

## Transmission of the Organism Associated with Citrus Greening Disease from Sweet Orange to Periwinkle by Dodder

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### ABSTRACT

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One of four periwinkle (*Vinca rosea*) plants connected by dodder (*Cuscuta campestris*) to a cultivar Madame Vinous sweet orange seedling infected with greening disease developed yellowing symptoms within 3 mo. Observations by electron microscopy of thin sections of leaf midribs of the plant with yellow symptoms revealed the presence of bacterially like organisms in the sieve tubes. These microorganisms were morphologically and

ultrastructurally indistinguishable from those found in tissues of greening-affected citrus. From these observations we conclude that the greening organism was transmitted from affected citrus to periwinkle by dodder. Transmission from periwinkle to periwinkle was subsequently achieved by graft inoculation.

*Additional key words:* bacteria, graminicute, peptidoglycan, procaryote.

The procaryote associated with greening disease of citrus was discovered in 1970 (12) shortly before the mycoplasma agent of citrus stubborn disease (13) now known as *Spiroplasma citri* (17). While at that time we and others (7,16) were able to culture the stubborn spiroplasma, all our attempts to grow the greening agent were negative. More recent media such as those developed for fastidious spiroplasmas (11,19), xylem limited bacteria (5) or *Legionella* (6) have not sustained the growth of the greening organism (GO). Hence over the last 10 yr, characterization of 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, electron-absorbing inner zone; a dark outer zone; and an intermediate, clear, electron-transparent zone. The thickness of the three zones was approximately 25 nm (250 Å). Such an envelope was far too thick to be a single unit membrane, and the supposed mycoplasma nature of the GO had to be questioned (4,16). The various geographical forms of greening (blotchy mottle, citrus decline, leaf mottling, yellow branch, and likubin) were all characterized by organisms with the same 25-nm (250 Å)-thick envelope system (2,9). Whereas the inner and outer dark, electron-dense zones were often parallel, they could also be clearly separated, suggesting that each was a single membrane (9). Each of the two electron-dense zones could be resolved into a triple-layered unit membrane, 9–10 nm (90–100 Å) thick (8), confirming an earlier report by Moll and Martin (14). The inner membrane appeared as the cytoplasmic membrane, the outer membrane as a cell wall. Elongated, electron-dense forms of the GO were most often the only structures present in the sieve tubes; they measured 0.15–0.25 μm in cross section. Occasionally round electron-clear structures, of 0.2–1.0 μm diameter, which seemed to be plasmolyzed were also observed. The round and elongated forms very probably represent different aspects of the same organism because they occasionally appeared to be connected (9). No peptidoglycan (PG) layer could rigorously be demonstrated between the inner and outer membranes of the GO (14,18). However, occasionally we noticed that at certain locations, the

inner layer of the outer membrane was somewhat thicker, more electron-dense than the other layers and reminiscent of the PG zone of certain Gram-negative bacteria (8). Since the GO was not available in culture, direct biochemical detection of PG could not be achieved. However, indirect indications for the occurrence of PG in the GO have been obtained from the effect of penicillin on greening-affected sweet orange plants. Penicillin solutions applied to roots of greenhouse grown seedlings (2) or injected into the trunk of orchard trees (1) resulted in a beneficial effect in that the treated plants produced more roots and larger symptomless shoots and leaves than untreated controls. For all these reasons, the GO is thought to be a bacterial and not a mycoplasma organism. Thus, the term "Graminicute-like organism" was proposed (2) to describe the organism. Finally, citrus plants infected with the Indian strain of GO develop symptoms over a wide temperature range (24–35 C) but plants carrying the African strain develop symptoms only below 27 C (4). In citrus, the GOs are present only in small numbers, but in dodder (*Cuscuta campestris*) the organism is able to multiply to quite high titers (10). We now report the successful transmission of the GO from sweet orange seedlings to periwinkle (*Vinca rosea*) plants by dodder and its further transmission from periwinkle to periwinkle by grafting.

