Detection of the Asian Huanglongbing (Greening) Liberobacter in Nepal by DNA-DNA Hybridization

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ABSTRACT. Leaf samples from the main citrus growing regions of Nepal were collected in December 1992 and April 1994, and the 52 samples were analyzed by DNA-DNA hybridization with probes In 2.6 and As 1.7 specific for Liberobacter asiaticum and L. africanum respectively, the causal agents of huanglongbing (HLB) (greening). Positive hybridization signals were obtained only with probe In 2.6, indicating that only L. asiaticum is present in Nepal. On the basis of these analyses, and also from the presence of leaf mottle and the occurrence of the psyllid vector, *Diaphorina citri*, we found that HLB and *D. citri* are present in many parts of Nepal, but only below 1,300 to 1,400 m elevation. Neither HLB nor *D. citri* were seen above this altitude, except in the Kathmandu area. Some areas or valleys at lower altitudes (Dumre, 450 m; Syangja, 850 m) are still HLB-free, probably because trees are produced locally and not imported from the highly contaminated Pokhara area, and because it is likely that the movement of *D. citri* is hindered by the mountainous nature of the country. Exocortis, bark scaling, tristeza virus-induced vein clearing, citrus canker lesions, budunion crease and phytophthora gummosis were also found.

Huanglongbing (HLB) (greening) was first seen in Nepal at the Pokhara Horticultural Research Station in the 1960s and is believed to have been introduced with planting material from India (2, 3). The presence of the Asian HLB agent, "*Candidatus* Liberobacter asiaticum", in the major citrus growing areas of India has recently been documented (1, 6). In India and Nepal, L. asiaticum is transmitted by the Asian psyllid, *Diaphorina citri* Kuwayama. In the Pokhara valley (900 m altitude), the insect reaches high populations in May, June and July (5).

To date, the presence of HLB in Nepal was based on symptomatology only. We have recently developed two DNA probes: Probe In 2.6 detects L. asiaticum (7, 8), and probe As 1.7 detects "*Candidatus* L. africanum" strains (4). We have used these probes to determine the presence of HLB in Nepal and to identify the liberobacter species involved.

MATERIALS AND METHODS

Citrus leaf samples were collected in various regions of Nepal during two surveys (December 1992, and April to May, 1994). They were kept at 4°C before they were taken to Bordeaux for DNA extraction and hybridization with probes In 2.6 and As 1.7 as described earlier (4, 7, 8).

RESULTS AND DISCUSSION

Twenty-three samples (1 to 23) were collected during the 1992 survey, and 29 samples (24 to 52) in 1994. Fig. 1 represents the dot-blot hybridizations obtained with probe In 2.6 and shows that the DNA from 25 leaf samples hybridized with probe In 2.6; no hybridization was obtained with probe As 1.7 (results not shown). These results indicate that the liberobacter present in Nepal is, as expected, L. asiaticum. Table 1 lists the samples that gave positive hybridization signals with probe In 2.6. Positive hybridization signals were weak (+ on Table 1), strong (3+) or very strong (5+). The leaf samples that gave positive hybridization signals showed HLB associated mottle and, in general, leaves with pronounced mottle, give strong hybridization signals. *D. citri* was seen or has been reported in the areas where the liberobacter has

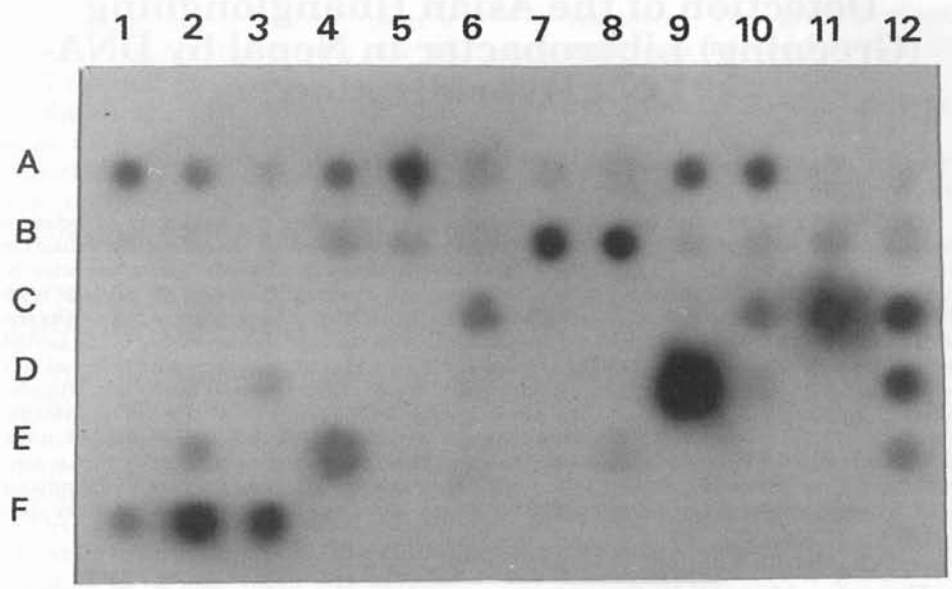

Fig. 1. Autoradiography of dot-blot hybridizations between leaf DNA and ^{32}P-labelled huanglongbing probe In 2.6. Each position represents a leaf sample from an individual citrus tree. Hybridization positive samples are identified in Table 1.

been detected. The 1992 survey was carried out in December; the trees had mature leaves, with or without mottle, and had not yet produced a new flush of growth. In 1994, the survey was undertaken in April, a time when most mature leaves on symptomatic trees had dropped and new young leaves were appearing. Therefore, it was more difficult to collect suitable leaves in April for HLB detection. Of 22 leaf samples collected in December 1992, 16 gave a positive hybridization signal, and only 10 of 29 in April 1994.

