Spiroplasma citri in Maryland: Isolation from Field-Grown Plants of Horseradish (*Armoracia rusticana*) with Brittle Root Symptoms

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ABSTRACT

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Horseradish plants with stunted young green leaves, extensive necrosis in old leaves, and a darkened phloem ring in roots were found in commercial horseradish fields in Maryland during September 1981. Spiroplasmas were consistently isolated in pure culture in vitro from surface-sterilized samples of roots from plants showing these symptoms. Results from serological tests of growth inhibition and enzyme-linked immunosorbent assay and from polyacrylamide gel electrophoretic analysis of cellular proteins indicated these spiroplasmas were strains of *Spiroplasma citri*, the cause of brittle root disease of horseradish in Illinois.

Spiroplasma citri has been known as a pathogen of plants in arid or semiarid regions. In the United States, *S. citri* has been known for several years to occur in California and Arizona, where it causes the "stubborn" disease of citrus and also infects several brassicaceous weeds and crop plants (4). In 1980, *S. citri* was discovered in and confirmed as the causal agent of brittle root disease of horseradish (*Armoracia rusticana* Gaertn., Mey., & Scherb.) in Illinois (14,26). This discovery greatly expanded the known geographic and climatic range of the pathogen and drew new attention to unconfirmed previous reports (5,18,19) of *S. citri* in the eastern United States.

In 1981, we learned that commercial cultivation of horseradish for processing had begun in Maryland in 1980. Because brittle root disease is a serious problem of horseradish elsewhere, we investigated whether *S. citri* might be present in horseradish in Maryland. We report that some Maryland horseradish plants showed symptoms of brittle root disease in 1981. Our investigation also revealed the presence of *S. citri* in such plants. The findings broaden concepts of the geographic and climatic range in which *S. citri* may be found and raise questions about the potential for spread of the pathogen to other crops and weed hosts on the eastern seaboard.

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MATERIALS AND METHODS

Farms in Charles County, MD, in which horseradish was commercially cultivated, were visited during September 1981. According to grower reports, a mixture of root pieces from several locations in the United States and Canada was used as planting stock on these farms. Plants in three fields on two farms were examined for brittle root disease. Two of the fields from which plant samples were taken had been under irrigation throughout the growing

season. The remaining field had received no irrigation.

Horseradish plants that appeared healthy and plants that showed stunting and foliar chlorosis or necrosis were removed from soil. Cross sections of roots were then examined for a darkened ring in the phloem region, which is a characteristic of brittle root disease (14,26). In the laboratory at Beltsville, samples were distributed randomly among four persons who independently performed the isolation experiments. The procedure and media used in attempts to isolate spiroplasmas in vitro from the plants were described previously (14). Cultures were incubated aerobically at 30–31 C. Broth cultures were examined for spiroplasmas by dark-field microscopy (×1,250), and agar cultures were examined for colonies under a stereoscopic microscope. Several spiroplasma isolates were triply cloned by a procedure described elsewhere (8). Cloned strains thus obtained from Maryland horseradish (MDHR) were designated MDHR1, MDHR2, MDHR3, MDHR4, and MDHR5.

Serological growth inhibition tests and polyacrylamide gel electrophoresis (PAGE) of cellular proteins were performed as described elsewhere (9,10,21) for identification of MDHR strains. Antisera used in serological tests were produced in rabbits. Known spiroplasmas or their antisera used in these tests included *S. citri* strain Maroc R8A2^T(= American Type Culture Collection [ATCC] 27556^T), *S. citri* strains BR3 and MBR8 (= ATCC 33479) from horseradish in Illinois (14), *Spiroplasma* sp. strain AS576 from Maryland honeybees (= ATCC 29416), *S. floricola* strain 23-6^T from flower nectar (= ATCC 29989^T), *Spiroplasma* spp. strains SR3 (= ATCC 33095) and brevi (= ATCC 33474) from flower nectar, and corn stunt *Spiroplasma* sp. strain 1747 (= ATCC 29051). *S. citri* Maroc R8A2 and corn stunt spiroplasma 1747 were obtained from the ATCC. The remaining strains were isolated in previous work (8,10,14).