### MATERIALS AND METHODS

**Description of plant material.** Both citrus and periwinkle plants were grown in a glasshouse at about 25 C in the day (16 hr) and 20 C at night, unless otherwise indicated.

Indian (Asian) greening was established in a cultivar Madame Vinous sweet orange (*Citrus sinensis* (L.) Osbeck) seedling by graft inoculation in 1970 with two leaf patches taken from 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).

African greening was established in a cultivar Madame Vinous sweet orange seedling by graft inoculation in 1970 with leaf material from a cultivar Hamlin sweet orange seedling that earlier had been graft inoculated with leaf inoculum taken from a cultivar Eloff sweet orange seedling experimentally infected with African greening (Nelspruit strain) by the African vector of greening, *Trioza erythrae* (Del Guercio).

The two Madame Vinous sweet orange seedlings inoculated in 1970 with African and Asian greening, respectively, were used for

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the dodder transmission experiments to be described here. They both showed typical symptoms of greening, and GO were present in their sieve tubes. They were free of other known virus and viruslike diseases of citrus since such agents are not transmitted through the seeds and since the two psyllid vectors *D. citri* and *T. erytrae* only transmit greening.

**Transmission procedure.** Dodder seeds were germinated on moist cotton wool and transferred onto two healthy periwinkle plants. 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 seedlings carrying either the Indian or the African strains of greening. After the dodder had formed haustoria within the citrus plant, the strands between it and the two periwinkles were cut, and the latter were kept for observation. The dodder continued to develop on the citrus seedling for 1 wk after which newly developed dodder strands were placed on four surrounding, 4-wk-old healthy periwinkles. These connections were maintained for 3 wk. The periwinkle plants were then freed of dodder strands and kept in the greenhouse at approximately 25 C. Subsequent dodder strands growing from remaining haustoria were removed to prevent weakening of the periwinkle by dodder.

**Electron microscopy.** The electron microscopy techniques that were used have been described previously (9). Briefly, 1-mm-long pieces of leaf midribs were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.5) for 6 hr and postfixed with 1% osmium tetroxide in the same buffer; samples were serially dehydrated in alcohol and embedded in Epon 812. Sections, made with an LKB ultratome III ultramicrotome, were contrasted with lead citrate and observed in a Siemens Elmiskop 101 electron microscope. When the triple-layered structure of the membranes had to be visualized, the samples were transferred from the osmium postfixation to 1% uranyl acetate for 12 hr, and the sections were contrasted with 2% uranyl acetate, followed by lead citrate.

**Graft inoculation of periwinkles.** Healthy 6-wk-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.

**Effect of temperature on periwinkles infected with the Indian or African strains of GO.** Two types of experiments were done.

**First experiment.** Six healthy periwinkles were graft inoculated with the Indian strain of the GO and six other plants with the African strain. After 10 days in the moist chamber, three plants of each batch were transferred to a temperature-controlled greenhouse at 25 C, while the three others were placed in a greenhouse compartment kept at 32 C. Symptom development was observed for 6 mo.

**Second experiment.** Three out of six periwinkles infected with the Indian strain of the GO and already showing acute symptoms were kept at 25 C; the three remaining plants were placed at 32 C. The same treatment was done with six periwinkles grafted with, and showing symptoms of, the African strain of the GO.

## RESULTS

**Indian greening. Transmission of the Indian strain of GO by dodder.** Three months after one of the four periwinkle plants attached to the Indian greening-affected Madame Vinous sweet orange seedling by dodder, it showed peculiar yellowing symptoms that we had never observed before. The initial periwinkles on which dodder was first allowed to grow and which were 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 25-nm (250 Å)-thick envelope characteristic of the GO was

