# Detection of the Asian Strains of the Greening BLO by DNA-DNA Hybridization in Indian Orchard Trees and Malaysian *Diaphorina citri* Psyllids

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ABSTRACT. A BLO DNA fragment (In 2.6) from a Poona (India) strain of the greening organism (GO) has been cloned and used as a probe for the detection of the BLO in field trees and psyllids in India, in February 1992. Fifty three samples from Orissa, Rajasthan, Uttar Pradesh, and Myssore areas, were tested by dot hybridization using $^{32}P$ - labelled probes. Most of the samples were also examined by electron microscopy (EM) for the presence or absence of the BLO. The results show that DNA-DNA hybridization is able to reliably detect the GO in all regions of India with a sensitivity at least equivalent to that of EM.

The probe was also used to detect the GO in individual *Diaphorina citri* insects collected on infected trees in Delhi and Bangalore. A very low percentage of the psyllids (less than 1%) was found contaminated with the GO. This was also the case with psyllids collected in Malaysia in May 1992 (5% infection). On the contrary, when *D. citri* was collected in Malaysia in September 1991, 39% of the insects were found infected with the GO, indicating a variation in the percentage of contaminated *D. citri* insects during the year.

These studies show that probe In 2.6 is a powerfull reagent to detect the GO in field trees as well as in individual insects, and will be most useful in epidemiology studies.

Following the report of Fraser *et al.* (3) and the demonstration by Capoor *et al.* (2) that the greening pathogen was vectored in India by the Asian citrus psylla, *Diaphorina citri*, it became generally admitted that greening was widespread throughout India. This belief was essentially based on symptomatology, particularly foliar zinc deficiency symptoms. Leaf mottle, a better symptom of greening, was generally overlooked. In the early 1970s, Laflèche and Bové (9) showed by electron microscopy (EM) that the greening pathogen was not a virus but a prokaryotic organism, first described as mycoplasma-like, but later recognized as bacterium-like (1, 5, 12). The first EM visualization of this newly discovered bacterium-like organism (BLO) in leaf material from India was reported in 1971 (12), following the visit of a group of citrus virologists to S. P. Capoor in Poona after the IOCV conference in Japan in 1969. A Mosambi sweet orange seedling experimentally infected with a Poona strain of the greening pathogen, was taken to Bordeaux, France, where it was shown to contain a phloem-restricted BLO morphologically identical to that seen in greening-affected leaves from South Africa, Reunion island and the Philippines (1). Later, Indian workers in Bangalore also detected the greening BLO by EM in Indian citrus leaves (11).

The Mosambi sweet orange seedling infected with the Poona strain of the BLO was used in Bordeaux as a source of inoculum. Through periodic graft transmissions to healthy sweet orange seedlings, the Poona BLO strain has been maintained in the Bordeaux glasshouse, ever since 1969. These graft inoculated seedlings carry only the greening BLO, as the initial Mosambi sweet orange seedling was infected with the BLO through *D. citri* vector transmission. In particular, they are free of citrus tristeza virus.

In 1983, African and Asian strains of the BLO were transmitted experimentally to periwinkle plants by dodder (4), and BLO-infected periwinkle leaves rather than citrus material, have been used thereafter for the development of BLO-specific detection reagents.

Since 1987, monoclonal antibodies (MAs) have been obtained, first against the Poona strain of the BLO, then against a BLO strain from Fujian (Chi-

na) and one from Nelspruit (South Africa) (6, 7, 8). Their use resulted in the discovery that different serotypes of the BLO occurred, even in nearby orchards. However, each MA reacted almost exclusively with the BLO strain used for immunization (homologous strain) and, hence, these MAs, even when combined in mixtures, were too strain specific for diagnostic purposes.

More recently, DNA restriction fragments of the Poona BLO strain have been cloned and sequenced (14, 15). A 2.6 kbp fragment was identified as the *rpl*KAJL*rpo*BC operon, a well-known operon in eubacteria, coding for four ribosomal proteins of the large ribosomal subunit, and for proteins β and β' of RNA polymerase. As ribosomal proteins as well as their genes are phylogenetically conserved, it was not surprising that the 2.6 kbp fragment, when used as a probe in Southern and dot-blot hybridizations, was able to detect not only the homologous Poona BLO strain, but also the seven other Asian strains tested (14). The probe was even able to detect the Nelspruit strain, the only South African strain tested, when hybridization was carried out at a stringency lower than that for the Asian strains (15). These results were obtained with greenhouse-grown plants maintained at the most favorable conditions for good symptom expression. We wanted to evaluate the adequacy of the 2.6 kbp DNA probe to detect the BLO in Indian orchard trees often growing under suboptimal conditions. This evaluation required that field trees unambiguously known to be infected by the BLO be available as positive control trees. At the beginning of this work, no such trees were known in India. As EM was the only technique by which infection of a tree by the BLO could be relatively quickly established, we have analyzed many of the orchard trees by both EM and dot-blot hybridization (dbH). Previous EM work in our laboratory has shown that the best material to detect the greening BLO consists of midveins from leaves showing blotchy mottle as described by McClean and Schwarz (10). This symptom has been found on greening-affected trees in all geographical areas where African or Asian greening occurs, and it is shown by all cultivars affected, even though mottle is sometimes less pronounced on mandarin than on sweet orange leaves. Therefore, occurrence of leaf mottle was an important factor in selecting orchard trees for BLO detection by EM and/or dbH.

