Distribution of *Xylella fastidiosa* within Sweet Orange Trees: Influence of Age and Level of Symptom Expression of Citrus Variegated Chlorosis

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ABSTRACT. The internal distribution of *Xylella fastidiosa* in 2-, 4- and 6-yr-old trees of Pera sweet orange with citrus variegated chlorosis (CVC) symptoms levels (I, II and III) was evaluated. The aim of this study was to determine the *X. fastidiosa* distribution within the citrus to support pruning height recommendations as a control method. Plants were segmented into five parts and three samples per segment were analyzed. The diagnostic methodology was based on PCR, using specific primers for *X. fastidiosa* strains that cause CVC. In plants with symptom levels II and III, independent of the age, the bacteria were detected in the trunk and even near the primary branches. Plants with level I symptom expression, mainly the 4-yr-old, showed the lowest frequency of positive PCR results in the woody tissue. The high frequency of positive PCR results in asymptomatic leaves suggests a need for a constant inspection in the orchard, since our data showed that previous identification of CVC symptoms facilitates integrated management of CVC.

Citrus variegated chlorosis (CVC) was detected in Brazil in 1987 and has spread quickly in all Brazilian citrus-growing regions. This disease is caused by the xylem-limited bacterium *Xylella fastidiosa* (4, 6), which is transmitted by sharpshooter leafhoppers (Hemiptera: Cicadellidae) (8). Because of the economic and social importance of Brazilian citrus culture, and the damage caused by CVC in Brazilian orchards, a great deal of effort has been done by researchers and growers to solve this problem. In São Paulo State, about 40% of the sweet orange plants show CVC symptoms, with the greatest percentage of plants having mild symptoms (FUNDECITRUS, unpublished data, 1997).

The systemic occurrence of *X. fastidiosa* and limitation to xylem vessels hinder the development of chemical or biological control. On the other hand, its apparent slow growth in plant and reduced movement against the flow of water in the xylem could be the basis for a possible control by eliminating infected branches, thus reducing the systemic infection as well as reducing the inoculum source within the orchard. Such practice has been done by some growers (13), but it is costly. Therefore, better knowledge of how *X. fastidiosa* is distributed within affected citrus would help growers make correct decisions about pruning. The objective of this work was to evaluate, using specific PCR-based diagnostic assays, the distribution of the *X. fastidiosa* in Pera sweet orange at different ages and levels of symptom expression of CVC growing in the field.

MATERIALS AND METHODS

Plant material and sampling methodology. Plants of different ages (2, 4 and 6 yr), and different CVC symptoms expression levels (EL) were analyzed: I = one branch with mild symptoms on one leaf); II = two branches with mild symptoms on several leaves; and III = branches with symptoms on all leaves. Three plants of each age and level of symptoms were used.

Five sections in the canopy were sampled as shown in Fig. 1: main trunk; primary branches; first, second and third secondary branches.

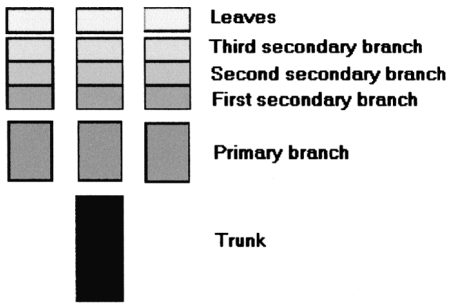

Fig. 1. Illustration of how citrus plants were segmented for sampling.

Woody tissue of each segment and leaves above the third secondary branch were sampled. Three samples per section were analyzed. Samples of the woody tissues and petioles of leaves were collected for DNA extraction.

Selected trees were taken from two orchards located in the North (Barretos) and Central (Araraquara) regions of São Paulo State, Brazil. Both orchards containing trees with the three EL symptoms and included trees without symptoms.

DNA extraction and PCR amplification. The samples were ground to a fine powder in liquid nitrogen and the DNA was extracted from tissues according to modifications of methodology described by Murray and Thompson (11) and used by Machado et al. (9). The DNA samples were redissolved in 15 µl of 1/10 TE (1 mM Tris-HCl pH8.0; 0.1 mM EDTA) containing 10 µg/µl RNAse DNA free and used for PCR analysis. Amplification of the samples was conducted in volumes of 25 µl with 1/10 volume of 10× buffer (100 mM Tris-HCl pH 8.3; 500 mM KCl; 25 mM $MgCl_2$; 0.01% gelatin); 200 mM of each dATP, dCTP dGTP dTTP nucleotides; 50 ng of DNA; 0.3 units of *Taq* polymerase; and primers CVC-1 and 272-2 int (12). The amplifications were performed in a thermocycler (RoboCycler™, STRATAGENE) programmed with a first denaturation at 94°C for 2 min followed by 30 cycles of 1 min at 94°C; 1 min at 60°C; 2 min at 72°C, with a final extension at 72°C for 10 min. Following PCR, aliquots of 10 µl of amplification products were analyzed by electrophoresis in 1.0% agarose gels containing 0.5 µg/ml of ethidium bromide.