clearly visible (Fig. 1C). In citrus, the envelope of the GO consists of an outer and an inner membrane, both of which are triple-layered structures (Fig. 1D). Similarly, the organisms in the infected periwinkle also possess an outer and inner membrane (Fig. 1E). In addition, between these two membranes an electron-dense layer similar to the peptidoglycan layer of certain Gram-negative bacteria was seen occasionally (Fig. 1E). Additional evidence for the presence of a peptidoglycan layer has been obtained by treating GO-infected periwinkle tissues with papain and lysozyme as described previously for *Escherichia coli* (15). This treatment showed that the envelope of the GO possesses an electron-dense layer between the inner and outer membrane. The membrane structure of GO was indistinguishable from that of *E. coli* used as a Gram-negative control but clearly differed from that of a Gram-positive organism (*Staphylococcus aureus*) examined at the same time (*unpublished*). Such a treatment has not been applied to GO infected citrus essentially because of the low number of GO in citrus. In periwinkles (Fig. 2A and B) as in citrus (Fig. 2C and D) both the round and elongated forms of the GO could be seen. The outer and inner membranes of the round forms were often separated over a certain length (Fig. 2B), suggesting that plasmolysis had occurred.

**Graft transmission and development of symptoms.** After 3 mo at 25 C, symptoms developed on the periwinkles that were topgrafted with yellowing shoots of the initial periwinkle infected through dodder. The symptoms appeared on the shoots located immediately below the graft insertion. The first symptom was yellowing around the secondary veins (Fig. 3A, leaf on left). This localized yellowing subsequently spread along the margins of the leaf (Fig. 3A, 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 8–60 days after onset of initial symptoms, some plants were completely yellowed; in other plants, only certain branches yellowed. 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 periwinkles were packed with organisms (Fig. 3B) while others were not.

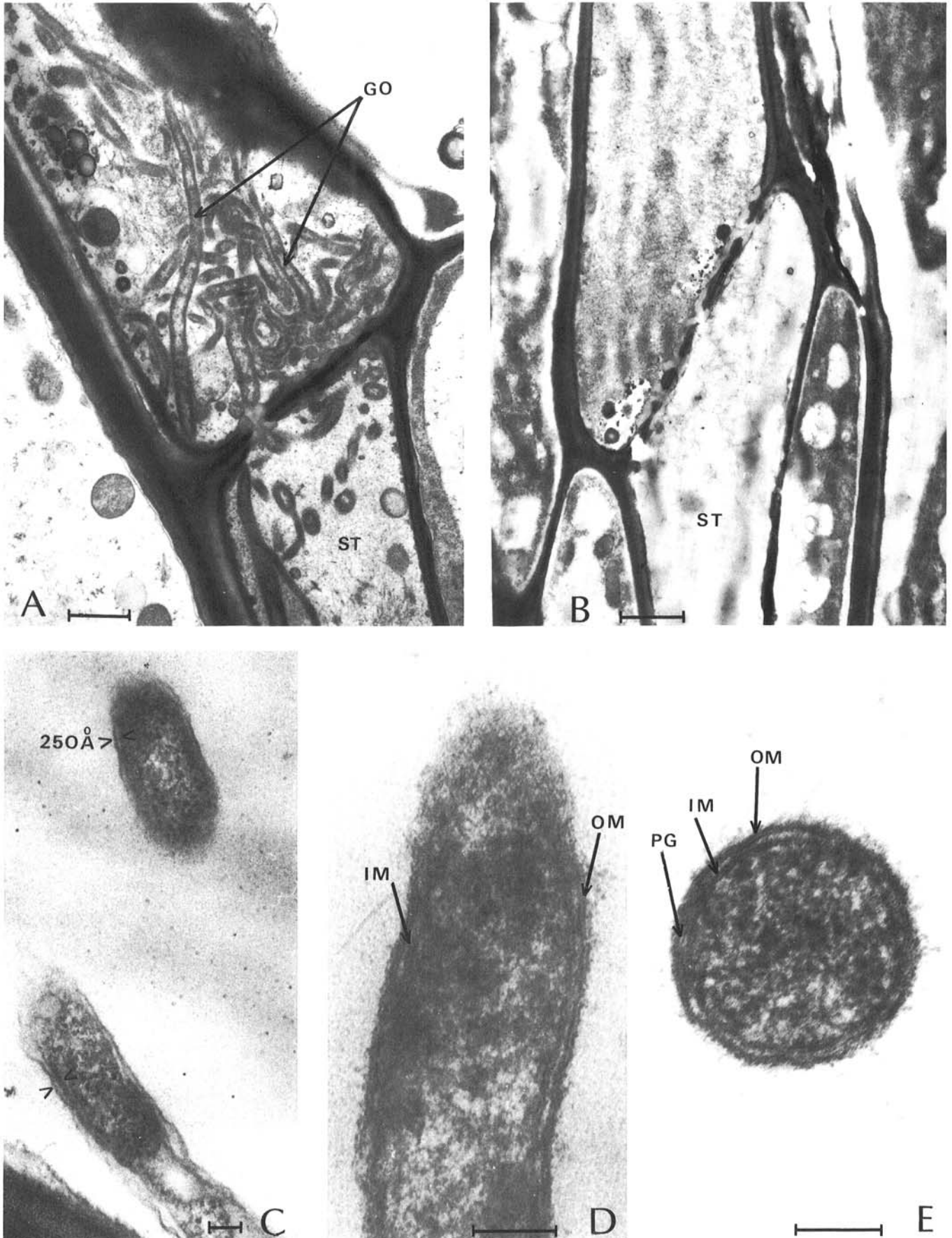
The time required to observe symptoms on plants after grafting could be reduced to 2 mo when periwinkle plants were grown at 32 C. Infected plants became stunted and flowers were generally smaller than those of healthy plants. Flowers showed no symptoms of virescence. The GO could be graft-transmitted to healthy periwinkle plants by bark inoculum from infected periwinkle plants, but top grafting with infected shoots was the preferred technique for successful transmission.

