Brittle Root Disease of Horseradish: Evidence for an Etiological Role of *Spiroplasma citri*

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ABSTRACT

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The symptoms of brittle root (BR) disease of horseradish include foliar chlorosis, stunting, and a discoloration in the phloem ring of affected plant roots. The disease has resulted in severe crop losses in Illinois horseradish in certain years since it was first reported in 1936, but no pathogenic agent has been conclusively implicated in the etiology of the disease until now. Dienes' staining of hand-cut sections of BR and nonbrittle root (NBR) horseradish roots revealed irregularly distributed blue-stained cells in the phloem of BR but not of NBR plants. Spiroplasmas were cultured in liquid media from all BR plants examined but from none of the NBR plants. Spiroplasmas were also isolated from *Circulifer tenellus* reared in captivity and allowed to feed on BR plants, and from horseradish plants initially free of BR symptoms to which infective leafhoppers had been given inoculation access. All spiroplasma isolates tested (including those from field-collected BR plants, from infective *C. tenellus* and from the plants on which infective leafhoppers were allowed inoculation access) were indistinguishable from *Spiroplasma citri* by serological growth inhibition and organism deformation tests, and by SDS-polyacrylamide gel electrophoresis. These results established for the first time a consistent association of a pathogenic agent with plants having BR symptoms and its absence from symptomless plants. *C. tenellus* injected with horseradish isolates of *S. citri* grown in pure culture transmitted the spiroplasma to horseradish test plants, which subsequently developed symptoms of BR. *S. citri* was reisolated from these plants. These results indicate an important etiological role for *S. citri* in BR disease of horseradish.

Additional key words: Armoracia rusticana, mycoplasmalike organism.

Brittle root (BR), possibly the most destructive disease of horseradish (23), was first reported in Illinois in 1936 (11). The disease is present almost every year as isolated, roughly circular patches of affected plants in some growers' fields, but epidemics and severe crop losses occurred in 1936, 1953–1954, 1975 (21), and 1979 (*unpublished*). Named for typical loss of root flexibility in affected plants, BR is also characterized by foliar chlorosis, stunting, development of a darkened ring in the phloem region of the root, and sometimes wilting. Affected plants usually die within a few weeks of symptom onset.

The cause of BR has never been demonstrated. In 1940, Kadow and Anderson (12) used the term "brittle root" to describe a disease in Illinois horseradish which, based on similarities in symptoms to those reported by Severin in California horseradish, they attributed to curly top (CT) virus.

In 1929, Severin (22) described a condition in a California horseradish field which he believed to resemble CT disease of sugar beet. He reported experimental transmission of the CT disease agent from infected sugar beet plants to horseradish and back to sugar beet via the beet leafhopper, *Circulifer tenellus* (Baker) (formerly *Eutettix tenellus*). Thornberry and Takeshita (24) reported the presence of *C. tenellus* carrying CT virus in Illinois horseradish fields during the 1953 BR outbreak.

During many years of work on BR disease, however, no pathogen has been associated consistently with diseased plants; no clear-cut evidence has been obtained for presence of a pathogen in plants with symptoms and its absence in plants without symptoms. Early in 1980, mycoplasmalike organisms (MLO) were found by E. Backus and D. L. McLean, University of California at Davis, in electron micrographs of tissue sections of a BR horseradish plant obtained from us. Subsequently, a spiroplasma was cultured in vitro from the same diseased plant by G. Nyland and B. C. Raju, University of California at Davis. The results of these and subsequent independent studies are reported in the preceding paper (20). As a result of these findings we have conducted a thorough investigation of the possible involvement of a spiroplasma in the horseradish BR disease. Here we report evidence that *Spiroplasma citri* was present in all examined horseradish plants having BR symptoms and was not found in any plant lacking such symptoms. We present methods for culture of the organism from plants and insects. We also report transfer of the spiroplasma by infective *C. tenellus* that had acquired the spiroplasma either by feeding on BR plants or by microinjection of the spiroplasma grown in pure culture. BR symptoms were obtained in, and *S. citri* reisolated from, test plants in both cases.

MATERIALS AND METHODS

Source of plants. Horseradish plants (*Armoracia rusticana* Gaertn., Mey., and Scherb.) used for spiroplasma isolation attempts were collected in June, July, August, and September 1980 from growers' fields in Madison and St. Clair counties near East St. Louis, IL (Table 1). Plants were selected and designated as 'brittle root' (BR), 'nonbrittle root' (NBR), or 'questionable brittle root' (QBR) diseased on the basis of foliar and root symptoms. A QBR designation was given when a plant appeared smaller than average, chlorotic, or wilted but lacked a distinct darkened ring in the root. Samples were sealed in plastic bags, transported in coolers to Urbana (and on one occasion to Beltsville,

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MD), and stored at 4 C for a maximum of 5 days prior to use.

Microscopy. Juice was expressed from portions of NBR and BR horseradish plants by squeezing leaf, stem, and root pieces over a glass slide and was examined by dark field microscopy with an ×100, iris diaphram-equipped oil immersion lens (total magnification ×1,250) on a Zeiss or Aus Jena photomicroscope. Broth cultures of spiroplasmas were monitored using the same optical systems. Helical spiroplasma cells were photographed after fixation with 4% glutaraldehyde. Colonies growing on agar-solidified LD8 medium (2,5) were photographed unstained at about ×5 magnification using substage illumination.

Hand-cut sections of petioles, stems, or roots of horseradish and other plants were examined with the light microscope. Dienes' stain (0.2%) was applied to the sections for 10 min, then replaced with distilled water (7). The presence of groups of cells with blue stain distributed irregularly in the phloem, and their absence in healthy controls, was taken as an indication of possible infection of tissue by an MLO.

