Acquisition of Spiroplasma citri Through Membranes by Homopterous Insects

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ABSTRACT

Several cicadellids and one membracid acquired *Spiroplasma citri* from a concentrated suspension by feeding through stretched Parafilm M membranes. Two species, *Circulifer tenellus* and *Scaphytopius nitridus*, retained the microorganism for life. After acquisition, individuals of both species could inoculate solutions of 5% phosphate-buffered sucrose with *S. citri*. In single instances, both species transmitted *S. citri* to sweet orange.

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The wall-free prokaryote, *Spiroplasma citri* Saglio et al., has been isolated repeatedly from stubborn-infected citrus (1, 2, 10) and from *Circulifer tennellus* (Baker) (7) collected in the field. Workers in England injected *Euscelis plebejus* (Fallen) with suspensions of *S. citri* and demonstrated the transmission of the microorganism to clover, citrus, and some other plants (2, 9, 10).

Feeding insects through membranes has been done before (4, 8) and has been useful in virus vector work (8). Recently Spaar et al. (11) reported that *E. plebejus* acquired *S. citri* through a membrane and transmitted it to chrysanthemum plants. We believed the membrane technique might indicate probable natural vectors of *S. citri* in California. This method retains the advantages of natural imbibition, high vector survival, and simple equipment. In addition to greenhouse-reared *C. tenellus* and *Scaphytopius nitridus* de Long, which are already suspected of being vectors (5, 6, 7), five Cicadellidae and one Membracidae collected from various plants in California citrus-growing areas were tested for their ability to acquire and retain *S. citri*.

MATERIALS AND METHODS.—The *S. citri* isolates used were: Cir I B, originally isolated from *C. tenellus* collected in the Riverside area from Russian thistle, *Salsola* sp.; M 5-22, originally cultured from a Madam Vinous sweet orange (MVSO) seedling, *Citrus sinensis* (L.) Osbeck, naturally infected with *S. citri* at the University of California's (U.C.) Moreno Farm east of Riverside; and Sca 101, originally isolated from *S. nitridus* experimentally fed for 15 days on stubborn-infected MVSO seedlings. These isolates should close serological relationship in ring-interface tests and cross reacted by growth-inhibition against Maroc and C-189 strain antisera (J. G. Tully, *personal communication*).

All isolates were cultured using conventional techniques and media (3). All serum was subjected to heat treatment (30 minutes, 56 C) and centrifugation (150 minutes, 100,000 *g*). Liquid cultures of *S. citri* 1.5 – 2.0 days old were used in this study.

Circulifer tenellus and *S. nitridus* colonies composed of spiroplasma-free individuals were reared in a greenhouse on healthy sugar beet and celery seedlings, respectively. In this study, we used laboratory colonies of *C. tenellus* from Prosser, Washington, the U.C. Farm at Moreno, and a curly top virus-free colony maintained for several years at U.C. Riverside. One population of *S. nitridus*, descendants of individuals originally collected near Riverside, was used. The other Homoptera, *Aceratagallia* spp. Kirkaldy, *Empoasca* spp. Walsh, *Hordnia circellata* (Baker), *Spissistilus festinus* (Say), *Acinopterus angulatus* Lawson, and *Erythroneura variabilis* Beamer, were field collected and used directly in our trials since numerous samples from such collections of these insects were consistently negative in *S. citri* isolation attempts.

Stretched Parafilm M was selected for the acquisition feedings from the various membranes tested (4, 8) since its impermeability to water prevented the suspension of *S. citri* from dripping into leafhopper cages beneath each reservoir. A unit constructed with 35-mm diameter plastic petri dish bottoms is schematically illustrated in Fig. 1. The reservoir chambers were cleaned with 75% ethanol and washed twice with sterile water before being sealed with Parafilm M and filled with feeding solution.

Suspensions of *S. citri* for feeding were obtained by concentrating 26-28 ml of liquid culture into a single pellet by centrifugation at 27,000 *g* for 30 minutes. Each pellet was resuspended in 4 ml of sterile 5% sucrose in 0.02 M phosphate buffer, pH 7.2. The *S. citri* concentrations were determined spectrophotometrically at 420 nm. The reservoir of each feeding chamber was filled with a 0.3 OD suspension from sterile syringes. The feeding units were transferred to a controlled-temperature chamber with 16 hours of high light (1,615 lux) at 30 C alternated with 8 hours of low light (1 lux) at 12-14 C. In long acquisition tests the *S. citri* suspensions were renewed every 10-12 hours.

