The Relationship of *Spiroplasma citri* and *Circulifer tenellus*

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ABSTRACT

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The causal agent of citrus stubborn disease, *S. citri*, multiplies in its natural leafhopper vector, *C. tenellus*. One day following injection, the total assayable colony-forming units (CFU) decreased by 0.5 $\log_{10}$ units and then gradually increased to a maximum of between 10^4 and 10^5 CFU per insect at 15 days after injection. No CFU were observed from insects injected with sterile liquid medium. *S. citri* also caused premature death of some *C. tenellus* whether acquisition was by injection, membrane feeding, or feeding on plants infected with *S. citri*. Cells of *S. citri* were first observed by electron microscopy in the salivary glands of *C. tenellus* 10 days after the insects were injected with *S. citri*. Large concentrations of *S. citri*, usually in irregular pockets, were observed adjacent to the acini of the salivary glands 15 days after injection. No bodies resembling *S. citri* were found in the salivary glands of healthy leafhoppers. Sequential electron microscopy studies of *S. citri* in *C. tenellus* following membrane feeding can be summarized as follows: *S. citri* enters the gut lumen after feeding, where a number of the cells of *S. citri* are digested or destroyed. Surviving cells pass through the gut wall into the epithelial cells where they may multiply. They continue to move toward and through the basement membrane of the intestine and into the hemocoel, where further multiplication occurs. *S. citri* is then transported by the hemolymph to the salivary glands, from which it can be injected into plants via salivary secretion during feeding.

The observed pattern of stubborn disease spread in the field suggested to some researchers that an insect was involved in the dispersal of the causal agent (1–3). Several reports of transmission by leafhoppers exist (6–8, 15–17). In 1973, Lee et al (10) cultured *S. citri* from beet leafhoppers, *C. tenellus*, collected from sweet orange seedlings and weeds at the University of California Moreno Farm where Calavan et al (1,2) had found a high incidence of stubborn disease in citrus seedlings. Oldfield et al (16) collected *C. tenellus* from several citrus orchards and non-citrus-growing areas in California and showed that the insects harbored *S. citri* and could transmit it directly to Madagascar periwinkle. They also demonstrated that *C. tenellus* could acquire *S. citri* from citrus naturally infected with *S. citri* and transmit it to periwinkle and citrus, thus showing that *C. tenellus* was a natural vector. Two other leafhoppers, *Scaphytopius nitridus* (DeLong) (6,8,15,17) and *S. acutus delongi* (7) have also been reported to be vectors of *S. citri*. There have been few studies, however, on the morphology, location, and movement of *S. citri* in its natural vectors. Russo et al (18) fed *S. nitridus* on 5% sucrose solutions containing *S. citri* and, 40 days after feeding, fixed, embedded, and sectioned individual *S. nitridus* for electron microscopic examination. They found *S. citri*

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in the intestine, salivary glands, and in either intact or degenerating somatic muscles.

Whitcomb et al (21) injected cultured *S. citri* into the aster yellows vector *Macrosteles fascifrons* (Stål.), and the corn stunt vector *Dalbulus elimatus* (Frolova), and demonstrated that *S. citri* could multiply in the bodies of these insects. Whitcomb et al (22) also demonstrated that cultured *S. citri* was pathogenic to *M. fascifrons*. In England, Markham and Townsend (14) and Townsend et al (20) injected cultured *S. citri* into individuals of the leafhopper, *Euscelis plebejus* (Fallén), which is known to vector European yellows type diseases, and found that it multiplied, but had little effect on the longevity of the leafhoppers.

This study was undertaken to determine the particulars of the interaction of *S. citri* and its natural vector *C. tenellus*. Specifically, we wanted to determine if *S. citri* could multiply within, and be pathogenic to, *C. tenellus* and to investigate the location of *S. citri* in *C. tenellus*.

