mesophilicum* competent cells

The competent cells preparation was adapted from Figueira *et al.* (2000). Bacterial cells were grown for 3∼4 days at 28°C on solid CHOI3 medium. One loop of bacterial cells was inoculated into 5 ml liquid medium and incubated at 28°C with shaking until the culture reached an optical density at 600 nm (OD_{600}=0.1). The medium was transferred into 50 ml of CHOI3 liquid medium and grown to mid-exponential growth phase (OD_{600}=1.0) at 28°C. Cells were collected by centrifugation (1.600×g) at 4°C for 10 min. The cell pellets were washed twice with chilled sterile water and then washed with 10% glycerol at 4°C and resuspended in 10% glycerol to concentration of approximately 10^9 cells/ml. Aliquots of 100 μl of cells were frozen in liquid N_2 and stored at -80°C.

Transformation of SR1.6/6 with pCM88 plasmid

The transformation of *M. mesophilicum* (SR1.6/6) was adapted from Figueira *et al.* (2000) and Marx and Lidstrom (2001). Aliquots of SR1.6/6 competent cells were mixed with 100 ng (1 μl) plasmid pCM88 and electroporated using a Gene Pulser (Bio-Rad, USA) apparatus with the following parameters: voltage, 2.5 kV; current, 25 mA; capacitance, 25 μF; and resistance, 400 Ω, resulting in a time constant of 8 to 10 ms. After pulse delivery, cells were immediately diluted by 1 ml chilled CHOI3 into a polypropylene tube incubated on ice for 15 min, then the cells were incubated at 28°C with constant shaking to allow for expression of antibiotic resistance. After 24 h, the transformed cell was plated on CHOI3 containing tetracycline and then incubated at 28°C for 4 days. Aliquots (1,000 μl) were recovered from storage in CHOI3 containing 30% glycerol at -80°C. For each experiment, one aliquot was cultivated for 24∼48 h on solid CHOI3 at 28°C. Samples of each culture were checked for green fluorescence to ensure quality control.

In vitro plasmid stability

To assess plasmid stability in SRGFP-derivative strain, bacteria containing the plasmid were first grown in CHOI3 medium supplemented with tetracycline for 18 h and then diluted to 10^3 CFU/ml in 25 ml of CHOI3 without antibiotic. Cultures were grown for approximately four generations (approximately 24 h∼6 h by generations), and cells (10^3 CFU/ml) from the 4^{th} generation were grown for another 4^{th} generations (five times), in a total of 120 h. A fraction of cultures at late log phase was used for a dilution series. Aliquots were plated on CHOI3 medium without tetracycline. A hundred random single colonies were inoculated using toothpick on CHOI3 agar supplemented with tetracycline. Colonies were counted and the percentage of clones carrying the plasmid was calculated.

Collection and maintenance of sharpshooter

Sharpshooter adults used in the study were collected using sweep nets on shoots of citrus trees in groves of northern São Paulo State, Brazil. The insects were transported to the laboratory on potted healthy citrus plants covered with fine net (tulle) bags and then kept in the greenhouse for 3∼5 days before the experiments (Almeida *et al.*, 2001).

Plant cultivation

Catharanthus roseus (L.) G. Don (cv. Peppermint Cooler) plants, an excellent experimental host for *X. fastidiosa* and endophytic bacteria (Monteiro *et al.*, 2001; Andreote *et al.*, 2006; Lacava *et al.*, 2006; Lacava *et al.*, 2007a, 2007b), was obtained commercially (Sakama Co., Brazil). *C. roseus* was used in experiment for plant colonization and transmission of *M. mesophilicum* by insect vectors. Plants cultivated *in vitro* and *in vivo* were used for different purposes. The plants were grown *in vivo* in MetroMix 510 and fertilized via irrigation with nitrogen:phosphorous:potassium (21:5:19) at 100 ppm nitrogen. Copper and iron were added to concentrations of 2 and 6 ppm, respectively. The greenhouse conditions were photoperiod of 14 h light at 28°C and 10 h dark at 18°C, and plants were watered regularly. To obtain *in vitro* axenic plants, seeds were sterilized by immersion in

sodium hypochlorite (1.5%) for 1 min, ethanol 70% for 1 min, and double rinsing in sterilized deionized water. Seeds were further germinated in pots containing MS media (Murashige and Skoog, 1962), in cultivation chamber, with temperature of 25°C and photoperiod of 16 h light and 8 h dark. The radiation intensity during the experiment was about 2.3 MJ/m^2.

