Culture of Corn Stunt Spiroplasma in a Simple Medium

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ABSTRACT

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A simple medium (C-3G) containing 20% agamma horse serum, 1.5% PPLO (pleuropneumonialike organism) broth, 12% sucrose, in distilled water met all the nutritional and physical requirements for the in vitro growth of corn stunt spiroplasma. The growth characteristics of one isolate established in C-3G medium was studied after triple cloning. A sigmoid type of growth pattern was observed by directly counting the cell helix under the dark-field microscope. The doubling time of the helices in the exponential phase was calculated to be 20 hr at 29 C in nonshaken cultures. Since the organism lost motility and helicity immediately when the pH in the culture medium dropped to 5.4 or lower, this suggested that the relatively short stationary phase (1-2 days) and the rapid decrease rate in the last phase (7×10^6 helices/hr) most likely resulted from low pH rather than from exhaustion of nutrients in senescing cultures. This suggestion was further supported by the observation that the growth yield could be enhanced by the addition of 0.06 M of HEPES (*N*-2-hydroxyethyl piperazine - *N*-2-ethanesulfonic acid) buffer to strengthen the buffer activity in C-3G medium. The granulated and occasionally fried-egg shaped colonies that appeared in the interior of medium solidified with 0.8% Oxoid Ionagar were characterized by a relatively smaller size, and somewhat diffused edge.

Additional key word: plant mycoplasmas.

A helical, motile, wall-free prokaryote termed a "spiroplasma" was previously examined in expressed juice from stunt-infected corn tissues by using dark-field microscopy, thin-sectioning, and freeze-etching electron microscopy (2, 3). Its etiologic role in corn stunt disease, however, was not proved until the recent success in isolation and cultivation of the organism in two artificial media: C-3 medium (1) and M-1 medium (14). Both C-3 and M-1 media are rather complex; they contain horse (or bovine) serum, fresh yeast extract, PPLO (pleuropneumonialike organism) broth, various carbon sources, and certain prepared tissue culture media. In order to study corn stunt spiroplasma (CS spiroplasma) biochemically and nutritionally, the culture medium therefore had to be simplied and chemically defined. We present here the simplest medium we have yet developed that is suitable for the routine isolation and cultivation of CS spiroplasma, and also suitable for the serial maintenance of another plant mycoplasma (*Spiroplasma citri*) which is associated with citrus stubborn disease (4, 9). The growth characteristics of the helices of CS spiroplasma in cell-free media, which had not been reported in previous studies, were examined.

MATERIALS AND METHODS

Diseased plants.—The Rio Grande strain of corn stunt was used throughout the experiment. Diseased plants were obtained by permitting the inoculative leafhopper vectors (*Dalbulus elimatus* Ball) to feed on 7-day-old corn seedlings (cultivar Ohio 28) and subsequently incubating the inoculated corn seedlings in the greenhouse for 5 wk. The inoculative vectors were obtained by allowing previously noninfective leafhoppers to feed on a diseased plant for 7 days, then incubating them on a healthy plant for an additional 3 wk. Only those plants that showed severe corn stunt symptoms were selected for the isolation.

Culture media.—Medium C-3G, which contained 20% (v/v) agamma horse serum (Grand Island Biological Co., 3175 Staley Rd., Grand Island, NY 14072), and 1.5% (w/v) Difco PPLO broth, 12% (w/v) sucrose in distilled water was prepared by dissolving PPLO broth and sucrose in distilled water and autoclaving at 120 C for 15 min. Agamma horse serum that previously had been inactivated at 56 C for 40 min and filtered through 0.22-μm Millipore membrane filters, was added when the autoclaved PPLO broth and sucrose solution cooled. In some cases, 0.06 M of HEPES (*N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid) buffer at pH 7.4 was included to strengthen the buffer activity in C-3G medium. The osmolality in HEPES-buffered C-3G medium was adjusted with sucrose to approximately 700 mosm. The osmolality and pH in culture medium were measured with an Osmett Precision Osmoter and a Corning Model 7 pH meter, respectively.