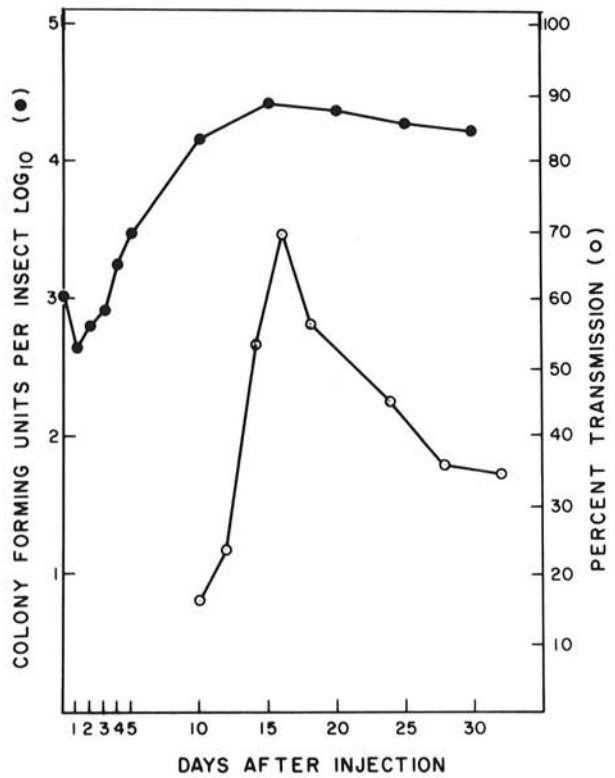


Fig. 1. Multiplication of *Spiroplasma citri* in *Circulifer tenellus* and its transmission to periwinkle after vector acquisition.

TABLE 1. Mortality of *Circulifer tenellus* after acquisition of *Spiroplasma citri*

Mode of acquisition of *S. citri*	Mortality (%) after: 1 wk	2 wk	3 wk	4 wk	5 wk	6 wk	7 wk
Injection with 2-day-old culture of *S. citri*	31	65	82	97	100	···	···
Control[a]	8	49	74	93	97	···	···
Statistical significance ($P<$)	0.01	0.05					
Feeding on *Beta geniculata* infected with *S. citri*	22	45	68	84	90	96	100
Control[b]	11	25	32	53	78	88	95
Statistical significance ($P<$)	0.05	0.05	0.01	0.01	0.01	0.05	0.05
Membrane feeding on buffered *S. citri* suspensions	4	15	42	65	73	85	96
Control[c]	2	8	21	39	57	78	87
Statistical significance ($P<$)			0.05	0.01	0.05		

[a] Nymphs of *C. tennellus* were injected with sterilized liquid medium.
[b] *C. tennellus* were fed on healthy *B. geniculata*.
[c] *C. tennellus* were fed on a buffered sucrose solution.

MATERIALS AND METHODS

Organism. An isolate of *S. citri*, MV101, (12) was obtained from field-grown periwinkle and single-colony cloned three times on semisolid medium. The isolate was confirmed to be *S. citri* on the basis of morphology, serology, and pathogenicity to citrus.

Culture media. Lee's improved liquid medium (9) was used throughout this study for the culture of *S. citri*. The compositions of this medium and of a solid medium used for colony-forming unit (CFU) assays are given previously (13).

Test insects. *C. tenellus* stock cultures were reared on sugar beet plants in a greenhouse at 30 ± 3 C. Stock colonies of leafhoppers used for these studies were tested frequently to ensure they remained spiroplasma-free.

Injection of insects with *S. citri*. Third instar nymphs of spiroplasma-free *C. tenellus* were anesthetized with carbon dioxide and injected with a 2-day-old culture of *S. citri* containing 10^8 CFU/ml at the rate of 0.03 − 0.05 μl per insect. Separate groups of insects injected with sterilized liquid medium were used as controls. Injected insects were caged on healthy sugar beet plants in the greenhouse at 30 ± 3 C for multiplication and longevity tests.

Acquisition of *S. citri* by the insects. *Membrane feeding.* Nymphs of spiroplasma-free *C. tenellus* were allowed to feed on solutions contained between two layers of stretched Parafilm M membrane. The feeding solution was prepared by centrifuging 100 ml of liquid from two-day-old cultures of *S. citri* at 27,000 *g* for 5 min and resuspending the pellet in 10 ml of pH 7.5 HEPES-buffered sucrose (HBS), 1.7% HEPES, and 7.5% sucrose. The HBS was sterilized by filtration through a 0.2-μm membrane filter. Sterilized HBS solution alone was used as a control for acquisition feeding experiments. The acquisition feeding time was 24 hr with the feeding solution changed at 12 hr. Following membrane acquisition feeding, insects were caged on healthy sugar beet plants in the greenhouse at 30 ± 3 C.