After the acquisition period, *C. tenellus* was caged on a single young MVSO seedling and a sugar beet plant caged

together. *Scaphytopius nitridus* was maintained on celery and MVSO seedlings caged together. After acquisition feeding, *Aceratagallia* spp., *S. festinus*, and *A. angulatus* were kept on alfalfa, and *Empoasca* spp., *E. variabilis*, and *H. circellata* were kept on sugar beet, celery, and grapevine, respectively. All experiments with plants were carried out in greenhouses maintained at 26-30 C.

The presence of *S. citri* in the fed insects and in exposed plants was confirmed by previously described isolation techniques (3, 7).

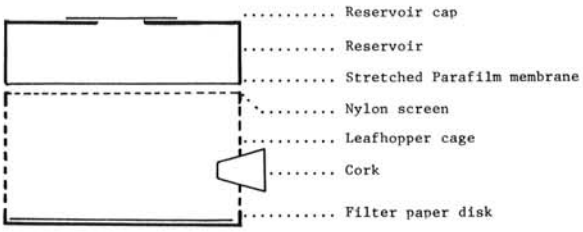

Fig. 1. Chamber used for feeding 15-25 leafhoppers through a membrane.

EXPERIMENTS AND RESULTS.—*Acquisition by Circulifer tenellus.*—Groups of adult beet leafhoppers were starved 8-12 hours and allowed to feed through membranes on a suspension of *S. citri* for 5 minutes to 8 hours, after which they were caged on healthy sugar beet plants. After 8-11 days, we attempted to isolate *S. citri* from groups of six to ten surviving leafhoppers. The results, summarized in Table 1, indicate a minimum acquisition time between 10 and 20 minutes. *C. tenellus* from all three populations were able to acquire *S. citri* through a membrane.

Adults of *C. tenellus* from the Prosser and Moreno populations were fed 48 hours on a fresh suspension of *S. citri* isolate Sca 101. Ten days after acquisition feeding *S. citri* was isolated from five groups of five leafhoppers.

Tests using 40-50 *C. tenellus* nymphs of the first two and last two instars demonstrated that some groups of each category acquired *S. citri* by feeding 36 hours on a suspension of M 5-22 (Table 2).

We isolated *S. citri* from groups of three-to-five adult *C. tenellus* 2 days after a 24-hour acquisition period. We also isolated it from groups of *C. tenellus* that died during the acquisition period.

TABLE 1. Threshold of acquisition of *Spiroplasma citri* through a stretched Parafilm M membrane by *Circulifer tenellus*

Acquisition period	Isolate	Post-acquisition period (days)	Isolations[a]
5 min	M 5-22	9	0/3
10 min	M 5-22	9	0/3
20 min	M 5-22	8	1/3
30 min	M 5-22	11	2/3
1 h	M 5-22	11	2/3
2 h	Cir I B	9	2/3
4 h	Cir I B	10	2/3
8 h	Cir I B	10	2/3

[a]Cultures of *S. citri* per groups of six-to-eight *C. tennellus* tested.

Retention by Circulifer tenellus.—To determine whether *S. citri* was permanently associated with *C. tenellus*, 120 greenhouse-reared adults from Moreno were given a 36-hour acquisition-feeding period, then transferred to a sugar beet and a MVSO seedling caged together. The surviving insects were transferred to new pairs of plants 13 and 33 days after acquisition. On the 25th and 40th days after acquisition, *S. citri* was isolated from groups of three *C. tenellus*. The seven leafhoppers still alive after 48 days were transferred daily, alternating them from a sugar beet to a young MVSO seedling. *Spiroplasma citri* was isolated from these last insects soon after they died: two insects at 50 days, two at 51, and one each at 54, 56, and 58 days after acquisition. It is possible that some of the insects may have acquired or reacquired *S. citri* from the single MVSO plant that became infected sometime between 33 and 48 days after the acquisition feeding. This seems unlikely, however, for there was little time for *S. citri* to build up in the newly infected MVSO.

Transmission to MVSO by Circulifer tenellus.—One to 2 months after the death of the last *C. tenellus* in the retention test, *S. citri* isolations were attempted from the newly growing shoots or leaves of all MVSO and sugar beets exposed to *C. tenellus*. *Spiroplasma citri* was isolated only from the MVSO plant exposed to 30 *C. tenellus* from the 33rd to the 48th day after acquisition feeding and was reisolated from the same plant 8 weeks and 18 weeks later. The infected plant showed mild stubborn symptoms.