In the work reported here we have evaluated dbH in comparison with EM for the detection of the greening BLO in Indian orchard trees. We have also applied hybridization to the detection of the BLO in individual *D. citri* psyllids from Malaysia.

## MATERIALS AND METHODS

**Leaf samples.** Leaves showing mottle were collected in January-February 1992 for EM and dbH. Leaves from trees without greening symptoms were also sampled; some came from normal, symptomless trees, others showed zinc deficiency symptoms or symptoms of Indian ringspot or mosaic virus. Healthy periwinkle leaves as well as periwinkle leaves and Hamlin sweet orange leaves infected with the Poona BLO strain were from the Bordeaux glasshouse, and were used as negative and positive controls respectively. The leaf samples from individual trees were kept in plastic bags at 4 C, no longer than 5 days before they were used. Leaves were washed and rubbed under tap water, and wiped dry with filter paper. The leaf midveins with about 1 mm of leaf blade on each side were removed with a scalpel. A few, 2-4 mm-long midvein pieces were fixed in 2% glutaraldehyde for EM. Two grams of midveins from each sample were used for DNA extraction. Midveins for DNA extraction were stored at -80 C before use.

**Psyllids.** Psyllids were collected with a mouth aspirator in September 1991 and May 1992 in Malaysia by Dr. Teo Chan Hock on greening affected trees from the same orchard. They were sent to Bordeaux by 48 hr-deliv-

ery mail. Psyllids were also collected in Indian orchards in February 1992.

**Methods.** DNA extraction from leaf-midveins and dbH with the 2.6 kbp DNA probe have been described previously (14). EM was carried out on longitudinal sections of phloem tissue as these are more suitable for BLO detection by EM than transverse sections. The EM techniques were as previously described (9). EM was carried out in Bordeaux, DNA for dbH was extracted at IARI, New Delhi, and dbH with the 32P-labelled probe was carried out in Bordeaux.

For detection of the greening BLO in *D. citri*, the dead psyllids were, upon arrival, individually crushed with a glass rod onto a nylon N+ membrane previously soaked in 5 SSC. The membrane was then placed for 30 min on a Whatman paper soaked in 0.4 N NaOH. The membrane was air dried and hybridized with the 2.6 kbp probe.

## RESULTS AND DISCUSSION

**Detection of the greening BLO in orchard-trees.** Fifty-three leaf samples were collected from individual trees in 18 orchards distributed throughout India, except Assam and the Punjab. Leaves of the major citrus cultivars grown in India were assayed: Chini, Mosambi and Sathgudi sweet oranges; Coorg, Kinnow and Nagpur mandarins, and Kagzi lime. Samples of the following citrus species were also analyzed: undetermined sweet orange varieties, rough lemon (major rootstock in India), *Citrus indica* and *Citrus macroptera*.

Leaf mottle was present in all areas surveyed. Forty-two of the 53 samples consisted of midveins from mottled leaves (Table 1). The remaining 11 samples came from trees without mottle or other symptoms of greening, i.e. normal, symptomless trees, trees showing

TABLE 1
DETECTION OF THE GREENING BLO BY ELECTRON MICROSCOPY (EM) AND DOT-BLOT HYBRIDIZATION (dbH) IN MIDVEINS OF MOTTLED LEAVES FROM VARIOUS CITRUS CULTIVARS IN INDIA

