Graft Transmission and Cultivar Reaction of Citrus to '*Candidatus* Liberibacter americanus'

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ABSTRACT

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Little is known about '*Candidatus* Liberibacter americanus', a causal agent of huanglongbing or greening disease in Brazil, or its interaction with citrus trees. Greenhouse experiments were conducted with the objective of determining conditions favorable for transmission from field affected trees to young potted plants, to evaluate the reaction of multiple citrus species to the disease, and to determine the efficiency of pathogen propagation from individual buds. Single buds or bark pieces of various sizes or bark plus wood (budstick) that were removed from symptomatic or asymptomatic branches were used as sources of inoculum. Transmission success was evaluated through polymerase chain reaction analysis of total DNA extracted from leaf samples. Beginning at 4 to 5 months after inoculation, infected plants manifested leaf mottling and symptoms similar to those of iron, manganese, and zinc deficiencies. Blotchy mottled leaves appeared only on sweet oranges and Murcott tangor. Pathogen transmission was higher for these citrus cultivars and species (31.2 to 65.2%) than for limes, mandarins, or Swingle citrumelo (2.0 to 25.0%). Deformed small fruits with brownish columellae also developed on sweet oranges. Only buds and budsticks served as sources of inoculum and the larger the piece of tissue, the higher the transmission efficiency. Experiments initiated during the winter showed lower rates of graft tissue survival but relatively higher percentages of pathogen transmission.

'*Candidatus* Liberibacter' is a genus of gram-negative bacteria composed of three species–'*Ca.* Liberibacter asiaticus', '*Ca.* Liberibacter africanus' and '*Ca.* Liberibacter americanus' (5,13). They are transmitted in the field by psyllids (2,9,17), colonize the phloem of the infected plant (7), and cause an important citrus disease named huanglongbing (HLB). The disease is also known as greening in South Africa, mottle leaf in the Philippines, dieback in India, and vein phloem degeneration in Indonesia (1). While the behavior of the first two species is well known and has been studied in several countries, the behavior of '*Ca.* Liberibacter americanus' has received little attention since it was first described in 2004 from HLB-affected trees in Brazilian citrus orchards (13). '*Ca.* Liberibacter asiaticus' has also been found in Brazil (3).

Independent of the Liberibacter species, HLB management has been achieved through the elimination of affected trees to reduce inoculum, chemical control of vector populations, and the use of healthy young trees for planting. The adoption of these practices is expensive because of the need to have all trees surveyed by well-trained inspectors and the cost of psyllid control (14).

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To improve HLB management, a better understanding of the complex interaction that exists among host, pathogen, and vector is necessary. One obstacle for this understanding is that no one has been able to isolate and culture the pathogen on artificial media (1). Therefore, all research that involves pathogen inoculation and transmission require the use of infected plants as direct sources of inoculum. Liberibacter can be transmitted to citrus plants with the insect vector (*Diaphorina citri* or *Trioza erytreae*), dodder (*Cuscuta* sp.), or grafting (2,5,9,17; Hsiang, 1956, cited by Bové [1]). The choice of method will depend on the research purpose. Psyllid transmission is the natural process that occurs in the field, but its experimental use is complicated because of the low efficiency of pathogen transmission and the need to use a large number of insects. Dodder and shoot grafting are artificial, but because of the high efficiency of pathogen transmission, these methods have been used to investigate some aspects of the disease including host reaction to pathogen infection, host range, and pathogen aggressiveness.

Since the first report of the presence of HLB in Brazil and the finding that a new species of Liberibacter was involved, several studies using graft inoculation have been carried out in our greenhouses. Here we present the results of three experiments: i) to determine the best affected twig tissue as a source of inoculum; ii) to evaluate the disease reaction in the main commercial Brazilian citrus cultivars and species used for fruit production or rootstocks; and iii) to determine the efficiency of pathogen propagation from individual buds through the usual process of producing young nursery trees for commercial planting.