For electron microscopy, spiroplasma cells from a log phase culture were negatively stained for 15 sec with 6% ammonium molybdate on Formvar-coated, 38-μm (400-mesh) grids and examined with a JEOL 100B electron microscope.

Isolation and cultivation of spiroplasma. The two media (DSM4 and LD8) employed for spiroplasma culture have been described previously (2,5). Root pieces were used as sources except in experiments designed to determine efficiency of spiroplasma isolation from various plant parts. When petioles and leaf blades were used, they were selected from the youngest foliage available. In one experiment, root pieces were surface sterilized prior to attempts to isolate spiroplasma; the tissue was dipped briefly into 70% alcohol, soaked for 2 min in 10% Clorox, and rinsed twice in sterile DSM4 medium. For routine isolations, when surface sterilization was not done, root pieces were washed thoroughly in tap water. Epidermis and central xylem were trimmed away with a flamed scalpel. The remaining root tissue (about 0.7 cm^3) containing the phloem was minced in 5 ml of sterile DSM4 medium. The chopped pieces were incubated in medium for 5–15 min, and the liquid was then passed through a sterile 0.45-μm Acrodisc filter. Primary cultures were made by transferring 0.2 ml of filtrate into 5 ml of sterile LD8 medium; a separate 0.1 ml aliquot of filtrate was seeded onto 1% Noble agar-solidified LD8 medium. Tubes and plates were incubated aerobically at 30–31 C. In some experiments, two primary culture tubes were prepared using each filtrate, one containing 200 ppm of ampicillin to inhibit bacterial growth and one without. The ampicillin had no apparent effect on spiroplasma growth.

Beet leafhoppers used as sources for isolation attempts were selected from a colony originally collected in August 1979 in St. Clair County, IL, and maintained in cages on sugar beet (*Beta vulgaris* L.) in the greenhouse. Leafhoppers fed on BR or NBR horseradish for 9 or 14 days were collected in plastic bags and frozen at −20 C until used. For isolation attempts, insects were thawed and homogenized singly or in groups of five or 15 in 2.5 ml sterile DSM4. Subsequent steps were identical to those used for plant tissues.

Presence of spiroplasmas in liquid media and within colonies on solid media was verified by dark field microscopy. Subcultures of spiroplasma isolates in liquid cultures were made at 48–60 hr intervals by transferring 0.1 ml into 5 ml of fresh sterile LD8 or DSM4 medium.

Five isolates were cloned by passing a 48-hr broth culture through a sterile 0.2 μm Acrodisc filter (Gelman Instrument Co., Ann Arbor, MI 48106), serially diluting the filtrate, plating selected dilutions on solid medium, and reintroducing organisms from a single colony into sterile broth medium (5).

Properties and identification of spiroplasma isolates. Prior to completion of serological tests for identification of the isolates obtained from horseradish, several tests were performed to ascertain the nature of the microorganisms obtained in cultures. Evidence for sterol requirement by six uncloned isolates based on growth inhibition by digitonin (8) was determined on solid LD8 medium containing 5% fetal bovine serum.

Filterability of the cultured cells of one isolate was determined by passing aliquots of a log phase culture through sterile Millipore or Acrodisc filters of pore diameters averaging 0.80, 0.65, 0.45, and 0.20 μm. Titer of each filtrate was determined by plating on agar-solidified LD8 medium incubated at 30–31 C and was expressed as colony-forming units (cfu) per milliliter.

A culture of one isolate which had been cloned twice was tested for ability to grow at 37 C by seeding duplicate LD8 plates with each of a 10-fold dilution series made in sterile DSM4 without serum. One plate of each dilution was incubated at 37 C and the other at 30 C for 2 wk.

Serological growth inhibition tests (3) were used to determine the major serological group of plant-associated strains to which the newly isolated spiroplasmas might belong (4). A filter paper disk soaked in antiserum to strain *S. citri* (Maroc R8A2) (Serogroup I, Subgroup A), AS576 (Serogroup I, Subgroup B), 23-6 (Serogroup II), or SR3 (Serogroup III) (6) was placed on solid DSM4 agar seeded 2 hr previously with a log phase culture of spiroplasma diluted in liquid DSM4 to yield 1,000–5,000 cfu per 6-cm-diameter plate. Growth of three singly cloned horseradish isolates and two uncloned isolates was compared with that of *S. citri* (Maroc R8A2) and strains 23-6 and SR3 from flowers. Zones of

TABLE 1. Results of attempts to isolate and cultivate in vitro spiroplasma from brittle root (BR) diseased and non-BR (NBR) diseased plants of horseradish

Exp. no.[a] and date[b]	Plant collection		Source (plant organ)	Surface sterilization of plant organs prior to isolation	No. of BR plants from which spiroplasma isolation was		No. of NBR plants from which spiroplasma isolation was	
	Location[c]	Date			Attempted	Positive	Attempted	Positive
I	Field 1	11 June	Roots	No	7	7	6	0
(17 June)			Roots[d]	Yes	7	7	6	0
II	Field 2	30 July	Roots	No	10	10	10	0
(6 August)								
III	Field 2	15 August	Roots	No	8	8	10	0
(20 August)								
IV	Field 3	11 Sept	Roots	No	6	6	6	0
(16 Sept)			Petioles	No	6	4	–[e]	–
			Leaves	No	6	5	–	–
Total plants					31	31	32	0
Total isolations					50	47	38	0

[a] Experiment I was performed at Beltsville, MD; the remaining isolations were performed at Urbana, IL. All plants were collected from fields in Illinois.
[b] Dates are in 1980.
[c] Farms in Madison and St. Clair counties in Illinois were sources of field-collected plants.
[d] The surface-sterilized root pieces in this experiment were taken from the same seven plants as those that were not surface sterilized and do not increase the total number of plants from which isolations were attempted.
[e] – Signifies no isolation was attempted.

inhibition from the edge of the disks were measured after 12 days of incubation at 31 C.

