

Association of a Spiroplasma with Brittle Root of Horseradish

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ABSTRACT

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Brittle root (BR) horseradish from Illinois was consistently distinguished from healthy horseradish by the dark discoloration of phloem tissue of the root of affected plants and also by the reaction of stained (Dienes' stain) phloem of leaf or root. Mycoplasma-like organisms were observed by electron microscopy in ultrathin sections of BR horseradish phloem, but not in phloem of healthy plants. A helical, motile, wall-free spiroplasma was isolated in 36 of 36 attempts from surface-sterilized leaves and roots of diseased horseradish. No spiroplasma was isolated from healthy horseradish obtained from Illinois and California. The spiroplasma isolate (HR-101) from horseradish was triple cloned and deposited at the American Type Culture Collection (ATCC 33451). The HR-101 spiroplasma isolate

resembled *Spiroplasma citri* in morphology, ultrastructure, and serology. Optimum temperature for the growth of the organism was 31 C. The organism also grew at 22, 25, 28, and 34 C, but not at 19 and 40 C. Growth of the HR-101 isolate required serum and was inhibited by digitonin. The organism can hydrolyze arginine. In serological tests by growth inhibition and deformation, the spiroplasma isolates from diseased horseradish were indistinguishable from *S. citri*. The spiroplasma in BR horseradish was effectively detected by enzyme-linked immunosorbent assay (ELISA) conducted with our antisera prepared against the HR-101 isolate or *S. citri*. Positive ELISA reaction also was obtained with extracts from stubborn-diseased citrus by using HR-101 spiroplasma antiserum.

Horseradish (*Armoracia rusticana*, Gaertn., Mey. & Scherb.), a perennial crucifer, is grown for its pungent roots. In the United States it is grown in the Mississippi River Valley near East St. Louis, IL; and near Eau Claire, WI, and Tulelake, CA (17). "Brittle root" (BR) (a name coined by growers) was reported to be the most important disease of horseradish in Illinois (12). It is characterized by the wilting of aerial parts, and brittle roots that break instead of bending and are much smaller than those of normal plants (11).

Severin (19) reported that the sugar beet curly top agent was present in field-grown horseradish in California and the symptoms he described were similar to those of BR. Severin (19) also demonstrated that beet leafhopper (*Circulifer tenellus* Baker) transmitted curly top agent from horseradish to sugar beets. Kadow and Anderson (12) concluded that the curly top agent was the cause of BR of horseradish based on the similarity of the symptoms observed on horseradish in Illinois and those reported by Severin (19) in California. Thornberry and Takeshita (20) suggested that BR was caused by a complex and provided circumstantial evidence that the beet leafhopper and the curly top agent were associated with the disease. Beet leafhoppers were first found in East St. Louis horseradish fields in 1936 (7). The evidence provided in previous reports had not clearly established the etiology of BR.

We were provided with living plants of horseradish with symptoms of BR to assist colleagues in Illinois in further investigations on the etiology of the disease. We report finding mycoplasma-like organisms in diseased horseradish sent to us from Illinois, *in vitro* isolation of a spiroplasma similar or identical to *Spiroplasma citri* from diseased plants and the use of enzyme-linked immunosorbent assay to detect horseradish plants affected by BR.

Following our isolation and serology of the spiroplasma, colleagues (8) in Illinois independently isolated a spiroplasma from BR horseradish plants and confirmed our work. Those results also are presented in the following paper.

MATERIALS AND METHODS

The principal source of healthy and BR horseradish plants was R. M. Goodman and C. E. Eastman, University of Illinois, Urbana. Plants were grown in an insect-proof greenhouse at the University of California, Davis. Field-grown horseradish plants from Tulelake, CA, were transplanted into the greenhouse and used for comparison in this study.

Cross and longitudinal sections of healthy and BR horseradish roots were made with a hand-held single-edge razor blade, observed for dark discoloration and photographed. Thin sections of healthy and infected horseradish leaf petioles and roots were cut into distilled water. The sections were transferred to 0.2% Bacto Dienes' stain (Difco) for 10 min, rinsed, and mounted in distilled water for observation (6). Dienes' stain has been reported to be useful in diagnosing diseases with which mycoplasma-like organisms (MLO) are associated.

Small pieces of midvein and lateral veinlets from BR and healthy horseradish plants were selected, cut into small pieces, fixed in 4% glutaraldehyde for 4 hr, and postfixed in osmium tetroxide. After dehydration in an acetone series, the tissue was embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate, counterstained with lead citrate, and examined with a Zeiss EM9 S-2 transmission electron microscope.

