Root Grafting and Mechanical Transmission of Citrus Exocortis Viroid Within a Citrus Budwood Multiplication Block

P. Broadbent, B. I. Gollnow, M. R. Gillings and K. B. Bevington

ABSTRACT. In 1968 a closely planted budwood multiplication block was established at the Agricultural Research Centre, Dareton using fully indexed clones. In 1985, during routine re-indexing of 860 mother trees, some lemon trees were found infected with citrus exocortis viroid (CEV). In 1970, an imported Monroe Lisbon clone had been planted in the block. This Lisbon clone was later found to be infected with CEV, and was removed in 1973. No hedging or pruning took place before its removal. As the oldest infections occurred immediately adjacent to those Monroe Lisbon trees (within 2 m), it is possible that transmission by root grafting occurred. Later contamination of other lemon trees, further removed from the original infections, was probably by mechanical transmission of CEV on secateurs and hedging equipment. Biological indexing on citron 60-13 or Arizona 861 and hybridisation of CEV cDNA probes to RNA dot blots have been used to re-index all trees. The results were generally similar, but some specific discrepancies occurred. The role of root grafting in transmission of CEV was studied by excavating root systems.
Index words. Indexing, cDNA probe, dot blots, hybridisations, RNA.

In the period 1974-1986, the New South Wales (NSW) Citrus Budwood Scheme (1, 4) has supplied 5.2 million buds from its multiplication unit of 800 trees (26 scion varieties) at the Horticultural Research Station at Dareton (NSW). In 1985-86, 534,500 buds were supplied to 93 growers and nurserymen throughout Australia. A backup multiplication block of 229 trees was established at Griffith (NSW) in 1977-78 and a replacement block of 411 trees at Dareton in 1982.

The Horticultural Stock and Nurseries Act (1974) ensures that most citrus trees sold are grown from budwood and seed obtained from approved sources in the NSW Budwood Multiplication Scheme. Approved sources must be genetically stable, true-to-type, of good horticultural performance and free of xyloporosis, psorosis, CEV and other citrus diseases, with the exception of tristeza virus, which is endemic in Australia. Re-indexing of mother trees on biological indicators has been undertaken every 10 years.

During this re-indexing contamination of previously healthy lemon mother trees with CEV was found. This paper details field observations, including studies of root grafting, tissue-graft indexing and molecular hybridisation studies, carried out to determine the extent of the CEV infection.

MATERIALS AND METHODS

With the exception of Eureka lemon trees, and several clones planted more recently, all trees in the multiplication block are on trifoliate orange rootstock. Fig. 1 shows the plan of the lemon budwood multiplication block at Dareton. Trees are planted 1.6 m apart in rows spaced at 6.6 m. Because of overcrowding, the Lambert Eureka lemon trees on rough lemon rootstock in Rows 21 and 22 were thinned to half density.

The Taylor Eureka lemons on Cleopatra mandarin rootstock in Row 19 were planted in 1979. They occupy a site previously planted in 1970 to an imported Monroe Lisbon lemon clone, which was found by indexing in 1973 to be infected with CEV and was therefore immediately removed from the multiplication block.

Trees were hedged in alternate years using a Patterson mechanical hedger with three hydraulically driven saw blades, with a 1.5 m cut.

