# Distribution of *Xylella fastidiosa* in Citrus Rootstocks and Transmission of Citrus Variegated Chlorosis Between Sweet Orange Plants Through Natural Root Grafts

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**ABSTRACT**

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To study translocation of *Xylella fastidiosa* to citrus rootstocks, budsticks from citrus variegated chlorosis (CVC)–affected cv. Pera sweet orange (*Citrus sinenesis* (L.) Osb.) were top grafted on 15 citrus rootstocks. Disease symptoms were conspicuous 3 months later on all 15 rootstocks tested. The presence of *X. fastidiosa* was confirmed by light microscopy, double-antibody sandwich enzyme-linked immunosorbent assays, and polymerase chain reaction in rootlets and main roots of CVC-symptomatic Pera sweet orange in 11 of the 15 rootstocks tested. These results suggest that bacterial translocation from the aerial plant parts to the root system occurs but is not essential for *X. fastidiosa* to induce symptoms in the aerial parts. Bacterial translocation to the roots was not correlated with CVC leaf-symptom severity in the Pera scion. To determine if CVC disease could be transmitted by natural root grafts, two matched seedlings of each of four sweet orange cultivars (Pera, Natal, Valencia, and Caipira) were transplanted into single pots. One seedling rootstock of each pair was inoculated by top grafting with a CVC-contaminated budstick while the other seedling rootstock was cut but not graft inoculated. Transmission of *X. fastidiosa* from an inoculated plant to a noninoculated plant sharing the same pot was observed in all four sweet orange cultivars tested. Transmission was confirmed by observation of natural roots grafts between the two plants, presence of *X. fastidiosa* in the root grafts, and disease development in the uninoculated plants. This is the first report of transmission of CVC disease through natural root grafts.

Additional keywords: citrus, plant-pathogenic bacteria, xylem, xylem-inhabiting bacteria

Citrus variegated chlorosis (CVC) was first observed at Alto Paraná, Missiones Province, Argentina, in 1984 (3) and at Macaubal, São Paulo State, Brazil, in 1987 (29). The disease already limits citrus production in Brazil (30) and is a threat to the world citrus industry (11,28). All commercial sweet orange (*Citrus. sinensis* (L.) Osb.) cultivars grown in Brazil (12,15,17) and most of the mandarins or tangerines and their hybrids are susceptible to the disease (18). The pathogen, *Xylella fastidiosa* Wells et al. (31), can be spread long distances through contaminated nursery trees (13,16). It is also naturally transmitted by sharpshooter leafhoppers (27). The pathogen has been observed in nature only in xylem vessels of infected plants (5,10,22) or in the mouthparts of the insect vectors (4,24).

The bacterium can be observed by light microscopy (21) and its identity can be confirmed by double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA; 6,8) and by polymerase chain reaction (PCR; 23) in extracts of petioles, small branches, and rootlets of CVC-affected sweet oranges. *X. fastidiosa* also has been observed in both branches and roots in other plants, including peach (1), plum (7), and coffee (20).

The objectives of this work were to determine if citrus rootstocks differed in the degree in which *X. fastidiosa* can colonize their root systems, and if this root colonization was required for symptom expression. We also wished to determine whether or not CVC disease could be transmitted between citrus plants by natural root grafts. Some portions of this work were presented previously (14).

## MATERIALS AND METHODS

**Plant material.** Seeds of 15 citrus rootstocks (Table 1) and of four sweet orange cultivars (Table 2) were extracted from fruit collected from healthy trees, sown in tubes containing commercial substrate PlantMax (Eucatex, São Paulo, Brazil), and grown for 3 months. Uniform seedlings were then selected for each rootstock and sweet orange cultivar and transplanted into pots. The pots contained soil substrate (65% subsoil, 20% composted bovine manure, 10% vermiculite, 3% humus [12]). The plants were cultivated for an additional 3 months before inoculation. For studies on root-graft transmission, two matched plants were transplanted without root pruning 8 cm apart in single pots 18 cm wide and 40 cm deep. All plants were maintained in a screen-protected greenhouse throughout the experiment to preclude unintended transmission of the pathogen by insect vectors.

