Detection of Greening BLO by Electron Microscopy, DNA Hybridization in Citrus Leaves with and without Mottle from Various Regions in India

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ABSTRACT. Leaf mottle is one of the symptoms of citrus greening disease. We have collected leaves with and without mottle in various regions of India. Detection of the greening BLO in midribs of these leaves was carried out by electron microscopy (EM), dot blot DNA hybridization (dbH) and ELISA. The DNA probe and the monoclonal antibodies (MAs) used were produced from the Poona strain of the BLO.

Fifty-seven of 59 leaf samples with mottle gave a positive reaction by EM, dbH or ELISA. None of the 11 leaf samples without mottle was positive by either one of the techniques. These results confirm that leaf mottle is a useful symptom in surveying for greening in India. Most samples found positive by EM were also positive by dbH, a technique more convenient than EM. MAs directed against the Indian Poona strain of the BLO detected the BLO in only one of four orchards tested, showing that these MAs are unsuitable for diagnostic purposes.

The presence of greening as based on detection of the greening BLO was confirmed in the following regions: Andhra Pradesh (Hindupur, Tirupati), Delhi, Karnataka (Bangalore, Coorg area), Maharashtra (Poona), Orissa (Angul, Subalda) and Rajasthan (Jhalawar).

Citrus decline or dieback disease was reported to have been present in India as early as the 18th century (3). During the 20th century it has spread alarmingly, especially since the 1940s, and by the early 1960s it had been recorded in all citrus growing areas of the country. It was generally accepted that the problem involved tristeza virus, zinc deficiency and some fungal parasites of twigs such as *Colletotrichum gloeosporioides*, *Curvularia tuberculata*, *Diplodia natalensis* and *Fusarium* spp. (3, 14). However, none of the above conditions could adequately explain the disease or its spread (5). In 1966, at the request of the government of India, Lilian Fraser made a study of citrus dieback disease in all major citrus areas of India, and concluded that dieback was caused by the "virus" responsible for greening disease, because dieback in India closely resembled greening in South Africa (4, 5, 6).

At the time when the above survey was carried out, the so-called greening "virus" had not yet been identified. In 1970-1971 French scientists in Versailles discovered that the agent associated with South Africa greening was not a virus, but a microorganism restricted to the sieve tubes of affected plants (11, 12, 15, 16). Furthermore these workers showed that a similar microorganism was also present in the phloem of a Mosambi sweet orange seedling infected with a Poona (Maharashtra) strain of Indian citrus decline or dieback (2, 15, 16). This result supported the conclusion of Fraser *et al.* (5) that indeed Indian citrus dieback was associated with an agent similar to that of greening disease. It has since been shown that the agent of greening is a bacterium with a cell wall of the Gram negative type (7, 8). It has not yet been cultured in cell-free medium, and it is therefore called a "bacterium-like organism" or BLO.

We have recently produced monoclonal antibodies (9) and DNA probes (17, 18) for the detection of the greening BLO. We have used these reagents to survey for the presence of greening in various regions of India. In previous studies we have found that leaf blotchy mottle as described by McClean and Schwarz (13) is more reliable than zinc deficiency patterns for greening diagnosis. This is also true in India. Indeed, we have shown recently that there is

a good correlation between mottle on leaves and the presence of the greening BLO in such leaves as determined by electron microscopy (EM) and dot blot hybridization (dbH) (1). Here we report the geographical distribution of greening in India as based on leaf mottle and detection of the BLO by EM, dbH and ELISA.

MATERIALS AND METHODS

Leaf samples. Leaves were collected from orchard trees in various parts of India (Fig. 1) in December 1990 for EM and ELISA, and in January-February 1992 for EM and dbH. They were treated as described elsewhere (1).

Methods. EM techniques and dbH with the 2.6 kbp probe from the Poona BLO strain have been described previously (17), as have the enzyme-linked immunosorbent assays (ELISA) with monoclonal antibodies 2D12 and 10A6 directed against the Poona strain of the BLO.

RESULTS AND DISCUSSION

Seventy leaf samples were collected in 21 orchards in the regions of India (Fig. 1, Table 1). Among the 59 samples with leaf mottle, 57 (96%) gave a positive reaction by EM, dbH or ELISA (Table 1). None of the 11 samples without mottle was positive by either one of the techniques. Most samples found positive by EM were also positive by dbH. These data indicate that leaf mottle is a useful symptom in surveying for greening. The presence of the greening BLO in trees with leaf mottle can now be confirmed by dbH, a technique more convenient than EM.

