

## *Nicotiana tabacum* as an Experimental Host for the Study of Plant–*Xylella fastidiosa* Interactions

S. A. Lopes, D. M. Ribeiro, P. G. Roberto, and S. C. França, UNAERP, Dep. Biotecnologia Vegetal, Av. Costábile Romano, 2201, Ribeirão Preto, SP; and J. M. Santos, FCAV, UNESP, Rod. Carlos Tonanni, Km 5, Jaboticabal, SP, Brazil

### ABSTRACT

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*Xylella fastidiosa* causes citrus variegated chlorosis (CVC). Information generated from the *X. fastidiosa* genome project is being used to study the underlying mechanisms responsible for pathogenicity. However, the lack of an experimental host other than citrus to study plant–*X. fastidiosa* interaction has been an obstacle to accelerated progress in this area. We present here results of three experiments that demonstrated that tobacco could be an important experimental host for *X. fastidiosa*. All tobacco plants inoculated with a citrus strain of *X. fastidiosa* expressed unequivocal symptoms, consisting of orange leaf lesions, approximately 2 months after injection of the pathogen. CVC symptoms were observed in citrus 3 to 6 months after inoculation. The pathogen was readily detected in symptomatic tobacco plants by polymerase chain reaction (PCR) and phase contrast microscopy. In addition, *X. fastidiosa* was reisolated on agar plates in 4 of 10 plants. Scanning electron microscopy analysis of cross sections of stems and petioles revealed the presence of rod shaped bacteria restricted to the xylem of inoculated plants. The cell size was within the limit typical of *X. fastidiosa*.

Citrus variegated chlorosis disease (CVC), caused by the xylem limited bacterium *Xylella fastidiosa*, has become a major problem for Brazilian citrus producers. In the state of São Paulo, responsible in 1996 for 83% of the national citrus production, the disease was present in all citrus growing areas (1). Despite eradication efforts, the percentage of infected plants, with variable levels of severity, increased from 15.7% in 1994 to 34% in 1996 (1).

After *X. fastidiosa* was identified as the causal agent of CVC (2,3) and three species of leafhopper were identified as vectors of the pathogen (7), pruning of symptomatic branches of infected trees to reduce inoculum sources, use of healthy stock material for planting, and application of insecticides to reduce vector populations in the field were adopted as control measures by growers. However, this has not impeded the rapid spread of the disease to new locations. This may indicate that not all growers implemented the control measures and/or that they are not totally effective.

It is becoming evident that only the understanding of the complex interactions among the plant, pathogen, and insect vectors is likely to lead to procedures that would permit effective disease control. The information generated with the complete genome sequence of the citrus strain of *X. fastidiosa* (14) might contribute to this understanding, revealing important information on potential pathogenicity genes.

One of the difficulties faced by those studying potential pathogenicity genes in *X. fastidiosa* is the lack of a good experimental host. Even though citrus is the target of these research efforts, citrus plants have proved not to be appropriate, mainly due to the extended periods, from 6 months to 1 year, required for symptom expression in inoculated plants (2,5,6). A plant species that more rapidly expresses external symptoms would represent a major step forward in this regard. We show here that tobacco plants inoculated with a citrus strain of *X. fastidiosa* induced unequivocal leaf symptoms significantly faster than citrus, demonstrating that tobacco may represent an important experimental host for the pathogen.

### MATERIAL AND METHODS

***X. fastidiosa* strain and inoculum preparation.** The strain used in this study was isolated from infected orange trees

(*Citrus sinensis* cv. Lima) located on São José farm in Taquaritinga, SP. Leaf petioles and midribs were removed from symptomatic leaves, surface-sterilized in 70% ethanol for 1 min followed by 3 min in 1% sodium hypochlorite, and washed twice with sterile water. Approximately 0.4 g of tissue was macerated in 1 ml of phosphate-buffered saline (PBS), plated on supplemented PW (SPW) agar (5), and incubated for 20 days at 28°C. Individual colonies were transferred to 50 ml of liquid SPW and incubated for 20 days for inoculum production. The inoculum consisted of liquid cultures containing 10<sup>8</sup> to 10<sup>9</sup> CFU/ml, estimated by 10-fold serial dilution plating.