**Plant inoculations.** To study translocation of *X. fastidiosa* to citrus rootstocks, four seedlings of each rootstock were cut at a height of 10 cm above the soil, and then one 5-cm length of a CVC-infected budstick of cv. Pera sweet orange was top grafted onto the rootstock (13). Budsticks were cut from selected trees which showed typical symptom expression for CVC disease. Another four matched seedlings of each rootstock were top grafted with healthy Pera budsticks as a control. To determine whether or not CVC disease could be transmitted by natural root grafts, one of two plants in each of eight pots per cultivar was inoculated using the same method. The other plant in the same pot was also cut but not inoculated.

**Symptoms and root-graft union observation.** After top-graft inoculations, the plants were observed regularly for CVC leaf symptoms (9,12). All graft-inoculated plant developed symptoms, and disease severity was estimated as follows. The total number of characteristic CVC leaf lesions was determined on the first five leaves of flushes which grew following graft inoculation. These values were recorded for four consecutive flushes as the variable *X*. The greatest number of lesions per flush among all varieties tested was denoted as *X* max. Data were transformed and a symptom severity index of $X + 0.01/X$ max + 0.1 was calculated. These symptom severity indices were thus relative to the cultivar which produced the strongest symptoms in the test, and were subjected to analysis of variance and mean separation using the Tukey test. The disease severity scale of Amorim et al. (2)

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was not used for this purpose because it is intended for grove assessments of disease severity and was impractical for our greenhouse experiments. After plants had shown typical leaf symptoms and the presence of *X. fastidiosa* had been confirmed in their aerial parts by DAS-ELISA and PCR, the plants were dug up to take root samples for DAS-ELISA and PCR assays, for reisolation of the bacteria, and for observation of natural root-graft unions.

***X. fastidiosa* identification.** Bacterial detection was carried out using light microscopy (21) and PCR (23). Xylem extracts were obtained for light microscopy by the syringe method (21). Leaf petioles or rootlets were excised and fitted to a plastic tube (2 to 4 cm long by 0.2 cm in diameter) attached to a syringe. Sterilized, distilled water was then pushed through the xylem by applying manual pressure to the syringe to release bacteria from the vessels. The resulting drops of xylem extract were dried on glass slides, stained with methylene blue, and observed at 400× with a light microscope for the presence of bacilliform cells or their agglomerates typical of *X. fastidiosa* (7,11,26).

The resulting drops of xylem extract were also collected and were centrifuged for 10 min at 12,000 × *g*. After the supernatant was removed, the pellet was dried for 10 min in a vacuum. The pellet was resuspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for PCR amplification.

Primers specific for *X. fastidiosa*, 272-1-int and 272-2-int (23), were used for PCR amplification, which was performed in a mixture containing 10 µl of the plant xylem extract, 0.5 µM each primer, 0.2 mM each dNTP, 1× reaction buffer, 2.5 mM $MgCI_2$, and 1 U of *Taq* DNA Polymerase (Life Technologies, Grand Island, NY). PCR was carried out for 34 cycles in a Programmable Thermal Cycler (PTC-100TM, MJ Research, Watertown, MA) with an initial denaturation for 4 min at 94°C. Each cycle consisted of denaturation for 1 min at 94°C, primer annealing for 1 min at 64°C, and extension for 1 min 30 s at 72°C. After 34 cycles, the reaction was completed by incubating for 10 min at 72°C. PCR products were identified after electrophoresis in a 1.0% agarose gel and staining with ethidium bromide.

The same leaves which had been used for PCR were used for DAS-ELISA assays performed according to the method described by Chang et al. (6). The leaf midribs and petioles or rootlets (about 1 g) were ground in 3 ml of phosphate-buffered saline (PBS) buffer in a Polytron homogenizer (Pro Scientific, Monroe, CT). The extracts were filtered through four layers of cheese cloth, and 100 µl of filtrate were placed in each well of the ELISA plates. DAS-ELISA for detection of the CVC-bacterium was carried out with a concentration of 5 µg of immunoglobulin G (IgG) per ml for coating the ELISA plates (4 h at 37°C) and with a 2,000-fold dilution of the alkaline phosphatase-conjugated IgG (4 h at 37°C).