| Cultivars | Number of samples | Number of samples with mottle | Detection of BLO in mottled leaves by dbH[z] | EM[z] | Spots on autoradiogram of Fig. 1 |
|---|---|---|---|---|---|
| Sweet orange | | | | | |
| Chini | 3 | 3 | 3/3 | 3/3 | D8, D9, D10 |
| Mosambi | 6 | 2 | 2/2 | 2/2 | E5, E7 |
| Sathgudi | 2 | 2 | 2/2 | 2/2 | C9, C10 |
| Other | 3 | 3 | 3/3 | 2/2 | B3, C6, C7 |
| Mandarin | | | | | |
| Coorg | 12 | 11 | 11/11 | 7/7 | B2; 4 to 7, 9 to 11 C1, C2, C5 |
| Kinnow | 6 | 4 | 4/4 | 3/3 | D11, D12, E1, E2 |
| Nagpur | 9 | 5 | 3/5 | 2/5 | A4, A5, A7 |
| Kagzi lime | 3 | 3 | 3/3 | 2/3 | A2, C8, C11 |
| Rough lemon | 4 | 4 | 4/4 | 2/2 | B8, D5, D6, D7 |
| *Citrus indica* | 2 | 2 | 2/2 | 0/1 | D3, D4 |
| *Citrus macroptera* | 1 | 1 | 0/1 | 1/1 | |
| Undetermined | 2 | 2 | 2/2 | 2/2 | C3, D1 |
| Total | 53 | 42 | 39/42 93% | 28/33 85% | |

[z]Figures are number of samples found positive by dbH or EM over total number of samples tested by dbH or EM.

zinc deficiency symtoms but no leaf mottle, or trees showing symptoms of virus diseases (ringspot, mosaic). Fig. 1 shows the autoradiography of a Nylon membrane on which the DNA preparations from the 53 field samples were applied. Three additional DNA preparations were blotted; one from healthy periwinkle leaves (negative control), one from BLO-infected periwinkle leaves and one from BLO-infected Hamlin sweet orange leaves (positive controls). The seedlings for these control preparations were growing in the Bordeaux glasshouse. Fig. 1 shows that no hybridization signals were obtained with the negative control DNA (E9) nor with the DNAs from the 11 field trees without symptoms of greening (A3, A6, C4, E3, B12 for instance). In contrast, positive hybridization signals were obtained with the positive control DNA from periwinkle (E10) or citrus (E11) leaves as well as with 39 of the 42 midvein samples from mottled leaves (Table 1). The intensities of the positive hybridization signals varied from very weak (E4, E7) to very strong (B6, C2 for instance) (Fig. 1). As EM is a cumbersome and more time-consuming technique than dbH, only 33 of the 42 samples from mottled leaves were examined by EM. In 28 of the 33 samples, the greening BLO could be detected by EM (Table 1). The 28 samples positive by EM were also positive by dbH except one (*Citrus macroptera*, Table 1 and Fig. 1, C4). Furthermore, dbH signals were obtained with samples from mottled leaves that were negative by EM. Such signals are for instance D3 (*C. indica*), C11 (Kagzi lime), B3 (sweet orange), and A4 (Nagpur mandarin). This shows that the BLO was present but was not detected by regular EM. Probably more extensive search for the BLO in a greater number of sections would have revealed its presence. Inversely, sometimes EM was positive, but dbH was negative; this is the case for the *C. macroptera* sample of Table 1 and Fig. 1 (C4). In this instance even though EM was positive, the number of BLOs detected was very low: only one BLO for several sections observed. These results indicate that EM and dbH may give erratic results for too low concentrations of the BLO.

The samples found positive by EM and/or dbH came all from trees with leaf mottle. Conversely, in the absence of mottle, EM and/or dbH were always negative. As shown in Fig. 1, this is

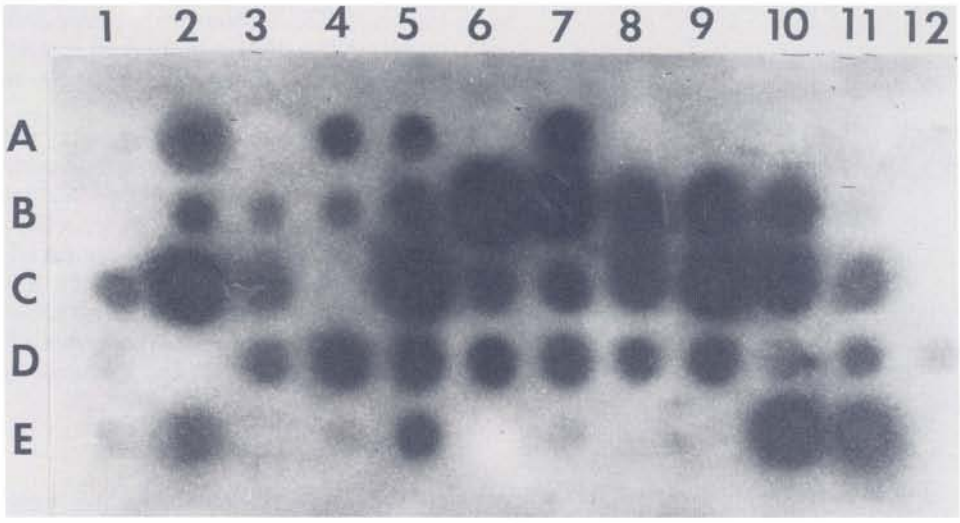


**Fig. 1. Dot blot hybridization between the 2.6 kbp probe and DNA extracted from 53 field samples. Field samples identified in Table 1 and controls in the text.**

