Evidence for a Mixed Infection of Spiroplasmas and Nonhelical Mycoplasmalike Organisms in Cherry with X-Disease

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ABSTRACT

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Spiroplasmas were isolated from 4 and 5% of total samples from individual Green Valley X-diseased cherry trees in two orchards in San Joaquin County, CA, in 1980; however, 53 and 78% of diseased trees in the orchards yielded spiroplasmas at some time of the year. In 1981, spiroplasmas were isolated from 40% of diseased trees and 60% of healthy trees in one orchard. All isolates were obtained during a 2- to 3-wk period shortly before fruit maturity; subsequent attempts to isolate spiroplasmas from the same trees were negative. The isolated spiroplasmas were serologically indistinguishable from *Spiroplasma citri*. Mycoplasmalike organisms (MLOs) were detected in high numbers in electron micrographs of phloem from X-diseased trees throughout the season, including times when no spiroplasmas were isolated. MLOs from diseased cherry were nonhelical as revealed with high voltage and standard electron microscopy. Helical MLOs were detected in sections of celery inoculated via leafhoppers (*Colladonus montanus*) with spiroplasmas isolated from X-diseased cherry. The results suggest that the X-disease agent is a noncultivatable, nonhelical MLO rather than a spiroplasma, and that both symptomless and X-diseased cherry in California are occasionally infected with *S. citri*.

X-disease of peaches and cherries is presumably caused by a mycoplasmalike organism (MLO), which is found in phloem cells of diseased, but not symptomless or healthy, trees (4,5,8,14). Gonot and Purcell (3) recently demonstrated that the leafhopper *Colladonus montanus* could acquire the X-disease agent from cherry trees in the field and transmit it to celery and cherry.

In 1978, the consistent isolation of spiroplasmas from celery, lettuce, and *Plantago* with X-disease was reported (10,15). Spiroplasmas were subsequently isolated from *C. montanus* fed on X-diseased celery (11), while no isolates were obtained from leafhoppers fed on healthy celery. Three spiroplasma isolates from celery with X-disease produced typical X-disease symptoms when injected into *C. montanus* after being subcultured three to five times (9), raising the possibility that the MLO associated with

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X-disease was actually a spiroplasma. Although evidence for MLO helicity was not directly reported in the electron micrographic studies, "undulating cylindrical tubules" (8) were observed in longitudinal sections of peach with peach X-disease raising the possibility that the MLOs may be predominantly helical.

Under field conditions, however, spiroplasmas were isolated from only 3% of samples from cherry trees with Green Valley X-disease (6), even though MLOs were observed in electron micrographs of phloem from all the diseased trees. Field isolations from peach with peach yellow leaf roll X-disease have also inconsistently yielded spiroplasmas (B. C. Raju, *unpublished*). These isolations were attempted from leaves, petioles, or fruit peduncles from different trees at various times during the growing season. However, Seemüller and Schaper (12,13) examined various pear tissues from the same trees at monthly intervals for the presence of the MLO associated with pear decline and found that the MLO populations fluctuated greatly in leaves and petioles during the growing season while populations remained constant in roots. Clark et al (2) reported a similar seasonal effect with ELISA detection of *Spiroplasma citri*. In July, sap from leaf mid-veins of infected trees had a positive ELISA reaction with antiserum toward *S. citri*, while no reaction occurred with infected trees sampled in January or March.

The purpose of this project was to determine if the MLO associated with X-disease is a cultivatable spiroplasma or a noncultivatable nonhelical MLO. Based on a 2-yr field study, data is presented on the percentage of individual diseased and healthy cherry trees from which spiroplasmas were isolated and on the optimum time of year for their isolation. The in vivo morphology of MLOs in phloem of X-diseased cherry was examined via high voltage electron microscopy and compared to the in vivo morphology of spiroplasmas.

MATERIALS AND METHODS

Two cherry orchards with Green Valley X-disease were selected for sampling in 1980 and two in 1981 as listed in Table 1. Nine to 15 visually healthy or diseased trees were sampled at least once per week from April through September in 1980 and from March through May in 1981. Isolations were attempted from fruit peduncles and/or leaf midveins as previously described (6) using DG-2 (6) broth medium. Primary spiroplasma isolates were cloned three times on solid DG-2 medium and compared to *S. citri* and a honeybee spiroplasma (BC-3) for reaction in the spiroplasma deformation test (16) by using *S. citri* antiserum.