Feeding on plants infected with S. citri. Nymphs of spiroplasma-free *C. tenellus* were fed on *Brassica geniculata* plants infected by *S. citri* for 2 days and then transferred to healthy sugar beets in the greenhouse at 30 ± 3 C. Insects fed on healthy plants of *B. geniculata* served as controls.

Colony-forming unit assays. Immediately after injection, and on predetermined subsequent days, 20 insects were collected. Each

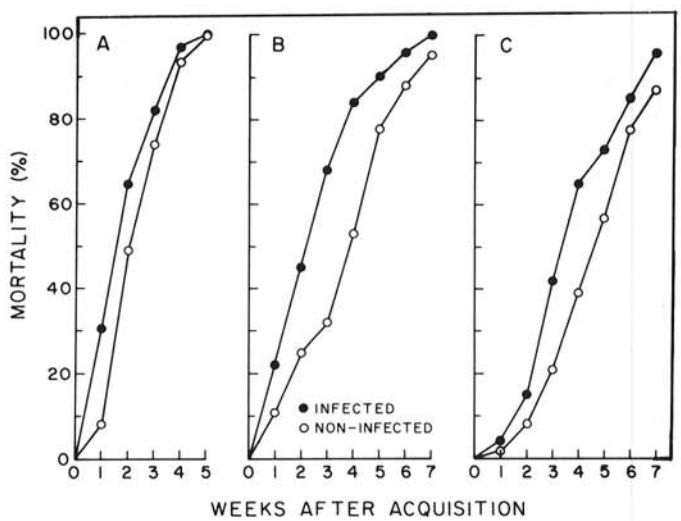


Fig. 2. Mortality of *Circulifer tenellus* free of *Spiroplasma citri* compared to *C. tennellus* following acquisition of *S. citri* by means of: **A,** injection; **B,** feeding on plants infected with *S. citri*; and **C,** membrane feeding on suspensions of *S. citri*.

insect was surface sterilized with 0.1% sodium hypochlorite for 5 min, rinsed three times in sterile distilled water, then ground with 1 ml of C3G medium (11) in 1-ml tissue grinders. C3G medium gave consistently higher recovery of *S. citri* from plant and insect tissues whereas Lee's improved medium produced the higher titer of organism necessary for membrane feeding and insect injection. The homogenate was centrifuged for 3 min at 3,000 *g* and 10 μl of the supernatant fluid was plated on solid medium and incubated for 10–20 days at 30 C. Developing colonies were counted under the microscope. Random colonies were picked for confirmation of *S. citri* by morphology and serology.

Mortality test. Following injection, membrane feeding or acquisition by feeding on plants infected by *S. citri*, groups of 200 *C. tenellus* were transferred to healthy sugar beet plants. Weekly counts of viable insects were made. All experiments were repeated three times.

Electron microscopy. Immediately after acquisition and every subsequent 5 days, 10 inoculated and 10 control inoculated leafhoppers were collected from each group. Salivary glands and intestinal tract were removed from the insects and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), at 4 C for 4 hr. Although sodium cacodylate-buffered glutaraldehyde is not the recommended fixative for preserving the shape of *S. citri*, it was the best for maintaining the insect cells. Since this study was to show the relationship of *S. citri* with insect tissues, the procedures for optimum preservation of the insect cells were chosen. The specimens were rinsed three times with 0.1 M phosphate buffer at pH 7.2 and postfixed at 4 C for 1.5 hr in 2% osmium tetroxide solution containing 3% sucrose. The fixed specimens were dehydrated in a graded series of ethyl alcohol and propylene oxide before being embedded in Spurr's medium (19).