for example the case of samples B12 (symptomless Coorg mandarin), E6 (symptomless Mosambi sweet orange), A12 (Mosambi sweet orange with zinc deficiency), and A8 (Mosambi sweet orange with ringspot symptoms). These results confirm the great value of leaf mottle for the diagnosis of greening in the orchard. However, there was a third situation; leaves were mottled but both EM and dbH were negative. This was the case of two of the Nagpur mandarin samples in Table 1 (A1 and A3, Fig. 1). Interpretation of such results is difficult. It could mean that the BLO was indeed present in these leaves as shown by leaf mottle, but in concentrations too low to be detected by EM or dbH. Or else the tree was free of greening, and leaf mottle was, in this case, unrelated to greening. It is known that leaf mottle is not a specific symptom of greening, as stubborn affected leaves may also show it, and leaf mottle sometimes results from root damage.

**Detection of the greening BLO in *D. citri* psyllids.** Fig. 2 shows the autoradiography of a Nylon membrane on which 115 *D. citri* psyllids were crushed and submitted to hybridization with the 2.6 kbp probe. Forty-five (39%) of these psyllids gave a positive hybridization signal. They were collected in Malaysia in September 1991. Among the psyllids captured in May 1992, on the same greening affected trees only 5% gave a hybridization signal (results not shown). Only a very low percentage (less than 1%) of psyllids were found contaminated in February 1992 in India. Uninfected *D. citri* psyllids raised in the Bordeaux insectarium have always given negative reactions. These results show that it is now possible to determine the percentage of BLO-contaminated psyllids within a population throughout the year.

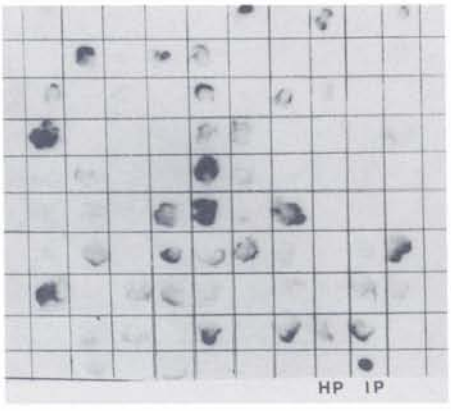


**Fig. 2. Crush blot hybridization of *D. citri* psyllids from Malaysia with the 2.6 kbp probe. HP: DNA from healthy periwinkle leaves, IP: DNA from Poona BLO-infected periwinkle leaves.**

## CONCLUSION

The 2.6 kbp DNA probe is able to detect the greening BLO in orchard trees with a sensitivity similar to, or perhaps somewhat higher than EM. As all Asian strains tested so far are detected by the 2.6 kbp probe, dbH is a good substitute for EM. It is cheaper, less cumbersome and less time-consuming. There is a good correlation between presence of leaf mottle and presence of the BLO as detected by EM or dbH. For detection of the BLO by dbH (or EM) only mottled leaves should be used at this time. The geographical distribution of greening in India based on dbH, EM, serology and leaf mottling is reported elsewhere in these proceedings (13). The 2.6 bkp probe is also able to detect the BLO in individual *D. citri* psyllids, and should be valuable in epidemiology studies.

Hybridization will however not solve all the problems. Even more sensitive techniques are required for detection of the BLO early in infection, when no symptoms have yet appeared. Gene amplification by PCR with primers deduced from the 2.6 kbp DNA fragment or from other BLO genes offers new perspectives and has already given very promising results.

## ACKNOWLEDGEMENTS

We are grateful to the Indo-French Center for the Promotion of Advanced Research through which this work was made possible.

We thank the following colleagues for their help during our survey: H. C. Dass, S.A.M.H. Naqvi (National Research Centre for Citrus, Nagpur, Maharashtra), S. J. Singh and K. J. Chandra (Bangalore, Karnataka), A. Narain, J. N. Das, B. Mohanti (Bubaneshwar, Orissa), S. N. Behera (Dhenkanal, Orissa), N. Khatna (Angul, Orissa), Dr. M.M. Mathur (Kota, Rajasthan), and Dr. V. V. Sulladmath (Gonikoppal, Karnataka).

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