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Relation of In Vivo Morphology to Isolation of Plant Spiroplasmas

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Abstract. Two procedures were developed to isolate plant spiroplasmas directly onto DG-2 agar plates or in DG-2 broth without subcultures or dilutions. The frequency of successful spiroplasma isolations was increased by centrifuging samples, after passing through a 0.45-μm filter, at 25,000 × *g* for 1 h. Spiroplasmas were obtained from peach, cherry, Madagascar periwinkle, and celery with typical symptoms of the Green Valley strain of X disease (GVX), from peach with typical symptoms of the peach yellow leaf roll strain of X disease (PYLR), from Madagascar periwinkle with typical symptoms of aster yellows (AY), from celery with atypical symptoms of GVX (mild GVX), from plantago with atypical symptoms of aster yellows (mild AY), and from stubborn-diseased citrus. Isolations were consistent (>90%) from plants with mild GVX, mild AY, and citrus stubborn, while isolations were inconsistent (0–9%) from plants with typical symptoms of GVX, PYLR, and AY. The role of the isolated spiroplasmas in plant disease was not determined in this study. All spiroplasma isolates were serologically indistinguishable from *Spiroplasma citri*. Spiroplasmas were seen in electron micrographs of 8 out of 9 examined plants from which spiroplasmas were isolated. However, electron micrographs of all 13 examined plants from which no spiroplasmas were isolated contained mycoplasma-like organisms (MLOs) but no spiroplasmas. These results indicate that there is a correlation between helical MLOs in vivo and successful isolation of spiroplasmas, and that plants may be infected with both *S. citri* and nonhelical mycoplasmas.

Several techniques have been used for isolation of plant spiroplasmas with varying degrees of success. Some of the first successful isolations of *Spiroplasma citri* were obtained by grinding infected tissues in a mortar and pestle containing the liquid culture medium [2,7]. However, animal mycoplasmologists have demonstrated that grinding of tissue may release mycoplasma-inhibiting substances [8]. Liao and Chen [4] detected a spiroplasmacidal factor in corn stunt–diseased corn tissues, and isolation of the corn stunt spiroplasma [4,12] was achieved by squeezing sap into liquid medium, making dilutions, and making subsequent passages every 2–5 days. Raju et al. [6,9] confirmed the requirement of dilutions and subpassages of the original inoculum for successful isolation of a spiroplasma from aster yellows–diseased plants. They reported that the effect of plant inhibitors on spiroplasmas was minimized by chopping a small amount of plant tissue in a large amount (20–25 ml) of medium and by repeated subpassages of the original sample. This paper reports a method for the isolation of plant spiroplasmas from homogenates of large amounts of plant tissue either plated directly onto solid media without dilutions or maintained without subpassages in liquid media. The successful isolation of spiroplasmas from some diseased plants is compared to the in vivo morphology of mycoplasma-like organisms (MLOs).

Materials and Methods

Diseased plants. The isolation procedures were developed using greenhouse-grown citrus infected with *Spiroplasma citri* and celery infected via leafhoppers (*Colladonus montanus*) with spiroplasma strain MP which was isolated in 1978 from a cherry tree with the Green Valley strain of X-disease (GVX); the disease in celery was termed "mild GVX", since the plant had atypical GVX symptoms.

Isolations were also attempted using the two procedures from field-grown cherry and peach trees with typical symptoms of GVX, field-grown orange trees with stubborn disease (from E. C. Calavan), field-collected periwinkle with aster yellows disease (AY) (from B. C. Raju), and from the following sources provided by A. H. Purcell: celery infected with spiroplasmas isolated from peach with the peach yellow leaf roll strain of X disease (PYLR), but which had atypical PYLR symptoms (termed mild PYLR), celery with typical PYLR, plantago infected with a spiroplasma isolated from plants with AY but which had atypical AY symptoms (termed mild AY), plantago with typical dwarf, Tulelake, and severe AY, and peach with typical PYLR.

