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The Endophyte *Curtobacterium flaccumfaciens* Reduces Symptoms Caused by *Xylella fastidiosa* in *Catharanthus roseus*

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Citrus variegated chlorosis (CVC) is a disease of the sweet orange [*Citrus sinensis* (L.)], which is caused by *Xylella fastidiosa* subsp. *pauca*, a phytopathogenic bacterium that has been shown to infect all sweet orange cultivars. Sweet orange trees have been occasionally observed to be infected by *Xylella fastidiosa* without evidencing severe disease symptoms, whereas other trees in the same grove may exhibit severe disease symptoms. The principal endophytic bacterial species isolated from such CVC-asymptomatic citrus plants is *Curtobacterium flaccumfaciens*. The Madagascar periwinkle [*Citrus sinensis* (L.)] is a model plant which has been used to study *X. fastidiosa* in greenhouse environments. In order to characterize the interactions of *X. fastidiosa* and *C. flaccumfaciens*, periwinkle plants were inoculated separately with *C. flaccumfaciens*, *X. fastidiosa*, and both bacteria together. The number of flowers produced by the plants, the heights of the plants, and the exhibited disease symptoms were evaluated. PCR-primers for *C. flaccumfaciens* were designed in order to verify the presence of this endophytic bacterium in plant tissue, and to complement an existing assay for *X. fastidiosa*. These primers were capable of detecting *C. flaccumfaciens* in the periwinkle in the presence of *X. fastidiosa*. *X. fastidiosa* induced stunting and reduced the number of flowers produced by the periwinkle. When *C. flaccumfaciens* was inoculated together with *X. fastidiosa*, no stunting was observed. The number of flowers produced by our doubly- inoculated plants was an intermediate between the number produced by the plants inoculated with either of the bacteria separately. Our data indicate that *C. flaccumfaciens* interacted with *X. fastidiosa* in *C. roseus*, and reduced the severity of the disease symptoms induced by *X. fastidiosa*. Periwinkle is considered to be an excellent experimental system by which the interaction of *C. flaccumfaciens* and other endophytic bacteria with *X. fastidiosa* can be studied.

Keywords: bioassay, *Citrus sinensis*, citrus variegated chlorosis, biocontrol

The bacterium *Xylella fastidiosa* (Wells *et al.*, 1987) 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 in the United States. These include Pierce's disease in grapevines, phony disease in the peach, and leaf scorches in the plum and almond (Hopkins and Purcell, 2002), the pecan (Sanderlin and Heyderich-Alger, 2000; Sanderlin and Melanson, 2006) and the pear in Taiwan (Leu and Su, 1993). Because strains of the bacterium evidence a wide host range in natural flora and are transmitted by common sharpshooter insects (Freitag, 1951; Freitag and Frazier, 1954), there are currently no adequate control measures.

Citrus-variegated chlorosis (CVC) is a disease that afflicts sweet orange [*Citrus sinensis* (L.)] trees, and is 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). This disease continues to show an increase in severity, with 35% of the sweet orange trees in São Paulo, Brazil currently evidencing yield losses (www.fundecitrus.com.br). The presence of asymptomatic sweet orange trees in otherwise heavily symptomatic groves in Brazil has resulted in novel approaches to the investigation of the control of CVC. As sweet orange scions are propagated clonally, these asymptomatic plants evidence the same genotype as diseased plants, and are located in the same groves under the same climatic and edaphic conditions, thereby suggesting that some other factor may be responsible for this apparent resistance to CVC. These trees are not simply resistant, as trees derived from them have been shown to become symptomatic when propagated from budwood and grown in heavily infested areas (Li *et al.*, 1997). One factor that may confer apparent resistance to CVC is the endophytic microbial community colonizing individual *C. sinensis* plants (Araújo *et al.*, 2002).

