Xylella fastidiosa subsp. *pauca* and *fastidiosa* Colonize *Arabidopsis* Systemically and Induce Anthocyanin Accumulation in Infected Leaves

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ABSTRACT

The bacterium *Xylella fastidiosa* is a multihost pathogen that affects perennial crops such as grapevine, sweet orange, and olive tree worldwide. It is inherently difficult to study these pathosystems owing to the long-term growth habit of the host plant. Thus, the availability of model plants becomes essential to accelerate discoveries with economic impact. In this study, we uncovered evidence that the model plant *Arabidopsis thaliana* can be colonized by two different *X. fastidiosa* subspecies, *pauca* and *fastidiosa*. We observed that these bacteria are able to move away from the inoculation point as high bacterial populations were found in distant tissues. In addition, confocal laser scanning microscopy analysis of bacterial movement inside the petiole revealed the ability of the

bacterium to move against the net xylem flow during the time course of colonization forming biofilm. These findings provide evidence for the capacity of *X. fastidiosa* to colonize *Arabidopsis*. Furthermore, leaves inoculated with *X. fastidiosa* showed a significant accumulation of anthocyanin. We propose that the *X. fastidiosa* subsp. *pauca* or *fastidiosa* colonization pattern and anthocyanin accumulation in the *Arabidopsis* ecotype Col-0 can be used as marker phenotypes to facilitate further studies aimed at improving genetic components involved in *X. fastidiosa*–host interaction.

Additional keywords: *Xylella*–*Arabidopsis* interaction.

Xylella fastidiosa is a xylem-limited plant pathogen that causes diseases in more than 100 plant species including many crops such as citrus, grape, almond, coffee, and plum among others (Rapicavoli et al. 2018). Until very recently this phytopathogen has caused crop damage only in the Americas; however, in 2013 it was found to cause the olive quick decline syndrome (OQDS), a disease responsible for the death of thousands of olive trees in southern Italy (Saponari et al. 2013). Since then, the bacterium has been found in other countries and is a reason for great concerns regarding food security in the European Union. *X. fastidiosa* is now considered a quarantine pathogen included in the European and Mediterranean Plant Protection Organization (EPPO) A 1 list (Mang et al. 2016).

Currently, *X. fastidiosa* is divided into five subspecies: *fastidiosa*, *pauca*, *multplex*, *sandyi*, and *morus* (Almeida and Nunney 2015).

Subspecies *fastidiosa* and *pauca* are the most widely studied as they cause economic damage in important crops such as grapevines in the North and Central America (*X. fastidiosa* subsp. *fastidiosa*) as well as citrus and olive (*X. fastidiosa* subsp. *pauca*) in South America and Italy (Bové and Ayres 2007; Luvisi et al. 2017a; Tumber et al. 2014). The existence of different level of host susceptibility is well recognized in these pathosystems. For instance, there are susceptible and resistant varieties of grape in response to *X. fastidiosa* (Baccari and Lindow 2010; Fry and Milholland 1990). Plant resistance and susceptible are also observed in citrus (Coletta-Filho et al. 2007; Niza et al. 2015) and olive (Giampetruzzi et al. 2016; Luvisi et al. 2017b), providing the opportunity to identify defense response-associated genes in the plant host that could impair *X. fastidiosa* colonization (Chakraborty et al. 2016; De Souza et al. 2007; Giampetruzzi et al. 2016; Lin et al. 2007; Rodrigues et al. 2013; Yang et al. 2011). Although these studies have contributed significantly to the knowledge regarding *Xylella*–plant interactions, the functionality of the genes identified remains elusive. A major impediment to advance current knowledge on the function of defense-associated gene in this pathosystem is the slow growth of the perennial hosts and the long incubation period for symptom development (Almeida et al. 2001; Coletta-Filho et al. 2007; Saponari et al. 2017). In addition, genetic transformation using perennial crops such as grapevines, citrus, and olive are very laborious due to the long juvenile period necessary to analyze the gene function during pathogen colonization (Mencuccini et al. 1999; Peña et al. 2001; Saporta et al. 2016).