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Culture medium. Medium DG-2 used in this study contained the following ingredients and had an osmolarity of 610 mOsm ± 20 mOsm.

Autoclavable base	
PPLO broth base	20 g
Sorbitol	50 g
Tryptone	1 g
Peptone	1 g
Glucose	1 g
Fructose	1 g
Sucrose	1 g
Phenol red (0.2%)	10 ml
Distilled water	800 ml
(Difco agar)	(10 g)
Adjust to pH 7.4 with 1N NaOH	
Filter-sterilized additives	
CMRL 1066 10X without L-glutamine and $NaHCO_3$ (Gibco)	10 ml
Fresh yeast extract (100%)	10 ml
Yeastolate (2% solution)	100 ml
Horse serum (heated at 56°C for 1 h) (Gibco GG-free)	170 ml
Penicillin (100,000 U/ml)	10 ml
Adjust to pH 7.4 with sterile 1 N NaOH	

Culture medium. The efficiency of medium DG-2 was compared to SP-4 [11] and ME-1 [6] for both isolation and maintenance of spiroplasma. For isolation, stubborn-diseased orange, cherry with GVX, and plantago with mild AY were surface-sterilized in 1.5% sodium hypochlorite for 2.5 min followed by three rinses in sterile water. Petiole and stem tissue (0.2 to 0.5 g) were homogenized in 5 ml phosphate-buffered sucrose (10% sucrose in 0.1 M phosphate buffer pH 7.4 and 618 mOsm) using a mortar and pestle. Samples were clarified by centrifugation at 500 × g (20°C) for 5 min, and the supernatant was filtered through a 0.45-μm membrane filter using positive pressure. Ten-milliliter aliquots of each sample were mixed with 2 ml liquid medium in a screw-cap tube (17 × 125 mm). Samples were incubated aerobically without passages at 28°C and were examined using dark-field microscopy when a color change from red to yellow-orange was apparent. Forty-eight-hour-old spiroplasma cultures from stubborn-diseased citrus and GVX-diseased peaches were used to test the efficiency of the media for routine maintenance of spiroplasmas. Aliquots (0.1 ml) of serial 10-fold dilutions were plated onto three replicate agar plates of SP-4, ME-1, and DG-2. Plates measured 35 × 10 mm and contained 2.5 ml agar medium. The ability of each medium to support growth of spiroplasmas was determined after incubation at 28°C for 14 days.

Isolation procedures. Young and medium-aged leaves were removed from the test plant and surface-sterilized as deseribed ahove. Up to 20 cm (approximately 1.4 g) of central leaf veins and petioles were ground in 8 ml phosphate-buffered sucrose in a mortar and pestle and clarified by centrifugation at room temperature at 500 × *g* for 5 min. The supernatant was passed through a 0.45-μm membranc filter using positive pressure, and 0.1-ml aliquots were directly plated on DG-2 medium and maintained as undiluted liquid cultures (procedure 1).

In procedure 2, the clarified filtered supernatant was recentrifuged in sterile screw-top centrifuge tubes at 25,000 × *g* at 17°C for 60 min. The pellet was resuspended in 0.5 ml DG-2 broth and 0.1 ml was inoculated onto agar plates and into 2.0 ml liquid DG-2. After drying by incubating at 28°C for 24 h, plates were placed inside closed plastic bags and inenbated aerobically at 28°C for up to 5 weeks. Plates were examined at 2-day intervals for the presence of colonies or yellow eolor and liquid tubes were examined for color changes and for the presence of spiroplasma using the dark-field microscope. Spiroplasma isolates from plants with "mild" and typical symptoms of AY, PYLR, and GVX were serologically compared to *Spiroplasma citri* using the spiroplasma deformation test with antiserum to the Morocco strain of *S. citri.*

