Quantification of Plant Pathogenic Spiroplasmas from Infected Plants

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ABSTRACT

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Quantification of spiroplasmas in host plant tissue was accomplished by centrifuging samples to remove possible spiroplasma inhibitors and plating the samples directly on a solid medium. Population estimates varied less than one log unit among different replications of a given sample and therefore, populations were determined by taking the average of four to six replications per sample. Populations of spiroplasmas ranged from a minimum detectable number of 50 colony-forming units per gram (cfu/g) to a maximum of 3.1×10^6 in foliage of celery, periwinkle, orange, corn, and plantago in the greenhouse. Populations in plants fed on by leafhoppers injected with *Spiroplasma citri* originally isolated from cherry with Green Valley X disease averaged 6.0×10^5 cfu/g but only averaged 50 cfu/g when the leafhoppers were fed on the cherry and allowed to transmit without injection to indicator plants. Populations of *S. citri* and the corn stunt spiroplasma were approximately 1 log unit lower in roots than in petioles of greenhouse plants. Spiroplasmas were present at detectable levels in all ages of host plants; however, the populations averaged 1.0–1.5 log units less in old leaves than in young leaves. Populations in field-grown broccoli naturally infected with *S. citri* averaged 4.8×10^5, while spiroplasma populations averaged 2.1×10^5 in plants infected with both *S. citri* and nonhelical vinca virescence mycoplasmalike organism (MLO). Electron micrographs of thick longitudinal sections revealed the presence of two distinct organisms: one filamentous and helical, the other globular in plants infected with both *S. citri* and the vinca virescence MLO. Only filamentous helical organisms were seen in plants infected with *S. citri*.

Spiroplasma citri is a procaryotic plant pathogen of the class Mollicutes (27), which causes citrus stubborn disease on sweet orange (*Citrus sinensis*), mandarin (*C. reticulata*), grapefruit (*C. paradisi*), and tangelo (*C. reticulata* × *C. paradisi*) (4). Spiroplasmas, which are serologically indistinguishable from *S. citri*, based upon the spiroplasma deformation test (32), are periodically associated with various diseased plants including pear (*Pyrus communis*) with pear decline (20,22), peach (*Prunus persica*) and cherry (*Prunus avium*) with Green Valley X disease (15), peach with peach yellow leaf roll X-disease (24,26) and various plants with aster yellows disease (20,25,26). Each of these diseases is of presumed mycoplasma etiology (8,9,11,12–14,21,25,31) with mycoplasmalike organisms (MLOs) consistently present in diseased plants.

The relation of the spiroplasmas isolated from some diseased plants to the MLOs is unknown. Clarification of the role of *S. citri* in pear, peach, and cherry as well as studies of infection in citrus would be enhanced by methods that would allow the quantification of spiroplasmas in plant tissues. Using quantification procedures, one could monitor the course of infections and distribution of the spiroplasmas within the infected plant, especially if the quantification procedures could detect low populations.

The success of the enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of bacteria (reviewed in 5) and viruses (reviewed in 2) suggested that it might be useful for the detection and quantification of spiroplasmas in plants. However, the initial screening of stubborn-diseased orange trees (cultivar Valencia) with ELISA (7) yielded variable results with a minimum detectable number of 10^5. Hafidi et al (10) obtained positive ELISA readings from seed coats, young leaves, and

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immature fruits at different times of the year. Saillard et al (28) reported consistently positive ELISA readings with young leaves of stubborn-diseased orange (cultivar Madame Vinous); however, results were negative with older leaves whose phloem contained fewer detectable organisms upon examination with the electron microscope (16). Archer and Best (1) reported that even when purified spiralin (the major membrane protein of *S. citri*) (33,34) was used as antigen, the minimum detectable number of spiroplasma cells was between 10^4 and 10^5. These results indicate that ELISA cannot be used for quantification, especially with low populations in infected plants.

Most of the isolation procedures that are generally used for spiroplasmas do not allow the quantification of the organisms in plants. Isolations are routinely performed in liquid media with several subpassages of the original inoculum (3) in order to minimize "spiroplasmostatic" factors (6,18,19) present in the plant. The recent development (J. W. Kloepper and D. G. Garrott, *unpublished*) of an isolation procedure for spiroplasmas, which allowed the direct plating of homogenates from a large sample of plant tissue, made possible the quantification of spiroplasmas, especially when present in low numbers.