In order to confirm a preliminary diagnosis of healthy or diseased, peduncles from the diseased and healthy trees were collected and prepared for routine electron microscopic examination for the presence or absence of MLOs (6). Additional studies on the in vitro morphology of MLOs were conducted by using high voltage electron microscopy (HVEM) with a Hitachi electron microscope operated at 650 kV. Samples for HVEM included celery infected via *C. montanus* with a spiroplasma isolated from X-diseased cherry. The samples for HVEM were fixed, dehydrated, and embedded in Spurr's plastic (6). Longitudinal sections 0.75- to 1.25-μm thick were cut by using glass knives and were placed on Pelco (Ted Pella, Inc., P.O. Box 510, Tustin, CA 92680) "clamshell grids." Grids were stained for 30 min with 20% uranyl acetate in absolute methanol followed by lead citrate for 5 min.

RESULTS

Spiroplasmas were isolated from only 4 and 5% of the total samples in 1980 from X-diseased cherry trees in orchards 1 and 2 (Table 1). However, 53 and 78%, respectively, of the individually sampled diseased trees in the two orchards yielded spiroplasma isolates from 30 April to 21 May. Spiroplasmas were isolated from 60% of symptomless trees in one of the orchards sampled in 1981 while 40% of diseased trees in the same orchard yielded spiroplasmas. Spiroplasmas were not isolated from any of the sampled diseased or healthy trees in another orchard during 1981 (orchard 3, Table 1). All spiroplasma strains were serologically indistinguishable from *S. citri* according to the spiroplasma deformation test (16).

MLOs were detected by electron microscopy in the phloem of all sampled diseased trees (Fig. 1), but in none of the symptomless or healthy trees. The MLOs, which were present throughout the season, including times when attempted spiroplasma isolations were negative, included filamentous and ovoid cells. HVEM of 1-μm-thick longitudinal sections of X-diseased cherry (Fig. 3) revealed no evidence for helicity of the ovoid of filamentous forms; however, helical MLOs (Fig. 2) were present in sections of celery infected via *C. montanus* with a spiroplasma isolated from X-diseased cherry.

DISCUSSION

The isolation of spiroplasmas from 40 to 78% (Table 1) of diseased trees in three orchards contrasts sharply with the total percentages of samples from which spiroplasmas were isolated (4 and 5%) in 1980 and indicates the importance of continually

TABLE 1. Isolation of spiroplasmas from healthy and X-diseased cherry trees in the field[a]

