Huanglongbing (Greening) in Vietnam: Detection of Liberobacter asiaticum by DNA-Hybridization with Probe In 2.6 and PCR-Amplification of 16S Ribosomal DNA

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ABSTRACT. Leaf samples were collected from 55 citrus trees from north, central and south Vietnam in January-February 1995, and analyzed by PCR amplification, DNA hybridization and immunofluorescence for the presence of the Huanglongbing (HLB) (greening) liberobacter. Forty-eight of the 55 samples hybridized with probe In 2.6 which is specific for Liberobacter asiaticum. No hybridization was obtained with probe As 1.7, specific for L. africanum. Forty-five of 47 samples analyzed by PCR gave the 1,160 bp amplicons expected from the liberobacter-specific primers used, and on XbaI digestion, the amplicons gave the two restriction fragments (640 bp and 520 bp) specific for L. asiaticum. The combined use of hybridization and PCR amplification was able to detect L. asiaticum in 53 of the 55 samples tested. Fourteen of 48 samples tested gave a positive immunofluorescence reaction with monoclonal antibodies 10A6 as well as 1A5, known to react with certain L. asiaticum serotypes only. Liberobacters were detected by electron microscopy in four samples tested. These samples were also found to be positive for L. asiaticum by hybridization and PCR. Finally, Hamlin sweet orange seedlings graft-inoculated with budwood sticks from four trees, which were later found positive for L. asiaticum by hybridization and PCR, developed symptoms. These results show that HLB is widespread throughout Vietnam. Symptoms of cristacortis, previously unrecorded in Vietnam, were also seen.

As Huanglongbing (HLB) (greening) is widespread throughout China and South-East Asia, it is not surprising that the symptoms of the disease have been reported from various regions of Vietnam where *Diaphorina citri* Kuwayama, the psyllid vector of the disease agent is known to be present (5).

We have recently developed techniques to detect the Asian HLB agent, "*Candidatus* Liberobacter asiaticum", in citrus: DNA hybridization with probe In 2.6, specific of L. asiaticum (1, 10, 11), and PCR amplification with L. asiaticum-specific primers OI1 and OI2C (6, 7). Similar techniques have also been developed for the specific detection of the African HLB agent, "*Candidatus* L. africanum" (6, 7, 9). We have applied these methods to citrus leaf samples that we collected during a survey in Vietnam in January and February 1995. Some of these samples were also used for detection of the liberobacter by electron microscopy and immunofluorescence. Budwood sticks, in addition to leaf samples, were collected from some of the trees and used for graft transmission assays of the HLB agent.

MATERIALS AND METHODS

Leaf samples, designated 1 to 55, were collected from 55 different citrus trees in various regions of north, central and south Vietnam from January 25 to February 14, 1995 (Table 1). All the trees had HLB or HLB-like symptoms, except tree 1. Attention was paid, whenever possible, to collect leaves affected by mottle, as this symptom is one of the most reliable ones for HLB diagnosis. The leaf samples were washed in tap water, surface dried and mailed by express mail to the INRA laboratory in Bordeaux. Before mailing, the leaf samples were kept in a refrigerator at 4°C for not more than 8 days. Some of the first leaf samples collected in northern Vietnam (samples 9, 11, 13, 14, 18, 19, 23) and one sample from the south (sample 39) underwent fer-

TABLE 1
DNA HYBRIDIZATION WITH PROBE IN 2.6, PCR AMPLIFICATION OF 16S RIBOSOMAL DNA AND IMMUNOFLUORESCENCE FOR THE DETECTION OF LIBEROBACTER ASIATICUM IN CITRUS CULTIVARS FROM VARIOUS PROVINCES OF VIETNAM

