ENDOPHYTIC BACTERIA ASSOCIATED WITH SHARPSHOOTERS, INSECT VECTORS OF *XYLELLA FASTIDIOSA* SUBSP. *PAUCA*

Principal Investigator:
Paulo Teixeira Lacava
Institute of Natural Sciences
Federal University of Alfenas
Alfenas, MG, Brazil 37130-000
ptlacava@unifal-mg.edu.br

Co-Principal Investigators:
Cláudia Santos Gai
Dept. of Genetics
University of São Paulo
Piracicaba, SP 13400-970

João Lúcio Azevedo
Dept. of Genetics
University of São Paulo
Piracicaba, SP 13400-970

Cooperators:
João Roberto Spotti Lopes
Dept. of Entomology
University of São Paulo
Piracicaba, SP 13400-970

Welington Luiz Araújo
Dept. of Genetics
University of São Paulo
Piracicaba, SP 13400-970

Thomas Albert Miller
Dept. of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted September 20, 2009 to October 8, 2010.

ABSTRACT

Xylella fastidiosa (Xf) subsp. P*auca* causes citrus variegated chlorosis (CVC) disease in Brazil, resulting in significant production losses in the citrus industry. *Xf* is mainly transmitted by three species of sharpshooters (Hemiptera: Cicadellidae) in Brazil; *Dilobopterus costalimai* (Young), *Acrogonia citrina* Marucci & Cavichioli and *Oncometopia facialis* (Signoret). We identified bacterial communities associated with the heads of surface-sterilized insect vectors of *Xf* that were collected from CVC affected citrus groves in Brazil. Bacteria were isolated and analyzed by amplified ribosomal DNA restriction analysis (ARDRA) and sequencing, revealing the presence, among the most abundant genera, of the well-known citrus endophytes *Methylobacterium* spp. and *Curtobacterium* spp. Specific PCR systems for the detection of these genera indicated high frequencies of presence of these bacteria in sharpshooters. The remaining bacterial community was compared in distinct vector species and at different period of the year by denaturing gradient gel electrophoresis (DGGE), showing its responsiveness to the climate change over the year. These results represent a new basis for the knowledge about the interaction symbiotic-pathogenic bacteria inside insect vectors and provides a basis for further work on the biocontrol of phytopathogens like *Xf*.

LAYPERSON SUMMARY

We report the bacterial communities associated with the heads of surface-sterilized insect vectors of *Xylella fastidiosa (Xf)* that were collected from citrus variegated chlorosis (CVC) affected citrus groves in Brazil. Many aspects can influence the transmission of a pathogen by an insect vector such as the low concentration of *Xf* cells in the citrus plants and the low number of colonized vessels in affected plants. The interaction between different bacteria inside the insect foregut can also influence the transmission, as once inside the foregut, bacterial interaction, such as competition for nutrients, space and other complex interactions could occur. *C. flaccunfaciens* is a potential candidate for biological control of CVC because an antagonism between *C. flaccumfaciens* and *Xf* was strongly indicated *in vitro* and *in vivo* including inhibition of growth of *Xf* and reduced severity of the disease symptoms in the presence of this phytopathogen. The ability demonstrated by *C. flaccumfaciens* to colonize plant tissues in the presence of *Xf* and the reduction of disease symptoms caused by *Xf* 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 *Xf* as demonstrated in the present study, they fulfill another requirement of candidates for biological control of *Xf*.

INTRODUCTION

Brazil is the largest producer of citrus fruit in the world, also supplying most of the international market for concentrated orange juice. By 2005, the percentage had increased to 43%, and citrus variegated chlorosis (CVC) was present in all citrus growing regions of Brazil (Bové & Ayers 2008). The disease is caused by the xylem-limited gram-negative bacterial pathogen, *Xylella fastidiosa (Xf)* subsp. *Pauca* (Hartung et al. 1994). Endophytic microorganisms live within plants without causing apparent harm to the host. We have studied the possible use of endophytes as vehicles to control both phytopathogens and insects (Azevedo et al. 2000). Endophytes colonize ecological niches similar to that of phytopathogens (Gai et al 2009a), which gives them access as possible candidates as biocontrol agents. In citrus, several endophytic bacteria were isolated and *Methylobacterium* spp. and *Curtobacterium flaccumfaciens* were further determined as the main endophytic species interacting with *Xf* (Araújo et al. 2001). These species vary in population density when CVC-affected and asymptomatic plants are compared. Later, Lacava et al. (2004) reported that the growth of *Xf* was inhibited by endophytic *C. flaccumfaciens* and stimulated by *Methylobacterium* sp. A similar effect was demonstrated by the reduced severity of the *Xf* colonization in plants priory colonized by *C. flaccumfaciens* (Lacava et al. 2007a). In Brazilian citrus groves, *Dilobopterus costalimai*, *Oncometopia facialis* and *Acrogonia citrina* are the most common sharpshooters found. The transmission efficiency of bacteria is a measure of the ability to successfully acquire bacteria from an affected plant and transmit to healthy ones. The efficiency of *Xf* transmission leafhopper species ranges from 0 to 100%, however the lower values are found when transmission among citrus plants is considered. The transmission rates for the main species associated with CVC vary from 1% to 5% (Redak et al. 2004). Many aspects can influence the transmission of a pathogen by an insect vector such as the low concentration of *Xf* cells in the citrus plants (Almeida & Purcell 2003) and the low number of colonized vessels in affected plants. The interaction between different bacteria inside the insect foregut can also influence the transmission, as once inside the foregut, bacterial interaction, such as competition for nutrients, space and other complex interactions could occur.