Positive and negative controls obtained from leaves with CVC symptoms and leaves from shoot-tip-grafting plant growing under greenhouse conditions (without CVC symptoms), respectively, were used for all the DNA extraction procedures, PCR amplification and gel electrophoresis. DNA from *X. fastidiosa* (isolates from coffee, periwinkle, plum and grape) and DNA from endophytic bacteria (*Methylobacterium* sp. and *Curtobacterium* sp. (W. Araújo, personal communication)) isolated from citrus were used to confirm primer specificity.

RESULTS

The primer pair used amplified the predicted 500 bp fragment from positive CVC controls, positive samples, and *X. fastidiosa* isolated from coffee. However, this primer pair did not amplify DNA from the negative control, 10 different citrus endophytic bacteria, or from *X. fastidiosa* isolated from periwinkle, plum and grape hosts (data not shown). This specific PCR amplification of *X. fastidiosa* causing CVC by CVC-1 and 272-2 int. primers is consistent with the results of Pooler and Hartung (12). However, only bacteria other than *X. fastidiosa* used were *Erwinia amylovora* and *Xanthomonas campestris*. Therefore, the specificity of these primers for CVC *X. fastidiosa* in plant was extended in this study. The known high sensitivity of PCR-based detection (10) and the high specificity of this primer pair to *X. fastidiosa* of CVC made it suitable for this study. Figure 2 illustrates PCR amplifications obtained from controls and asymp-

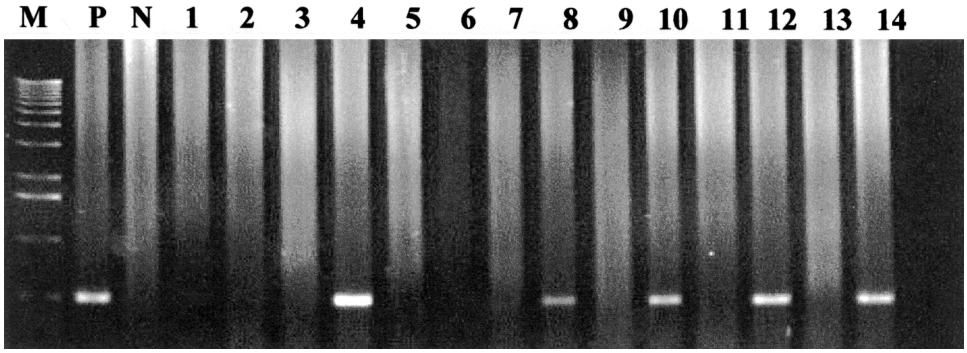

Fig. 2. Electrophoretic profiles of DNA amplified via PCR with CVC 1/ 272-2 int primers. M = 1 Kb DNA Ladder (Gibco); P = positive; N = negative control; lanes #1 to #14 = asymptomatic leaves samples taken from plants with 4-yr-old and symptom expression level I.

tomatic leaves taken from plants with 4-yr-old and symptom expression level I.

Two-year-old plants with symptom expression levels I, II and III. The results are summarized in Table 1. The frequency of positive PCR results was directly related to the level of symptom expression (mild to severe). However, the frequency decreased from leaves (upper third secondary branch) to trunk. Thus, the samples collected from all over the plant showed, through the positive PCR amplifications, the presence of *X. fastidiosa* in levels II and III, with a higher degree in samples from level III tress. In level I trees, 51.8% of leaf samples were PCR positive. The samples collected from trunk and primary branches were PCR negative.

Four-year-old plants with symptom expression levels I, II and III. Positive PCR results were obtained from each part of the plant analyzed with level III symptoms (Table 1). However, positive PCR results were observed only in 20% of the samples collected from the trunk. While, none of the samples collected from the trunk and primary branches from plants with symptom expression level II was PCR positive PCR result, only 3.7% of the samples collected from the first and second secondary branches were PCR positive.

On the other hand, in level I plants, 44.4% of the leaves and 7.4% of the samples collected from the third secondary branch were PCR positive. No DNA fragments were amplified from samples collected in the second and first secondary branches, primary branch and trunk.