**Plants and inoculation.** Seeds of a native variety of *Nicotiana tabacum* (accession RP1) were sown on the commercial substrate Plantmax (Eucatex, Paulínia, SP). Thirty-day-old plantlets were transplanted to 0.5-liter pots containing a 3:1 mixture of sandy soil and decomposed cow manure, or to foam trays containing Plantmax. The plants were inoculated 30 and 45 days later by injecting 0.1 ml of the inoculum or cell-free SPW medium through insulin needles at four locations at the base of each plant. Four-month-old citrus plants (cv. Caipira), grown in 3-liter pots containing the soil and manure mixture described above, were also included in the experiments. The Caipira cultivar, like most of the Brazilian sweet orange cultivars, is known to be susceptible to CVC and to express leaf symptoms under field and greenhouse conditions. The stems of these plants were pruned to approximately 15 cm above the ground 3 weeks before the first inoculation. The inoculum was injected at several locations just below the only branch left after pruning. All tobacco and citrus plants were watered twice a day and maintained in an insect-proof greenhouse throughout the experiments. A water pad cooling system maintained the greenhouse temperature below 32°C. Three experiments were conducted, with inoculation dates of 10 and 25 November 1998 for the first, 26 February and 15 March 1999 for the second, and 30 April and 15 May 1999 for the third.

Corresponding author: S. A. Lopes  
E-mail: slopes@unaerp.br

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**Detection of *X. fastidiosa* in plant tissues.** Sixty days after the second inoculation, stem segments, petioles, and leaf midribs of tobacco and citrus plants were removed, surface-sterilized, and macerated in 1 ml of sterile PBS as described. The stems were removed only from the tobacco plants, 2 to 3 cm above the injection site. A sample of 100  $\mu$ l of the macerate was diluted 10- and 100-fold in PBS, plated on PW agar (4) and incubated at 28°C for 30 to 45 days. A sample of 500  $\mu$ l was centri-

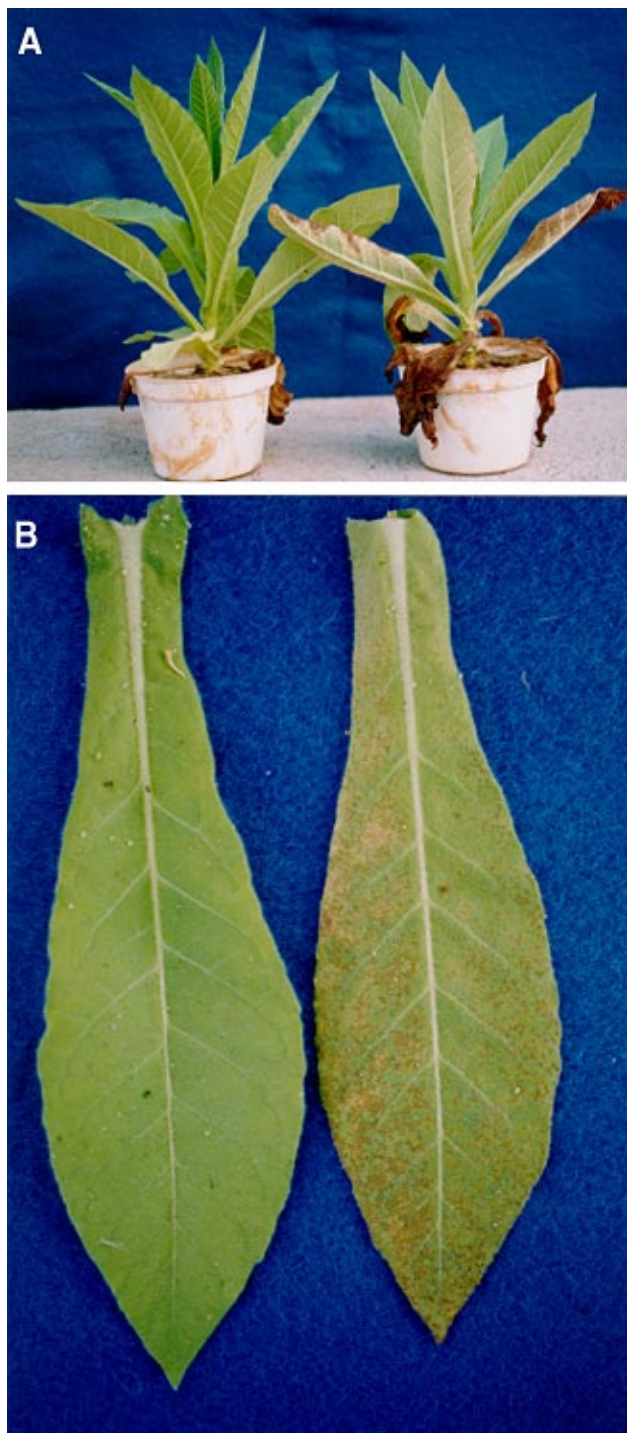
fuged for 2 min at  $10,600 \times g$  in a microcentrifuge, washed in extraction buffer (0.5 M Tris, pH 8.2, 140 mM NaCl, 2% acid washed polyvinyl-pyrrolidone [PVP] 10,000, and 0.05% Tween 20), resuspended in 20  $\mu$ l of Milli Q sterile water (Millipore Corp., Bedford, MA), and boiled for 10 min. Of this mixture, 5  $\mu$ l was added to 20  $\mu$ l of the PCR master mix consisting of 1 $\times$  reaction buffer (Amersham Pharmacia Biotech, Piscataway, NJ), 3.5 mM  $MgCl_2$ , 0.1% Triton X100, 0.2 mM of each dNTP,