**African greening.** Similar results were obtained when the Madame Vinous sweet orange seedling infected with African greening was used for transmission to dodder. One of four periwinkles that had been attached to citrus developed yellowing symptoms. However, the periwinkles that became infected either directly by dodder transmission or by subsequent graft transmission had to be kept below 27 C for symptom expression, as discussed below.

**Effect of temperature on symptom expression in periwinkles infected with the Indian or the African strain of GO. Experiment 1.** The three periwinkle plants infected with the African strain of the GO failed to develop symptoms when they were kept at 32 C for 6 mo while those placed at 25 C showed symptoms after 2–3 mo.

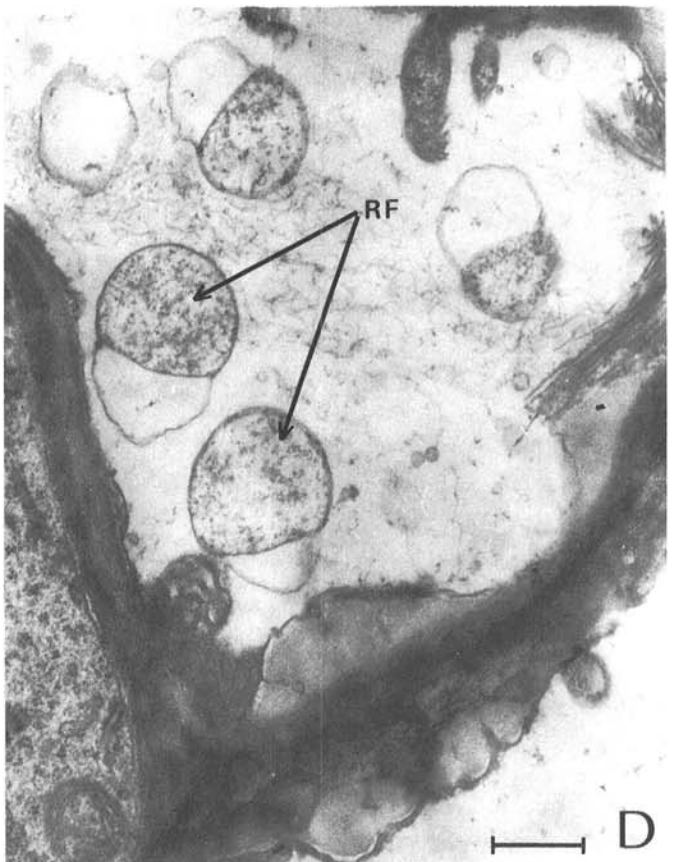
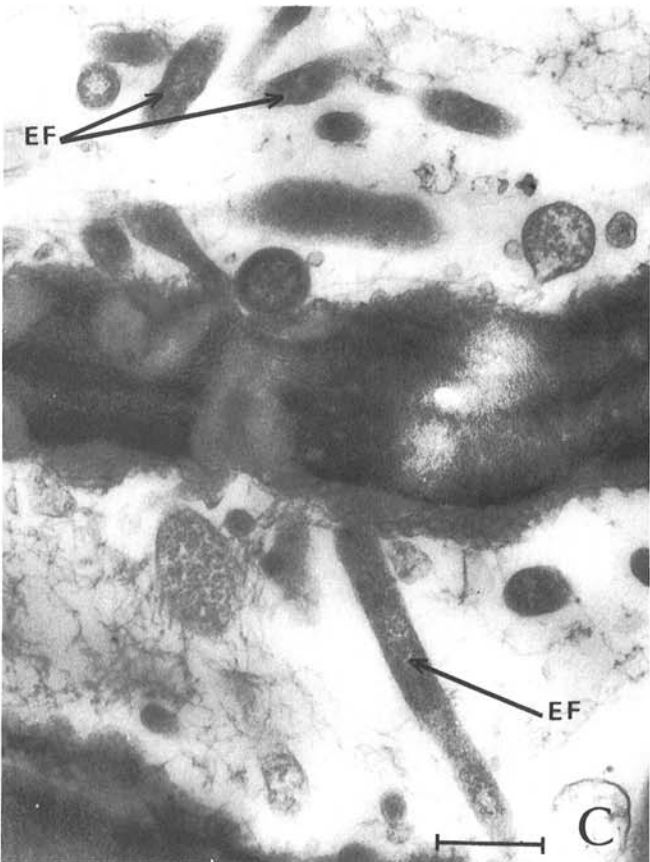
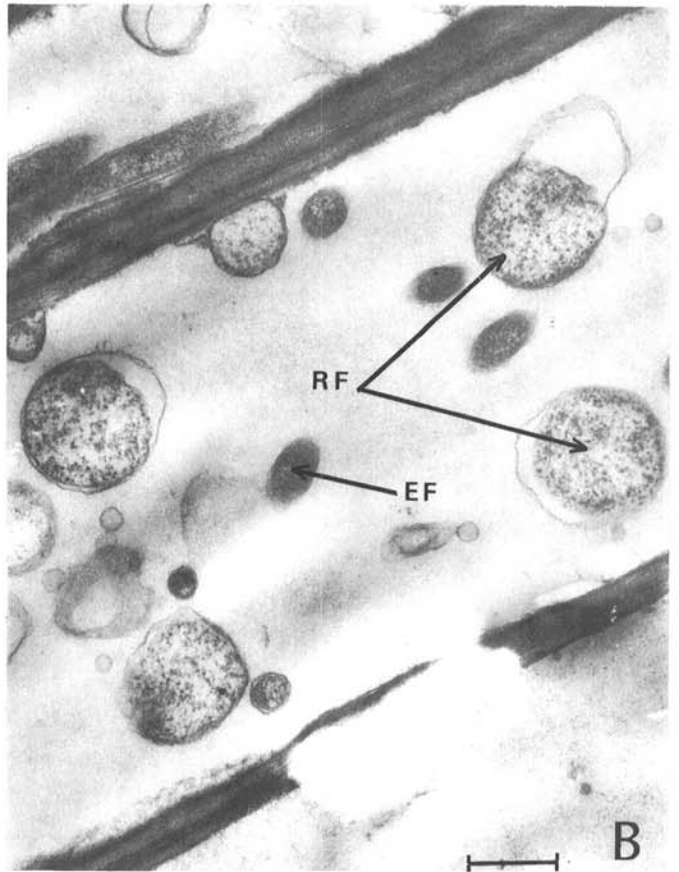
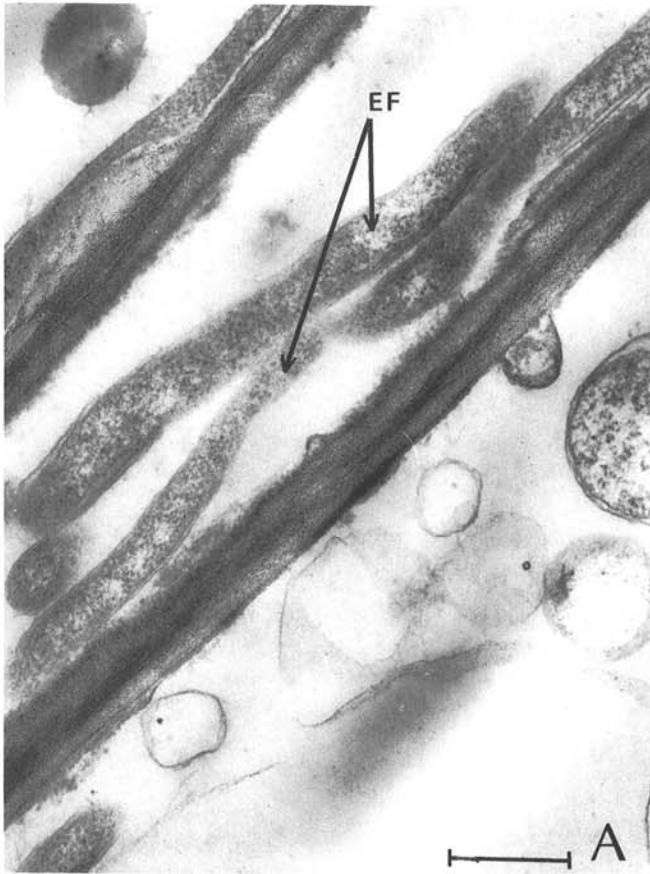
The three periwinkle plants infected with the Indian strain of the GO and kept at 32 C developed symptoms after 6 wk. Only two of those kept at 25 C developed symptoms after 3 mo.

