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Immunogold Localization of Xylem-Inhabiting Bacteria Affecting Citrus in Argentina and Brazil*

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ABSTRACT. Citrus leaf tissue exhibiting symptoms of pecosita from Misiones, Argentina and citrus variegated chlorosis (CVC) from Minas Gerais, Brazil was collected and fixed for immunogold labelling. Various fixation and embedding procedures were tested in order to facilitate identification of *Xylella fastidiosa* in ultrathin sections of affected citrus using immunogold labelling. Fixation with 2.5% glutaraldehyde in cacodylate or phosphate buffers and embedding in London Resin White proved most reliable for localization of bacteria. Polyclonal antisera for the identification of the causal organisms of ragweed stunt (RW), alfalfa dwarf (AD), Pierce's disease (PD-LH2), periwinkle wilt (PW), plum leaf scald (PLS), elm leaf scorch (ELS) and two antisera to CVC (CREC-22) and (CREC-26) were used in localization studies. *X. fastidiosa* was present in tissues affected with both diseases. The highest density of gold labelling was obtained using the homologous CVC antisera CREC-22 and CREC-26. High labelling densities also occurred using antisera to PLS and PD-LH2. A lower density of labelling was achieved with RW, PW and AD antisera. Antiserum to ELS reacted only to the lightly stained fibrillar matrix which surrounded bacteria in the vessel lumina.

Citrus variegated chlorosis (CVC) and pecosita are similar diseases affecting sweet orange in Brazil and Argentina, respectively. CVC was the first of these two bacterial diseases to be identified and has been spreading rapidly in citrus nurseries and commercial groves in Brazil since 1987 (3, 8). The symptomatology of these two diseases is similar. Chlorotic areas are visible on the abaxial side of the leaf between lateral veins and resemble zinc deficiency. Small, slightly raised, light brown lesions are usually visible on the adaxial side of the leaf and are located in the center of the chlorotic areas. These chlorotic spots often elongate and coalesce. Bacteria with rippled cell walls have been found in the xylem vessels of stems, petioles, mid veins, and fruit peduncles (3, 4, 13). These xylem-inhabiting bacteria are morphologically and serologically similar to *Xylella fastidiosa* and have been associated with both diseases (3, 4, 9).

Immunogold labelling *in situ* has been used successfully to locate and identify bacteria, bacterial polysaccharides and fungi in diseased tissues (1, 12, 14). A number of antisera to different strains of *X. fastidiosa*, including

the CVC strain have been prepared and have proved successful to varying degrees in detection of the CVC bacterium in citrus (2, 9). The purpose of this study was to utilize *in situ* immunogold labelling techniques for the detection and identification of *X. fastidiosa* in citrus xylem tissues and to determine which *X. fastidiosa* antiserum is best suited for this type of detection.

MATERIALS AND METHODS

Fixation and embedding. Citrus leaf midrib tissue was excised in 4mm pieces from symptomatic leaves and treated with one of four different fixation procedures: a) 2.5% glutaraldehyde (GLU) in 0.066M phosphate buffer pH 6.8. b) 2.5% GLU in 0.1M cacodylate buffer pH 7.2. c) 3% GLU in 0.066M phosphate buffer pH 6.8, followed by a 1% osmium tetroxide fixation in 0.066M phosphate buffer. d) 4% formaldehyde and 0.2% GLU in PIPES buffer pH 7.2, followed by 1% osmium tetroxide in PIPES buffer. Samples in fixative were put under slight vacuum for 30-60 sec and left in the fixatives for 4 hr at room temperature. The samples were removed from the fixative and washed 3X in the same buffer. Post-fixation in c & d above in 1% osmium tetroxide was done for 2-4 hr at room temperature. Samples were washed

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3X in the appropriate buffer. All samples were then dehydrated through an ethanol series before embedding in London Resin White (L. R. White), or through an acetone series before embedding in Spurr's resin. Samples from all four fixation procedures were embedded in both L. R. White and Spurr's embedding resins.