The culture medium (ME-5) used for primary isolations and subsequent subculturing had the following composition (100 ml): 2.0 g of PPLO broth (Difco), 0.2 g of sucrose, 0.1 g of glucose, 5.0 g of sorbitol, 15 ml of heat-inactivated (30 min at 56 C) GG-free horse serum, 10 ml fresh yeast extract, 1 ml of CMRL-1066, 1 ml of TC-199, and 2.0 mg of phenol red (Difco). All ingredients except horse serum, yeast extract, CMRL-1066 and TC-199 were dissolved in distilled water to give a total volume of 73 ml and sterilized by autoclaving at 121 C for 20 min. Sterile serum, yeast extract, CMRL-1066, and TC-199 were added aseptically to the cooled sterile basal medium. Solid medium was prepared by adding 0.8% Noble agar (Difco). Horse serum, yeast extract, CMRL-1066, and TC-199 were obtained from Grand Island Biological Company (Santa Clara, CA 95050).

The isolations were made in liquid ME-5 medium as follows:

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young leaves or roots (0.2–0.5 g) of BR diseased and healthy horseradish were surface sterilized in 1.5% sodium hypochlorite solution, washed four times in sterile glass-distilled water and rinsed once in liquid ME-5 medium. The pieces of tissue were chopped into smaller pieces in 10 ml of medium with a sterile single-edge razor blade and left in the medium for 1 min. The liquid medium was passed through a 0.45- μ m (pore diameter) membrane Nalgene® (Nalgene Company, Rochester, NY 14602) filter by using very slight suction. Three tenths to 1 ml of the filtrate was added to 5 ml of medium in a screw-capped tube and incubated at 30 C. Three uninoculated tubes with culture medium were included as controls in each test.

After the presence of spiroplasma in the medium was confirmed by dark-field microscopy, the organisms were subcultured one or more times in the same medium and then transferred to ME-5 agar medium. The inoculated petri plates were placed in a BBL anaerobic system (BBL, Cockeysville, MD 21030) fitted with a CO₂ gaspak and incubated at 30 C. After 7–10 days of incubation, the plates were examined and the colony morphology was studied.

The isolates were filter cloned three times by passing a broth culture through a 0.2 μ m (pore diameter) membrane filter and plating dilutions of the filtrate on ME-5 agar medium. After incubation for 6 days at 30 C, a single colony was picked off the agar surface, placed in liquid medium; the process repeated twice. Spiroplasma isolate HR-101 was triple cloned and deposited at the American Type Culture Collection where the accession number is ATCC 33451.

Partial characterization of spiroplasma isolate, HR-101. The morphology of individual organisms from liquid culture was observed by dark-field light microscopy, and by transmission electron microscopy of negative stained cells and ultrathin sections of pelleted cells. The negative staining was done as follows: a drop of liquid medium containing spiroplasmas was mixed well with a drop of 2% potassium phosphotungstate (KPT) on a Parafilm® sheet. Grids coated with a collodion membrane were allowed to touch the mixture for a few seconds, dried thoroughly, and examined with Zeiss EM9 S-2 transmission electron microscope. For ultrathin sections, the cells were harvested from liquid cultures, processed, stained, and observed with the transmission electron microscope as described earlier (16).

Growth of the spiroplasma isolate HR-101 at various temperatures was determined as follows: five tubes each containing 9 ml of ME-5 liquid medium were inoculated with 1 ml of 48-hr

culture and incubated for 3 days in separate incubators (General Electric) set at 19, 22, 25, 28, 31, 34, 37, 40, and 43 C. Titers were determined by duplicate plating on ME-5 agar medium. The plates were held for 6–7 days at 31 C, and the numbers of colonies per plate were recorded.

Sterol requirement of the isolated organisms was determined by studying the ability of HR-101 isolate to grow in serum-free ME-5 liquid medium, and by growth inhibition by digitonin (9). Inhibition of growth by digitonin was determined by placing agar disks containing digitonin on inoculated agar plates. The digitonin disks were prepared by wetting sterile paper disks (0.6-mm-diameter) in a 1.5% ethanolic solution of digitonin and allowing them to air-dry in a laminar-flow hood.

The ability of spiroplasma HR-101 to hydrolyze arginine was studied by inoculating ME-5 liquid medium containing 20 mM arginine with 48-hr culture and incubating at 31 C. The inoculated tubes were observed for a color reaction and change in pH.