Location	Sample designation (sample no.)	No. of positive hybridization samples/Total no. samples analyzed	No. positive PCR samples/Total no. of samples analyzed	No. positive hybridization or PCR samples/Total no. of samples analyzed	Positive immunofluorescence with MA 10A6 and 1A5 (sample no.)
NORTH VIETNAM					
Ninh Binh province					
Dong Giao State farm	1 to 5[z]	4/5	4/5	4/5	2, 3
Xuan Mai Citrus Station	6 to 16[y]	10/11	6/7	11/11	6, 11
Hoa Binh province					
Thanh Ha State farm	17, 18[x]	1/2	1/1	2/2	
Van Canh suburb of Hanoi	19 to 23[w]	3/5	3/3	4/5	21
CENTRAL VIETNAM					
Hue province					
Thien An Monastery	24[v]	1/1	1/1	1/1	
Nan Dong State farm	25 to 28[u]	3/4	4/4	4/4	25
SOUTH VIETNAM					
Dong Nai province					
Tan Uyen district					
Tan Uyen village	29[t]	1/1	1/1	1/1	
Chau Thanh district					
Chau Thanh state farm	30[s]	1/1	1/1	1/1	30
Tien Giang province					
Chau Thanh district					
Thoi Son village					
Le van Be orchard	31, 32[r]	2/2	2/2	2/2	
Vinh Long province					
Binh Minh district					
Phong Hoa village					
Bui Van Tuoc orchard	33, 34[q]	2/2	2/2	2/2	

TABLE 1 (CONTINUED)
DNA HYBRIDIZATION WITH PROBE IN 2.6, PCR AMPLIFICATION OF 16S RIBOSOMAL DNA AND IMMUNOFLUORESCENCE FOR THE DETECTION OF LIBEROBACTER ASIATICUM IN CITRUS CULTIVARS FROM VARIOUS PROVINCES OF VIETNAM

Location	Sample designation (sample no.)	No. of positive hybridization samples/Total no. samples analyzed	No. positive PCR samples/Total no. of samples analyzed	No. positive hybridization or PCR samples/Total no. of samples analyzed	Positive immunofluorescence with MA 10A6 and 1A5 (sample no.)
Thanh Dong village					
Batan orchard	35, 36, 37[p]	3/3	3/3	3/3	
Ben Tre province					
Mo Cai district					
Thanh Ngai village					
Tran van Loc orchard	38, 39[o]	2/2	1/1	2/2	38
Tran Thanh Hien orchard	40[n]	1/1	1/1	1/1	40
Chau Thanh district					
Tien Long village					
Le van De orchard	41, 42[m]	2/2	2/2	2/2	42
Can Tho province					
Cai Be district					
My Loi village					
Phan van Bay orchard	43[l]	0/1	1/1	1/1	
Chau Thanh district					
Dong Thanh village					
Nguyen hung Hung orchard	44 to 47[q]	4/4	4/4	4/4	44, 46
Le van Thuong orchard	48[t]	1/1	1/1	1/1	
To van Pho orchard	49 to 52[i]	4/4	4/4	4/4	51
Le van Nhuong orchard	53[h]	1/1	1/1	1/1	
Can Tho University	54, 55[g]	2/2	2/2	2/2	55

TABLE 1 (CONTINUED)
DNA HYBRIDIZATION WITH PROBE IN 2.6, PCR AMPLIFICATION OF 16S RIBOSOMAL DNA AND IMMUNOFLUORESCENCE FOR THE DETECTION OF LIBEROBACTER ASIATICUM IN CITRUS CULTIVARS FROM VARIOUS PROVINCES OF VIETNAM

Location	Sample designation (sample no.)	No. of positive hybridization samples/Total no. samples analyzed	No. positive PCR samples/Total no. of samples analyzed	No. positive hybridization or PCR samples/Total no. of samples analyzed	Positive immunofluorescence with MA 10A6 and 1A5 (sample no.)
TOTAL		**48/55**	**45/47**	**53/55**	**14/48**

