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Recovery of Rickettsialike Bacteria from Xylem of Healthy and Young Tree Decline-Affected Citrus Trees*

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Young tree decline (YTD), sometimes referred to as sandhill decline or blight, causes extensive destruction of citrus trees on rough lemon rootstock in Florida. The disease, which is not apparent until trees come into bearing or later, is characterized by zinc-like deficiency symptoms of foliage in portions of the tree, thin foliage, delayed flush, twig dieback, leaf roll, wilting during periods of slight water stress, vessel plugging, and zinc accumulation in the wood (Smith, 1974). Etiology of YTD is unknown and the complete syndrome has not been reproduced by transmission (Feldman *et al.*, 1976) or by propagation. A rickettsialike bacterium (RLB) was found in vascular fluid of YTD-affected as well as in some apparently healthy citrus trees (Feldman *et al.*, 1977) but its involvement in the etiology of the disease has not been established. We report now additional examinations of vascular fluid by electron microscopy (EM) to locate portions of the tree where sufficient numbers of RLB might be recovered for identification, culturing, serology, or for inoculum for transmission studies.

MATERIALS AND METHODS

Source trees. Sixty-one YTD-affected and 25 apparently healthy trees on rough lemon rootstock were selected from 14 groves in the ridge area of central Florida. Numbers of groves of each cultivar were: 6 Valencia, 2 Hamlin, 2 Pineapple, and 1 Jaffa sweet orange, 1 Orlando Tangelo, 1 Marsh grapefruit, and 1 Thompson grapefruit. Because of extensive vessel plugging in moderate and severely affected trees, samples were obtained from YTD-

affected trees in early stages of decline. No attempt was made to compare influence of scion variety on recovery of RLB. Samples were collected from February 1977 to November 1978.

Vacuum extraction of twigs and roots. Generally 8-12 sections of roots or twigs, approximately 1 x 15 cm, were randomly collected from each source tree. Samples were washed in dishware detergent, rinsed, dipped in 70 per cent ethanol for 3 minutes and flamed. Glassware and buffer were sterilized by autoclaving; knives, pruning shears, and tygon tubing were dipped in 95 per cent ethanol for 10 minutes and rinsed in sterile water. Root pieces and twigs, with the bark removed from upper and lower ends, were extracted with 0.02 M phosphate buffer (pH 7.0) as previously described (Feldman *et al.*, 1977). After vacuum extraction, an aliquot of vascular fluid was removed for culturing and remainder was centrifuged in a conical tube at 500 g for 30 minutes. The pellet was used for EM examination.

Extraction of vascular fluid by centrifugation. Root pieces and twigs were aseptically prepared as described above except after vacuum infiltrating 0.5 to 1 ml of sterile phosphate buffer, the twigs or root pieces were placed in sterile centrifuge tubes, generally 3/tube, and centrifuged at 3500 g for 30 minutes. After centrifugation, fluid from each tube was combined, an aliquot taken for culturing and remaining fluid centrifuged to recover pellet for EM examination.

Vacuum extraction of stock and scion. Collection of vascular fluids from stock and scion, including pioneer roots, of grove trees was accomplished by the following procedure: Area of tree

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selected for extraction was scrubbed with a wire brush, washed with 95 per cent ethanol, and rinsed with sterile water. Using a sterile drill bit, two 6-mm-diameter holes 20 to 30 mm deep were then drilled one above the other ca 100 to 150 mm apart. An L-shaped copper tube, enlarged at the upper end to serve as a reservoir, held 30 ml of 0.02 M sterile phosphate buffer (pH 7.0). A 500-ml vacuum flask with side arm was attached to the lower hole in the tree by an L-shaped glass tube and the other end of the tube was inserted 4 cm below the neck of the stoppered vacuum flask so that the glass tube emptied directly into a sterile 15-ml conical centrifuge tube. A generator-driven vacuum pump was then connected to the side arm of the vacuum flask. After collecting approximately 12 ml of fluid, the procedure was repeated for collection of a second sample from opposite side of the tree. Tubes containing xylem fluid were capped and stored on ice until processed several hours later. Contents of both centrifuge tubes were combined, an aliquot was removed for culture, and remainder centrifuged as above to recover pellet for examination by EM.

