Current Status of the Etiology of Pear Decline

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ABSTRACT

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Samples from diseased pear were collected from each of 10 trees from June 1980 to April 1981. Pear psylla were also collected from pear at the same time. Helical and motile spiroplasmas were isolated from two of 10 trees four times in 330 isolation attempts. Spiroplasma also were isolated from psylla collected during early December 1980, but not from 30 other collections made at other times of the year. No spiroplasma was isolated from healthy pear grown from seed, or from healthy or diseased periwinkle. The isolates from pear were serologically and culturally indistinguishable from *Spiroplasma citri*. No transmission to healthy pear occurred when pear psylla were injected with these spiroplasma isolates. No symptoms were seen in pear and periwinkle on which field-collected psylla were caged. Dienes' stain did not clearly distinguish healthy from pear decline-affected pear tissue. An infectious agent has been transmitted via dodder from pear with pear decline symptoms to periwinkle. Diseased periwinkle could be distinguished from healthy with Dienes' stain. Based on inconsistent isolations of spiroplasmas from pears and on results from pathogenicity studies, we conclude that the spiroplasma isolated from pear is not the causal agent of pear decline.

Pear decline, a destructive disease of pear trees (*Pyrus communis* L.), was first found in British Columbia in 1948 (20). During the past 30 yr, the disease has killed many producing pear trees in North America (19–22,32), Czechoslovakia (4), France (18), Greece (1), Italy (1), Switzerland (29), West Germany (3,11), and Yugoslavia (12). The pear decline agent was transmitted by grafting (5,30) and by the pear psylla, *Psylla pyricola* Foerster (15). Mycoplasmalike organisms (MLO) have been associated with diseased trees (3,4,14,19) and *P. pyricola* (13). *P. pyricola* transmitted an infectious agent, apparently an MLO, from decline-affected pear to periwinkle (16).

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The most obvious symptoms of pear decline are either quick decline and collapse of the tree or slow decline and leaf curl. Quick decline is most often associated with oriental rootstocks like *P. serotina* Rehd. and *P. ussuriensis* Maxim. Trees on oriental rootstocks suffer phloem necrosis at the graft union (2). When the tree is under stress, it may suddenly wilt and die within a few days. Slow decline is characterized by reduced growth of shoots and fruit spurs. Affected trees in early stages of symptom development have red or purple, downward-curled fall foliage in contrast to the normal yellow color of senescent flat, healthy leaves. Trees with chronic symptoms have small leaves with upward-curled margins and lose their leaves prematurely. Trees affected by slow decline produce abnormally short shoots but may live for many years or may die within a few years after infection. The leaf curl symptom of pear decline is most commonly seen in trees on decline-tolerant rootstocks such as seedlings of *P. betulifolia* Bunge., *P. calleryana* Decne., and *P. communis*. Young trees that originally show leaf

curl symptoms may express slow decline in subsequent years. Terramycin® (Pfizer Inc., Brooklyn, NY 11206) effectively controls pear decline in California (23) and in Connecticut (19). We isolated spiroplasmas from decline-affected pear trees in 1978 (24) but could not repeat this consistently. Hence, we investigated the seasonal effect on isolations during 1980–1981 by collecting plant and insect samples at various times throughout the year from the same trees. We report here our isolation methods and results, and the serological characteristics of the isolates we obtained from pear and *P. pyricola.*