[a]1: Hamlin sweet orange; 2 to 5: Valencia sweet orange.
[b]6 to 10: Xa Doai sweet orange; 11, 14 to 16: Vandu sweet orange; 12: pummelo; 13: Rangpur lime.
[c]17: Vandu sweet orange; 18: lemon.
[d]19 to 21: Canh mandarin; 22: pummelo; 23: lemon.
[e]24: sweet orange.
[f]25, 26: Hong Nhieu mandarin; 27: sweet lime; 28: lemon.
[g]29: Duong da Lang pummelo.
[h]30: Ortanique tangor.
[i]31: King mandarin; 32: Mat sweet orange.
[j]33: acid lime; 34: Nam Roi pummelo.
[k]35: sweet orange; 36, 37: Thieu mandarin.
[l]38, 39: Mat sweet orange.
[m]40: Thieu mandarin.
[n]41: acid lime; 42: King mandarin.
[o]43: King mandarin.
[p]44 to 46: King mandarin; 47: Duong mandarin.
[q]48: King mandarin.
[r]49: Nam Roi pummelo; 50: Mat sweet orange; 51, 52: Duong mandarin.
[s]53: Mat sweet orange.
[t]54, 55: Mat sweet orange.

mentation and turned brown during shipment. These samples were unsuitable for PCR as their DNA could not be amplified, whatever primers were used. They were, however, suitable for DNA hybridization. Therefore, hybridization was conducted on all 55 samples, but PCR on only 47 (see Table 1).

DNA hybridization with probe In 2.6, specific of L. asiaticum, and probe As 1.7, specific of L. africanum, were carried out as described earlier (9, 10, 11), while PCR was conducted as described by Jagoueix et al. (6). The amplicons obtained by PCR were submitted to Southern blot hybridizations with a ^{32}P-labelled probe specific for liberobacter 16S ribosomal DNA (16S rDNA). In this way, some of the amplicons, barely visible upon ethidium bromide staining of the agarose gels, could be clearly seen upon autoradiography.

The use of immunofluorescence on thin sections of leaf midribs has been described previously (2, 3, 4). Electron microscopy for the detection of liberobacters in ultrathin sections of citrus leaf midribs was done according to Laflèche and Bové (8). For graft transmissions, budwood sticks were collected from trees 35, 39 and 55, and side-grafted onto Hamlin sweet orange seedlings in Bordeaux. The inoculated plants were kept in the glasshouse at 25 to 30°C. Budwood for graft-inoculation was also collected from a cristacortis-affected Duong mandarin on Mat sweet orange rootstock (tree 99) which also showed foliar symptoms of HLB.

RESULTS AND DISCUSSION

Fifty-five samples were collected in various provinces of Vietnam (Table 1) and the names of the cultivars corresponding to these samples are given in the legend of Table 1. None of the 55 samples gave a hybridization signal with the ^{32}P-labelled probe As 1.7, specific of L. africanum (results not shown).

Fig. 1 represents the results of dot-blot hybridizations between the

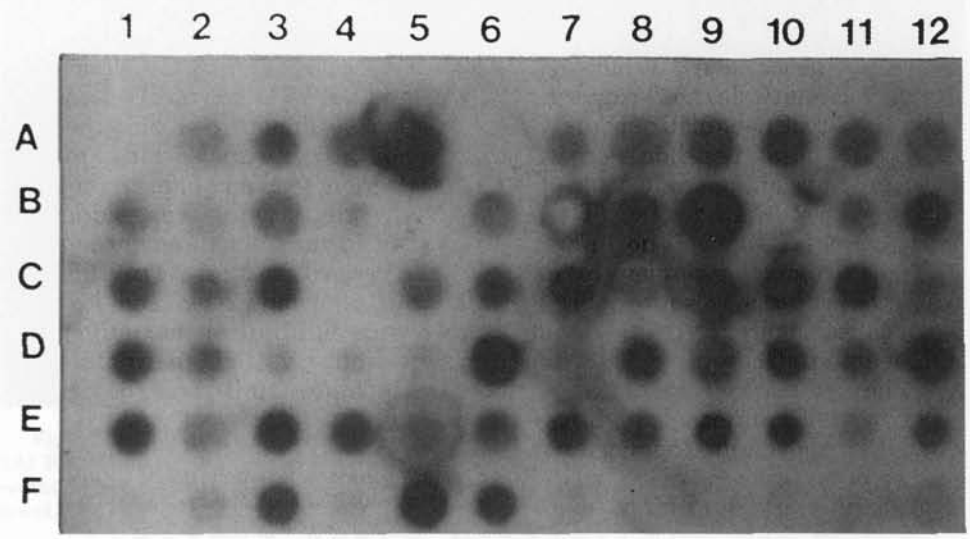

Fig. 1. Audoradiography of dot-blot hybridizations between the leaf DNAs of the 55 samples and the ^{32}P-labelled probe In 2.6. Each position represents a leaf sample from an individual tree. Sample designation is a follows: samples 1 to 12: lane A, 1 to 12; samples 13 to 24: lane B, 1 to 12; samples 25 to 36: lane C, 1 to 12; samples 37 to 48: lane D, 1 to 12; samples 49 to 55: lane E, 1 to 7.