The model plant *Arabidopsis* is widely used to study many aspects of plant–pathogen interactions (Windram et al. 2012; Witzel et al. 2015) owing to the large and readily available collection of mutants (Alonso et al. 2003), fast growth habit and established genetic transformation protocols (Clough and Bent 1998), and comprehensive literature on plant responses to pathogens (Schlauch 2011). Thus, we sought to establish a robust procedure to investigate the function of *X. fastidiosa* defense-associated gene using *Arabidopsis* as a potential host plant. Here, we show that the *Arabidopsis* ecotype

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Col-0 can be a model host for two different subspecies of *X. fastidiosa*, subsp. *pauca* and *fastidiosa*. These bacteria colonize the xylem vessel of the floral stem, roots, and leaves during the time course of colonization. Moreover, we found a significant accumulation of anthocyanin in leaves inoculated with *X. fastidiosa* providing an opportunity to use this phenotype as a quantitative marker for plant colonization. This newly discovered pathosystem will facilitate further studies aimed at improving genetic resistance against *X. fastidiosa*.

MATERIALS AND METHODS

A. thaliana ecotypes and *X. fastidiosa* inoculation. The bacterial strains *X. fastidiosa* subsp. *pauca* 9a5c (Simpson et al. 2000) and *X. fastidiosa* subsp. *fastidiosa* Temecula-1 (Van Sluys et al. 2012) were cultured on Periwinkle wilt (PWG) media (Davis et al. 1981) for 5 days at 28°C in a BOD incubator (TE-391, Tecnal, Piracicaba, SP). Bacterial cells were harvested from the plates with 1 ml of phosphate buffered saline (PBS) buffer and centrifuged for 1 min at 16,000 × g. Cell pellets were suspended in sterile, deionized water to an optical density (OD) of 0.2, 0.7, or 2.0 to inoculate different *Arabidopsis* ecotypes (Col-0, Tsu-1, and Van1). Additionally, the bacterial concentration in the inoculum was determined using the serial dilution plating method. Briefly, each 10-fold dilution was plated on Petri dishes containing PWG and incubated at 28°C. After 30 days, the number of colony forming units (CFU) on each plate was counted to estimate the inoculum concentration (CFU/ml) used for the experiments. Statistical significance between the means ($n = 8 \pm$ standard error) was calculated with the Student's *t* test ($P \leq 0.05$). All the experiments were conducted at least two times with similar results.

Seeds of Col-0, Tsu-1, and Van-1 were sown in a multiplant substrate (Terra do Paraíso, Holambra, SP) with vermiculite in a 5:2 (vol/vol) proportion. Plants were grown in a Conviron growth chamber, at 22°C, 60 ± 5% relative humidity, and 12-h photoperiod with light intensity of 100 μmol m⁻² s⁻¹. Plant inoculation was done according to the procedure described by Rogers (2012) with some modifications. Briefly, 4-week-old plants were used for inoculation and plants were not irrigated 1 day before and 1 day after the inoculation to optimize the inoculation efficiency. Four young, fully expanded rosette leaves were inoculated by dropping 5 μl of the inoculum on the midrib at the petiole-leaf junction. The petiole tissue under the drop was pricked seven to eight times using an insulin needle 13 mm × 0.38 mm (Becton Dickinson, Franklin Lakes, NJ). Water-inoculated plants were used as a mock control. Plants were kept well-watered during the experimental time.

***X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *fastidiosa* detection.** A total of 24 plants were inoculated with either water or bacterial inoculum from both strains. The tissue from rosette base was collected at 7, 14, and 21 days after inoculation (dai). DNA was extracted using the protocol developed by Doyle and Doyle (1990). DNA quality and quantity were determined by agarose gel electrophoresis and NanoDrop 8000 (Thermo Scientific, Waltham, MA), respectively. PCR-based diagnostic was done for both bacterial subspecies using the primers RST31 (5'-GCGTTAATTTTC GAAGTGATTTCGATTGC-3') and RST33 (5'-CACCATTTCGTA TCCCGGTG-3') as described by Minsavage et al. (1994). Two independent experiments were conducted with similar results.

To confirm the *X. fastidiosa* colonization, samples of the floral stem 0.5 cm above the rosette base were collected at 14 dai, immersed into Karnovsky buffer (Glutaraldehyde 2.5%, Paraformaldehyde 2.5% in 0.1 M sodium cacodylate buffer, pH 7.2), and kept at 4°C for further evaluation using scanning electron microscope (SEM). Samples were cut with a microtome SM2010R (Leica, Wetzlar), immersed into the same buffer, and dehydrated for 10 min in each acetone solution of increasing concentration (50, 70, 90, 95, and 100%). Subsequently, samples were dried in Balzers CPD030 desiccator, coated with gold using Balzers SCD050 sputter coater, and observed under a SEM (TM3000, Hitachi, Japan). Pictures were

taken using the LAS X software (Leica) and analyzed in Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