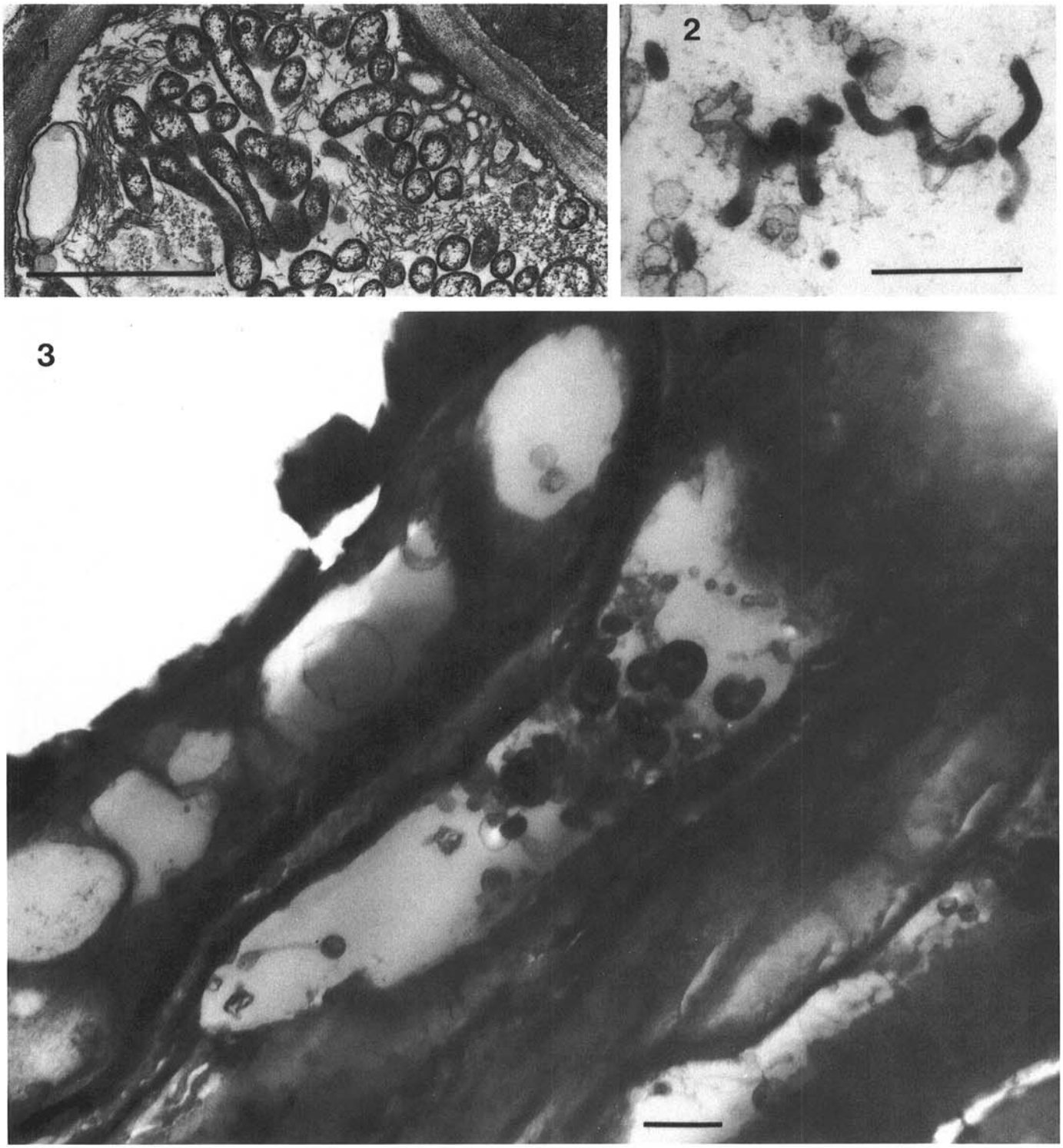
Year	Orchard number	Source trees[b]	Diseased or healthy	Trees sampled (no.)	Trees yielding spiroplasmas	Successful spiroplasma isolations (mo/day)	Spiroplasma-infected trees (%)	Total: Trees sampled throughout season (no.)	Total: Positive isolations (no.)	Total: Samples yielding spiroplasmas (%)
1980	1	*Prunus avium*/ *P. avium*	Diseased	15	8	4/30, 5/14 5/16, 5/21	53	289	11	4
	2	*P. avium*/ *P. mahaleb*	Diseased	9	7	5/14, 5/21	78	229	11	5
1981	3	*P. avium*/ *P. mahaleb*	Diseased	15	0	None	0	...[d]	...	...
			Healthy[c]	10	0	None	0	...	...	...
	4	*P. avium*/ *P. mahaleb*	Diseased	10	4	4/1, 4/14	40	...	...	...
			Healthy[b]	10	6	4/1,4/14	60			

[a] Samples were collected at least once weekly from April–September 1980 and from March–May 1981.
[b] Tree/rootstock.
[c] The initial diagnosis of healthy in April was confirmed at harvest time by presence of symptomless fruit, and MLOs were not detected in phloem of healthy trees later in the season.
[d] Isolations in 1981 were restricted to the fruit-ripening period.

isolating at regular time intervals from individual trees when searching for spiroplasmas in woody plants. The optimum time for spiroplasma isolation apparently varies slightly from year to year and may relate to chemical changes in phloem, which occur with the onset of fruit ripening.

