

# Transmission Efficiency of *Xylella fastidiosa* subsp. *pauca* Sequence Types by Sharpshooter Vectors after In Vitro Acquisition

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## ABSTRACT

*Xylella fastidiosa* subsp. *pauca* is genetically diverse and has many vector species. However, there is limited information on vector specificity and efficiency for different sequence types (STs) within the pathogen subspecies. Both STs of *X. fastidiosa* and vectors differ in their associations with plants; therefore, assessment of vector competence should include the standardized vector acquisition ability of bacteria from artificial diets. This work aimed to adapt and validate an in vitro acquisition system for strains of *X. fastidiosa* that cause citrus variegated chlorosis, and to compare the transmission efficiency of STs of subsp. *pauca* by different species of sharpshooter vector. First, acquisition and transmission of ST13 by *Bucephalagonia xanthophis* and *Macugonalia leucomelas* was tested using an artificial diet with bacteria grown on minimum defined medium (*X. fastidiosa* medium) with or without

1% galacturonic acid (GA). Subsequently, four sharpshooter species (*B. xanthophis*, *M. leucomelas*, *M. cavifrons*, and *Sibovia sagata*) were compared as vectors of ST13 acquired from artificial diets, and four STs of subsp. *pauca* (11, 13, 65, and 70) were tested for acquisition and transmission by *M. leucomelas*. The artificial system allowed efficient acquisition and transmission of ST13 to plants, with no differences between the media tested. ST13 was transmitted more efficiently by *B. xanthophis* and *M. leucomelas* when compared with *M. cavifrons* and *S. sagata*. Different STs influenced acquisition and transmission rates by *M. leucomelas*. The differences in vector competence, despite the standardized acquisition system, suggest that ST–vector foregut or vector–plant interactions may influence bacterial acquisition, retention and inoculation by the insect.

*Xylella fastidiosa* (Wells et al. 1987) is a vector-borne xylem-limited bacterium that colonizes many plant species and causes severe diseases in some agricultural crops such as Pierce’s disease in grapevines (PD), citrus variegated chlorosis (CVC), coffee leaf scorch, plum leaf scald, and olive quick decline syndrome (Hopkins and Purcell 2002; Saponari et al. 2014). Due to its restricted location in the xylem vessels of plants, the bacterium depends on transmission by xylem sap-feeding insects, including sharpshooter leafhoppers (Hemiptera: Cicadellidae: Cicadellinae) and spittlebugs (Hemiptera: Cercopoidea) (Redak et al. 2004).

Compared with other vector-borne phytopathogenic bacteria, *X. fastidiosa* is unique in its mode of transmission and wide vector range (Almeida and Nunney 2015; Redak et al. 2004). After acquisition from plants, cells of *X. fastidiosa* attach to specific portions of the foregut of vectors (precibarium and cibarium), where multiplication and biofilm formation occurs (Almeida and Purcell

2006), allowing persistent transmission by adults but no transstadial passage in nymphs (Almeida and Purcell 2003; Purcell and Finlay 1979). Transmission is noncirculative, with no latent period between acquisition and inoculation (Purcell and Finlay 1979).

The rather wide vector range suggests low vector specificity, which appears to be guided mainly by the xylem-sap-feeding habit of demonstrated vectors (Almeida and Nunney 2015; Purcell 1989). However, not all xylem-sap-feeding species tested for transmission of almond and citrus strains of *X. fastidiosa* were confirmed as vectors, despite being tested in large numbers (Lopes and Krugner 2016; Lopes et al. 2009), although that may be due to vector–plant interactions. In addition, there is marked variation in transmission rates among vector species and strains of *X. fastidiosa*. In the case of PD in California, for example, *Graphocephala atropunctata* Signoret (Hemiptera: Cicadellidae) is a very efficient vector of grape strains of *X. fastidiosa*, with transmission rates >90% (Hill and Purcell 1995), compared with only 20 to 30% by *Homalodisca vitripennis* Germar (Hemiptera: Cicadellidae) (Daugherty and Almeida 2009).

A comparison of transmission rates of *X. fastidiosa* causing CVC by 13 different vector species ranged from <1 to 30% (Lopes and Krugner 2016; Marucci et al. 2008). Factors related to vector–plant and *X. fastidiosa*–plant interactions have been proposed to explain the reported variations in transmission efficiency. The interaction may be associated with higher populations of *X. fastidiosa* in infected (source) plants (Hill and Purcell 1997), with a vector preference for infected plants (Daugherty et al. 2011; Marucci et al. 2005) and for parts of the infected plants with higher bacterial population for acquisition (Daugherty et al. 2010). Sharpshooter feeding preference is influenced by various factors, particularly the nutritional state of the host plant, which may vary seasonally, or even within a single day (Mizell et al. 2008).

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\*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures are published online.

Other aspects directly related to vector–*X. fastidiosa* interactions, including bacterial adhesion, propagation, and biofilm formation on the foregut of the vector, may be involved in the determination of vector specificity and transmission efficiency. Attachment of the bacterium to the cuticular lining of the foregut is mediated by adhesion proteins present in the bacterial cell wall, which interact with the insect cuticular surface; chitin is used as a carbon source and leads to changes in gene expression associated with biofilm formation (Killiny and Almeida 2009a; Killiny et al. 2010; Labroussaa et al. 2017). Mutants of *X. fastidiosa* for adhesion, chitinase, and quorum-sensing genes show impaired biofilm formation and transmission by sharpshooters (Killiny and Almeida 2013; Labroussaa et al. 2017). Therefore, it is plausible that differences in vector competence reported in the literature may be associated with genetic variation among strains of *X. fastidiosa* or vector species that influence bacterial adhesion or propagation in the vector.