Isolation of bacterial community from sharpshooter

Heads of five insects were cut off, while they were still alive, and macerated, one by one, in 1 ml of saline solution (NaCl 0.8%). An aliquot of 100 µl of this solution was plated on TSB 5% medium (Tryptone Soy Broth, Difco Lab, USA). Plates were incubated at 28°C for 5 days and colonies were counted and classified according to morphological groups, and the number of colony forming unit per insect head (CFU/insect head) was determined. One representative of each morphological group was identified by sequencing of the 16S rDNA.

DNA extraction and PCR for 16S ribosomal DNA sequence

DNA from bacteria was extracted from fresh cultures of each isolate using the QIAGEN DNA Extraction kit (QIAGEN, USA). PCR was carried out in a 50 µl reaction volume containing 1 µl DNA from bacterial, 0.4 µM each primer: R1378; CGGTGTGTACAAGGCCCGGGAACG and PO27F; GAGAGTTTGATCCTGGCTCAG (Heuer *et al.*, 1999), 200 µM of each dNTPs, 3.75 mM of $MgCl_2$, 5 U *Taq* DNA polymerase (Invitrogen, Brazil) in 10 mM Tris-HCl (pH 8.3) and 10 mM KCl. The amplification protocol consisted of an initial step at 94°C for 4 min, followed by 30 amplification cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min with a final extension at 72°C for 10 min. Amplification products were separated by electrophoresis by spotting 5 µl of the PCR reaction mixture onto 1% agarose gel and visualized under ultraviolet light after staining with ethidium bromide (0.5 µg/ml).

Colonization of *C. roseus* plants by endophytic SRGFP strain

The ecology of *M. mesophilicum* strain SRGFP was accessed by inoculation of a cell suspension of the strain in *C. roseus* seedlings cultured *in vitro*. After 30 days of disinfected seeds germination, five seedlings were removed from pots and inoculated by cutting the roots ends and immersing on bacterial suspension (10^5 CFU/ml) during 1 h. Plants were then cultivated in new pots containing MS media during additional 30 days. Colonization patterns were then verified by isolation of the inoculated strain in CHOI3 medium and by analysis of fluorescence microscopy. Examination was performed using a Leica MZ FLIII fluorescence stereomicroscope (Leica, France) using a combination of DAPI (365 nm) and GFP (510 nm) filters. Images were combined using the Overlay module in Metavue (Universal Imaging Corporation, USA). For control, five plants were treated with sterilized NaCl 0.8% solution.

Acquisition of SRGFP strain by sharpshooters

A cell suspension of SRGFP strain (10^5 CFU/ml in NaCl 0.8% solution) was supplemented with glutamine (5%) and offered to insects by membrane feeding system (Purcell and Finlay, 1979). Briefly, insects were confined in an acrylic tube filled with bacterial suspension (200 µl) and closed at both ends with a paraffin membrane. Insects were kept on the feeding system for 12 h (during light period), under 25°C~28°C.

Transmission of SRGFP strain by *B. xanthophis*

To simulate the transmission of endophytic bacteria in a manner similar to the way *X. fastidiosa* subsp. *pauca* is spread in plants, the endophyte *M. mesophilicum* expressing the GFP protein (SRGFP) was delivered by xylem-feeding leafhopper, *B. xanthophis*.

After an acquisition time, insects were transferred to clean plants of *C. roseus* cultivated in a greenhouse. These plants were 30 days old (days after germination) and in total, 50 plants and 50 insects were used (one insect per plant). Insects were confined on plants in cages, which permitted the insect access to plant leaves for 45 days.