For *X. fastidiosa* isolation, the rosette base of three plants containing the petioles were harvested and kept in a 15 ml tube. Then, sample surface sterilization was processed in four steps: (i) rinsing with sterile water for 1 min; (ii) rinsing with 70% ethanol for 2 min; (iii) rinsing with 1% sodium hypochlorite for 2 min; and (iv) washing twice with sterile and deionized water. Each rinsing step included vortexing and removing the solution before the next step. Surface-sterilized plant material was placed on a sterile Petri dish, sliced using a sterile scalpel, placed in a 2 ml microtube, and homogenized in 1 ml of PBS buffer using a sterile glass stirring rod and a vortex. Ten-fold serial dilutions of the homogenized suspension were diluted 1 × 10⁻⁴ times by transferring 100 μl of the homogenate to a new tube containing 900 μl of PBS. Aliquots of 10 μl of each dilution were plotted to either PW or buffered charcoal yeast extract (BCYE) solid medium (Wells et al. 1981); the plates were incubated at 28°C until the appearance of colonies (approximately 15 days). Colonies were identified as *X. fastidiosa* based on fastidious growth, coloring, and PCR using primers specific to *X. fastidiosa* (Minsavage et al. 1994).

Evaluation of *X. fastidiosa* colonization of *Arabidopsis*. A total of 48 plants were inoculated with either *X. fastidiosa* subsp. *pauca* or *X. fastidiosa* subsp. *fastidiosa* as described above and the presence of bacterial cells in systemic tissues were determined by qPCR. Rosette, floral stem (3 cm above the rosette base), and roots (3 cm below the rosette base) were harvested at 7, 14, and 21 dai and weighed to estimate the bacterial population by gram of tissue. qPCR reactions to detect *X. fastidiosa* subsp. *pauca* were setup in a total volume of 25 μl containing 5 μl of HOT FIREPol Probe qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 525 nM of the primers CVC-1 (5'-AGATGA AAA CAATCATGCG AAA-3') and CCSM-1 (5'-GCG CAT GCC AAG TCC ATA TTT-3'), 500 nM TAQCVC probe (5'-(6FAM)AAC CGC AGC AGA AGC CGC TCA TC (TAMRA)p-3') (Oliveira et al. 2002), and 200 ng of DNA template. qPCR reactions to detect *X. fastidiosa* subsp. *fastidiosa* were setup in a total volume of 25 μl containing SsoAdvanced Universal Probes Supermix (Bio-Rad, Berkeley, CA), 0.4 μM of the probe (5'-TGGCAGGCAGCAACGATACGGCT-3') marked with FAM at the 5'-end and BHQ1 in the 3'-end (Sigma-Aldrich, St. Louis, MO), 0.2 μM of primers HL5 (5'-AAGGCAATAAACGCGCACTA-3') and HL6 (5'-GGTTTTGCTGACTGGCAACA-3') (Francis et al. 2006), and 200 ng of DNA template. A negative control (DNA from mock-inoculated plants) and a positive control (bacteria genomic DNA) were included in all experiments. The amplification parameters were used according to Oliveira et al. (2002) (50°C for 2 min, 95° for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C) and capture of the signal was obtained in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). All samples were processed in duplicates (technical replicates) and the experiment was repeated at least twice (biological replicates) with similar results.

A standard curve for *X. fastidiosa* quantification was prepared using as a reference the molecular weight of 1 genome of *X. fastidiosa* that is equal to 2.94 × 10⁻⁶ ng (Muranaka et al. 2013; Guan et al. 2013). Considering that 1 genome corresponds to 1 cell, it is possible to estimate the number of cells according to the amount of *X. fastidiosa* DNA (e.g., 294 ng corresponds to 1 × 10⁸ cells). The standard curve was produced using known concentrations of genomic DNA of *X. fastidiosa* obtained from 10-fold serial dilutions from 1 × 10⁻² to 1 × 10⁻⁸. DNA from *X. fastidiosa* was mixed with DNA of healthy *Arabidopsis* plants (100 ng/μl), aiming to simulate the natural conditions where the plant cells are infected with *X. fastidiosa* and DNA extraction is performed for diagnostic and bacterial quantifications (Muranaka et al. 2013; Guan et al. 2013). Table 1 shows the cycle threshold (Ct) obtained for each range point for each bacterium. The standard curve for *X. fastidiosa* subsp. *pauca* detection was [$y = -2.8607x$ (DNA copy number) + 35.432] with $R^2 = 0.9944$ and the standard curve for *X. fastidiosa*

subsp. *fastidiosa* detection was [$y = -3.3964x$ (DNA copy number) + 39.154] with $R^2 = 0.9987$. Based on the comparison with controls, an increase in fluorescent signal below a threshold of 36 PCR cycles was considered positive (i.e., Ct < 36). Two independent experiments were performed with similar results and statistical significance among the means was calculated using analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$).