There is considerable genetic and phenotypic variability within *X. fastidiosa*, which is taxonomically subdivided into subspecies (recent work proposes five subspecies: *fastidiosa*, *multiplex*, *pauca*, *sandyi*, and *morus*) (Nunney et al. 2014). Even within subspecies, there is variation in nucleotide sequence that allows further grouping of strains into sequence types (STs) using a multilocus sequence typing (MLST) approach. STs are particularly numerous in *X. fastidiosa* subsp. *pauca* in South America (Coletta-Filho et al. 2017). Strains and STs of *X. fastidiosa* and vectors might differ in their abilities to interact with plants; therefore, studies aimed at comparing bacterial acquisition and retention in vectors need to be performed with acquisition of bacteria from artificial diets, thus removing the potentially confounding effect of source plant interactions. Killiny and Almeida (2009b) established an artificial diet system for acquisition of *X. fastidiosa* subsp. *fastidiosa* by sharpshooters that excluded the use of source plants. By using the in vitro acquisition system, they demonstrated that the product of pectin degradation serves as a molecular cue that induces transcriptional changes and leads to vector colonization and subsequent transmission of *X. fastidiosa*.

The application of the artificial diet system for transmission studies with other subspecies of *X. fastidiosa* needs validation. There are genetic and biological differences among the subspecies in addition to the vector species involved in the transmission process. Therefore, this study aimed to adapt and validate an in vitro acquisition system for sharpshooter vectors of strains of *X. fastidiosa* subsp. *pauca* causing CVC. A second objective was to apply the established technique to investigate vector competence in acquisition and transmission of different STs of subsp. *pauca* by the sharpshooters.

## MATERIALS AND METHODS

**Insect vector collection and rearing.** Four laboratory-reared sharpshooter species (*Bucephalognia xanthophis* Berg, *Macugonalia cavifrons* Stal, *M. leucomelas* Walker, and *Sibovia sagata* Signoret (Hemiptera: Cicadellidae)) were used in the transmission assays. Approximately 50 to 60 individual adults of these species were collected from plants of *Lagerstroemia indica* L. (Lythraceae), *Duranta repens* L. (Verbenaceae), *Vernonia condensata* Baker (Asteraceae), and *Hibiscus* spp. (Malvaceae) growing in Piracicaba, São Paulo State, Brazil. The collected insects were placed within separate rearing cages (32 by 32 cm [base] by 50 cm [height]) covered with antiaphid screen and containing an appropriate host plant for oviposition and development, in a greenhouse equipped with a pad-fan cooling system and a heater activated by a thermostat for temperature control (25 ± 5°C, with a photoperiod of 12 h). Plants of *V. condensata* were used for rearing *B. xanthophis* and *S. sagata*, whereas plants of *Ocimum basilicum* L. (Lamiaceae) were used for rearing *M. leucomelas* and *M. cavifrons*. Plants of *V. condensata* were obtained from 40-cm stem cuttings planted in 3-liter plastic pots (15 by 20 cm [height by diameter]) containing a potting mix composed of shredded pine bark, peat, and expanded

vermiculite (Tropstrato HT Vida Verde; Mogi Mirim, São Paulo, Brazil). Seed of *O. basilicum* (ISLA PRO Seeds, Porto Alegre, Rio Grande do Sul, Brazil) were planted in Styrofoam trays and transplanted to 1.2-liter pots (11 by 14 cm [height by diameter]) using the same potting mix. The plants of *V. condensata* and *O. basilicum* were grown in a vector-proof greenhouse covered with antiaphid screen (25 ± 5°C with a photoperiod of 12 h) and fertilized twice a week via irrigation water with ammonium nitrate (33% N, 1% K) at 0.08 g/liter and calcium nitrate (15.5% N, 18% Ca) at 0.91 g/liter; and once a week with magnesium sulfate (1% K<sub>2</sub>O, 11.8% S, 9% Mg) at 0.33 g/liter, potassium nitrate (12% N, 43% K<sub>2</sub>O, 1% S, 1% Mg) at 0.36 g/liter, monoammonium phosphate (12% N, 61% P<sub>2</sub>O<sub>5</sub>) at 0.14 g/liter, iron (5% Fe ortho-ortho, 6% Fe chelated EDDHA) at 0.09 g/liter, copper sulfate (24% Cu, 1% S) at 0.03 g/liter, and zinc sulfate (20% Zn, 10% S) at 0.02 g/liter. The plants were offered to the insects when the stem (*O. basilicum*) or young shoots (*V. condensata*) were approximately 20 cm long (6 to 7 weeks after planting). To ensure that the insects used in the experiments were free of *X. fastidiosa*, the adults were removed from the cages after 2 weeks, leaving only the eggs and nymphs on the plants. First-generation sharpshooters that fed on *V. condensata* (a previously proven nonhost plant of *X. fastidiosa*) (Marucci et al. 2003) were used in the experiments. For the sharpshooters that were placed on plants of *O. basilicum*, the newly hatched nymphs were transferred to healthy plants from the same species, using only the second-generation insects for the experiments.

**Plants used for transmission assays.** Healthy seedlings of *Catharanthus roseus* L. (Apocynaceae) were used as test plants for inoculation in the transmission assays, because this species is known to be a host of different strains of *X. fastidiosa* (Purcell and Saunders 1999), grows fast from seed, and shows symptoms earlier than citrus (Monteiro et al. 2001). Seed collected from *C. roseus* in Piracicaba, São Paulo were planted in Styrofoam trays and transplanted to 1.2-liter pots (11 by 14 cm [height by diameter]) using the same potting mix, vector-proof greenhouse, and fertilization described above. The plants were inoculated at 10 to 12 weeks after sowing (2 weeks after transplanting), when the stem was approximately 10 cm long. Inoculated plants were sprayed with dimethoate (Perfekthion; BASF S.A., Guaratinguetá, SP, Brazil) and kept in a separate vector-proof greenhouse (25 ± 5°C, with a photoperiod of 12 h) until the infection of *X. fastidiosa* could develop to a detectable level using polymerase chain reaction (PCR).