Annual Inspections. Annual inspections of trees in the multiplication

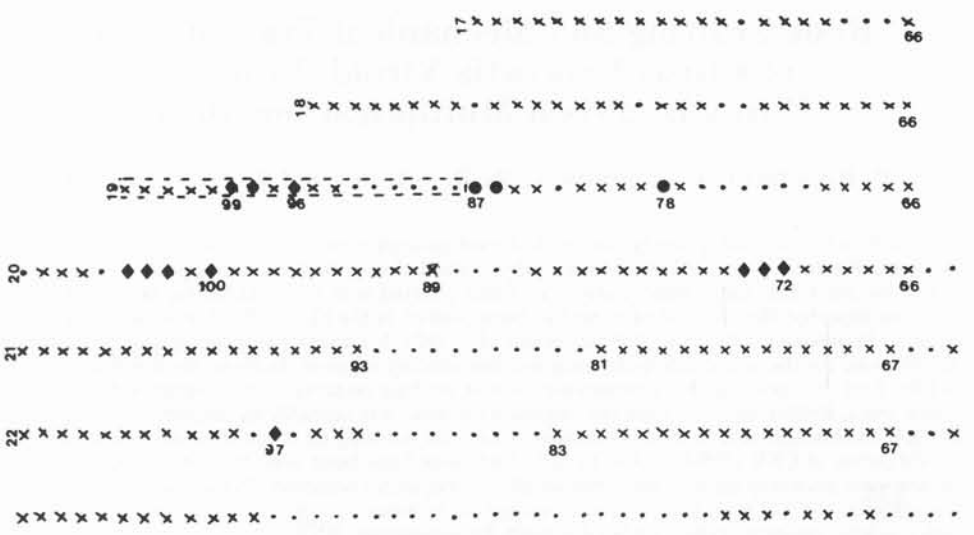

Fig. 1. Plan of the lemon section of the NSW Citrus Budwood Multiplication Block at Dareton. Trees are identified by Row (left margin) and by Tree (below symbol). R_{19}, $T_{66\text{-}87}$ are Prior Lisbon Lemon; R_{19}, $T_{94\text{-}104}$ are Taylor Eureka lemon; R_{20}, $T_{66\text{-}84}$ are Taylor Eureka lemon; R_{20}, $T_{89\text{-}108}$ are Villafranca lemon; R_{21}, $T_{67\text{-}81}$ and $T_{93\text{-}109}$ are Lambert Lisbon lemon; R_{22}, $T_{67\text{-}83}$ and $T_{93\text{-}109}$ are Lambert Lisbon lemon. : : : : = Site previously occupied by CEV-infected Monroe Lisbon lemon; ♦ CEV positive by citron reaction; ● = CEV positive by dot blot hybridisation and citron reaction; • = no tree.

block are made by a horticulturist and plant pathologist to ensure that trees are visually healthy and true-to-type.

Observation for Citrus Exocortis Viroid (CEV) Symptoms. Since initial plantings of clones in the multiplication unit were on trifoliate orange rootstock which shows bark scaling and dwarfing symptoms of CEV, these were observed for disease. Eureka lemon trees could not be grown on this stock due to the yellowring incompatibility.

Tissue-graft indexing on Etrog citron. Eight rooted cuttings, or scions of Arizona 861 or USDCS 60-13 citron on rough lemon roots, were grafted with two buds each from the candidate mother tree. Budwood was collected from four positions on the outside of the tree canopy in February (late summer). A known source of CEV (Queensland, Villafranca Accession No. 4339) was used as a positive control. Great care was taken to avoid contaminating indicator plants with CEV. Grafting was done using a new razor blade for each budwood source and pruning tools were disinfected with a 1% solution of sodium hypochlorite between each manipulation.

Indicator plants were held at 28-32 C day and 24-26 C night temperatures and examined for symptoms of CEV infection after each new flush of citron growth had matured. Indicators were cut back 3 or 4 times to induce further growth flushes, with pruning tools again being disinfected with 1% sodium hypochlorite between manipulations.

Dot-blot hybridisation procedure.—Sample preparation and extraction of nucleic acids. Leaf and bark samples were collected in late summer in 1985 and 1986. Samples were ground to a fine powder under liquid nitrogen and stored at –80 C until extraction. Rootstock bark (two 2x10 cm patches cut from immediately below the budunion) and scion bark (from twigs 1 cm in diameter) were pared into thin strips before grinding. The 1985 sampling included rootstock bark and scion bark, whereas in 1986 only scion bark was extracted.

Nucleic acids were extracted by the method of Schwinghamer and Broadbent (10) in 1985 and by a modification of the method of Flores *et al.* (5) in 1986. In the latter method, 2.5 g of finely chopped fresh or frozen ground tissue were added to a 50 ml polypropylene tube containing: 5 ml phenol [equilibrated with 0.1 M Tris-HCl, pH 8.0, and containing 0.1% w/v 8-hydroxyquinoline], 4 ml of extraction medium [4% SDS, 0.1 M NaCl, 0.01 M EDTA and 0.1 M Tris, pH 8.9], 1 ml 20% polyvinyl pyrrolidone and 25 µl β-mercaptoethanol. The tissue was ground using an Ultra Turrax homogenizer for 30 sec at half speed. Three ml of 1 x STE [100mM NaCl, lmM EDTA, 50mM Tris, pH 7.2] were added to the homogenate and the mixture incubated on ice for 1 hr with occasional inversion. The phases were separated by a 15 min centrifugation at 9,000 rpm in a Sorvall SS34 Rotor. The aqueous phase was extracted with 2 ml of phenol for 10 min and then with an equal volume of chloroform/isoamyl alcohol [24:1 v/v] for a further 20 min. After centrifugation as above, the nucleic acids were precipitated from the aqueous phase by the addition of 2½ volumes of cold (–20 C) absolute ethanol. Tubes were held at –20 C for 1 hr and the nucleic acids collected by centrifugation as above. The pellet was washed with cold 70% ethanol, 100mM Na acetate and air-dried overnight.