DNAs from the 55 samples and the ^{32}P-labelled probe In 2.6. Forty-eight of the 55 samples gave positive hybridization signals. Of the remaining seven samples, four were found positive by PCR: samples 6 (A6 on Fig. 1), 17 (B5), 22 (B10) and 28 (C4) (Fig. 2 and 3). Two of the seven samples, 19 (B7) and 43 (D7), gave inconclusive hybridization signals. Sample 19 was one of the eight samples which had turned brown during shipment and could not be amplified by PCR. Sample 43 gave a positive PCR signal, but only after Southern blot hybridization with the ^{32}P-labelled 16S rDNA probe (result not shown). Only one of the seven samples was negative by both hybridization and PCR, as well as by immunofluorescence. This was sample 1 from a Hamlin sweet orange on Cleopatra mandarin which was severely affected by phytophthora gummosis but free of HLB symptoms.

Results of the PCR amplifications of the 55 samples with primers OI1 and OI2C defined from the 16S ribosomal DNA sequence of L. asiaticum are shown in Fig. 2. As expected from the primers used (6), the size of

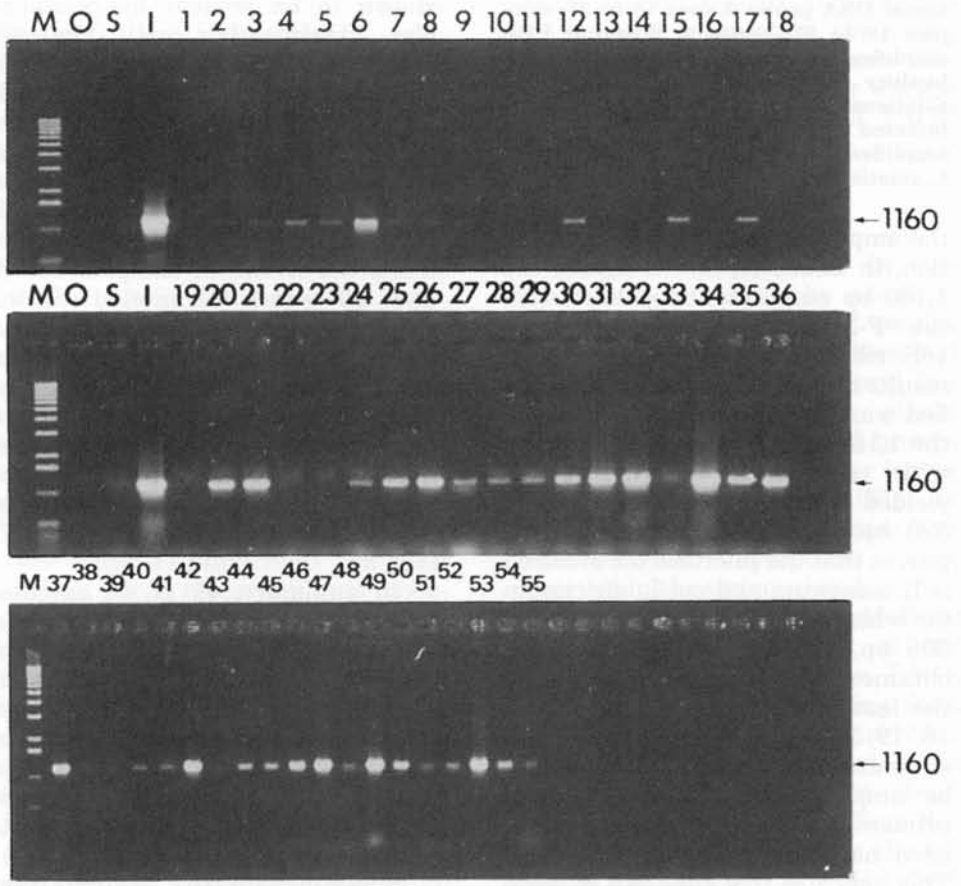

Fig. 2. DNA amplification with primers OI1 and OI2c specific for liberobacter 16S ribosomal DNA, followed by agarose (0.7%) gel electrophoresis. Amplification with the DNAs from samples 1 to 55: lanes 1 to 55; with water: lane 0; with DNA from glasshouse grown healthy citrus: lane S; with DNA from L. asiaticum (Poona strain)-infected citrus: lane I. 1160: size (bp) of DNA amplified with primers OI1 and OI2C on L. asiaticum DNA. M: DNA size markers.

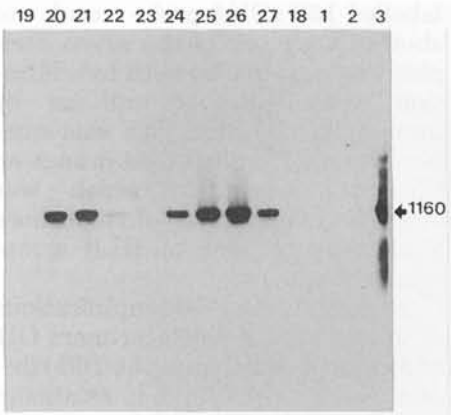

Fig. 3. Autoradiography after Southern-blot hybridization between amplicons from samples 18 to 27 and the ^{32}P-labelled, liberobacter-specific, 16S ribosomal DNA probe. Lanes 18 to 27: samples 18 to 27. Lanes 1, 2 and 3: PCR amplification respectively with water, healthy citrus DNA, and DNA from L. asiaticum (Poona strain) from HLB trees infected citrus. 1,160: size (bp) of DNA amplified with primers OI1 and OI2C on L. asiaticum DNA.

