Spiroplasmas from Plants with Aster Yellows Disease and X-Disease: Isolation and Transmission by Leafhoppers

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ABSTRACT

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Spiroplasma citri was isolated infrequently in vitro from surface-sterilized plants affected with aster yellows (AY) disease and from aster leafhoppers, *Macrosteles severini* (= *M. fascifrons*), that had fed on AY-affected *Plantago*. Similarly, spiroplasmas were isolated from celery, cherry, and peach affected by X-disease and from leafhoppers (*Colladonus montanus*) that had fed on X-diseased celery. No spiroplasmas were isolated from plant, insect, or media controls. In transmission trials, *M. severini* was injected with spiroplasmas that had been cultured from AY-diseased plants. Similarly, the leafhoppers *C. montanus* and *Scaphytopius nitridus* were injected with spiroplasma from X-diseased plants. No apparent transmissions occurred with most isolates (AY, 36 isolates and X-disease, 23 isolates). However, typical AY and X-disease symptoms were produced in *Plantago* and celery, respectively, with three separate uncloned isolates from AY-diseased plants and with three uncloned isolates from X-diseased plants. Plants with typical AY or X-disease symptoms were good sources for efficient leafhopper transmission, but spiroplasmas were only infrequently cultured in many attempts. Some spiroplasma isolates from AY-affected plants or X-diseased plants caused disease symptoms that were not typical of either AY or of X-disease in *Plantago* or celery, respectively. A spiroplasma was isolated readily from plants with "atypical" symptoms, but none of the three species of leafhoppers that were tested transmitted spiroplasma after acquisition feeding on "atypical" plants. Spiroplasma isolates reacted identically to *Spiroplasma citri* in spiral deformation and growth inhibition tests. *S. citri* from stubborn-affected oranges injected into *C. montanus* and *S. nitridus*, which subsequently inoculated celery, induced disease symptoms distinct from either "atypical" symptoms or from symptoms of AY or X-disease.

Additional key words: mycoplasma, mollicute.

Numerous studies support a mycoplasmalike organism (MLO) etiology of aster yellows (AY), but attempts to culture the AY agent in vitro have been unsuccessful (31). In the past few years, several researchers independently have reported the isolation of spiroplasma from plants or insects infected with AY-like agents (3,7,12,23) or the X-disease agent (11,28). Insect vector transmissions of spiroplasma isolated from such plants also have been reported (7,13,17,18). Uncertainty over the etiological role of these spiroplasmas prevails (26,31).

We present here details of our isolations of helical mollicutes from AY- and X-disease-affected plants and leafhoppers and our attempts to transmit these isolates via leafhoppers to healthy plants and to resolve their pathological role. Some preliminary results of this study have been reported (17,18,23,28).

MATERIALS AND METHODS

Leafhopper and plant sources. Three strains of AY disease agents, dwarf aster yellow (DAY), severe aster yellows (SAY), and Tulelake aster yellows (TLAY) (6), were maintained in broad-leaf plantain, *Plantago major* L., and aster, *Callistephus chinensis* Nees, in a greenhouse at Berkeley via transmission by the "shortwinged form" of the aster leafhopper, *Macrosteles severini*, recently described by Hamilton (9) as a new species of what was formerly considered *M. fascifrons* (Stål). Two types of X-disease, peach yellow leaf roll (PYLR) (8), and Green Valley (GV) (8,27), were maintained in the greenhouse in celery, *Apium graveolens* L. 'Tall Utah 52-70,' via transmission by the leafhopper *Colladonus montanus* (Van Duzee). Noninfectious colonies of *C. montanus* and *Scaphytopius nitridus* (DeLong) were reared on celery, *M. severini* and *Euscelidius variegatus* (Kirshbaum) on oats, and *Circulifer tenellus* (Baker) on sugar beet.

Isolation. Media (ME-1 and ME-2) and isolation techniques have been previously reported (22). In some isolation attempts, surface-sterilized plant stem tips or insects were chopped into small segments in 20 ml of broth medium followed by immediate or periodic (usually every 2 days) blind passages from original inoculum at 10-fold dilutions. In later isolation attempts, blind passages were discontinued. Culture media were examined at various intervals for growth by using dark-field microscopy.

Solid ME-1 medium with 0.8% Ionagar (Difco) was used for cloning isolates (21). One triple-cloned isolate (TLAY-A15) was deposited in the American Type Culture Collection, Rockville, MD (ATCC 33210).