Inoculation of Spiroplasma citri into sucrose solution by Circulifer tenellus.—About 100 adult *C. tenellus*, fed 48 hours on a suspension of *S. citri* isolate M 5-22, were caged on healthy sugar beet and MVSO plants for 23 days. Then, two groups of 20 of these leafhoppers were fed 24 hours on 2-ml aliquots of a sterile 5% sucrose solution prepared in 0.02 M phosphate buffer, pH 7.2. *Spiroplasma citri* was isolated from one of these solutions and from the heads and abdomens of the 20 *C. tennellus* used to inoculate the same sucrose solution.

TABLE 2. Acquisition of *Spiroplasma citri* through a stretched Parafilm M membrane by nymphs of *Circulifer tenellus* and *Scaphytopius nitridus*

Species	Nymphal stage	Post-acquisition period (days)	Isolations[a]
C. tenellus	First two	10	3/5
	Last two	10	3/5
S. nitridus	First two	10	4/5
	Last two	10	3/5

[a]Cultures of *S. citri* per groups of six-to-eight insects tested.

Tests with Scaphytopius nitridus.—Ten minutes were sufficient for starved *S. nitridus* adults to acquire *S. citri*, and after a feeding period of 36-48 hours, *S. citri* was retained by *S. nitridus* adults for at least 20-22 days (Table 3). Nymphs of the first two and the last two instars were able to acquire *S. citri* through membranes (Table 2). A group of late instar nymphs that acquired *S. citri* through a membrane during a 36-hour feeding was then allowed to feed on an MVSO seedling 28 days. Strongly developed stubborn symptoms appeared on the MVSO 16 weeks later and *S. citri* was cultured from it.

TABLE 3. Acquisition and retention of *Spiroplasma citri* by species of Cicadellidae and Membracidae.

Species	*S. citri* isolated after indicated no. of days post-acquisition[a]			
	0	8	12-14	20-22
Cicadellidae				
Aceratagallia spp.	2/3	1/3	1/3	1/3
Acinopterus angulatus	2/3	1/3	1/3	0/3
Empoasca spp.	0/3	0/3	0/3	0/3
Erythroneura variabilis	0/2	1/2		
Hordnia circellata	1/3	2/3	1/2	1/3
Scaphytopius nitridus	2/3	2/2	2/3	3/3
Membracidae				
Spissistilus festinus	3/3		1/2	2/2

[a]Cultures obtained per groups of insects tested.

Two groups of 12 adults each were fed 38 hours on a *S. citri* isolate M 5-22 suspension, then were kept on healthy celery and MVSO for 20 days before feeding 24 hours on buffered sucrose solutions. *Spiroplasma citri* was isolated from two sucrose solutions and from the separated heads and abdomens of the insects in both groups. *Spiroplasma citri* was also cultured from groups of three-to-five *S. nitridus* on days 2, 4, and 6 following an acquisition-feeding period of 12-24 hours, thus indicating a short or absent lag period in *S. nitridus*.

Acquisition by other homopterous species.—Several adults of the other homopterous species listed above were allowed to feed through membranes for 24-28 hours. Isolations of *S. citri*, attempted periodically from groups of two-to-six individuals each, showed that all the insects used, except *Empoasca* spp., acquired *S. citri* and retained it for 8-22 days (Table 3).

DISCUSSION.—Among the insects fed through membranes, *C. tenellus* and *S. nitridus* are of special interest, the former because it has been implicated as a possible stubborn disease vector (7) and the latter because it readily feeds and reproduces on citrus (6) and has recently been shown to transmit *S. citri* to *Vinca rosea* L. (5). Both leafhoppers acquired *S. citri* by membrane feeding for 10-20 minutes. Once acquired, whether as a nymph or an adult, *S. citri* was retained for life by the leafhoppers.

The demonstration that five other homopterous species were able to acquire *S. citri* through membranes increases to 11 the total number of insects known to be able to harbor this microorganism within their bodies (2, 12). Some significance is attributed to acquisition by feeding through membranes since it is more nearly natural than microinjection (2, 9, 10). Nevertheless, only three of the 11 insect species have been shown to transmit *S. citri* to citrus plants and cause stubborn disease. Markham et al. (10) reported transmission of the organism causing little-leaf (stubborn) disease to two of 49 citrus plants by the leafhopper, *E. plebejus,* following microinjection with *S. citri.* In our study, *C. tenellus*, which is known to visit citrus plants and to carry *S. citri* in the field (7), readily acquired *S. citri* through membranes, but transmitted it to only one of six exposed MVSO plants.

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