The Greening Organism is a Gram Negative Bacterium

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ABSTRACT. One of four periwinkle plants connected by dodder to a Madame Vinous sweet orange seedling infected with Indian greening developed yellowing symptoms within 6 months. Electron microscopy of leaf midribs of chlorotic leaves revealed the bacteria-like organisms in the sieve tubes morphologically and ultra structurally identical to those found in greening-affected citrus, and indicated that the greening organism (GO) was transmitted from affected citrus to periwinkle by dodder. The GO was graft-transmitted from periwinkle to periwinkle and GO multiplied more actively in periwinkle than in citrus.

The cytochemical treatments previously developed to visualize the PG layer of *E. coli* were applied to the GO. Papain digestion revealed an additional layer between the outer and the inner membrane of the GO which disappeared after lysozyme digestion and is therefore thought to be PG.

The presence of PG in the envelope of the GO is direct proof for the bacterial nature of the GO and since the structure of the GO envelope was found to be similar to that of *E. coli*, but quite different from that of *Staphyloccocus aureus*, the GO is very probably a Gram-negative bacterium.

Index word. Cuscuta.

The procaryote associated with greening disease of citrus was discovered in 1970 (11). Since then, all our attempts to grow the greening organism (GO) have failed even with recent media used to grow fastidious spiroplasmas (10, 17) xylem-limited bacteria (5) or legionellas (6). Hence, over the last ten years characterization of the greening organism (GO) had to be done on the organisms *in situ* within the sieve tubes of affected citrus plants. We showed that the envelope surrounding the organism comprised three zones: a dark inner zone, a dark outer zone and an intermediate electron-transparent zone. The thickness of the three zones was approximately 250Å (2, 7, 15). Each of the two dark zones could be resolved into a triple-layered unit membrane 90-100 Å thick (8). The inner membrane appeared as the cytoplasmic membrane; the outer membrane as a cell wall. No peptidoglycan (PG) layer could be demonstrated between the inner and outer membranes of the GO (13, 16). However, indirect indications for the occurence of PG in the GO have been obtained from the beneficial effect of penicillin applied to the roots of sweet orange seedlings in the greenhouse (4) or injected into the trunk of orchard trees (1). Because of the thickness of its envelope and the effect of penicillin, the GO was thought to be a bacterial and not a mycoplasmal organism.

In 1967, De Petris (14) studied the envelope of *E. coli* using digestion of the cells with papain. After such a treatment, he was able to show that the envelope of *Escherichia coli* was composed of three different zones: an inner triple-layered cytoplasmic membrane, a dark intermediate layer and an outer triple-layered membrane. He further demonstrated that the intermediate layer, present only after papain digestion, was composed of peptidoglycan (PG) and could therefore be hydrolysed by lyzozyme. To further study the structure of the GO's cell wall, the same experiments could be envisaged. However citrus material, on the basis of the low number of GO's present in the sieve tubes was not suitable for cytochemical

studies. In 1977, *et al.* (9) reported that the GO was able to multiply to quite high titers in dodder. We have therefore used dodder to transmit the GO to periwinkle plants. The organism multiplies more actively in periwinkles than in citrus and keeps its characteristic morphology. We have therefore used greening infected periwinkle to study the cell wall constitution of the GO.

MATERIALS AND METHODS

Description of plant material. Citrus and periwinkles were grown in a glasshouse at about 25C during the day (16 hours) and 20C at night.

Indian greening: In 1970, a Madame Vinous sweet orange seedling was graft inoculated with two leaf patches taken on a Mosambi sweet orange seedling that had been experimentally infected with Indian greening (Poona strain) by the Asian vector of greening, *Diaphorina citri* (Kuwait), as described previously (3).

The Madame Vinous sweet orange seedlings inoculated in 1970 with Asian greening was used for the dodder transmission experiment described here. It showed typical symptoms of greening and the GO was present in the sieve tubes. It was free of other known virus and virus-like diseases of citrus since such agents are not transmitted through seed and since the psyllid vector *D. citri* only transmits greening.

Bacterial strains. *E. coli* and *Staphylococcus aureus* were grown in LPG medium (yeast extract 5 g/liter, peptone 5 g/liter, and glucose 10 g/liter) at 37 C.