**Strains of *X. fastidiosa*.** Five strains representing four different STs of *X. fastidiosa* subsp. *pauca* were used in the experiments. The strains were isolated from citrus with symptoms of CVC (T-19/C13, representing ST11; 9a5c and U24d, both classified as ST13; and BS1, representing ST65) in the State of São Paulo, and from hibiscus (Hib4, representing ST70) in Brasília, Federal District, Brazil (Coletta-Filho et al. 2017). After primary isolation, all isolates were triple cloned, typed by MLST, and stored at –80°C at the Sylvio Moreira Center of Citrus (Instituto Agrônômico), Cordeirópolis, São Paulo, Brazil. When needed, strains were recovered on Periwinkle wilt gelrite (PWG) (Hill and Purcell 1995), and the colonies cultured at 28°C in the dark for 15 days.

**Evaluation of the test plant *C. roseus* as a host for STs of *X. fastidiosa*.** The ST 9a5c (ST13) was the only CVC strain previously shown to infect *C. roseus* (Monteiro et al. 2001); therefore, we mechanically inoculated plants of *C. roseus* with the other strains representing STs 11, 13, 65, and 70 to confirm their ability to colonize this host. For inoculum production, the strains were grown on PWG medium (two to three transfers) at 28°C in the dark for 7 to 10 days. Colonies were suspended in phosphate-buffered saline (PBS) using a platinum loop to agitate the colony surface and to obtain a turbid suspension, with a concentration ranging from 10<sup>7</sup> to 10<sup>8</sup> CFU/ml. The cell suspensions of each isolate were inoculated using the pinprick method (Hopkins 1985) at two points on the stem of 9 or 10 plants of *C. roseus*, delivering 7 µl of suspension per inoculation point. At 60 days after

inoculation, four or five leaves close to the inoculation site were sampled for detection of *X. fastidiosa* by conventional PCR, using the RST31/RST33 primer set (Minsavage et al. 1994). The percentage of positive plants in each treatment were analyzed using a  $\chi^2$  test and generalized linear models (GLM) using the package *stats* in R software (R Core Team 2017). A GLM was used because the data did not meet the assumptions for a linear regression analysis; it was not normally distributed, and had heterogeneity of variance. A Shapiro-Wilk test was performed to analyze normality. Tukey's honestly significant difference (HSD) means separation ( $\alpha = 0.05$ ) was used for multiple comparisons when significant effects of treatments were detected, using the *multcomp* package in R (Hothorn et al. 2008).

**Test of artificial diet conditions to deliver cell suspensions of *X. fastidiosa* to vectors.** The citrus strain 9a5c (ST13) was chosen as it is the type strain of *X. fastidiosa* subsp. *pauca* and is widely used in research. The bacterial colonies were scraped from the PWG medium and suspended in PBS as previously described; the resulting suspension was plated on a minimum defined medium for this bacterium (*X. fastidiosa* medium [Xfm]), which was tested in two ways: (i) solid Xfm (Almeida et al. 2004) and (ii) solid Xfm plus 1% galacturonic acid (GA) (Killiny and Almeida 2009b). After incubation at 28°C in the dark for 10 to 12 days, the colonies on the Xfm and Xfm+GA media were scraped and suspended in a liquid artificial diet (0.7 mM L-glutamine, 0.1 mM L-asparagine, and 1 mM sodium citrate, pH 6.4) (Killiny and Almeida 2009b), with bacterial concentration adjusted to  $10^8$  CFU/ml (optical density at 600 nm = 0.6 nm).

The diet containing bacterium was offered to the sharpshooter species *B. xanthophis* and *M. leucomelas* through a membrane feeding system, mounted on clear plastic tube 1 cm in diameter and cut in approximately 3-cm sections. Each tube section was covered with a stretched parafilm membrane (Parafilm; Bemis, Oshkosh, WI) at the upper end, onto which was deposited a 35- $\mu$ l suspension of the liquid diet + *X. fastidiosa*, and which was covered by another layer of parafilm in order to form a diet sachet. The sharpshooters were introduced through the lower end of the tube into the membrane feeding system (1 individual per tube), which was subsequently closed with a stopper, and the insects were allowed an acquisition access period (AAP) of 6 h under fluorescent light (150 W) at  $25 \pm 2^\circ\text{C}$ . The negative control consisted of sharpshooters exposed to the artificial diet without *X. fastidiosa*.

After the AAP, the number of dead insects in each treatment was counted and the surviving individuals of each species were grouped by treatment (Xfm, Xfm+GA, and the control), and maintained on plants of *V. condensata* for 24 h. *V. condensata* is not a host for *X. fastidiosa*; therefore, the process allows washing of ingested bacteria that may not have attached to the vectors. Subsequently, insects from each treatment were redistributed into groups containing one to three individuals (depending on the mortality during the AAP and gut-washing period) and confined on 12 healthy plants of *C. roseus* for an inoculation access period (IAP) of 72 h, using rectangular (5.5 by 4.0 cm) clear plastic hinged cages (model G320; Gary Plastic Packaging Corp., New York) with a circular opening covered with antiaphid screen for ventilation. The insect mortality was recorded every 24 h during the IAP to verify the suitability of the test plant (*C. roseus*) for the sharpshooter species. Both the IAP and the previous period on *V. condensata* were conducted under controlled conditions ( $25 \pm 2^\circ\text{C}$  with a 14-h photoperiod under fluorescent light [150 W]).

The experiment was a complete randomized block design with fixed effects of block, sharpshooter species, and treatment (culture medium). However, due to experiment restrictions, block was repeated temporally on three occasions between May and September 2017. We considered two sharpshooter species and three levels of media (liquid diet + *X. fastidiosa* suspension from cultures grown on Xfm or Xfm+GA, and a diet without *X. fastidiosa* [control]). In the experiment, each level of species was crossed with each level of

media. Furthermore, each treatment was replicated four times within a block. Therefore, each block had 24 experimental units (four replications  $\times$  two levels of species  $\times$  three levels of medium).