**Experiment 2.** Infected periwinkles that had developed symptoms at 25 C were placed at 32 C. With the African strain of the GO, the yellowing symptoms disappeared progressively, the leaves becoming green again, and leaves on new shoots were devoid of symptoms. With the Indian strain of the GO, the leaf symptoms did not regress at 32 C; on the contrary, they became more severe and newly developing shoots were affected.



**Fig. 1.** Electron micrographs of an ultrathin section through sieve tubes of leaf midribs from: **A**, Greenling-affected periwinkle showing elongated forms of greenling organism (GO). ST = sieve tube, bar = 1  $\mu$ m. **B**, Uninfected periwinkle. ST = sieve tube, bar = 1  $\mu$ m. **C**, Greenling-affected periwinkle with GO showing the typical 25 nm (250  $\text{\AA}$ )-thick envelope. Bar = 0.1  $\mu$ m. **D**, Greenling-affected citrus with elongated form of GO showing the triple layered structure of both the inner (IM) and outer (OM) membranes. Bar = 0.1  $\mu$ m. **E**, Greenling-affected periwinkle with transverse section through an elongated form of GO showing a faint peptidoglycane like layer (PG) between the tripled layered inner (IM) and outer (OM) membranes. Bar = 0.1  $\mu$ m.





**Fig. 2.** Electron micrographs of an ultrathin section through leaf midribs of greening-affected periwinkles (**A and B**) and citrus (**C and D**). Bar = 0.5  $\mu$ m. **A and C**, Elongated forms (EF) of the greening organism (GO). **B and D**, Round forms (RF) showing a separation between inner and outer membranes. EF = cross section through elongated forms of GO.