Dodder transmission procedure. Dodder (*cuscuta spp.*) seeds were germinated on moist cotton wool and transferred onto an healthy periwinkle plant. After about 15 days, when the shoots were approximately 10 cm long, connections were established by attaching the dodder strands to the Madame Vinous sweet orange seedling carrying the Indian strain of greening. After the dodder had formed haustoria within the citrus plant, the strands between it and the periwinkle were cut, and the latter was kept for observation. The dodder continued to develop on the citrus seedling for 1 week after which newly developed dodder strands were placed on four surrounding, 4-week-old healthy periwinkles. These connections were maintained for three weeks. The periwinkle plants were then freed of dodder strands and kept in the greenhouse at approximately 25C. Subsequent dodder strands growing from remaining haustoria were removed to prevent weakening of the periwinkle by dodder.

Graft inoculation of periwinkles. Healthy 6-week-old periwinkles were top-grafted with small yellowing shoots from infected periwinkles. After grafting, the periwinkles were kept for 10 days in a moist chamber and thereafter in the greenhouse.

Papain treatment. The papain solution contained 0.5 mg of enzyme per ml of 0.18 M phosphate buffer pH 7.5 containing 0.02 M EDTA and 0.01 M cysteine.

Treatment of bacterial cultures: Mid log phase cultures of *E. coli* or *S. aureus* used respectively as Gram negative and Gram positive models were centrifuged for 15 minutes at 10,000xg. The pellet was resuspended in the papain solution and incubated for 20 hours at 32 C; the suspension was centrifuged again and fixed for electron microscopy as described below.

Treatment of greening affected periwinkles: The midribs of affected leaves were cut in one mm long fragments and put in the papain solution. A vacuum infiltration of the enzyme solution was carried out for 10 minutes. The

midrib fragments were then transferred to a fresh solution of papain and incubated for 48 hours at 32 C.

Lyzozyme treatment. The lyzozyme solution contained 1 mg of enzyme per ml of 0.1 M Tris pH 8. Sections of glycol methacrylate (GMA) embedded *E. coli* cultures or GO infected periwinkle midribs were floated on 10% hydrogen peroxide for 15 minutes at room temperature, washed with distilled water, and incubated on the lyzozyme solution for 15 minutes at 32 C. The sections were washed with distilled water and stained with a 2.5% aqueous solution of uranyl acetate for 5 minutes followed by lead citrate for 5 minutes.

Electron microscopy. Conventional fixation: The electron microscopy techniques that were used have been described previously (8). Briefly, 1-mm long pieces of leaf midribs were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer pH 7.5 for 6 hours, post-fixed with 1% osmium tetroxide in the same buffer; samples were dehydrated in alcohol and embedded in Epon 812. Sections, made with an LKB ultrotome III ultramicrotome, were stained with lead citrate and observed in a Siemens Elmiskop 101 electron microscope.

Fixation after papain digestion: The fixation was as above except that the samples were transferred from the osmium post-fixation to 1% uranyl acetate for 12 hours and the sections were stained with 2% uranyl acetate, followed by lead citrate. When lyzozyme treatment was done, the samples were embedded in glycol methacylate GMA instead of Epon 812 as described by Leduc and Bernhard (12).

RESULTS

Transmission of the Indian strain of GO by dodder. One of four periwinkle plants attached to an Indian greening-affected Madame Vinous sweet orange seedling showed peculiar yellowing symptoms after three months that we had never observed before. The initial periwinkle on which dodder was first allowed to grow and which was kept for observation failed to develop any symptoms during the year after removal of the dodder strands.

Electron microscopy. Thin sections of leaf midribs from the initial periwinkle as well as from the four periwinkles used in the transmission experiment were examined by electron microscopy. Procaryotic microorganisms were present in the sieve tubes of the periwinkle plant with symptoms (fig. 1A) but not in any of the three plants without symptoms that had been attached to citrus (fig. 1B). Neither were microorganisms observed in periwinkle on which the dodder was originally grown. At higher magnification, the 250 Å-thick envelope characteristic of the GO was clearly visible (fig. 1C). As in citrus the organisms in the infected periwinkle also possess an outer and inner membrane (fig. 1D).

Graft transmission and development of symptoms. After three months at 25C, symptoms developed on the periwinkles that were topgrafted with yellowing shoots of the initial periwinkle infected by dodder. The symptoms appeared on the shoots located immediately below the graft insertion. The first symptom was yellowing around the secondary veins (fig. 2A, leaf on left). This localized yellowing subsequently spread along the margins of the leaf (fig. 2A, leaf at right) so that eventually the whole leaf became yellow. Other leaves of the same shoot also gradually developed symptoms until the whole shoot was affected. Eventually additional shoots became yellow. Within 8 to 60 days after onset of initial symptoms,