**Analysis of acquisition and transmission efficiencies.** Acquisition rates were determined based on the percentage of individuals from each treatment that acquired *X. fastidiosa* per block. A model was developed to estimate transmission probabilities by single insects in this experiment as heterogenous groups (one to three individuals) were used to inoculate test plants. Let  $z_{ijk}$  be the response variable that assumes values of 1 if the plant is infected or 0 otherwise at  $i$ th block,  $j$ th species, and  $k$ th treatment. Thus,  $Z_{ijk} \sim \text{Bernoulli}(\pi_{ijk})$  is a particular case of binomial distribution, where  $\pi_{ijk} = P(Z_{ijk} = 1)$ . However, to estimate this probability, we considered a weight factor that is given by:

$$w_{ijk} = (\text{number of insects that acquired the bacterium} / \text{total number of insects}) \in [0, 1] \quad (1)$$

The linear predictor that incorporates the information regarding the block, species, and treatment weighted by  $w_{ijk}$  is given by:

$$\eta_{ijk} = \beta_0 + \beta_{1i}\text{Block}_i + \beta_{2j}\text{Species}_j + \beta_{3k}\text{Treatment}_k + \gamma_{jk}(\text{Species:Treatment})_{jk} + \text{offset}(w_{ijk}) \quad (2)$$

where  $\beta_0$  is the intercept;  $\beta_{1i}$ ,  $\beta_{2j}$ , and  $\beta_{3k}$  are the main effects of the  $i$ th block ( $i = 1, 2, 3$ ),  $j$ th species ( $j = 1, 2$ ), and  $k$ th treatment ( $k = 1, 2, 3$ ), respectively;  $\gamma_{jk}$  is the interaction effect between  $j$ th species and  $k$ th treatment, and  $w_{ijk}$  is a known term that is included as offset. We used the likelihood procedure to estimate the parameters of the models. The model selection was based on the likelihood-ratio test (difference of the deviances) for nested models with  $\alpha = 0.05$ .

The acquisition rates and estimated probabilities of transmission were checked for normality using the Shapiro-Wilk normality test. Because this assumption of normality was not met, GLM was used to analyze the data by two-way analysis of deviance with a logit link function using the package *stats* in R software (R Core Team 2017). The factors considered in the linear predictor were block, species, culture medium, and the interaction between species and culture medium. If significant main or interaction effects were observed, a multiple comparison of means was performed with Tukey's HSD test using the *multcomp* package (Hothorn et al. 2008).

**Analysis of mortality rates.** To analyze insect mortality, we used a logistic regression model, in which sharpshooter species and treatment (diet) were considered as fixed factors in the linear predictor. Variable selection was done by a likelihood ratio test ( $\alpha = 0.05$ ). A generalized linear mixed model effects approach was used to analyze the mortality data for each species evaluated over time. We included an individual random effect to account for within experimental unit correlation:

$$\log[\pi_{ijkl} / (1 - \pi_{ijkl})] = (\beta_0 + b_i) + \beta_{1j}\text{Block}_j + \beta_{2l}\text{Species}_l + \beta_{3k}\text{Treatment}_k + \beta_{4t}\text{Time}_t \quad (3)$$

where  $\pi_{ijkl}$  is the probability of  $l$ th species of insect dying in the  $j$ th block,  $k$ th treatment, and at the  $t$ th time;  $\beta_0$ ,  $\beta_{1j}$ ,  $\beta_{2l}$ ,  $\beta_{3k}$ , and  $\beta_{4t}$  are the fixed effect parameters of the model; and  $b_i$  is a random variable that is assumed to have a normal distribution with mean zero and variance  $\sigma^2$ . The most appropriate model was selected as described for the transmission test. The statistical analysis was performed using R software (R Core Team 2017) in the packages *hnp* (Moral et al. 2017) and *ggplot2* (Wickham 2016) to investigate the goodness of fit and to build the graphical output, respectively.

**Effect of vector species on in vitro acquisition and transmission of *X. fastidiosa*.** The previously described artificial diet system was used for acquisition of ST13 (strain 9a5c) by vector species *B. xanthophis*, *M. cavifrons*, *M. leucomelas*,

and *S. sagata*. The bacterium was grown only on Xfm before being offered to the vectors in the artificial liquid diet. After an AAP of 6 h, insects of each species were placed on plants of *V. condensata* for a 24-h gut-washing period, and transferred in groups of three individuals to test plants of *C. roseus* for an IAP of 48 h. The experiment was conducted in a complete randomized block design, with blocks repeated temporarily on three occasions between October 2017 and January 2018. Each block comprised 20 to 21 individuals (replicates) of each sharpshooter species, in addition to 12 individuals of each species that were submitted to a 6-h AAP on artificial diets without bacterial suspensions (negative control). Acquisition and transmission rates of the different species were compared by one-way analysis of deviance using the GLM approach and Tukey's HSD test for multiple comparison of means as described for the previous experiment. However, the probability of transmission by single vectors was estimated as described by Swallow (1985) because homogenous groups of individuals ( $n = 3$ ) were used to inoculate the test plants.

**Effect of STs of *X. fastidiosa* on vector transmission.** STs 11, 13, 65, and 70 were compared with respect to acquisition and transmission by *M. leucomelas*, one of the most efficient vectors of *X. fastidiosa* in citrus (Lopes and Krugner 2016). In this experiment, ST13 strain U24d was used. The STs were plated onto Xfm and incubated at 28°C in the dark for 12 days. Cell suspensions were offered to the vectors in the artificial diet during a 6-h AAP. After the AAP, the insects were submitted to a 24-h foregut-washing period on plants of *V. condensata* and individually transferred to plants of *C. roseus* for an IAP of 48 h. The experiment followed a randomized complete block design, with blocks repeated temporarily on three occasions between November 2017 and March 2018. Each block comprised 18 to 20 sharpshooters (replicates) for each of the four STs tested. As a negative control, groups of 15 individuals were submitted to a 6-h AAP on artificial diets without bacterial suspension in each block. The proportion of individuals that acquired bacteria of *X. fastidiosa* (acquisition rate) and the proportion of infected plants (transmission rate) were analyzed by one-way analysis of deviance using the GLM approach and Tukey's HSD test for multiple comparison of means, as described above.