The nucleic acids were resuspended in 400 µl 1 x STE overnight at 4 C and clarified by centrifugation. Samples were sometimes extracted again with phenol/chloroform, but this was usually not necessary, as samples could be used for polyacrylamide gel electrophoresis or dot blotting without apparent interference by contaminating pigments.

RNA dot blots and hybridizations. One eightieth of the nucleic acids from 2.5 g of tissue were denatured and fixed to nitrocellulose essentially by the method of White and Bancroft (12). Serial dilutions of each sample were made and also dotted. Positive controls (nucleic acids from citron leaves inoculated with RNA from a known CEV source, Taylor Eureka lemon R19 T96 (Gillings *et al.* (7)) and negative controls (nucleic acids from citron leaves inoculated with RNA from an uninoculated citron indicator) were included on every dot blot.

A full-length positive sense DNA copy of CEV-A cloned into M13mp93 was used to prepare single stranded ^{32}P labelled complementary (negative sense) DNA probes (11). Probes were prepared by Biotechnology Research Enterprises S.A. Pty. Ltd.

Hybridisation of the probes to dot blots was by a modification of the method of Maniatis *et al.* (8). Both the dot-blot and hybridisation procedures are fully described in Gillings *et al.* (7).

Root grafting. To determine if root grafting between adjacent trees was a possibility, a trench 1.5 m deep x 0.5 m wide was dug with a backhoe on each side of a row of lemon trees in the budwood multiplication block. The soil type was a coarse Tiltao sand, so it was easily washed from the root systems by a high pressure jet of water, without breaking the feeder roots. The course of pioneer roots was traced for their entire length, to determine the extent to which root systems overlapped along the tree row.

RESULTS AND DISCUSSION

In 1970, an imported Monroe Lisbon clone was planted in the budwood multiplication block at the Dareton Agricultural Research Centre. During routine indexing on biological indicators in 1973, this clone was found to be infected with CEV, and was immediately removed from the block.

In 1983-84, during re-indexing of mother trees, 14 lemon trees of four clones were shown to be CEV-infected using Etrog citron indicators (table 1). Symptoms on Etrog citron were severe, and included the characteristic leaf epinasty and corking of the midrib.

Inspection of field trees showed Prior Lisbon trees 86 and 87 and Villafranca trees 100, 102, 103 and 104 to be infected and expressing rootstock scaling symptoms (fig. 1). Symptom expression on trifoliate orange rootstock in NSW often takes four years or more from the time of inoculation. Apart from the scaling symptoms, infected field trees were not distinguishable from their uninfected counterparts in tree height, girth or canopy appearance, suggesting recent infection of mature trees.

Trees giving positive reactions on Etrog citron indicators or showing bark scaling of the trifoliate orange rootstock were immediately removed from the block. Some trees, including Prior Lisbon trees 71, 72 and 74 (fig. 1) were removed on the basis of bark scaling, but were not confirmed as CEV-infected by indexing on Etrog citron or hybridisation tests using a cDNA probe for CEV (table 1). The minor bark scaling of these rootstocks may have been caused by sunburn of the recently exposed bark.

As the infected budlines were free of CEV when planted in the budwood multiplication block, sources of contamination were sought. Prior Lisbon trees 86 and 87 were planted immediately adjacent (1.6 and 3.2 m respectively) to a CEV-infected Monroe Lisbon lemon clone, whereas the Villafranca lemon trees were in an adjacent row 6.6 m away (fig. 1). The proximity of these infected trees to the Monroe Lisbon, and the fact that no

Fig. 2. Overlapping roots from adjacent trees which have grafted, allowing possible transmission of CEV.

hedging, pruning or budcutting took place while the Monroe Lisbon was *in situ*, suggested that transmission of CEV may have occurred by root-grafting.