Major serogroup affiliation was confirmed and serological subgroup affiliation was determined by organism deformation tests (16,25). The procedure was that reported previously (4), except that sterile microtiter plates (Costar) were used, limiting the total volume of antiserum and antigen to 0.1 ml. Antiserum to *S. citri* (Maroc R8A2) was tested against four uncloned isolates from field-collected BR horseradish, against one isolate from beet leafhoppers previously permitted to feed on field-collected BR horseradish plants, against one isolate from test plants to which BR-fed leafhoppers had been given inoculation access and subsequently showed BR symptoms, against one isolate from test plants to which spiroplasma-injected leafhoppers had been given inoculation access and subsequently showed BR symptoms, and against *S. citri* (Maroc R8A2) as a control.

Polyacrylamide gel electrophoresis (PAGE) of cellular proteins. Organisms were grown in broth medium for 48 hr at 31 C, harvested by centrifugation, washed, and solubilized according to Mouches et al (18). Electrophoresis was performed after the method of Laemmli (15) in 9% acrylamide gels with 4% acrylamide stacking gels in an Aquebogue vertical slab gel electrophoresis apparatus (Aquebogue Machine Shop, Aquebogue, NY 11931).

Transmission of the BR-associated spiroplasma. For tests of transmission from plant to plant, 230 beet leafhoppers reared from eggs on sugar beet plants in the greenhouse were transferred to a cage containing three field-collected horseradish plants with BR symptoms. The same number was placed into a cage with three field-collected NBR horseradish plants. A third cage contained BR plants, but no insects. Two days later, six NBR horseradish plants ~6 wk old were put into each of the three cages. The horseradish test plants were cloned from NBR set roots by cutting root pieces into three sections with each section potted separately. This procedure yielded three plants of identical genotype from each original root. In this experiment, one member of each plant clone was placed in each of the three cages, a distribution which allowed detection of any naturally occurring BR in test plants. During the 2-day inoculation access period the cages were kept in an insectary at a constant temperature of ~22 C. After this period, the plants were held for an observation period in a greenhouse room with a daytime temperature of about 31 C. Symptoms were recorded after 52 days, and plants were used for isolation attempts after 55 days.

For transmission tests with cultured spiroplasmas, a suspension of the organisms was prepared from a 48-hr culture in LD8 medium. The spiroplasmas were sedimented at 15,000 rpm for 30 min in a Sorval SS-34 rotor and resuspended in an equal volume of 7 mM sodium phosphate pH 7.0 containing 3 mM NaCl and 0.3 M sucrose (PBS-sucrose). In the first experiment, the horseradish *S. citri* isolate used had been subcultured 10 times to a total dilution of 10^{-19}; in the second experiment, cultured spiroplasmas in the 19th subculture (10^{-37} dilution) were used. Insects anesthetized by CO_2 were injected through hand-drawn glass needles attached to a hydraulically operated system consisting of oil-filled polyethylene tubing and a tuberculin syringe connected to an ISCO microapplicator (Instrumentation Specialties Co., Inc., Lincoln, NE 68504). Each insect received 0.2–0.3 μl of spiroplasma suspension in PBS-sucrose or PBS-sucrose only. Injected leafhoppers were held for 14–15 days on sugar beet or horseradish and then transferred singly or in groups of two to six onto horseradish test plants for an inoculation access period of 6–7 days. One plant of each plant clone was used for inoculation access by leafhoppers injected with cultured spiroplasmas, another was used for inoculation access by leafhoppers injected with buffer and the third was left without exposure to insects. All phases of these tests were conducted in a growth chamber with 16 hr of light each 24 hr and a temperature regime of 28 C (day) and 22 C (night). Attempts to reisolate spiroplasma from test plants were made within 4–6 wk after the beginning of the inoculation access period. In all isolation attempts from experimentally inoculated and control plants, the samples were coded so that the individual doing the isolations did not know the treatments until after data were recorded.

RESULTS

Symptomatology. Initial field symptoms of BR in horseradish include chlorosis and downward curling of the leaf edges. Affected plants in the field usually grow more slowly than adjacent symptomless horseradish (Fig. 1). If plants are uprooted at this time, a discoloration is evident just external to the cambial ring, in the region of the secondary phloem (Fig. 2). Originally yellow-tan in color, the ring deepens to brown and finally black as disease develops. Infected roots characteristically break easily when bent, in contrast to the very flexible roots of NBR plants. There is a pronounced reduction in the number of lateral roots. Plants affected early in the season usually die within a few weeks of the appearance of symptoms, as foliage becomes necrotic from the edges inward. Root tissues remain alive longer, but usually succumb to root rot caused by secondary organisms. The resulting losses in yield and root quality are so great in epidemic years that the disease may become economically devastating. Plants affected late in the season may survive until fall harvest. Secondary roots taken from these symptomless survivors and used as planting stock the following spring often fail to sprout or, if they do, they develop symptoms and die early in the season.

Presence of spiroplasmas in tissues. No cells resembling spiroplasmas were seen in juice expressed from any of the horseradish samples. However, indirect evidence of infection by a mycoplasmalike organism was provided by Dienes' staining (7) of hand-cut sections of horseradish petioles and roots. Root sections of BR plants contained scattered patches of cells in the phloem region which stained a bright blue, while phloem of NBR roots

Fig. 1. Patch of brittle root (BR)-affected plants in a horseradish field in Madison County, IL. BR plant foliage is chlorotic, with necrotic and downward curling edges.