Enzyme-linked immunosorbent assay (ELISA) (1,6) was used for additional estimation of serological relatedness of spiroplasmas. Spiroplasma antigens for use in ELISA were prepared by the method of Raju et al (25). Cultures were centrifuged at 15,000 *g* for 30 min at 4 C and washed three times in phosphate-buffered saline containing 10% sucrose (PBS+S). Samples were sonicated at 50% pulse, 80% output, in a Branson Sonifier Cell Disrupter 200 for 3 min. Preparations in PBS+S were adjusted to 10 μg protein per milliliter by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA), which is based on the technique of Bradford (3). The ELISA test was carried out according to the procedure of Clark and Adams (6) with slight modification. The wells of flat-bottomed Micro-ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 2 μg/ml purified immunoglobulin G (IgG). IgG was conjugated to alkaline phosphatase and used at a dilution of 1:200. After 60 min at room temperature, the reaction was stopped by adding 50 μl of 3 M NaOH per well. Results were determined by diluting well contents 1:5 in distilled water and measuring the absorbance of each sample at 405 nm in a Gilford recording spectrophotometer.

RESULTS

Most horseradish plants observed in fields in Maryland appeared healthy and showed no yellowing or necrosis of foliage as described for brittle root disease in Illinois (14). Some plants in the irrigated fields examined, however, bore only a few young but stunted green leaves at the center of the plant crown; these leaves were often surrounded by the remains of large mature leaves that had completely necrosed and dried. Cross sections of the roots of such plants usually revealed a darkened ring in the phloem region similar to that described for brittle root disease (14,26). Generally, such roots broke easily when bent; in contrast, normal roots were relatively flexible. Plants with these foliar and root symptoms were designated as probably "brittle root" (BR)-diseased. A designation of "questionable brittle root" (QBR) was given to stunted plants lacking a complete darkened ring in the root phloem or having irregular darkened rings in root tissue other than phloem. Plants lacking foliar and root symptoms were designated "non-brittle-root" (NBR)-diseased. These designations correspond in most respects to those used in previous work (14).

Spiroplasmas were isolated from 31 horseradish plants collected at two farms in Charles County, MD (Table 1). Cultures were obtained in both liquid and solid (1% agar) LD8 medium. The initial experiments were performed at Beltsville and the results were confirmed by isolations from coded samples sent to the laboratory in Urbana. All 23 plants designated BR yielded spiroplasma cultures, whereas none of 33 NBR plants yielded spiroplasma. Eight of 13 QBR plants yielded spiroplasma cultures. Spiroplasmas were isolated only from plants collected in irrigated fields. No plants with probable brittle root symptoms were observed in the unirrigated field, and the one QBR plant collected from that field failed to yield spiroplasma cultures (Table 1).

In size, shape, and motility, the MDHR spiroplasmas were typical of known spiroplasmas. During log-phase increase in broth medium, both cloned and uncloned Maryland horseradish spiroplasma isolates were helical, motile cells about 0.15 μm in diameter and 2 to about 5 μm long. Colonies produced on LD8 agar medium had granular centers usually surrounded by smaller "satellite" colonies. Colonies contained numerous

Table 1. Results of attempts to isolate and cultivate in vitro spiroplasmas from horseradish plants grown commercially in Maryland

Locations[a]	Isolations attempted/isolations positive[b] BR plants (no.)	QBR plants (no.)	NBR plants (no.)
Farm A			
Field 1	17/17	10/7	12/0
Field 2	...[c]	1/0	9/0
Farm B			
Field 3	6/6	2/1	12/0
Totals	23/23	13/8	33/0

[a] Farms A and B are in Charles County, MD. Field 1 of farm A and field 3 of farm B were irrigated. Field 2 of farm A had not been irrigated.

[b] BR = plants judged probably brittle root-diseased on the basis of foliar symptoms and the presence of a darkened ring in the phloem region of cross-sectioned roots, QBR = stunted plants lacking a complete darkened ring in the root phloem or having irregular darkened rings in root tissue other than phloem, and NBR = plants lacking foliar and root symptoms of disease.

[c] No plants with BR symptoms were observed in field 2.