OBJECTIVES

The aims of this work were:

1. Access the bacterial population associated with the main sharpshooters responsible for the transmission of *Xf* in citrus.
2. Evaluate the diversity of heterotrophic bacteria by amplified ribosomal DNA restriction analysis (ARDRA).
3. Compare the bacterial community colonizing insects from distinct species and collected from citrus at distinct period of the year by denaturing gradient gel electrophoresis (DGGE).

RESULTS

A total of 17.230 bacterial colonies were counted in plating from different species and at distinct sampling time. These colonies were classified into three distinct morphological groups. The concentration of bacteria (CFU/insect head) belonging to each morphological group. The populations of group G1 (*Methylobacterium*) remained constant for *O. facialis* and *D. costalimai* in March and May, decreasing in June. From *A. citrina* the presence of G1 bacteria decreased from May. Group 2 (*Curtobacterium*) was higher in March for *O.. facialis* and *D.. costalimai*, decreased in May and was undetected in *D. costalimai* in June and group G3 other bacteria. The population of *Curtbacterium* spp. in *A. citrina* remained constant during the period of analysis.. A subsample of the total number of colonies obtained (120 colonies) was subjected to the genotypic characterization by ARDRA. In total, 16 cleavage patterns were observed, determining the ribotypes constituting the heterotrophic bacterial communities from sharpshooter heads. Among these ribotypes, the colonies from the two targeted groups, *Curtobacterium* spp and *Methylobacterium* spp., has revealed a fidelity in the pattern of the 16S rRNA gene cleavage, allowing this approach to measure the proportion of these bacteria within the sampled colonies (**Figure 1**). Other bacterial haplotypes were characterized randomly and sequences have shown the affiliation of the isolates to the genera *Bacillus*, *Brachybacterium*, *Brevibacillus*, *Brevundimonas*, *Nocardia*, *Paenibacillus*, *Pseudoclavibacter*, *Rhodococcus*, *Sphingomonas* and *Staphylococcus*.. DNA samples directly extracted from insect heads were used for specific amplification of DNA from bacteria affiliated with the genera *Curtobacterium* and *Methylobacterium*. The number of positive samples was unique to each sharpshooter species.. The detection of DNA from *Curtobacterium* spp. was positive in 89.6% of the samples from *O. facialis*, 39.1% of *D. costalimai* and 70% of *A. citrina*. In a similar analysis for *Methylobacterium* spp. the numbers of positive PCR were 51.7% for *O. facialis*, 8.7% for *D. costalimai* and 20% of the *A. citrina*. Another point found in this work was the comparative phylogeny of isolates from both targeted groups with endophytic isolates obtained in previous works. The sequences UIA-12-R, UIA-13-R, UIA-14-R and ER1.6 are *Curtobacterium flaccumfaciens* and AR1.6/11, SR1.6/2 and SR3/27 are *Methylobacterium* species isolated from citrus. Sequences from DGGE bands were also included in the clustering and it was possible to observe a clustering of some sharpshooter-associated bacteria with endophytes for both bacterial groups; *Curtobacterium* spp. (**Figure 2**) and *Methylobacterium* spp (**Figure 3**). Hence, besides the partial grouping of vector bacteria and endophytes, clusters containing only bacteria from one source were found. The DGGE analysis showed variable fingerprinting according to the period when insects were collected. The first separation was observed for samples collected in June, with a further sub-branching for samples from May and March (**Figure 4**). These variations were based on changes in the abundance of bands and also in the intensity of similar bands found in distinct samples..