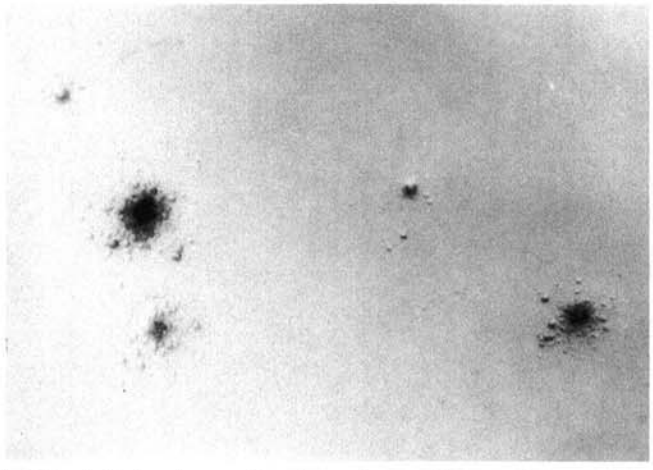

Fig. 1. Typical spiroplasma colony formation on solid mycoplasma medium (× 250). Five colony-forming units are present in the figure.

The purposes of this project were to use isolation on solid medium to gain quantitative data on the range of spiroplasma populations in various diseased plants, to quantify the relative distribution of spiroplasmas within infected plants, to test the quantification procedure with field plants, and to compare populations of *S. citri* in field-collected plants infected only with *S. citri* with populations in plants infected with *S. citri* and the noncultivable, nonhelical vinca virescence (23, and G. N. Oldfield, *unpublished*) MLO. A portion of this work was previously reported in an abstract (J. W. Kloepper and D. G. Garrott, *unpublished*).

MATERIALS AND METHODS

General quantification procedure. Plant tissues to be tested were removed from greenhouse plants, surface sterilized 1 min in 70% ethanol followed by 2.5 min in 2.5% sodium hypochlorite, rinsed three times in sterile distilled water, weighed, and homogenized in 10 ml of phosphate-buffered 10% sucrose (PBS) by using a mortar and pestle. When leaves were sampled, the petiole and leaf midveins were separated from the remaining leaf material, weighed, and homogenized. The isolation procedure was as previously reported (15) with a low-speed centrifugation at 500 *g* at 10 C followed by passage through a 0.45 μm membrane filter and an aseptic centrifugation of 25,000 *g* at 10 C. Pellets were resuspended, and serial 10-fold dilutions were prepared in DG-2 (15) broth. Aliquots (0.1 ml) of each dilution were plated onto either DG-2 or LD-8 (17) agar plates (3 ml medium in 35 × 10 mm petri dishes). Plates were dried at 27 C for 24 hr, placed inside plastic bags to maintain moisture, and incubated aerobically until colonies developed (7–14 days). Colonies (Fig. 1) were counted at the appropriate dilution, and populations were calculated as colony-forming units per gram of plant tissue (cfu/g).

Plant hosts sampled. The various host plants sampled are listed in Table 1 with the original host of each spiroplasma isolate, the symptoms of each plant, and the leafhopper vector used to infect the plants sampled in this study. Plants sampled included plants with only spiroplasma infections and plants with spiroplasmas and typical symptoms of diseases with MLO etiology (aster yellows and

TABLE 1. The range of spiroplasma populations: Greenhouse plants sampled and the original hosts of the *spiroplasmas*[a]