1 unit of *Taq* DNA polymerase, and 12 pmols each of the primers XF5F (5'-CAACGTGTAGCGCAAACG) and XF5R (5'-CGTCTGCTATTATTGCATTG). This primer pair was designed based on a consensus sequence of the citrus strain of *X. fastidiosa* obtained in our lab as part of the sequencing work of the genome project. The primers showed high specificity for *X. fastidiosa* when tested against cell lysates of a diverse population of more than 50 nonidentified species of bacteria isolated from several plant species (8). PCR amplified products were separated in 1% agarose gels, stained with ethidium bromide, and photographed using standard procedures (10). PCR assays were conducted at least twice.

**Cell observation using phase contrast and scanning electron microscopy.** Cross and longitudinal sections of stems, petioles, and leaf midribs of symptomatic and control tobacco plants were prepared with a razor blade and observed under phase contrast and scanning electron microscopy (SEM). For phase contrast observations, cross sections of about 0.5 mm thick were mounted in water on glass slides and observed under Plan 10 $\times$  and 40 $\times$  objectives with a Eclipse E400 Nikon microscope (Nikon Corp., Tokyo, Japan). For SEM observations (13), sections about 1 cm long were fixed in 3% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.4) for 72 h at 5°C, then rinsed with cold pure buffer and postfixed with buffered 2% osmium tetroxide for 12 h at 5°C. The samples were rinsed again with the same buffer, then dehydrated with an eight-step graded series of ethanol during a 6-h period at room temperature and dried at the critical-point with carbon dioxide. Cross and longitudinal sections of the dried samples were mounted onto stubs with adhesive copper tape on top. The edges of the tape were bordered with colloidal graphite. Finally, the samples were sputtered with gold-palladium and observed in a JEOL JSM 5410 SEM operated at 15 kV.

## RESULTS AND DISCUSSION

On citrus, leaf chlorosis typical of CVC was detected in all 10 inoculated plants included in the first inoculation experiment, but in none of the controls (Table 1). The symptoms were first observed in three plants 80 days after the second inoculation. At this time, five plants, including those with symptoms, were positive when analyzed by PCR using the primer pair XF5F/XF5R. CVC symptoms continued to appear in the remaining plants until approximately 180 days after the second inoculation, when samples from all plants were PCR positive. Temporary symptom remission was observed in all plants from July to mid-October, which is in agreement with field observations. No citrus plants were included in the second experiment. In the third experiment, 5 months after the



**Fig. 1.** (A) Tobacco (left) control and (right) inoculated with the citrus strain of *Xylella fastidiosa* 80 days after the second inoculation. (B) Detached leaves from the control and the symptomatic plant showing detail of leaf lesions.

second inoculation, leaf chlorosis typical of CVC was observed in just one plant. The pathogen was isolated from the symptomatic and from one asymptomatic plant. Populations of viable *X. fastidiosa* cells were estimated in  $2.1 \times 10^5$  CFU/g of petiole and midrib tissues of the symptomatic and  $1.9 \times 10^4$  CFU/g of tissue of the asymptomatic plant.