Isolates of *S. citri* from the seeds of stubborn-diseased navel orange trees were obtained from E. C. Calavan, University of California, Riverside, and from J. Kloepper, University of California, Berkeley. These were maintained in ME-1 and ME-2 broth medium and injected into leafhoppers in several experiments.

Insect injections. Spiroplasma isolates were injected into noninfective late-instar nymphs of *M. severini* with hollow glass needles (8). Each injected dose that was drawn into the needle averaged 0.05 μg. In most experiments, each isolate was injected into at least 100 leafhoppers.

Injected leafhoppers were confined on healthy *Plantago* or celery seedlings for 2 wk and then were transferred to a new plant at weekly intervals. Beet leafhoppers were transferred alternately to beet and celery to prolong their survival. Test plants were sprayed with dimethoate (Cygon 25 WP; American Cyanamid Co., Princeton, NJ) after exposure to leafhoppers. The plants were recaged for at least 12 days and then were resprayed with

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dimethoate and held uncaged in a greenhouse (21–27 C) where they were monitored for AY or X-disease symptoms for 8 wk or longer.

Serological tests. Antisera to spiroplasma isolates were produced in New Zealand white rabbits as previously described (21). Serological comparisons were made of spiroplasma isolates from AY or X-diseased plants to the corn stunt (CS) spiroplasma (I-747 isolate: ATCC 29051), *S. citri* (ATCC 27563), honeybee spiroplasma (AS-576: ATCC 29416), suckling mouse cataract agent (SMCA: ATCC 29335) obtained from Joseph Tully, NIAID, Frederick, MD, and tulip tree flower spiroplasma (TT-15 isolate: ATCC 33214) by using spiral deformation (32) and growth inhibition (4) tests. Plant material for ELISA was prepared as described before (20,21). Antiserum prepared against the TLAY-A15 isolate was processed and used in ELISA according to methods previously described (16).

TABLE 1. Isolation in ME-1 medium of spiroplasmas from plants with aster yellows disease

Aster yellows strain[a]	Plant sources	No. of isolations[b]/ No. of plants	(%)
TLAY	Celery	1/20	5.0
	Aster	4/52	7.7
	Lettuce	0/19	0.0
	Plantago	2/30	7.0
SAY	Celery	0/28	0.0
	Aster	2/33	6.0
	Plantago	0/25	0.0
DAY	Aster	0/30	0.0
	Plantago	2/47	4.0
FAY[b]	Lettuce	2/28	7.0
	Periwinkle	4/20	20.0

[a] Strains: Tulelake, TLAY; severe aster yellows, SAY; dwarf aster yellows, DAY; and field-collected, FAY.
[b] Equal numbers of healthy plants and of uninoculated media were included in each attempt as controls. No spiroplasma was detected in controls.

TABLE 2. Summary of transmissions of spiroplasma isolated from aster yellows-infected plants and insects to *Plantago*

Isolate (strain)[a]	Passage level[b]	Insects injected (no.)	Symptoms[c]	Average incubation period (wk)[d]	Range of latent period (days)[e] min	max
RB (TLAY)	5	75	Atypical	5.5	83	91
RM (TLAY)	2	100	Atypical	5	35	52
RP (TLAY)	3	100	Typical	2	...[f]	41
RR (SAY)	3	100	Typical	2	17	27
RS (FAY)	4	100	Typical	2	16	26
RV (TLAY)	4	100	Atypical	4.5	16	24
RW (SAY)	5	100	Atypical	4.5	45	52
RZ (TLAY)	4	100	Atypical	4	26	45
BA (TLAY-I)	4	100	Atypical	4.5	?	16
BB (DAY-I)	3	100	Atypical	3-4	?	19
BC (TLAY)	5	100	Atypical	7	29	37
BQ (DAY-I)	4	100	Atypical	5-7	22	32

[a] Isolates cultured from plants affected by the following strains of aster yellows (AY): Tulelake (TLAY), severe (SAY), dwarf (DAY), field-collected (FAY). Insect isolates are designated with the suffix "I" and were obtained from *Macrosteles severini* that had fed on plants affected by the indicated AY strains.
[b] Number of subcultures following original isolation.
[c] "Atypical" symptoms were initial vein clearing, leaf distortion, and thickening and reddening of older leaves. "Typical" symptoms were etiolated and twisted leaves, proliferation, stunting, and phyllody of flowers.
[d] The approximate average time between the start of feeding access of the insects and the first appearance of symptoms of AY.
[e] The minimum and maximum latent periods were calculated from the date of injection until the first or the last day of access to the first plant which developed AY. The maximum latent period assumed transmission occurred on the last day of access to the first plant to develop AY in a series of weekly transfers of injected insects.
[f] Indicates transmission occurred on the first plant.