**Isolation and culture.** *X. fastidiosa* was cultured by immersion of petioles or rootlets from symptomatic trees in 70% ethanol for 1 min followed by 3 min in 1% sodium hypochlorite. The plant samples were then rinsed for 5 min in sterile distilled water and cut into approximately 1-mm pieces which were placed into 1 to 2 ml of periwinkle wilt (PW) (6) broth and centrifuged at 700 × *g* for 2 to 3 min to help release bacteria from the xylem. Supernatant aliquots of 1 ml were then inoculated into liquid or on solid PW media and incubated microaerobically in the dark at 28°C. After bacterial colonies had grown, they were confirmed to be *X. fastidiosa* by colony morphology, DAS-ELISA, and PCR.

## RESULTS

**Symptoms and distribution in rootstocks.** Both CVC-affected and healthy Pera sweet orange twigs, top grafted on the 15 rootstocks, began to sprout 5 to 8 days after grafting. None of the plants from healthy twigs developed CVC symptoms. The plants from the CVC-affected twigs and from the inoculated sweet orange seedlings started to develop CVC leaf symptoms on their first flushes (3 to 5 cm from the graft union) 2 months after grafting, when the shoots were about 60 cm long. Symptoms included chlorotic variegation with lesions appearing on the upper surface of the leaves. Erumpent blisters on the lower side of the leaves, which were congruent with the lesions on the upper side of the leaves, were conspicuous one additional month later.

In spite of the fact that some of the 15 citrus rootstocks used in this study are considered to be resistant to CVC disease and others are considered to be tolerant to the CVC disease (14), all trees produced from CVC-affected Pera sweet orange twigs produced typical CVC leaf symptoms. Nevertheless, there were significant differences in leaf-symptom severity among the trees on different rootstocks, showing that this severity did not depend only on rootstocks or cultivars but on citrus scion–rootstock combination (17). Pera plants grafted on susceptible mandarin cultivars Sunkat and Wilking, on tolerant or resistant mandarin cultivars Oneco and

**Table 1.** Distribution of *Xylella fastidiosa* in roots of citrus variegated chlorosis (CVC)-affected cv. Pera sweet orange grafted on various rootstocks

| | | ***X. fastidiosa* diagnosis in roots[x]** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **DAS-ELISA** | | | | **PCR** | | | |
| **Rootstock[y]** | **Symptoms[z]** | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** |
| Sunkat mandarin (*Citrus sunki*) S | 0.9928 a | + | + | – | – | + | + | – | – |
| Wilking mandarin (*C. reticulata*) S | 0.9647 a | + | + | + | + | + | + | + | + |
| Oneco mandarin (*C. reticulata*) T | 0.8914 ab | – | – | – | – | – | – | – | – |
| Cleopatra mandarin (*C. reticulata*) R | 0.8856 ab | – | – | – | – | – | – | – | – |
| Rangpur lime (*C. limonia*) R | 0.8478 ab | – | – | + | – | – | – | + | – |
| Orlando tangelo (*C. reticulata* × *C. paradisi*) S | 0.8012 b | + | + | + | – | + | + | + | – |
| Swingle citrumelo (*C. paradisi* × *P. trifoliata*) T | 0.6110 c | + | + | – | – | + | + | – | – |
| *C. amblycarpa* T | 0.5819 c | – | – | – | – | – | – | – | – |
| Troyer citrange (*C. sinensis* × *P. trifoliata*) T | 0.5623 c | – | – | + | – | – | – | + | – |
| Batangas mandarin (*C. reticulata*) S | 0.5521 c | – | + | – | + | – | + | – | + |
| Volkamer lemon (*C. volkameriana*) R | 0.5296 c | + | – | – | – | + | – | – | – |
| Thornton tangelo (*C. reticulata* × *C. paradisi*) R | 0.4857 c | – | + | + | – | – | + | + | – |
| Rough lemon (*C. jambhiri*) R | 0.4631 c | – | – | – | – | – | – | – | – |
| Caipira sweet orange (*C. sinensis*) S | 0.2215 d | + | + | + | + | + | + | + | + |
| Trifoliate orange (*P. trifoliata*) T | 0.1420 d | + | – | – | + | + | – | – | + |

[x] Four plants tested; + and – indicate positive and negative reactions, respectively. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was considered positive if the absorbance at 405 nm was three times that of the healthy control. Polymerase chain reactions (PCR) with an amplification product of approximately 500 base pairs were considered positive.

