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AETIOLOGY OF CITRUS GREENING DISEASE

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SUMMARY.

Greening disease of citrus is characterized by the presence of procaryotic organisms in the sieve tubes of infected plants. These procaryotes have often been called mycoplasma-like. We have previously shown that the envelope of the organism was composed of two membranes, each with a triple-layered structure: an inner membrane (cytoplasmic membrane) and an outer membrane. Penicillin treatment of greening-affected plants results in remission of symptoms, suggesting the presence of a peptidoglycan (PG) layer in the envelope of the organism. However, when observed by conventional electron microscopy, no PG layer could be detected in the envelope of the greening organism (GO). Recently, we were able to transmit the GO from citrus to periwinkles by dodder. In periwinkles, GO multiply to high titres and, therefore, characterization studies can be carried out directly on the organisms *in situ*. Using papain treatment of GO in greening-infected periwinkles, we were able to visualize a PG-like layer in the envelope of the GO. This layer was removed by lysozyme treatment. In these respects, the structure of the GO envelope was nearly identical to that of *E. coli*, a Gram-negative bacterium, but was different from that of *Staphylococcus aureus* (a Gram-positive bacterium) treated in the same way.

From the presence of a membranous PG-containing cell wall, the GO appears to be a true bacterium of the Gram-negative type, and not a mycoplasma.

KEY-WORDS: Citrus greening disease, Peptidoglycan; Cytology, Periwinkle, Aetiology.

INTRODUCTION.

The procaryote associated with greening disease of citrus was discovered in 1970 [12]. Since then, all our attempts to grow the greening organism (GO) have been negative, even with recent media used to grow fastidious spiroplasmas [11, 18], xylem-limited bacteria [4] or legionellas [5]. Hence, over the last ten years, characterization of the 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 250 Å [2, 6, 16]. Each of the two dark zones could be resolved into a triple-layered unit membrane 90-100 Å thick [7]. The inner membrane appeared as the cytoplasmic membrane and the outer membrane as a cell wall. No peptidoglycan (PG) layer

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could be demonstrated between the inner and outer membranes of the GO [14, 17]. However, indirect indications for the occurrence of PG in the GO have been obtained from the beneficial effect of penicillin applied to the roots of sweet orange seedling in the greenhouse [3] 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 [15] studied the envelope of *Escherichia coli* using digestion of the cells with papain. After such a treatment, he was able to show that the envelope of *E. 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, only present after papain digestion, was composed of PG and could therefore be hydrolysed by lysozyme. To further study the structure of the GO cell wall, the same experiments were envisaged. However, citrus material, on the basis of the low number of GO present in the sieve tubes, was not suitable for cytochemical studies. In 1977, Ghosh *et al.* [10] reported that the GO was able to multiply to quite high titres in dodder. We therefore used dodder to transmit the GO to periwinkle plants [8, 9]. The organism multiplied more actively in periwinkles than in citrus and kept its characteristic morphology [8, 9]. We therefore used greening-infected periwinkle to study the cell wall constitution of the GO by papain and lysozyme treatments. Part of this work was recently presented at the International Conference of Citrus Virologists [9].

Materials and methods.

Description of plant material. — Transmission procedure by dodder and graft inoculation of periwinkles have been outlined earlier [8, 9].

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

Papain treatment.

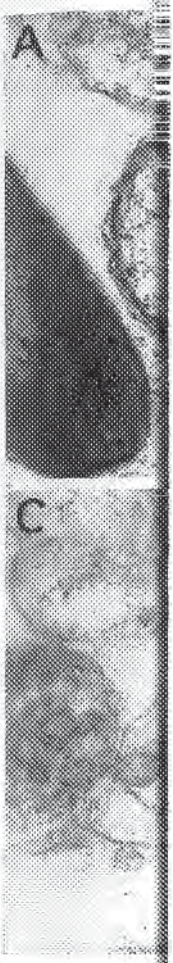
The papain solution contained 0.5 mg of enzyme per ml of 0.18 M phosphate buffer pH 7.5 containing 0.02 M EDTA and 0.01 M cysteine.