CONCLUSIONS

The bacterial communities associated with vector insects and plants differ in abundance through the yearly season.. Endophytic bacteria could influence disease development by reducing the insect transmission efficiency due to competition with pathogens in host plants and also in insect foreguts. In addition the bacterial communities in the foregut of insect vectors of *Xf* changed with time, environmental conditions and in different insect species. However, members of the genus *Curtobacterium* were consistently detected in the sharpshooters foregut and are commonly isolated from the xylem of citrus plants, and because of this, they may be candidates for biological control. Since members of the genus *Curtobacterium* were consistently detected in the insect vectors of *Xf* as demonstrated in the present study, they fulfill another requirement of candidates for biological control of *Xf*, i.e. they can colonize both the insect vectors of *Xf* and citrus plants.

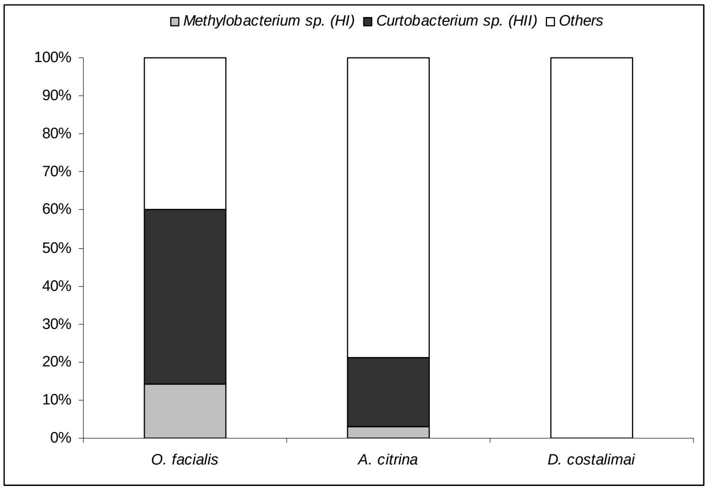

Figure 1. Proportion of the ARDRA ribotypes I and II (cleavage made with endonuclease *Alu*I), related to *Methylobacterium* spp and, *Curtobacterium* spp. respectively.

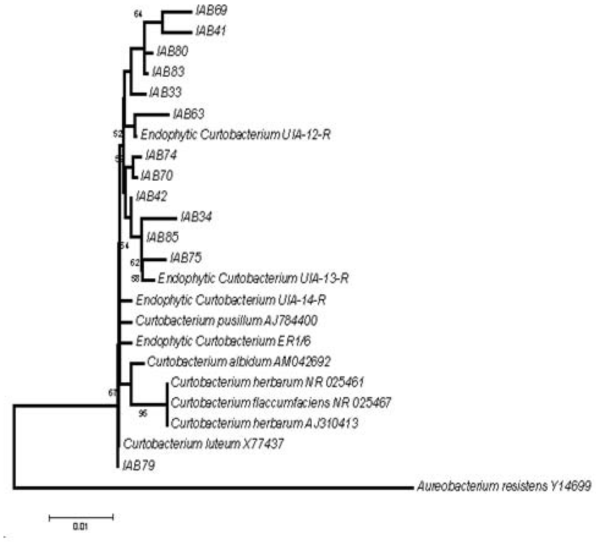

Figure 2. Neighbor-joining clustering among sharpshooters and plant associated bacteria sequences with types strains of *Curtobacterium* spp. A partial fragment of the 16SrRNA gen was used for comparisons.

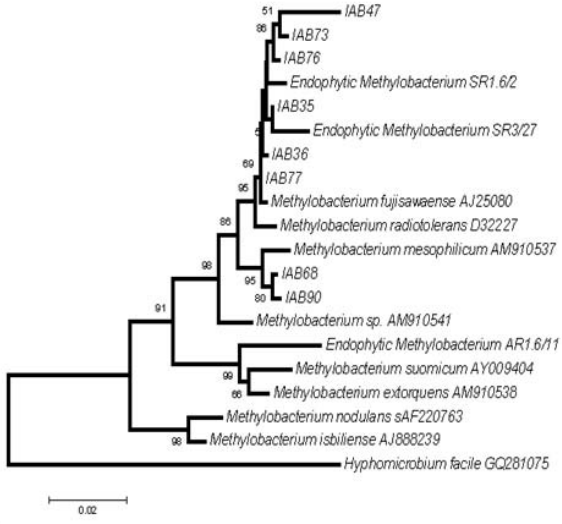

Figure 3. Neighbor-joining clustering among sharpshooters and plant associated bacteria sequences with types strains of *Methylobacterium* spp. A partial fragment of the 16SrRNA gen was used for comparisons.