Electron microscopy. The following plants from which spiroplasmas were isolated were examined for the presence of spiroplasmas and/or MLOs in phloem cells: 1 orange tree with citrus stubhorn, 1 vinca with typical AY, 4 cherry trees with typical GVX, 1 plantago with inild AY, and 2 celery with mild GVX. In addition, the following plants from which no spiroplasmas were isolated were examined: 7 celery with typical PYLR, 4 vinca with typical AY, 3 cherry trees with typical GVX, and 1 plantago with typical AY. Leaf inid-veins and/or petioles were immersed in 4% glutaraldehyde–4% formaldehyde in 0.08 M cacodylate buffer. pH 7, cnt into 1- to 2-mm segments, and fixed for 4 h at room temperature. Segmcnts were postfixed overnight at 4°C in 1% osminm tetroxide in 0.08 M cacodylatc buffer, pH 7, dehydrated in a graded acetone series, and embedded in Spurr plastic. Sections of 190–240 mm were stained with urauyl acetate and lead citrate.

Results

Culture medium. DG-2 broth was superior to both ME-1 and SP-4 broth for the isolation of spiroplasmas without dilutions from the same plant tissues (Table 1). Spiroplasmas isolated in DG-2 broth were periodically not isolated in SP-4 or ME-1. The incubation time required for spiroplasmas isolated from plants with mild AY was the same using DG-2 and ME-1; however, the incubation timc was less using DG-2 than ME-1 for isolation from plants with typical GVX, mild AY, and stubborn-diseased citrus. DG-2, SP-4, and ME-1 agar plates were all useful for maintenance of isolated spiroplasmas.

Isolation procedures. Spiroplasmas were consistently (>90% of attempts) isolated from celery with mild GVX (atypical symptoms which included slight interveinal chlorosis and brittleness of leaves), plantago with mild AY (atypical symptoms which included slightly club-shaped new leaves), and orange with stubborn disease (Table 2). Procedure 2 consistently yielded spiroplasma colonies on solid DG-2 medium without dilutions, while some isolations were negative using procedure 1. Colony morphologies, which were similar to those reported by Townsend, Burgess, and Plaskitt [10], included lens-shaped without a dark center region and granular with satellite colonies. Isolations from large amounts of plant tissue (more than 0.12 g/ml) were frequently obtained only

Table 1. Isolation of plant spiroplasmas in various liquid media without subcultures.

Host	Medium	Isolation in broth: + or −	Isolation in broth: Average incubation time required[a] (weeks)
Stubborn-diseased orange	SP-4	−	—
	DG-2	+	4
	ME-1	+/−[b]	5.3
Plantago with mild AY[c]	SP-4	+	4
	DG-2	+	2
	ME-1	+	2
Cherry GVX[d]	SP-4	+/−	5
	DG-2	+	3.5
	ME-1	+/−	4
Celery mild GVX[e]	SP-4	+	6
	DG-2	+	4.5
	ME-1	+	5

[a] Average time until spiroplasmas were detected using dark-field microscopy. Isolations were repeated six times.
[b] +/− indicates that spiroplasmas were sometimes not isolated.
[c] Plant was inoculated via *Macrosteles fascifrons* with a spiroplasma isolated from plant with typical aster yellows symptoms; however, the plant developed atypical "mild" symptoms. Plant was provided by A. H. Purcell.
[d] Green Valley strain of X disease.
[e] Plant was inoculated via *Colladonus montanus* with a spiroplasma isolated from a cherry tree with typical GVX symptoms; however, the celery developed atypical "mild" symptoms. Plant was provided by A. H. Purcell.

