Shifting from Seedling Mandarin Trees to Grafted Trees and Controlling Huanglongbing and Viroids: a Biotechnological Revolution in Nepal

C. Regmi[1], R. P. Devkota[2], K. P. Paudyal[3], S. Shrestha[1], A. J. Ayres[4], N. Murcia[6], J. M. Bové[5] and N. Duran-Vila[6,7]

[1]Nepal Academy of Science and Technology, Kathmandu, Nepal
[2]ECARDS-NEPAL, P.O.B. 8115, Kathmandu, Nepal
[3]National Citrus Research Program, NARC, Paripatle, Dhankuta, Nepal
[4]Fundecitrus, Araraquara, S. P., Brazil
[5]Université de Bordeaux 2 & INRA, 33883 Villenave d'Ornon, BP 81, France
[6]I.V.I.A., Moncada (Valencia), Spain
[7]Centro de Estudios Rurales y de Agricultura Internacional (CERAI), Valencia, Spain

ABSTRACT. Poverty in Nepal, largely a rural phenomenon, is widespread, with 30.8% of the population living below the poverty line. Agriculture is the main source of livelihood of the Nepalese people who are living below poverty. Citrus, the first cash crop, is grown in small to very small orchards producing less than 10 tons/ha. More than 90% of the trees are seedlings of a local mandarin and therefore they are essentially free of most graft-transmissible diseases. Huanglongbing (HLB) and the Asian psyllid vector, *Diaphorina citri*, were reported in Nepal in the mid-1960s and, in the absence of any control measures, have continued to spread ever since. Here we report the information available regarding the presence of HLB in several important citrus-growing areas (Armalakur, Bandipur, Dhankuta/Karmitar, Kathmandu, Lamjung, Paripatle, Pokhara, Sindulimadi and Syangja) and the identification of four citrus viroids (*Citrus exocortis viroid*, *Hop stunt viroid*, *Citrus viroid-III*, and *Citrus viroid-V*) in the experimental citrus station of Pokhara.

Citrus rehabilitation, as part of a program to improve food security for the Nepalese population, was started in 2004, and is based on (i) producing disease-free citrus trees, grafted on adequate rootstocks, in covered, insect-proof nursery facilities, (ii) demonstration orchards with grafted trees, (iii) control of HLB by trunk applications of systemic insecticides and/or guava interplants, (iv) selection of viroid-free budwood sources, and (v) transfer of technology to the farmers.
Index words: Citrus viroids, HLB.

Poverty in Nepal, largely a rural phenomenon, is widespread, with 30.8% of the population living below the poverty line. Agriculture is the main source of livelihood of the Nepalese people. Nepal extends in altitude from less than 100 m in the South to more than 8000 m in the North. The mid-hills region, between 600 and 1600 m, has the best climatic conditions for citrus. It is the dominant fruit crop as well as the first cash crop, and it has been recommended by the Agriculture Perspective Plan as "priority crop" throughout the mid-hills. Orchards are small to very small; yet they cover a surface of almost 20,000 ha. Productivity is low with less than 10 tons/ha and farmers have virtually no access to technology. In particular, the use of grafted trees is largely unknown, more than 95 % of the trees being seedlings. A seedy local mandarin is the major variety, but local sweet orange trees are also grown.

Since commercial citrus plants are grown as seedling trees, they are essentially free of graft-transmissible diseases and they are tolerant to tristeza even though *Citrus tristeza virus* (CTV) is endemic in the region. However, many trees perform poorly and *Phytophthora* root rot is a major problem. In addition, Huanglongbing (HLB) and the Asian psyllid vector, *Diaphorina citri*, have been reported in Nepal since the mid-1960s and, in the absence of any control measures, have continued to spread ever since. Pokhara, half way between the eastern and

western limits of the country, has been the initial focus of the disease, probably by the introduction of budwood from neighboring India (3, 4) where the disease is widespread (2, 14). Since 1992, when molecular techniques for liberibacter detection were used for the first time in Nepal, the disease has been detected or confirmed in many parts of the country. Here we report the information available regarding the presence of HLB in several important citrus- growing areas and the identification of citrus viroids in the experimental citrus station of Pokhara.

In 2004, a citrus rehabilitation project, based on use of disease-free planting material, shifting from seedling trees to grafted trees and controlling HLB, has been initiated by ECARDS as part of a program to improve food security for the Nepalese population.

MATERIALS AND METHODS

Surveys of citrus for graft-transmissible diseases. In December 1992, April-May 1994, October 2004, October 2005, and May and December 2006, surveys for graft-transmissible diseases of citrus were conducted in various parts of Nepal. Symptomatic leaf samples from trees suspicious of HLB were collected for liberibacter detection by DNA hybridization or PCR. The altitudes of the orchards where samples were collected were recorded with an altimeter.