Polymerized blocks were trimmed and 85nm (gold) ultrathin sections were cut with a Riechert ultracut E microtome and placed on 200 mesh formvar coated nickel electron microscope grids.

Antisera and immunolabelling.

Antisera to the Pierce's disease (PD-LH2), ragweed stunt (RW), plum leaf scald (PLS), periwinkle wilt (PW), alfalfa dwarf (AD), and elm leaf scorch (ELS) were prepared as previously described (11). Antiserum to citrus variegated chlorosis (CREC-22) was prepared by culturing bacteria on modified periwinkle wilt media (2, 5). Bacteria were removed from plates of 14-day-old cultures and resuspended in sterile 0.1M phosphate buffer to approximately 10^8 CFU/ml and placed at -20°C . Antiserum to CREC-26 was produced as previously described (7). Injection of rabbits and production of polyclonal antisera for CREC-22 was carried out using previously described methods (7). Primary antibodies PD-LH2, PW, AD, PLS, ELS, RW, CREC-22 and CREC-26 or a control antiserum, *Xanthomonas campestris* pv. *citriumelo* were all diluted 1/1000 in 0.2M Tris-HCl with 0.1% bovine serum albumin (Tris-BSA) pH 8.2. A second control was performed utilizing a 15nm goat anti-rabbit (GAR) gold-IgG probe diluted 1/25 in Tris-BSA buffer without the use of primary antibodies.

The sections were etched for 5 min with a 0.4M solution of sodium metaperiodate and rinsed on two drops of double distilled water. Sections were incubated for 10 min on a drop of 0.2M Tris pH 8.2 containing 1% BSA as a blocking agent, blotted on filter paper and immediately placed on the diluted primary antibodies. The grids were incubated on primary antibodies and controls for 30 min at 37°C and rinsed on 3

drops of Tris-BSA buffer for 5 min each. After rinsing, the grids were incubated at 37°C for 20 min on a secondary 15nm GAR gold-IgG diluted 1/25 in Tris-BSA buffer. After incubation on the secondary antibody, the grids were again rinsed on three drops of Tris-BSA buffer for 5 min each and three times on drops of distilled water. The grids were stained with 7% aqueous uranyl acetate for samples that were embedded in L. R. White or with 15% uranyl acetate in methanol for those embedded in Spurr's. Final staining was done with Reynolds lead citrate.

RESULTS

Electron microscopy. Bacteria morphologically similar to *X. fastidiosa* were observed in the xylem elements of thin sections examined from affected citrus from Argentina and Brazil. A large number of bacteria were observed in the petioles, midveins and fruit peduncles examined. Bacteria were not evident in healthy field and greenhouse samples.

In sections through diseased petioles, bacteria were often located within a lightly stained fibrillar matrix (Fig. 1A). Some xylem vessels from diseased tissues contained small irregular spindle-shaped bacteria (Fig. 1B). Similar bacteria have been described by other researchers as degenerating forms of *X. fastidiosa* (6, 10). A larger number of bacteria were evident in sections of diseased tissues from Brazil. Higher populations of *Xylella* in affected citrus from Brazil were also observed with membrane entrapment immunofluorescence (Brlansky *et al.* unpublished) using equal weights of affected tissues from both areas.

Fixation and embedding. Fixation procedures a and b containing 2.5% GLU in 0.1M cacodylate or 0.066M phosphate buffers allowed for the best localization of bacteria with all antisera tested. Labelling specificity was greatly improved when post-fixation with osmium tetroxide was omitted. Osmium tetroxide evidently blocked localization sites and allowed only for minimal recognition