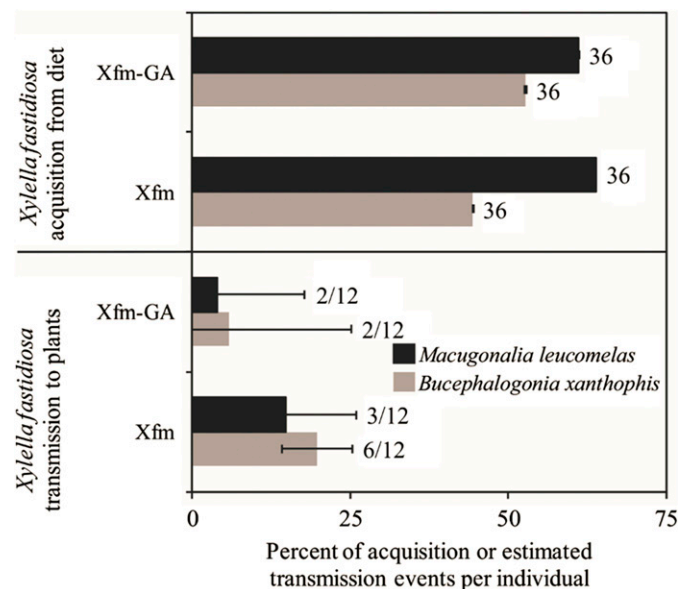
**Detection of *X. fastidiosa* in insects and plants.** After the IAP on the test plants, the DNA was extracted from individual sharpshooter heads following a cetyltrimethylammonium bromide protocol (Rogers and Bendich 1988) and quantified based on absorbance obtained using an Epoch Spectrophotometer (Biotek Instruments, Inc., Winooski, VT). DNA concentrations were standardized (20 to 100 ng/μl) for the quantitative (q)PCR, which was conducted using the XF16Sf/XF16Sr primer set (Li et al. 2013) combined with the Maxima SYBR green/ROX qPCR master mix (2x) (Thermo Fisher Scientific, São Paulo, Brazil). The PCR cycles were as follows: a denaturation cycle at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing or extension at 62°C for 45 s, conducted on the ViiA7 Real-Time PCR System (Applied Biosystems, Foster, CA). Each reaction had a positive control (from an insect known to be infective) and a negative control (milli-Q water). All samples were run in duplicate (technical replications). A standard curve was developed using a series of ninefold dilutions of DNA extracted directly from the colonies of *X. fastidiosa* (676 ng/μl); the bacterial DNA was diluted in solutions of total DNA extracted from heads of healthy sharpshooters, as proposed by Francis et al. (2006). These standard dilutions were used to establish a calibration curve by plotting the cycle threshold (CT) value (y axis) against the log<sub>10</sub> of the copy number of the 16S ribosomal RNA (rRNA) gene (x axis). The relationship was obtained between the CT value and the amount of 16S rRNA copy input, stated by the regression equation  $y = -3.426 \times \log(16S \text{ rRNA copy number}) + 35.05$ ,  $R^2 = 0.997$ . Based on the values obtained from the standard curve, all the samples that had CT values  $\leq 30$  were considered positive.

The test plants used in the transmission assays, as well as those mechanically inoculated, had four to five leaves sampled (on the branch where the sharpshooter fed) approximately 60 to 90 days after inoculation. The petioles were removed for DNA extraction following the method described by Murray and Thompson (1980). Presence of *X. fastidiosa* in test plants by was evaluated by conventional PCR using the RST31/RST33 primer set and reaction conditions as described by Minsavage et al. (1994).

## RESULTS

**Incidence of artificial infection of plants with STs of *X. fastidiosa* subsp. *pauca*.** All STs used for artificial inoculation resulted in detectable infection in plants of *C. roseus* at 60 days after mechanical inoculation. The percentages of infected plants (and numbers inoculated) were 50% ( $n = 10$ ) and 67% ( $n = 9$ ) for STs 11 (strain T-19/C13) and 70 (strain hib4), respectively, and 60% ( $n = 10$ ) for STs 13 (strain U24d) and 65 (strain BS1). There was no statistical difference between the infection rates for the different STs ( $\chi^2 = 0.56$ ;  $df = 3$ ;  $P = 0.90$ ).

**Acquisition and transmission of *X. fastidiosa* subsp. *pauca* by vectors using an artificial diet.** *X. fastidiosa* ST13 (strain 9a5c) was acquired by both sharpshooter species from the artificial diet based on both media (Xfm and Xfm+GA). The acquisition rates, measured by the percentage of qPCR-positive individuals, were 44.4 and 63.8% after feeding on Xfm for *B. xanthophis* and *M. leucomelas*, respectively, and 52.7 and 61.1%, respectively, after feeding on Xfm+GA (Fig. 1). There was no statistical difference between the sharpshooter species ( $\chi^2 = 0.09$ ;  $df = 1$ ;  $P = 0.25$ ) or between the two media used to culture *X. fastidiosa* ( $\chi^2 = 0.001$ ;  $df = 1$ ;  $P = 0.83$ ). *X. fastidiosa* was not detected in the