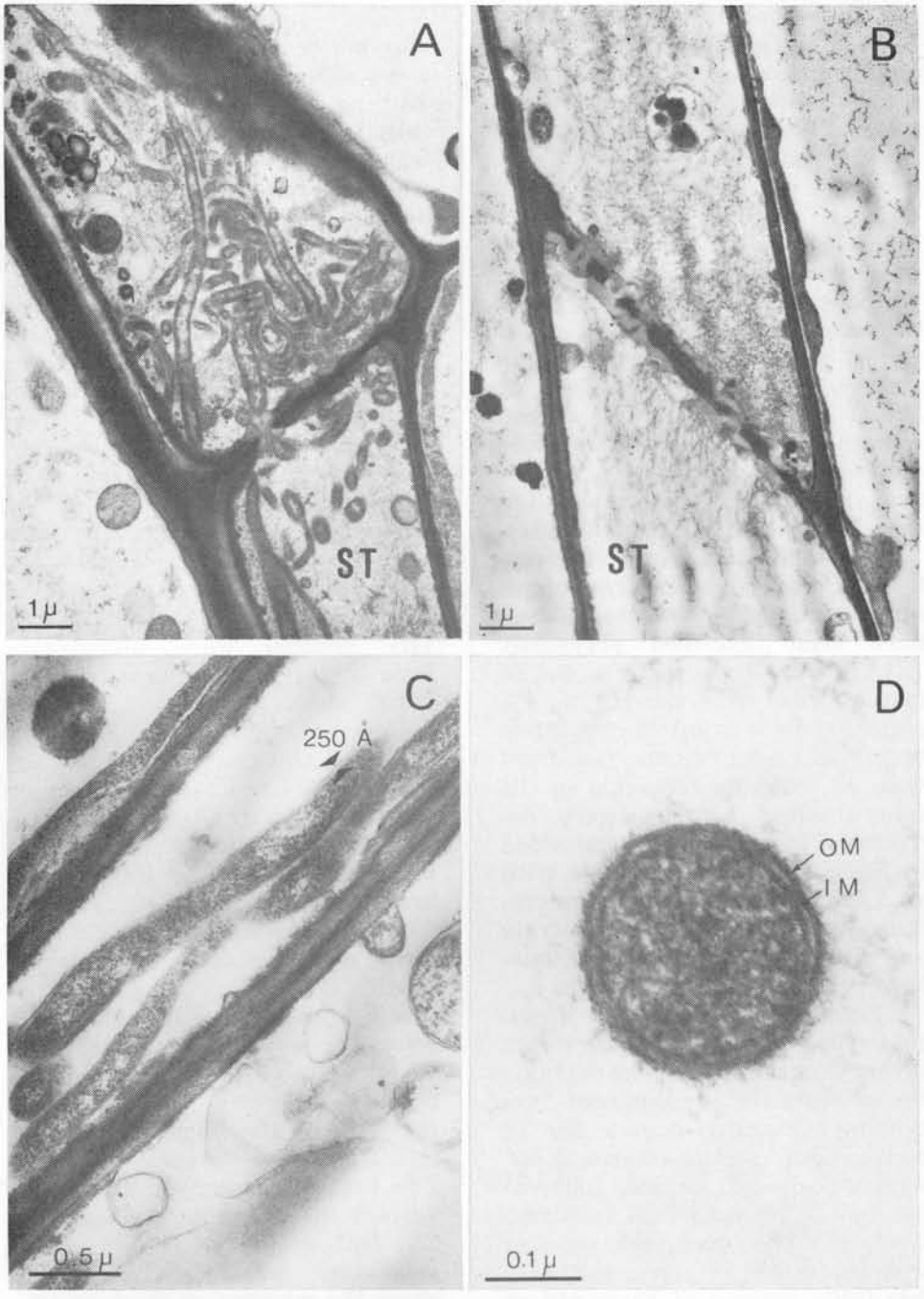

Fig. 1. Electron micrographs of ultrathin section through sieve tubes of leaf midribs from: A) Greening affected periwinkle showing elongated forms of greening organism (GO), ST = sieve tube; B) Uninfected periwinkle, ST = sieve tube; C) Greening affected periwinkle with GO showing the typical 250 Å thick envelope; D) Greening affected periwinkle showing the triple layered inner (IM) and outer (OM) membranes of the organism.