Monoclonal antibodies (MAs) directed against the Poona strain of the greening BLO when used in ELISA, detected the greening BLO in only one of four orchards tested (Table 1, Bangalore), even though all four orchards were affected by greening as determined by EM and dbH. The ELISA reactions, in samples from this orchard, were highly positive, as the optical densities at 405 nm were between

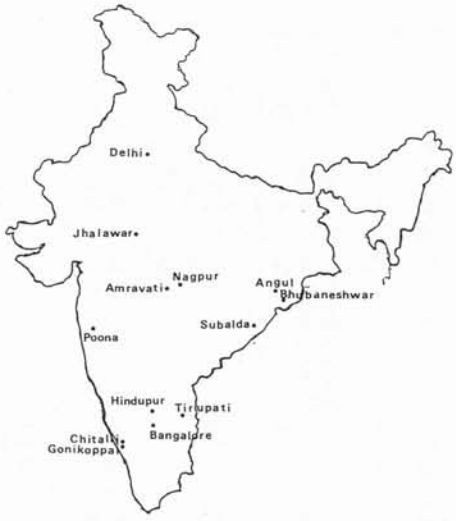

Fig. 1. Map of India showing locations from where samples for greening analysis were collected.

1 and 2. The orchard in which the MAs detected the greening BLO is the 20-yr-old varietal collection of the Indian Institute of Horticultural Research (I.I.H.R.) near Bangalore in Southern India. Many trees of this collection have died. On the basis of leaf mottle, EM, dbH and ELISA it is clear that greening is present and is probably responsible for the severe decline of trees in this orchard. Within 5 km of this orchard, a 4-yr-old Chini sweet orange orchard, at Kasakatapura village, was also found by EM and dbH to be severely affected by greening. However ELISA reactions were negative (Table 1). These results show that two nearby orchards are infected with two different strains of the BLO, one strain being detected by the anti-Poona BLO MAs and hence related to the Poona BLO strain, the other giving no reactions. Obviously, as pointed out previously (10), these MAs are unsuitable for diagnostic purposes as they recognize only certain strains of the BLO.

The region around Hindupur, 100 km North of Bangalore, is severely affected by greening (Table 1). Sweet orange and Kagzi lime are the major cultivars. The Coorg region, west of Bangalore, around Gonikoppal and

TABLE 1
NUMBER OF LEAF SAMPLES WITH AND WITHOUT MOTTLE WHICH TESTED POSITIVE BY ELECTRON MICROSCOPY (EM) DOT BLOT HYBRIDIZATION (dbH) OR ELISA IN VARIOUS REGIONS OF INDIA

REGION Nearby town	Cultivars[a]	Number of leaf samples tested With +M	 Without –M	Samples positive by EM/samples tested +M	 –M	Samples positive by dbH/sample tested +M	 –M	Samples positive by ELISA/samples tested +M	 –M	Positive samples/ samples tested +M	 –M
ANDHRA PRADESH											
Tirupati	Sat. sw. or. (1)	3	1	3/3	0/1		ND_b	0/3	0/1	3/3	0/1
Hindupur	Sat. sw. or.	4		3/3		4/4			ND	4/4	
	Kagzi l. (2)	1		1/1		1/1			ND	1/1	
DELHI (I.A.R.I.)	Kinn. m. (3)	4	1	3/3	0/1	4/4	0/1		ND	4/4	0/1
	Mos. sw. or. (4)	2	3	2/2	0/3	2/2	0/3		ND	2/2	0/3
KARNATAKA											
Bangalore (I.I.H.R.)	S. or. (5)	1		1/1			ND	1/1		1/1	
	C. indica (6)	4		1/3		2/2		2/2		4/4	
	R. lem (7)	6		4/4		3/3		1/1		6/6	
	Kagzi l.	1		0/1		1/1			ND	1/1	
	lemon	1		1/1			ND		ND	1/1	
	undet. (8)	1		1/1		1/1			ND	1/1	
Kasakatapura	Ch. sw. or. (9)	7		5/5		3/3		0/4		7/7	
Gonikoppal	Coorg. m. (10)	6		3/3		6/6			ND	6/6	
	und. sw. or. (11)	1		0/1		1/1			ND	1/1	
	R. lem.	1		1/1		1/1			ND	1/1	
Chitalli	Coorg. m.	5	1	4/4	ND	5/5	0/1		ND	5/5	0/1
	C. macrop. (12)	1		1/1		0/1				1/1	
	undet.	1		1/1		1/1			ND		1/1
MAHARASHTRA											
Amravati	Nagpur m. (13)	3		3/3			ND	0/3		3/3	
ORISSA											
Angul	Nagpur m.	2		0/2		0/2			ND	0/2	
	Kagzi l.	1		1/1		1/1			ND	1/1	
Subalda	Nagpur m.	1		0/1		1/1			ND	1/1	