MATERIALS AND METHODS

Plants, sources of inoculum, and inoculations. Experiments were conducted from October 2004 to August 2006 in a greenhouse where temperatures did not exceed 32°C. Depending on the experimental purpose, inoculated plants were either 6-month-old Valencia sweet orange (*Citrus sinensis*) or a diverse group of citrus used for fruit production, all grafted on Rangpur lime (*C. limonia*), or a group of four citrus species used as rootstocks. All plants were produced in 4-liter plastic bags in screened nurseries, required by law in the state of São Paulo, and fertilized as necessary. The sources of inoculum were symptomatic or asymptomatic branches of 5-year-old Valencia sweet orange trees of a block located on a farm severely affected by the disease. Prior to branch removal, 10 to 20 leaves were collected from each tree, processed for DNA extraction (0.5 g of leaf midribs) using the cetyltrimethylammoniumbromide (CTAB) method of Murray and Thompson (10), and analyzed by polymerase chain reaction (PCR) with the primers A2/J5 for '*Ca.* Liberibacter asiaticus' (6) and GB1/GB3 for '*Ca.* Liberibacter americanus' (12). Branches were removed from the trees and transported to the greenhouse the day of inoculation. With the exception of the experiment to determine the rate of pathogen propagation during the process of young tree formation, all plants were inoculated on the scion 10 to 15 cm above the bud union. The efficiency of pathogen transmission or propagation was compared by using a chi-square test.

Best source of inoculum. The experiments to determine the best tissue and the best size of a symptomatic branch for use as inoculum involved seven treatments consisting of one individual bud or individual branch segments of 2 or 4 cm containing only bark or bark plus wood (budstick). Similar experiments were reported by Van Vuuren (15) for '*Ca*. Liberibacter africanus'. Segments were detached from the branch region containing mottled leaves or the peduncle of deformed fruits with aborted seeds. Leaf mottling and fruit deformation are characteristic symptoms of HLB (4,8). For inoculation, a longitudinal cut was made at the stem. The cut size was compatible with the size of the segment to be grafted. Plants that would receive buds or budsticks were cut to a depth approximately one-third of the stem diameter. On the plants that would receive just bark segments, the cut was only through the bark. In all cases, the grafting tissues were inserted within the cut regions and firmly fixed by wrapping them with 2 cm wide transparent plastic strips. The plastic strips were maintained on the stems for 40 days. Each treatment contained 40 plants. The plants were observed regularly and evaluated for symptom development at 6, 8, and 12 months and evaluated by PCR at 12 months after inoculation. For the PCR analysis, samples consisting of three to five mature leaves removed from each plant were washed and processed as described. Two experiments were conducted, one from October 2004 to October 2005 and another from July 2005 to June 2006.

Citrus reaction to HLB. To evaluate citrus responses to liberibacter infection, inoculum consisting of two, 4 cm long budsticks was grafted as described above onto the stems of 25 plants each of Hamlin, Natal, and Pera sweet oranges (*C. sinensis*), Murcott tangor (*C. reticulata* × *C. sinensis*), and Ponkan mandarin (*C. reticulata*) and 50 plants each of Rangpur (*C. limonia*) and Tahiti limes (*C. latifolia*), Sunki (*C. sunki*) and Cleopatra (*C. reshni*) mandarins, and Swingle citrumelo (*Poncirus trifoliata* × *C. paradisi*). All plants were maintained in the greenhouse and pruned at 7 months after inoculation by cutting the stem 10 cm above the inoculation site. Plants were observed for symptom expression at 4, 6, and 8 months after pruning and evaluated by PCR at 8 months. The experiment was conducted from February 2005 to June 2006.