MATERIALS AND METHODS

Pear decline-affected Bartlett trees, which had been marked during September–October 1979 in a commercial orchard near Placerville, CA, were the principal sources of plants and *P. pyricola* used in our isolation attempts. Pear grown from seed in an insect-proof greenhouse were used as controls or as test plants in transmission attempts. Three twig or stem samples from the 10 previously marked trees were individually collected once a week from June to December 1980 and twice a month from January to April 1981. Fruit samples also were collected from August to December 1980. Monthly from June to November 1980 and twice a month from December 1980 to April 1981, *P. pyricola* were collected from pear and nearby apple trees with a beating tray. Plant and insect samples in plastic bags were carried in an ice chest to the laboratory where isolations were made on the same or the following day. At least 50 psylla were collected on each date; half of them were used for isolation and the other half were placed on periwinkle (*Vinca minor* L.) or Bartlett pear seedlings in an insect-proof greenhouse. We used dodder (*Cuscuta compestris*) in attempts to transmit a disease-producing agent (16) from greenhouse-grown pear trees that had pear decline symptoms to periwinkle test plants. Dodder seeds were germinated on moist filter paper in petri plates at room temperature. When the stems were 6–7 cm long they were twisted around young stems of diseased 'Precocious' pear (obtained from H. Schneider, University of California, Riverside) grown in a greenhouse. Young periwinkle plants were later placed next to diseased pear and connection was established through dodder for at least 3–4 wk. Dodder was later removed from periwinkle and the plants observed for symptoms.

Thin cross and longitudinal sections of healthy and decline-affected pear leaf petioles and stems were cut in distilled water. The sections were transferred to 0.2% Bacto Dienes' stain (Difco) for 10 min, rinsed, and mounted in distilled water for observation (9,10,26).

The isolations were made in ME-1 or ME-2 medium (Table 1) as follows: Leaf samples were surface-sterilized in 1.5% sodium hypochlorite for 2.5 min, washed three times in sterile glass-distilled water, and rinsed once in liquid medium. Petiole and mid-vein tissue (0.5–0.8 g) were homogenized in 10 ml of medium in a sterilized mortar and pestle. The medium was quickly pipetted into a sterilized screw-capped tube and centrifuged at 2,500 *g* at 20 C for 5 min; the supernatant was passed asceptically through a Nalgene® (Nalgene Company, Rochester, NY 14602) filter (0.45-μm pore diameter) by using very slight suction. Three-tenths to 1 ml of the filtrate was added to 5–10 ml of medium in a screw-capped tube and incubated at 30 C. Isolations were also made using the procedures described before (26). Two uninoculated tubes with culture medium and healthy checks were included as controls. During the winter, leaf buds and inner bark of stems were used for isolations instead of leaf tissue.

Isolations from green fruit were made as follows: The fruit pedicle was gently removed and the end that was attached to the fruit was cut off and the isolations were made as described above. During October–December, seeds were collected from 5–10 mature fruits, surface sterilized in sodium hypochlorite, rinsed in water, and in medium, and homogenized in 10–15 ml of culture medium in a sterilized mortar and pestle. After 30 min, samples were clarified by centrifugation (2,500 *g*, 5 min, 20 C) and the procedure continued as described above. Plates containing agar medium (ME-1 plus 0.8% ion agar) were also inoculated with 0.1–0.2 ml of inoculum and incubated at 30 C. Usually two or three small aborted seeds were obtained from a fruit taken from a diseased tree.

For each attempted isolation from pear psylla, 10–25 adult insects were used and the isolation procedures were the same as described above.

When a color change (from red to orange-yellow) was observed in the medium, the presence of spiroplasma was confirmed by dark-field microscopy. The organisms were subcultured one or more times in ME-1 broth medium and then transferred to ME-1 agar medium. The inoculated plates were sealed with Parafilm® and incubated at 30 C. After 8–10 days of incubation, the plates were examined and the colony morphology studied.

An isolate (PR-T_6) filter-cloned three times (26,27) was used for characterization studies. Ultrastructural studies were done by using negative staining and transmission electron microscopy as previously described (26,27). Growth of the spiroplasma isolate at 19, 22, 25, 28, 31, 34, 37, 40, and 43 C, sterol requirement, and the ability of PR-T_6 isolate to hydrolyze arginine were tested according to the procedures already described (26).