RESULTS

Isolation of spiroplasmas from AY-infected plants and insects. Spiroplasmas were isolated from a very low percentage of diseased plant materials (Table 1) and infective *M. severini*. No spiroplasmas were isolated from surface-sterilized healthy plant materials, noninfective insects, or uninoculated control media. The number of successful isolations varied with the type of AY, host plant, and the type of tissue used. Most isolations were obtained from young shoot tips or leaves. Only six isolations were made from groups of infective insects in 72 attempts.

Initially, we combined and chopped plant stem tips and young leaves obtained from several plants in a small amount of medium (5–8 ml) for primary isolations and blind subcultures were made on alternate days. Round bodies (blebs) with filamentous protrusions were seen after 2 or 3 wk of incubation. After 10–12 subcultures from primary isolations, helical forms were seen in some of these subcultures. Nonhelical filaments were also observed in spiroplasma cultures. In many cases, including all isolations from noninfective leafhoppers, nonhelical bodies occurred but helical bodies usually did not. Spiroplasmas usually were observed in positive cultures after 3–12 blind subcultures during 16–26 days of incubation at 27–28 C. A change in medium color from red to yellow caused by the drop in pH (from 7.4 to 6.0) indicated heavy growth of spiroplasma.

Transmission by leafhoppers. Our early attempts to transmit cultured spiroplasma with injected leafhoppers were not successful. None of the 31 spiroplasma cultures obtained from AY-affected plants (DAY—eight isolates, SAY—nine isolates, TLAY—eight isolates, and field-grown plants with AY symptoms—four isolates) was transmitted by injected leafhoppers. The plants were held 10–12 wk after exposure to leafhoppers but none developed symptoms, nor was spiroplasma isolated from such symptomless plants in several attempts with randomly-selected inoculated plants. Broth culture media inoculated with extracts from DAY-plants (three attempts) or TLAY-plants (one attempt), and which subsequently contained only nonhelical bodies, were centrifuged and the pellets were injected into leafhoppers, but no symptomatic plants resulted.

The first *Plantago* inoculated with our spiroplasma isolates to develop typical AY symptoms had been fed upon by 100 leafhoppers that had been injected with a third-passage uncloned isolate (RP) of spiroplasma from aster with TLAY. Through an oversight, the leafhoppers placed on this plant were not transferred for 41 days, and AY symptoms (Fig. 1A) were already evident at that time. Therefore, the combined insect latent period and incubation period of the causal agent was <7 wk. Five of 20 leafhoppers caged singly on test plants of *Plantago* transmitted the AY agent. Two additional isolates induced typical SAY/TLAY symptoms (Fig. 1B): isolate RR (Table 2) from SAY-infected aster, and isolate RS from field-collected aster with AY symptoms. Spiroplasmas were reisolated in 7 of 13 attempts from diseased *Plantago* that had been experimentally inoculated by leafhoppers injected with the cultured spiroplasmas.