Antisera against two spiroplasma isolates from horseradish, HR-101 and HR-105, were produced in female New Zealand white rabbits (16). Antisera were produced previously in rabbits by the same methods against the following spiroplasma isolates: *S. citri* (18) (ATCC 27563), corn stunt spiroplasma strain I-747 (1) (ATCC 29051) honey bee spiroplasma strain AS-576 (ATCC 29416), flower strains SR-3 (ATCC 33095) and 23-6 (5) (ATCC 29989), and suckling mouse cataract agent (22) (ATCC 29335).

The serological relationships of the spiroplasma isolates from horseradish with several other spiroplasmas were studied by the growth-inhibition (3) and deformation (24) tests with some modifications (16).

Enzyme-linked immunosorbent assay (ELISA) for the detection of spiroplasma in BR horseradish. Antisera prepared against HR-101 isolate and *S. citri* were used in this study. BR diseased and healthy horseradish from Illinois, horseradish from Tulalake, CA, and healthy and stubborn diseased Valencia sweet orange (*Citrus sinensis* L.) were included in ELISA. Plant samples from leaf and stem tissue were processed for ELISA as previously described (15).

Gamma globulin was purified from the antisera by precipitation with ammonium sulphate and filtration through DE-52 cellulose (Whatman, Ltd.) in a column preequilibrated with phosphate-buffered saline. Conjugation with alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO 63178) was accomplished with glutaraldehyde at a final concentration of 0.06%. Flat-bottom Micro-ELISA® plates (Dynatech Laboratories, Inc., Alexandria,

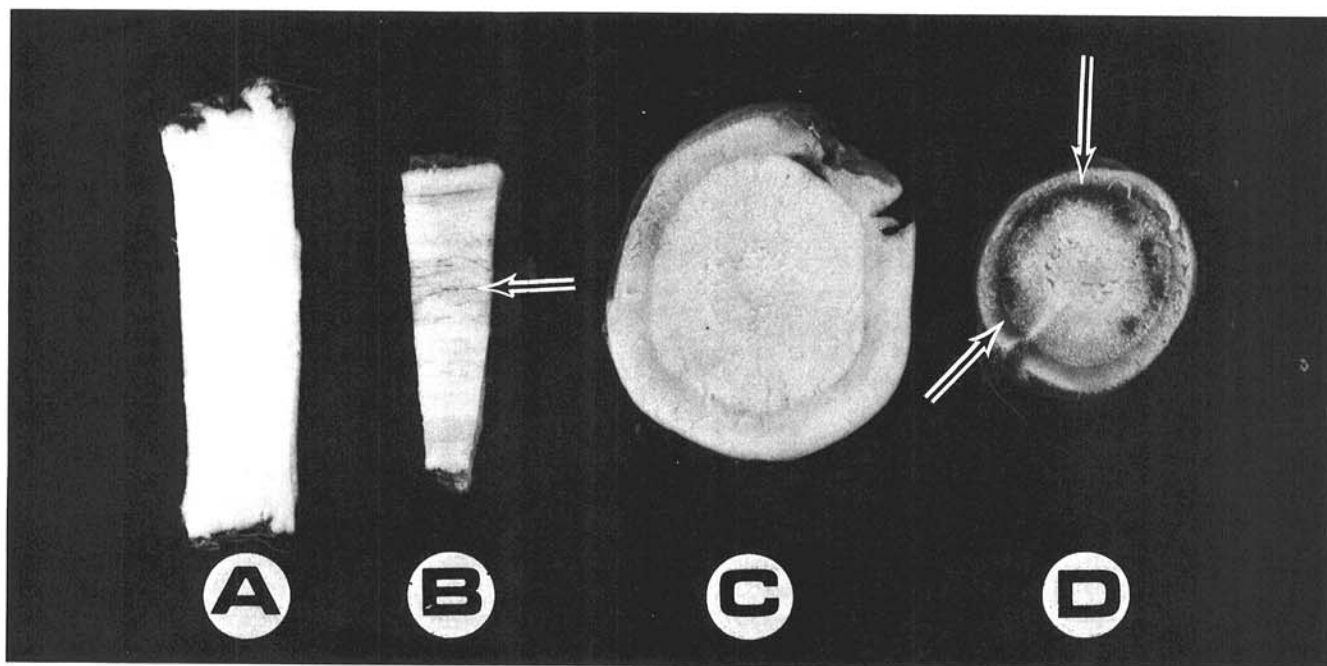


Fig. 1. Photographs of freehand longitudinal and cross sections of horseradish (*Armoracia rusticana*) roots. **A and C**, Sections of root of healthy plant in which no discoloration near phloem was observed. **B and D**, Sections of a brittle-root-affected plant in which discoloration was observed (arrows).