the amplicons was 1,160 bp. In addition, in Southern blot analysis, the 1,160 bp amplicons hybridized with the ^{32}P-labelled, liberobacter specific 16S ribosomal DNA probe. These results indicate that the DNA amplified was Liberobacter DNA. Finally, the 1,160 bp amplicon, when treated with restriction enzyme *XbaI*, yielded two fragments (640 bp and 520 bp) (results not shown). This proves that the liberobacter involved is L. asiaticum and not L. africanum for which three fragments (520 bp, 506 bp, 130 bp) would have been obtained (6). As mentioned above, the leaves of samples 9, 11, 13, 14, 18, 19, 23 and 39 fermented during shipment and their DNA could not be amplified, even with universal primers for 16 S ribosomal DNA, used as controls for amplification. This indicates that the DNA of these samples was probably too degraded for PCR amplification, even though it was still suitable for DNA hybridization (Fig. 1). The 1,160 bp amplicons were absent from the lanes of these eight samples (Fig. 2). Similarly, the 1,160 bp DNA was also absent from lane 1. The DNA of this sample, which came from the tree without HLB symptoms but with phytophthora gummosis, could be amplified with the universal, control primers (result not shown) but not with the liberobacter specific primers OI1 and O12C. Of the remaining 47 samples, 45 gave the liberobacter specific 1,160 bp amplicons upon PCR amplification with primers OI1 and OI2C. For some of the 45 positive samples, the 1,160 bp amplicons were hardly visible on the agarose gel (Fig. 2, samples 2, 3, 7, 8, 10, 22, 38 and 43) but they could be shown to be present by Southern blot hybridization with the ^{32}P-labelled, liberobacter specific 16S rDNA probe. This is illustrated in Fig. 3 for sample 22. Its amplicon could not be seen on the agarose gel (Fig. 2, lane 22), but it was revealed by its hybridization with the 16S rDNA probe (Fig. 3). On the contrary, the DNAs of samples 18, 19 and 23 which fermented during shipment, could not be amplified and no amplicons were seen (Figs. 2 and 3). With 37 samples, the amplicons were readily distinguishable on the gel (Fig. 2) and they gave strong hybridization bands upon Southern blot analysis. This is illustrated for samples 20, 21, 24, 25, 26 and 27 (Fig. 3).