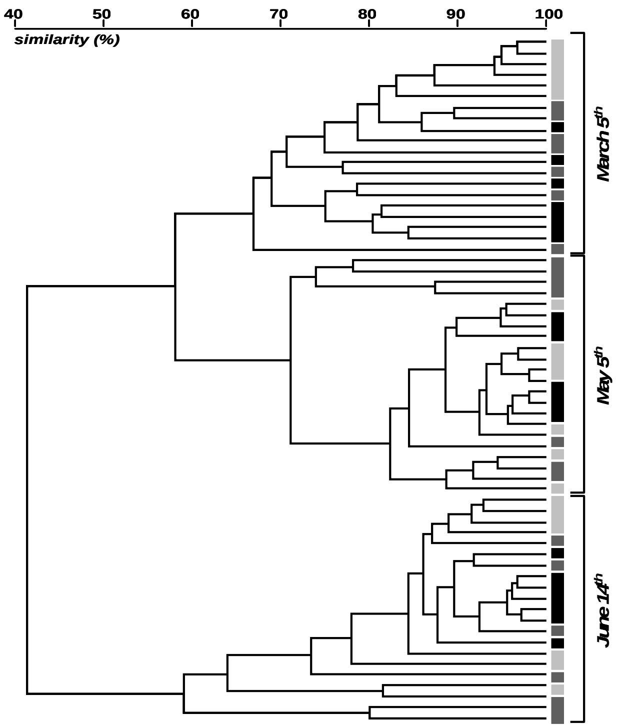

Figure 4. Clustering of DGGE fingerprinting generated by UPGMA based on the similarity calculated by the Pearson correlation. Sharpshooters species are represented by dark gray bars (*O. facialis*), light gray (*A. citrina*) and black bars (*D. costalimai*).

REFERENCES CITED

Almeida, R.P, and Purcell, A.H. 2003. Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). J. Econ. Entomol. **96:** 264–271.

Araújo, W.L., Maccheroni Jr, W., Aguilar-Vildoso, C.I., Barroso, P.A.V., Saridakis H.O., and Azevedo, J.L. 2001. Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Can. J. Microbiol. **47:** 229–236.

Azevedo, J.L., Maccheroni Jr., W., Pereira, J.O., and Araújo, W.L. 2000. Endophytic microorganisms: A review on insect control and recent advances on tropical plants. EJB: Electronic Journal of Biotechnology. 15 April 2000. http://www.ejb.org/content/vol3/issue1/full/3/4.

Bové, J.M., and Ayres, A.J. 2008. Etiology of three recent diseases of citrus in São Paulo State: Sudden death, variegated chlorosis and huanglongbing. IUBMB Life. **59:** 346–354.

Gai, C.S., Lacava, P.T., Maccheroni Jr, W., Glienke, C., Araújo, W.L., Miller, T.A., and Azevedo, J.L. 2009a. Diversity of endophytic yeasts from sweet orange and their localization by scanning electron microscopy. J. Basic Microb. **49:** 441–451.

Hartung, J.S., Beretta, J, Brlansky, R.H., Spisso, J., and Lee, R.F. 1994. Citrus variegated chlorosis bacterium: axenic culture, pathogenicity, and serological relationships with other strains of *Xylella fastidiosa*. Phytopathology. **84:** 591–597.

Lacava, P.T., Araújo, W.L., Marcon, J., Maccheroni Jr, W., and Azevedo, J.L. 2004. Interaction between endophytic bacteria from citrus plants and the phytopathogenic bacteria *Xylella fastidiosa*, causal agent of citrus-variegated chlorosis. Lett. Appl. Microbiol. **39:** 55–59.

Lacava, P.T., Li, W.B., Araújo, W.L., Azevedo, J.L., and Hartung, J.S. 2007. The endophyte *Curtobacterium flaccumfaciens* reduces symptoms caused by *Xylella fastidiosa* in *Catharanthus roseus*. J. Microbiol. 45: 388–393.

Redak, R.A., Purcell, A.H., Lopes, J.R.S., Blua, M.J., Mizell III, R.F., and Andersen, P.C. 2004. The biology of xylem fluid feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. Annu. Rev. Entomol. **49:** 243–270.

FUNDING AGENCIES

Funding for this project was provided by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Foundation for Research Support of the State of São Paulo; n. proc. 06/55494-4).