Pathogen propagation. To determine if the bacteria were present in the bud and perpetuated in the new branch, we followed the same procedures used by certified nurseries to propagate young trees. Buds from symptomatic and asymptomatic branches and buds from asymptomatic trees were grafted under the bark of the stem of 4-month-old Rangpur lime seedlings and fixed in place with transparent plastic strips as described. The strips were maintained for 20 days. The plants were pruned after 25 days by removing the Rangpur stem just above the young branch originating from the bud. A total of 120 plants were used per treatment. Plants were evaluated for symptom expression at 6, 9, and 12 months after inoculation and evaluated by PCR at 12 months. Two experiments were carried out, one from October 2004 to September 2005 and another from July 2005 to July 2006.

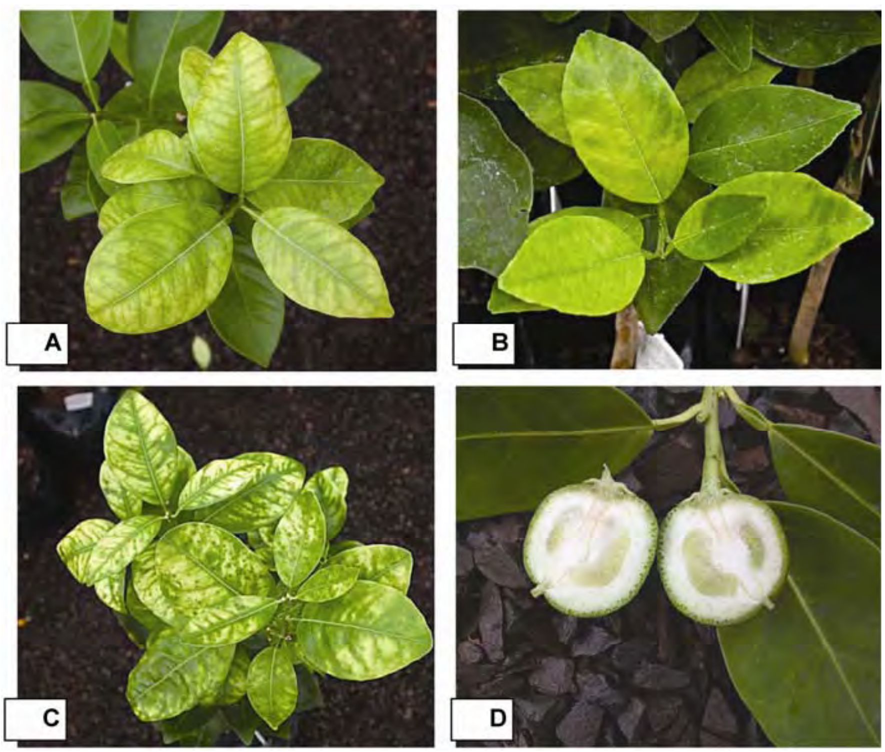

Fig. 1. Distinct pattern of leaf and fruit symptoms shown by huanglongbing (HLB)-infected young sweet orange plants graft inoculated with segments of symptomatic branches of field trees affected by '*Candidatus* Liberibacter americanus'. **A,** Leaf symptom similar to iron deficiency first observed 4 to 5 months after inoculation. **B,** Blotchy mottled leaves, characteristic of HLB, first observed approximately 6 months after inoculation. **C,** Leaf symptom similar to zinc deficiency first observed approximately 10 months after inoculation. **D,** Sectioned deformed fruit showing brownish internal columella.

RESULTS

In all experiments, symptoms were first observed on infected plants (as determined by PCR) approximately 4 to 5 months after inoculation or pruning. Symptoms were characterized by a yellowing of the apical leaves that is similar to deficiencies of manganese and iron (Fig. 1A). Blotchy, mottled leaves showing a diffuse and asymmetrical light chlorosis (Fig. 1B) appeared approximately 6 months after grafting or pruning and continued to appear up to 1 year, peaking around 8 months. As occurs in the field, mottled leaves could easily be detached from the plant or abscised spontaneously, especially during or after the colder months of the year. In general, by 10 months after inoculation, all infected plants expressed symptoms similar to intense zinc deficiency, characterized by a strong yellowing sometimes associated with the presence of small, green islands on the blade of the apical leaves (Fig. 1C). These symptoms resembling mineral deficiency did not disappear with foliar applications of micronutrients. Small deformed fruits with brownish columella were also observed in several sweet orange plants (Fig. 1D). PCR tests were positive only with samples from plants showing mottled leaves or from plants showing leaves with symptoms of mineral deficiency (Fig. 1).