Table 2. Growth inhibition tests of Maryland horseradish spiroplasma strains of *S. citri*, *S. floricola*, and *Spiroplasma* spp. strains SR3 and brevi

Spiroplasma strain	Zone (mm) of growth inhibition with indicated antiserum: *S. citri* (Maroc R8A2^T)	*S. floricola* (23-6^T)	SR3	Brevi
Maroc R8A2^T	8	0	0	0
MDHR1[a]	8	0	0	0
MDHR2	8	0	0	0
MDHR3	8	0	0	0
MDHR4	9	0	0	0
MDHR5	10	0	0	0

[a] MDHR1–MDHR5 refer to triply cloned spiroplasma strains isolated from horseradish field-grown in Maryland.

helical, motile cells.

In serological growth-inhibition tests with cloned strains isolated from Maryland horseradish, positive reactions were obtained with antisera against *S. citri* (Table 2), which comprises one of several distinct subgroups (IA) in a major serogroup (I) of the genus *Spiroplasma* (9,10,16). No growth inhibition of MDHR spiroplasma strains was observed when antisera to flower spiroplasma strains 23-6^T, SR3, and brevi (representing serogroups II, III, and V, respectively) were employed. These tests identified the MDHR strains as members of serogroup I. Further tests were required to determine subgroup affiliation within that serogroup (9).

In ELISA, antiserum against an Illinois horseradish isolate of *S. citri* (strain BR3) reacted specifically with all four strains of spiroplasma from Maryland horseradish (MDHR1–4) tested, with the type strain of *S. citri* (Maroc R8A2^T), and with strains BR3 and MBR8 from Illinois horseradish (Table 3). It did not react with corn stunt spiroplasma 1747 (subgroup IC), with the honeybee spiroplasma AS576 (subgroup IB), or with flower spiroplasma *S. floricola* (strain 23-6^T). These results indicated that the MDHR spiroplasma strains were members of the *S. citri* subgroup.

PAGE patterns of proteins from the three Maryland horseradish spiroplasma strains examined were identical or nearly so to one another and to the PAGE pattern of proteins from *S. citri* (Maroc R8A2) (Fig. 1). Other work (7,14,21) has shown that PAGE protein patterns of various strains of *S. citri* are nearly identical to one another but distinct from those of other spiroplasmas. Our results (Fig. 1) thus confirm that the MDHR spiroplasmas are strains of *S. citri*.

DISCUSSION

Our study revealed that a spiroplasma was associated with certain diseased plants of horseradish in Maryland in 1981. Isolation of the spiroplasma from surface-sterilized tissue samples clearly indicated that it was an internal parasite, and plants from which the spiroplasma was consistently isolated showed darkening in the phloem region of roots and other symptoms described previously (14) for the brittle root disease of horseradish in Illinois. Isolation of spiroplasmas from a high percentage of plants designated QBR may reflect the difficulty in diagnosing the disease based on symptoms late in the growing season. Consistent isolation of the spiroplasma from plants with symptoms of brittle root disease and our failure to isolate spiroplasma from apparently healthy plants strongly suggested a causal association between the spiroplasma and brittle root disease in Maryland. Results indicated for the first time that brittle root disease can occur in horseradish outside of Illinois.

Results from serological tests indicated that the MDHR spiroplasmas were strains of *S. citri*, the causal agent (14,26) of brittle root disease in Illinois. PAGE patterns of proteins from the Maryland horseradish spiroplasmas confirmed that the MDHR spiroplasmas were strains of *S. citri*. Although this study does not provide unequivocal proof that the isolated spiroplasma is the cause of the disease symptoms observed in Maryland horseradish, the evidence, when interpreted in the light of previous work (14,26), strongly indicates that strains of *S. citri* induced BR disease in Maryland horseradish during 1981.

Previous reports of isolating *S. citri* from field-grown plants in states on the U.S. East Coast (18,19) have been questioned (28,29). Before 1980, *S. citri* was generally thought to be largely limited to certain arid or semiarid regions of the world (eg, California, Arizona, Mideast countries, and the Mediterranean region), where it is known to cause "stubborn" or "little-leaf" disease of citrus and to infect a number of plant species including several weeds and crop plants in the family Brassicaceae (4). Even though in 1979 *S. citri* had been found in plants of turnip (*Brassica rapa* L.) and in the leafhopper *Circulifer tenellus* in Washington State (G. N. Oldfield, *personal communication*), it was still believed to be principally an inhabitant of the Southwest in the United States. The discovery of *S. citri* in Illinois (14,26), however, greatly altered notions about the geographic and climatic ranges in which this pathogen might occur and provided a new light in which earlier reports (5,18,19) of *S. citri* in the northeastern United States might be examined (13,20).