Isolation and cultivation.—The technique for the isolation was based on the one described by Chen and Liao (1). Briefly, a piece of diseased stem tissue (0.4 cm $\times$ 1.2 cm) was sterilized superficially with 75% ethyl alcohol, momentarily held over an alcohol flame, and then placed in a sterile petri dish. The stem juice was squeezed out and mixed with 0.8 ml of fresh C-3G medium, 0.1 ml of which was transferred into a culture tube containing 3.5 ml of C-3G medium. In order for growth to continue, 0.2 ml of the

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primary culture was transferred within 1-2 days into a new culture tube. Two wk of incubation normally were required for the organism to reach its maximum cell concentration in the secondary culture at 29 C. The established cultures were serially transferred at 6-day intervals. Occasionally 0.1-ml portions of dilutions of 6-day-old liquid culture were added into a tightly sealed petri dish (5 cm × 0.5 cm, Falcon Plastics, Fisher Scientific Co. 15 Jet View Dr. Rochester, NY 14624) and mixed with 2.5 ml of solid C-3G medium containing 0.8% Oxoid Ionagar unless otherwise indicated. All the solid cultures were kept in a partially anaerobic chamber using Gas Paks (BBL, Div., Becton, Dickinson, & Co. Cockeysville, MD 21030) at 29 C. Colony formation was examined with a dissecting microscope or phase-contrast microscope.

Enumeration of helices.—Normally, 20 μg of phenol red/ml was added to C-3G medium; growth of CS spiroplasma was indicated by the acid production that mediated a color change (from red to yellow) in culture media. Since the helical, motile filaments of CS spiroplasma could be observed easily with a dark-field microscope, a simulated haemocytometric method was designed to quantitate the cell growth. To do this, 5 μliters of culture was microsampled and deposited on a microslide. These portions of culture then were fully covered with a thin coverslip (18 mm × 18 mm, Fisher Scientific Co., 15 Jet View Dr., Rochester, NY 14624) and directly counted under the dark-field microscope (× 1,500, Nikon Model M inverted microscope). Care was taken to evenly distribute 5 μliters of sample under the exact area of one coverslip. For each sample, five visual fields were randomly selected, counted and recorded. Since the total area of one coverslip was equivalent to 324 mm^2 (= 18 cm × 18 cm) and the area of one visual field was determined to be 0.0165 mm^2 by using a grid ocular micrometer, the number of cells counted in a visual field represented $1/1.96 \times 10^4$ (= 0.0165 mm^2/324 mm^2) portions of the 5-μliter sample. The total number of cells in 5 μliters of culture then was calculated by multiplying the average cell number per visual field by the constant 1.96×10^4. The cell aggregate observed in aged cultures included a large number of helices. Prior to counting, the aged cultures were shaken on a Vortex mixer for 2 min and diluted tenfold in fresh C-3G medium. Most of the cell aggregates were dispersed during Vortex-type mixing and serial dilution. Occasionally, one or two tiny aggregates per visual field still remained in the diluted cultures. In these cases, the helices in the aggregate were individually counted.

RESULTS

Medium C-3G, (20% agamma horse serum, 1.5% PPLO broth, and 12% sucrose) fulfilled all the nutritional and physical requirements for the growth of CS spiroplasma. In ten attempts seven isolates were successfully isolated and established in C-3G medium. Growth of *Spiroplasma citri* also was obtained in this simple medium for at least thirty passages. The pH and osmolality in C-3G medium were determined to be 7.4 and 700 ± 15 mosm, respectively. Retarded growth of CS spiroplasma was observed consistently in primary cultures. Since the organism lost viability gradually in primary cultures, it was essential to transfer the organism from the primary culture into fresh medium within a short period of time. It was found that 2-3 days were the limit. Beyond that, the helical shapes of the cells became distorted and their numbers were greatly reduced. None of the seven isolates established in C-3G medium was observed to grow continuously in primary cultures. A relatively long period of about 2 wk was needed for the organism to reach its maximal growth in secondary cultures.

One of the seven isolates that were established was serially transferred in C-3G medium at 6-day intervals and used for the study of growth characteristics at the tenth passage. This isolate was purified by three filter-cloning procedures. For the measurement of the growth curve, 100 ml of C-3G (or HEPES-buffered C-3G) medium was inoculated with 5 ml of 6-day-old culture and incubated at 29 C. The cell concentration and pH change in culture medium were measured daily for 14 days.