Table 1 shows that the citrus cultivars that were infected with the liberobacter, comprised Local and Kinnow mandarins, Local and Junar sweet oranges, Rangpur lime and lemon.

Except for the Kathmandu area (1,300 to 1,400 m), Liberobacter-infected trees were not found at an altitude above 1,300 to 1,400 m and, similarly, the psyllid vector, *D. citri* was not seen, nor has it been reported, above that altitude. Areas above 1,400 m where HLB and *D. citri* were not seen, were: 1) Lumle (1,750 m), west of Pokhara, 2) Paripatle horticultural station (1,450 m) near Dhankuta in the east of Nepal, 3) Bijayachap (1,300 m) above Sindhulimadi (which itself, is at 600 m and has both HLB and *D. citri*), and 4) Tansen horticultural farm (1,300 m), north of Butwal on the road to Pokhara.

The areas above 1,300 to 1,400 m are probably free of HLB because the psyllid vector, *D. citri*, is absent, either because it has never been introduced in these areas or, more likely, because it does not complete its life cycle at these altitudes, although there is at present no evidence for this.

There are also areas below 1,300 to 1,400 m where no HLB has been detected. Such areas include: 1) the Syangja district (850 m), 20 miles south of Pokhara, where *D. citri* is present. The apparent absence of HLB is probably due to the fact that the individual growers produce their own nursery trees and do not introduce trees from Pokhara. 2) At

TABLE 1
LIBEROBACTER ASIATICUM-INFECTED CITRUS CULTIVARS FROM VARIOUS REGIONS OF NEPAL, AS DETECTED BY DNA HYBRIDIZATION WITH PROBE IN 2.6

Location (altitude)	Sample number[a]	Position of hybridization dot on Fig. 1	Dot intensity
KATHMANDU AREA			
Shankuthree, Kabre district (1,300 m)			
Local mandarin			
Tree 1	20	B7	+++
Tree 2	21	B8	+++
Tree 3	22	B9	+
Tree 4	23	B10	+
POKHARA AREA (900 m)			
Pokhara Horticulture Station			
Local mandarin[y]			
Tree 1	1	A1	+++
Tree 2	4	A4	+++
Tree 3	5	A5	+++
Tree 4	35	C10 and F1	+
Tree 5	36	C11 and F2	+++
Kinnow mandarin[y]	40	D3	+
Kinnow mand./Troyer c.	17	B5	+
Junar sweet orange[y]			
Tree 1	2	A2	+++
Tree 2	3	A3	+
Tree 3	37	C12 and F3	+++
Rangpur lime	16	B4	+
Hamta			
Local mandarin[y]	6	A6	+
Lamachaur			
Local mandarin			
Tree 1	46	D9	++++
Tree 2	47	E4	+++
Batulechaur			
Local mandarin	31	C6	+++
ARMALAKUR			
Local mandarin			
Tree 1	7	A7	+
Tree 2	8	A8	+
Lemon	9	A9	+++
Kinnow mandarin[y]	10	A10	+++
POKHERE (West of Pokhara) (950 m)			
Local mandarin seedling	49	D12	+++
SINDHULI (600 m)			
Sweet orange seedling	24	B11	+
BIHMAN (400 m)	25	B12	+

[a]Samples 1 to 23 are from the December 1992 survey, samples 24 to 52 from the April 1994 survey.
[y]Grafted on *Poncirus trifoliata*.

Dumre (490 m) near Damauli, along the road from Pokhara to Kathmandu, where there is a 2-ha orchard of 20-yr-old seedling manda-

rin trees on the slopes of a hill which showed no symptoms of HLB in 1992 and 1994. According to the local extension service engineers, *D. citri* has never been seen in this site, in spite of the low altitude. Here again the farmer grows his own nursery trees, no trees having ever been imported from Pokhara. The movement of the vector into these areas has probably been hindered by the mountainous nature of the country.

If some areas of Nepal are to be kept free of HLB, it is essential that all the nurseries in the Pokhara area be closed and that movement of trees out of the Pokhara area be prohibited.

During the survey, problems other than those related to HLB were encountered. At the Paripatle horticultural station, budwood from selected sweet orange trees was grafted on *Poncirus trifoliata* and rough lemon. Several trees on *P. trifoliata* were stunted in comparison with the same sweet orange selection on rough lemon. The stunted trees showed clear-cut symptoms of exocortis bark-scaling on the trifoliate rootstock. Old Pineapple sweet orange trees on unrecorded rootstocks, possibly Rangpur lime, showed pronounced budunion-crease symptoms. Karna Khatta trees had severe lesions of Phytophthora gummosis. Small-fruited acid lime trees had tristeza virus-induced symptoms of vein clearing. At Bijayachap (1,300 m), only accessible by a mountain track, many locally produced 16 yr-old sweet orange seedling trees in the demonstration farm, were free of HLB but were badly affected by Phytophthora gummosis; there were citrus canker lesions on lime leaves.

In Nepal, there are still many seedling trees with the use of grafted trees being relatively recent. The locally produced seedling trees of sweet orange and mandarin, have the advantage of being free of virus and virus-like diseases, including sometimes HLB, but they are often affected by Phytophthora gummosis. Citrus in Nepal can be greatly improved not only by trying to avoid HLB but also by improved nursery and horticultural practices.

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