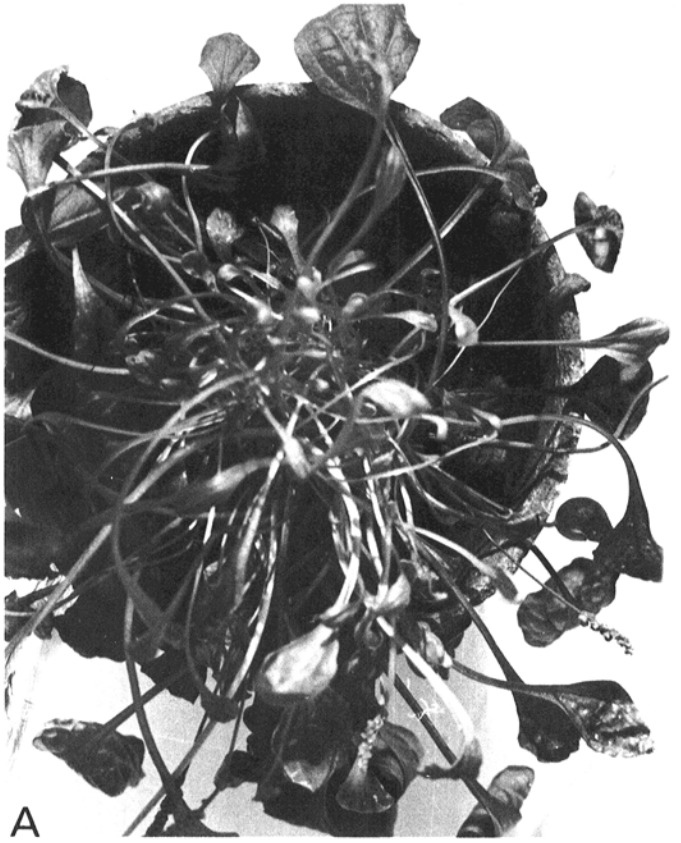
The original inoculum of isolate RP which had been frozen (−18 C) after an aliquot had been injected, was thawed and reinjected into an additional 100 noninfective nymphs of *M. severini*. The second injection of isolate RP resulted in 10% weekly transmission (2/20) by individual leafhoppers. Noninjected control insects from the same colonies did not infect healthy plants. Spiroplasmas from one additional in vitro passage of isolates RR and RS beyond the passage that resulted in typical AY was injected into aster leafhoppers but was not transmitted to *Plantago* in either case, based on the disease-free appearance of the plants of *Plantago* after exposure to injected leafhoppers.

Most spiroplasma isolates that induced plant disease after injection into *M. severini* produced disease symptoms (Fig. 1B) "atypical" of AY in *Plantago* (Table 2). After an incubation of 6 wk,

the youngest leaves of a test plant exposed to the ninth weekly transfer of injected leafhoppers developed asymmetrical vein clearing and leaf distortion that is a typical early symptom of AY; however, symptoms were never apparent on the flowers of this plant or three subsequently inoculated plants that had the same "atypical" symptoms (Fig. 1B) when compared with healthy control plants (Fig. 1C). Numerous additional spiroplasma isolates also induced symptoms "atypical" of AY (Table 2). These symptoms appeared 4–5 wk after insect inoculation. Isolate BB from insects induced a leaf rugosity and distortion similar to, but more pronounced than, DAY in *Plantago*; moreover, the flower stalks were stunted but without the phyllody typical of AY (Fig. 2). Spiroplasmas were reisolated in 11 of 16 attempts from *Plantago* with "atypical" symptoms. The minimum latent and incubation periods of isolates inducing "atypical" symptoms were also compared to isolates that caused "typical" AY (Table 2).

Although spiroplasma could be isolated readily from plants with "atypical" symptoms, noninfectious *M. severini* fed on these plants subsequently were not able to infect *Plantago*. In separate trials, more than 100 noninfective nymphs of *M. severini* were confined for 2 wk or more on plants of *Plantago* with "atypical" symptoms of AY after infection by isolates BA (three replicates), BB, or BQ (two replicates each). All insects were subsequently transferred to test plants of *Plantago* weekly. The same procedure was used for plants with "atypical" symptoms induced by isolates RM, RW, and RV except that leafhoppers were allowed 4-wk acquisition feedings before weekly transfer to test plants. None of the insects that had fed on plants with "atypical" symptoms transmitted the causal agent of "atypical" symptoms to any subsequent test plants. The leafhopper *Euscelidius variegatus* was confined for 11 days on "atypically" symptomatic *Plantago* infected with isolate BQ. None of the 14 adults of *E. variegatus* that survived the first transfer to healthy *Plantago* or the 11 adults that survived an additional 2 wk transmitted isolate BQ.