In 2006, during a visit to the Agricultural Citrus Experiment Station at Pokhara, samples of three sweet orange cultivars (Valencia, Rubi and Washington navel) grafted on trifoliate orange were collected for citrus viroid indexing. A symptomless, 32-yr-old seedling mandarin tree, selected in 2004 in the Paripatle citrus experiment station (Eastern Nepal) as a mother tree for the production of grafted mandarin trees, was also indexed for viroids.

HLB liberibacter detection by DNA hybridization or PCR. The leaf samples collected in 1992 (23 samples) and 1994 (29 samples) were analyzed by DNA hybridization with probe In 2.6, specific for *Candidatus* Liberibacter asiaticus as well as probe As 1.7, specific for *Ca*. L. africanus, as described earlier (9). The samples from 2004 (8 samples), 2005 (14 samples), and 2006 (38 samples) were submitted to PCR detection with primers specific for *Ca*. L. asiaticus and *Ca*. L. americanus according to method used by Teixeira, et al. (12).

Viroid analysis. Samples (5 g) of bark tissue were powdered in liquid nitrogen and homogenized in 5 ml of extraction medium (0.4 M Tris-HCl pH 8.9; 1% (w/v) SDS; 5 mM EDTA pH 7.0; 4% (v/v) β-mercaptoethanol) and 15 ml of water-saturated phenol (10). The total nucleic acids were partitioned in 2 M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl, pH 7.4; 10 mM KCl; 0.1 mM $MgCl_2$). Aliquots of the nucleic acid preparations (20 µl equivalent to 300 mg fresh weight) were subjected to 5% non-denaturing PAGE and the gel was stained with ethidium bromide. The RNAs separated by 5% PAGE were electroblotted (400 mA for 2 h) to positively-charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA), immobilized by UV cross-linking and hybridized with viroid specific probes. Digoxigenin (DIG)-labeled DNA probes were synthesized by PCR using as a template a cloned plasmid containing full-length viroid monomeric DNA, as described by Palacio-Bielsa et al., (5) for *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus viroid-III* (CVd-III) and *Citrus viroid-IV* (CVd-IV). A probe specific for the newly described *Citrus viroid V* (CVd-V) (11) was synthesized using primers CVd-V-h (5'-TCGACG AAGGCCGGTGAGCA-3') and CVd-V-c

(5'-CGACGACAGGTGAGTACTCTCTA C -3') homologous and complementary to positions 88-107 and 64-87, respectively, of the viroid reference sequence. Prehybridization (at 60ºC for 2-4 h) and hybridization (at 60ºC overnight) were performed in 50% formamide and and 5XSSC buffer containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking reagent. After hybridization, the membranes were washed twice in 2X SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1X SSC, 0.1% SDS at 60ºC for 60 min, and revealed with an anti-DIG alkaline phosphatase conjugate and the chemiluminiscence substrate CSPD (Roche Applied Science) (DIG-labeled probes).

Viroid identification. RT-PCR was performed as described by Bernad and Duran-Vila (1). First-strand cDNA synthesized at 60ºC using 27-mer primers specific for each viroid and Thermoscript reverse transcriptase (Invitrogen®). In order to recover full-length viroid DNA, second strand synthesis and DNA amplification were performed using a set of two contiguous 18-mer forward and reverse primers specific for each viroid in 50-µl reactions containing 1.0 mM $MgCl_2$, 0.12 mM dNTPs, 0.5 µM of each primer and 1 U of *Taq* DNA polymerase. PCR parameters consisted of a 5-min denaturation at 94°C followed by 35 cycles of 94°C (30 s), 60°C (30 s), 72°C (1 min) and finishing with a 5-min extension step at 72°C. Electrophoretic analysis in 2% agarose gels confirmed the synthesis of the expected DNA products that were sequenced. When the sequences contained in determinations the amplification product was ligated in the pGEM-T vector (Promega) and the recombinant plasmids were used to transform DH5α *E. coli* cells. Uncloned amplicons synthesized by RT-PCR and/or recombinant plasmids were sequenced with an ABI PRISM DNA sequencer 377 (Perkin-Elmer). Multiple sequence alignments were performed with Clustal W (13).