Treatment of bacterial cultures. — Midlog phase culture of *E. coli* or *S. aureus* used as Gram-negative and Gram-positive models, respectively, were centrifuged for 15 min at 10,000 *g*. The pellet was resuspended in the papain solution and incubated for 20 h 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 1-mm-long fragments and put in the papain solution. A vacuum infiltration of the enzyme solution was performed for 10 min. The midrib fragments were then transferred to a fresh solution of papain and incubated for 48 h at 32° C.

Lysozyme treatment. — The lysozyme 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 periwinkles midribs were floated on 10% hydrogen peroxide for 15 min at room temperature, washed in distilled water and incubated on the lysozyme solution for 15 min at 32° C. The sections were washed with distilled water and stained with a 2.5% aqueous solution of uranyl acetate for 5 min followed by lead citrate for 5 min.

GMA = glycol methacrylate.
GO = greening organism.
PG = peptidoglycan.



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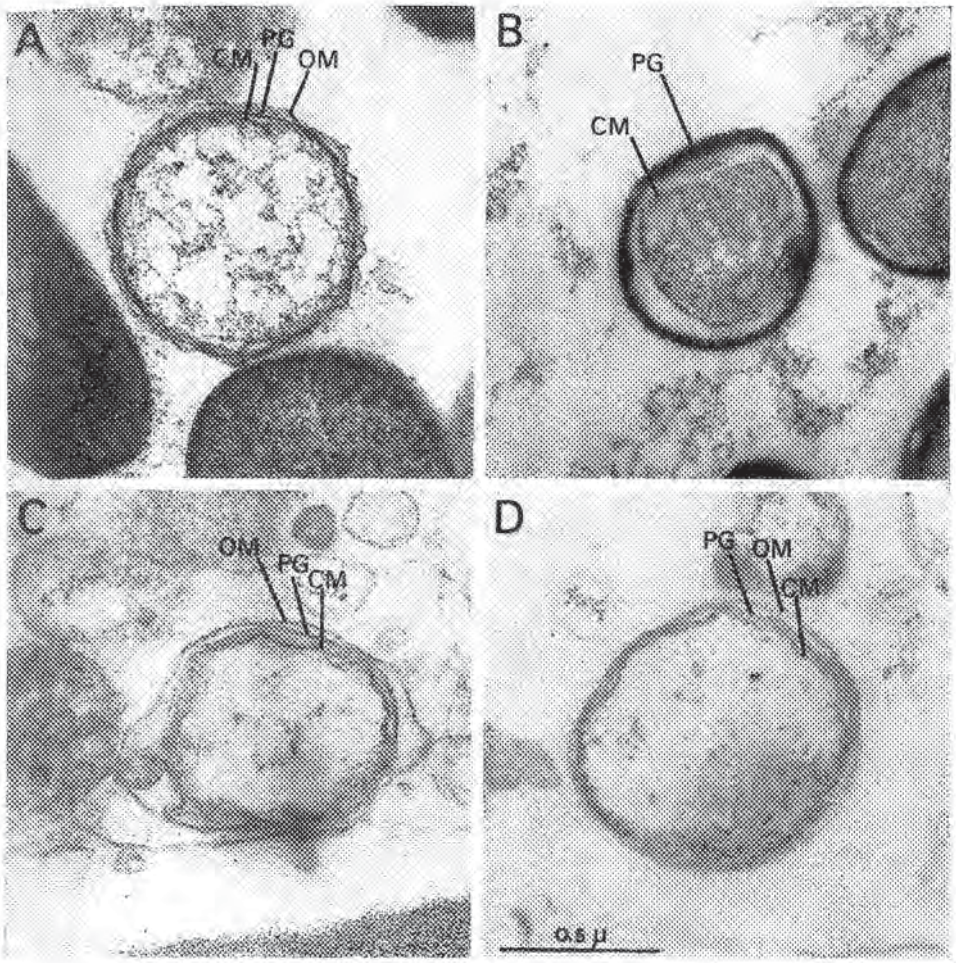