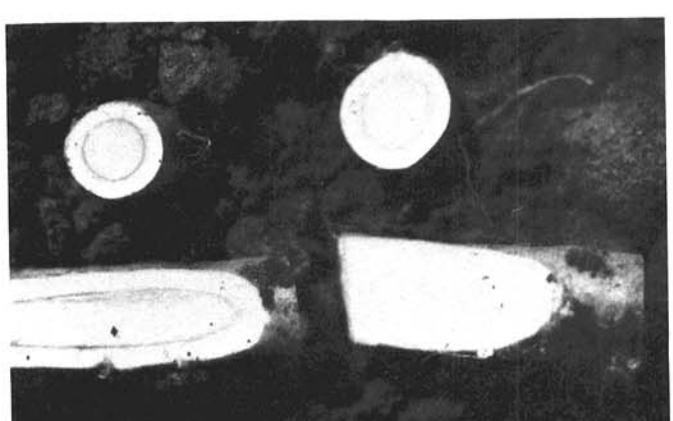


Fig. 2. Cross sections of horseradish roots. The root on the left shows the darkened phloem characteristic of the brittle root (BR) disease, while the root on the right lacks BR symptoms.

remained colorless or appeared to have a uniform pale blue background, also seen in the background of some diseased plants (Fig. 3). The deep blue patches in the phloem appeared to be due to the uptake of stain by the cell protoplasm rather than to coloration of the cell wall. Since Dienes' stain is commonly used to stain mycoplasma and spiroplasma colonies on agar, we interpreted the observation of such staining in BR roots as evidence for the presence of such organisms within phloem cells. In addition, stem sections of *Catharanthus roseus* (L.) G. Don infected with the aster yellows agent (presumed to be an MLO) and *C. roseus* infected with *S. citri* were examined; they also contained patches of blue phloem cells, while corresponding stem sections from healthy *C. roseus* did not. Phloem sections of *Lycopersicon esculentum* L., infected with the planta macho viroid (9), were free of blue-stained phloem cells, as were their healthy counterparts.

Isolation of spiroplasmas from plant tissues. A total of 31 BR and 32 NBR horseradish plants was collected from growers' fields for spiroplasma isolation attempts during the 1980 growing season (collection dates: 11 June, 30 July, 20 August, 9 September). Spiroplasmas were cultured from all BR plants, but from none of the NBR plants (Table 1). Isolation attempts from three plants designated QBR (with similar foliar symptoms, but no darkened ring in the root) were negative. In addition, one young horseradish plant grown from seed (field-grown horseradish is normally propagated vegetatively from root pieces) and two young plants regenerated by tissue culture (17) from leaf pieces of plants, one parental plant having symptoms of brittle root and the other without such symptoms (all courtesy of M. Meyer, University of Illinois), gave negative results in isolation experiments. No spiral cells were seen in sterile control media.

The effect of surface sterilization of root pieces was examined by repeating isolation attempts from sterilized root tissues of seven BR horseradish plants and six NBR plants. Results were unchanged; spiroplasmas were isolated from all the BR plants and from none of the NBR plants (Table 1).

When different organs of the same horseradish plants were used as sources in isolation attempts, roots were found to be the best sources of spiroplasmas (Table 1). Organs other than roots did not always yield cultures of spiroplasmas even from plants showing definite root and foliar symptoms; the reason for this is unclear.

Dienes' stain was used to check histological reactions of 20 field-collected horseradish plants which were also used in isolation experiments. A positive Dienes' stain reaction was recorded, and spiroplasmas were isolated, from each of the 10 BR plants, while the 10 NBR controls were all negative for both staining and culturing. Results were not as clear-cut, however, with plants grown in the greenhouse for several months. On one occasion a positive staining reaction was obtained in a root from which no spiroplasma could be isolated, and Dienes' reactions of several other root sections from these plants were difficult to interpret.

Properties of spiroplasma isolates. Dark field microscopy of spiroplasma cultures revealed the presence of helical cells which exhibited rapid flexional and rotational, but not translational, mobility in broth medium (Fig. 4). All isolates were similar in cell size and spiral morphology, though the morphology of all isolates varied with the type of medium and the age of the culture. In log phase culture, the spiroplasma cells were helical filaments about 0.15 μm in diameter and usually about 1–4 μm in length. A negatively stained preparation of one isolate examined by electron microscopy confirmed the helical morphology (Fig. 5).

All isolates produced colonies on agar-solidified LD8 medium. Colony morphology varied between isolates; however, colonies from any given isolate were all similar. Colonies ranged from very compact masses having granular texture and many satellite colonies (Fig. 6) to symmetrically diffuse, nongranular types having few or no satellites. "Fried-egg" shaped colonies were not