Table 2. Frequency of isolation of various plant spiroplasmas.[a]

Disease	Host plant	Isolation	No. of plants isolated from	Isolation frequency
Citrus stubborn	Orange	+	1	Consistent[a]
Mild GVX[b]	Celery	+	4	Consistent
Mild aster yellows	Plantago	+	3	Consistent
Typical GVX	Celery	+/−	3	1/10[c]
	Vinca	+/−	4	1/28
	Peach	+/−	10	4/58
	Cherry	+/−	194	16/459
Typical PYLR[d]	Celery	−	45	0/45
	Peach	+/−	43	2/72
Typical aster Yellows	Vinca	+/−	34	4/53
	Plantago	−	12	0/48
	Lettuce	−	7	0/14

[a] Isolations made using procedure 2, described in text, using DG-2 broth and solid medium. Consistent = >90% successful isolations.
[b] GVX = the Green Valley strain of X disease. Plant was inoculated via *Colladonus montanus*. Spiroplasmas were isolated from a cherry tree with typical GVX symptoms. However, the celery developed atypical "mild" symptoms. Plant was provided by A. H. Purcell.
[c] Number successful spiroplasma isolations/number of attempted isolations.
[d] PYLR = the peach yellow leaf roll strain of X disease.

on solid medium and not in undiluted liquid medium; however, spiroplasmas were consistently isolated in undiluted liquid medium when less than 0.12 g host tissue/ml broth was used. Spiroplasma colonies developed on solid media in 7–11 days, when plants infected with mild strains of GVX and AY were used, while spiroplasmas were detected in liquid media in 14–21 days. Spiroplasma isolates from stubborn-diseased citrus were detected after 12–14 days on solid media compared with 24–28 days in liquid medium.

Spiroplasmas were inconsistently isolated from plants with typical symptoms of AY, PYLR, and GVX (Table 2). All spiroplasma isolates were serologically indistinguishable from *Spiroplasma citri*. The percentages of successful isolations from diseased plants ranged from 0% with AY plantago infected and grown in the greenhouse to approximately 9% with AY field-collected vinca. The isolated spiroplasmas from plants with typical symptoms were detected in liquid medium after 21–35 days' incubation, while spiroplasma colonies developed on solid medium in 14–21 days. The colonies on agar medium were indistinguishable from colonies which developed from plants exhibiting mild symptoms. No spiroplasmas were isolated from healthy control plants which were included in each isolation series.

Electron microscopy. Cells of MLOs resembling spiroplasmas (Fig. 1) observed by previous workers [1,3] were observed in eight of nine examined plants from which spiroplasmas were isolated (Table 3). Spiroplasmas were present in large concentrations in plants with mild disease symptoms in which they were the predominant morphological form. Plants, with typical symptoms, from which spiroplasmas were isolated contained both rounded MLOs and filamentous forms which resembled spiroplasmas (Fig. 2). In contrast, only nonhelical MLOs were observed in all 13 examined plants from which spiroplasmas were not isolated (Fig. 3).

Discussion

The procedure reported here for the isolation of plant spiroplasmas eliminates the previously reported requirement for dilutions or subcultures of the origi-

Table 3. Isolation of spiroplasmas compared with in vivo morphology.[a]

Presence or absence	Diseased plants from which spiroplasma isolations were: Positive		Negative[b]	
Spiroplasma observed in electron micrographs	Orange with citrus stubborn	1		
	Celery with mild GVX[c]	2		
	Plantago with mild AY[d]	1	None	
	Cherry with typical GVX	4		
	Total	8	Total	0
No spiroplasma observed in electron micrographs	Vinca with typical AY	2	Celery with typical PYLR[e]	5
			Vinca with typical AY	4
			Cherry with typical GVX	3
			Plantago with typical AY	1
	Total	2	Total	13

[a] Transmission electron microscopy was used as described in the text to determine the in vivo morphology.
[b] Mycoplasma-like organisms were observed in plants which did not contain spiroplasmas in electron micrographs.
[c] GVX = the Green Valley strain of X disease.
[d] AY = aster yellows.
[e] PYLR = the peach yellow leaf roll strain of X disease.