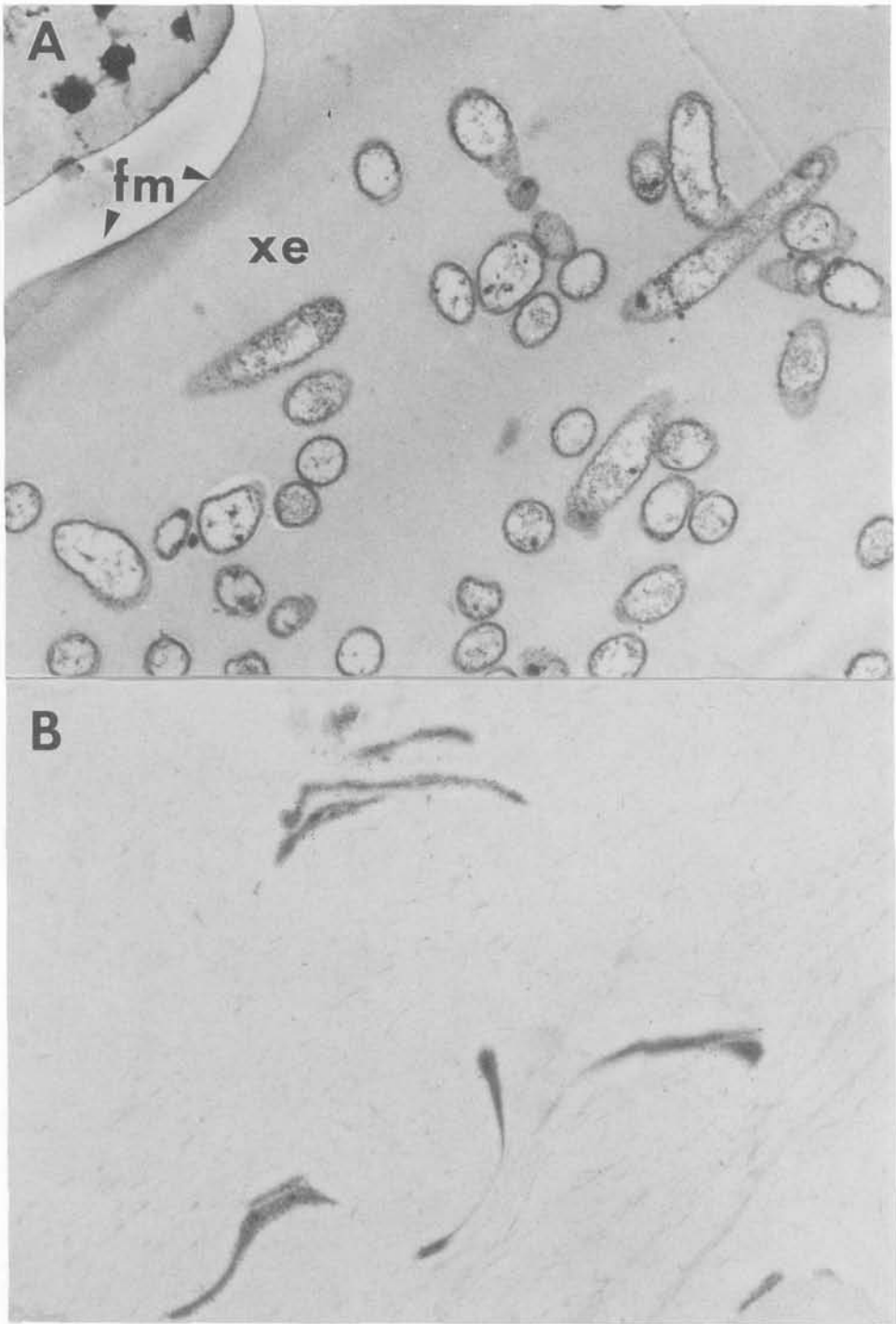


Fig. 1. A. Transmission electron micrograph (TEM) of a ultrathin section through a petiole of a citrus variegated chlorosis-affected sweet orange from Brazil. Bacteria morphologically similar to *X. fastidiosa* are evident in the xylem element (xe) and arrows indicate the fibrillar matrix (fm) (X21,000). B. Electron micrograph of spindle shaped degrading bacteria in a petiole of pecosita affected citrus from Argentina (X13,950).