Endophytes are microorganisms which do not visibly harm the host plant, but which 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 (Hallmann *et al.*, 1997). Indeed, intensive study has demonstrated that some endophytic microorganisms have the ability

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to control pathogens (Sturz and Matheson, 1996; Duijff *et al.*, 1997; Krishnamurthy and Gnanamanickam, 1997; M'Piga *et al.*, 1997; Sharma and Nowak, 1998; Sturz *et al.*, 1998). In some cases, bacterial endophytes can also accelerate seedling emergence and promote the establishment of plants under adverse conditions (Chanway, 1997), and enhance plant growth and development (Lazarovits and Nowak, 1997; Pillay and Nowak, 1997; Bent and Chanway, 1998).

Bacteria of the genus *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). Araújo *et al.* (2002) isolated the strain of *C. flaccumfaciens* used in this study from the internal tissues of trees without symptoms of CVC from otherwise CVC-symptomatic orange groves. *C. flaccumfaciens* was isolated more frequently from CVC-asymptomatic than from CVC-symptomatic orange and tangerine plants. Both CVC-symptomatic and asymptomatic orange trees were demonstrated to be infected by *X. fastidiosa*, with the intensity of the amplification product greater from the extracts of symptomatic plant samples (Araújo *et al.*, 2002). Also, Lacava *et al.* (2004) suggested, on the basis of *in vitro* interaction experiments, that the growth of *X. fastidiosa* could be inhibited by endophytic *C. flaccumfaciens.*

Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don, has been identified as an excellent experimental host for *X. fastidiosa* (Monteiro *et al.*, 2001). Symptoms of *X. fastidiosa* infection in periwinkle include shortened internodes, reduced flowering, stunting, and leaf chlorosis with occasional scorch symptoms and wilting (Monteiro *et al.*, 2001). In comparison with the sweet orange, the Madagascar periwinkle is significantly easier to maintain in a greenhouse, and symptom induction following inoculation with *X. fastidiosa* is both more rapid and more reliable. The Madagascar periwinkle has also been utilized to study the interactions between *X. fastidiosa* and other endophytic bacteria (Andreote *et al.*, 2006; Lacava *et al.*, 2006; Lacava *et al.*, 2007).

The principal objective of this study was to determine, using periwinkle plants, whether there were *in planta* interactions between *X. fastidiosa* and *C. flaccumfaciens.* Such an experimental system is required in order to evaluate the potential use of this endophytic bacterium for the biocontrol of CVC. Symptoms were reduced or entirely prevented by the co-inoculation of *C. flaccumfaciens* with *X. fastidiosa*. We also developed a PCR-based assay for *C. flaccumfaciens* and employed it to complement our existing assay for *X. fastidiosa* in infected plants.

Materials and Methods

Bacterial cultures

X. fastidiosa was isolated from the sweet orange on solid PW medium (Davis *et al.*, 1981). The strain of endophytic *C. flaccumfaciens* utilized in these experiments was obtained from the collection of the Laboratório de Genética de Microrganismos (Depto. de Genética, ESALQ/USP, Brazil). It was isolated previously from CVC-asymptomatic citrus plants on solid tryptic soy agar amended with benomyl (50 mg/ml) (Araújo *et al.*, 2002).

Plant care and inoculations of Catharanthus roseus

C. roseus plants were commercially acquired and raised in a well-screened greenhouse. The plants were grown 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. Ambient light was supplemented with 4 h of high pressure sodium lighting. Inoculations with *X. fastidiosa* and *C. flaccumfaciens* were conducted via stem puncture with bacterial cultures at a concentration of -10^8 CFU/ml each (Li *et al.*, 2001) when the young *C. roseus* plants were -12 cm tall. Inoculations were performed when the sun was high and the soil mix was dry to facilitate the uptake of the inoculum. Ten droplets, each containing 10 ul of inoculum, were positioned separately on the stem of the periwinkle plant, and a 21-gauge syringe needle was utilized to puncture the stem through the droplets. The plants were observed until the inoculum was taken up by the plants. Six replicate plants were inoculated for each treatment in a completely randomized block design. Control inoculations with sterile PW medium were also conducted. Interactions among the bacteria and the plants were evaluated on the basis of the whole plant response to inoculation. Sixty days after inoculation, the disease symptoms were evaluated (Monteiro *et al.*, 2001) and the number of flowers/plant and the heights of the plants were recorded. Data were analyzed using the SAS® software package (SAS Institute, USA). The Tukey-test was utilized for means comparison after analysis of variance showed sig-