Experiments designed to determine the best portion of a symptomatic tissue to use as inoculum revealed, in both experiments, significant variation in efficiencies of pathogen transmission (chi-square values of 30.75 [$P < 0.0001$] and 27.10 [$P = 0.0001$]) among treatments (Table 1). In no case was the pathogen transmitted when bark alone was used as a source of inoculum. Successful transmission occurred only with tissues containing the xylem (wood) and, in this case, the larger the tissue segment, the higher the transmission efficiency. Transmission efficiencies were 2.5, 10.0, and 25.0% in the first experiment and 2.6, 17.1, and 19.0% in the second experiment for individual buds (approximately 1.5 cm long) and 2- and 4-cm twigs, respectively. Transmission with 2 cm long fruit peduncle was 15.0% in the first experiment and 37.5% in the second experiment. In relation to graft tissue survival, with exception of buds, the experiment initiated during the winter resulted in lower rates, with an average of 66.4% versus 96.8% in the experiment initiated during the summer (Table 1).

In the experiment to evaluate citrus reaction to HLB, the first yellow leaves were observed on the new shoots 4 months after pruning. The appearance and development of leaf symptoms were similar to those described above (Fig. 1). Typical blotchy mottling appeared only on sweet oranges and Murcott tangor leaves (Fig. 1B). Less intense mottled leaves also appeared on Cleopatra mandarin. On the remaining cultivars, only general leaf chlorosis, typical of mineral deficiencies, was observed. Several plants that developed these symptoms, especially Sunki mandarin, Rangpur lime, and Swingle citrumelo, tested negative by PCR. Late symptoms in all PCR-positive plants included defoliation with yellow and curved leaves at the apex (Table 2).

The percentage of tissues that survived the grafting process varied from 40% in Ponkan mandarin to 98% in Swingle citrumelo. There was significant variation in pathogen transmission among citrus cultivars and species (chi-square 85.04, $P < 0.0001$). '*Ca.* Liberibacter americanus' was transmitted at higher percentages to sweet oranges (31.2 to 65.2%) and Murcott tangor (44.4%), followed by Cleopatra (25.0%) and Ponkan (20.0%) mandarins, Tahiti (11.1%) and Rangpur (7.7%) limes, Sunki (2.9%) mandarin, and Swingle citrumelo (2.0%). Among the three sweet oranges tested in this experiment, Hamlin was the most susceptible with 65.2% of infection.

The experiments conducted to determine if the pathogen would be propagated in the scion originating from buds grafted on Rangpur lime rootstock resulted in a low percentage of symptomatic young trees (Table 3). The pathogen was only detected in 1 of 115 plants in the first experiment and in 2 of 97 in the second experiment. In no case was the pathogen detected in scions originating from buds that were removed from the asymptomatic portion of HLB-affected trees or from asymptomatic trees. As observed in previous experiments, the rate of bud survival was affected by the season in which the experiment was initiated, with an overall average of 75.2% in the winter against 94.1% in the summer.

DISCUSSION

'*Ca.* Liberibacter americanus' is the new species of Liberibacter that has been confirmed only in Brazilian orchards to date. Because of its recent description, an inability to culture the pathogen, and its limited geographic distribution, little is known about the organism or its interactions with citrus trees. This study revealed some basic information on pathogen transmission, citrus responses to pathogen infection, and pathogen propagation to young trees.