Infected plant sampled for quantification study	Symptoms on plant sampled[b]	Original plant host of spiroplasma	Symptoms on original plant host	Leafhopper used to transmit spiroplasmas	Leafhoppers injected or not[c]	Spiroplasma population (cfu/g)[d] in sampled plant	
						Average[e]	Maximum
Celery (cultivar Utah Pascal)	Atypical with mottled chlorosis and brittle, upturned leaves	Cherry	Green Valley X disease	*Colladonus montanus*	Injected	6.0×10^5	2.6×10^6
Periwinkle[f] (*Catharanthus roseus*)	Typical Green Valley X disease	Cherry	Green Valley X disease	*Colladonus montanus*	Not injected	50	8.1×10^2
Orange (cultivar Valencia)	Typical stubborn disease	Orange	Citrus stubborn disease	*Circulifer tenellus*	Not injected	3.0×10^3	2.5×10^4
Corn[f] (*Zea mays*) (Golden Cross Bantam hybrid)	Typical corn stunt disease	Corn	Corn stunt disease	*Euscelidius variegatus*	Injected	2.7×10^3	2.5×10^4
Plantain[f] (*Plantago major*)	Atypical with asymetric leaves and loss of seed production	Periwinkle	Aster yellows disease	*Macrosteles fascifrons*	Injected	1.1×10^6	3.1×10^6
Periwinkle[f] (*Catharanthus roseus*)	Typical aster yellows disease	Periwinkle	Aster yellows disease	*Macrosteles fascifrons*	Not injected	1.8×10^4	4.0×10^4

[a] All of the spiroplasmas, with the exception of the corn stunt spiroplasma, were serologically indistinguishable from *S. citri* using the spiroplasma deformation test (32).

[b] "Atypical" indicates that the described symptoms of the sample plant are different from the typical symptoms of the particular disease of the original host plant (column 3).

[c] "Injected" indicates leafhoppers were injected with spiroplasmas cultured from the original plant and were then fed on the sample plant. "Not injected" indicates leafhoppers were fed directly on the original plant and were then transferred to the healthy sample plant, which subsequently developed the indicated symptoms.

[d] Colony-forming units per gram.

[e] Average of three replications per sample.

[f] Plant kindly provided by A. H. Purcell, Entomology, University of California, Berkeley.

Green Valley X disease), which were kindly provided by A. H. Purcell.

Range of spiroplasma populations. In order to determine how many replications of one sample are necessary to calculate a reliable estimate of the spiroplasma population, the variability of the quantification procedure was measured. Five replications of leaf petioles from one stalk of plant 1 (Table 1) were sampled as described above, and the population of each sample was determined. The range of spiroplasma populations in plants 1–6 (Table 1) was determined by using three replicate samples from leaf petioles and midveins of the youngest fully elongated leaves.

Spiroplasma population distributions in plants. The population of spiroplasmas in petioles were compared with that in roots using celery infected with *S. citri* isolated from celery, orange with citrus stubborn disease, and corn with corn stunt disease. Four replicate samples from petioles and roots of each plant were used as described above to calculate the spiroplasma populations. The distribution of spiroplasmas in foliage was determined by calculating the populations in new, medium-aged, and old celery and corn leaves. Leaf midveins were sampled from four corn plants infected with corn stunt disease and three celery plants infected with *S. citri* isolated from cherry.

***S. citri* populations in field plants infected with a noncultivable MLO.** Broccoli plants were collected from a field plot in Riverside, CA, with symptoms of *S. citri* (45) alone, vinca virescence disease (23, and G. N. Oldfield, *unpublished*) symptoms alone, and symptoms of both *S. citri* and vinca virescence. To confirm the presence or absence of spiroplasmas and/or nonhelical MLOs, tissue from each of five plants with each of the symptom combinations was fixed in 4% glutaraldehyde + 4% formaldehyde in 0.08 M cacodylate buffer, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in a graded acetone series, and embedded in Spurr's plastic. Longitudinal sections (190–240 nm thick) were stained with uranyl-acetate and lead citrate for electron microscopic examination. Populations of spiroplasmas in each combination of symptoms were determined by using two petiole and leaf midvein samples from five plants per symptom group.

TABLE 2. Spiroplasma population distribution in petioles compared to roots

Plant sampled	Average population[a] (cfu/g)[b]	
	Petioles	Roots
Celery with *S. citri* from cherry	7.8×10^5	5.3×10^4*
Orange with citrus stubborn disease	3.0×10^3	$<1.0 \times 10^2$*
Corn with corn stunt disease	1.7×10^3	3.6×10^2

[a] Average of four replications per treatment. * Indicates significantly lower mean compared to mean population in petioles ($P = 0.05$) according to the *t*-test.
[b] Colony-forming units per gram.