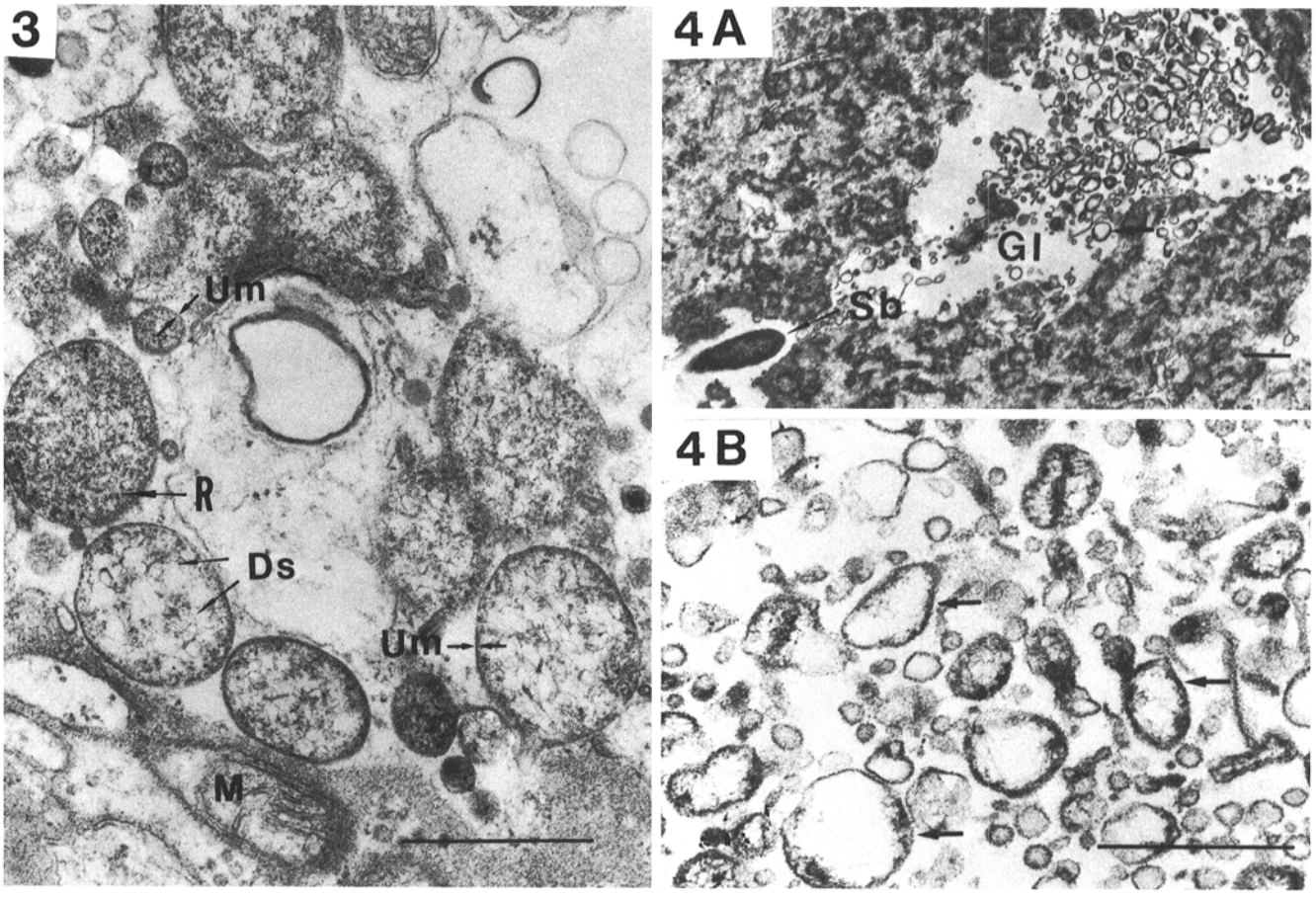
Ultrathin sections of all embedded specimens were cut with a diamond knife on a Sorvall MT-2 (Porter-Blum) ultramicrotome. Sections were mounted on 102-μm (150-mesh) Formvar-coated grids and stained with uranyl acetate and lead citrate. All preparations were examined with a Hitachi HU-12 electron microscope operating at 75 kV.