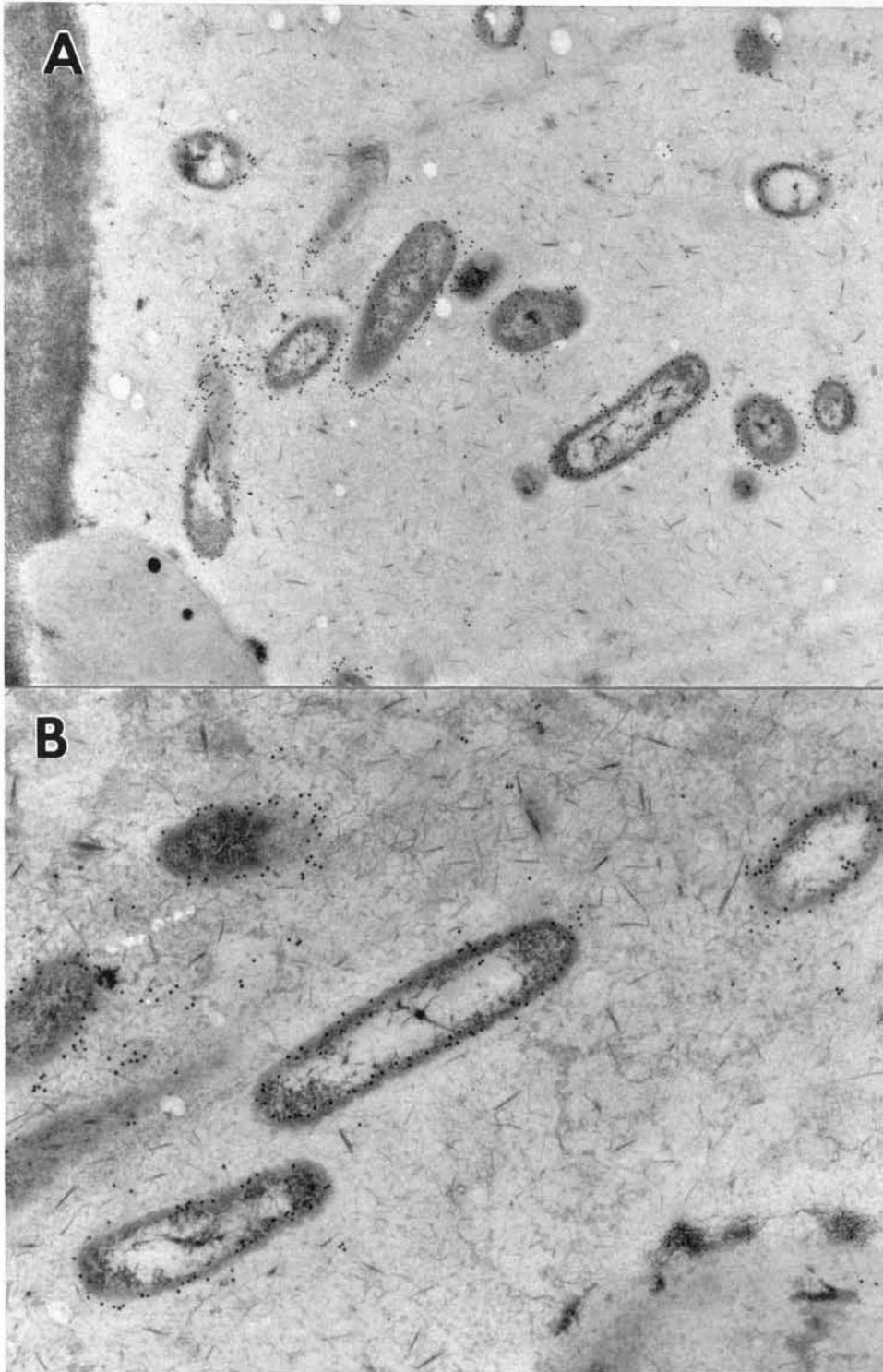


Fig. 2. A. Transmission electron micrograph (TEM) of *X. fastidiosa* in a thin section from a pecosita-affected citrus incubated on antiserum (CREC-22) to citrus variegated chlorosis (CVC) diluted 1/1000 and then on a 15 nm GAR gold-IgG complex (X16,275). The antibody shows a good affinity for the bacterial cells in xylem elements. B. TEM of *X. fastidiosa* in CVC-affected citrus after incubation on homologous antiserum (CREC-26) (X30,000). The antiserum shows a high density of labelling on the bacterial cell wall with little to no background labelling.