In contrast to the lack of transmission of a disease agent by *M. severini* from "atypical" AY plants, the plants infected with isolates RP, RR, and RS (which produced typical symptoms of AY) served as source plants for transmission by noninfectious *M. severini*. A large fraction (18/20) of individual *M. severini* fed for 2 wk on *Plantago* infected with isolate RP transmitted TLAY agent after a minimum latent period of 3 wk and a median latent period of ~6 wk. In two separate tests conducted with 20 insects per test, in which 66 and 95% (respectively) of the leafhoppers transmitted, the median latent period for *M. severini* fed for 1 wk on our



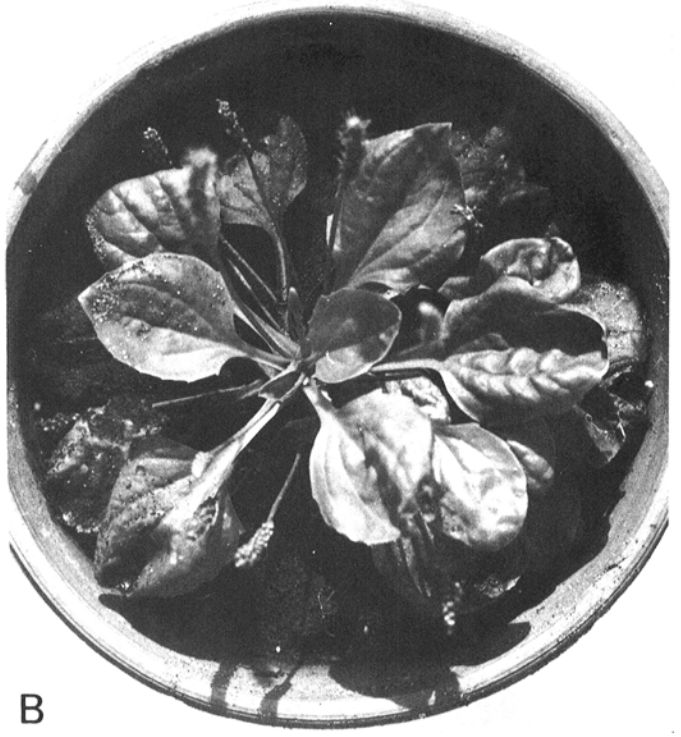


Fig. 1. *Plantago major*: **A,** with severe aster yellows (SAY) induced by isolate RP; **B,** with "atypical" symptoms (leaves mottled but with little rugosity), induced by isolate RB of SAY, and **C,** healthy.

greenhouse-maintained AY strains was about 3 wk. Using the same procedures for acquisition and subsequent testing and using *M. severini* for AY transmission, leafhoppers that were fed upon plants infected with isolates RR or RS transmitted after a minimum latent period of 3 wk. Thus, although the symptoms produced by the isolates RP, RR, and RS were typical for AY, transmission by injected leafhoppers was less efficient compared to that of our greenhouse isolates of AY following natural feeding acquisition.

Isolation and transmission of spiroplasmas from plants and leafhoppers with X-disease. Spiroplasmas were isolated from celery, *Plantago*, lettuce, periwinkle, and peach affected by X-disease (Table 3). Spiroplasmas were isolated from leafhoppers (*C. montanus*) infected with greenhouse-maintained PYLR type of X-disease in three of 44 attempts. Spiroplasmas were also isolated from field-grown peach and cherry trees with symptoms of PYLR and GV types of X-disease, respectively (Table 3). No spiroplasmas were isolated from symptomless plants or insects in the greenhouse or insectary or from field trees of peach and cherry without symptoms.

TABLE 3. Isolations of spiroplasma from X-diseased plants

Type of X-disease	Plant[a]	No. of isolations/ No. of plants	(%)
Peach yellow leaf roll	Peach (field)[b]	6/40	15
	Peach	1/5	20
	Periwinkle	0/18	0
	Celery	3/60	5
Green Valley	Cherry (field)	12/54	22
	Peach (field)	2/16	13
	Periwinkle	1/28	4
	Celery	0/15	0

[a] Plants from greenhouse at Berkeley or Davis unless designated "field." Healthy plant samples and uninoculated media were included as controls in all attempts and were negative.

[b] Spiroplasmas from field-collected peach and cherry were isolated only in the early spring (March-May).