RAJASTHAN Jhalawar	Nagpur m. Mos. sw. or.	2	4 1	2/2	0/2 0/1	2/2	0/4 0/1		ND ND	2/2	0/4 0/1
		59	11	41/48	0/8	39/42	0/10	4/14	0/1	57/59	0/11

[a]Abbreviations – 1,4,9: Sathgudi, Mosambi, Chini sweet orange; 3,10,13: Kinnow, Coorg, Nagpur mandarin; 2: Kagzi lime; 5: sour orange; 6: *Citrus indica*; 7: rough lemon; 8: undetermined cultivar; 11: undetermined sweet orange; 12: *Citrus macropetra*.
[b]ND = not done.

Chitalli (Fig. 1), was well known for its Coorg mandarins. Over the last hundred years, coffee plantations have taken over, partly as the result of citrus decline. Today, the only mandarin trees left are scattered through coffee plantations and practically all are affected by severe greening (Table 1). The regional citrus center of I.I.H.R. grows a large acreage of Coorg mandarin trees of various ages. The greening BLO could be detected by EM and dbH in the mottled leaves of these trees, including those less than 2 yr-old (Table 1, Gonikoppal and Chitalli). Citrus cultivation under these conditions is a difficult challenge.

The Nagpur region is one of the major citrus growing areas in India, with the Nagpur mandarin being almost exclusively cultivated. It is, however, affected by a particular decline, the Nagpur mandarin dieback, which is quite different from greening, most notably by the absence of leaf mottle. Greening is definitely present in the area (Table 1, Amravati; Garnier and Bové, unpublished), but the involvement of greening in the Nagpur mandarin dieback requires further investigation.

Many nursery plants are sent from the Nagpur area to other regions of India, such as Rajasthan. The presence of the greening BLO in Nagpur mandarins leaves with mottle could be demonstrated in Rajasthan (Table 1). However, trees with typical Nagpur mandarin dieback and without leaf mottle, gave negative results in EM and dbH (Table 1, Jhalawar).

In the State of Orissa, Nagpur mandarin is grown northwest of Bhubaneshwar in the Angul area and south of Bhubaneshwar on the steep slopes of the Jirango hills. In one of the orchards near Angul, a Kagzi lime with leaf mottle was found to carry the greening BLO as both EM and dbH were positive (Table 1, Angul). In the same orchard two Nagpur mandarin trees with mottled leaves gave unexpectedly negative results by both EM and dbH. This is one of the very few cases where the BLO could not be detected in mottled leaves. In the Jirango hills, Nagpur mandarin is grown as wild seedling trees, more like forest trees than fruit trees! In this isolated area, near Subalda, the greening BLO could be clearly detected by dbH even though EM was negative. Many *Murraya paniculata* plants grow wild under the citrus trees and serve as host plants for the insect vector of the BLO, the psyllid *Diaphorina citri*. This situation undoubtedly favors spread of the BLO within this relatively isolated area.

The greening BLO could also be detected in northern India. On the farm of the Indian Agricultural Research Institute (IARI) in New Delhi, many trees showed greening symptoms and the BLO could be detected by EM and dbH in mottled leaves of Kinnow mandarin and Mosambi sweet orange trees.

The 1992 survey did not include the Poona area in Maharashtra. Since from previous work by Capoor and coworkers it is known that many trees in the area have died from greening. Unfortunately the States of Punjab and Assam could not be surveyed, for security reasons.

In conclusion, greening was present in all the areas surveyed. This result is not only based on symptomatology, and leaf mottle in particular, but also on the detection of the greening BLO by EM and dbH. This is the first time that the presence of greening in many regions of India has been confirmed by laboratory techniques. Greening is responsible for the destruction of citrus in southern Karnataka (Bangalore, Coorg area), southern Andhra Pradesh (Hindupur, Tirupati), and western Maharashtra (Poona). The role of greening in the dieback syndrome of Nagpur mandarin trees in nothern Maharashtra (Nagpur) remains to be evaluated. Finally, the Asian vector of the greening BLO, *Diaphorina citri*, was present in all the areas surveyed and is largely responsible for the spread of the disease throughout India.

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