Table 1. Sequence of primers used in this work to amplify the intergenic region of the ribosomal operon and to detect *Xylella fastidiosa* and *Curtobacterium flaccumfaciens*

Primer	Target	Sequence 5'-3'	Reference
R1378(-)	16S rRNA genes	CGGTGTGTACAAGGCCC	This work; modified from Heuer *et al.* (1999)
F985PTO(-)	16S rRNA genes	AACGCGAAGAACCTTAC	This work; modified from Heuer *et al.* (1999)
Cf1	*C. flaccumfaciens*	ATCAGGAGCTTGCTCCTGTG	This work
Cf2	*C. flaccumfaciens*	GGCTGGCACGTAGTTAGCC	This work
272-1 int	*X. fastidiosa*	CTGCACTTACCCAATGCATCG	Pooler and Hartung (1995)
272-2 int	*X. fastidiosa*	GCCGCTTCGGAGAGCATTCCT	Pooler and Hartung (1995)

nificance at $P<0.05$ (Steel and Torrie, 1980).

Design of primers and extraction of DNA from plants

Primers for the ribosomal operon of Gram-negative bacteria (Table 1) were employed to amplify the intergenic region of the ribosomal operon of *C. flaccumfaciens*. We modified the universal primers F985PTO and R1378 (Heuer *et al.*, 1999) via the removal of 1 and 7 nucleotides, respectively, from the sequences of the 3' ends of these primers to create the primers F985PTO(-) and R1378(-), which were then utilized to amplify the intergenic region of the ribosomal operon of *C. flaccumfaciens* (Table 1). The PCR product was isolated and purified with a Geneclean Spin Kit (Qbiogene, USA), cloned into the TOPO TA cloning vector pCR2.1, and the resultant plasmids were introduced into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen, USA). Plasmid DNA was purified with an RPM kit (Qbiogene). DNA sequencing was conducted at the Biotechnology Center, University of Maryland, College Park, MD, USA. The primer pair Cf1/Cf2 (Table 1) was empirically designed on the basis of this sequence.

Total DNA was extracted from the midribs (about 200 mg) of *C. roseus* leaves. The midribs were sliced with a razor blade and placed in 2.0 ml extraction tubes, then pulverized in a Fast-Prep bead mill (Qbiogen, USA) using AP-1 extraction buffer from the DNeasy Plant kit (Qiagen, USA). The bead mill was operated at setting 4 for 4 cycles of 40 sec each, and the sample tubes were placed on ice between cycles to prevent them from overheating. After the extracts were pulverized, the DNeasy Plant protocol was followed to purify the DNA, which was resuspended in 100 ul of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0).

PCR assay conditions

Standard format PCR assays were conducted with primers 272-1-int and 272-2-int specific for *X. fastidiosa* (Pooler and Hartung, 1995) and the Cf1 and Cf2 primers specific for *C. flaccumfaciens* (Table 1) in a final reaction volume of 40 μl. The amplification conditions used were as follows: one cycle of 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1.5 min, with a final 10 min extension cycle at 72°C. The PCR products were visualized via staining with ethidium bromide after electrophoresis on agarose gels. The expected amplification products were 472 bp for *X. fastidiosa* (Pooler and Hartung, 1995) and 436 bp for *C. flaccumfaciens*. The sensitivity of the PCR assay for *C. flaccumfaciens* was estimated by plating the serial dilutions used for PCR. DNA was extracted from *C. roseus* petioles as previously described (Li *et al.*, 2003).

Results

Interaction of bacteria with inoculated Catharanthus roseus

Plants inoculated with sterile PW medium (negative control) and plants inoculated with *C. flaccumfaciens* alone evidenced no statistically significant differences ($P<0.05$) with regard to the number of flowers/plant, but the number of flowers/plant was reduced in plants inoculated with *X. fastidiosa* alone ($P<0.05$) (Fig. 1A). Interestingly, the number of flowers produced/plant on plants doubly-inoculated with both *X. fastidiosa* and *C. flaccumfaciens* was an intermediate between the number of flowers produced by the uninoculated control and those treated with *X. fastidiosa* (Fig. 1A), but was not statistically different from either of the other treatments (Fig. 1A).

