MANIPULATION OF ENDOPHYTIC BACTERIA FOR SYMBIOTIC CONTROL OF *XYLELLA FASTIDIOSA*, CAUSAL AGENT OF CITRUS VARIEGATED CHLOROSIS

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ABSTRACT

Methylobacterium mesophilicum, originally isolated as an endophytic bacterium from citrus plants, was genetically transformed to express GFP (Green Fluorescent Protein). 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* (*Xf*p), was verified by insects feeding from fluids containing the GFP bacterium, 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 *Xf*p 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 transmission of *Xf*p, the bacterial pathogen causing Citrus variegated chlorosis, by insect vectors.

INTRODUCTION

Citrus variegated chlorosis (CVC) is a disease of sweet orange [*Citrus sinensis* (L.)] trees caused by *Xylella fastidiosa* subsp. *pauca* (*Xf*p) (Schaad et al. 2004). The disease continues to increase in severity, with 35% of the sweet orange trees in São Paulo, Brazil currently showing loss of yield. Endophytes 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). Many endophytic bacteria have been isolated from sweet orange (Araújo et al. 2002) but our research has focused on the genus *Methylobacterium*, which occupies the same ecological niche as *Xf*p in the xylem vessels of plants (Araújo et al., 2002; Lacava et al. 2004). The genus *Methylobacterium* is described as a main player in the interaction between the endophytic community and the pathogen *Xf*p (Araújo et al., 2002; Lacava et al. 2004). Cicadellinae leafhoppers, or sharpshooters, are considered xylem-fluid feeders (Young et al., 1968) and a clear association has been observed between their xylem-feeding habit and ability to transmit *Xf* (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 nurseries 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 (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. Bacteria of the genus *Methylobacterium* are known to occupy the same niche as *Xf*p 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.

OBJECTIVES

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 *Xf*p.

RESULTS

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 twenty generations in 120 h, 95%, decreasing the stability on 0,25 % per generations approximately (Fig 1). The original bacterial community of *B. xanthophis* was comprised of five groups: *Methylobacterium* sp., Actinomycetes, *Curtobacterium* sp., *Sphingomonas* sp. and *Bacillus* sp. (Fig 2). The *Methylobacterium* genus occurred naturally in *B. xanthophis*. 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 3). The insects used in transmission experiments were monitored for the presence of the SRGFP strain 24 hours after acquisition. Bacteria isolation from insect heads revealed the average population density of *M. mesophilicum* of 4.6 10^3 CFU/insect $head^{-1}$, suggesting that the bacteria is capable of colonizing the foregut of the insect as it was not washed way by the sap flux. The ability of the sharpshooter *B. xanthophis* in transmitting *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 $CFU.g^{-1}$ of fresh tissue. 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 by the same way it transmits *Xf,* with an efficiency of transmission of 13.3%.

CONCLUSIONS

The colonization and transmission of *M. mesophilicum* in the same host tissues and insect vector of *Xf*p 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.