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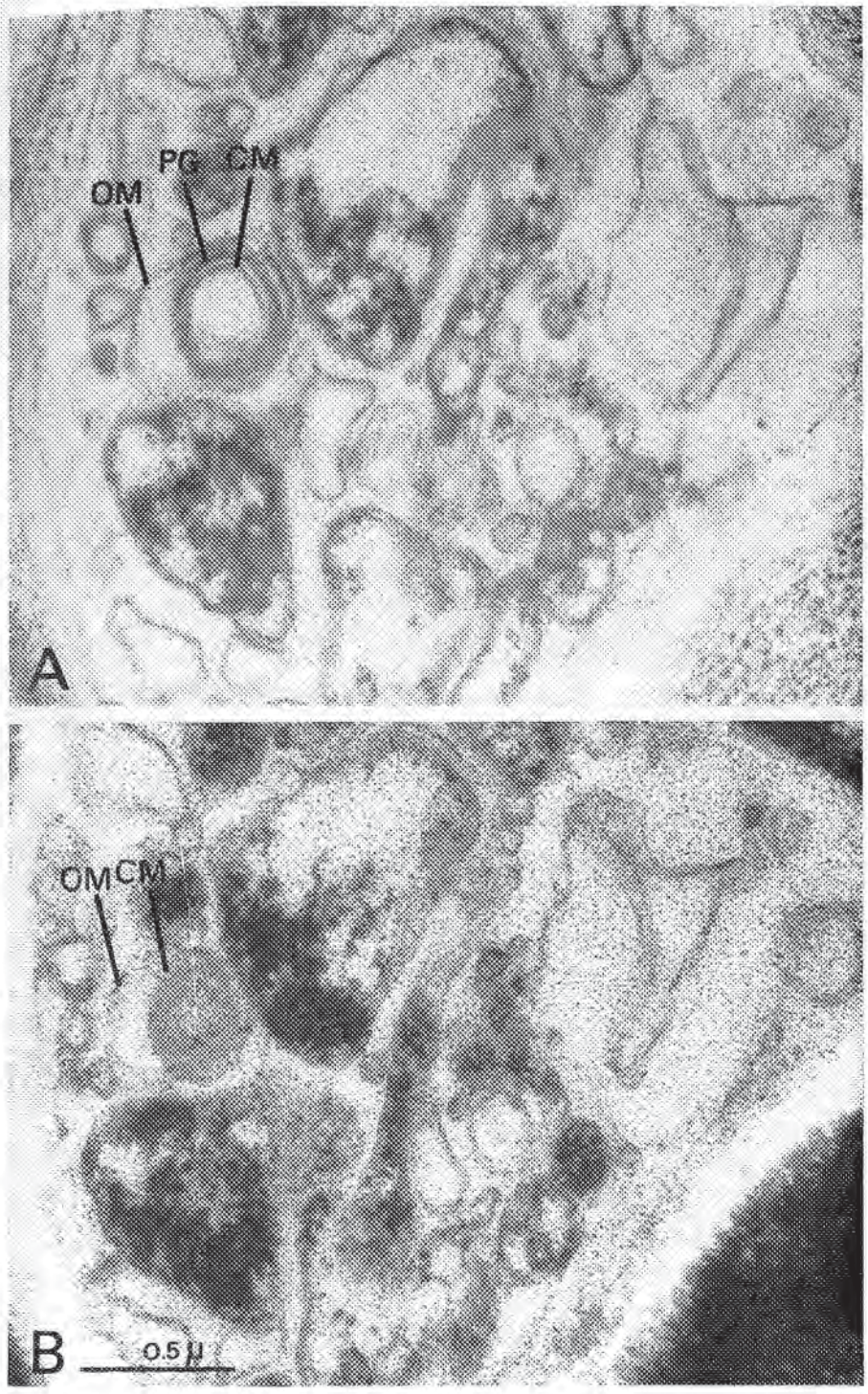