Six-year-old plants with symptom expression levels I, II and III. Trunk and primary branch PCR negative for trees of all symptom expression levels. On the contrary, DNA fragments were amplified from preparations from other parts of the plant (Table 1). Symptom level I plants were identical to 2 and 4-yr-old plants, where positive PCR results were obtained whit DNA extracted from leaves without symptoms.

DISCUSSION

When elimination of CVC-symptomatic branches was initiated in 1994 (13) as a means to control CVC, it was done without sufficient information about the distribution of *X. fastidiosa* within the plant according to its age or symptom levels. Limbs, trunks, leaves and fruits of CVC affected trees were analyzed by serological tests, but positive results were obtained only symptomatic leaves and limbs (2), but not in leaves that were 50 cm below the

TABLE 1
PCR-BASED DIAGNOSIS OF *XYLELLA FASTIDIOSA* IN PERA SWEET ORANGE WITH CVC EXPRESSION LEVELS I, II AND III

	Expression level					
	I		II		III	
Tree age and segments analyzed[z]	No. samples analyzed	% + PCR[y]	No. samples analyzed	% + PCR[y]	No. samples analyzed	% + PCR[y]
2-yr-old						
Leaf	27	51.8	27	44.4	27	100
Third secondary branch	27	22.2	27	33.3	27	81.4
Second secondary branch	27	3.7	27	14.8	27	70.3
First secondary branch	27	3.7	27	11.1	27	51.8
Primary branch	27	0	27	3.7	27	25.9
Trunk	12	0	12	3.7	12	11.1
4-yr-old						
Leaf	27	44.4	27	66.6	27	88.8
Third secondary branch	27	7.4	27	40.7	27	62.9
Second secondary branch	27	0	27	3.7	27	22.2
First secondary branch	27	0	27	3.7	27	33.3
Primary branch	27	0	27	0	27	18.5
Trunk	12	0	12	0	12	20
6-yr-old						
Leaf	27	66.6	27	100	27	100
Third secondary branch	27	29.6	27	37	27	85
Second secondary branch	27	11.1	27	33.3	27	29.6
First secondary branch	27	18.5	27	18.5	27	22.2
Primary branch	27	0	27	0	27	0
Trunk	12	0	12	0	12	0

[z]See Fig. 1. Average of three plants analyzed.
[y]+ PCR results = % of samples with specific amplified DNA fragment.

last symptomatic leaf on a branch (3). The lack of more detailed information was probably the cause of several failures with this approach as related by some growers.

Plants with severe CVC symptoms (EL III) are systemically colonized by *X. fastidiosa* based on PCR analyses (Table 1). In younger plants, EL III is probably the result of a previous contamination in the nursery by vector or by infected bud wood.

The negative-PCR results with samples from the trunk and primary branches of 6-yr-old plants with EL III can be attributed to a non-sufficient number of sampling, since *X. fastidiosa* is unevenly distributed in infected plants (1) and for the size of sampled sections (trunk and primary branch) that were too large.

Although with somewhat less frequency, EL II plants were PCR positive over the plants segments analyzed (Table 1), with the exception of the trunk and primary branches of 4- and 6-yr-old plants. Expression level I was characterized by a lower frequency of *X. fastidiosa* detection within the citrus plants. This probably is related to a low bacterial population level in these plants, especially in the 4-yr-old trees. Citrus plants naturally infected by *X. fastidiosa* take over 6 mo to show CVC symptoms (8), and the colonization of *X. fastidiosa* in older plants can occur more slowly and with minor economic damage (5). Rodas (13) documented a lack of success with pruning of 2-yr-old plants, and such observations were confirmed by our dates (Table 1).

The high frequency of positive PCR results in asymptomatic leaves (Table 1) can be explained by the long incubation period necessary for the expression of CVC symptoms and by the frequent transmission of bacteria by the vectors. However, a constant inspection of the orchard is necessary, since that success in pruning is related to previous CVC symptoms identification and correct pruning of the infected branch.

Infected citrus is a primary source of inoculum for in-grove *X. fastidiosa* spread (7) and the vectors have a preference for citrus plants (14). The integrated agricultural practices of controlling the vector and reducing the inoculum in the orchard are helping growers manage CVC (5). *X. fastidiosa* was detected throughout plants with EL II and III symptoms, irrespective of their ages. In contrast, 4-yr-old plants with EL I presented a minor percentage of distribution of *X. fastidiosa* within the plant.

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