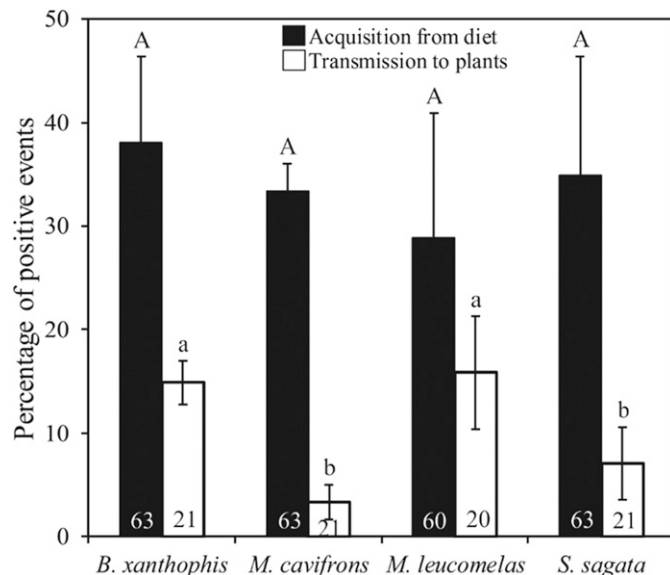
**Fig. 1.** Influence of culture media on the acquisition and transmission efficiencies of *Xylella fastidiosa* subsp. *pauca* sequence type 13 (strain 9a5c) by *Bucephalagonia xanthophis* and *Macugonalia leucomelas*. The bacterium was grown on *X. fastidiosa* medium (Xfm) or Xfm + galacturonic acid (Xfm+GA) and suspended in the artificial diet for an *in vitro* acquisition access period (AAP) of 6 h. After the AAP, the insects were fed on a nonhost of *X. fastidiosa* (*Vernonia condensata*) for 24 h, and subsequently confined in groups of one to three individuals on healthy plants of *Catharanthus roseus* for a 72-h inoculation access period. The acquisition efficiency is represented by the percentage of insects that acquired the bacterium from the diet. Transmission probabilities by single insects within heterogeneous groups (one to three insects per plant) were estimated by the model described in Materials and Methods. Values after the columns represent the total number of insects tested for acquisition and the number of infected plants over the total number inoculated, in the case of transmission. Bars represent the standard error of the mean.

insects fed on the diet without bacteria (negative control;  $n = 36$ ), confirming that the sharpshooters used in the experiment were initially free of the pathogen.

After acquisition of bacteria grown on Xfm, estimated transmission rates per individual vector using plants of *C. roseus* were 14.7 and 19.8% for *M. leucomelas* and *B. xanthophis*, respectively. When *X. fastidiosa* was grown on Xfm+GA, the rates of transmission per individual were 3.9% for *M. leucomelas* and 5.7% for *B. xanthophis* (Fig. 1). However, no statistical difference was observed between the growth media used for *X. fastidiosa* ( $\chi^2 = 3.4$ ;  $df = 1$ ;  $P = 0.065$ ) or between sharpshooter species ( $\chi^2 = 2.3$ ;  $df = 1$ ;  $P = 0.125$ ). No transmission occurred with insects fed on the negative control diet.

Mortality rates of *B. xanthophis* and *M. leucomelas* during the 6-h AAP on the liquid diets were  $\leq 15\%$ ; there was no significant effect of sharpshooter species ( $\chi^2 = 0$ ;  $df = 1$ ;  $P = 1$ ) and no interaction between species and diet treatment ( $\chi^2 = 0.46$ ;  $df = 2$ ;  $P = 0.792$ ). There was an effect of diet treatment ( $\chi^2 = 6.6$ ;  $df = 2$ ;  $P = 0.037$ ), with lower mortality on artificial diets containing *X. fastidiosa* grown on either Xfm (4.1%) or Xfm+GA (5.6%) when compared with the diet not containing *X. fastidiosa* (negative control) (15.3%). These results show that the high bacterial concentrations ( $10^8$  CFU/ml) used in the liquid diet did not affect the survival of the sharpshooters. During the 72-h IAP on plants of *C. roseus*, the effect of species was marginally significant ( $\chi^2 = 3.8$ ;  $df = 1$ ;  $P = 0.05$ ), with *M. leucomelas* having lower mortality compared with *B. xanthophis* (Supplementary Fig. S1).

**Effect of vector species and artificial diet on transmission of *X. fastidiosa*.** All sharpshooter species (*B. xanthophis*, *M. cavifrons*, *M. leucomelas*, and *S. sagata*) acquired ST13 (strain 9a5c) from the artificial diet. The acquisition rates ranged from 28.8% (*M. leucomelas*) to 38.1% (*B. xanthophis*) but there was no significant difference among species ( $\chi^2 = 4.0$ ;  $df = 3$ ;  $P = 0.26$ ) (Fig. 2). All sharpshooter species transmitted ST13 to plants of



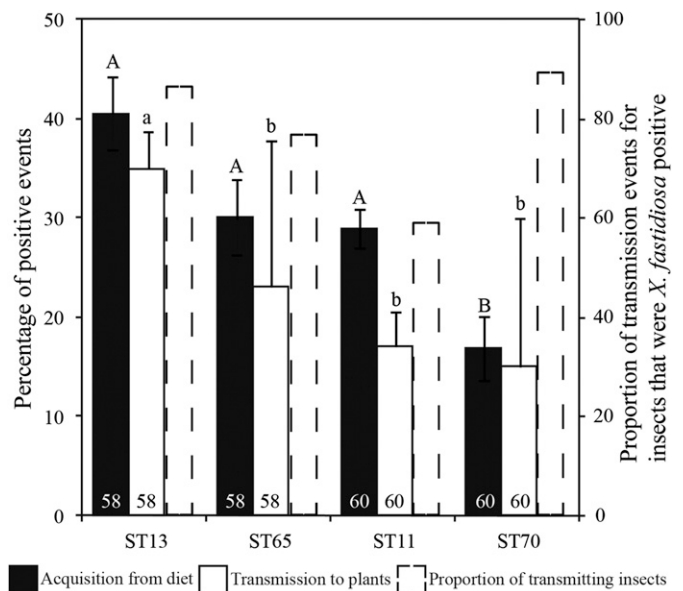
**Fig. 2.** Effect of sharpshooter species on the acquisition and transmission efficiencies of *Xylella fastidiosa* subsp. *pauca* sequence type 13 (9a5c strain). After growth on *Xylella fastidiosa* medium (Xfm), the bacterial cells were suspended in an artificial diet for an in vitro acquisition access period (AAP) of 6 h by vectors *Bucephalagonia xanthophis*, *Macugonalia cavifrons*, *M. leucomelas*, and *Sibovia sagata*. After the AAP, the insects were fed on a nonhost of *X. fastidiosa* (*Vernonia condensata*) for 24 h, and subsequently confined in groups of three individuals on healthy plants of *Catharanthus roseus* for a 72-h inoculation access period. The acquisition efficiency is represented by the percentage of insects that acquired the bacterium from the diet. Transmission probabilities by single insects were estimated according to Swallow (1985). Values and bars in the columns represent the total number of samples tested and the standard error of the mean, respectively.