The growth pattern of the helices of CS spiroplasma and relative pH change in C-3G medium are shown in Fig. 1. The sigmoid type of growth, resembling the life cycle of bacteria and other mycoplasmas, clearly involved four phases. Each phase was characterized by a characteristic rate of population change. In the lag phase the growth rate was zero. The duration of the lag phase varied from several hours to 2 days depending upon the growth stage

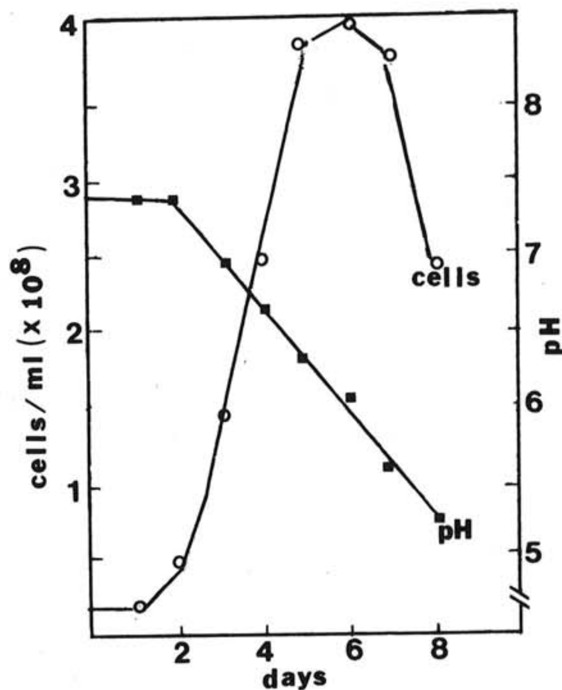

Fig. 1. The growth pattern of the helices of corn stunt spiroplasma in C-3G culture medium containing 12% sucrose, 1.5% PPLO (pleuropneumonialike organism) broth, 20% agamma horse serum, and distilled water. Note that the rapid decrease of helices followed immediately when the pH in C-3G medium dropped to 5.4.

of inocula. In the exponential (logarithmic) phase, the growth rate reached the constant maximal value. The doubling time in this phase was 20 hr at 29 C for a nonshaken culture. The duration of the exponential phase was about 3-4 days. After the stationary phase, the helices became very slender (up to 15 μm long) and lost helical configuration. The decrease of helices in the last phase was suprisingly rapid: the titer dropped by a factor of about 7×10^6 helices/hr. The helices seemed to be very sensitive to high concentrations of hydrogen ion. As shown in Fig. 1, the number of helices decreased immediately when the pH in culture medium dropped to 5.4.

The growth patterns of the helices of CS spiroplasma in C-3G and HEPES-buffered C-3G medium are compared in Fig. 2. A somewhat longer exponential phase (about 7 days) was observed in HEPES-buffered medium. The growth yield was apparently enhanced by the addition of 0.06 M HEPES buffer of pH 7.4 in culture media. Although the organism took 2 days longer to reach its maximum yield in HEPES-buffered medium, the concentration of helices in the stationary phase in HEPES-buffered medium was almost twice that in HEPES-free medium. However, the rapid decrease in number in the HEPES-buffered medium also followed immediately when the pH dropped to 5.4.

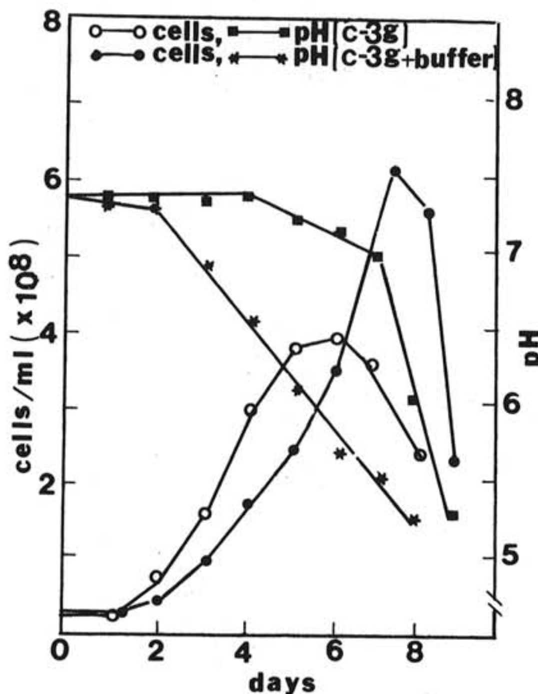

Fig. 2. The comparative growth pattern of the helices of corn stunt spiroplasma in C-3G medium with and without 0.06 M of HEPES (*N*′-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid) buffer. Medium C-3G contained 12% sucrose, 1.5% PPLO (pleuropneumonialike organism) broth, 20% agamma horse serum, and distilled water. Note the higher growth yield and longer maintenance of adequate pH in HEPES (*N*′-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid)-buffered medium.