## DISCUSSION

The bacteria-like organism in greening-affected citrus plants has several distinctive characteristics. It is located exclusively in sieve tubes, and is surrounded by two triple-layered membranes, an inner (cytoplasmic) membrane and an outer membrane that resembles a typical Gram-negative bacterial cell wall. Both elongated filaments and round forms are seen in the sieve tubes and appear to be related forms (9). The inner and outer membranes of some presumably plasmolyzed cells are clearly separated from each other. The symptomatic periwinkles receiving GO, respectively, from the Indian or African greening-affected Madame Vinous sweet orange seedlings, contained organisms, similar in all respects to those found in greening-affected citrus. Temperature experiments conducted with infected periwinkles gave the same results than those obtained previously (4) with greening-affected citrus: symptom expression occurs at both 25 and 32 C with the Indian strain of GO, but only at 25 C with the African strain. This suggests that the two strains of GO have not only been transmitted from citrus to periwinkle by dodder, but, in addition, they have conserved their heat sensitivity or tolerance during the transmission event. We have also presented evidence for the probable presence of a peptidoglycan layer between the two membranes of the GO in periwinkle; from experiments involving penicillin treatment, peptidoglycan has also been inferred to be present in GO in citrus (2).

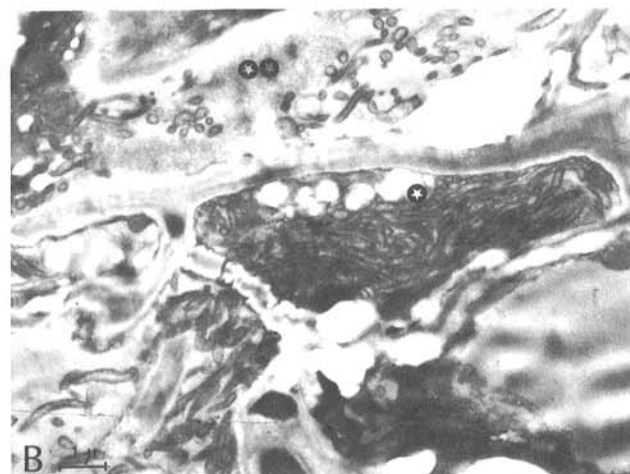
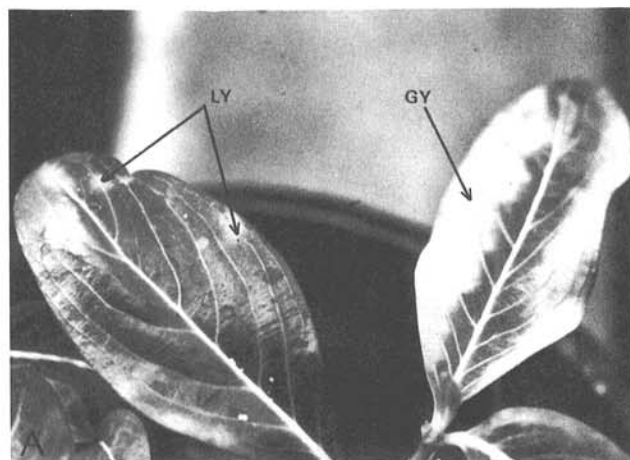
Hence, from the presence of a characteristic microorganism in the sieve tubes of only those periwinkles that were connected

through dodder to greening-affected citrus seedlings, the close ultrastructural similarity of the microorganisms in citrus and in periwinkle, the identical behavior of both infected citrus and periwinkles regarding symptom expression as influenced by temperature, we conclude that the microorganism present in the symptomatic periwinkles are GO and were transmitted from greening affected citrus to periwinkle by dodder.

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 GOs presumed bacterial nature. Even though the GO seems to move slowly from one sieve tube to the other, partially explaining why disease development in periwinkle is slow compared to that caused by *Spiroplasma citri*, the symptomatic periwinkles contained a large number of infected sieve tubes and numerous organisms in them. Therefore, they are potentially a more convenient material than citrus for further studies. However, culturing the organisms in the different media used previously with citrus material has been so far unsuccessful (*unpublished*).

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**Fig. 3. 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 the greening organism (GO, \*) and one with fewer GO (\*\*).

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