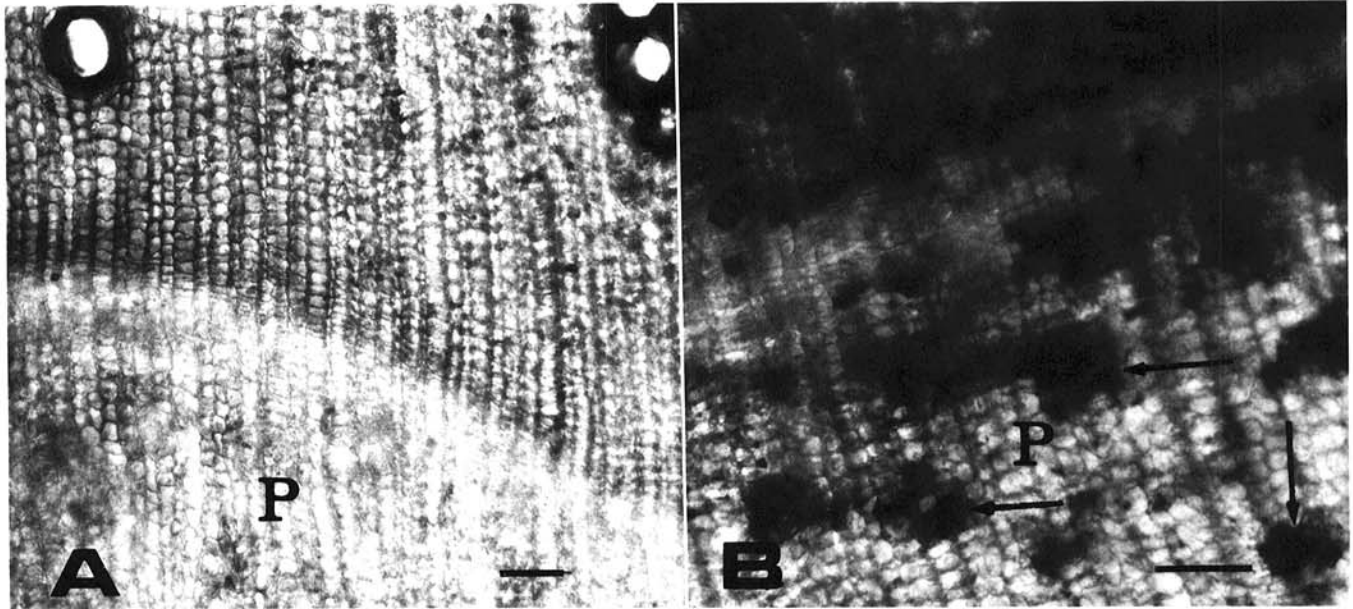


Fig. 2. Light micrographs of cross sections of horseradish petioles treated with Dienes' stain. **A**, Section of a petiole of a healthy plant in which the phloem (P) remains unstained. **B**, Section of a petiole of a brittle root plant in which the phloem stained blue (arrow). Scale bar = 40 μm .

TABLE 1. Isolation in ME-5 medium of helical, motile spiroplasmas from brittle root-affected horseradish (*Armoracia rusticana*)

Plant part ^a	Samples (no.)		Spiroplasma isolates (no.)	
	Healthy ^b	Diseased ^c	Healthy	Diseased
Leaves	35(15) ^d	21(7)	0	21
Roots	20(10)	15(5)	0	15

^aAll plant parts tested were surface sterilized in 1.5% sodium hypochlorite.

^bPlants were obtained from Urbana, IL, and Tulelake, CA.

^cField-collected plants from farms in Madison County and St. Clair County in Illinois.

^dNumbers in parentheses indicate the number of plants from which the samples were taken.

VA 22314) were used and the tests were carried out as described by Clark and Adams (2). The plate wells were coated with gamma globulin at a concentration of 4 $\mu\text{g}/\text{ml}$ and 1/800 dilution of stock enzyme conjugate labeled with alkaline phosphatase was used. The enzyme substrate (*p*-nitrophenyl phosphate, Sigma 104-105) at a concentration of 0.65 mg/ml was allowed to react for 30 min at room temperature and read directly (with no NaOH) in a Titertek[®] Multiskan ELISA reader (Flow Laboratories, Inc., Inglewood, CA 90302) at 405 nm.