Antisera against two spiroplasma isolates (PR-T_1 and PR-T_6) were produced in New Zealand white rabbits (25). Antisera were produced previously in rabbits by the same methods against the following spiroplasma isolates: *S. citri* (28) (ATCC 27563), corn stunt spiroplasma strain I-747 (6) (ATCC 29051), honeybee spiroplasma strain AS-576 (8) (ATCC 29416), and flower strain TT-15 (27) (ATCC 33214). The serological relationships of the spiroplasma isolates from pear and *P. pyricola* were studied by growth-inhibition (7,8) and deformation (31) tests with some modification (27).

Noninfective *P. pyricola* nymphs and adults raised from eggs and maintained on healthy pear seedlings were used for injection and transmission studies. Insects (35–80 per experiment) were injected with subcultures of the spiroplasma isolate using hollow glass needles. The inoculum for psylla injection was prepared as follows: 30 ml of culture medium containing log-phase growth of spiroplasma was centrifuged (21,000 *g*, 15 min, at 4 C) and the pellet suspended with a mixer in 1–3 ml of 10% sucrose in phosphate buffer (0.1 M, pH 7.5). Inocula for use in insect injection prepared by these methods contained approximately 4–5 $\times$ 10^8 colony-forming units per milliliter.

Insects to be injected were anesthetized in CO_2 bubbled through water. Inoculum was spotted in small drops on Parafilm® stretched

TABLE 1. Constituents (per liter) of culture media for isolation and growth of spiroplasma from pears

Constituent[a]	Medium ME-1	Medium ME-2
1. PPLO broth base	21 g	21 g
2. Tryptone	1 g	1 g
3. Peptone	1 g	1 g
4. Glucose	1 g	1 g
5. Fructose	1 g	1 g
6. Sorbitol	50 g	
7. Sucrose	1 g	10 g
8. HEPES buffer (0.06 M)	600 ml	600 ml
9. Phenol red	0.02 g	0.02 g
10. GG-free fetal bovine serum	200 ml	200 ml
11. Fresh yeast extract solution (25%)	100 ml	100 ml
12. Schneider's Drosophila medium	1 ml	
13. CMRL-1066 medium	1 ml	
14. TC-199 medium (IX)	1 ml	1 ml
15. Earle's balanced salt solution		1 ml

[a] Constituents 1–9 were autoclaved at 121 C and 1.27 kg/cm^2 for 20 min after the pH was adjusted to 7.4 (ME-1) or 7.6 (ME-2). Constituents 11–15 were filter sterilized (0.2-μm membrane filter) and added to the autoclaved and cooled medium. Fetal bovine serum was heat-inactivated in hot water at 56 C for 1 hr before use. Constituents 1–3 and 9 were obtained from Difco Laboratories, Detroit, MI 48232; constituents 4–8 were obtained from Sigma Chemical Co., St. Louis, MO 63178; and constituents 10–15 were obtained from Grand Island Biological Company, Santa Clara, CA 95050.

over solid ice in a petri dish and drawn by capillary into needles pulled by machine (Roderick Craig, *unpublished*) from 3-mm o. d. flint glass tubing. The inoculum was injected through the intersegmental membrane of the venter of the abdomen by gentle air pressure. The average dose, calculated by gravimetric methods, was 0.02 μl.

Injected *P. pyricola* were placed on healthy Bartlett pear seedlings and transferred to new plants at weekly intervals. Test plants were sprayed with dimethoate (Cygon 25 WP, American Cyanamid Co., Princeton, NJ 08540) after exposure to *P. pyricola*, held in a greenhouse (21–27 C), and monitored periodically for pear decline symptoms. Isolations from the young leaves of test plants were made according to methods described previously (24,26).