Leaves were then surface sterilized (serial tissue immersion in ethanol 70% for 5 min, sodium hypochlorite 1.5% for 5 min, ethanol 70% for 5 min, twice rinsing in sterilized deionized water), macerated in NaCl 0.8% solution, and serial dilutions were plated onto solid CHOI3 culture medium supplemented with tetracycline (50 µg/ml). Plates were incubated at 28°C for seven days. Identification of the inoculated strain was made first by visualization of *Methylobacterium*-like colonies (pink pigmented) in culture plates. To ensure that *Methylobacterium* colonies observed were those acquired and transmitted by vectors, plates were also observed under UV light, which resulted in visualization of GFP protein

Table 1. Plasmidial stability of pCM88 on *M. mesophilicum*. The percent of reaming colonies caring out the pMC88 was obtained from randomly collected samples after 24, 48, 72, and 120 h of culture cells of strain SR1.6/6 growing without antibiotic tetracycline

Generation number	Reaming colonies caring out pCM88 (%)	SD[a]
0 (0 h)	100	0
4 (24 h)	99	1
8 (48 h)	98	3.21
12 (72 h)	95	2.08
20 (120 h)	95	1

[a] SD for four replicates

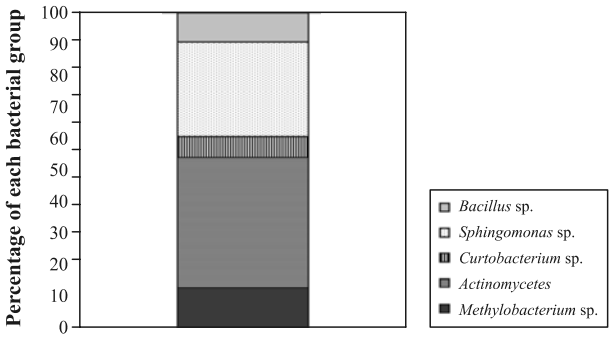

Fig. 1. Most dominant group of bacteria isolated from *B. xanthophis*.

present in the SRGFP strain.

Insects used for transmission of *M. mesophilicum* to *C. roseus* plants were monitored for the presence of strain, SRGFP, by isolation. Heads from all insects were separated, surface sterilized, and macerated in NaCl 0.8% solution and plated onto solid CHOI3 culture medium supplemented with tetracycline (50 μg/ml). The identification of inoculated strain was made as described previously for isolation from plants.

For negative control, five insects were fed with similar solution free of bacterial cells.

Results

Transfomation of endophytic *M. mesophilicum* strain and *in vitro* plasmid stability

When the pCM88 was introduced into the strain *M. mesophilicum* SR1.6/6, up to 10^2 transformants per μg of plasmid DNA were obtained (now called SRGFP), indicating a high efficiency of transformation. The analysis of randomly selected SRGFP transformants revealed that pCM88 was stably maintained in medium without antibiotic, expressing both the resistance to tetracycline and the *gfp* gene, after 20 generations in 120 h, 95%, decreasing the stability on 0.25% per generations approximately (Table 1).

Isolation of bacterial community from sharpshooter

To evaluate the bacterial community of insect heads, five insects were used. After isolation, a total of 2.14×10^3 bacteria with an average of $3.56\times10^2\pm23.2$ bacteria per insect head were isolated. The original bacterial community of *B. xanthophis* was comprised of five groups: *Methylobacterium* sp., Actinomycetes, *Curtobacterium* sp., *Sphingomonas* sp., and *Bacillus* sp. (Fig. 1). The *Methylobacterium* genus occurred naturally in *B. xanthophis*.