TABLE 3. Spiroplasma population distribution in young, mid-aged, and old leaves

Leaf age	Spiroplasma population (cfu/g)[a]	
	Corn with corn stunt disease[b]	Celery infected with *S. citri* isolated from cherry[c]
Young	3.7×10^3*	1.2×10^5*
Medium	1.1×10^3*	5.4×10^4*
Old	1.5×10^2*	3.4×10^3*

[a] Colony-forming units per gram. * Indicates significant difference ($P = 0.05$) between means of populations from different leaf ages according to the *t*-test.
[b] Average of four replicate plants.
[c] Average of three replicate plants.

RESULTS

Range of spiroplasma populations. The spiroplasma populations in foliage varied nearly 4 log units, from 50 cfu/g to a maximum of 3.1×10^6 cfu/g, among the different plant hosts sampled (Table 1). Populations in celery of a spiroplasma (serologically indistinguishable from *S. citri*) that was originally isolated from cherry with Green Valley X disease (GVX) and injected into *Colladonus montanus* averaged 6.0×10^5 cfu/g. However, when the leafhoppers were fed on GVX-diseased cherry and allowed to transmit to periwinkle without injection, populations averaged only 50 cfu/g in the plants that also developed typical symptoms of the MLO-caused disease.

There was no significant variation in calculated populations of different replications of the same host sample. Populations of *S. citri* in celery ranged from 8.0×10^5 to 1.0×10^6 and varied less than 1 log unit among the five replications.

Spiroplasma population distributions in plants. The average spiroplasma population in petioles was approximately 1 log unit higher than the population in roots (Table 2) with each of the sampled plants. No *S. citri* colonies developed on agar plates inoculated with homogenates of tissues from roots of orange with citrus stubborn disease. The minimum detectable number of spiroplasmas was 1.0×10^2 with the weight of tissues used in this particular experiment.

Spiroplasmas were present in detectable numbers in leaves of all ages of corn with corn stunt and celery with *S. citri* (Table 3). However, populations decreased with increasing age of leaves with differences of 1 to 1.5 log units from young and old leaves.

***S. citri* populations in field plants infected with a noncultivable MLO.** Field-collected broccoli with symptoms of only *S. citri* infection (4) had an average spiroplasma population of 4.8×10^5 cfu/g (Table 4), while plants with symptoms of both infection by *S.*

TABLE 4. Spiroplasma populations in field broccoli infected only with *Spiroplasma citri* compared with broccoli infected with both *S. citri* and a noncultivatable mycoplasmalike organism associated with vinca virescence disease[a]

Signs and Symptoms[b]	Population (cfu/g)[c]					
	Replication[d]					
	1	2	3	4	5	Average[f]
S. citri alone	2.8×10^5	2.2×10^5	2.6×10^5	1.5×10^6	1.6×10^5	4.8×10^5
S. citri and Vinca virescence[e]	4.7×10^5	4.7×10^5	2.7×10^3	5.4×10^2	6.0×10^4	2.0×10^5
Vinca virescence[e] alone (symptom control)	0	0	0	0	0	0

[a] Plants were collected in September, 1980, in Riverside, California.
[b] Symptom readings were verified using electron microscopy to detect the presence or absence of spiroplamas and/or MLOs (see details in text).
[c] Colony-forming units per gram.
[d] Average of two samples per replication. Each replication represents a separate plant.
[e] Disease of presumed MLO-etiology endemic to Riverside County, CA(23 and G. N. Oldfield, *unpublished*).
[f] Means not significantly different ($P = 0.05$).

citri and vinca virescence disease (23,24) averaged less than half as many spiroplasma (2.1×10^5 cfu/g). Spiroplasma populations varied less than 1 log unit among different replications of plants infected only with *S. citri* (Table 4), while there was a wide fluctuation of over 2.5 log units in populations of plants infected with both spiroplasmas and noncultivable MLOs. No spiroplasmas were isolated from the control plants with symptoms of only vinca virescence.