Inspection of root systems in this closely planted block showed that root grafting had occurred between adjacent trees. The root systems of adjacent trees overlapped and individual pioneer roots extended more than 2 tree spaces (3.4 m) along the tree row (fig. 2). There were numerous instances where woody roots from adjacent trees were closely appressed. The distorted pattern of growth at the point of contact indicated that some roots had been in contact for a considerable period of time. Most root contacts had not resulted in union, but in a small proportion of cases some degree of union was evident (fig. 2). As two or more intersecting roots may partially envelop each other and seem quite rigid, yet be separated by a degraded layer of bark (3), root grafting was confirmed by bark stripping and dissection. It therefore seems likely that infections close to the original Monroe Lisbon occurred via root grafting.

Other more distant infections (fig. 1) within the lemon block may have occurred by transmission of CEV on pruning tools or hedging equipment that had previously been used on the root-graft infected trees. The ragged cuts made by the hedging equipment could have resulted in the deposition of infected bark or wood and subsequent "graft" transmission of CEV. Mechanical hedging of trees in the mother tree block is done in September (spring) whereas the major bud-cutting takes place in late summer (February), at a time when the titres of CEV are at their highest. However the climate at Dareton is hot and arid and desiccation and death of injured tissues in the orchard could severely limit the numbers of infections resulting from cuts (2).

The only trees to be contaminated by CEV in the mother tree block are lemon clones. Garnsey and Weathers (6) showed Eureka lemon to be very easily infected by contaminated knives, whereas sweet orange and grapefruit were less easily infected.

Calavan *et al.* (2) found extensive spread of CEV from infected to healthy trees in mechanically pruned lemon orchards, provided adequate inoculum was available. These authors found that trees adjacent to infected trees were twice as subject to infection as other trees, suggesting that some transmission by root grafting occurred in that orchard.

Since further mechanical and root-graft transmissions of CEV may have occurred in the Dareton budwood multiplicaiion block during the time taken for the biological indexing, this block has been continually reindexed since 1984. In some cases, buds taken from different parts of the canopy of an individual tree failed to give positive reactions on all biological indicators, suggesting that some trees were in the early stages of infection, or that the viroid was unevenly distributed.

To circumvent these difficulties, we used hybridisation assays to test for the presence of CEV-RNA in field trees. Such a technique is potentially very rapid.

A typical hybridisation experiment to dot blots of RNA from field plants is shown in fig. 3. Known positives from infected indicator plants or field samples gave good responses after overnight exposure. Negative controls (Etrog citron inoculated with healthy citron RNA) were included to assess the degree of non-specific hybridisation. Field samples were considered positive if they gave significantly more response (2 to 3 times) than the negative controls. In the experiments illustrated, only one minor positive was detected (fig. 3). This tree has not yet shown a positive reaction on Etrog citron. It may be that the buds used for biological indexing were taken from as yet uninfected portions of the tree in question.