RESULTS

Spread of HLB. Huanglongbing (HLB) and the Asian psyllid vector, *D. citri*, have been reported in Nepal in the mid 1960s. Pokhara, half way between the eastern and western borders of the country, was the initial focus of the disease as determined by symptomatology. In 1992 and 1994, a molecular technique, DNA hybridization, was used for the first time in Nepal for liberibacter detection (9), and presence of the disease was confirmed in the following regions: Kathmandu area (Shankuthree, Kabre, districts, ~1300 to ~1400m), Pokhara area (Hamta, Lamachaur, Batulechaur, Pokhere, ~900m), Armalakur area, and Sindhulimadi area (~600m). Except for the Kathmandu area, liberibacter-infected trees were not found at an altitude above ~1300m to ~1400m, and similarly, the psyllid vector, *D. citri*, was not seen above this altitude. Areas above 1400m where HLB and *D. citri* were not seen, were: Lumle (~1750m) west of Pokhara, Paripatle horticultural station (~1200m to ~1350m) near Dhankuta in eastern Nepal, Bijayachap (~1300m) above Sindhulimadi (which itself is at ~600m and has both HLB and *D. citri*) and Tansen horticultural farm (~1300m), southwest of Pokhara. During the 1992-1994 surveys, there were also areas below ~1300m where no HLB was detected. Such areas included the Syangja district ~850m), south of Pokhara, where, interestingly, *D. citri* was present, and Dumre (~450m), near Bandipur, on the road from Kathmandu and Pokhara, where a beautiful 2-ha orchard of 20-yr-old seedling mandarin trees on the slope of a hill had no signs of HLB, and *D. citri* had never been seen. In these two areas, the farmers grew their own seedling nursery trees, no trees having been

imported from the heavily contaminated Pokhara valley.

The October 2004 survey established that the Bandipur area was now also contaminated with HLB, and in particular, *D. citri* had become established in the above 2-ha orchard at Dumre, in the meantime. The Dumre orchard was again visited in December 2006. By then, HLB had devastated the whole plantation, most trees being severely affected, as confirmed by PCR. Similarly, the Syangja district, near Pokhara, that was free of HLB in 1994, was found to be severely affected in December 2006. The Lamjung region, northeast of Pokhara, was surveyed for the first time in December 2006. Practically all mandarin trees in the orchards surveyed were yellow, with characteristic HLB leaf and fruit symptoms, and all leaf samples collected for liberibacter detection tested positive. The farmers in the region had never heard of HLB. At the Pokhara agricultural station, where HLB had first appeared in the 1960s, and confirmed by DNA-hybridization in 1992 and 1994, a Murcott tangor plot was found in December 2006 to be heavily affected by HLB, the disease being confirmed by PCR. The major result of the October 2005 survey was the finding that the Dhankuta/Karmitar region (~1100m), free of HLB in 1994, had become contaminated with HLB, including the Paripatle horticultural station (~1200m to 1350m), where yellowish seedling mandarin trees in the lowest parts of the station gave positive PCR reactions. Finally, during the May 2006 survey, many sweet orange and mandarin trees in the horticultural station at Kirtipur, near Kathmandu, showed severe symptoms of HLB. Many of these trees have been cut down, but many still remain, as seen in January, 2008.

In the PCR tests carried out since October 2004, both *Ca*. L. asiaticus and *Ca*. L. americanus have been sought. Only the Asian liberibacter has been detected. All tests for *Ca*. L. americanus have been negative.

Identification of citrus viroids. In December 2006, during a visit to the Agricultural Station at Pokhara, sweet orange trees of three different cultivars grafted on trifoliate orange and growing in an experimental plot showed poor growth and mild exocortis-like symptoms in the rootstock (Fig. 1). Northern blot hybridization analysis showed that the three cultivars were infected with CEVd, HSVd, CVd-III and CVd-V. Washington navel sweet orange was also infected with CBLVd. The results regarding CVd-IV were inconclusive but suggest that Valencia sweet orange is probably infected with CVd-IV. (Fig. 2). The samples were subjected to RT-PCR using CEVd, HSVd and CVd-III specific primers and the uncloned amplicons and/or recombinant plasmids were sequenced. The consensus CEVd sequences showed the highest identities (Table 1) with the reference sequence of Class A defined by Visvader and Symons (15, 16). The consensus HSVd sequences showed the highest identities (Table 1) with CVd-IIa variants and contained in the Variable domain the sequence motif characteristic of non-cachexia variants (6, 8). The consensus sequences of CVd-III were identical to variant CV-IIIb (7).