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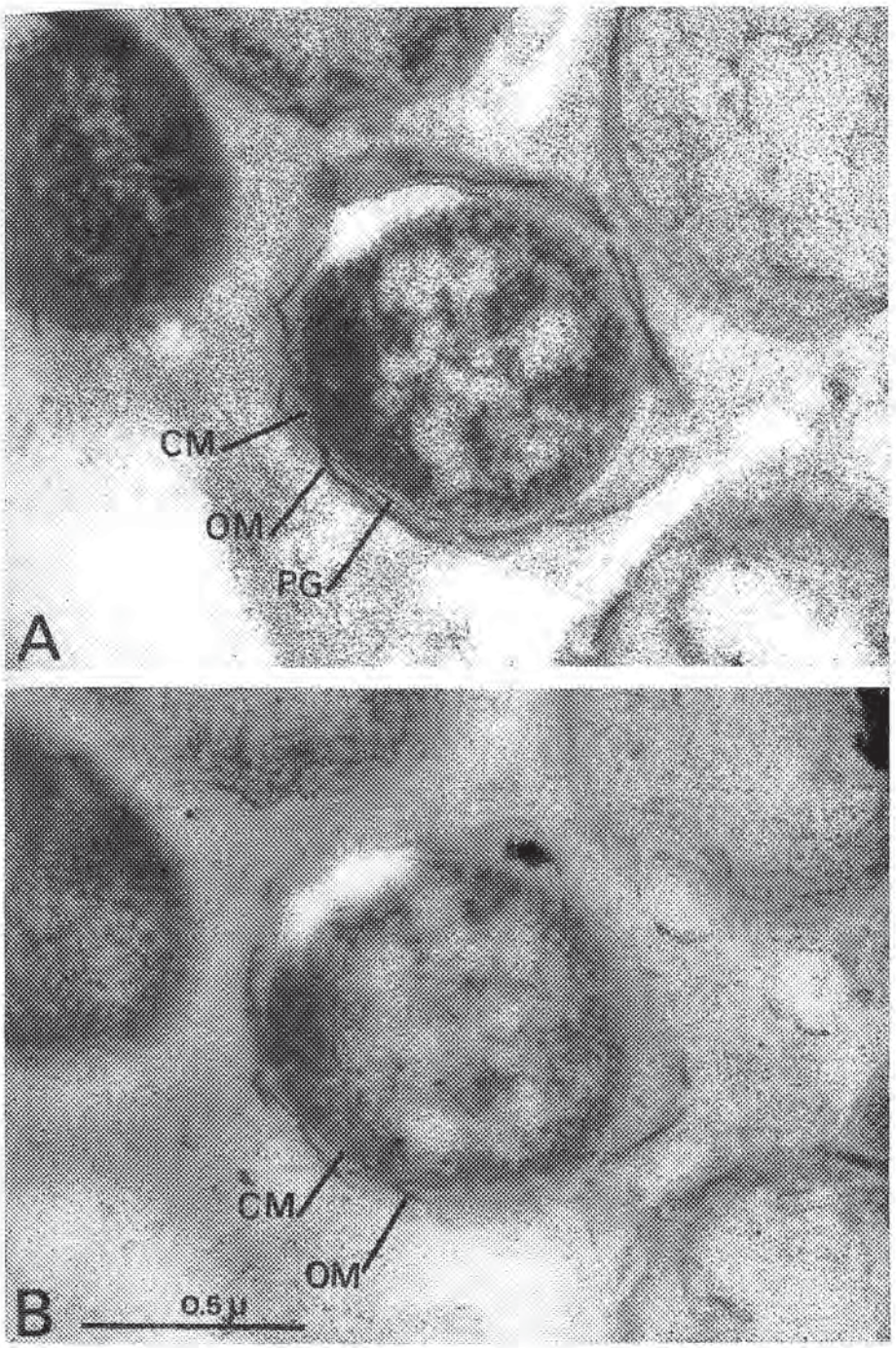

FIG. 3

Electron microscopy.

