

# Graft-Transmissible Dwarfing in Australian Citrus

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**ABSTRACT.** Sweet orange trees on trifoliate orange rootstock, dwarfed by graft inoculation in earlier field trials were tested to determine whether viroids were associated with the dwarfing symptoms. Tests used were symptomatology in citron (Arizona 861), gynura, and chrysanthemum, hybridisation with a nucleic acid probe specific for citrus exocortis viroid (CEV) and polyacrylamide gel electrophoresis (PAGE). CEV was present in some, but not all, dwarfing budlines. The commercial application of dwarfing budlines is discussed relative to these findings.

*Index words.* Dwarfing budlines, PAGE, dot blot hybridisations, cDNA probe, citrus exocortis viroid, trifoliate orange.

High-density plantings of dwarfed citrus trees have numerous advantages: earlier productivity, effective and economical pest and disease control, ease of harvesting, more efficient usage of fertilizers and irrigation water and the potential for further mechanisation of orchard practices using smaller, less expensive equipment (8). Dwarf trees are better suited to high-density plantings than vigorous scion-rootstock combinations because of fewer problems with excessive crowding and competition (4, 17).

In the absence of a proven dwarfing rootstock for citrus, the possibility of using a graft-transmissible dwarfing agent has been investigated in Australia over the last 30 yr (15).

When tree losses due to *Phytophthora* root rot necessitated a change to the *Phytophthora*-resistant rootstock trifoliate orange in New South Wales, it was observed that trees on this stock were often of variable size and quite small. Many of the dwarfed trees showed bark scaling of the trifoliate orange rootstocks (exocortis) (2), but other dwarfed trees possessed smooth butts, or butts with corky pustules, intermittent and sparse scaling or gummy pitting (10, 11).

Budwood from these healthy but dwarfed trees was used for extensive direct propagation and graft transmission trials. Closely spaced orange trees on trifoliate orange or citrange rootstocks, dwarfed by bud inocula-

tion with the graft-transmissible dwarfing agent(s), have outyielded conventional plantings (1, 3, 8, 21) without any apparent adverse effects on tree health, yield, fruit size and quality. No bark-scaling symptoms of CEV have been observed in any of the field trials and trees are uniform. Tree-spacing effects on productivity of high-density planted dwarfed orange trees are being examined (13, 14).

The promising results for dwarfing orange trees on trifoliate orange and citrange rootstocks, using these graft-transmissible dwarfing budlines, have not been repeated for lemons (Prior Lisbon and Taylor Eureka) on any of five rootstocks (6).

Transient bark-scaling symptoms on the trifoliate orange rootstocks of the original mother trees (table 1), the leaf curling reaction of Etrog citrons inoculated with these dwarfing budlines (5, 19), and the susceptibility of the rootstocks trifoliate orange, citranges and rangpur lime to graft-transmissible dwarfing (3) suggested an association with CEV. Studies to determine the cause of graft-transmissible dwarfing form the basis of this paper.

## MATERIALS AND METHODS

The origins and and symptomatology of the dwarfing budlines are given in table 1. Fig. 1 is a schematic representation of the experiments carried out and presented in this paper.

TABLE 1  
ORIGINS OF DWARFING BUDLINES

| Accession no.    | Symptoms in original tree <sup>y</sup>  |
|------------------|---|
| 033 <sup>z</sup> | W.N./tri, planted 1947. Scalybutt (scaling) symptoms in tri. Dwarfed.   |
| 3531             | W.N./tri, planted 1933. Few bark scales, pustules and gummy pitting in tri., discontinuous ring at budunion, scaling of tri. inarches, dwarfed. |
| 3532             | W.N./tri, planted 1933. Few transient bark scales, dwarfed.   |
| 3534             | W.N./tri, planted 1933. No scaling, strongly benched, dwarfed.  |
| 3536             | W.N./tri, planted 1933. Transient bark scales and pustules, dwarfed.  |
| 3538             | Marsh G.F./tri, planted 1949. No bark symptoms, intermediate size.  |
| 3539             | Marsh G.F./tri, planted 1949. No bark symptoms, dwarfed.  |

<sup>z</sup>Reference citrus exocortis viroid isolate.

<sup>y</sup>W.N./tri = Washington navel on trifoliolate orange rootstock; G.F. = Grapefruit.