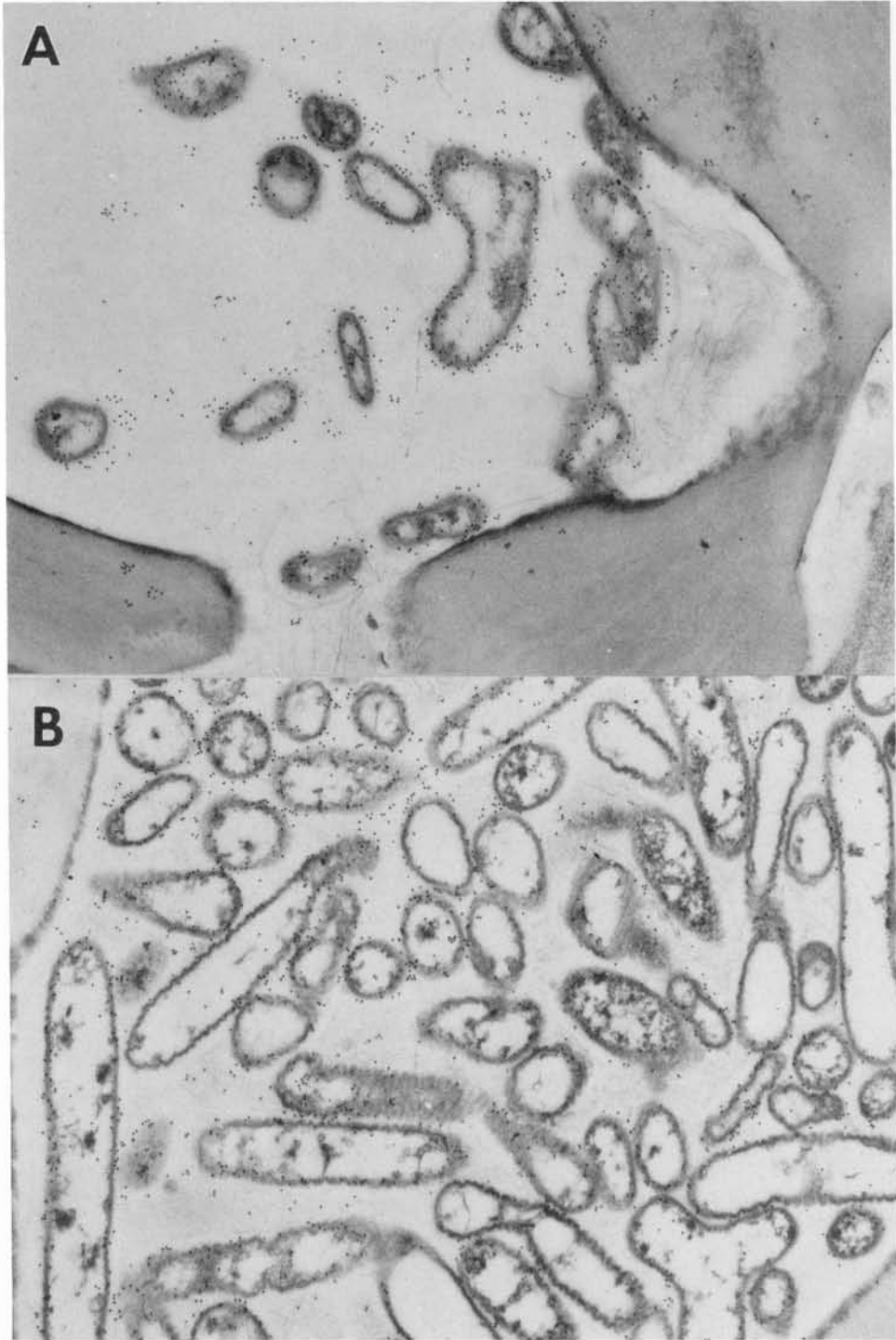


Fig. 3. A. Transmission electron micrograph of a thin section through a leaf petiole from a pectosita-affected citrus tree. The section was incubated on antiserum prepared to Pierce's disease of grape (PD-LH2) (X21,000). A high density of labelling was evident, but background labelling with PD-LH2 was slightly higher than with the homologous antisera. B. A thin section of tissue from a citrus variegated chlorosis-affected citrus tree incubated on antiserum to plum leaf scald (PLS) (X21,000).