Confocal laser scanning microscopy (CLSM). *X. fastidiosa* subsp. *fastidiosa*-expressing green fluorescent protein (GFP) (Newman et al. 2003) was inoculated onto the midrib at the petiole-leaf junction in 4-week-old plants. A total of 152 transversal cross-sections were performed for 18 petioles at 7, 12, 17, 24, and 31 dai. Petiole cross-sections were observed under a TCS SPE CLSM (Leica) equipped with a 63× water-immersion objective and filter sets to detect fluorescent protein (GFP and CFP) and chlorophyll. Sequential scanning was conducted to avoid misinterpretations of overlapping fluorescent channels. Pictures were taken using a LAS X software (Leica) and analyzed in Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

Plant fresh and dry weight measurements. Fifteen plants were inoculated with either bacterium or water (mock control) as described above. Five weeks after inoculation, rosettes were harvested and immediately weighed. Subsequently, the same plants were dehydrated in an oven incubator set at 110°C for 20 h to obtain the dry weight. Two independent experiments were conducted, and statistical analysis was performed using ANOVA followed by Tukey's test ($P < 0.05$).

Anthocyanin detection assay. *Arabidopsis* plants were inoculated as described above and the floral stems were regularly cut at the base until the time for anthocyanin quantification. This procedure ensured the robustness of the assay. The amount of anthocyanin accumulated in inoculated leaves was determined according to Chen et al. (2013). Three to four leaves were frozen in liquid nitrogen and ground to a fine powder, followed by the addition of 1 ml of methanol containing 1% HCl and overnight incubation at 4°C in the dark. Anthocyanin was separated from organic elements and cell components by adding chloroform and centrifuged at 16,000 × g for 10 min. The aqueous phase of the supernatant was transferred to a new tube containing 1 ml of 60% methanol 1% HCl: 40% deionized water solution. The absorbance was measured at 530 nm for anthocyanin and at 657 nm for chlorophyll using a spectrophotometer (Spectronic 20, Bausch & Lomb). The concentration of anthocyanin was determined according to the formula $A_{530\text{ nm}} - (0.25 \times A_{657\text{ nm}})/\text{g}$ of fresh weight (Chen et al. 2013). Statistical significance among the means ($n = 15$ in three biological replicates) was calculated with ANOVA and Tukey's test ($P < 0.05$).

RESULTS

***X. fastidiosa* subsp. *pauca* colonization of *Arabidopsis* ecotypes.** Previous studies have demonstrated that different *Arabidopsis* ecotypes support variable population levels of *X. fastidiosa*

TABLE 1. Standard curves for *Xylella fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *fastidiosa* detection by quantitative PCR in *Arabidopsis thaliana*

<i>X. fastidiosa</i> cells per reaction ^a	CVC-1/CCSM-1	HL5/HL6
	Ct ^b (<i>X. fastidiosa</i> subsp. <i>pauca</i>)	Ct ^b (<i>X. fastidiosa</i> subsp. <i>fastidiosa</i>)
10 ⁸	13.2	12.0
10 ⁷	15.4	15.0
10 ⁶	17.6	19.1
10 ⁵	20.9	22.1
10 ⁴	23.6	25.9
10 ³	27.1	29.0
10 ²	30.1	32.1

^a *X. fastidiosa* DNA were diluted in *A. thaliana* DNA extracts.
^b Values represent the mean of three replicates. Cycle threshold (Ct) > 36 is considered to be negative.

subsp. *fastidiosa* Temecula-1 strain, where the ecotype Col-0 had the smallest bacterial population and Tsu-1 was the most susceptible ecotype (Rogers 2012). Thus, we verified whether the *X. fastidiosa* subsp. *pauca* 9a5c strain also shows similar trends in bacterial growth within the ecotypes Col-0, Tsu-1, and Van-0. We observed that *X. fastidiosa* subsp. *pauca* is able to efficiently survive at a high level in all ecotypes (Fig. 1A). Interestingly, unlike *X. fastidiosa* subsp. *fastidiosa* (Rogers 2012), *X. fastidiosa* subsp. *pauca* population was significantly higher (2- to 10-fold) in Col-0 compared with its survival in the other ecotypes (Fig. 1A). To verify whether this apparently contrasting result was due the different inoculum concentration used in both experiments (i.e., 0.2 OD_{600nm} used by Rogers (2012) and 0.7 OD_{600nm} used in this study), we repeated the experiment with several inoculum concentrations (0.2, 0.7, and 2.0). *X. fastidiosa* subsp. *pauca* colonization of Col-0 does not differ in response to inoculation dose as we detected similar amounts of bacterial cells from all tissue samples (Fig. 1B). In fact, the inoculum OD is very dependent of the time of bacteria growth on the plate. In this study, we used bacteria that grew from 5 to 6 days on solid media, otherwise the amount of exopolysaccharide produced by old cells can mask the real number of living cells in OD measurement and generate not reproducible results after plant inoculation. Altogether, these results indicate that Col-0 is a good reference ecotype to study *X. fastidiosa* subsp. *pauca* colonization of plants and it was chosen for further analyses.