RESULTS

In tests utilizing Dienes' stain we were not able to clearly identify diseased and healthy pear petioles and stems in 50 samples. The phloem of diseased tissue stained light blue, but the phloem of some healthy tissue stained the same. Healthy material came from plants grown from seed in an insect-proof greenhouse and the diseased leaves were showing typical field symptoms of pear decline. Unlike pear tissue, tissues from periwinkle plants that developed disease symptoms (16) after transmission by dodder could be readily distinguished from healthy by observing petiole and stem sections stained with Dienes' stain. The phloem of diseased periwinkle stained distinct blue whereas sections of healthy tissue remained unstained. The blue stain in phloem was observed in 25 of 25 diseased stems and leaves of periwinkle, but not in that of equal numbers of healthy samples. Diseased periwinkle plants showed general yellowing of leaves, veinal chlorosis, and small leaves. Some of the leaves showed wavy (zig-zag) mid-veins and the plants produced small flowers compared to healthy. No phyllody, virescence, or proliferation was observed.

Helical and motile spiroplasmas were isolated from pear trees showing symptoms of pear decline in only four of 330 isolation attempts from June 1980 to April 1981. Two of 10 trees yielded spiroplasmas on 4 and 10 September 1980, when the leaves had just begun to develop leaf curl and reddening symptoms. We were unable to isolate spiroplasma or other mollicutes from the remaining eight trees, which were showing the same type of symptoms. During October and November all the marked trees clearly showed typical pear decline symptoms, but we were not able to isolate spiroplasma from any tree. The two trees that yielded spiroplasmas during the first and second weeks of September 1980 yielded no organisms later. No spiroplasma was isolated from greenhouse-grown healthy pear seedlings or from uninoculated media.

No spiroplasma or mycoplasma was isolated from 660 diseased fruit or seeds collected at various times from 10 different trees in 66 attempts.

Spiroplasma was isolated from pear psylla in two of 32 attempts. Positive isolations were obtained from psylla collected only during early December but not from other collections. No symptoms were seen in any of the periwinkle or pear on which field-collected psylla were placed.

No spiroplasma was isolated in 41 attempts from leaves, stems, flowers, or roots of symptomatic periwinkle that had been connected with dodder to decline-affected pear.

Spiroplasmas were seen in primary liquid culture after 12–16 days of incubation at 30 C. Growth of the organism was indicated by a change in color of the culture medium from red to yellow and the presence of spiroplasma was confirmed by dark-field microscopy. All four of the isolates formed typical "fried-egg" shaped colonies in ME-1 or ME-2 agar medium after 7–8 days of incubation at 30 C. Helical and nonhelical organisms were seen by dark-field microscopy in the water mounts made from agar colonies.

Morphologically and ultrastructurally, the spiroplasma isolates resembled spiroplasmas from other sources. In our preliminary studies with negatively stained organisms we observed a "cap"-like structure on the main body of spiroplasma from pear. Further work is necessary to confirm the consistent presence of this structure.

The isolate PR-T_6 grew well at 28 and 31 C. Growth was also observed at 22, 25, and 34 C, but not at 19, 37, 40, and 43 C. The growth pattern of PR-T_6 at various temperatures was similar to that of our horseradish isolate HR-101 (26), which was also identical to *S. citri* in serological tests. Inhibition zones 9–10 mm wide were seen around digitonin disks placed on the agar medium and the organism was unable to grow in ME-1 liquid medium without serum. Isolate PR-T_6 utilized arginine. The color change in the medium was similar to that observed with HR-101 (26).

The isolates PR-T_1 and PR-T_6 were serologically identical to *S. citri* by growth-inhibition and deformation tests (Tables 2 and 3). The inhibition zones of pear isolates and *S. citri* were almost the same in homologous and heterologous reactions (Table 2). Like *S. citri*, pear isolates showed some serologic cross-reactions with corn stunt spiroplasma, honeybee, and flower isolates. In deformation tests also the pear isolates were indistinguishable from *S. citri* (Table 3). Similar results were obtained with isolates from *P. pyricola*. There was no transmission to pear by the injected *P. pyricola* and none of the pears showed symptoms. In 11 experiments, we injected a total of 266 pear psylla with cultures ranging from the second to the ninth passage, but with no success. No spiroplasma was isolated in 24 attempts from pears fed upon by the injected *P. pyricola*.