RESULTS

Cross sections of horseradish roots from plants with typical BR symptoms showed pronounced discoloration in the vicinity of the phloem (Fig. 1D). Longitudinal sections showed the dark discolorations extending lengthwise through the root (Fig. 1B). Discoloration of phloem was observed in 10 of 10 diseased roots we examined. No discoloration was observed in comparable numbers of healthy roots (Fig. 1A and C) of plants obtained from Illinois or California. BR-affected material could be distinguished from healthy by observing sections stained with Dienes' stain. The phloem of healthy stem or root sections remained unstained (Fig. 2A) whereas many areas in the phloem of diseased sections stained distinct blue (Fig. 2B). The xylem tissue in sections from both healthy and diseased tissue stained light purplish blue. Of the 21 samples of leaves from apparently diseased plants that we tested, color reaction was observed in 19. No color reaction was seen in any of 25 healthy horseradish leaf samples from Illinois and California that were tested similarly. The blue color also was observed in phloem tissue of 15 of 15 diseased roots, but not in that of 20



Fig. 3. Electron micrograph of phloem cells of the petiole of brittle root horseradish showing mycoplasma-like organisms (MLO) in a sieve tube. Note the absence of cell wall (arrow). Scale bar = 0.5 μm .

samples of healthy roots.

Electron microscopy of ultrathin sections of leaves from BR horseradish plants revealed the presence of MLO in the phloem (Fig. 3). No MLO were found in the phloem of leaves and roots of healthy plants from Illinois and California. The MLO were bound by a unit membrane, possessed no cell wall, and contained ribosomelike granules and fine electron-dense strands.

Helical, motile spiroplasmas were isolated *in vitro* from surface-sterilized BR horseradish. No spiroplasmas were isolated from surface-sterilized healthy horseradish from Illinois or California (Table 1) or uninoculated control medium. In each attempt, spiroplasmas were isolated from samples of leaves and roots of horseradish plants showing typical BR symptoms (Table 1). Spiroplasmas were usually observed in the primary liquid culture after 10–14 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 caused by a drop in pH from 7.5 to 6.0. The presence of spiroplasma was confirmed by dark-field microscopy. The spiroplasma isolates from horseradish were similar to those obtained from other sources in helical cell shape, motility, and size. The organisms were 0.1–0.2 μm in diameter, from 2 to $\sim 8 \mu\text{m}$ in length, and exhibited flexional and rotational motility in liquid culture medium.

The spiroplasma isolates from diseased horseradish formed typical “fried-egg”-shaped colonies (Fig. 4) in ME-5 agar medium after 6–7 days of anaerobic incubation at 30 C. When samples from such colonies in liquid medium mounts were examined with a dark-field microscope, helical and nonhelical organisms were seen.

Negatively stained organisms from liquid medium (4-day-old culture) showed helical and nonhelical filaments (Fig. 5A) as described with *S. citri* (4). Transmission electron microscopy of ultrathin sections revealed the absence of any cell wall or envelope enclosing the spiroplasma. The cells were bound by a unit membrane and electron-dense strands (presumably DNA) were seen inside the cells (Fig. 5B).

Horseradish isolate (HR-101) grew well at 28 and 31 C with the optimum growth at 31 C (Fig. 6). Growth was also observed at 22, 25, and 34 C. Very little or no growth was seen at 37 C and no growth occurred at 19, 40, and 43 C. The organism reached the maximum growth in about 6–7 days with a doubling time of 4–5 hr. The doubling time was determined by using a hemocytometric method with dark-field microscopy (13).

No growth of HR-101 isolate occurred in ME-5 medium without serum. Inhibition zones 10–11 mm wide were observed in agar

cultures when a digitonin disk was placed on agar medium. The phenol red indicator in ME-5 liquid medium changed from red to yellow by 6 days after inoculation with HR-101 spiroplasma isolate. The pH of ME-5 shifted from 7.4 to 5.5 by 6 days after inoculation and remained at 5.5. In ME-5 medium containing 20 mM arginine the phenol red indicator also changed from red (pH 7.4) to yellow (pH 5.6) by day 6; however, 5 days later the color reverted from yellow to red and the pH returned to 7.2. Our study indicates that HR-101 hydrolyzed arginine.

The HR-101 isolate and three other horseradish isolates we tested were serologically indistinguishable from *S. citri*. The inhibition zones observed with horseradish isolates and *S. citri* were the same with homologous and heterologous antisera in the growth inhibition test (Table 2). No antigenic relationship between HR-101 and the flower isolates SR-3 and 23-6 or the suckling mouse cataract agent was indicated (Table 2). The growth inhibition test (Table 2) indicated that *S. citri* and horseradish isolate HR-101 share some common antigens with corn stunt spiroplasma (I-747) and honeybee spiroplasma (AS-S76). Previously published serological studies (5,23) indicate that *S. citri*, corn stunt, and honeybee spiroplasmas share some common antigens.