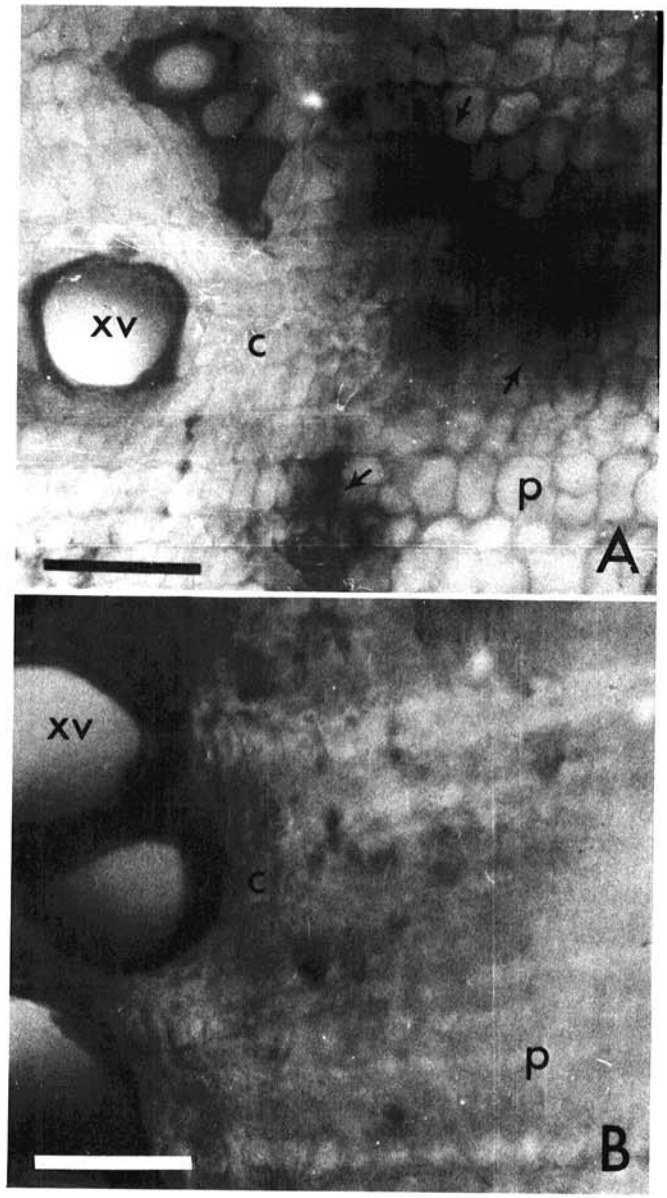
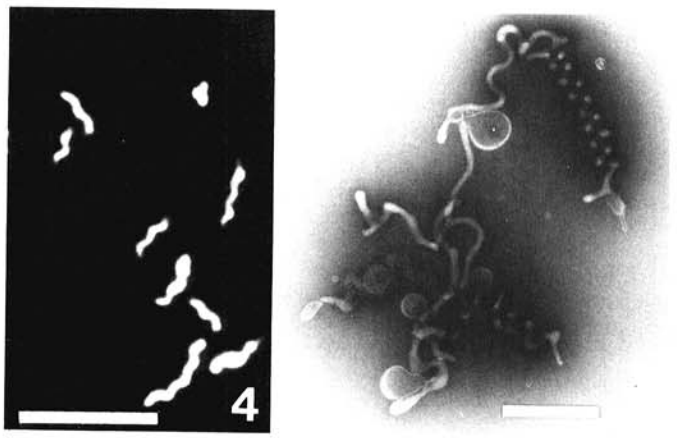


Fig. 3. Hand-cut razor blade sections of brittle root (BR)-infected or uninfected horseradish roots, stained with 0.2% Dienes' stain for 10 min. **A,** Irregularly distributed patches of blue-stained cells in the phloem (arrows) of BR-affected plants were interpreted as possible evidence of the presence of a mycoplasmalike organism with the tissue. **B,** Xylem vessels absorb stain in all sections, but the phloem of symptomless plants lacks the blue-stained cells. xv = xylem vessels, c = cambium, p = phloem. Bar represents 5 μm.



Figs. 4 and 5. Spiroplasma isolate from horseradish plants with BR disease. **4,** Dark field micrograph of a fixed (4% glutaraldehyde) broth culture. Bar represents 2.5 μm. **5,** Electron micrograph of negatively contrasted (6% ammonium molybdate) unfixed cells from broth culture. Bar represents 1 μm.

seen on solid LD8 medium, but were observed on rare occasions when spiroplasmas were plated onto agar-solidified DSM4 medium. Selected colonies from seven isolates, removed from petri plates to glass slides and crushed under coverslips for light microscopic examinations, consisted of a central core of immobile, filamentous cells surrounded by a ring of highly mobile, helical organisms. One isolate was shown to be filterable through pore diameters averaging from 0.80 to 0.20 μm, with only a 100-fold loss of titer in the latter case (Table 2).

No colonies were produced on any of the dilution plates of a doubly cloned isolate incubated at 37 C for 2 wk. A duplicate series of plates incubated at 30 C had colonies visible to the unaided eye after 7 days, and after 14 days the titer of the original culture was calculated from these plates as 7.7×10^9 cfu/ml.

Each of six isolates examined was strongly inhibited by digitonin, as was *S. citri* (Maroc R8A2), suggesting a sterol requirement typical of spiroplasmas (8).

Isolations from insects. Spiroplasmas were cultured from the combined group of 15 beet leafhoppers which had fed on BR horseradish for 9 days but not from the group of 15 insects exposed to NBR horseradish for an equal period of time. Twenty-two leafhoppers from each treatment collected after a 14-day acquisition access period were divided into groups of 15, five, one, and one for isolation attempts. Of those leafhoppers from BR plant cages, spiroplasmas were cultured from the groups of 15 and 5 insects but not from either single insect. Spiroplasmas were not found in culture attempts from any of the NBR-fed insects.

Transmission of the BR-associated spiroplasma from plant to plant. In a test of the transmissibility of the horseradish spiroplasma via the beet leafhopper, after 52 days four of the six NBR test plants in the cage with BR horseradish and leafhoppers had developed a dark brown phloem discoloration; three of the four plants had chlorotic and stunted leaves, while the leaves of the fourth plant, though dwarfed, remained green until 1 mo later when the leaves had yellowed and collapsed. Fifty-five days after the beginning of the experiment, root samples were taken for culturing attempts. Spiroplasmas were isolated from each of the four plants showing root symptoms. Culture attempts were negative from the symptomless plants in the cage with BR sources and leafhoppers and from the 12 test plants in the other two cages.