Overall, the most appropriate source of inoculum to be used for transmission was 4 cm long budsticks removed from symptomatic branches. Transmission efficiencies ranged from 25.0 to 65.2%. Single buds and 2 cm long budsticks were less effective (10.0 to 17.1% transmissions). This is in contrast to the results obtained with '*Ca.* liberibacter africanus' in South Africa, where one to four buds transmitted the pathogen to 50% of the inoculated Madame Vinous sweet orange seedlings (15). We also found peduncle budsticks were an effective method to transmit the pathogen (15.0 and 37.5%), but in one experiment tissue survival was low (20%).

Tissue survival is a factor that should be considered in graft transmission experiments. Survival was lower during the cooler months of the year and variable among the varieties and species of citrus tested, which may be explained in these cases by distinct levels of tissue compatibility or by the relative sensitivity to cold-induced dormancy. The temperature also seemed to affect pathogen transmission, but contrary to tissue survival, transmission efficiency was higher during the winter.

Inoculated experimental plants showed characteristic leaf and fruit symptoms similar to those observed on field trees affected by HLB. Symptoms on leaves were variable, depending on citrus cultivar or species, and in many cases, were confusing. The characteristic blotchy mottling appeared only on sweet oranges and Murcott tangor with the remaining species showing only yellow leaves similar to those induced by mineral deficiencies. Because of this wide symptom variation during evaluations of greenhouse experiments, all plants showing any kind of yellow leaves should be evaluated by PCR.

The experiment designed to evaluate citrus reaction to '*Ca.* Liberibacter americanus' indicated that all tested materials are susceptible, but the level of susceptibility is highly variable, similar to that observed with '*Ca.* Liberibacter asiaticus' and '*Ca.* Liberibacter africanus' (4).

Table 2. Graft transmission efficiency of '*Candidatus* Liberibacter americanus' and symptom expression on distinct citrus cultivars and species used as scion (fruit production) or rootstock 15 months after inoculation

Cultivar/species	Use	Total[a]	Symptoms[b]	Transmission[c]
Hamlin sweet orange	Scion	23/25	10 mtl, 5 chl	15 (65.2)
Pera sweet orange	Scion	11/25	4 mtl	4 (36.4)
Natal sweet orange	Scion	16/25	3 mtl, 5 chl	5 (31.2)
Murcott tangor	Scion	18/25	8 mtl, 1 chl	8 (44.4)
Cleopatra mandarin	Rootstock	16/50	4 mtl, 2 chl	4 (25.0)
Ponkan mandarin	Scion	10/25	2 chl	2 (20.0)
Sunki mandarin	Rootstock	34/50	11 chl	1 (2.9)
Tahiti lime	Scion	45/50	5 chl	5 (11.1)
Rangpur lime	Rootstock	39/50	15 chl	3 (7.7)
Swingle citrumelo	Rootstock	49/50	8 chl	1 (2.0)
Chi-square				85.04 ($P < 0.0001$)

[a] Number of plants with viable grafted tissues from the total number of grafted plants.
[b] Number of plants showing mottled (mtl) or chlorotic (chl) leaves similar to symptoms of iron and zinc deficiencies (see Fig. 1).
[c] Total and percentage of plants that tested positive by polymerase chain reaction (PCR) with primers specific for '*Ca.* Liberibacter americanus' (12). The PCR tests were positive only with samples from symptomatic plants and included all plants with mottled leaves.