### Biological indexing.

**Graft transmission.** Dwarfing budlines were indexed on rooted cuttings of citron (cv. 60-13 or Arizona 861) in a high-humidity glasshouse with a 32 C 12-h day and a 25 C night temperature. Six citron indicators were each inoculated with two buds from individual dwarfing budlines collected in June (winter) 1986 from the Somersby field trial (planted in 1955 and described in Long *et al.*, (15)). The plants were maintained at high nitrogen status and cut back intermit-

tently to promote new growth. Pruning shears used for sampling or pruning plants were disinfected between plants in 1% sodium hypochlorite solution (18). Razor blades used for budding were discarded between samples.

**Infectivity assays.** Total nucleic acids were extracted from citron tissue containing the dwarfing isolates by the leaf press method of Gillings *et al.*, (12). The extracts were assayed for infectivity by slash inoculation of *Gynura aurantiaca* DC and *Chrysanthemum morifolium* Ramat. (cv. Bonnie Jean), which had been propagated as rooted cuttings. Herbaceous hosts were kept under the same environmental conditions as the citron indicators.

### Biochemical indexing.

**Polyacrylamide gel electrophoresis (PAGE).** Total nucleic acids extracted from citron indicators 16 weeks after bud inoculation were subjected to polyacrylamide slab gel electrophoresis (PAGE), as described by Gillings *et al.*, (12).

**Dot-blot Hybridisation.** Complementary DNA (cDNA) to severe CEV-RNA was obtained from 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.*, (16). Both the dot-blot and hybridisation procedures are fully described in Gillings *et al.*, (12). Hybridisation assays were carried out on total nucleic acids extracted from citron leaves 16 weeks after inoculation.

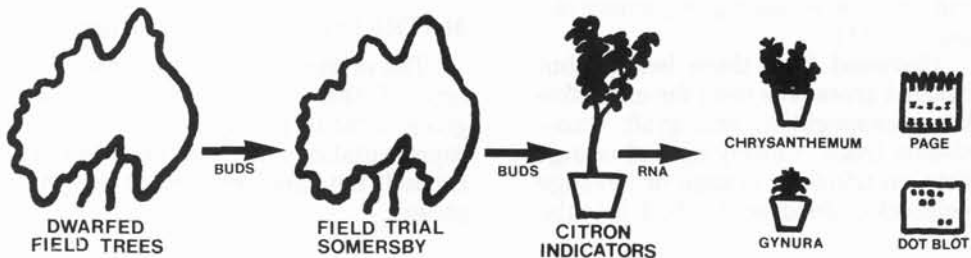


Fig. 1. Schematic representation of the experiments described.

## RESULTS

Some of the original trees from which the dwarfing budlines were derived have shown a transient exfoliation of small pieces of rootstock bark or pustule formation, as distinct from the extensive bark scaling of trifoliolate orange exhibited by the reference CEV isolate (table 1). Inoculated orange trees in 10 subsequent transmission trials (see introduction) have been uniformly dwarfed, but have not shown any rootstock bark scaling or pustule formation (fig. 2). All dwarfing budlines are free of psorosis, but carry tristeza virus (as do all citrus trees in Australia), and clone 3539 is infected with cachexia (xyloporosis) (10).

Inoculation of citron indicators kept under ideal conditions for symptom expression of CEV resulted in severe leaf-curling symptoms for 4339 (CEV positive control) and 3536 in 4-5 weeks (fig. 3a) and mild leaf 'kinking' symptoms for isolates 3531, 3532, 3534, 3538 and 3539 in 4-6 months (fig. 3b and 3c, table 2). Mild symptoms became obvious in leaves 2-3 cm long as a downward curling of



Fig. 2. Budunion of a Washington navel orange tree on trifoliolate orange inoculated with dwarfing budline 3532.

the leaves or an angular downward change in direction of the midrib. The symptoms became less obvious as the leaves matured, or resulted in an 'S' bending of each leaf (fig. 3b).

Hybridisation assays of total nucleic acids extracted from the leaves of these inoculated citrons at 16 weeks postinoculation gave a positive dot-blot response to a CEV-cDNA probe for isolates 4339 and 3536, but not for other isolates (fig. 4), suggesting that CEV was either absent or at very low titre in isolates 3531, 3532, 3534, 3538 and 3539 at that time.

