Transmission of *Spiroplasma citri* by *Circulifer tenellus*

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ABSTRACT

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The beet leafhopper, *Circulifer tenellus*, can transmit *Spiroplasma citri* isolate MV101 acquired either by injection, membrane feeding, or feeding directly on host plants infected with *S. citri*; however, minimum length of the latent period varied with the three methods. Minimum latent periods were 10, 16, and 24 days for injection, feeding on plants infected with *S. citri*, and membrane feeding, respectively. The minimum acquisition access period for transmission was 6 hr; one of 16 test plants became infected. During a 48-hr period, the transmission rate did not increase with longer acquisition access periods. The minimum transmission time was 2 hr, when one of 16 test plants had become infected. Transmission increased with increased transmission periods to a maximum after 48 hr when 12 of 16 test plants had become infected. Number of leafhoppers also affected the transmission of *S. citri*. When a single insect was used, 2–4% of the test plants became infected. For groups of 2, 5, 10, and 20 leafhoppers, the percentage of infected plants was 14–18%, 20–25%, 66–75%, and 70–80%, respectively. Transmission data showed that after the fourth transfer in a culture medium, *S. citri* isolate MV101 lost pathogenicity and/or transmissibility. No transovarial transmission of *S. citri* by *C. tenellus* could be demonstrated.

Citrus stubborn disease has spread for several decades in the hot, arid areas of California and other citrus-growing areas of the world. Among the leafhopper species that have been reported to be vectors (4–6,15,16), *Circulifer tenellus* is thought to be the most important natural vector in California. This opinion is based on its geographical distribution, wide host range, frequency with which field-collected *C. tenellus* harbor *S. citri*, and experimental transmission studies (1–3,4,10,13–15,17). In spite of its importance, there is a lack of information detailing vector-pathogen relationships between *C. tenellus* and *S. citri*. Therefore, this study of the transmission of different *S. citri* isolates, the transmissibility of *S. citri* after continuous subculture in a culture medium, and other characteristics of *S. citri* transmission by *C. tenellus* was initiated.

MATERIALS AND METHODS

Organisms. *Spiroplasma citri* isolates used in this study included C189, C3B, MV101, and Maroc. Isolate C189 originally isolated from a cultivar Madame Vinous sweet orange seedling, C3B isolated from field-collected *C. tenellus*, and MV101 isolated from field-grown periwinkle plants have all been single-colony cloned three times on semisolid media. The Maroc isolate was obtained from the American Type Culture Collection as culture 27551.

Culture media. Lee's improved liquid medium (9) was used throughout this study for the culture of *S. citri*. This medium consists of 0.5 g PPLO broth, 0.1 g dry yeast extract, 0.5 g tryptone, 2.5 g sorbitol, 1.0 g sucrose, 0.2 g glucose, 0.2 g fructose, 0.04 g KCl, 0.03 g KH_2PO_4, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.14 g NaCl, 0.02 g Na_2HPO_4, 0.05 g Na_2CO_3, 1.5 g HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid), 0.04 g succinic acid, 0.08 g L-asparagine, 0.08 g L-arginine, 0.08 g L-glutamine, 0.08 g L-lysine HCl, 0.05 g L-cysteine, 0.05 g L-glycine, 0.05 g L-methionine, 0.05 g L-proline, 1.0 mg adenosine, 1.0 mg cytidine, 1.0 mg guanosine, 1.0 mg thymidine, 1.0 mg uridine, and 70 ml of double glass-distilled water. The pH of the medium was adjusted to 7.5 with 2 N NaOH. After being autoclaved at 121 C and 1.02 kg/cm^2 (15 lb) for 15 min and then cooled, the medium was completed by adding 3 ml of 25% (v/v) fresh yeast extract and 20 ml of fetal bovine serum previously adjusted to pH 7.5 and sterilized by filtration through 0.20 μm millipore filters. Solid medium used for colony-forming unit (cfu) assays consisted of 12 g of sucrose, 3 g of PPLO agar, 10 ml of horse serum, 250 μg of amphotericin B, 0.1% thallium acetate, 10^{-2} g of penicillin, and double glass-distilled water to a final volume of 100 ml. The final pH of this medium was adjusted to 7.5.

Test insects and plants. Beet leafhoppers, *C. tenellus*, were reared on sugar beet plants in a greenhouse at 30 ± 3 C and transferred to new sugar beets every other month. Stock leafhopper colonies were tested monthly for contamination by *S. citri*. Plants used in this study as hosts or to assay for *S. citri* were: sugar beet (*Beta vulgaris*, Crassa group); Madagascar periwinkle (*Cartharanthus roseus*, Burpee's dwarf mixture 3588); and shortpod mustard (*Brassica geniculata*).