Table 1. Transmission efficiency of '*Candidatus* Liberibacter americanus' from symptomatic twigs and fruit peduncle and symptom expression on Valencia sweet orange 1 year after inoculation

	First experiment (summer)			Second experiment (winter)		
Grafted tissues	Total[a]	Symptoms[b]	Transmission[c]	Total	Symptoms	Transmission
Single bud	39	1 chl	1 (2.6)	40	1 mtl	1 (2.5)
Branch budstick 2 cm	40	4 mtl	4 (10.0)	35	6 mtl	6 (17.1)
Branch budstick 4 cm	40	7 mtl, 3 chl	10 (25.0)	21	4 mtl	4 (19.0)
Branch bark 2 cm	38	0	0	31	0	0
Branch bark 4 cm	34	0	0	27	0	0
Peduncle budstick 2 cm	40	4 mtl, 2 chl	6 (15.0)	8	3 mtl	3 (37.5)
Peduncle bark 2 cm	40	0	0	24	0	0
Chi-square			30.75 ($P < 0.0001$)			27.10 ($P = 0.0001$)

[a] Number of plants with viable grafted tissues from a total of 40 plants per treatment.
[b] Number of plants showing mottled (mtl) or chlorotic (chl) leaves, similar to symptoms of iron and zinc deficiencies (see Fig. 1).
[c] Total and percentage of plants that tested positive by polymerase chain reaction (PCR) with primers specific for '*Ca.* Liberibacter americanus' (12). The PCR tests were positive only with samples from symptomatic plants.

Table 3. Efficiency of '*Candidatus* Liberibacter americanus' propagation on new shoots grown from buds of symptomatic or asymptomatic branches, during the process of young nursery tree production, 1 year after grafting

	First experiment (summer)			Second experiment (winter)		
Branch status of bud origin	**Total[a]**	**Symptoms[b]**	**Pathogen propagation[c]**	**Total**	**Symptoms**	**Pathogen propagation**
Symptomatic	115	1 (0.9)	1 (0.9)	97	2 (2.1)	2 (2.1)
Asymptomatic (from affected trees)	113	0	0	100	0	0
Asymptomatic (from asymptomatic trees)	111	0	0	74	0	0
Chi-square		1.95 (P = 0.3765)			3.61 (P = 0.1641)	

[a] Number of plants with viable grafted tissues from a total of 120 plants per treatment.
[b] Number of plants showing mottled leaves (see Fig. 1).
[c] Total and percentage of plants that tested positive by polymerase chain reaction (PCR) with primers specific for '*Ca.* Liberibacter americanus' (12). The PCR tests were positive only with samples from symptomatic plants (see Fig. 1).

Among the materials used as scions, the most affected by '*Ca.* Liberibacter americanus' were the sweet oranges and the Murcott tangor, in agreement with field observations. Among the materials used as rootstocks, the most affected was Cleopatra mandarin. The commercial use of Cleopatra has increased recently in São Paulo State as a preventive measure against the citrus sudden death disease (11). The implication of its relatively high level of susceptibility on the development of HLB symptoms on the scion canopies is not known. Field observations indicate no rootstock effect, but no detailed comparative study has yet been conducted. In South Africa, the highest percentage of HLB fruit symptoms occurred where *Poncirus trifoliata* was used as rootstock. The effect was attributed to an influence of *P. trifoliata* on extending the flushing rhythm of the tree, and thus, extending the vector breeding and feeding periods, increasing the chances of transmitting the pathogen (16).

A low efficiency of pathogen propagation was observed in the experiments where the process to produce young nursery trees was used. Positive results were obtained on scions developed from buds of symptomatic branches but not from asymptomatic branches of the same affected trees or from asymptomatic trees. This indicates that the chances of disseminating '*Ca.* Liberibacter americanus' by planting asymptomatic nursery young trees that became infected by bud grafting are low. Indeed, currently in São Paulo State, this kind of dissemination is unlikely to occur since all nursery citrus buds and rootstocks are produced under screen. Nevertheless, the possibility cannot be discarded that, in the past, when young nursery trees were produced in open areas, some plants might have been infected by psyllas.

This work shows the results of citrus plants inoculated with '*Ca.* Liberibacter americanus' only. Ongoing experiments with '*Ca.* Liberibacter asiaticus' have shown a higher rate of pathogen transmission and the development of a less intense symptom of mineral deficiency (results not shown) on the inoculated plants.

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