Plants inoculated with *X. fastidiosa* were not as tall after 60 days as were the uninoculated PW medium controls (Fig. 1B). Plants doubly-inoculated with *X. fastidiosa* and *C. flaccumfaciens* grew to the same height as those inoculated with PW medium ($P<0.05$) (Fig. 1B).

After sixty days, the plants inoculated with sterile PW medium (negative control) demonstrated no symptoms of disease, nor did the plants inoculated with *C. flaccumfaciens* alone. However, plants inoculated with *X. fastidiosa* alone did develop characteristic disease symptoms, including stunting, reductions in the number of flowers, reductions in leaf size, and wilting (Fig. 2). These symptoms were observed previously in *C. roseus* plants inoculated with *X. fastidiosa* subsp. *pauca* (Monteiro *et al.*, 2001). Plants doubly-inoculated with *X. fastidiosa* and *C. flaccumfaciens* developed no disease symptoms (Fig. 2).

Specificity and sensitivity of primers for detection of C. flaccumfaciens

The specificity of primers Cf1 and Cf2 for *C. flaccumfa-*

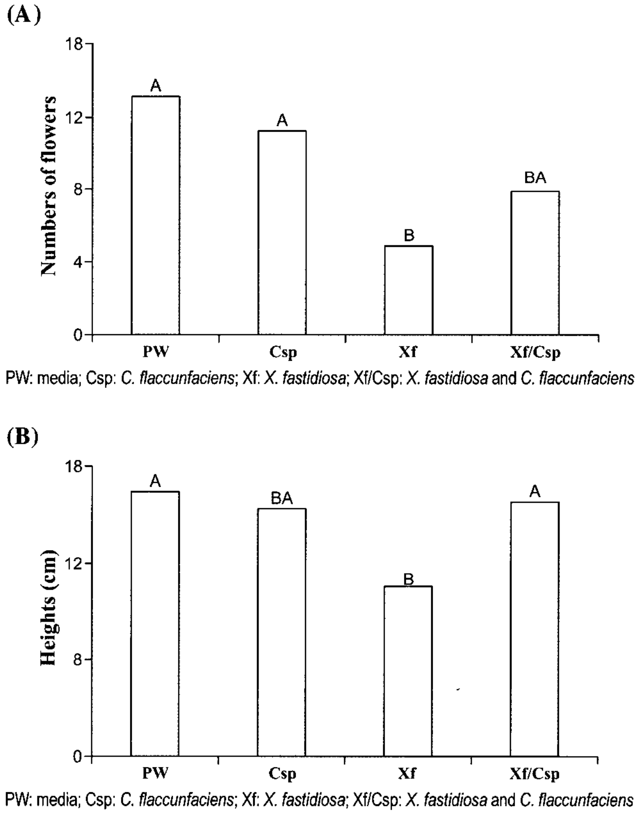

Fig. 1. (A) Number of flowers/plant produced by *C. roseus* and (B) height of plants 60 days post inoculation with *C. flaccumfaciens*, *X. fastidiosa* and *C. flaccumfaciens* and *X. fastidiosa* inoculated together. Treatments with the same letter are not different, as shown by Tukey's test at a significance level of 5%.

Fig. 2. Disease symptoms induced in *Catharanthus roseus* plants 60 days after inoculation with *X. fastidiosa* (right). A symptom-free plant doubly-inoculated *X. fastidiosa* and *C. flaccumfaciens* is also shown (left).

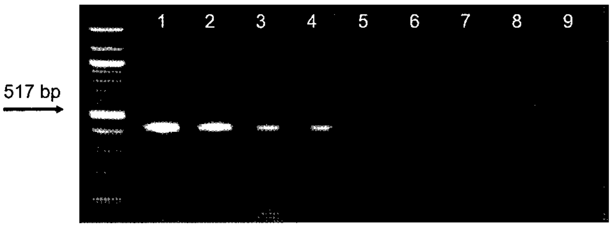

Fig. 3. Sensitivity of the PCR with the Cfl/Cf2 primer set for the detection of C. *flaccumfaciens* (Cfl). 100 bp DNA ladder. Lanes 1-8, 10-fold serial dilution of a single colony beginning with 10^8 CFU/ml in lane 1; the dilution series was plated in parallel to estimate the number of viable CFU/PCR assay. Lane 9 negative control (water). The PCR product is 436 bp.