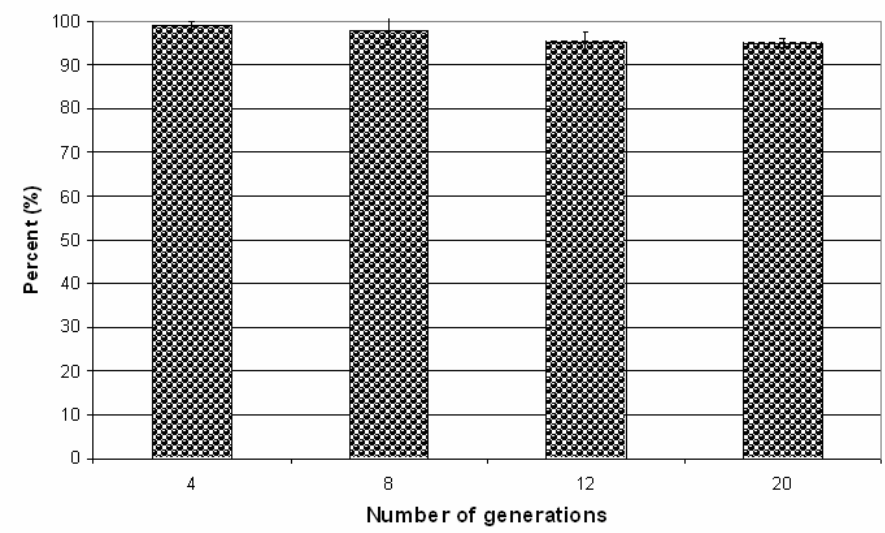

Figure 1. Plasmidial stability of pCM88 on *Methylobacterium mesophilicum*. The percent was obtained collecting random samples after 24, 48, 72 and 120 hours of culture cells of SRGFP strain growing without antibiotic tetracycline.

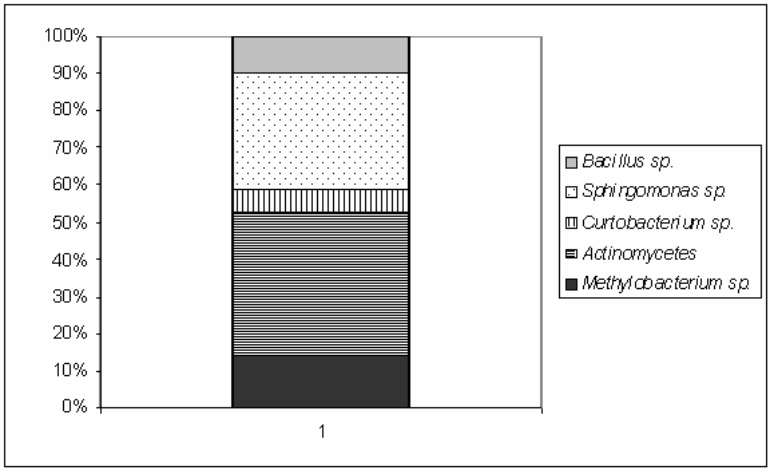

Figure 2. Most frequently group of bacteria isolated from *Bucephalogonia xanthophis*.

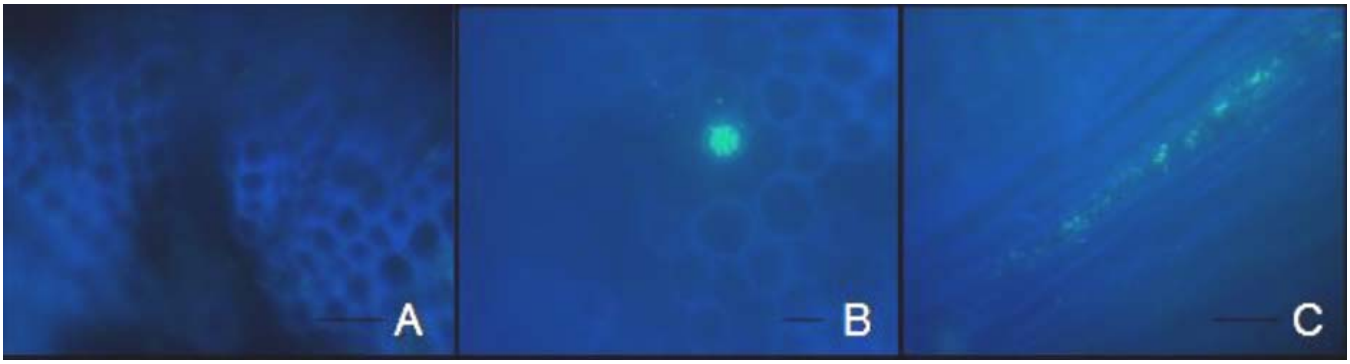

Figure 3. Fluorescent microscopy evidencing the ecological niche occupied by endophyte *Methylobacterium mesophilicum* in *Catharanthus roseus* plants 45 days after inoculation. A) Xylem vessels of a control plant, Scale bar = 10µm; B) Colonized xylem vessel, Scale bar = 5µm; C) Xylem vessels longitudinal cut, Scale bar = 10µm.

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