DISCUSSION

Although Dienes' stain could detect MLO in diseased plants (9,10,26), we were not able to use it to distinguish healthy and diseased pears. We have obtained the same type of results with X-disease affected cherry, nectarine, and peach (B. C. Raju and G. Nyland, *unpublished*). Unlike pear, healthy and diseased periwinkle (infected from symptomatic pear) can be easily distinguished by using Dienes' stain.

The percentage of our spiroplasma isolations from diseased pear was low. Only two of 10 trees individually processed yielded spiroplasmas. We were not able to isolate any mollicutes from the remaining eight trees even though they had excellent pear decline symptoms. No mycoplasmas or spiroplasmas were isolated in 66

TABLE 2. Serological relationships of spiroplasma isolates from pear (*Pyrus communis*) with other spiroplasmas based on the growth-inhibition test.

Antigen (isolate)[a]	Width of inhibition zone (mm) with antiserum to				
	PR-T_1	PR-T_6	*S. citri*	CSS	TT-15
PR-T_1	10	8	12	2	4
PR-T_6	10	9	11	2	5
S. citri	12	9	12	3	6
CSS-1-747	4	3	4	13	3
TT-15	6	4	5	4	12
AS-576	5	4	5	4	12

[a] PR-1 and PR-6 are spiroplasma isolates from pear; *S. citri* = *Spiroplasma citri*; CSS = corn stunt spiroplasma; TT-15 = tulip tree flower spiroplasma from California, and AS-576 = honeybee spiroplasma.

TABLE 3. Serological relationships of *Spiroplasma citri* and isolates of *Spiroplasma* from pear (*Pyrus communis*) based on the deformation test

Antigen (isolate)[a]	Reciprocal antibody titer with antiserum to	
	PR-T_1	*S. citri*
PR-T_1	16,384	8,192
PR-T_6	16,384	8,192
S. citri (MO)	8,192	8,192
S. citri (CA)	16,384	8,192

[a] PT-T_1 and PR-T_6 are spiroplasma isolates from pear; *S. citri* (MO) and *S. citri* (CA) are isolates of *S. citri* from Morocco and California, respectively.

attempts from seeds collected from decline-affect trees (Table 2). *S. citri* can be readily isolated from diseased citrus fruits. We could isolate spiroplasmas only during the early stage of symptom development, but not at other times when the plants were showing pronounced symptoms. A similar situation was observed with X-disease-affected peach and cherry (*unpublished*, and J. W. Kloepper, *personal communication*) from which spiroplasmas could be isolated only for a short period of time of the year. When we mixed one leaf from a tree that yielded spiroplasma with four leaves (individually collected from four other trees that yielded no spiroplasma) we were able to isolate spiroplasma from this mixed sample. Predictably, we isolated spiroplasma more frequently when we mixed samples from different trees (24). However, in this study we used samples from each tree separately. More than 20 different media formulations were tried for the initial isolation attempts. We found the media reported here to be superior to others we have used for isolations from other fruit trees and for isolating *S. citri* from stubborn-affected citrus. By using similar methods, Kloepper and Garrott (17) also isolated spiroplasma from diseased fruit trees.

Our results indicate that a spiroplasma identical to *S. citri* in morphology, ultrastructure and serology is present in some diseased pears in California during the early stage of pear decline symptom development. By using the same media and techniques, we also isolated a spiroplasma from decline-affected pear from Connecticut during 1979 but not during 1980 in one of several attempts. This isolate like PR-T_6 was indistinguishable from *S. citri*. The exact role of this organism in pear decline is not known. Results from our attempts to transmit several California isolates to pear via *P. pyricola* were negative. On the basis of our data, we conclude that the isolated spiroplasma is not the causal agent of pear decline and that *S. citri* may occur as part of a mixed infection with the pear decline agent in some plants.

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