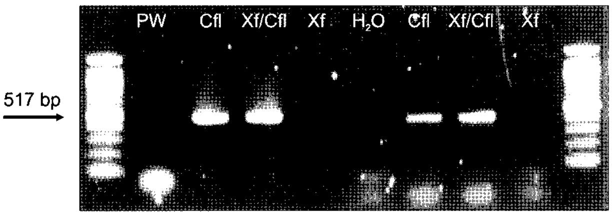

Fig. 4. Detection of *C. flaccumfaciens* in artificially inoculated *C. roseus* by PCR with the Cfl/Cf2 primer sets. PW, media control; Cfl, *C. flaccumfaciens*; Xf/Cfl, *X. fastidiosa* and *C. flaccumfaciens*, Xf, *X. fastidiosa*; H_2O, negative control.

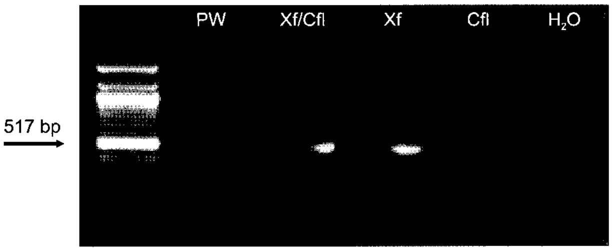

Fig. 5. Detection of *X. fastidiosa* in artificially inoculated *C. roseus* via PCR with the 272-1 int/272-2 int primer sets (Pooler and Hartung, 1995). PW, liquid media control; Cfl, *C. flaccumfaciens*; Xf/Cfl, *X. fastidiosa* and *C. flaccumfaciens*, Xf, *X. fastidiosa*; H_2O, negative control.

ciens was evaluated via PCR using bacterial DNA isolated from a large number of endophytic bacteria isolated from *C. sinensis*: *M. mesophilicum*, *M. extorquens*, *M. radiotolerans*, *M. zatamanii*, and *M. fujisawaense* (Araújo *et al.*, 2002), *Pantoea agglomerans* and *Enterobacter cloacae* (Andreote *et al.*, 2004), *Bacillus* sp. (Araújo *et al.*, 2001), as well as the phytopathogens *X. fastidiosa* and *Xanthomonas axonopodis* pv. *citri*. Extracts containing *Candidatus Liberibacter asiaticus* (Jagoueix *et al.*, 1997), the causal agent of citrus greening or huanglongbing disease, were also prepared from greenhouse-grown infected plants, and tested against these primers. The expected amplification products were detected only in the *C. flaccumfaciens* extracts (data not shown). The sensitivity of this primer set was estimated via standard PCR using serial dilutions of cultured bacteria. The predicted amplicons were observed through the 10^{-6} dilution, which contained 110 viable cells in the amplification reaction, based on the dilution plating which was conducted in parallel with the PCR reactions (Fig. 3).

Detection of C. flaccumfaciens and X. fastidiosa in C. roseus by PCR

Sixty days after the inoculation of *C. roseus* plants, *C. flaccumfaciens* was detected via PCR using the Cf1 and Cf2 primers (Fig. 4). *X. fastidiosa* was also specifically detected using primers 271-int and 272-int in extracts of *C. roseus* inoculated with *X. fastidiosa* (Fig. 5).