RESULTS

Multiplication of *S. citri* in *C. tenellus*. The number of CFU recovered from leafhoppers injected with *S. citri* is shown in Fig. 1. The mean of the number of CFU formed from 20 individual insects was calculated. One day following injection, the CFU decreased by 0.5 $\log_{10}$ units and then gradually increased to a maximum of 10^4–10^5 CFU per insect at 15 days after injection. Fifteen days postinjection also corresponds with the period of maximum transmission by *C. tenellus* of *S. citri* to periwinkle (Fig. 1). From 15–30 days after injection, the number of CFU remained about constant. No CFU were recovered from insects injected with the sterilized liquid medium.

Mortality of *C. tenellus* injected with *S. citri*. Two days after injection, 200 active *C. tenellus* were transferred to a new set of sugar beets, thereby eliminating those dead or dying of injection wounds or bacterial infection. Numbers of viable insects were statistically different between infected and noninfected leafhoppers 1 and 2 wk after injection (Table 1). However, by the third week after injection, there was no significant difference statistically. Results of the mortality studies are given in Table 1 and Fig. 2A. Infection of leafhoppers was confirmed by isolation and culture of *S. citri* (in liquid medium) from injected leafhoppers.

Mortality of *C. tenellus* fed on healthy plants and *S. citri* infected plants. After acquisition feeding, 200 leafhoppers from both groups were transferred to healthy sugar beets and live insects were



Figs. 3 and **4. 3.** *Spiroplasma citri* in the acini of the salivary glands of *Circulifer tenellus* 10 days after injection. Um = unit membrane, M = mitochondrion, Ds = a network of DNA-like strands, R = ribosomes. **4. A,** Gut lumen of *Circulifer tenellus* one day after membrane feeding. Note some of the *Spiroplasma citri* cells appear to be degenerating (arrows). Gl = gut lumen, Sb = symbiotic bacterium. **B,** An enlarged portion of Fig. 4A. Bars are 500 nm.

counted weekly. Longevity of the insects is shown in Table 1 and Fig. 2B. Significant differences in the numbers of infected and noninfected leafhoppers were observed between 1 and 7 wk after acquisition feeding (Table 1).

Mortality of *C. tenellus* fed on a suspension of *S. citri* through membranes. After 24 hr of feeding, 200 insects from each group were transferred to sugar beets and the numbers of live insects were counted weekly. The mortality of each group is shown in Table 1 and Fig. 2C. There was no significant difference in longevity between infected and noninfected leafhoppers at 1, 2, 6, and 7 wk after acquisition feeding. During weeks 3, 4, and 5 after feeding, however, there was a significant difference in mortality between infected and noninfected leafhoppers (Table 1).

Sequential observations of *S. citri* in salivary glands of *C. tenellus* after injection. *Spiroplasma citri* was first detected in salivary glands of *C. tenellus* 10 days after injection. Numerous pleomorphic bodies of various sizes were observed in these sections (Fig. 3). The spherical bodies of *S. citri* are 410–500 nm in diameter and contain fibrillar nuclear material and ribosomes. The organism is surrounded by a unit membrane 7.0–7.5 nm thick.

Fifteen days after injection, large concentrations of *S. citri*, usually in irregular pockets, were observed close to the acini of the salivary glands. *S. citri* was not found in the salivary glands of healthy leafhoppers.

Sequential observations of *S. citri* in the intestine of *C. tenellus* after membrane feeding. One day after membrane feeding, both complete and disrupted *S. citri* cells were found in the gut lumen of *C. tenellus* (Fig. 4). In the intestinal epithelial cells, *S. citri* could be observed 15 days after membrane feeding (Fig. 5A).

Between 15 and 25 days after membrane feeding, a number of pleomorphic bodies of *S. citri* were located partially embedded in the wall of epithelial cells (Fig. 5B), immediately outside the wall of epithelial cells (Fig. 5C and D), and within "vesicles" between the epithelial cells and the basement membrane (Fig. 5E).

Observation of *S. citri* in the salivary glands of *C. tenellus* after membrane feeding. The earliest observation of *S. citri* in salivary glands of *C. tenellus* occurred 25 days after acquisition (Fig. 6A). The size and shape were similar to those found in the intestine. In 30-day samples, *S. citri* cells were abundant and concentrated close to the acini membrane of salivary glands (Fig. 6B). Cells resembling *S. citri* were never observed in salivary glands of healthy leafhoppers.