Conversely, CEV was not detected in Lambert Eureka lemon tree

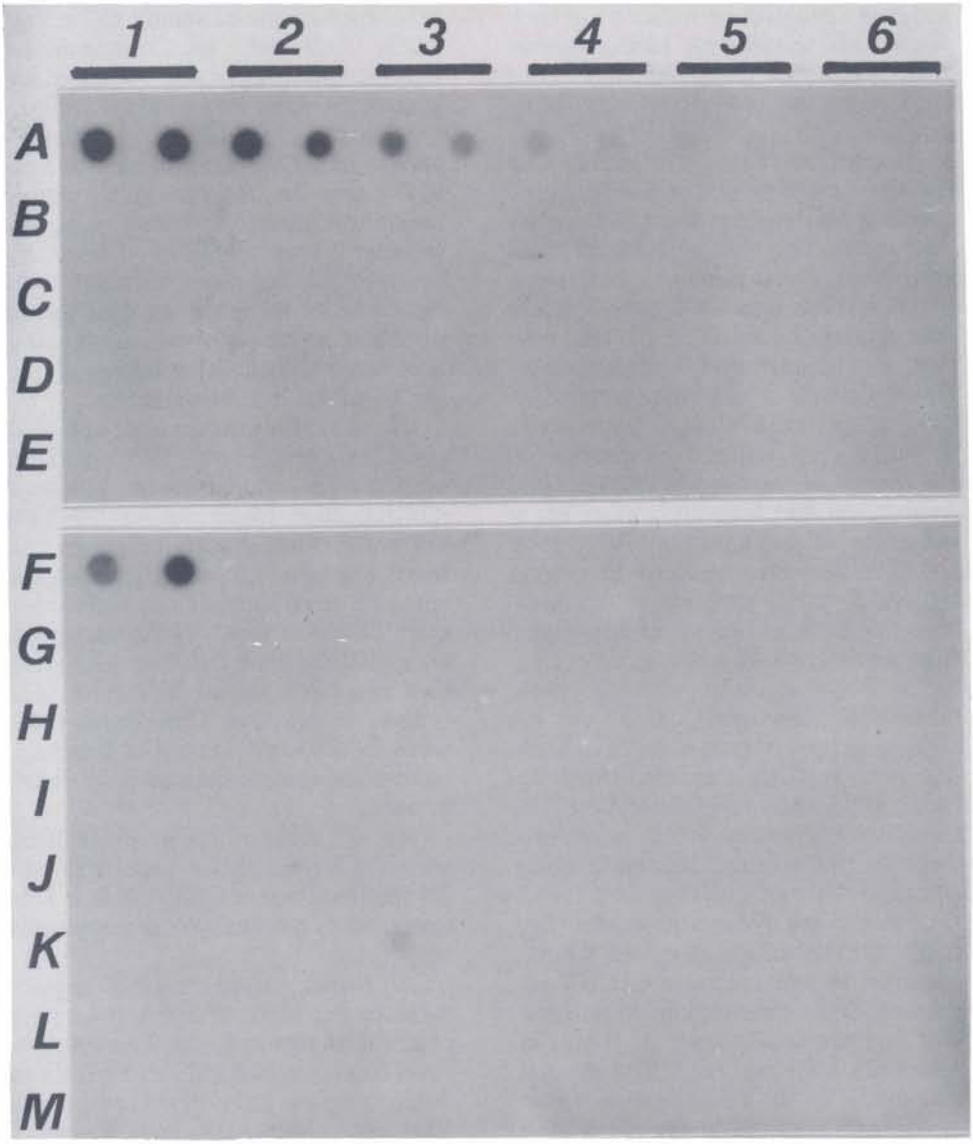

Fig 3. Dot Blot hybridisation assay for CEV in field trees. Nucleic acid from twig bark of field trees was probed with ^{32}P labelled CEV-cDNA. A1-A3: 1:2 serial dilutions of RNA from citron with a known source of CEV within the mother tree block, Taylor Eureka lemon 3402 R 19/T96; F1: Taylor Eureka lemon 3402 R19/T96; F2-F3: Buffer only; A4-A6, F4-F6: 1:2 serial dilutions of RNA from citron mock inoculated with RNA from healthy citrons. Rows B-E and G-M: All field samples were loaded undiluted and as 1:2 dilutions. Only one positive was detected (at position K3).

97 in Row 22, although this tree gave a positive response on Etrog citron. There may be extremely low titres of CEV in this tree, or as above, uneven distribution of the viroid. In general the hybridisation and biological indexing are in accord, yet the discrepancies between the two procedures caution against reliance on any one detection system.

A number of valuable lessons were learned from the contamination by CEV of trees in the budwood multiplication block. These are:

1. Only fully indexed clones should be included in a budwood multiplica-

tion block for "approved" scions. To meet the industry demand for varieties which are not fully indexed or are virus-infected, a miscellaneous selected budwood multiplication block has been established, at a site removed from the approved trees.

2. All imported clones should be fully indexed before inclusion in a local multiplication scheme.

3. Although trees in a hedgerow system produce larger quantities of budwood in the early years of budwood production, the disadvantages are a greater possibility of root grafting, more trees to be indexed for the quantity of budwood supplied and difficulties in disinfecting pruning equipment between trees.

4. Disinfection of all hedging and budwood cutting tools by 1-2% sodium hypochlorite is essential (9).

The contamination of a few lemon trees in the NSW budwood multiplication block has resulted in a revision of the operations of the scheme including work practices (viz. budcutting, hedging, tree spacing), and indexing procedures. Support from industry was increased to improve facilities, equipment and staffing.

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