Electron microscopy of thick longitudinal sections of the sampled plants in Table 4 revealed helical and nonhelical MLOs (Fig. 2) in plants with presumed mixed infections. Plants with only *S. citri* symptoms contained only filamentous helical MLOs (Fig. 3), while plants with only vinca virescence symptoms contained only nonhelical MLOs (Fig. 4). Filamentous, helical MLOs, in plants with *S. citri* symptoms averaged 120 nm in diameter, while nonhelical MLOs associated with vinca virescence symptoms averaged 320 nm in diameter. These results verified the infection states that were suggested by the symptoms noted above.

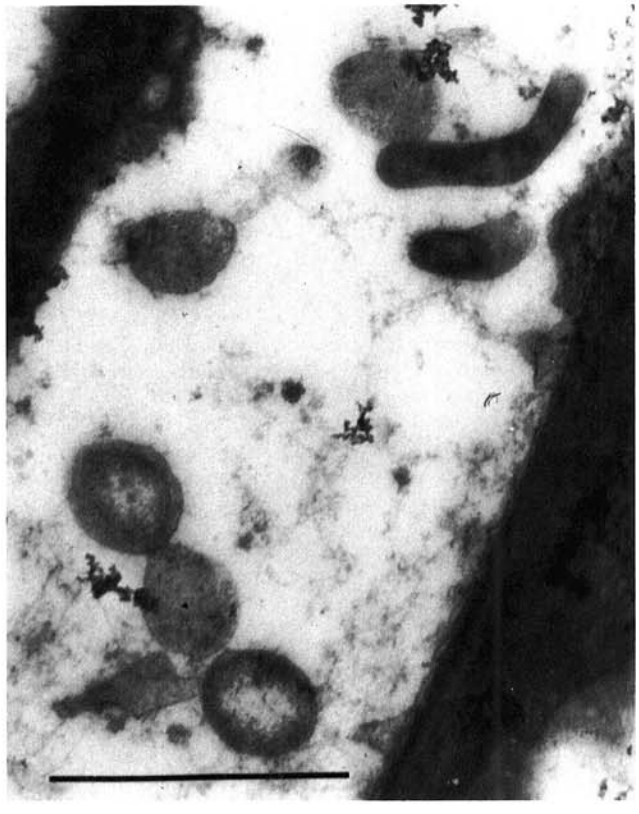

Fig. 2. Longitudinal sections (240 nm thick) of field-collected broccoli infected with both *Spiroplasma citri* and the vinca virescence mycoplasmalike organism (bar indicates 1 μm).

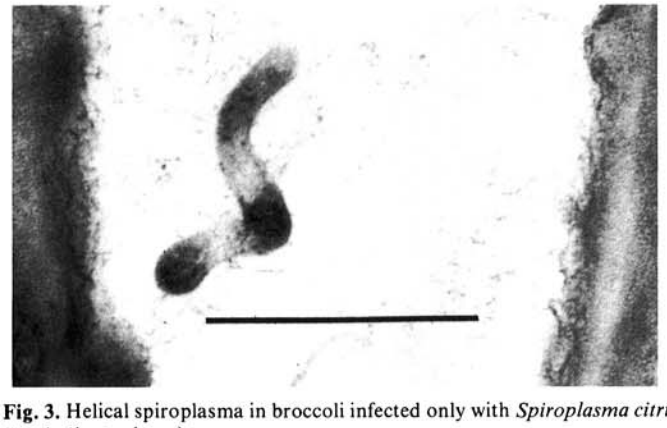

Fig. 3. Helical spiroplasma in broccoli infected only with *Spiroplasma citri* (bar indicates 1 μm).

DISCUSSION

The plate-count procedure reported here is an accurate and sensitive method for the quantification of spiroplasma populations within plant hosts. A spiroplasma population can be accurately estimated by using only three to four replications, since the variation in populations among replications of one sample was within 1 log unit, which is similar to results of plate-count procedures with bacterial plant pathogens. The procedure detects populations three to four log units below the minimum detectable level with ELISA.