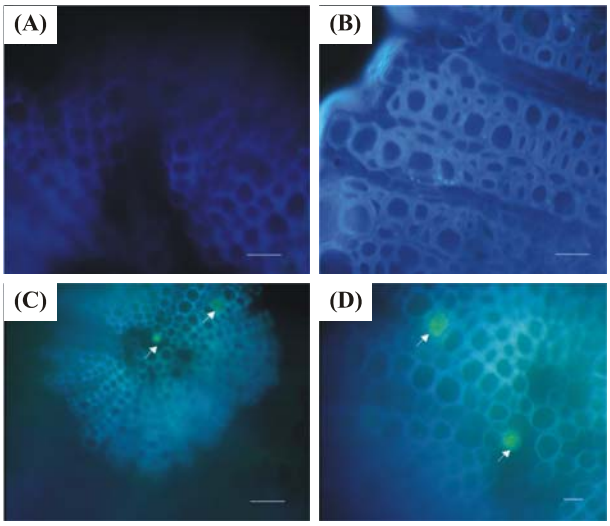

Fig. 2. Fluorescent microscopy evidencing the ecological niche occupied by endophytic *Methylobacterium mesophilicum*, expressing GFP in *Catharanthus roseus* plants. Xylem vessels observed under a fluorescence microscope (Leica MZ FLIII) 45 days after inoculation. Images are the result of the overlay of images produced using filters DAPI and GFP. A and B) Xylem vessels of a control plant, scale bar=10 μm. C and D) Colonized xylem vessel, scale bar=10 μm and 5 μm.

Colonization of *C. roseus* plants by endophytic SRGFP strain

The ecological niche occupied by the endophytic bacterium *M. mesophilicum* on *C. roseus* plants was determined by visualization with fluorescent microscope, of *in vitro* cultivated plants, 45 days after bacterium inoculation. A preferential colonization of plant xylem by this bacterium is clearly observed in fluorescence microscopy (Fig. 2C and D). Fig. 2A and B show vessels from control plants, where no fluorescent cell can be observed.

Transmission experiment of SRGFP strain

The insects used in transmission experiments were monitored for the presence of the SRGFP strain 24 h after acquisition. Bacteria isolation from insect heads revealed the average population density of *M. mesophilicum* of $1.64\times10^2\pm11.33$ CFU/insect head suggesting that the bacteria are capable of colonizing the foregut of the insect as they were not washed way by the sap flux.

The ability of the sharpshooter *B. xanthophis* in transmi-

Table 2. Evidence of the transmission of *M. mesophilicum* expressing GFP (SRGFP) to healthy plants (*C. roseus*) by insects (*B. xanthophis*). Plants were inoculated by insects, which acquired the fluorescent bacteria from membrane system (see 'Materials and Methods'). Endophytic bacteria were isolated from inoculated plants after 45 days of inoculation and fluorescent bacteria were counted. The average of SRGFP in inoculated plants was calculated as colony forming unit (CFU)/g of fresh tissue

Number of inoculated plants	Number of plants positive to the presence of SRGFP	Transmission Rate	SRGFP in plants (CFU/g fresh tissue)
45	6	13.3%	2.8×10^3

tting *M. mesophilicum* was accessed by insect acquisition of endophytic strain SRGFP and further feeding in *C. roseus* plants cultivated in greenhouse. Forty-five days after the insect feeding on plants, leaves on which insects were trapped, were submitted to bacterial isolation. The population density of *M. mesophilicum* found in *C. roseus* leaves 45 days after insect transmission presented an average of 2.8×10^3 CFU/g of fresh tissue.

In analyzing inoculated plants, from 45 plants used in insect traps, six presented the SRGFP strain colonizing inner tissues endophytically. It indicates that *B. xanthophis* is able to transmit the endophytic bacteria in the same way it transmits *X. fastidiosa*, with an efficiency of transmission of 13.3% (Table 2).

Discussion

The strain of *M. mesophilicum* SR1.6/6 was effectively transformed with the pCM88 plasmid. Figueira *et al.* (2000) obtained similar result $\sim10^3$ cell/μg of DNA using *M. extorquens*, another species of *Methylobacterium*, with pRK310 plasmid. *In vitro*, the pCM88 plasmid was stably maintained in SRGFP strain for at least 20 generations of cells growing in medium without antibiotic, suggesting that any error that could affect its inheritance and stability functions did not occur. In addition, the SRGFP strain was stable after the bacteria colonized the plant and insect tissues, therefore, representing an important tool to assess both the establishment of the inoculated strain in host plant and the transmission by *B. xanthophis*. The results from this study also suggest that the pCM88 plasmid was stably maintained *in planta* and sharpshooter for at least 180 generations in the transmission assay.