Extraction of wood chips. After bark removal, wood chips were collected from stock or scion by boring three or four 25 x 50 mm holes. Aliquots of 30 g of wood chips were ground with 150 ml 0.02M phosphate buffer (pH 7.0) in a Waring blender for 2 minutes. The resultant slurry was filtered through 4 layers of cheese cloth and the filtrate centrifuged 100 g for 10 minutes to remove starch and other debris. Supernatant was centrifuged in a conical tube at 500 g for 30 minutes and the pellet recovered for EM examination.

Preparation of sample for EM. The pellet obtained from each of the above preparations was infiltrated with 4 ml warm 8 per cent agar solution, cut into five to eight pieces after solidification, fixed in glutaraldehyde (3 per cent in 0.1 M phosphate buffer pH 7.0), post-fixed in osmium tetroxide (2 per cent in 0.1 M phosphate buffer pH 7.0), rinsed with fresh buffer, and dehydrated with in-

creasing concentrations of acetone at 4 C. The material was infiltrated with Spurr's embedding medium, sectioned, stained with uranyl acetate and lead citrate, and viewed with a Philips 201 Electron Microscope. Presence or absence of RLB in a given tissue sample was determined by viewing three to six grids, each with eight serial sections, from a single pellet piece.

Isolations. Vascular fluid obtained from roots, twigs, stock or scion by either vacuum extraction or by centrifugation was used to attempt isolation of the RLB. Additional isolations were also made from tissue samples of roots, twigs, petioles, midribs, and feeder roots. These were washed with dishware detergent, rinsed with deionized water, surface sterilized in 95 per cent ethanol for 5 minutes, and rinsed with sterile water. Larger twigs and roots were flamed after ethanol dip, after which the bark was removed. Tissues were then squeezed in a garlic press and placed in 3 to 5 ml JD-3 broth (Davis *et al.*, 1978) for subsequent plating. Generally, 0.05 to 0.1 ml vascular fluid or 0.1 to 0.2 ml of JD-3 broth from the tissue squeezing was plated on the following media: nutrient agar (Difco), medium 523 and D-5 (Kado and Heskett, 1970), and JD-3 agar, the latter being specific for culturing the RLB of Pierce's disease of grapevines. Cultures were maintained at 28 C and were examined daily after 5 days incubation.

RESULTS

General description of RLB. Morphology of the bacterium appears to be similar to described RLB of phony peach (Nyland *et al.*, 1973) and Pierce's disease of grape (Hopkins and Mollenhauer, 1973) (fig. 1A). Bacteria ranged in size from 0.3-0.6 x 0.9-2.3 μ m and generally possessed a rippled cell wall ca 20 to 30 nm thick that appeared to be composed of a double trilaminar membrane and an R-layer (fig. 1B). A fibrillar matrix (fig. 1B) and fimbriae (fig. 1B, C) were sometimes associated with the outer cell wall. Occasionally,

the bacterium was found encapsulated in a translucent matrix (fig. 1D).

Recovery of RLB from roots and twigs. The RLB were inconsistently recovered from vascular fluid from roots and twigs of YTD-affected trees (table 1) as well as from subsequently sampled RLB-positive trees. When RLB were found, generally one, and rarely three to six, were present in the 24 to 48 serial sections from a single pellet. The bacterium was found more frequently in root than in twig samples collected in the fall but frequency of recovery was essentially the same in roots and twigs when samples were collected in spring (table 1). Frequency of recovery from twigs was approximately the same during fall and winter (50 per cent) and least during summer (25 per cent).

Vacuum extraction of vascular fluid from twigs and roots was more effective in recovering RLB than was centrifugation. Of the 23 YTD-affected trees sampled, RLB were recovered from 14 by vacuum extraction and from seven by centrifugation.

Recovery of RLB from trunks. The bacterium was found in only one of 29 samples of vacuum-extracted vascular fluid obtained from the scion trunks of YTD-affected trees (table 1). The organism was not recovered from vascular fluid from trunk stocks and pioneer roots nor from wood chips from trunks (stocks or scions.)

Isolations. RLB could not be cultured from vascular fluid of stock, scion, roots, or twigs nor from tissue samples of midrib, petiole, feeder roots, twigs, or roots although many other bacteria were recovered. No attempt was made to identify the various bacteria isolated. Characteristics of the various bacterial colonies cultured from vascular fluid of healthy and YTD-affected trees appeared somewhat similar, although generally more bacteria were isolated from YTD-affected trees.