DISCUSSION

There have been previous reports that *S. citri* can multiply in several leafhoppers including *M. fascifrons*, *D. elimatus*, *Draeculacephala* sp., *D. pseudoobscura*, and *E. blebejus* (14,20–22), none of which are known to be natural vectors of stubborn disease. This study, therefore, was undertaken to elucidate particulars of the relationship between *S. citri* and one of its natural vectors, *C. tennellus*. We believe that the mycoplasmalike bodies observed in the intestine and salivary glands of diseased leafhoppers are *S. citri* for the following reasons: all test insects reared on healthy sugar beets under controlled conditions were confirmed to be spiroplasma-free by frequent culturing; mycoplasmalike bodies were found only in leafhoppers that had either fed on, or were injected with, suspensions of cultured *S. citri*; no mycoplasmalike bodies were found in noninjected leafhoppers or leafhoppers injected with, or fed on,

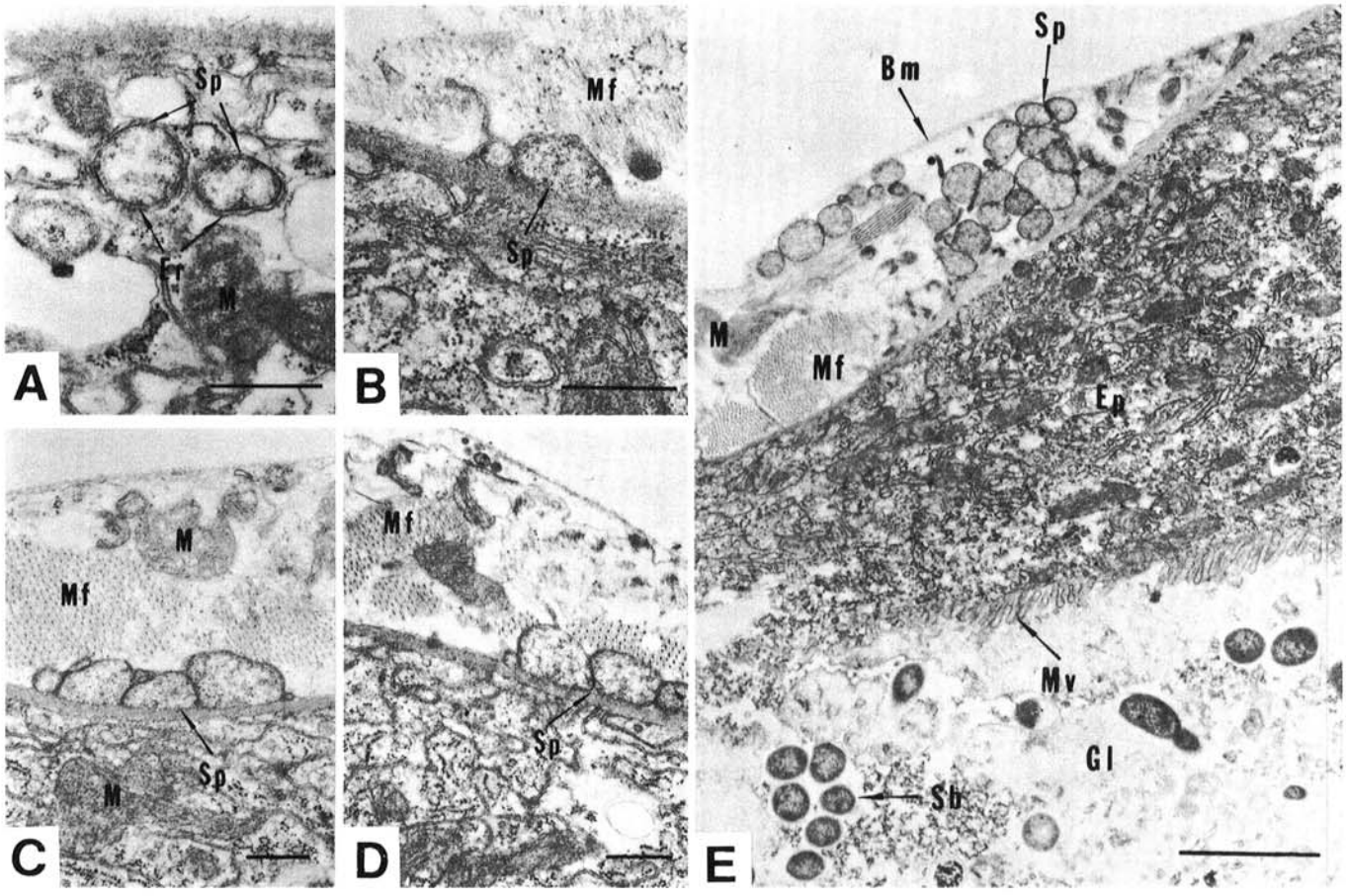


Fig. 5. *Spiroplasma citri* (Sp) within intestinal cells of *Circulifer tenellus* 15 to 20 days after membrane feeding. **A,** *S. citri* in the epithelial cells 15 days after membrane feeding. **B,** *S. citri* partially embedded in the wall of epithelial cells 15 days after membrane feeding. **C** and **D,** *S. citri* immediately outside the wall of epithelial cells 20 days after membrane feeding. **E,** *S. citri* adjacent to the basement membrane of the intestine 20 days after membrane feeding. Bm = basement membrane, Er = endoplasmic reticulum, M = mitochondria, Mf = muscle fibers, Ep = intestinal epithelial cells, Mv = microvilli, Gl = gut lumen, Sb = symbiotic bacteria. Bars for A–D = 500 nm. Bar for E = 2 μ.

sterilized culture media; and *S. citri* could be readily cultured from insects that had fed on, or were injected with, suspensions of cultured *S. citri* but not from those that had similarly received sterilized culture media.