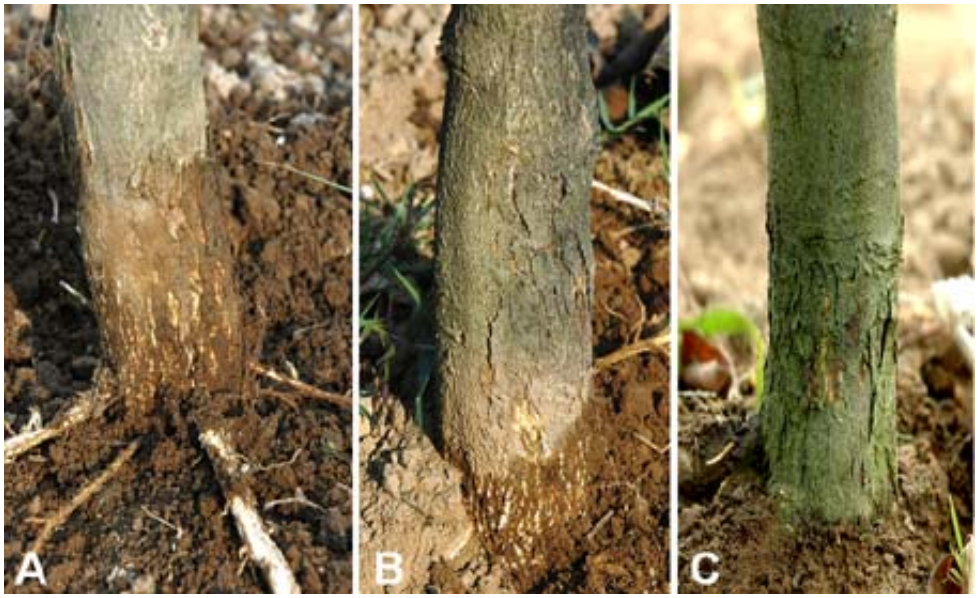

Fig. 1. Symptoms on the trifoliate orange rootstock of trees growing in the Pokhara Agricultural Experimental Station: A) Valencia sweet orange; B) Ruby sweet orange; and C) Washington navel sweet orange.

TABLE 1
SEQUENCE IDENTITIES OF VIROIDS IDENTIFIED IN NEPAL WITH THE TYPE MEMBERS OF CEVd, HSVd and CVd-III

Source	Sequence identities[1]						
	CEVd		HSVd		CVd-III		
	CEVd-A (M30868)	CEVd-B (M30870)	CVd-IIa (AF213503)	CVd-IIb (AF 213501)	CVd-IIIa (S76452)	CVd-IIIb (AF184147)	CVd-IIIc (AF184149)
Valencia orange	96.7	92.4	98.7	96.0	96.3	100	95.2
Ruby orange	96.7	93.0	99.3	95.6	96.0	100	95.5
Wash. navel orange	98.6	93.5	98.0	94.3	96.3	100	95.5

[1] The identities were calculated by comparison with reference sequences of which GeneBank accession numbers are shown.

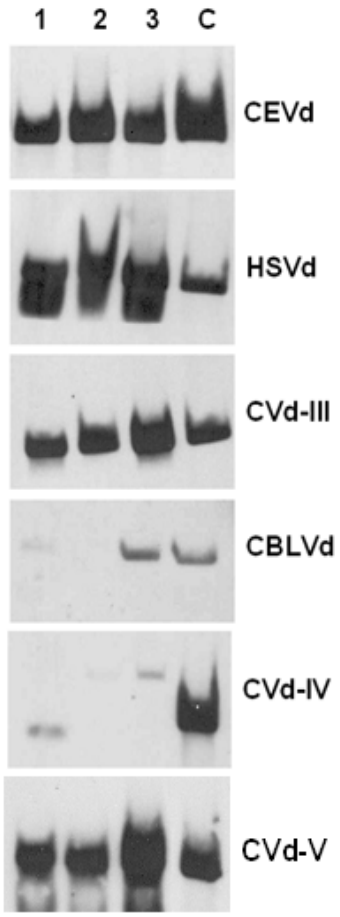

Fig. 2. Northern blot hybridization analysis using viroid-specific probes. Samples were: 1) Valencia sweet orange; 2) Ruby sweet orange; 3) Washington navel sweet orange; C) Viroid-infected control.

The Citrus Rehabilitation Project. The situation that the citrus industry of Nepal is facing can only be rehabilitated through what can be viewed as a biotechnological revolution. The major change needed is to produce and grow high quality plants grafted on suitable rootstocks instead of seedling trees, a change that was implemented in virtually all citrus growing areas of the world more than a century ago. The project has the objective to demonstrate to technicians and growers the advantages of growing grafted plants as a means to: i) overcome the long juvenile periods of seedling trees; ii) control *Phytophthora* root rot; and iii) live with HLB through preventive control. The strategy started in 2004 consisted of : a) selection of viroid-free and HLB-free budwood sources; b) production of disease-free citrus trees, grafted on adequate rootstocks, in covered, insect-proof nursery facilities, built for that purpose; c) establishment of demonstration orchards with grafted trees; d) control of HLB by (i) removal of HLB-affected trees and trunk applications of systemic insecticides and/or guava interplants; and e) transfer of technology to the farmers.

A symptomless 32-yr-old mandarin tree was selected. The tree was indexed on Etrog citron, and sPAGE analysis confirmed viroid-free. Budwood collected from this tree was graft-propagated on *Citrus volkameriana* and *Poncirus trifoliata* rootstocks to produce, under insect-proof conditions, mother trees for the production of mandarin buds for the production of grafted mandarin trees. The first grafted trees for the establishment of small demonstration orchards were distributed to selected farmers in the summer of 2007.

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