[y] Letters following the rootstock name indicate the disease severity evaluation for these rootstocks when evaluated as seedlings: S = susceptible, T = tolerant, and R = resistant (13).

[z] CVC leaf symptoms. Number of lesions per leaf on the first five leaves of each of the first four flushes after top grafting, transformed into (X + 0.01)/(X max + 0.1); Tukey test ($P < 0.05$).

Cleopatra, and on the resistant cultivar Rangpur lime showed the highest leaf-symptom severity, without any significant differences in CVC leaf-symptom severity among these plants (Table 1). Similarly, Pera plants grafted on the susceptible mandarin cultivar Batangas; the tolerant cultivars Swingle citrumelo, *C. amblycarpa*, and Troyer citrange; and the resistant cultivars Volkamer, Rough lemon, and Thornton tangelo all showed the same intermediate leaf-symptom severity. The lowest CVC leaf-symptom severity was observed on Pera plants grafted on the susceptible Caipira sweet orange and the tolerant trifoliate orange.

Presence of *X. fastidiosa* was confirmed by DAS-ELISA and PCR in rootlets and main roots of CVC symptomatic Pera sweet orange on 11 of the 15 rootstocks tested (Table 1). The presence of *X. fastidiosa* in the aerial parts (leaves, petioles, and stems) of these plants was also confirmed by DAS-ELISA and PCR. Therefore, the bacterium was distributed throughout entire plants and moved downward to the root system. However, *X. fastidiosa* was not detected by microscopy, ELISA, or PCR in the root system of symptomatic Pera sweet orange plants grafted on four of the rootstocks tested. These observations were made 6 months after the Pera scions began to display disease symptoms.

**Isolation and bacterial morphology.** *X. fastidiosa* was readily cultured from petioles, twigs, and stems of symptomatic Pera sweet orange plants grafted on various rootstocks and from symptomatic sweet orange seedlings in this study. The bacterial colonies were observed 10 to 15 days after inoculation of culture media. The appearance of the colonies and the bacterial cell morphologies were similar to those of other *X. fastidiosa* strains (31). It was not possible to isolate *X. fastidiosa* from citrus roots and rootlets because of the presence of rapidly growing bacterial contaminants which survived the decontamination procedure and overgrew all isolation plates before colonies of *X. fastidiosa* could be observed.

**Transmission by natural root grafts.** Transmission of *X. fastidiosa* from an inoculated plant to a noninoculated plant sharing the same pot was observed in all four cultivars tested after 2 years of cocultivation of the plants in the same pot. Symptoms of CVC were present in the uninoculated plants in four, three, two, and one of eight pots for each of the sweet orange cultivars Caipira, Pera, Valencia, and Natal, respectively (Table 2) for an overall transmission rate of 31% (10/32). In all uninoculated plants with symptoms of CVC, *X. fastidiosa* was observed by light microscopy and its presence was confirmed by DAS-ELISA and PCR. The 22 plants that did not develop symptoms during the cocultivation period also did not have positive results for *X. fastidiosa* by light microscopy, ELISA, and PCR assays. Natural root grafts were observed between the inoculated and noninoculated but symptomatic plants and the presence of *X. fastidiosa* in various plant parts was confirmed by DAS-ELISA and PCR assays (Table 2, Fig. 1).