On tobacco, all inoculated plants included in three experiments, but none of the controls, developed leaf symptoms (Fig. 1). The symptoms were characterized by small, dark orange lesions, appearing initially at the margins of the older leaves. The symptoms were detected first in a few plants around 6 weeks after the second inoculation, and 2 weeks later in all inoculated plants. Lesion coalescence resulted in burning of the older leaves, which was more severe on plants grown in the substrate Plantmax in foam trays than in the 3:1 mixture of sandy soil and cow manure. No visible damage was observed in the root system. Samples from all plants were PCR positive. Temporary seasonal symptom remission was not observed on tobacco plants.

Attempts were made to isolate *X. fastidiosa* from tobacco. The presence of fast-growing contaminants did not allow detection of *X. fastidiosa* in any of the stem samples of the first and second experiments. In the third experiment, samples consisted of extracts of stems as well as petioles and midribs and were diluted 10- and 100-fold before plating. In the first attempt, 60 days after the second inoculation, no colonies typical of *X. fastidiosa* grew on SPW. The plant stems were then allowed to regrow for another 60 days. The new branches showed leaf symptoms as described, and small colonies similar in color and shape to those isolated from symptomatic citrus were isolated from 4 out of 10 plants, in two attempts from petiole and midrib and in one attempt from stem samples. Consistently fewer contaminant bacteria grew on petiole and midrib samples than on stem samples. When cell lysates of the recovered bacteria and of known *X. fastidiosa* were tested by PCR with the primer pair XF5F/XF5R, a product of 490 bp was amplified from all four

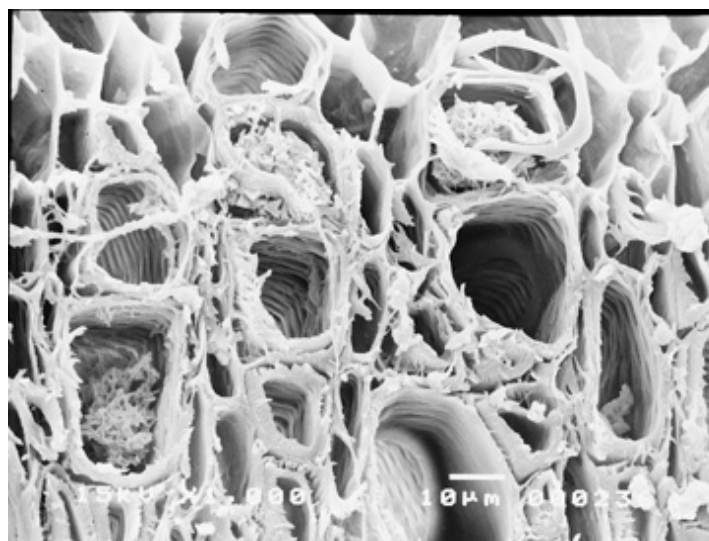
samples and the positive control. Population of viable *X. fastidiosa* cells varied from  $4.9 \times 10^4$  to  $6.6 \times 10^5$  CFU/g of tobacco tissue.

Phase contrast and scanning electron microscopy (SEM) were also employed for pathogen detection in plant tissues. Under phase contrast microscopy, masses of bacterial cells could be observed streaming from the edge of the tissues. The masses of bacteria were more easily detected from stem than from leaf midrib cross sections, and after removing the epidermis and the layer of cortical cells that involves the plant vascular system. Careful analysis by SEM of stem cross sections revealed the presence of rod shaped cells, 0.45 to 2.82  $\mu$ m, inside many of the xylem vessels of symptomatic but not of control plants (Fig. 2). Cell shape and dimensions are consistent with those described for *X. fastidiosa* (15).