The results of deformation tests are shown in Table 3. As in the growth inhibition tests the horseradish isolates were indistinguishable from *S. citri*. The isolates HR-101 and HR-105 showed some antigenic relatedness to corn stunt spiroplasma and honeybee spiroplasma, whereas no reaction occurred with the flower isolates 23-6 and SR-3, or the suckling mouse cataract agent.

ELISA distinguished between BR-affected and healthy

TABLE 2. Serological relationships of spiroplasma isolates from horseradish with other spiroplasmas based on the growth-inhibition test

Antigens ^a	Antisera							
	HR-101	HR-105	<i>S. citri</i>	CSS	AS-576	23-6	SR-3	SMCA
HR-101	12 ^b	9	10	2	3	0	0	0
HR-102	12	8	10	ND ^c	ND	ND	ND	0
HR-104	11	8	10	ND	ND	ND	ND	ND
HR-105	11	9	9	2	ND	ND	ND	ND
<i>S. citri</i>	12	9	10	2	4	0	0	0
CSS	4	3	3	10	2	0	0	0
AS-576	3	3	2	1	9	0	0	0
23-6	0	0	0	0	0	12	0	0
SR-3	0	0	0	0	0	0	10	0
SMCA	0	ND	ND	ND	0	0	0	10

^aHR-101, HR-102, HR-104, and HR-105 are spiroplasma isolates from brittle root horseradish; *S. citri* = *Spiroplasma citri*; CSS = corn stunt spiroplasma; AS-576 = honey bee spiroplasma from Maryland; 23-6 = flower isolate from Maryland; SR-3 = flower isolate from Connecticut; and SMCA = suckling mouse cataract agent.

^bWidth of inhibition zones with indicated antiserum. No inhibition zone was observed with preimmune control serum.

^cND, not done.

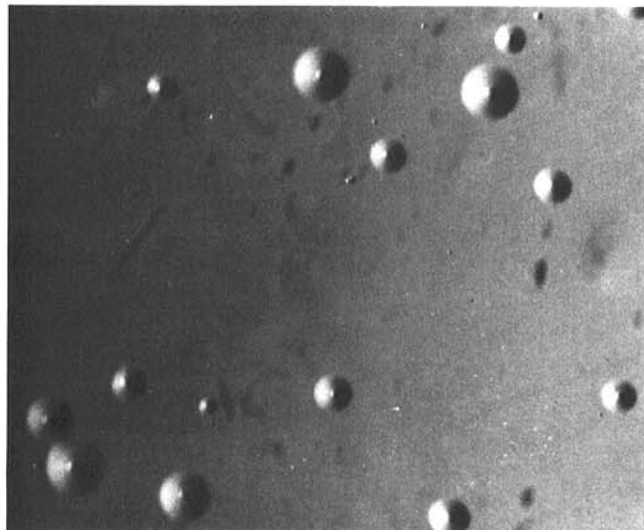


Fig. 4. “Fried-egg” type colonies produced by HR-101 spiroplasma isolate on ME-5 agar medium after 7–8 days of incubation at 30 C ($\times 10$).

TABLE 3. Serological relationships of spiroplasma isolates from horseradish with other spiroplasmas based on the deformation test

Antigens ^b	Reciprocal antibody titer with indicated antiserum ^a							
	HR-101	HR-105	<i>S. citri</i>	CSS	AS-576	23-6	SR-3	SMCA
HR-101	16,384	4,096	16,384	128	256	8	16	16
HR-102	16,384	8,192	ND ^c	ND	ND	ND	ND	16
HR-105	8,192	8,192	8,192	ND	ND	ND	ND	ND
<i>S. citri</i>	16,384	8,192	16,384	256	256	8	16	8–16
CSS	512	256	256	8,192	128	8	ND	8
AS-576	256	256	128	128	16,384	16	ND	16–32
23-6	8	8	16	8	16	4,096	8	8
SR-3	16	8	8	8	8	8	8,192	8
SMCA	16	8	8	16	8	8	8	8,192

^aPreimmune control sera had a titer of 4–8.

^bHR-101, HR-102, HR-105 = spiroplasma isolates from brittle root horseradish; *S. citri* = *Spiroplasma citri*; CSS = corn stunt spiroplasma; AS-576 = honeybee spiroplasma from Maryland; 23-6 = flower isolate from Maryland; SR-3 = flower isolate from Connecticut; and SMCA = suckling mouse cataract agent.