Transmission of BR-associated spiroplasma cultured in vitro. Two separate experiments gave the same results (Table 3). Plants fed upon by *C. tenellus* injected with cultured spiroplasma developed symptoms of BR disease (Fig. 8). Spiroplasmas reisolated from these plants were indistinguishable from the injected isolates (Tables 4 and 5, Fig. 7). NBR plants fed upon by buffer-injected insects and plants not exposed to insects did not develop BR symptoms, and spiroplasmas were not recovered in culture from such plants. One of the reisolated spiroplasma cultures has been deposited with the American Type Culuture Collection (ATCC 33479).

Identification of spiroplasma isolates. A close antigenic relationship between the horseradish brittle root spiroplasma and *S. citri* was shown by serological tests. Growth inhibition tests showed that the brittle root isolates were related to *S. citri* and to honeybee strain AS576 and should be considered members of Serogroup I (6) (Table 4). Results of organism deformation tests clearly showed these isolates to be strains of *S. citri* (Table 5).

We examined the electrophoretic patterns of proteins of spiroplasma isolates from field-collected BR horseradish, from beet leafhoppers made infective by acquisition feeding on BR plants, and from horseradish to which the BR-associated spiroplasma had been transmitted by the beet leafhopper after either acquisition feeding or microinjection. The protein patterns of all BR isolates were indistinguishable from one another and from that of *S. citri* (Maroc R8A2) (Fig. 7).

TABLE 2. Ultrafiltration of a horseradish spiroplasma isolate

Average filter pore size (μm)	Titer of filtrate (colony-forming units/ml)
Unfiltered	1.8×10^8
0.80	1.6×10^8
0.65	4.5×10^7
0.45	1.5×10^7
0.20	1.6×10^6

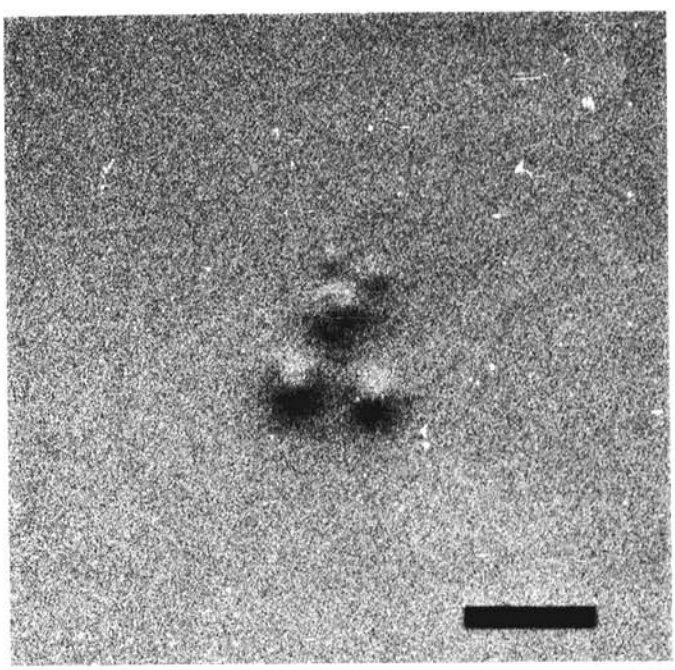


Fig. 6. Brittle root spiroplasma colony on 1% Noble agar-solidified LD8. "Fried-egg" morphology is not characteristic of these isolates on solid LD8 medium. Bar represents 0.1 mm.

TABLE 3. Microinjection of *Circulifer tenellus* with in vitro-cultivated horseradish isolates of *Spiroplasma citri* and their transmission to NBR horseradish plants

Experiment and date of injection	Isolate[a]	Insects per plant	Insects tested[b]	No. of test plants	No. of plants with BR symptoms[c]	No. of plants from which *S. citri* was isolated[d]
1. 15 July 1980	BR3	1	17	17	1	1
	Buffer control	1	16	16	0	0
2. 4 Aug. 1980[e]	BR7	1–6	62	34	25	25
	Buffer control	1–5	38	25	0	0

[a] Isolate BR3 (experiment 1) was in the 10th subculture at a dilution of 10^{-19} of the original 5-ml filtrate in primary isolation. Isolate BR7 (experiment 2) was in the 19th subculture, a final dilution of 10^{-37}.

[b] In experiment 1, leafhoppers were confined to test plants for 1 wk; in experiment 2, colonies were serially transferred three times at weekly intervals to new test plants.

[c] First symptoms were observed 20 and 24 days after inocultion in the first and second experiments, respectively.

[d] Isolations were made 4–6 wk after the beginning of the inoculation access period.

[e] In the second experiment, four of the 12 groups of leafhoppers were incubated on NBR horseradish and eight on sugar beet. The type of plant used for incubation made no difference in the relative number of successful transmissions.

DISCUSSION

The symptoms of brittle root (BR) in Illinois horseradish closely parallel symptoms attributed by Kadow and Anderson (12) to the curly top (CT) virus. The foliar symptoms of both BR and CT diseases are indicative of generalized stress on aerial portions of the plant. 'QBR' plants with similar foliar chlorosis, stunting, and wilting may easily be misdiagnosed as BR when, in fact, their roots have rotted or have been dislodged from the soil or otherwise stressed by causes unrelated to BR. Thus, it seems reasonable to assume that the effects of BR on the leaves of horseradish may be due in large measure to root dysfunction. Since we believe foliar symptoms to be unreliable in diagnosis of BR, we pulled suspect plants from the ground and cut their roots to verify diagnosis by the presence or absence of discolored phloem. This symptom too may sometimes lead to uncertainty of diagnosis, since some horseradish plants normally have a greyish-tan phloem ring, and the discoloration due to BR disease may be hard to discern early in disease development. However, as the disease progresses, the darkening of the ring in combination with external symptoms and root brittleness become unmistakable indicators of BR disease.