***X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *fastidiosa* colonization of Col-0.** Next, we compared *X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *fastidiosa* inoculation efficiencies in Col-0 to verify whether the behavior of these bacteria differ in our experimental setup. Indeed, *X. fastidiosa* subsp. *pauca* was more able to infect Col-0 than *X. fastidiosa* subsp. *fastidiosa* as the index of detection for these bacteria was 80 to 100% and 50 to 80%, respectively, during time course of colonization (Fig. 2A and B). This result is in agreement with the findings reported by Rogers (2012).

To rule out the possibility that the positive detection of bacteria was due to survival of bacterial cells from the inoculum, the detection analysis was performed in distant tissues using SEM. As this analysis was performed using tissue above the rosette base, we confirmed that plants positive for colonization was a consequence of the movement of the bacteria in the xylem. SEM revealed that *X. fastidiosa* subsp. *pauca* cells colonized the xylem stem above the rosette base at 14 dai (Fig. 2C). In addition, bacterial isolation in BCYE medium at 7 and 21 dai confirmed that those were living cells (2.7×10^6 CFU/g of tissue; SE = $\pm 2.2 \times 10^5$).

***X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *fastidiosa* systemically colonize Col-0.** It is well-known that

Fig. 1. Colonization of *Arabidopsis* ecotypes by *Xylella fastidiosa* subsp. *pauca*. **A**, Evaluation of *X. fastidiosa* subsp. *pauca* population in three different ecotypes (OD_{600nm} = 0.7). **B**, *X. fastidiosa* subsp. *pauca* population in Col-0 is not affected by different concentration of inoculum. Results in the graphs are shown as means of two independent biological replicates ($n = 12$) ± standard error (SE). Statistical difference among the means of different genotypes was determined by analysis of variance followed by Tukey's test ($P \leq 0.05$). FW = fresh weight. OD = optical density.

X. fastidiosa spp. are able to systemically colonize susceptible hosts such as citrus, grapevine, and olive trees (Newman et al. 2003; Niza et al. 2015; Saponari et al. 2017). Thus, to verify whether *X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *fastidiosa* can also colonize Col-0 systemically, we enumerated the bacterial population in different parts of the plant during the time course of colonization. As expected, a large bacterial population was observed in the rosette base, which is the sample closest to the inoculation point. At this region, *X. fastidiosa* subsp. *pauca* population remained at the same level (6 logs) (Fig. 3A), while the *X. fastidiosa* subsp. *fastidiosa* population ranged from 5 to 6 logs throughout the experimentation time (Fig. 3C). The bacterial population in the floral stem was of similar size for both strains (4 to 5 logs), but significant variation during the time course of colonization was observed to *X. fastidiosa* subsp. *fastidiosa* (Fig. 3A and C). No variation in bacterial population sizes in roots was observed along the time course of colonization; however, the population of *X. fastidiosa* subsp. *pauca* was 10 times larger than that of *X. fastidiosa* subsp. *fastidiosa*. These results demonstrate that Col-0 can be colonized systemically by both strains of *X. fastidiosa* as bacteria were found in all distant tissues analyzed (i.e., rosette base, floral stems, and roots [Fig. 3]). An illustration of the plant tissues used for the experiments is shown in Figure 3B.

***X. fastidiosa* subsp. *fastidiosa* moves against the xylem net flow in Col-0 petioles.** As *X. fastidiosa* subsp. *fastidiosa* is able to move against the xylem net flow in its grapevine host (Meng et al. 2005; Li et al. 2007), we sought to evaluate whether *X. fastidiosa* subsp. *fastidiosa* have the same behavior in Col-0. Petioles were inoculated with *X. fastidiosa* subsp. *fastidiosa*-GFP (Newman et al. 2003) and subsequently cut into a series of transversal sections at 7, 12, 17, 24, and 31 dai (Fig. 4A). Overall, the bacterial population increased during the time course of colonization as the green fluorescence from the bacterium in the xylem bundles increased over time (Fig. 4C). At 7 dai, we detected *X. fastidiosa* subsp. *fastidiosa* until the fourth section and at 17 dai, we detected the bacterium at the ninth section (Fig. 4C). From 24