As was the case with isolates from AY-affected plants, our early attempts to transmit spiroplasmas from X-diseased plants were not successful. Fourteen isolates cultured in either ME-1 or ME-2 media for five to 12 subcultures were not transmitted by groups of 100 *C. montanus* that had been injected with these isolates. Neither were six other spiroplasma isolates that had been subcultured for one to three passages. However, insects injected with three separate uncloned isolates obtained from celery, *C. montanus*, and peach, respectively, transmitted the X-disease agent to celery plants which subsequently developed typical symptoms of X-disease (Fig. 3A). The injected inoculum of each of these three isolates represented a $>10^{-5}$ dilution of the plant or insect tissue used in the original isolation. Celery fed on by insects injected with isolates BL or BM had typical PYLR symptoms (Fig. 3A) after an incubation period of ~5–6 wk. Another spiroplasma isolate (BP) from a PYLR-affected peach seedling that had been leafhopper-inoculated with PYLR in the greenhouse was also transmitted to celery by injected *C. montanus*. The inoculum of isolate BP injected into leafhoppers represented a dilution of 8×10^{-7} after five passages in vitro.

Some spiroplasma isolates from plants with PYLR that were injected into leafhoppers induced disease symptoms in celery that were not typical of PYLR in celery. Isolates BS from celery and BR from peach were subcultured five or six times, including cloning on solid media, and injected into *C. montanus* and *S. nitridus*. The oldest leaves of the celery plants on which the injected insects fed became brittle and cupped upward, with faint marginal chlorosis after an incubation period of 10–12 wk (Fig. 3B). Unlike typical PYLR in celery, however, these plants had not developed more severe chlorosis or necrosis even after 29 mo. Spiroplasmas were readily isolated from these plants. In contrast, in the same

Fig. 2. *Plantago major*: (left to right) healthy, severe aster yellows (SAY)- affected, and infected by an isolate of *Spiroplasma citri* from orange.

greenhouse, celery plants infected with isolates BL, BM, and BP developed severe chlorosis, stunted new foliage (Fig. 3A), and rapid dieback of roots, leading to the collapse and death of the plants 12–14 wk after inoculation, which is typical of our greenhouse-maintained PYLR strain (27). *M. severini* injected with spiroplasma isolated from "atypically" symptomatic celery (BR) transmitted spiroplasma to *Plantago*. Spiroplasma were reisolated from the leafhopper-inoculated *Plantago*, which had the same "atypical" symptoms as depicted in Fig. 1B.

Nymphs of *C. montanus* and *S. nitridus* from noninfective colonies that were caged for 2 wk on celery with "atypical" symptoms subsequently did not transmit spiroplasmas as judged by the lack of symptoms and the failure to reisolate spiroplasmas from plants inoculated by leafhoppers that had fed initially on celery with "atypical" symptoms.

Pathogenicity of *S. citri* to celery and *Plantago major*. Isolates of *S. citri* from southern California were tested for transmission and pathogenicity to celery and *P. major* and to compare disease symptoms produced to symptoms produced by our spiroplasma isolates from X-diseased or AY-affected plants. The isolates of *S. citri* used were isolated from stubborn-diseased orange and had indistinguishable serological characteristics resembling those of the type (Morocco) strain of *S. citri* (D. G. Garrot, *personal communication*). Groups of *M. severini* were injected and tested

Fig. 3. Foliage of celery: **A,** typical X-disease symptoms, **B,** "atypical" symptoms of X-disease (isolate BR), **C,** healthy, and **D,** infected with an isolate of *Spiroplasma citri* from orange.

for ability to transmit *S. citri* to *Plantago*. *Plantago* inoculated by the injected *M. severini* developed "atypical" symptoms (18): marginal necrosis of young leaves that caused crumpled, distorted mature leaves; stunted flower stalks with fewer flowers, but without phyllody; and overall stunting of growth (Figs. 1B and 2). Spiroplasmas serologically identical to *S. citri* were isolated from such plants in several attempts. Thus, *S. citri* from orange was pathologically indistinguishable from the spiroplasma causing "atypical" AY symptoms. Similarly, several hundred *M. severini* fed for 1 wk on *Plantago* infected by *S. citri* did not subsequently transmit it to healthy *Plantago*.

On the other hand, celery plants inoculated by any of the *C. montanus*, *C. tenellus*, and *S. nitridus* that had been injected with *S. citri* from stubborn-diseased orange did not develop "atypical" X-disease symptoms. The first sign of disease induced by *S. citri* in celery was a marginal necrosis of young leaflets about 3–6 wk after leafhopper inoculation. The continued or subsequent reappearance of such necrosis was rare. Celery plants infected with *S. citri* occasionally had darker than normal older leaves with slightly swollen leaf veins compared to healthy check plants (Fig. 3D). No chlorosis, curling, or stiffness of the leaflets that were distinctive "atypical" X-disease symptoms were noted in any of three separate leafhopper injection experiments with isolates of *S. citri*.