*C. roseus*, with higher rates of transmission by *B. xanthophis* (14.9%) and *M. leucomelas* (15.8%) compared with *M. cavifrons* (3.3%) and *S. sagata* (7.1%) ( $\chi^2 = 35.6$ ;  $df = 3$ ;  $P < 0.001$ ) (Fig. 2). Bacteria of *X. fastidiosa* were not detected in the negative control insects of any of the four species (fed on a diet not amended with *X. fastidiosa*); also, *X. fastidiosa* was not detected in the plants on which these insect fed.

**The effect of STs of *X. fastidiosa* on acquisition and transmission efficiency by *M. leucomelas*.** The sharpshooter *M. leucomelas* was chosen for this assay because of its relatively low mortality rate on the test plant species *C. roseus*. *M. leucomelas* acquired all STs tested through the artificial diet system. The acquisition efficiency was significantly affected by ST ( $\chi^2 = 30.1$ ;  $df = 3$ ;  $P < 0.001$ ), with higher rates for STs 11, 13 (strain U24d), and 65 (28.9, 30, and 40.4%, respectively) compared with ST 70 (15%) (Fig. 3). *M. leucomelas* transmitted all four STs to plants of *C. roseus* but the transmission rate per individual was significantly higher for ST13 (34.9%) compared with STs 70, 11, and 65 (15, 17, and 23%, respectively) ( $\chi^2 = 30.4$ ;  $df = 3$ ;  $P < 0.001$ ) (Fig. 3). However, when only the proportion of qPCR-positive insects that transmitted *X. fastidiosa* was considered, the values obtained were 58.9, 76.6, 86.2, and 89% for STs 11, 65, 13, and 70, respectively (Fig. 3).

## DISCUSSION

Vector transmission of *X. fastidiosa* consists of three steps: bacterial acquisition, retention in the foregut of vectors, and inoculation into plants (Lopes et al. 2009). We adapted and implemented the technique of in vitro bacterial acquisition used in the PD pathosystem (*X. fastidiosa* subsp. *fastidiosa*) (Killiny and Almeida 2009b) for our studies with *X. fastidiosa* subsp. *pauca* and the CVC-related sharpshooter vectors. Conventional transmission studies



**Fig. 3.** Effect of *Xylella fastidiosa* subsp. *pauca* sequence types (STs) on the acquisition and transmission efficiencies by the vector *Macugonalia leucomelas*. After growth of STs 11, 13 (strain U24d), 65, and 70 on *X. fastidiosa* medium (Xfm), the bacterial cells were suspended in an artificial diet for an in vitro acquisition access period (AAP) of 6 h. After the AAP, the insects were fed for 24 h on a nonhost of *X. fastidiosa* (*Vernonia condensata*), and were then confined individually on healthy *Catharanthus roseus* plants for a 48-h inoculation access period. Acquisition efficiency (black bars) is represented by the percentage of insects that acquired the STs from the diet, and transmission efficiency (white bars) by the percentage of infected (polymerase chain reaction [PCR]-positive) plants out of the total number inoculated by the sharpshooters. The dashed line bars show the proportion of quantitative-PCR-positive insects that transmitted *X. fastidiosa* to the plants. Values and bars in the columns represent the total number of insects tested and the standard error of the mean, respectively.

that use citrus as a source of inoculum for CVC strains (subsp. *pauca*) are challenging to perform because of the difficulty of obtaining citrus plants with consistently high bacterial populations (Almeida et al. 2001), which reduces pathogen acquisition efficiency by vectors (Hill and Purcell 1995). In addition, the proportion of xylem vessels colonized by the bacteria is relatively low in citrus compared with other host plants (Alves et al. 2004), and sharpshooter species tested discriminate against plants with CVC symptoms, showing a low rate of sap ingestion when confined on those plants (Marucci et al. 2005).

The results reported here demonstrate that the in vitro acquisition technique allows the transmission of *X. fastidiosa* subsp. *pauca* to plants of *C. roseus*. The use of different culture media (solid Xfm or Xfm+GA) for bacterial growth prior to insect acquisition did not result in alteration of the colony phenotypes of strain 9a5c (data not shown). There was also no significant difference in the acquisition and transmission efficiency when the bacterium was grown on medium with or without GA, which is a component (monomer) of pectin. Killiny and Almeida (2009b) observed higher transmission efficiency with subsp. *fastidiosa* grown on Xfm medium containing pectin compared with nonamended Xfm medium. They also observed a more adhesive phenotype when the Temecula strain (subsp. *fastidiosa*) was grown on medium with pectin. Later, in studies involving in vitro acquisition and transmission of a polygalacturonase (PglA) mutant strain, Killiny and Almeida (2013) observed similar transmission efficiencies over time for the wild-type strain grown on medium with pectin and for the mutant strain grown in the presence of GA. Their results showed that processing of GA, or PglA activity resulting in breaking pectin into the respective monomer, is required for efficient transmission of *X. fastidiosa* subsp. *fastidiosa* by vectors when fed on artificial diet. The bacterial strain (9a5c) used in this study lacks a functional PglA (Simpson et al. 2000), as do other strains of *X. fastidiosa* subsp. *pauca* for which data are available (including U24d [ST13, used in this study], Fb7 [ST69], and J1a12 [ST11] all from citrus and 3124 [ST16] from coffee) (Supplementary Fig. S2). We expected that addition of GA would lead to induction of a more adhesive phenotype of 9a5c, resulting in higher transmission efficiency by sharpshooters, but this was not observed. We suggest that GA in 9a5c (ST13) may not serve as an environmental cue as it does in subsp. *fastidiosa*.