In summary, 45 of 46 samples from trees with HLB symptoms and suitable for PCR amplification gave positive, liberobacter-specific PCR reactions, and the liberobacter species involved was L. asiaticum. In the DNA hybridization experiments reported above (Fig. 1), the liberobacter species detected was also L. asiaticum.

Summing up the results from DNA hybridization and/or PCR (Table 1), 53 of the 55 samples were positive for L. asiaticum by either one or the other of the two molecular techniques; 48 of 55 had a positive

hybridization and 45 of 47, a positive PCR. Forty-one were positive by both hybridization and PCR; four (samples 6, 17, 22, 28) were positive by PCR but negative by hybridization, and one was negative by PCR but positive by hybridization (sample 16). These results show the value of combining DNA-hybridization and PCR amplification for the detection of liberobacter in citrus.

Liberobacter cells were seen by electron microscopy in the sieve tubes of all four samples examined (samples 17, 30, 32, 41) (results not shown). These samples were also positive by the two molecular techniques.

Longitudinal sections of leaf midribs from 48 of the 55 samples were analyzed by immunofluorescence with the various monoclonal antibodies available (2, 3, 4). Fourteen of the 48 samples gave positive immunofluorescence reactions only with monoclonal antibodies 10A6 (4) and 1A5 (2) (Table 1). These 14 samples were also positive by the molecular techniques. These results show, as reported previously (3), that there are different serotypes of L. asiaticum of which some react with the monoclonal antibodies 10A6 and 1A5, while others do not.

The Hamlin sweet orange seedlings graft-inoculated with budwood sticks from trees 35, 39, 55 and 99 developed characteristic HLB-symptoms within 1 yr after inoculation. The leaf samples from trees 35 and 55 were positive by hybridization and PCR; sample 39 was positive by hybridization. No leaf sample was taken on tree 99, but this Duong mandarin tree showed HLB symptoms in the field and the nearby Duong mandarin tree (tree 52) was positive by hybridization and PCR.

Thus, on the basis of hybridization, PCR, immunofluorescence, electron microscopy and graft transmission experiments, the presence of HLB in Vietnam has been experimentally established for the first time. The liberobacter species involved is L. asiaticum.

Table 1 shows that HLB is present in all the major citrus production areas of north, central and south Vietnam surveyed. The cultivars affected include sweet oranges, mandarins, pummelos, lemon, Rangpur lime, sweet lime and tangor.

The wide distribution of HLB in Vietnam is probably due to two major reasons: (i) use of infected nursery plants and (ii) transmission by *D. citri*. An example of the efficiency of *D. citri* in spreading L. asiaticum is given by tree 30 of Table 1, an Ortanique tangor, one of 2-yr-old mother trees at the Chan Thanh state farm (Dong Nai province) propagated from certified budwood introduced from Corsica in 1990-1991 and planted in the field in 1993, 30 km from any other citrus trees. Within 2 yr of planting it, and other trees, showed typical HLB leaf mottle and was found to be infected with L. asiaticum by hybridization, PCR, immunofluorescence (Table 1) and electron microscopy. Adults of *D. citri* were seen on the tree.

In the course of this survey, a number of citrus diseases, some previously unrecorded in Vietnam, were observed: (i) exocortis on trifoliate, Carrizo citrange and Troyer citrange rootstocks at Xuan Mai citrus station (north Vietnam); (ii) tristeza-induced vein clearing on acid lime (tree 33) and Mat sweet orange (tree 32), the latter probably a severe strain; (iii) cristacortis stem pitting in three areas on Duong mandarin marcott trees (tree 47) and Duong on Mat rootstock (trees near trees 49-52 and 54-55) in the southern province of Can Tho; (iv) phytophthora gummosis on sweet orange and mandarin in many orchards in the north (e.g., Dong Giao state farm); (v) Rio Grande gummosis on pummelo in the south (Bin Van Truc); and (vi) citrus canker on mandarin fruit (Tran van Loc, south Vietnam).

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