^cND, not done.

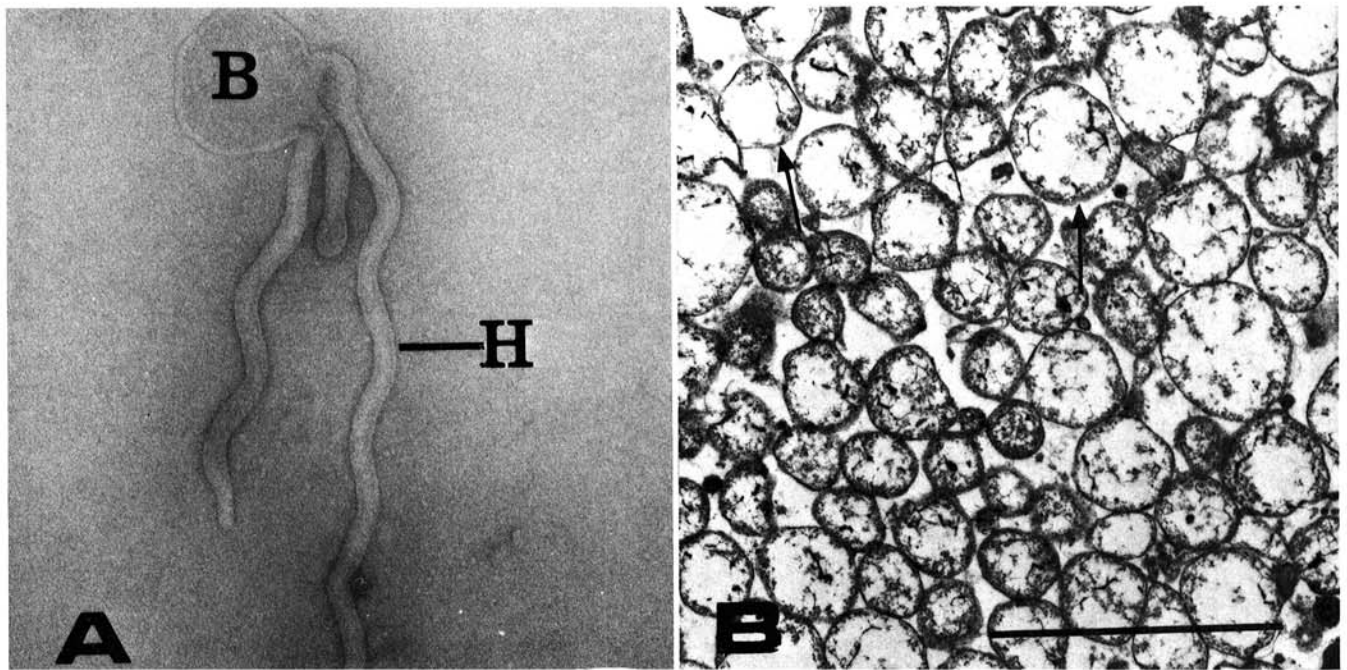


Fig. 5. Electron micrographs of HR-101 spiroplasma isolate cultured from brittle root horseradish. **A**, A negatively stained organism ($\times 10,000$) from a 4-day-old liquid subculture showing helical filament (H) and a main or central body (B). **B**, Ultrathin section of HR-101 spiroplasma isolate. Note the presence of a unit membrane (arrow) and the absence of a cell wall. Scale bar = 0.5 μm .

TABLE 4. Absorbance values ($A_{405\text{nm}}$) obtained from enzyme-linked immunosorbent assay with horseradish (*Armoracia rusticana*) and citrus (*Citrus sinensis*) using antisera prepared against horseradish spiroplasma isolate HR-101 and *Spiroplasma citri*

Source of antigen sample	Absorbance ^a			
	HR-101 ^b		<i>S. citri</i> ^c	
	Min	Max	Min	Max
Healthy horseradish	0.022	0.028	0.024	0.029
Brittle root horseradish	0.104	0.410	0.119	0.436
Healthy citrus	0.025	0.031	0.028	0.034
<i>S. citri</i> -infected citrus	0.116	0.524	0.201	0.640
Extraction buffer	0.002	0.014	0.008	0.017

^a Each absorbance value ($A_{405\text{nm}}$) is the mean of three determinations. Values from diseased plants are significantly different ($P = 0.01$) from those of healthy plants and of extraction buffer controls.