Extract from citrons inoculated with dwarfing budline 3536 (and having severe symptoms of exocortis disease) showed RNA bands with the same electrophoretic mobility as CEV from citrons inoculated with a known source of CEV (accession 4339). No characteristic CEV band was obtained from citrons inoculated with control 2006 or citrons inoculated with 3531, 3532, 3534, 3538 or 3539 and displaying mild symptoms. No other RNA species in the viroid size range has been uniquely identified with the dwarfing budlines.

When preparations of total nucleic acids from citrons showing mild and severe symptoms were assayed for infectivity on gynura and chrysanthemum plants under hot conditions, gynura plants developed symptoms in 4 weeks when inoculated with 4339 (CEV) or dwarfing budline 3536, but not for the other dwarfing budlines. The only chrysanthemums to show symptoms were those inoculated with 4339 or those inoculated with 3536 at 3 and 7 weeks, respectively.

Non-denaturing gels were run on total nucleic extracts of gynura taken from plants 6 weeks after inoculation with 3536, 3538, 4339 and 2006 (control). CEV was detected in extracts of gynuras inoculated with 3536 and 4339. Sequential gels were run on total nucleic acid extracts of chrysanthemum taken from plants 15 weeks after inoculation with 3536 and 2006 (control). CEV was detected in the 3536-inoculated plants.



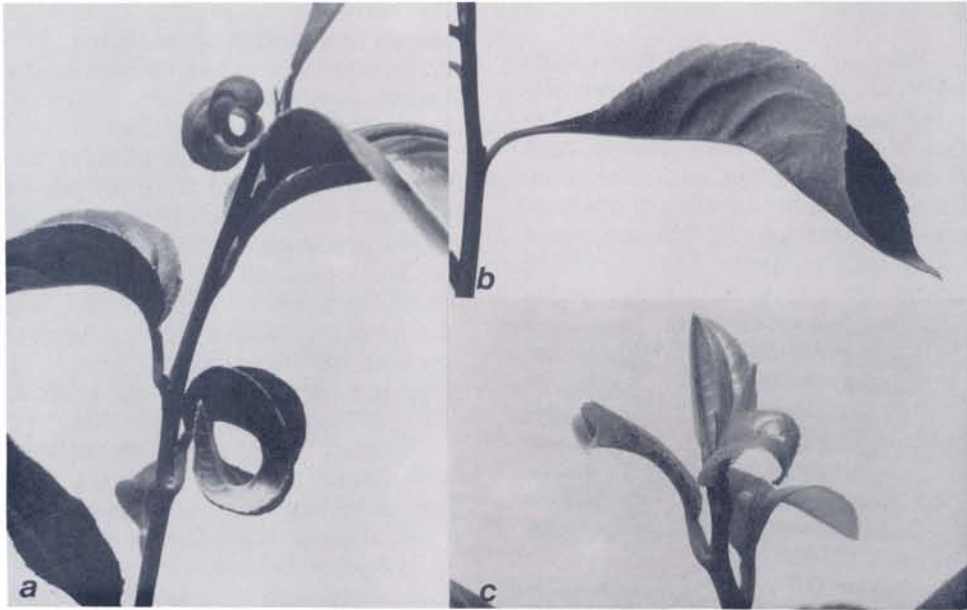


Fig. 3. Leaf curl in citron (Arizona 861) inoculated with dwarfing budline 3536 (a), angular downward change in direction of the midrib of a mature leaf (b) and minor curling of young leaves (c) of citron inoculated with dwarfing budline 3532.

## DISCUSSION

The results obtained in this study confirm those of Schwinghamer and Broadbent (19, 20). These authors also found CEV in citron indicators bud-inoculated with material from dwarfing budline 3536, but could detect little or no homology to CEV from citrons bud-inoculated with material from dwarfing budlines 3531, 3532, 3534, 3538 and 3539. However, Schwinghamer and Broadbent (20) did succeed in isolating CEV from budlines 3531, 3532 and 3539 when total nucleic acids from citrons budded with this material were puncture-inoculated onto chrysanthemum plants. The presence of CEV was confirmed in chrysanthemums with severe symptoms both by PAGE and hybridization assay. The infectivity of these isolates (3531, 3532 and 3539) on chrysanthemum was low; symptom onset taking 6-17 weeks compared to 3-8 weeks for typical citron/CEV extracts. Visvader and Symons (22) also used mechanical transfers to chrysanthemum to isolate and sequence the CEV in dwarfing budlines 3532 and

3536. While isolates 3532 and 3636 did contain CEV sequence variants (23), the changes in nucleotide sequences (22) are not sufficient to prevent detection by a CEV probe under the hybridisation conditions we used.