Lack of a good experimental host has been an obstacle for studies of plant-*X. fastidiosa* interactions. Hundreds of *X. fastidiosa* clones deficient or over-expressing pathogenicity genes are likely to be tested in order to carefully define the molecular basis of pathogenicity and viru-

lence. Citrus plants require extended and variable periods before symptom expression (2,5,6), as confirmed in this work. Symptom expression in citrus also seemed to be significantly influenced by temperature. Plants inoculated in November/December 1998 (spring/summer) required 80 days to 6 months to develop symptoms; whereas only one in six plants inoculated in April and May (fall) developed symptoms after 5 months. It is possible that the delay in symptom expression resulted from a reduction in multiplication rate of the pathogen caused by the lower temperatures registered in the greenhouse on 30 and 31 May, 2 and 22 to 25 June, and 9 and 10 August. The average lowest temperature on those days was 6.3°C. Influence of low temperature on temporary symptom remission and bacterial recovery has been described for grapevine affected by *X. fastidiosa* (11,12).

The results presented here indicate that tobacco, an experimental model plant for plant molecular biologists, could also be used as an experimental host for the study of pathogenicity genes in *X. fastidiosa*. The inoculated plants expressed symptoms faster than citrus, and temporary symptom



**Fig. 2.** Scanning electron micrograph of a cross section of a stem of symptomatic tobacco inoculated with the citrus strain of *Xylella fastidiosa*, showing masses of rod-shaped bacteria limited to the xylem vessels.

**Table 1.** Infection of citrus and tobacco plants artificially inoculated with the citrus strain of *Xylella fastidiosa* in three experiments conducted under greenhouse conditions

Experiment	Inoculation dates	Treatment	Citrus		Tobacco	
			Symptoms <sup>a</sup>	PCR <sup>b</sup>	Symptoms <sup>c</sup>	PCR
First	10 and 25	Inoculated	10/10	10/10	12/12	12/12
	Nov 1998	Control	0/10	0/10	0/10	0/10
Second	26 Feb and	Inoculated	... <sup>d</sup>	...	24/24	24/24
	15 Mar 1999	Control	...	...	0/24	0/24
Third	30 Apr and	Inoculated	1/6	1/6	10/10	10/10
	15 May 1999	Control	0/6	0/6	0/10	0/10

<sup>a</sup> Chlorosis typical of citrus variegated chlorosis disease present in several leaves 6 month after the second inoculation.

<sup>b</sup> Polymerase chain reaction based on amplification of a 490-bp DNA segment using the primer pair XF5F/XF5R.

<sup>c</sup> Orange leaf lesions progressing to leaf burning first observed approximately 6 weeks after the second inoculation.

<sup>d</sup> Not tested.

remission was not observed. The pathogen was detected by PCR, isolation, phase contrast, and scanning electron microscopy, 2 to 3 cm above the site of inoculation in the stem and in the leaf petiole and midribs, indicating that the bacteria were able to colonize the plant tissues. Plant colonization and symptom expression were confirmed in another inoculation experiment (results not shown) using the strain 9a5c of *X. fastidiosa*, which was originally isolated in 1992 from a CVC-affected Valencia orange tree (3). Strain 9a5c was isolated on BCYE agar plates (16) from stems of symptomatic tobacco, above and below the inoculation site, including the root system. Also, the pathogen was detected in macerated tissues by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) using commercial polyclonal antibodies (Sanofi Pasteur, France) produced against the citrus strain of *X. fastidiosa*.

The tobacco presented here as a new host for the citrus strain of *X. fastidiosa* consists of a native variety of *N. tabacum* not used for commercial planting. It was the only host that presented high susceptibility among more than 20 plant species investigated (9). The susceptibility was not limited to the citrus pathogen. Preliminary results indicate that the strains that affect Brazilian coffee and plum crops can also colonize the xylem and induce leaf symptoms in tobacco. This finding may open unique opportunities for comparative studies of distinct strains of *X. fastidiosa*. Tobacco susceptibility also may prove useful to study pathogen transmission by insects, allowing, for example, a more

complete screening of potential vectors among the diverse populations of leafhoppers observed in crops of citrus, coffee, and plum.

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