Endophytes have the ability to penetrate and to colonize plants systematically, inhabiting the apoplastic space (Quadt-Hallmann *et al.*, 1997) and plant vessels (Hallmann *et al.*, 1997; Newman *et al.*, 2003). Specific niches may be occupied according to endophyte species considered. *M. extorquens* was described colonizing intercellular space (Sy *et al.*, 2005). Andreote *et al.* (2006) described *M. mesophilicum* colonizing *C. roseus* and *Nicotiana clevelandii* (Clevelands tobacco) plants. These authors presented the data of *in vitro* inoculated plants revealing the biofilm formation by *M. mesophilicum* in roots of both plants, which may supply the basis for a further endophytic colonization. Here, we used a longer period of plant incubation after inoculation, verifying colonization of the inner tissues, with preferential colonization for the xylem.

Membrane-feeding sharpshooters acquire larger numbers of bacteria than insects feeding on diseased plants, mainly due to the higher concentration of bacteria available for insects, besides the lack of vacuum in the liquid inside membranes, which facilitates the feeding. Although the membrane system does not duplicate the natural environment and may lead to an (over or under) estimation of transmission rate, it is useful to demonstrate the transmission of bacteria by an insect.

The transmission efficiency of CVC strain of *X. fastidiosa* varies widely, depending on vector species and host plant (Purcell, 1989; Marucci *et al.*, 2003; Redak *et al.*, 2004). *B. xanthophis* has a transmission efficiency of 12%, while other important species have transmission efficiencies in the range from 1% to 5%. (Krügner *et al.*, 2000; Marucci *et al.*, 2003).

Concentration of *X. fastidiosa* subsp. *pauca* in citrus is $10^4\sim10^5$ CFU/g of fresh tissue (Oliveira *et al.*, 2002; Marucci *et al.*, 2003) and transmission efficiency by insect vectors is 12% (Krügner *et al.*, 2000). In this study, we used the model plant *C. roseus* and the concentration of *M. mesophilicum* was low, which does not lead to blockage of xylem vessels. Although the concentration of pathogen and endophyte varies in different plants, their transmission presented equivalent rates, 12% to *X. fastidiosa* subsp. *pauca* and 13.3% to *M. mesophilicum*, suggesting that transmission efficiency is determined by the insect itself.

As shown in this study, the transgenic endophytic *M. mesophilicum* has most of the prerequisites, listed by Durvasula *et al.* (2003), for a successful strategy using paratransgenesis. For example, *M. mesophilicum* that colonize citrus plants (Araújo *et al.*, 2002; Lacava *et al.*, 2004) and *B. xanthophis* is amenable to isolation, culture, and transformation with foreign genes. Also, the use of GFP, which does not affect the fitness of the bacteria, as a marker gene makes transfer of bacteria and the plasmid traceable (Valdivia *et al.*, 1996), was done with success in *M. mesophilicum* in this work.

Bextine *et al.* (2004, 2005) reported the transmission of *Alcaligenes xylosoxidans* subsp. *denitrifican*, commonly associated to insects digestive system, by the insect vector of *X. fastidiosa* in grapevines, *Homalodisca coagulata*, and suggested that the bacteria could be an effective delivery agent of a symbiotic control strategy (Durvasula *et al.*, 1997) for controlling *X. fastidiosa*, causal agent of Pierce's Disease (Almeida and Purcell, 2003).

Many aspects can influence the transmission efficiency, such as phytopathogen populations in feeding plant (Almeida and Purcell, 2003; Alves *et al.*, 2003) and the interaction between bacterial communities residing vector foregut and inoculated plant. Furthermore, bacteria community present in plants and insects could influence disease development by reducing the insect transmission efficiency due to competition with pathogens or by symbiotic control of *X. fastidiosa*.

The colonization and transmission of *M. mesophilicum* in the same host tissues and insect vector of *X. fastidiosa* subsp. *pauca* makes it possible to study the potential interactions between these bacteria in the insect body and makes *M. mesophilicum* an interesting candidate for the symbiotic control of the CVC agent, e.g., through a paratransgenesis approach (Beard *et al.*, 1998; Bextine *et al.*, 2004).

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