Conventional fixation. – The electron microscopy techniques which were used have been previously described [7]. Briefly, 1-mm-long pieces of leaf midribs were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer pH 7.5 for 6 h and 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 contrasted 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 h, and the sections were contrasted with 2% uranyl acetate, followed by lead citrate. When lysozyme treatment was to be done, the samples were embedded in glycol methacylate GMA instead of Epon 812, as described by Leduc and Bernhard [13].

Results.

Structure of the envelope of the GO after papain digestion. — As shown by De Petris, when an *E. coli* culture is treated with papain, a PG layer located between the outer and the inner membranes can be observed (fig. 1A). On the contrary, when *S. aureus* (Gram-positive bacterium) is treated in the same way, only two layers are observed: a cytoplasmic membrane and an outer thick cell wall containing PG (fig. 1B). When submitted to the same treatment, the envelope of the GO appears similar to the *E. coli* envelope, composed of three layers: the cytoplasmic membrane, the outer membrane and an intermediate dark layer ressembling the PG layer of *E. coli* (fig. 1C and D).

Lysozyme digestion of papain treated GO. — When one out of two serial sections of papain-digested leaf midribs is submitted to lysozyme digestion, the intermediate layer revealed after papain digestion (fig. 2A) disappeared (fig. 2B). The inner and outer membranes appeared fuzzy but were still visible. The same result was obtained when papain-treated *E. coli* (fig. 3A) were submitted to lysozyme digestion (fig. 3B).

Discussion.

In citrus as in periwinkle, the GO has several distinctive characteristics: it is located exclusively in the sieve tube and is surrounded by two triple-layered membranes, an inner (cytoplasmic) membrane and an outer membrane. The different forms of the GO (filamentous and round forms), already described in citrus [8], are also present in periwinkles.

The presence of two membranes in the envelope of the GO occurring in citrus and periwinkle distinguishes the organisms from mollicutes and lends credence to the presumed bacterial nature of the GO.

The availability of periwinkles highly infected with GO allowed us to develope cytochemical experiments in order to study the envelope of the organism. The envelope of the GO was shown to be different from the envelope of *S. aureus* (Gram-positive bacterium), but similar to the envelope of *E. coli* (Gram-negative bacterium).

On the basis of its membrane-like cell wall, including the presence of a PG layer, explaining its penicillin sensitivity, the GO appears to be a true bacterium of the Gram-negative type.

RÉSUMÉ

Étiologie de la maladie du « greening » des agrumes

La maladie du « greening » des agrumes se caractérise par la présence de micro-organismes procaryotes dans les tubes criblés du phloeme des plantes infectées. Ces procaryotes ont souvent été appelés « mycroplasma-like ». Nous avons montré précédemment que l'enveloppe de l'organisme était composée de deux membranes possédant chacune une structure trilamellaire : une membrane interne (membrane cytoplasmique) et une membrane externe. La rémission des symptômes obtenues après traitement des plants infectés par de la pénicilline suggère la présence de peptidoglycane (PG) dans l'enveloppe de l'organisme. Toutefois, lorsque l'organisme est observé en microscopie électronique, aucun feuillet de PG n'est visible.

Récemment, nous avons pu transmettre l'organisme du « greening » des plants de citrus à des plants de pervenches au moyen de la cuscute. Dans les pervenches, les organismes sont présents en plus grand nombre que dans les citrus et, de ce fait, les études de caractérisation peuvent être réalisées directement sur l'organisme *in situ*. En traitant par de la papaïne, des nervures de pervenches infectées par le « greening », nous avons pu visualiser un feuillet ressemblant à du PG dans l'enveloppe de l'organisme. Ce feuillet disparaît après traitement par du lysozyme.

La structure de l'enveloppe du « greening » après ces traitements est presque identique à celle de *Escherichia coli* (une bactérie à Gram négatif), mais différente de celle de *Staphylococcus aureus* (une bactérie à Gram positif).

Du fait de la présence d'une paroi membranaire contenant du PG, l'organisme du « greening » semble être une vraie bactérie de type Gram-négatif et non un mycoplasme.

Mots-clés : Greening des agrumes, Peptidoglycane ; Cytologie, Pervenche, Citrus, Étiologie.

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