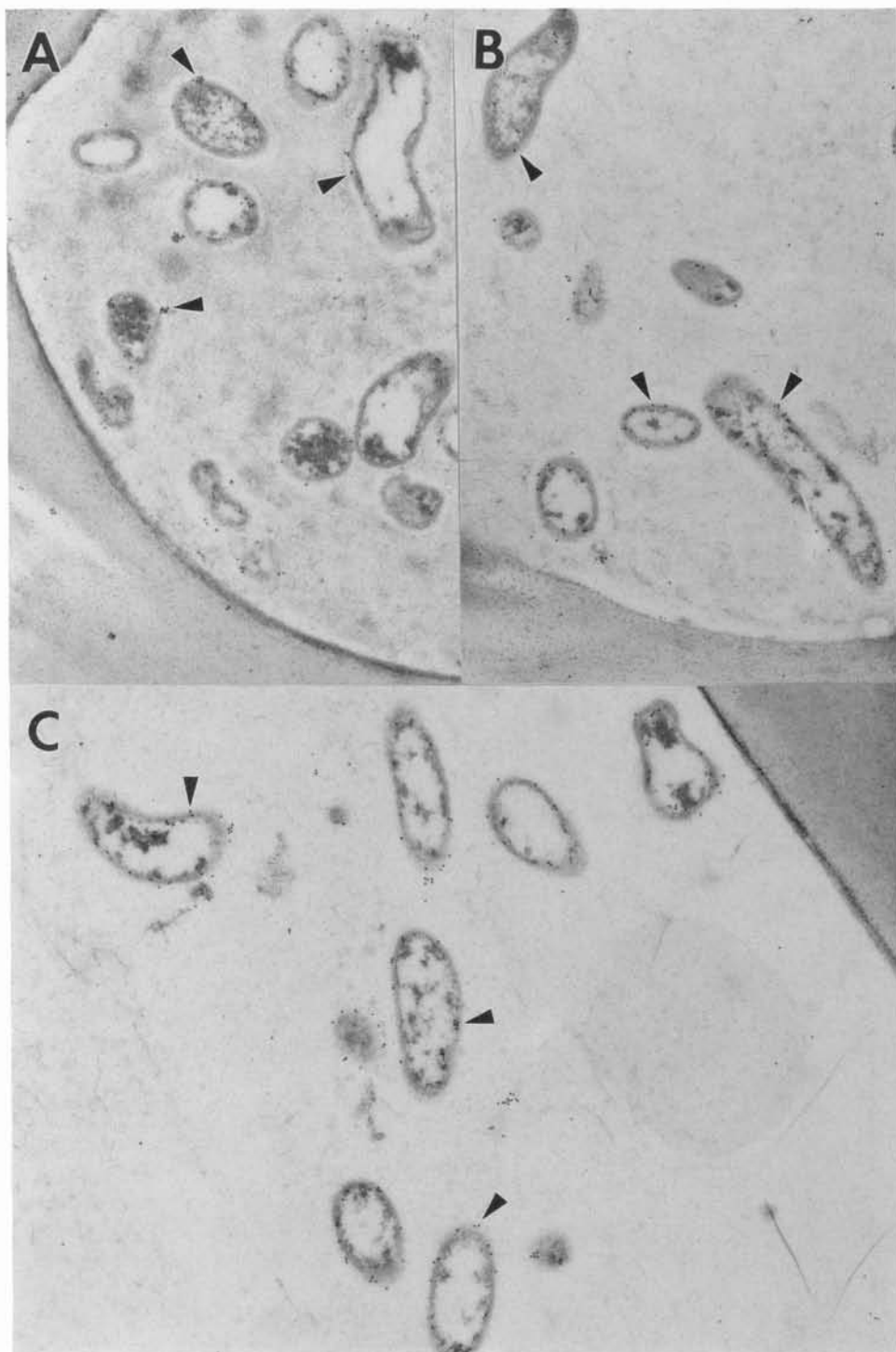


Fig. 4. Thin sections from citrus variegated chlorosis (CVC)-affected citrus which was incubated on antisera to: A. ragweed (RW) (X18,600). B. alfalfa dwarf (AD) (X21,000). And C. periwinkle wilt (PW) (X21,000). Arrows indicate typical gold particles and a low density of labelling is evident.



Fig. 5. A. Thin section from citrus variegated chlorosis (CVC)-affected citrus which was incubated on antiserum to elm leaf scorch (ELS) (X24,000). The antiserum only reacted to the fibrillar matrix which surrounded bacteria in the vessel lumina. B. Thin section of tissue from a CVC-affected citrus tree incubated on antiserum to *X. campestris* pv. *citrumelo*, no labelling was observed (X16,275).

and binding of the primary antibodies to the target protein. L. R. White, a hydrophilic embedding resin, allowed for excellent localization of bacteria compared to Spurr's epoxy resin. Spurr's epoxy resin did not allow good penetration of primary antibodies even when etching was performed with a saturated solution of sodium metaperiodate for 20 min.

Immunolabelling. All antisera to *X. fastidiosa* strains reacted with bacteria in ultrathin sections of affected citrus from Argentina and Brazil. The best localization in sections from tissues with either disease was obtained with homologous antisera prepared to CVC, CREC-22 and CREC-26 (Fig. 2A, B). Two other antisera PLS and PD-LH2 also provided good localization, although both antisera gave a small amount of non-specific labelling (Fig. 3A, B). A lower density of labelling was observed with antisera to RW, PW and AD (Fig. 4A, B, C). Antiserum to ELS did not directly attach to bacteria as did the other antisera tested, although it did label the fibrillar matrix which surrounds the bacteria in the vessel lumina (Fig. 5A). Antisera to *X. campestris* pv. *citrumelo* used as a control did not attach to bacteria in thin sections from diseased tissues (Fig. 5B). The GAR gold-IgG control gave a similar negative labelling pattern.