Inoculation of beet leafhoppers. *Injection*. Spiroplasma-free third instar nymphs of *C. tenellus* were anaesthetized with carbon dioxide and injected with 2-day-old cultures of *S. citri* in the abdominal cavity, using glass needles. Injections were administered at a rate of 0.03 to 0.05 μl per insect of culture fluid containing 10^8–10^9 cells per milliliter. Control groups of insects were injected with sterilized liquid medium without added antibiotics. Injected insects were caged on healthy sugar beets in the greenhouse at 30 ± 3 C.

Membrane feeding. Nymphs of spiroplasma-free *C. tenellus* were transferred from sugar beets into cardboard cages. One side of the cage was covered with fine nylon screen and the opposite side with black paper. Two layers of stretched parafilm M membrane containing feeding solution between the layers were closely attached to the surface of the nylon screen. A light source was suspended 15 cm above the Parafilm surface of the cage to attract insects to the feeding solution.

Acquisition feeding time was 24 hr with the feeding solution changed after 12 hr. The feeding solution was prepared by centrifuging 100 ml of liquid from 2-day-old cultures of *S. citri* at 27,000 *g* for 5 min and resuspending the pellet to a concentration of 10^8–10^9 cells of *S. citri* per ml with HEPES-buffered sucrose (HBS)

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solution containing 1.7% HEPES and 7.5% sucrose adjusted to pH 7.5. 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.

After the membrane acquisition feeding, insects were caged on healthy sugar beets in the greenhouse at 30 ± 3 C to complete the latent period.

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

Transmission tests. Following acquisition by feeding on plants infected with *S. citri*, *C. tenellus* were transferred to healthy sugar beet plants. At predetermined intervals, the insects were transferred from the sugar beet plants to 5- to 6-cm-tall periwinkle seedlings (10 insects per plant). Following a 2-day transmission feeding, all test plants were fumigated twice with methyl bromide (30 ml for 2 hr in a 1.81 m^3 [64 ft^3] chamber) and transferred to the greenhouse at 30 ± 3 C. Symptom development was checked every other day and positive transmissions were confirmed by culturing *S. citri*.

RESULTS

Latent period. Three acquisition methods were employed to determine the latent period: injection of healthy *C. tenellus* nymphs with inoculum from 2-day-old MV101 cultures; feeding nymphs of *C. tenellus* through a Parafilm membrane on MV101 organisms suspended in HBS solution for 24 hr; and feeding nymphs of *C. tenellus* on *S. citri*-infected *B. geniculata* for 2 days. Following acquisition, 10 leafhoppers were caged on each periwinkle plant for 2 days and then transferred to new test plants at successive 2-day intervals. Length of the latent period varied with the method of acquisition (Table 1). The minimum latent period using injected insects was 10 days, when five of the 30 plants used in the test ultimately became infected by *S. citri*. Maximum numbers of plants became infected when the transmission feeding occurred 16 days after injection. The minimum latent period for membrane feeding was 24 days and maximum infection occurring 32 days after membrane feeding. The minimum latent period for *S. citri* in leafhoppers that acquired the organism from infected plants was 16 days. Maximum infection was observed 24 days after acquisition feeding on infected plants.

Acquisition access period. To test the length of acquisition feeding on the ability of *C. tenellus* to transmit *S. citri*, leafhoppers were fed for specified periods on plants experimentally infected with *B. geniculata*. Following acquisition access periods (2, 4, 6, 8, 10, 12, 24, 48, and 72 hr), the insects were moved from the inoculum source plant to healthy sugar beet plants for 24 days and then transferred to periwinkle seedlings for 2 days (10 insects per plant).

The effect of acquisition feeding periods on transmission is shown in Table 2. It is evident that <4 hr of acquisition feeding was insufficient for transmission. The minimum acquisition access period was 6 hr; one of 16 test plants became infected. Acquisition feeding periods >48 hr were of little or no value for increasing transmission. Specifically, eight of 15 plants became infected after 24 hr acquisition, 11 of 15 after 48 hr, and 9 of 15 after 72 hr.

Transmission periods. Following a 2-day acquisition feeding on experimentally infected plants of *B. geniculata*, beet leafhoppers were held on sugar beet plants for 24 days. The insects were subsequently fasted for 1 hr and then 10 insects per plant were transferred to periwinkle seedlings for 1/2, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hr. The effect of length of transmission feeding period on transmission is shown in Table 2. The minimum transmission feeding period was 2 hr, after which the transmission increased with increased feeding time to a maximum at 48 hr.