Mild reactions were also observed when chrysanthemums were stab-inoculated with total nucleic acids from citrons budded with the dwarfing isolates (20). Chrysanthemums with mild symptoms did not show a band by PAGE or positive response to CEV probes. The mild symptoms on chrysanthemum were, however, suggestive of a viroidlike infection.

It must be assumed that CEV is present in dwarfing budlines 3531, 3532 and 3539, but at extremely low titres, and that inoculation onto chrysanthemum enhances CEV to detectable levels.

If CEV is at such low titres, it seems unlikely that CEV is the primary causal agent of dwarfing. Although we have not succeeded in detecting other viroidlike RNA's in the dwarfing budlines, there is circumstantial evidence that such RNA's may exist. Firstly, after

TABLE 2  
DETECTION OF CITRUS EXOCORTIS VIROID (CEV) IN DWARFING BUDLINES

| Accession <sup>z</sup><br>no. | Biological indexing                           |  | Biochemical indexing  |                               | Infectivity assays                  |   |
|-------------------------------|---|--|-----------------------|-------------------------------|-------------------------------------|---|
|                               | Severe<br>symptoms<br>on citron<br>at 7 weeks | Mild<br>symptoms<br>on citron<br>at 24 weeks | PAGE on<br>citron RNA | CEV<br>probe to<br>citron RNA | Symptoms<br>on gynura<br>at 4 weeks | Symptoms<br>on chrysan-<br>themum<br>at 4 weeks |
| 2006 <sup>y</sup>             | 0/8   | 0/8  | —                     | —                             | —                                   | —   |
| 3531                          | 0/8   | 2/8  | —                     | —                             | —                                   | —   |
| 3532                          | 0/8   | 1/8  | —                     | —                             | —                                   | —   |
| 3534                          | 0/8   | 3/8  | —                     | —                             | —                                   | —   |
| 3536                          | 4/4   | severe only                                  | +                     | +                             | +                                   | —   |
| 3538                          | 0/8   | 2/8  | —                     | —                             | —                                   | —   |
| 3539                          | 0/10  | 5/10   | —                     | —                             | —                                   | —   |
| 4339 <sup>x</sup>             | 2/2   | severe only                                  | +                     | +                             | +                                   | —   |

<sup>z</sup>Buds taken from several field sources per accession number.

<sup>y</sup>Control (healthy tree).

<sup>x</sup>Known source of CEV.