DISCUSSION AND CONCLUSIONS

Attempts to culture as well as to find and recover large numbers of RLB from YTD-affected trees were not successful. Inconsistent recovery of RLB from twigs and root pieces and the lack of recovery from stock, wood chips, and scion trunks (the latter from only one of 29 trees) still leaves unresolved the possible involvement of RLB in the etiology of YTD. Our inability to recover larger numbers of RLB may be due to vessel plugging (Nemec, 1975; Vandermolén, 1974), or to the adhesive characteristics of the matrix or fibrillar material frequently found associated with the bacteria (fig. 1B, D), or to the normally small numbers of RLB in citrus.

Certain observations and data are worth noting and perhaps justify a consideration for pursuing additional investigations with this bacterium, *i.e.*: (1) there appears to be some correlation between presence of RLB, particularly from roots sampled in the fall, and incidence of YTD; (2) RLB were recovered from xylem in which plugging and gum formation (Nemec, 1975; Vandermolén, 1974) indicated a disturbance in the water conducting system in a manner similar to the RLB-associated Pierce's disease of grape (Esau, 1948); (3) tetracycline treatments are reported to produce some remission of YTD symptoms (Tucker *et al.*, 1974); (4) Pierce's disease has been transmitted to grape from YTD-affected trees using *Oncometopia nigricans* Walker, a common sharpshooter that feeds on grape and citrus (Hopkins *et al.*, 1978); (5) wild grape, a common host of Pierce's disease RLB has been observed (Hopkins *et al.*, 1978) in and adjacent to many groves with YTD-affected trees; and (6) RLB in xylem fluid from YTD-affected trees can be detected by immunofluorescence (Lee *et al.*, 1978) using labeled antiserum prepared against the Pierce's disease RLB.

The inability to culture the RLB from citrus, however, is not consistent with the hypothesis that the RLB is the same