We also investigated the possibility of specificity among STs of *X. fastidiosa* subsp. *pauca* and sharpshooter species by using an in vitro acquisition system to eliminate the effect of host plant on sharpshooters or on the strain of *X. fastidiosa*. No significant variation in acquisition rates of ST13 was observed among the four species of sharpshooter tested but a higher transmission efficiency was observed for *B. xanthophis* (14.9%) and *M. leucomelas* (15.8%). The results obtained for individual transmission rates for these species are within the range already reported for *B. xanthophis* (5 to 12%) and *M. leucomelas* (16%) (Lopes and Krugner 2016; Marucci et al. 2008) in studies using acquisition from source plants. Because the acquisition rates were similar, the variations in transmission efficiency among the species were likely due to factors related to vector–plant interactions. The sharpshooter species might vary in their preference for feeding sites or probing behavior on *C. roseus*, with potential effects on the inoculation of this test plant species. In alfalfa, for example, vector competence is determined at least in part by preferred feeding sites of sharpshooter vectors in relation to the spatial distribution of *X. fastidiosa* within the infected plant. Higher transmission efficiency by *Draeculacephala minerva* Ball was observed, which prefers the basal portion of the stems where the bacterium have higher populations (thus favoring bacterial acquisition), compared with *G. atropunctata*, which prefers the upper parts of the stems (Daugherty et al. 2010). Possible variations in characteristics of the cibarium and precibarium among vector species (Almeida et al. 2005) may have implications on bacterial multiplication on these retention sites and detachment for inoculation. Although extensive multiplication of *X. fastidiosa* in the

foregut of the insect is not required for inoculation (Hill and Purcell 1995), higher cell populations have been shown to be correlated with vector transmission efficiency (Cornara et al. 2016).

STs 11, 13, and 65 of *X. fastidiosa* subsp. *pauca* were acquired by *M. leucomelas* with similar efficiency but ST70 from hibiscus had a lower acquisition rate. The transmission rate measured by the proportion of infected plants was higher for ST13 but not statistically different among the other STs. The variation in acquisition rate by the same sharpshooter species after feeding on diets with standardized concentrations of *X. fastidiosa* subsp. *pauca* suggest differential abilities of STs to colonize the foregut of the vector, whereas the differences in transmission rate to plants of *C. roseus* may be related to both vector–ST and ST–plant interactions. However, these STs were detected by PCR in similar proportions of plants of *C. roseus* when mechanically inoculated, suggesting that the differences in the transmission rates by *M. leucomelas* are not related to the ability of STs to infect and colonize this test plant.

The process of colonization of *X. fastidiosa* in the insect vector is complex and consists of three steps: (i) initial adhesion to the foregut and beginning of colonization, (ii) biofilm maturation, and (iii) detachment of bacterial cells from the foregut (Killiny and Almeida 2009a). It is possible that there is variation in the interaction of the genetically distinct strains tested (different STs) with vectors, resulting in differences in acquisition efficiency. These four STs showed marked phenotype differences when grown on PWG medium (Supplementary Fig. S3), with ST13 having most bacterial aggregates and biofilm formation in vitro (M. B. Esteves, personal observation). These in vitro growth characteristics may be related to genes important for vector colonization. The lower acquisition rate of ST70 by *M. leucomelas* may be due to the lower adhesion capacity of this strain to the insect which may, in turn, be related to the absence or abundance of adhesins on the cell surface. Similarly, considering the percentage of qPCR-positive insects that transmitted the bacterium to plants, it is evident that ST70 showed an efficient inoculation rate (89%) (i.e., nearly all the individuals that acquired this ST from the artificial diet were able to transmit it to test plants). ST11 was more efficiently acquired but showed a lower rate of inoculation by positive insects (approximately 60%). Therefore, acquisition, retention, and inoculation are different processes and may have different efficiencies for any particular ST–vector species combination.

By using the in vitro acquisition technique, we verified experimentally the lack of vector specificities among the four Cicadellinae species evaluated for transmission of ST13 or among the different STs of subsp. *pauca* tested for transmission by *M. leucomelas*. However, there is significant variation in transmission efficiencies depending on the sharpshooter species or bacterial strain tested. This is the first record of *M. cavifrons* and *S. sagata* acting as vectors for *X. fastidiosa* subsp. *pauca*. In a previous study using infected citrus for acquisition, *M. cavifrons* failed to transmit a CVC strain of *X. fastidiosa* (Lopes and Krugner 2016).

Our results provide a basis for new studies that include other strains of *X. fastidiosa* subsp. *pauca* or other subspecies not yet tested. In addition, the use of the in vitro bacterial acquisition system allows the study of mutants of several genes of interest, as already demonstrated for the PD pathosystem (Killiny and Almeida 2013). With the implementation of the artificial acquisition system for various STs and vector species associated with CVC, it is possible to reduce the experimental limitations of using citrus plants as a source of inoculum. We can now use large bacterial populations to improve transmission efficiency, and standardize the amount of inoculum offered to insects. Thus, transmission studies can be leveraged and optimized for different pathosystems, which have strains of *X. fastidiosa* that vary in genetic and biological characteristic, and a diversity of vectors. It should be noted that acquisition from an artificial diet circumvents factors that, in nature, influence not only vector feeding but also bacterial populations in infected plants, with potential impacts on accuracy of the estimates of

acquisition (and transmission) efficiency. Nevertheless, if the results are interpreted with caution, it is a valuable tool for comparative studies among *X. fastidiosa* strains or mutants and leafhopper species aimed at investigating specificity and mechanisms related to bacterial acquisition, retention, and inoculation by vectors.

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