The multiplication pattern of *S. citri* in *C. tenellus* is very similar to that of *S. citri* in *M. fascifrons* (21). As a result of this multiplication, *S. citri* can cause premature death of its natural vector, *C. tennellus*, regardless of the mode of acquisition. The time and degree to which *S. citri* affects vector longevity, however, does vary with the method of spiroplasma acquisition. *S. citri* injected into the abdominal cavity of the leafhopper moves directly into the hemolymph and is transported to the salivary glands within 10 days (Fig. 3). This coincides with the time of the first transmission of *S. citri* by *C. tennellus* (13) and the period of time of significant mortality differences between infected and noninfected leafhoppers (Table 1). Thus, the hemolymph appears to act as a medium for movement and multiplication of *S. citri*.

Maximum mortality for membrane-fed leafhoppers, however, was 3–5 wk following acquisition. This 3- to 5-wk time period again coincided well with the 32-day latent period before maximum transmitting ability (13) of the membrane-fed leafhoppers. Multiplication of *S. citri* during this 3- to 5-wk period may also account for the increased mortality of the leafhopper vector. Examination of intestine from *C. tennellus* revealed the presence of *S. citri* in four different, but sequential, sites between 15 and 25 days after membrane feeding. These observations would suggest the following pathway for the movement of *S. citri* from the gut into the hemocoel and ultimately the salivary glands. *S. citri* enters the gut lumen as a result of feeding. Most cells of *S. citri* are digested or destroyed in the gut lumen (Fig. 4). Some of the surviving cells pass through the gut wall into the epithelial cells (Fig. 5A-D) where they may multiply. They move further toward the basement membrane of the intestine (Fig. 5E). Finally, the organism enters the hemocoel where further multiplication may occur. Once in the hemocoel, *S. citri* is transported to the salivary glands (Fig. 6) where *C. tenellus* can then inject *S. citri* into plants via salivary secretions during the feeding process.