to 31 dai, the bacterial population increased dramatically as visualized by the intense green fluorescence in the xylem bundles (Fig. 4C), which is characteristic of biofilm formation (Newman et al. 2003). *X. fastidiosa* subsp. *fastidiosa* migration in the xylem of the petiole followed the *Arabidopsis* xylem organization (Nieminen et al. 2004) (Fig. 4B), which was found inside some vessels of the lateral bundles from 7 to 24 dai, but also colonizing the entire central bundle at 31 dai (Fig. 4C). These results confirm that *X. fastidiosa* subsp. *fastidiosa* is able to move and colonize the xylem bundles of the petiole and reach the xylem of the rosette base of Col-0. Bacterial population size was also quantified by qPCR at 35 dai (4.4×10^7 cells/g of tissue; SE = $\pm 6 \times 10^6$).

***X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *fastidiosa* induce anthocyanin accumulation in Col-0 leaves.** In order to identify plant phenotypes associated with *X. fastidiosa* colonization, we measured the dry matter content (DMC) of bacterial- and mock-inoculated plants. However, we have not observed differences among the treatments (Fig. 5A). Interestingly, we noticed an increased purpling in bacterial-inoculated leaves compared with mock control (Fig. 5B). Thus, we designed experiments to identify the best conditions to obtain reproducible results with potential to use anthocyanin accumulation as a phenotypic marker for *X. fastidiosa* colonization of *Arabidopsis*. First, we verified a consistent anthocyanin accumulation in leaves inoculated with either *X. fastidiosa* subsp. *pauca* or *X. fastidiosa* subsp. *fastidiosa* (Fig. 5B) compared with mock control when the floral stem (or its regrowth) of the plant was removed. Second, purpling of the infected leaves, indicative of anthocyanin accumulation in this tissue, occurred as early as 1 week after inoculation. The variation in purpling timing depended upon slight variations in bacterial inoculum concentration, plant developmental stage, and environmental conditions. Third, quantification of anthocyanin accumulation in these leaves revealed that bacterium-inoculated leaves contained a significantly higher amount of anthocyanin than the mock-inoculated leaves (Fig. 5B). Following this procedure, the accumulation of anthocyanin proved to be a reliable and quantifiable phenotype to assess Col-0 colonization by both strains of *X. fastidiosa*.

DISCUSSION

Until very recently, *X. fastidiosa* subsp. *pauca* had been found only in Latin America causing diseases mainly in citrus and coffee in Brazil (Coletta-Filho et al. 2017). However, recent detection of *X. fastidiosa* subsp. *pauca* in some European countries including serious damages in olive trees in Italy underscores the need for developing alternative model host for this subspecies to advance knowledge on this pathosystem. Thus, in this study we focused on *Arabidopsis*, as it was previously demonstrated to be a suitable host for *X. fastidiosa* subsp. *fastidiosa* (Rogers 2012) and the well-documented genetic basis for its interaction with pathogens (Windram et al. 2012; Zhang et al. 2017).

We verified that, different from what was observed for *X. fastidiosa* subsp. *fastidiosa* (Rogers 2012), the best ecotype for *X. fastidiosa* subsp. *pauca* is Col-0 as the bacterial population was significantly higher than the Tsu-1 and Van-0 ecotypes independently of the inoculum concentration (Fig. 1). Subsequently, we observed that the efficiency of inoculation in Col-0 was higher to *X. fastidiosa* subsp. *pauca* (80%) compared with *X. fastidiosa* subsp. *fastidiosa* (50%) (Fig. 2). These results suggest that *X. fastidiosa* subspecies might have different behavior in *Arabidopsis* ecotypes. Nevertheless, when bacterial populations were analyzed in different parts of the plant (xylem of rosette base, floral stem, and roots) both subspecies were able to systemically colonize Col-0. As expected, the bacterial population was higher in the rosette base, which is the closest to the inoculation point, than that of other parts of the plant; however, the *X. fastidiosa* subsp. *fastidiosa* population decreased significantly during the time course

Fig. 2. *Xylella fastidiosa* colonizes Col-0. Detection of *X. fastidiosa* subspecies **A**, *pauca* and **B**, *fastidiosa* in Col-0 by PCR at 7, 14, and 21 days after inoculation (dai). **C**, Scanning electron micrographs showing the presence of *X. fastidiosa* in xylem vessels of Col-0 floral stem at 14 dai in transversal (left) and longitudinal (right) sections. **D**, Micrographs of floral stem of mock-inoculated plants.

of colonization in rosette base and floral stem whereas *X. fastidiosa* subsp. *pauca* population remained constant (Fig. 3). One could infer that resistance response in Col-0 could be more efficient toward *X. fastidiosa* subsp. *fastidiosa* than to *X. fastidiosa* subsp. *pauca*.