The successful isolation of *S. citri* from celery plants with or without disease symptoms was less frequent (~50%) and more sporadic than were isolations of spiroplasmas from celery having "atypical" symptoms of X-disease (no failure in over 20 attempts). In several additional attempts to transmit *S. citri* to celery, there was no evidence of transmission based on the lack of symptoms and failures to isolate *S. citri* from inoculated plants. However, after several months, two such celery plants yielded *S. citri* in two of four isolation attempts (D. G. Garrot, *personal communication*). Neither plant ever developed disease symptoms. It appears that *S. citri* from stubborn-diseased orange was only weakly pathogenic to celery under our greenhouse conditions (17–36 C, average 23–25 C), whereas our spiroplasma isolates resembling *S. citri* from X-diseased plants produced a quite distinct syndrome in celery that was not typical of X-disease.

Serological tests. In spiral deformation tests, spiroplasmas we cultured from plants with AY were deformed at the same dilution end point for deformation of 50% of helical forms as was *S. citri* using antisera against one of our isolates (ATCC 33210) from a TLAY-affected *Plantago* (Table 4). In reciprocal tests using antisera produced against *S. citri*, there was only a twofold difference in spiral deformation titers and no significant difference in the width of growth inhibition zones against the homologous *S. citri* or our isolate from TLAY-affected *Plantago* (Table 4). The same tests against other serogroup I spiroplasmas clearly indicated much larger differences indicative of heterologous reactions (Table 4). ELISA tests of *Plantago* with symptoms "atypical" of AY readily distinguished ($A_{405\ nm} > 0.5$) these plants from healthy *Plantago* ($A_{405\ nm} \leq 0.03$) using antisera against spiroplasmas cultured from TLAY-affected *Plantago*. Less than 5% of *Plantago* or aster plants with typical AY symptoms had ELISA readings $A_{405\ nm} > 0.08$ and none exceeded 0.10.

ELISA tests of celery infected with *S. citri* and showing leaflet necrosis symptoms gave low readings ($A_{405\,nm}$) ranging from 0.08 to 0.12 with antisera prepared against spiroplasma cultured from celery with symptoms "atypical" of X-disease. The same antisera produced readings ranging from 0.43 to 1.60 for tissue samples from celery plants with "atypical" symptoms of X-disease and below 0.03 for healthy celery controls. ELISA tests of celery infected with *S. citri* with antisera against *S. citri* also yielded low readings ranging from 0.08 to 0.11, whereas readings of samples from celery with "atypical" symptoms were 0.33. These results suggest a low titer of stubborn-derived *S. citri* in celery. All ELISA tests of three isolates of stubborn-derived *S. citri* stationary-phase cultures (~10^8 cfu/ml) or cultures of spiroplasma isolates that produced "atypical" symptoms either of AY- or X-disease produced high $A_{405\ nm}$ readings (>1.80). Spiroplasma cultured from X-diseased plants were not significantly different from isolates of *S. citri* in spiral deformation or growth inhibition tests.

TABLE 4. Serological comparisons of spiroplasma from various sources

Antigen/antiserum	Spiral deformation titer[a]	Growth inhibition zone (mm)
TLAY/TLAY[b]	16,384	14
SAY/TLAY	16,384	13
DAY/TLAY	16,384	13
FAY/TLAY	16,384	14
SC/TLAY	8,192–16,384	12
CSS/TLAY	128	3
HBS/TLAY	32	3
TFS/TLAY	16–32	2
SMCA/TLAY	16	2
SC/SC	8,192	13
TLAY/SC	4,096	10
SAY/SC	4,096	11
DAY/SC	4,096	12
FAY/SC	4,096	11
CSS/SC	256	4
HBS/SC	64	3
TFS/SC	16–32	3
SMCA/SC	16	2

[a]The highest dilution of the antiserum that distorted the helicity and motility of an average of 50% of the helices of spiroplasma.

[b]TLAY—Tulelake AY spiroplasma, SAY—severe AY spiroplasma, DAY—dwarf AY spiroplasma, FAY—field-collected AY spiroplasma, SC—*Spiroplasma citri*, CSS—corn stunt spiroplasma, HBS—honeybee spiroplasma, TFS—tuliptree flower spiroplasma, and SMCA—suckling mouse cataract agent.