The ability to isolate spiroplasmas from leaf and peduncle tissues of individual trees only during a 2- to 3-wk period agrees with previous reports on the cycling of the MLO associated with pear decline (12,13) and with *S. citri* in stubborn-diseased citrus (2). Spiroplasmas previously isolated from trees with X-disease in 1978 and 1979 were obtained during a limited time period, similar to the 3-wk period reported here (B. C. Raju, *unpublished*; D. G. Garrott, *unpublished*).

The presence of spiroplasmas in 60% of symptomless trees in one orchard in 1981 (Table 1) suggests that the etiological agent of X-disease is not a spiroplasma. The isolation of spiroplasmas from only 4 and 5% of total X-diseased cherry samples (all of which were from trees containing MLOs) supports the conclusion that the



Figs. 1–3. 1, Filamentous and ovoid mycoplasmalike organisms (MLOs) in phloem of cherry with Green Valley X-disease. Section is a 70-nm-thick cross section. **2,** High-voltage (650 kV) electron micrograph (HVEM) of helical MLOs in a longitudinal section (~1 μm) of celery infected with a spiroplasma isolated from cherry with Green Valley X-disease. **3,** HVEM of a longitudinal section (~1 μm) of cherry with Green Valley X-disease. Note presence of filamentous and ovoid MLOs in central sieve tube member (STM). A second MLO-containing STM, partially obscured by callus is visible in lower right. Bars indicate 1 μm.

spiroplasmas are distinct from the MLO. The cherry spiroplasmas are serologically similar to *S. citri*, which is widely distributed in California (1) and has a wide experimental host range including cherry (1). Therefore, the association of spiroplasmas with X-diseased trees may simply be a chance coinfection with *S. citri*, which also occurs in symptomless trees.

Our data do not completely preclude the possibility that the spiroplasmas isolated from symptomless trees are an early form of the MLO, which causes X-disease, and that the healthy trees may develop X-disease symptoms in subsequent years as a result of the spiroplasma infection. We believe that this possibility is unlikely, since no MLOs were seen later in the season in the same symptomless trees from which spiroplasmas were isolated in April. However, MLOs in diseased trees, which are often not detected with electron microscopy in April, develop into detectable populations by early May (D. G. Garrott, *unpublished*) and increase in concentration throughout the season.

We previously presented evidence that both nonhelical MLOs and spiroplasmas were present at some times of the season in X-diseased phloem (6) by using electron microscopy of thick cross sections. Morphological characterization of MLOs as revealed by cross sections is dependent upon the observer's experience, comparison to known morphotypes, and generally requires interpretation. Longitudinal thick sections (7) proved useful for clearly visualizing helical MLOs; however, MLOs that appear to be nonhelical in the same sections are again open to interpretation. One plausible interpretation is that apparently ovoid MLOs are actually transverse sections of filamentous forms as was originally suggested with MLOs associated with peach X-disease (4). The interpretation could be continued to suggest that filamentous forms may indeed be helical and hence, the spiroplasmas and MLOs associated with X-disease are the same organism. HVEM used in this study allowed the clear elucidation of nonhelical MLOs (Fig. 3) and supports the suggestion by Sinha and Chiykowski (14) that spherical and ovoid MLOs associated with X-disease are distinct from filamentous forms. The clear presence of helical MLOs (visualized by using HVEM) in plants from which spiroplasmas were isolated (Fig. 3) contrasts with the inability to detect helicity in diseased cherry, which did not yield spiroplasmas.

The results of isolation and electron microscopy studies suggest that healthy, symptomless, and X-diseased cherry are occasionally infected with *S. citri*, which does not contribute to the symptoms of X-disease. This suggestion has important consequences for disease diagnosis. An ELISA system has recently been developed (B. C. Raju and G. Nyland, *unpublished*) using a spiroplasma isolate as the antigen source, and was tested for the detection of X-disease. If our suggestion that the spiroplasma is not the pathogen is correct, such an ELISA system would be of limited value for diagnosing X-disease. The final determination of the pathological role of cherry spiroplasmas will depend upon results of tests currently in progress comparing the pathogenicity of the isolates with *S. citri* and with the X-disease agent.

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