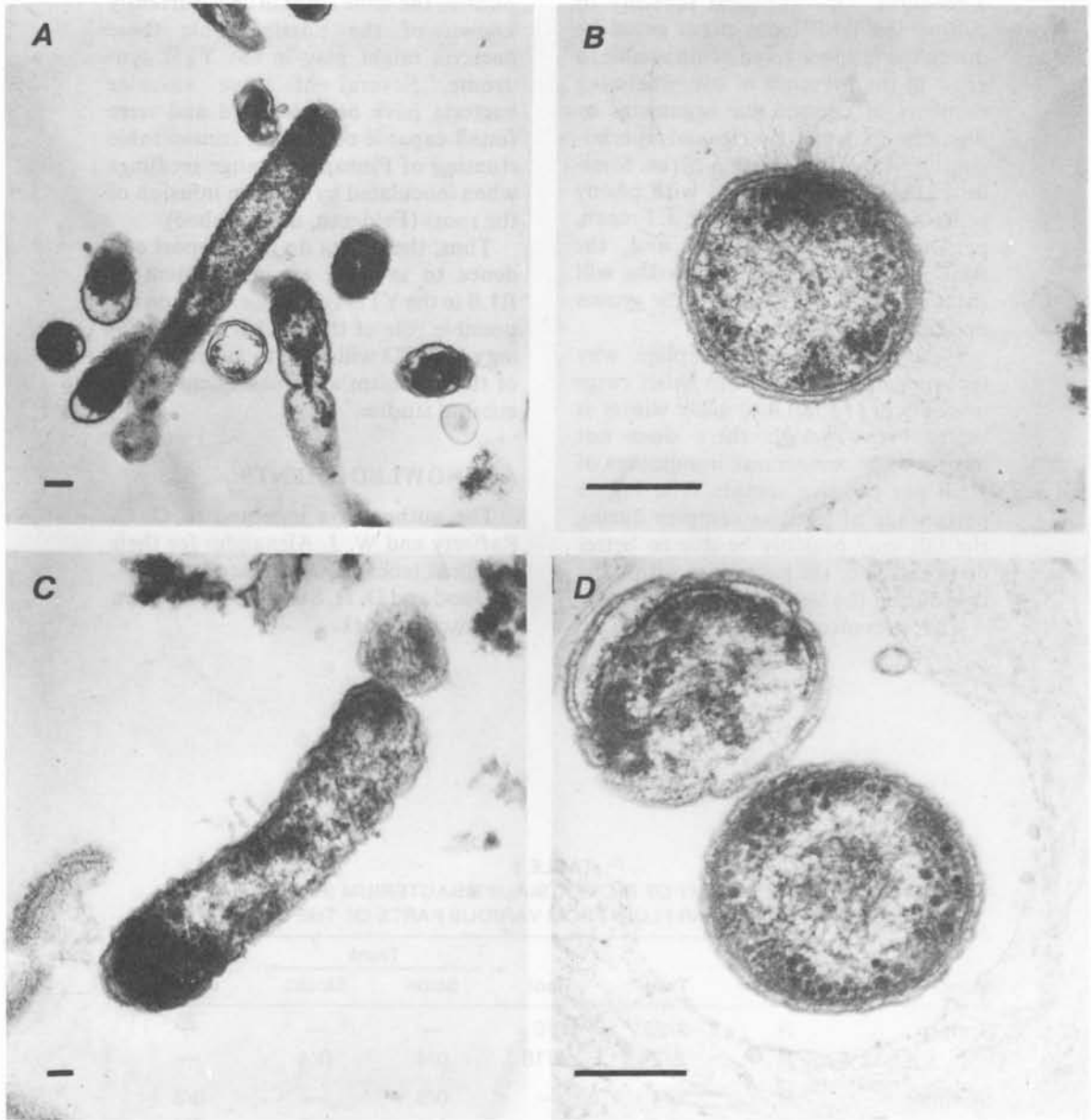


Fig. 1. Rickettsialike bacteria (RLB) in vascular fluid recovered by vacuum extraction of twigs and roots from TYD-affected citrus trees: (A) RLB with fibrillar material; (B) cross section of RLB showing fibrillar material and fimbriae (note double trilaminar wall and R-layer); (C) RLB with several fimbriae projecting from outer cell wall; (D) cross section of RLB encapsulated in a translucent matrix. Bar = 0.2 μ m.

as the Pierce's disease organism since the latter can be readily cultured on JD-3 medium. This apparent inability to culture the RLB from citrus could be due to the few recovered being unable to grow in the presence of overwhelming numbers of competitive organisms or possibly to some nutritional specialization of these RLB from citrus. Similarly, the RLB associated with phony peach and plum leaf scald (W. J. French, personal communication), and the RLB associated with periwinkle wilt (McCoy *et al.*, 1978) cannot be grown on the JD-3 medium.

It is not possible to explain why recovery of the RLB from either twigs or roots in the fall and early winter is better even though there does not appear to be an increase in numbers of RLB per positive sample. The higher percentage of positive samples during the fall may possibly be due to better distribution of the bacterium within the tree during the latter part of summer.

The presence of other bacteria in

vascular fluid, generally with larger numbers in YTD-affected trees, complicates the issue since little is currently known of the possible role these bacteria might play in the YTD syndrome. Several of these vascular bacteria have been isolated and were found capable of causing considerable stunting of Pineapple orange seedlings when inoculated by vacuum infusion of the roots (Feldman, unpublished).

Thus, these data do not support evidence to indicate an involvement of RLB in the YTD syndrome. Data on the possible role of these RLB in the etiology of YTD will have to await culture of the organism and subsequent transmission studies.

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TABLE 1
FREQUENCY OF RECOVERY OF RICKETTSIALIKE BACTERIUM (RLB) BY VACUUM EXTRACTION OF VASCULAR FLUID FROM VARIOUS PARTS OF THE CITRUS TREE*

| Season | Tree condition† | Twig | Root | Trunk | | Wood chips§ |
|--------|-----------------|--------|-------|-------|--------|-------------|
| | | | | Scion | Stock‡ | |
| Spring | H | 2/23** | 0/6 | — | — | — |
| | D | 8/24 | 5/16 | 0/4 | 0/4 | — |
| Summer | H | 0/4 | — | 0/5 | — | 0/3 |
| | D | 4/16 | — | 0/8 | — | 0/4 |
| Fall | H | 1/10 | 0/4 | 0/2 | 0/5 | 0/2 |
| | D | 12/24 | 10/14 | 0/10 | 0/11 | 0/6 |
| Winter | H | 0/5 | — | — | 0/2 | — |
| | D | 9/17 | — | 1/7 | 0/6 | — |

* Determinations by electron microscopy from samples collected Feb. 1977 to Nov. 1978.

† H = healthy; D = YTD-affected.

‡ Including pioneer roots.

§ Obtained from stock or scion; bacteria recovered by centrifugation.

** Number with RLB/total number of samples.

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