Plants with unquestionable BR symptoms always yielded spiroplasmas, while NBR plants never did. The correlation was noted regardless of the source of plants, age of plants, or time of year that the samples were collected. This was the first instance in the history of research on BR disease that a pathogen was consistently present in plants with BR symptoms and absent in plants without such symptoms. Further evidence supporting the association of a spiroplasma with BR disease was provided by the isolation and cultivation of a serologically identical spiroplasma

TABLE 4. Growth inhibition tests of horseradish spiroplasma isolates and *Spiroplasma citri* (Maroc R8A2), and spiroplasma strains 23-6, SR3, and AS576

Spiroplasma strain	Zone (mm) of growth inhibition with indicated antiserum[a]			
	Maroc	AS576	23-6	SR3
S. citri (Maroc)	7	7	0	0
BR2[b]	11	7	0	0
BR3	12	9	0	0
BR5	12	10	0	0
BR6	11	9	0	0
BR7	12	10	0	0
23-6	0	0	3	0
SR3	0	0	0	4

[a] Read after 12 days on DSM4 medium.
[b] BR2-BR7 refer to horseradish spiroplasma isolates.

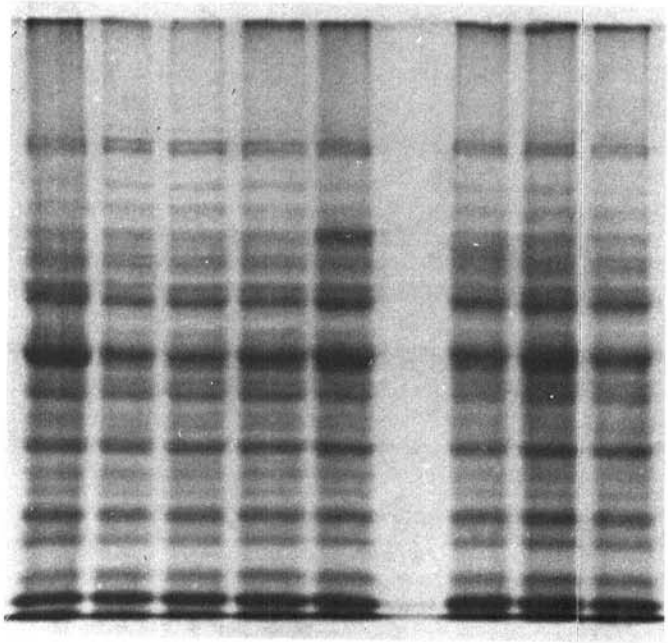


Fig. 7. Polyacrylamide gel electrophoretic patterns of proteins from (left to right) *Spiroplasma citri* strains (Maroc R8A2); horseradish strains BR1, BR3, BR6, and BR7 (from BR diseased field plants); BLH4, from *Circulifer tenellus* caged on BR diseased horseradish for 14 days; TBR4, from horseradish test plants exposed to beet leafhoppers that had fed previously on BR horseradish; and MBR8, from horseradish test plants exposed to beet leafhoppers microinjected with cultured BR7 isolate.

TABLE 5. Cross-serological organism deformation tests of several isolates of horseradish brittle root (BR) spiroplasma and *S. citri* (Maroc R8A2)

Antigen (strain or isolate)[a]	Reciprocal of antibody titer with indicated antiserum	
	Maroc	BR3
S. citri (Maroc)	8,000–16,000	16,000
BR3	8,000–16,000	16,000
BR7	8,000–16,000	–[b]
TBR4	16,000	–
BLH4	16,000	–
MBR8	8,000–16,000	–

[a] BR3 and BR7 are spiroplasma isolates from field-collected brittle root (BR) diseased horseradish, BLH4 is an isolate from *Circulifer tenellus* caged on BR diseased horseradish for 14 days, TBR4 is an isolate from horseradish test plants exposed to beet leafhoppers which had fed previously on BR horseradish, and MBR8 is an isolate from horseradish test plants exposed to beet leafhoppers microinjected with cultured BR7 isolate.
[b] – Signifies no deformation test was attempted.