In these experiments, we observed symptom expression in the terminal leaves of shoots 60 cm in length produced from infected buds 60 days after grafting in the greenhouse. CVC symptoms developed at a similar rate under field conditions on new flushes from CVC-affected branches of Pera or other sweet orange cultivars (9; and W. B. Li, *unpublished*). When CVC-affected trees were severely pruned, the new flushes that emerged rapidly developed CVC symptoms in the same season. More than 600 5-year-old CVC-affected trees of Pera sweet orange, half grafted on Rangpur lime (*C. limonia* Osb.) and half on Cleopatra mandarin (*C. reticulata* Blanco), were pruned to about 50 cm above the graft union on 28 August 1998 at the Bebedouro Citrus Experiment Station, Sao Paulo, Brazil. New flushes came out on the trunk and principal branches 1 week after prun-

**Table 2.** Transmission by natural root grafting of citrus variegated chlorosis (CVC) in sweet-orange seedlings in a greenhouse

| | Detection/identification[y] | | |
|---|---|---|---|
| **Cultivar** | **DAS-ELISA** | **PCR** | **Symptom expression[z]** |
| Caipira | (+) (–) | (+) (–) | No |
| | (+) (+) | (+) (+) | Yes |
| | (+) (+) | (+) (+) | Yes |
| | (+) (–) | (+) (–) | No |
| | (+) (+) | (+) (+) | Yes |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (+) | (+) (+) | Yes |
| Pera | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (+) | (+) (+) | Yes |
| | (+) (+) | (+) (+) | Yes |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (+) | (+) (+) | Yes |
| | (+) (–) | (+) (–) | No |
| Valencia | (+) (+) | (+) (+) | Yes |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (+) | (+) (+) | Yes |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| Natal | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |
| | (+) (+) | (+) (+) | Yes |
| | (+) (–) | (+) (–) | No |
| | (+) (–) | (+) (–) | No |

[y] DAS-ELISA = double-antibody sandwich enzyme-linked immunosorbent assay and PCR = polymerase chain reaction; + and – indicate positive and negative reactions, respectively. The symbol on the left refers to the inoculated plant and the symbol on the right refers to the uninoculated plant. [z] Foliar symptoms of CVC present in the uninoculated plant. All inoculated plants were symptomatic.

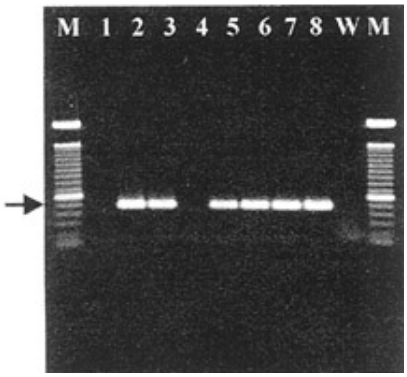


**Fig. 1.** Amplification of bacterial DNA using *Xylella fastidiosa*-specific primers (272-1-int and 272-2-int). Two M lanes contained 100-bp ladder DNA. Extracts: healthy leaves of cv. Caipira sweet orange, leaves from the inoculated plant showing symptoms of CVC, roots of the inoculated plant, roots from healthy Caipira sweet orange, the natural graft union between the two plants, the roots of the noninoculated plant, and CVC-symptomatic leaves of the noninoculated plant were assayed in lanes 1 through 7, respectively. *X. fastidiosa* from liquid culture was assayed in lane 8 as the positive control. Lane W contained the only water polymerase chain reaction negative control.

ing. In some cases, after 1 month, these flushes were more than 1 m long and symptoms of CVC disease began to appear in the top foliage. Thus, *X. fastidiosa* can move upward in sweet orange flushes at a rate of 1 cm/day (60 cm/2 months) in our experimental conditions in a greenhouse and 3 cm/day (100 cm/1 month) in field conditions in northern Sao Paulo State. This also suggests that our soil substrate and greenhouse conditions provided a reasonable approximation of field conditions.

## DISCUSSION

We have demonstrated that translocation of *X. fastidiosa* to the root system occurs in citrus. However, *X. fastidiosa* was not observed by either ELISA or PCR in the root systems of Pera grafted on 4 of 15 rootstocks. The scion Pera was symptomatic on all 15 rootstocks tested; therefore, this could mean that bacterial translocation from the aerial plant parts to the root system is not essential for *X. fastidiosa* to induce symptoms in the aerial parts. However, it is possible that *X. fastidiosa* was in fact present in all of the root systems but was not detected by the ELISA and PCR assays used. Also, *X. fastidiosa* is thought to be irregularly distributed in citrus plants (6), which would be consistent with our results. When field-collected roots were taken from CVC-affected sweet orange trees with severe disease symptoms from different citrus production regions, we detected *X. fastidiosa* in most but not all cases, even for the same scion cultivar grafted on the same rootstock.