DISCUSSION

Localization results indicate that ultrathin sections of citrus affected

with pecosita from Argentina and CVC from Brazil contain bacteria that are morphologically similar and cross react with antisera prepared against various strains of *X. fastidiosa*. Fixation procedures utilizing glutaraldehyde in either phosphate or cacodylate buffer, gave similar results. Post-fixation with osmium tetroxide, however appears to block or inhibit antibody recognition of tissue antigens and should be avoided. Antibodies easily penetrated L. R. White embedding medium and it was preferred over Spurr's resin even when Spurr's was etched with saturated sodium metaperiodate.

The best immunogold localization occurred using antisera produced to cultures of bacteria isolated from affected citrus from Brazil (CREC-22 and CREC-26). The high labelling densities of both CVC and pecosita bacteria with antisera prepared against CVC bacteria suggests these bacteria may be closely related. Antisera prepared against other isolates of *X. fastidiosa* also reacted with the bacteria associated with CVC and pecosita, but the lower labelling density suggests a more distant relationship with these isolates.

Immunogold labelling proved to be a useful tool for examining possible serological relationships of various *X. fastidiosa* strains, provided that good antisera is available. This technique also proved useful in detecting antibody reactions other than those with whole bacterial cells, such as that seen with the antiserum to ELS that labelled primarily the fibrillar matrix.

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