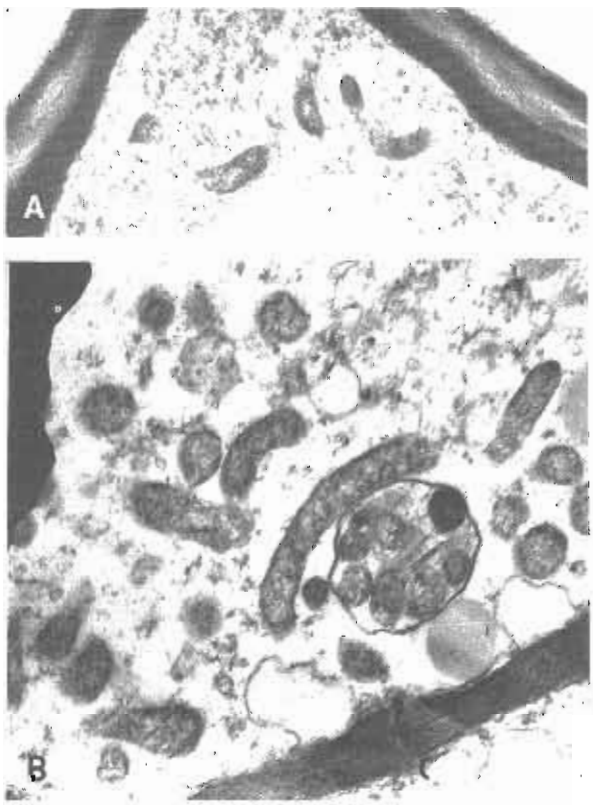

Fig. 1. Helical filaments in 190- to 240-nm sections of phloem cells from celery with atpical symptoms of the Green Valley strain of X disease (mild GVX), at magnification of 11,000 (A) and 16,000 (B).

nal inoculum [3,6,9]. Grinding of more than 0.12 g plant tissue per ml phosphate-buffered sucrose often failed to yield spiroplasmas in undiluted broth, although isolates were obtained on solid DG-2. In addition to reducing the number of required subcultures for a successful isolation, the procedure reported here increases sensitivity by concentrating low populations of spiroplasmas during centrifugation. The ability to isolate spiroplasmas present in plants in low populations is increased by the grinding of plant tissues since more total phloem sap is released than by previously used methods of cutting tissues in growth medium.

DG-2 broth is preferable for routine isolations of spiroplasmas to DG-2 agar plates since broth tubes containing spiroplasmas consistently undergo a color change from red to yellow. Agar plates with spiroplasma colonies, however, sometimes do not change color, which then requires the viewing of all plates under a dissecting scope for detection of colonies. Agar plates are useful when the quantification of spiroplasmas in a sample is desirable or when one is isolating over 0.12 g plant tissue per ml grinding medium. Isolations directly on solid DG-2 reduce the number of subcultures required to triple clone a spiroplasma. Medium DG-2 was superior to both SP-4 and ME-1 for the isolation of the tested spiroplasmas. The usefulness of DG-2 for isolation of spiroplasmas not included in our tests should be examined as should its usefulness compared to other spiroplasma media.

The consistent isolations of spiroplasmas from plants with citrus stubborn, mild GVX, or mild AY and the observation of spiroplasmas in phloem tissues of these plants with the electron microscope suggest that the plants contained MLOs which were predominantly helical. In contrast, the irregular isolation of spiroplasmas from plants with typical GVX, AY, and PYLR symptoms combined with no