Leafhopper numbers and transmission. The effect of *C. tenellus* numbers on transmission of *S. citri* is shown in Fig. 1. When exposed to single insects, 2–4% of the test plants were found to become infected with *S. citri*. Groups of 2, 5, 10, and 20 leafhoppers resulted in 14–18, 20–25, 66–75, and 70–80% of the test plants becoming infected, respectively. These experiments were all done with leafhoppers that had been injected with *S. citri*.

***S. citri* isolates and transmission.** Nymphs of *C. tenellus* were injected with 2-day-old cultures of C189, C3B, Maroc, and MV101 isolates. The MV101 culture had been transferred only twice following the original triple cloning, while the other three isolates have been passaged continually for several years. Sixteen days after injection, the leafhoppers were caged on healthy periwinkle seedlings for 2 days. Infectivity data are shown in Table 3. Isolates C189, C3B, and Maroc all failed to infect or were not transmitted to periwinkle even though *S. citri* could be reisolated from *C. tenellus* after injection. Isolate MV101, on the other hand, was transmitted and was infectious under these conditions.

Continuous culturing of *S. citri* and transmission. Nymphs of *C. tenellus* were injected with the MV101 isolate after different transfers. The triple-cloned *S. citri* isolate MV101 was considered the first transfer. Transfers were made every 2 days up to 100

TABLE 1. The transmission of *Spiroplasma citri* to periwinkle by *Circulifer tenellus* following acquisition by injection, membrane feeding, or feeding on infected *Brassica geniculata*

	Transmission[a] (plants infected/plants in test) following acquisition by:					
Days after acquisition	Injection		Membrane feeding		Feeding on infected *B. geniculata*	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
0	0/15	0/15	0/15	0/15	0/15	0/10
2	0/15	0/15	0/15	0/15	0/15	0/10
4	0/15	0/15	0/15	0/15	0/15	0/10
6	0/15	0/15	0/15	0/15	0/15	0/10
8	0/15	0/15	0/15	0/15	0/15	0/10
10	2/15	3/15	0/15	0/15	0/15	0/10
12	4/15	3/15	0/15	0/15	0/15	0/10
14	7/15	9/15	0/15	0/15	0/15	0/10
16	11/15	10/15	0/15	0/15	4/15	3/10
18	6/15	11/15	0/15	0/15	9/15	5/10
24	6/14	7/15	3/15	1/15	11/15	8/10
28	5/12	4/13	5/15	3/15	9/14	7/10
32	4/11	4/12	8/13	7/15	9/14	8/10
36	...	...	4/12	5/14	7/12	6/10

[a]Ten insects per periwinkle test plant were used for transmission.

TABLE 2. Effect of acquisition and transmission feeding periods on the transmission of *Spiroplasma citri* to periwinkle by *Circulifer tenellus*

Acquisition feeding period (hr)	Transmission feeding period (hr)	Transmission[a] (infected plants/plants tested)
2	48	0/16
4	48	0/16
6	48	1/16
8	48	2/16
10	48	2/16
12	48	7/15
24	48	8/15
48	48	11/15
72	48	9/15
48	1/2	0/16
48	1	0/16
48	2	1/16
48	4	1/16
48	6	5/16
48	8	6/16
48	12	5/16
48	24	10/16
48	48	12/16
48	72	9/16

[a]Ten insects per periwinkle test plant were used for transmission.

transfers by pipetting 0.1 ml of culture into 5 ml of fresh culture media. Results of this study are shown in Table 4. For the first and second transfers, transmission and/or infectivity remained the same. With each subsequent transfer, however, the number of plants that became infected decreased until it reached zero after the fourth transfer. Inocula from transfers 5–100 produced no infected plants even though *S. citri* could be reisolated from 90–100% of the injected *C. tenellus*.

Transovarial passage of *S. citri*. Experiments to determine transovarial transmission of *S. citri* were conducted with leafhoppers reared on sugar beets from eggs laid by infective *C. tenellus* females. Infective females acquired *S. citri* either by injection, membrane feeding, or feeding on plants infected with *S. citri*. Hatching leafhoppers were transferred to periwinkle test plants immediately, 2, 4, and 6 wk after emerging. Ten to 15 insects were placed on each plant, where they remained for 2 days. After this transmission feeding, all insects were killed by fumigation and the test plants were transferred to a greenhouse at 30 ± 3 C and observed daily for symptom development. No plants became infected as a result of these experiments.