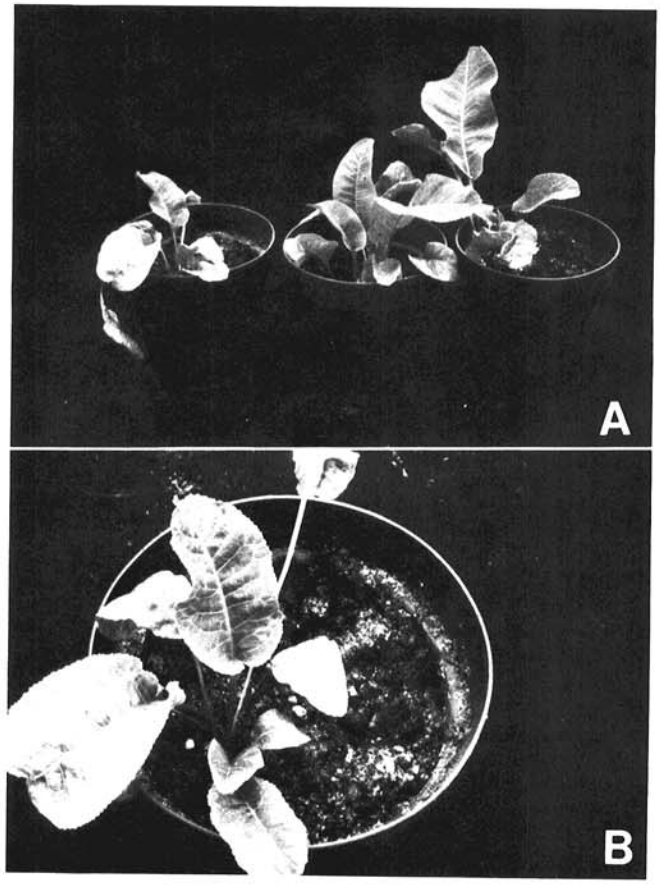


Fig. 8. Horseradish test plants cloned from one set root. **A,** From left to right, a plant exposed to beet leafhoppers microinjected with *Spiroplasma citri* (horseradish strain BR7 in PBS-sucrose), a plant exposed to leafhoppers microinjected with PBS-sucrose, and a plant not exposed to any insects. BR foliar symptoms of chlorosis, downward curling, and smaller size are evident. **B,** Close-up of the plant exposed to leafhoppers microinjected with spiroplasma. When cut, the roots of this plant were found to have a darkened phloem ring.

from beet leafhoppers allowed to feed on BR source plants, and from test plants exposed to leafhoppers carrying the BR agent, but not from their respective controls. Finally, transmission of axenically cultured horseradish spiroplasma by injected leafhoppers, the subsequent development of BR symptoms in test plants, and the reisolation of the same spiroplasma from the plants provided evidence that this spiroplasma, identified as *S. citri*, is the causal agent of BR disease.

Our work has not eliminated the possibility that other disease agents may be present in Illinois horseradish. Curly top virus may be present in some plants; however, in two separate experiments a total of 300 beet leafhoppers collected from a horseradish field with a high incidence of BR failed to transmit curly top virus to sugar beet plants (*unpublished*). Turnip mosaic, arabis mosaic, and cauliflower mosaic viruses also occur in horseradish (10,23). The occasional inconsistency between Dienes' staining and the presence of spiroplasmas could be explained by the possible presence in some plants of an MLO that cannot be cultured in the media tested. *Fusarium oxysporum* and an uncharacterized yellow-pigmented bacterium are routinely isolated from field-grown horseradish (B. J. Jacobsen, *personal communication*). However, none of these agents are consistently associated with the brittle root disease.

It was evident in our initial work that the spiroplasma of BR horseradish inhabits the interior of the plant and is not a surface resident, since neither surface sterilization nor trimming away the epidermal layers affected the success of isolation. Reaction to the Dienes' stain only in cells of the phloem cylinder supported this observation.

Variability in Dienes' stain reaction in sections of greenhouse-maintained plants indicates a need for refinement of the technique before it can be used reliably as a diagnostic tool in BR research. Also, Dienes' stain would be expected to give a positive test in the presence of any MLO within the tissue. A method to diagnose possible BR root sets prior to the onset of definite symptoms would be very useful; we are currently testing the use of ELISA for this purpose.

Seven spiroplasma isolates from BR-infective insects and from field-collected or greenhouse-inoculated plants were characterized to varying degrees. Serological tests and PAGE data showed that the horseradish BR spiroplasma was *S. citri*. Though similar in cell morphology, serological reactions, and PAGE, isolates differed in colony morphology and growth rates in broth media. These individual traits may be explained by a major genotypic difference between isolates or might be due to the presence of viruses or plasmids in some isolates and not in others. In fact, culture filtrates of two of three isolates tested for virus presence using an indicator lawn of *S. citri* (Maroc R8A2) produced plaques (*unpublished*).

Horseradish, a member of the family Cruciferae, is not a suprising addition to the host range of *S. citri*, as this spiroplasma has been isolated from a number of cruciferous hosts in California (19). Other recent reports of the association of *S. citri* with aster yellows disease are still under investigation (14). Until these reports are clarified, the presence of *S. citri* in horseradish from Illinois is the only confirmed case of natural occurrence of this spiroplasma in the United States east of Arizona. Although horseradish is grown commercially in California, Wisconsin, Pennsylvania, and Europe and by private gardeners in many states and countries, the BR disease has not been confirmed outside of Illinois.

Many questions of epidemiological importance remain to be answered, and the development of control measures for brittle root is essential. Our ability to isolate spiroplasma from BR-diseased horseradish, a consistent feature of the disease, has provided a reliable assay for evaluating greenhouse experiments and monitoring disease in the field. Reports of the insect vectors and alternate hosts of *S. citri* in California (1,13) have served as excellent guides for our attempts to answer the questions of spiroplasma pathogenicity, methods of disease spread, and possible reservoirs of the organism in Illinois and neighboring states. *C. tenellus* and *Scaphytopius acutus* (Say), both reported to be vectors of *S. citri* in California (13), occur with varying frequency in Illinois. We do not know, however, whether one of these species or another not yet implicated is the important vector in field spread of the disease. There are many cruciferous weeds adjacent to growers' horseradish fields, and edible leaf crops of the same family are commercially grown in the area. Illinois currently provides over half of the United States' supply of horseradish (21), for use both as a condiment and as a source of the enzyme horseradish peroxidase. Brittle root epidemics are erratic; most years are characterized by little or no incidence of the disease. However, a severe outbreak can result in yield losses of up to 100% in some horseradish fields (*unpublished*). In the year following a brittle root epidemic, scattered plants may develop BR symptoms and die early in the season. In light of the association of a spiroplasma with the BR disease, it seems likely that some of the set roots harvested the previous fall may harbor the spiroplasma which is then reintroduced to the field the following year within its host root.

Symptoms of BR in the field, particularly in the foliage, are somewhat variable and may differ on occasion from those produced experimentally in the greenhouse or growth chamber. Experiments are in progress to examine the role of environmental conditions and the possibility that other pathogens may play a secondary role in causing BR symptoms.

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