DISCUSSION

Other investigators have reported the isolation of spiroplasmas from AY-diseased plants (12,13) and AY-infective leafhoppers (3) and from plants affected by clover phyllody disease in Europe (7). We suggest that in our experiments in which typical AY- or X-disease resulted following the inoculation of leafhoppers with spiroplasma cultures, the isolated spiroplasmas (serotyped as *S. citri*) did not have a direct role in causing AY or X-disease.

The morphological, cultural, and serological characteristics of the spiroplasmas we isolated from either X-diseased or AY-diseased plants were indistinguishable from the type strain of *S. citri* and in our tests differed from fresh isolates of *S. citri* from stubborn-diseased orange only in pathogenicity to celery. Spiroplasmas isolated from X-diseased plants were also cultured more consistently from celery than were isolates of *S. citri* from citrus. A recent report (1) of the failure to isolate *S. citri* from inoculated celery is similar to our results in which *S. citri* usually induced subtle symptoms in celery and from which isolations were difficult.

The low overall frequency of spiroplasma isolations and the many negative ELISA tests from X-diseased or AY-affected plants also failed to establish a consistent association of spiroplasmas resembling *S. citri* with either AY or X-disease. Failures of leafhoppers injected with spiroplasma to transmit agents causing typical AY or X-disease with a few exceptions further suggest that the spiroplasmas we isolated are not the causal pathogens of AY or X-disease, but are coinfecting agents in plants in which symptoms of AY or X-disease predominate.

We can only speculate as to why, during several experiments with both AY and X-disease, the inoculated plants developed typical symptoms of these diseases and served as transmission sources in a way typical of AY and X-diseased plants, respectively. In each experiment, conducted with separate colonies of noninfectious insects on different dates (although perhaps notably all within the same 7-wk period), control test plants remained healthy; thus, the possibility of insectary or greenhouse contamination seems very unlikely. In addition, the repeated transmission of a frozen in vitro culture over 6 wk after its first transmission further confirmed the infectivity of this "typical" isolate (RP).

The possibility that the causal agents of the AY or X-disease

survived or multiplied along with spiroplasmas present in the same plants cannot be discounted. It is possible that unnoticed temporary changes in media constituents or cultivation procedures or conditions may have allowed the survival or growth of the AY or X-disease agents along with spiroplasmas. However, numerous other spiroplasma isolates that were injected into leafhoppers during the same 3-mo period were not transmitted. Because of the ordinarily poor leafhopper transmission of *S. citri*, it is difficult to distinguish whether specific isolates are truly not transmissible (31).

Previous workers (5,26) have shown that the infectivity of AY extracts can be prolonged with various media constituents, but the infectivity of insect extracts of the AY agent in these or other media have not exceeded 3 days. Our inocula that produced typical X-disease remained viable for several weeks. Extracts containing the AY agent concentrated from plant sources have not been very infectious (2). Our primary isolates from plants were greatly diluted by media rather than concentrated. The X-disease agent has also been reported to retain infectivity in extracts for only a few days (8,30). Evidence for limited multiplication of the X-disease agent in vitro was obtained by Nasu et al (15), but they did not report any serial subculture of the organism. For aster yellows, insect extracts seem to have a dilution end point of $\sim 10^{-4}$ (25, and A. H. Purcell and A. H. Finlay, *unpublished*). Because the cultures we used for injection inoculum were greater than a 10^{-6} dilution of the plant material used for isolation, the AY agent may have multiplied in vitro rather than merely surviving in those inoculations leading to typical AY symptoms. Similar arguments apply to the X-disease agent, although its dilution end point from insect extracts is perhaps as high as 10^{-5} (19,30).

We conclude that AY or X-diseased plants from which we were able to isolate spiroplasmas were infected with both *S. citri* and the AY or X-disease agents, respectively. Kloepper and Garrott (10,11) reached similar conclusions on the basis of a correspondence of helical morphology and ability to isolate spiroplasma from some plants but not from others. Other recent reports (7,14,24,29) also support the hypothesis that plants with symptoms of AY or similar diseases from which spiroplasmas that fall into the present definition of *S. citri* can be isolated are multiply-infected with *S. citri* and other pathogenic mollicutes. Why multiple infections of distinct mollicute pathogens are encountered as frequently as they are is a perplexing unanswered question.

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