^b Antiserum prepared against a spiroplasma isolate from brittle root-affected horseradish.

^c Antiserum prepared against *S. citri*.

horseradish plants as shown in Table 4. The absorbance values ($A_{405\text{nm}}$) for healthy horseradish plants were similar to the values of the control extraction buffer (Table 4). In ELISA, positive correlation of color reaction was obtained with 12 of 14 horseradish plant samples showing typical BR symptoms and 10 of 10 samples of a citrus plant infected with *S. citri* that were tested by using antisera prepared against either HR-101 or *S. citri*. Negative reaction was observed with healthy horseradish and citrus samples. The $A_{405\text{nm}}$ values of the diseased horseradish and citrus samples varied somewhat, but were in most cases at least four times higher than those of the samples from healthy plants.

DISCUSSION

The evidences that MLO are associated with BR horseradish are: tests with Diene's stain showed a consistent blue staining of phloem of BR horseradish, but not of phloem of healthy plants; presence of MLO in electron micrographs of the diseased, but not of healthy plant material; isolation of spiroplasma in vitro from 36 of 36 root and leaf samples from 12 plants of BR horseradish, but not from 25 non-BR plants; and consistent detection of BR horseradish plants by ELISA with antiserum against the spiroplasma isolated from

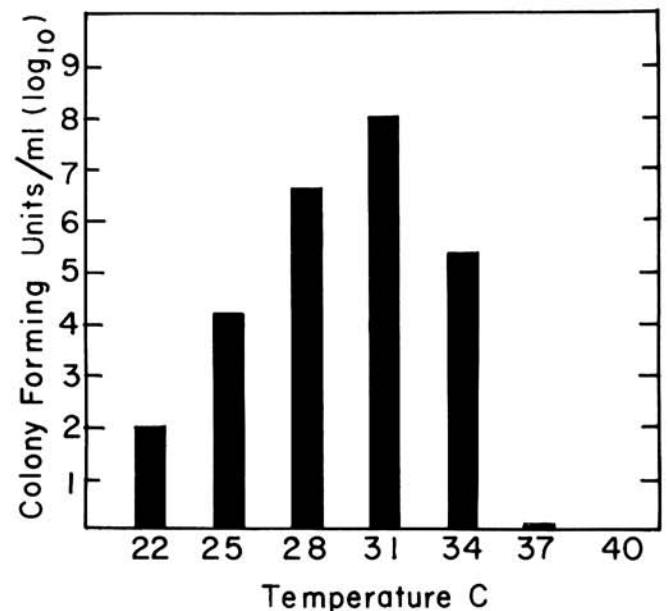


Fig. 6. Titer of spiroplasma isolate HR-101 cultured from brittle root horseradish at 22, 25, 28, 31, 34, 37, and 40 C.

diseased plants.

Our isolates, as is the case with all spiroplasmas so far isolated from plants and insects, were inhibited by digitonin, required sterol, possessed unit membranes and no cell wall, had helical and nonhelical filaments and motility, and produced characteristic "fried-egg"-shaped colonies on agar. Fletcher et al (8) observed "fried-egg"-shaped colonies only on rare occasions when horseradish isolates were plated onto agar medium. However, HR-101 almost always produced "fried-egg"-shaped colonies on solidified ME-5 agar medium. Since Fletcher et al (8) used a different medium, it is possible that medium composition is important in the formation of typical "fried-egg"-shaped colonies by horseradish isolates.

Like *S. citri* (18) horseradish spiroplasma isolate grew well at 31 C. Unlike the flower and insect spiroplasmas (16), no growth of

HR-101 was observed at 37 C. The ability of HR-101 to hydrolyze arginine indicates that arginine deaminase pathway may be operative in horseradish isolates as it is in *S. citri* (10,21).

Based on the results of growth inhibition and deformation, we consider the horseradish isolates to be identical to *S. citri*. The results of ELISA (Table 4) further indicate that *S. citri* is present in BR horseradish in Illinois. The ELISA can be used as a diagnostic tool for BR and in locating possible reservoir hosts and vectors of BR. Brittle root symptoms were not seen in commercially grown horseradish in California and *Spiroplasma* was not isolated from plants collected near Tulalake, CA, even though *S. citri* has been isolated from a number of other cruciferous hosts in California (14).

Stubborn disease-affected citrus and *S. citri*-infected herbaceous hosts exhibit stunting, chlorosis, and wilting (14) like the BR-affected horseradish. However, further evidence of the suspected role of *S. citri* in the etiology of BR horseradish awaits the results of pathogenicity tests now in progress.

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