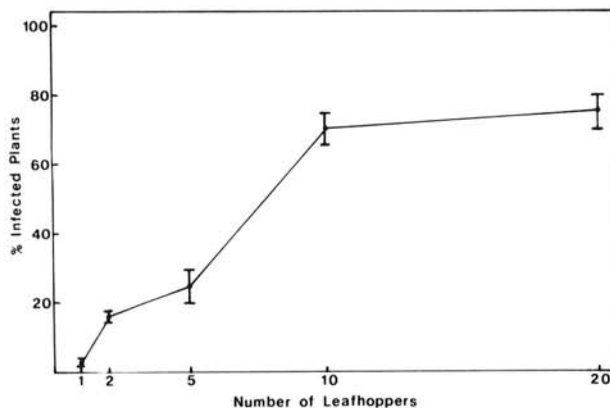

Fig. 1. Effect of numbers of beet leafhopper (*Circulifer tenellus*) vectors on transmission of *Spiroplasma citri*.

TABLE 3. Transmission of different isolates of *Spiroplasma citri* to periwinkle by *Circulifer tenellus*

S. citri isolates	Infected plants/plants tested[a]			
	Exp 1	Exp 2	Exp 3	Total
C189	0/15	0/15	0/15	0/45
C3B	0/15	0/15	0/15	0/45
Maroc	0/15	0/15	0/15	0/45
MV101	10/15	12/16	11/15	33/46

[a]Transmission period was 2 days; 10 insects per plant were used for transmission.

TABLE 4. Effect of continuous transfer of a culture of *Spiroplasma citri*, isolate MV101, on its infectivity or transmission to periwinkle by *Circulifer tenellus*

No. of transfers	No. infected plants/no. plants tested[a]		
	Exp 1	Exp 2	Total
1	9/15	11/15	20/30
2	11/15	10/15	21/30
3	10/15	7/15	17/30
4	0/15	1/15	1/30
5	0/15	0/15	0/30
10	0/15	0/15	0/30
24	0/15	0/15	0/30
50	0/15	0/15	0/30
100	0/15	0/15	0/30

[a]Transmission access period was 2 days; 10 insects per plant were used for transmission.

DISCUSSION

The results of this study demonstrate that the beet leafhopper, *C. tenellus*, could transmit *S. citri* regardless of the acquisition method. Minimum length of the latent period did, however, vary with the mode of acquisition (Table 1). The shortest latent period occurred when *C. tenellus* acquired *S. citri* by direct injection, while the longest latent period occurred after membrane feeding by *C. tenellus* on suspensions of *S. citri*. There are at least two possible explanations for these differences. The first is that *S. citri* is injected directly into the hemocoel where it multiplies and subsequently translocates to the salivary glands from which the leafhopper can then transmit *S. citri*. The *S. citri* therefore does not have to pass through the foregut into the midgut and through the gut wall into the hemocoel as shown to be the case with natural feeding (11,12). This abbreviation of the pathway would require a shorter latent period than the usual pathway of *S. citri* that must be traversed when insects feed directly on *S. citri*-infected plants. Secondly, both the physical and chemical environment of the culture medium differ from those of hemolymph, so it is reasonable that *S. citri* would require a longer latent period to adapt to the insect hemolymph after membrane feeding before it could multiply and spread into the salivary glands.

Additional transmission data showed that the minimum acquisition feeding period for transmission was 6 hr and the minimum transmission period was 2 hr. This would place very stringent requirements on the possible use of an insecticide to minimize the movement of stubborn disease in citrus groves.

After the fourth passage on artificial cell-free medium following triple cloning, isolate MV101 lost either its pathogenicity or transmissibility (Table 4). This phenomenon is thought to have occurred with the other isolates of *S. citri* (C189, C3B, and Maroc), which have been nonpathogenic or nontransmissible to citrus or periwinkle for years (Table 3). Some phytopathogenic bacteria have also displayed this phenomenon (6–8).

Possible explanations for the loss of pathogenicity or transmissibility due to continuous passage in artificial media are numerous and may include that: *S. citri* may not replicate in the vector or it may replicate so slowly that the concentration of organisms is too low for transmission to occur; *S. citri* can no longer invade the salivary glands of *C. tenellus*; even though the vector can transfer *S. citri* to the plant, the organism is unable to survive or multiply in plant tissue; the mode of pathogenicity has been altered (ie, *S. citri* no longer produces a toxin, etc.); or that because *S. citri* looses pathogenicity or transmissibility after only a few passages (four transfers following triple cloning), the involvement of a "plasmid" in pathogenicity could be postulated.

More extensive studies are needed to evaluate the above hypotheses.

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