Quantification of spiroplasma populations appears to be independent of the host plant used, since populations were successfully quantified in celery, periwinkle, plantain, citrus, and corn. This is an advantage over other possible quantification procedures such as a most probable number technique, which would use liquid media and would, therefore, be sensitive to inhibitors present in extracts of some plants (6,18,19). It is conceivable, however, that our method may prove to be limited by inhibitors in some plant species, which could reduce the number of viable spiroplasmas in a sample before centrifugation.

The sensitivity of the quantification procedure allows it to be used for studying the distribution of spiroplasmas within infected plants. Greenhouse celery, orange, and corn plants contained consistently lower spiroplasma populations in roots (Table 2) than in petioles. In contrast, field studies of the MLO associated with

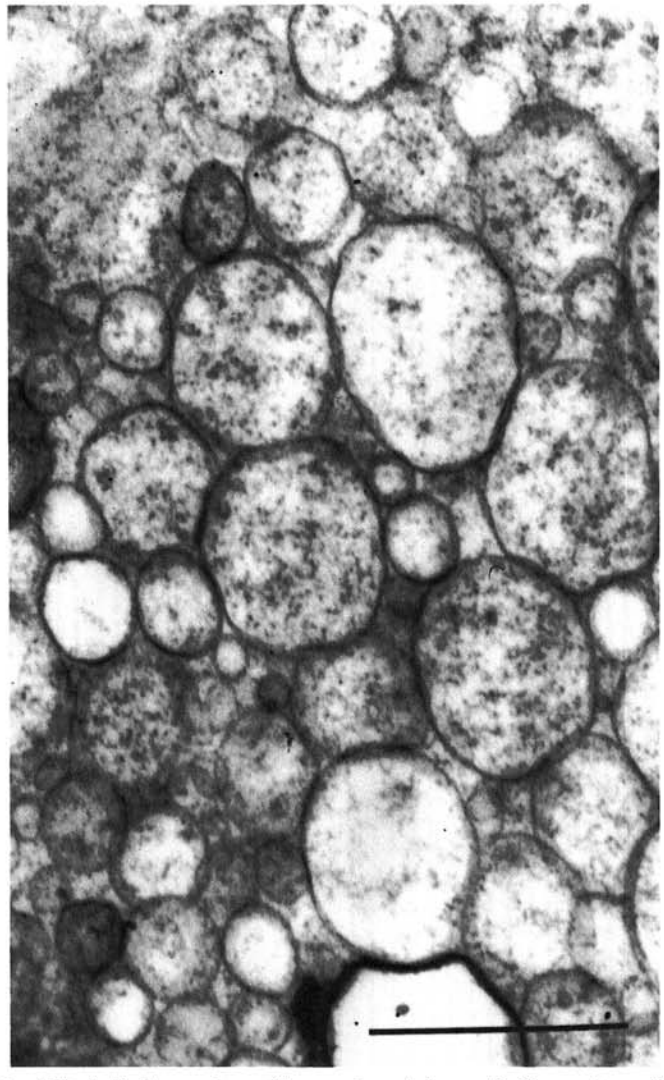

Fig. 4. Nonhelical mycoplasmalike organisms in broccoli with symptoms of vinca virescence disease. The section is a longitudinal section prepared identically to those shown in Figs. 2 and 3 (bar indicates 1 μm).

pear decline and apple proliferation diseases (30,31) suggested that higher populations occurred in roots, where MLOs were consistently detected, than in foliage, where MLOs were sporadically detected. The detection of reduced spiroplasma populations in old plant leaves compared to young leaves agrees with previous observations (29), and the procedure now quantifies this difference at 1–2 log units with the tested plants (Table 3).

The fluctuations of over 2.5 log units in individual plants infected with both *S. citri* and the MLO associated with vinca virescence disease (Table 4) suggest that spiroplasmas are compromised in some manner when they are present along with another MLO in infected plants. In contrast, there was little variation among replications of broccoli plants infected only with *S. citri* (which agrees with the greenhouse data) and the average spiroplasma population was twice the average for plants with mixed infections (Table 4). It is uncertain if the fluctuations in the case of dual infections occur from spiroplasmas "dying out" after reaching maximum levels early in the season or if the spiroplasmas never reach the maximum populations. This question and other epidemiological questions can now be addressed by using the procedure reported here.

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