X. fastidiosa subsp. *fastidiosa* bacterial population increased in the entire petiole during colonization (Fig. 4). For instance, small cell aggregates were observed only at the point closest to the inoculation site at 7 dai. The entire petiole was colonized after 17 dai and by 24 dai, extensive colonization with biofilm-like structures in the xylem was observed in all petiole sections (Fig. 4). This finding demonstrates that *X. fastidiosa* subsp. *fastidiosa* moves against the water flow in the xylem to colonize distant tissues. As the

pathogenicity of *X. fastidiosa* is associated with its capacity to move and systemically colonize the plant host (Caserta et al. 2017; Chatterjee et al. 2008; De La Fuente et al. 2013), our data suggest that Col-0 is a host for *X. fastidiosa* and these phenotypes can be easily used to test mutants of specific genes in Col-0 that could be associated to *X. fastidiosa* interaction.

Although the bacterial movement and colonization are excellent phenotypes to evaluate Col-0 mutants or to screen for strains of *X. fastidiosa* with different level of virulence, we sought to establish a quick visual tool to facilitate phenotyping of infected plants. Following this concept, we investigated some phenotypes that could be associated to *X. fastidiosa* colonization of *Arabidopsis*. During

Fig. 3. Quantification of *Xylella fastidiosa* populations in different Col-0 organs. The population of *X. fastidiosa* subsp. *pauca* and *fastidiosa* was analyzed by qPCR at 7, 14, and 21 days after inoculation (dai) in the rosette base, floral stem, and roots. **B.** Cartoon illustrating the inoculation point and the plant materials collected for bacterial enumeration. Results in the graphs are shown as means of two independent biological replicates ($n = 12$) $\pm$ standard error (SE). Statistical difference among the means of different genotypes was determined by analysis of variance followed by Tukey's test ($P \leq 0.05$). FW = fresh weight.

the course of experimentation, we observed that only inoculated plants with the two subspecies of *X. fastidiosa* showed purpling in the inoculated leaves, characteristic of anthocyanin accumulation (Fig. 5) (Himeno et al. 2014). These symptoms were more evident when the floral stems were clipped off from the rosette. Using this procedure, the anthocyanin accumulation was significantly and consistently higher in inoculated leaves. In addition, both *X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *fastidiosa* induced similar anthocyanin accumulation response. Curiously, anthocyanin accumulation is a characteristic symptom in phytoplasma-infected plants (Lee et al. 2000). Phytoplasma shares some similarities with *X. fastidiosa* as they are both plant-pathogenic bacteria that inhabit the vascular system (*Phytoplasma* in the phloem and *X. fastidiosa* in the xylem) and are transmitted by insect vectors (Oshima et al. 2013). Using anthocyanin-deficient *Arabidopsis* mutants, it has been demonstrated that anthocyanin accumulation is associated with reduction of leaf cell death caused by phytoplasma colonization, indicating that anthocyanin accumulation might be important for phytoplasma survival (Himeno et al. 2014). In addition, other studies have shown that plant immune responses repress anthocyanin biosynthesis in order to activate plant defenses against bacterial or fungi pathogens (McLusky et al. 1999; Schenke et al. 2011; Serrano et al. 2012). Studies involving *Arabidopsis* immune responses also have shown that synthetic immune-elicitor such as flg22 and EF-Tu repress anthocyanin accumulation (Schenke et al. 2011; Serrano et al. 2012). In addition,

it is known that *A. thaliana* grown in low phosphorus (P) conditions increases anthocyanin content (Sanchez-Calderón et al. 2006) and *X. fastidiosa* infection causes P deficiency in many crops (Andersen and French 1987; De La Fuente et al. 2013; Silva-Stenico et al. 2009). These facts lead us to speculate that P deficiency could also occur in *A. thaliana* infected with *X. fastidiosa* and as a consequence inducing anthocyanin accumulation. Nevertheless, the role of anthocyanin accumulation in *Arabidopsis*–*X. fastidiosa* interactions needs to be further investigated.