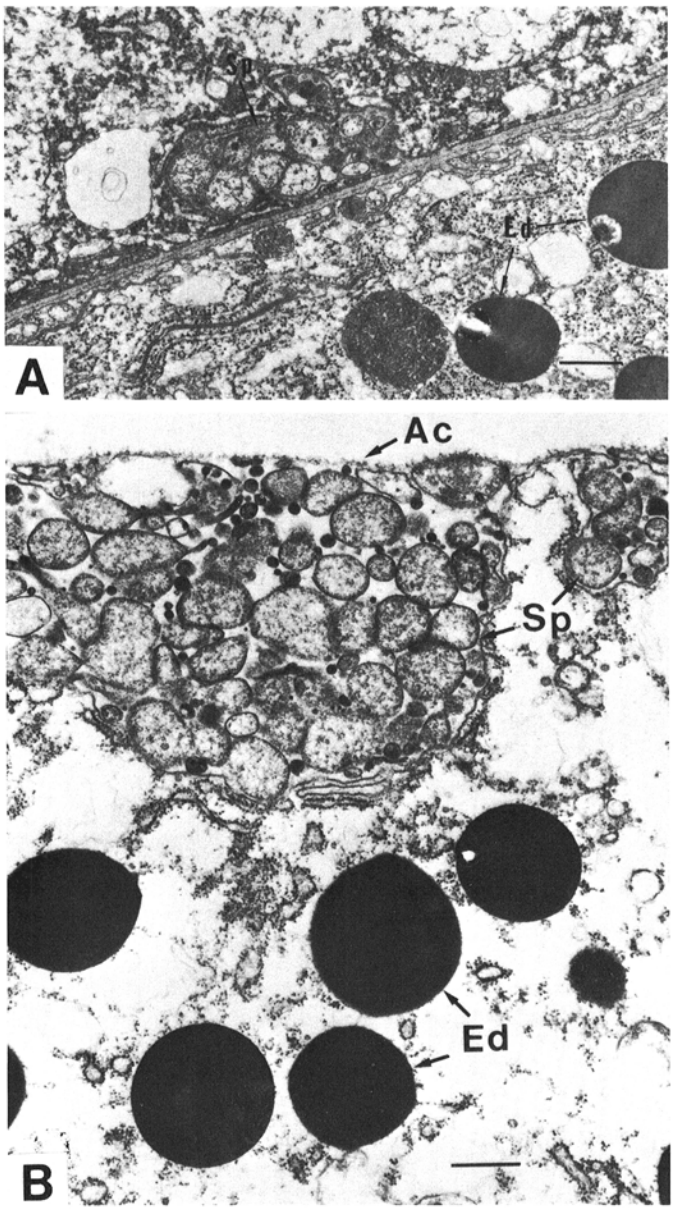


Fig. 6. *Spiroplasma citri* (Sp) inside the salivary glands of *Circulifer tenellus* after membrane feeding. **A,** Twenty-five days after membrane feeding. **B,** A large aggregation of *S. citri* in the acini (Ac) of the salivary glands 30 days after membrane feeding. Ed = electron-dense bodies. Bars are 500 nm.

In the case of acquisition by feeding on plants infected with *S. citri*, increased mortality of the vectors during the entire 1- to 7-wk period was significant. This might be attributed to an inhibitor or toxin produced in the plant as a result of infection with *S. citri*. This substance may be directly produced by the spiroplasma or by the plant in response to infection with *S. citri* and is toxic to *C. tenellus*. Similar results were obtained during early studies measuring the effect of the Western X disease pathogen of stone fruit and corn stunt disease spiroplasma on their vectors. The Western X disease agent was shown to cause premature death of its vector, *Colladonus montanus*, following acquisition of the agent from infected celery plants (5). Also, the corn stunt agent was shown to cause premature death of *Dalbulus elimatus* after it fed on corn stunt-affected plants (4).

The simultaneous occurrence of maximum multiplication, maximum transmission ability, and high mortality suggested that the titer of *S. citri* in its insect vector plays a major role in transmission and mortality. It is interesting to note that the time at which *S. citri* reaches its highest titer in *C. tenellus* is also the time of maximum transmission and mortality. From an evolutionary point of view, it seems unusual that *S. citri* would be pathogenic to its vector. One would expect that selection pressure would favor a less acute relationship. However, the numbers of *S. citri* present in *C. tenellus* prior to reaching peak titer would appear to be sufficient to ensure natural spread of the disease in the field.

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