Discussion

X. fastidiosa reduced the number of flowers generated by *C. roseus* (Fig. 1A), reduced the height of *C. roseus* (Fig. 1B), and induced disease symptoms including stunting, leaf malformation, and wilting in *C. roseus* (Fig. 2). These symptoms are as previously described for *X. fastidiosa* subsp. *pauca* and *C. roseus* (Monteiro *et al.*, 2001). None of these symptoms were observed in plants inoculated with *C. flaccumfaciens*, although the *C. roseus* plants were colonized by *C. flaccumfaciens* as evidenced by PCR. When *C. flaccumfaciens* was inoculated into *C. roseus* simultaneously with *X. fastidiosa*, no disease symptoms developed, and the height of the plants was not significantly different from that of the non-inoculated controls. The number of flowers generated by our doubly- inoculated plants was an intermediate between the number produced by plants inoculated separately with either bacterium. Our data indicate that *C. flaccumfaciens* interacted with *X. fastidiosa* in *C. roseus,* and reduced the severity of the disease symptoms induced by *X. fastidiosa*. This may be attributable to the induction of systemic resistance by *C. flaccumfaciens,* as has also been suggested for cucumber plants inoculated with *Pseudomonas syringae* and *Erwinia tracheiphila* (Raupach and Kloepper, 1998). Alternatively, the results could be explained by a direct *in vivo* interaction between the bacteria. Antagonism between *C. flaccumfaciens* and *X. fastidiosa* was strongly indicated on the basis of the frequency of isolation of *C. flaccumfaciens* (Araújo *et al.*, 2002), in addition to the *in vitro* interactions between *X. fastidiosa* and *C. flaccumfaciens*, including the inhibition of the growth of *X. fastidiosa* by cell-free supernatants of nutrient medium in which *C. flaccumfaciens* had been grown (Lacava *et al.*, 2004). Consistent with these observations, three bacteriocins evidencing activity against *X. fastidiosa* have been recently described from *C. flaccumfaciens* (Cursino, 2005). Therefore, we interpreted our results as indicating an interaction between *C. flaccumfaciens* and *X. fastidiosa* in inoculated host plants under our controlled conditions. These results reinforce the idea suggested by Araújo *et al.* (2002) and Lacava *et al.* (2004), that this endophytic bacterium, which colonizes a niche similar to that of *X. fastidiosa*, could contribute to the reduction of CVC symptoms in the field. The Madagascar periwinkle, *C. roseus*, provides a convenient experimental plant by which the interactions of *X. fastidiosa* subsp. *pauca* and *C. flaccumfaciens,* as well as other endophytic bacteria, might be studied (Andreote *et al.*, 2006; Lacava *et al.*, 2006; Lacava *et al.*, 2007).

In a previous study *C. flaccumfaciens* was most frequently isolated from sweet orange trees that evidenced no symptoms of CVC, but were infected by *X. fastidiosa.* Using denaturing gradient gel electrophoresis, a high molecular weight G+C band was detected in extracts from such asymptomatic plants, but not in extracts of symptomatic plants (Araújo *et al.*, 2002). This is consistent with the presence of *C. flaccumfaciens* in these samples (Araújo *et al.*, 2002). The development of specific primers for the detection of the endophytic bacterium, *C. flaccumfaciens,* in plant tissues via PCR (Fig. 4) provides a much more convenient and specific method for the detection of this pathogen in plant tissues. *C. flaccumfaciens* was detected in *C. roseus* extracts 60 days after inoculation using the Cf1/Cf2 primer pair in a PCR assay. In a parallel experiment, in which both *C. flaccumfaciens* and *X. fastidiosa* were inoculated into *C. roseus*, both the endophyte and the pathogen were detected via PCR. These data demonstrate that *C. flaccumfaciens* has the ability to colonize plant tissues in the presence or absence of *X. fastidiosa*. This is a prerequisite for the use of this bacterium as a biocontrol agent. Additionally, in the case of biocontrol of *X. fastidiosa* and CVC disease, it would be desirable if *C. flaccumfaciens* could be transmitted by budwood, but this has yet to be determined. We are currently investigating the use of a quantitative PCR assay to characterize the interaction of these two bacteria *in vivo*. Periwinkle is an excellent experimental host for the study of interactions between *X. fastidiosa* and antagonistic bacteria, due to its small size and ease of growth in a greenhouse setting. These methods should be applicable to diseases induced by *X. fastidiosa* in a wide range of fruit and nut crops.

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