There was no correlation between bacterial translocation from the aerial plant parts to the root system and the disease severity in the aerial parts. There was also no correlation between the symptom severity observed and the ELISA values summarized in Table 1. This is consistent with other work (19). *X. fastidiosa* was detected in the roots of plants grafted on Caipira sweet orange, which showed the lowest disease severity. However, *X. fastidiosa* was not detected in the roots of the mandarin cultivars Oneco and Cleopatra, which showed the highest disease severity. Finally, bacterial translocation was not related to the rootstock's resistance to CVC (14; Table 1). The presence of *X. fastidiosa* was confirmed by DAS-ELISA and PCR in roots of some of the plants grafted on resistant rootstock cultivars Rangpur lime and Volkamer lemon, as well as on susceptible rootstocks, such as Sunkat and Batangas mandarin cultivars. However, *X. fastidiosa* was detected in the roots of all the plants grafted on susceptible Caipira sweet orange and Wilking mandarin. This suggests that bacterial translocation may be facilitated in some rootstocks.

We observed *X. fastidiosa* in roots of Pera sweet orange trees grafted on 11 citrus rootstocks, starting about 6 months after the beginning of disease-symptom expression in the aerial parts. We did not sample roots for the presence of *X. fastidiosa* earlier. Our results are consistent with another recent study on pathogenicity of *X. fastidiosa* (19), in which the presence of the bacterium at root tips of Caipira sweet orange seedlings was confirmed at almost the same time that the aerial plant parts developed typical CVC leaf symptoms. This was 3 to 4 months after inoculation in the trunk with a pure bacterial culture in liquid PW medium, as compared to the graft inoculations performed in this work. Taken together, these results suggest that *X. fastidiosa* can move downward rapidly, perhaps as fast as it moves upward. The mechanism used by *X. fastidiosa* to move systemically is unknown.

To our knowledge, this is the first report of transmission of *X. fastidiosa* through natural root grafts. We do not know the rate at which natural root grafts between citrus trees occurs in Sao Paulo State. The probability of natural root grafting should increase if citrus plant spacings are decreased. Further experiments on transmission of CVC by root grafting should be carried out in commercial citrus orchards. Rootstock cultivars may vary in their tendency to form root grafts and therefore influence the rate of transmission of *X. fastidiosa* by root grafts. In São Paulo State, some citrus producers plant two citrus trees in each planting hole in order to obtain a bigger canopy for better production in a short time. This practice is essentially the same as in our experiment using potted citrus plants; therefore, transmission of CVC between such trees through natural root grafts could be expected.

We also note that, in commercial nursery operations, rootstocks are often planted at a rate of 3 plants/m. This distance is similar to the distance between the citrus plants in the pots used in our experiment; therefore, there is a possibility for root graft transmission in nurseries if the budwood used has not been adequately tested and shown to be free of *X. fastidiosa*. Such transmission could occur even if plants were grown in a greenhouse to avoid insect transmission of the pathogen.

Whether or not transmission of CVC disease via root grafts in commercial citrus groves is a problem, our results are very important for other diseases caused by *X. fastidiosa*. Requeima do Café or coffee leaf scorch (20) is caused by a strain of *X. fastidiosa* that is very closely related to the citrus strain (X. Qin et al., *in preparation*). In São Paulo State, coffee is grown in hedgerows at the rate of 3 plants/m. We have observed *X. fastidiosa* in the root systems of *Coffea canephora* var. *robusta*, the rootstock used by the coffee industry (V. S. Miranda et al., *in preparation*). Thus, transmission of Requeima do Café by natural root grafts is a concern, and we are currently testing this idea experimentally. We note also that oleander (*Nerium oleander* L.), the host of a newly described leaf scorch disease, is also grown in hedgerows in California where the disease occurs. Oleander leaf scorch has been shown to be caused by a strain of *X. fastidiosa* (25); therefore, the possibility of root-graft transmission in this pathosystem should also be carefully evaluated.


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