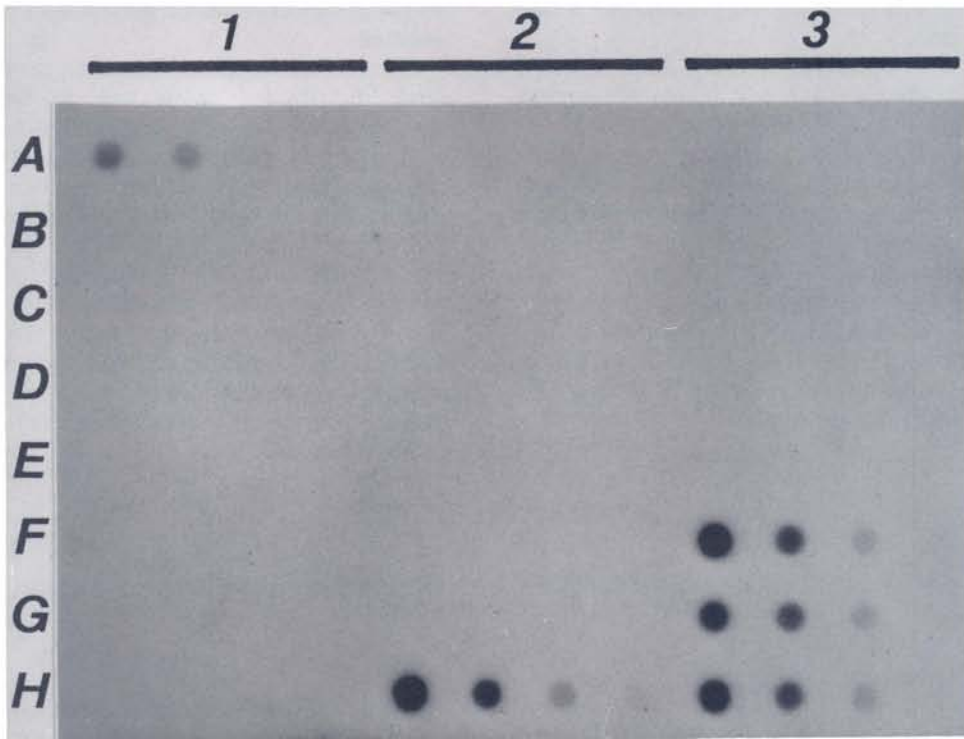


Fig. 4. Dot-blot hybridisation assay for citrus exocortis viroid (CEV) in citrons bud-inoculated with various dwarfing budlines. Nucleic acid from citron leaves was probed with <sup>32</sup>P labeled CEV-cDNA, and the filter washed for 1 h at 58 C in 2X SSC, 0.5% SDS (12). The filter was exposed to X-ray film at -80 C with intensifying screens for 90 h. Samples were from independent extractions and were loaded as 1:5 dilution series. A1-A2) RNA from a citron inoculated with a known CEV source (Taylor Eureka 3402, R19 T96), A3) RNA from a citron inoculated with RNA from a healthy citron; G3 and H3) RNA from citrons inoculated with a known CEV source, (Villafraanca 4339); B1, B2, and B3) RNA from citrons inoculated with a non-dwarfing (healthy) control of Bellamy navel orange 2006. C1, D1, and E1) RNA from citrons inoculated with dwarfing budline 3534; F1, G1, and H1) RNA from citrons inoculated with dwarfing budline 3539; C2, D2, and E2) RNA from citrons inoculated with dwarfing budline 3531; F2 and G2) RNA from citrons inoculated with dwarfing budline 3532; C3, D3 and E3) RNA from citrons inoculated with dwarfing budline 3538; and H2 and F3) RNA from citrons inoculated with dwarfing budline 3536.

transmission of 3531, 3532 and 3539 to chrysanthemum some plants exhibit mild symptoms suggestive of viroid infection, yet no homology to CEV is detected. Secondly, CEV has never been isolated from dwarfing line 3538, even though this line graft-transmits the dwarfing characters.

Duran-Vila, *et al.* (9) suggested that "the dwarfing factor utilized in controlling citrus growth in Australia . . . may be the result of the expression of a particular permutation of the reservoir of viroid-like RNAs apparently resident in citrus populations. Natural combinations . . . of such RNAs may very well be critical to the segregation of the biological expression or intensity of the exocortis disease."

Resolution of the causal agent(s) of graft-transmissible dwarfing must await the identification of the agent isolated from the dwarfing budlines (causing mild symptoms in chrysanthemum) and inoculation of the agent(s), singly and in combination, into citrus. Such pathogenicity tests should resolve whether the disease syndrome known as exocortis, and causing bark scaling and dwarfing, is caused by CEV or by a complex of different viroids.

These mixtures, whether of viroid-like RNA species (19) or CEV variants must be extremely stable in budwood of the dwarfing budlines, as trees in 10 field transmission trials have been uniform and exhibited the same budunion conformation and absence of bark-scaling symptoms in trifoliate orange rootstock. Mild, moderate and severe isolates have not been separated by graft transmission, although Roistacher *et al.* (18) separated mild isolates from severe by knife transmission. While mild and severe isolates are both mechanically transmitted, Calavan, *et al.* (7) suggested that severe isolates may spread more readily by cultural operations in lemon orchards than the mild isolates.

Thus, a management problem may arise if dwarfed trees are to be mechanically hedged and hedging equipment is subsequently used on pathogen-free trees without surface sterilization of equipment (15). The dangers of transmission of CEV in the nursery are acute and guidelines for the use of graft-transmissible dwarfing (8), including inoculations of orchard trees, must be adhered to prevent contamination of pathogen-free trees in citrus budwood multiplication schemes.

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