The presence of *S. citri* in Maryland is a new element in understanding the presence of this pathogen in the eastern United States, but its origin in Maryland horseradish is unknown. It is not possible to state whether horseradish became infected with *S. citri* after it was planted in fields in Maryland in 1980 and 1981 or whether some horseradish roots were already infected when planted.

Because the known plant host range of *S. citri* spans more than 20 families (4,23), it is important to consider the potential for spread of the pathogen to other crops and weeds as well as within horseradish fields in the East. Although *C. tenellus*, a

Table 3. Absorbance (A_{405}) of Maryland horseradish spiroplasma antigen preparations in enzyme-linked immunosorbent assay with antiserum against *S. citri* strain BR3 from Illinois horseradish[a]

Spiroplasma strain	Source of strain	A_{405}[b]
MDHR1	Maryland horseradish	0.133
MDHR2	Maryland horseradish	0.146
MDHR3	Maryland horseradish	0.266
MDHR4	Maryland horseradish	0.308
S. citri (BR3)	Illinois horseradish	0.231
S. citri (MBR8)	Illinois horseradish	0.290
S. citri (R8A2^T)	Sweet orange tree	0.188
AS576	Honeybee, Maryland	0.002
1747	Corn stunt diseased corn	−0.008
S. floricola (23-6^T)	Tulip tree flower, Maryland	−0.009

[a] Spiroplasmas were grown in liquid LD8 (9) except for strain 1747, which was grown in LD8A (9), washed three times in phosphate-buffered saline containing 10% sucrose, and sonicated to disrupt cells.

[b] A_{405} represents the average absorbance at 405 nm of two wells of the same sample minus the average absorbance of the buffer in control wells. Absorbance was read after diluting well contents 1:5 in water.

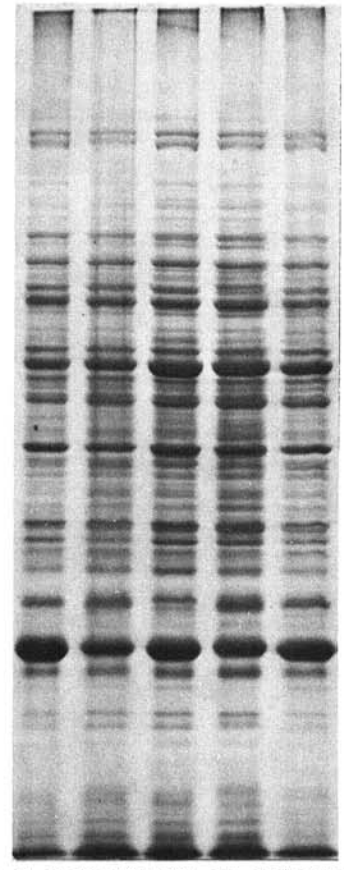

Fig. 1. Polyacrylamide gel electrophoretic patterns of proteins from (left to right) *Spiroplasma citri* strain Maroc R8A2^T from sweet orange in Morocco, strain MDHR4 from diseased horseradish in Maryland, strain MBR8 from diseased horseradish in Illinois, and from strains MDHR1 and MDHR3 isolated from diseased plants of horseradish in Maryland.

natural vector of *S. citri* (17), occurs east of the Mississippi River in Illinois (12,14,24) and Florida (11), it has not been reported in Maryland. However, curly top virus, for which the only known vector is *C. tenellus*, was reported in North Carolina, Virginia, and Maryland in 1958 (15,27). The leafhopper *Macrosteles fascifrons* (vector of the aster yellows agent) was recently reported capable of acquiring and transmitting *S. citri* by feeding on plants (22). Because this insect is abundant in the eastern United States (2), it would be of interest to determine its possible role in natural spread of *S. citri*. Although the origin of *S. citri* in Maryland horseradish is not known, the discovery of *S. citri* in field-grown plants in Maryland encourages further investigation of this pathogen and its potential vectors in the East.

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