There is a large body of literature regarding putative candidate genes involved in resistance or susceptibility of well-established crop hosts against *X. fastidiosa* (Chakraborty et al. 2016; De Souza et al. 2007; Giampetruzzi et al. 2016; Lin et al. 2007; Parker et al. 2016; Rodrigues et al. 2013; Sabella et al. 2018; Yang et al. 2011). For instance, it has been shown that genes involved with *X. fastidiosa* subsp. *fastidiosa* movement and biofilm formation, important for bacterial pathogenicity, are induced in the presence of calcium (Parker et al. 2016). Moreover, genes encoding calcium transporters are significantly induced in the susceptible plant *Olea europaea* ‘Ogliarola salentina’ infected with *X. fastidiosa* subsp. *pauca* (Giampetruzzi et al. 2016), suggesting that calcium may contribute to *X. fastidiosa* colonization. However, there is no report on the functional analysis of these genes in the host plant to validate this hypothesis. In addition, some genetic mechanisms have been suggested as being involved with resistance to *X. fastidiosa*, for example the activation of pattern-recognition receptors, which recognize pathogen-associated molecular patterns and trigger cell defense responses (Magalhães et al. 2016; Rapicavoli et al. 2018). The induction of members of these receptors was found in the resistant and tolerant hosts for *X. fastidiosa* subsp. *pauca*; *Citrus reticulata* ‘Ponkan’ (Rodrigues et al. 2013) and *Olea europaea* ‘Leccino’ (Giampetruzzi et al. 2016), respectively, and in the moderately tolerant host *Vitis vinifera* ‘Thompson Seedless’ (Wallis and Chen 2012) in response to *X. fastidiosa* subsp. *fastidiosa*

Fig. 4. Temporal movement of *Xylella fastidiosa* subsp. *fastidiosa* in Col-0 petioles analyzed by confocal laser scanning microscopy. **A**, Cartoon illustrating the petiole sections used to analyze the bacterial migration out of the inoculation point (red circle). **B**, Micrograph showing the vascular tissue organization in *Arabidopsis* petioles. **C**, Representative micrographs of transversal sections of the petioles showing green fluorescence from *X. fastidiosa*-GFP inside the xylem bundles, the arrows point to fluorescent bacterial cells. Number on the top of the pictures correspond to the sections in panel A. dai = days after inoculation.

Fig. 5. Dry matter content and anthocyanin accumulation in Col-0 inoculated leaves. **A**, Percentage of dry matter content and **B**, anthocyanin accumulation of plants inoculated with *Xylella fastidiosa* subsp. *pauca* (on the left) or *X. fastidiosa* subsp. *fastidiosa* (on the right). Pictures were taken at 3 weeks after inoculation with *X. fastidiosa* subsp. *pauca* or *X. fastidiosa* subsp. *fastidiosa* and water (Mock). Results in the graphs are shown as means of two independent biological replicates ($n = 20$) $\pm$ standard error (SE). Statistical difference among the means was determined by analysis of variance followed by Tukey's test ($P \leq 0.05$).

colonization (Chakraborty et al. 2016). It is important to note that the identified candidate genes in these pathosystems have high similarity to leucine-rich repeat receptor-like kinases (LRR-RLKs) from different plant species, including *Arabidopsis* (Magalhães et al. 2016). In addition, induction of genetic mechanism involved in cell wall lignification is associated with impaired *X. fastidiosa* colonization in resistant or tolerant varieties (Giampetruzzi et al. 2016; Niza et al. 2015; Rodrigues et al. 2013; Sabella et al. 2018). These are only a few examples of genes or genetic mechanisms involved in *X. fastidiosa* susceptibility or resistance already identified for this widespread phytopathogen with no functional validation of the genes involved. The lack of a tractable genetic system has been a bottleneck to advance functional analysis to demonstrate the possible role of these genes in plant immune response to *X. fastidiosa* in a timely manner.

It was previously demonstrated that *Arabidopsis* could be an alternative host for *X. fastidiosa* subsp. *fastidiosa* (Rogers 2012); however, this model plant has not been used to study key genes associated to plant-*X. fastidiosa* interactions, despite of the extensive use of *Arabidopsis* to improve the knowledge of genetic mechanisms for many other phytopathogens (Panchal et al. 2016; Windram et al. 2012; Witzel et al. 2015). The experimental conditions used before by Rogers (2012) has not allowed for the discovery of the phenotypes we identified, highlighting the importance of defining detailed protocols to capitalize on the *Arabidopsis* resources. With some modifications in the experimental procedure, we showed that Col-0 is a useful host for both *X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *fastidiosa* where systemic colonization, bacterial movement, and anthocyanin accumulation are reproducible phenotypes that can be used to uncover the knowledge of genetic mechanisms involved in *Xylella*-plant interaction.

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