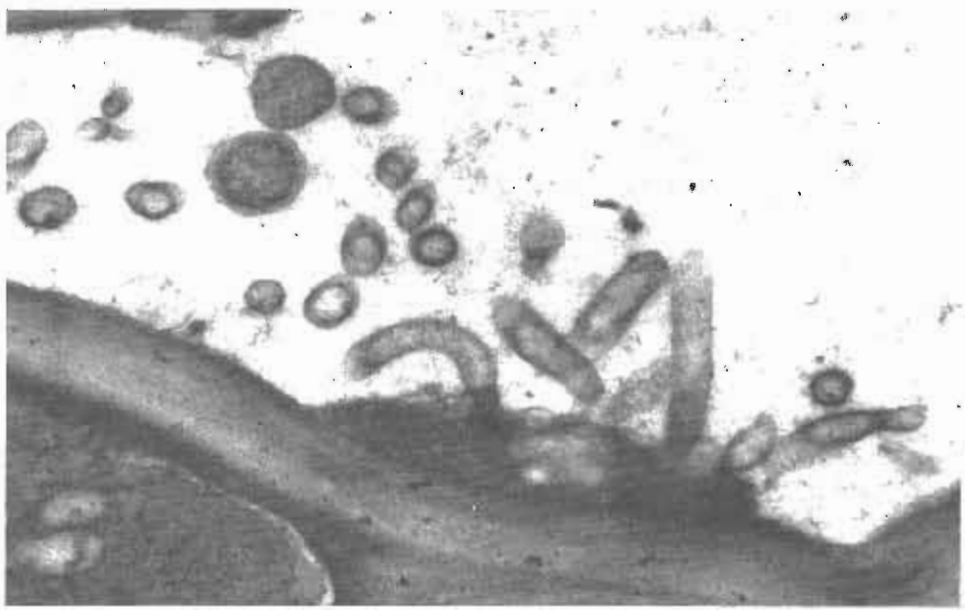

Fig. 2. Phloem cells of pedicles of cherry with typical sympoms of GVX at × 24,000.

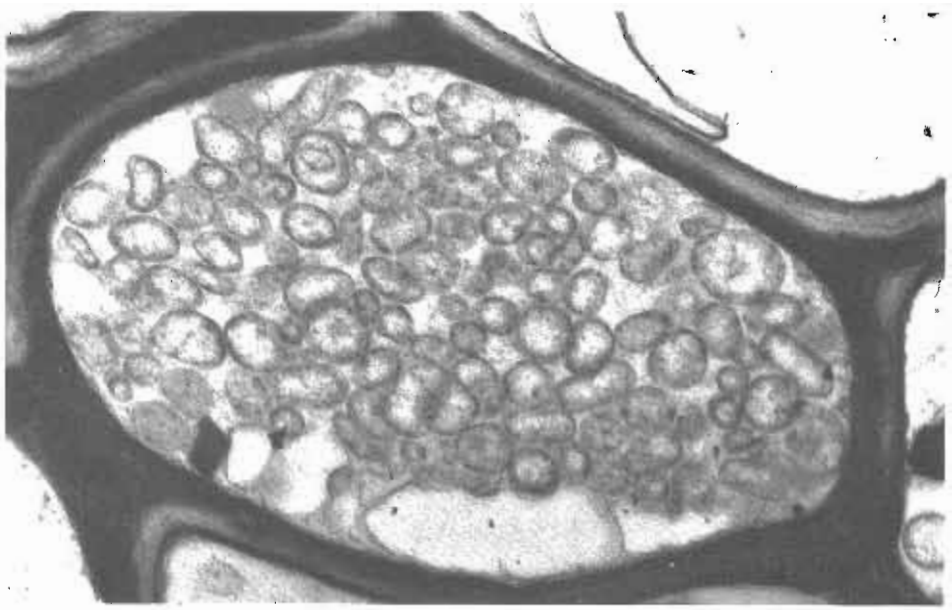

Fig. 3. Nonhelical mycoplasma-like organisms in phloem cells of celery petiolcs with typical symptoms of thc peach yellow leaf roll strain of X disease at × 21,000.

observation of spiroplasma-like bodies in phloem of these plants suggests that the MLOs were predominantly nonhelical and that spiroplasma isolates are obtained only when helical MLOs are present in the phloem. It is uncertain at this time if the apparently nonhelical MLOs in plants with typical PYLR, AY, and GVX are the same organisms as the helical MLOs. However, due to the inability to isolate these nonhelical MLOs, whose presence in tissue samples was confirmed with the EM, it is likely that they are not spiroplasmas. We believe that the data presented here could be explained by mixed infections of MLOs and *Spiroplasma citri* which have been previously demonstrated [3,5].

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