Fig. 2. A) Greening affected periwinkle showing earlier localized yellowing symptoms (LY) around the secondary veins (leaf on left) and more generalized yellowing (GY) (leaf on right); B) Ultrathin section from a greening-affected periwinkle leaf showing one sieve tube highly packed with GO (arrow).

some plants were completely yellowed; in other plants, only certain branches became yellow. Based on electron microscopic observations, high concentrations of the GO were present in leaves with symptoms but not in unaffected leaves, even when other parts of the plant were affected. Density of GO was often high on one side of a sieve plate and low on the other. Some sieve elements of affected

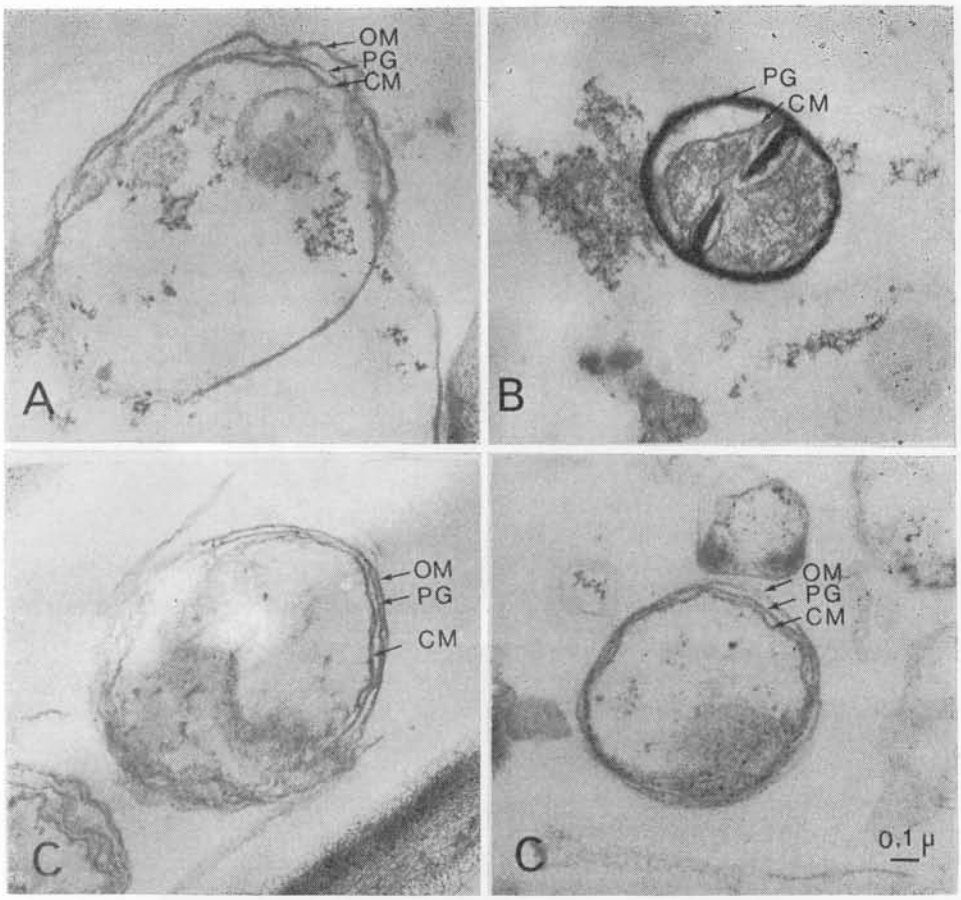

Fig. 3. Papain digestion of A) Escherichia coli; B) Staphylococcus aureus; C) Greening organisms in the sieve tubes of an affected periwinkle. PG = peptidoglycane; OM = outer membrane; CM = cytoplasmic membrane.

periwinkles were packed with organisms (fig. 2B) while others were not.

The time required to observe symptoms on plants after grafting could be reduced to two months when periwinkle plants were grown at 32C. Infected plants became stunted and flowers were generally smaller than those of healthy plants. Flowers showed no symptoms of virescence. The GO could be graft-transmitted to healthy periwinkle plants by bark inoculum from infected periwinkle plants, but top-grafting with infected shoots was the preferred technique for successful transmission.

Structure of the envelope of the GO after papain digestion. As shown by De Petris (14), when an *E. coli* culture is treated with papain, a PG layer located between the outer and the inner membranes could be observed (fig. 3A). On the contrary when *S. aureus* (Gram positive bacterium) is treated in the same way, only two layers are observed: a cytoplasmic membrane

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Fig. 4. Papain plus lyzozyme digestion of sections of GO infected periwinkle midribs; A) Papain treated section showing a GO with a PG layer.

Fig. 4. Papain plus lyzozyme digestion of sections of GO infected periwinkle midribs; B) Lyzozyme treatment of the same organism: the PG layer has been hydrolyzed.