The cellular morphology of CS spiroplasma in liquid cultures was investigated with both dark-field microscopy and electron microscopy. The helical, motile morphology present in the culture medium was identical with that observed in expressed juice from stunt-infected corn plants (2, 3). The majority of the constituent bodies found in the dark-field microscope were narrow, helical filaments ranging from 3-8 μm in length and 0.20-0.25 μm in diameter. As the culture aged, the helical filaments increased in length, branched, and became aggregated. The aggregate form was similar to that observed in the sedimented cell pellet. When the liquid culture was examined under the dark-field microscope, the helical filaments exhibited two types of motility, a screw-type of rotational movement, and a flexional movement. The screw-type of movement was reversible, and was characterized by rapid spinning about the long axis of the spiral. The flexional movement was manifested principally by continuous curling, bending, or undulating. Neither rotational movement nor flexional movement resulted in a net change of position. The helical filaments floated freely and with a wave-like motion in the suspending medium. A large, round or irregular body (bleb body) was frequently found at the end or in the middle of each filament. When negatively stained with 2% PTA (phosphotungstic acid) and examined under the electron microscope the helix seen in two cases had a blunt tip in one end and a pointed tip in another. Occasionally the branching of the filament also was observed.

Small, granulated colonies (Fig. 3-A, B) were found in C-3G medium containing 0.8% Oxoid Ionagar two weeks after inoculation. The amount of growth became greater when solid cultures were incubated with a BBL Gas Pak. The colonies were diffuse and lacked a clearly defined edge (Fig. 3 C) in C-3G medium containing Oxoid Ionagar less than 0.5%. It is suggested that the newly reproduced cells at the periphery of colonies were able to penetrate and diffuse in the soft agar medium. On the contrary, if agar concentration was increased up to 1.4%, which obviously impeded penetration by the motile organism, tiny colonies formed only on the surface of medium. Generally colonies were observed in the interior of solid media when inocula (diluted broth cultures) were mixed with solid media during inoculation. It was found that mixing of inocula with solid media allowed more colonies to develop than developed by spreading inocula on the surface. Acid production also occurred in solid cultures as the formation of colonies always was accompanied by a color change (from red to yellow) in the media. Each colony, when observed by dark-field microscopy, had a mass of helical cells somewhat resembling that formed in aged liquid cultures or in sedimented cell pellets.

DISCUSSION

None of the seven isolates of CS spiroplasma obtained in this study grew continuously in primary cultures. For growth to continue, primary cultures had to be transferred within 2-3 days after inoculation. We suggest that a spiroplasmacidal factor or an inhibitor from diseased corn tissue extract was introduced during the primary inoculation. Two lines of evidence strongly

supported this hypothesis: (i) The growth of an established isolate (ATCC No. 29051) in C-3G medium could be blocked by the addition of corn tissue extract at a dilution up to 1/160 (6); (ii) The organism could grow continuously in primary cultures if inocula obtained from inoculative leafhopper vectors were used (T. A. Chen, B. C. Raju, and C. H. Liao, *unpublished*). These results indicated that the spiroplasmacidal factors demonstrated in corn tissue extract were absent in inoculative leafhopper vectors.

At the same time as we succeeded in culturing CS spiroplasma in C-3 medium (1), Williamson and Whitcomb (14) reported cultivation of this organism in another artificial medium (M-1 medium). No retarded growth in primary cultures was observed. We were interested to know why there was no difficulty cultivating the organism in primary cultures throughout their experiments. The organism (ATCC No. 27954) they isolated can grow equally well in C-3G medium (not shown in results). This would exclude the assumption that M-1 medium contained certain essential nutrients which are deficient in C-3G medium. They prepared inocula by centrifuging the diseased plant sap at high force (16,000 *g*, 30 min), then washing and resuspending the pellet in fresh medium. These treatments probably removed most of spiroplasmacidal factors (in soluble form) in the plant sap and allowed them to cultivate this organism in primary cultures.

Medium C-3G contained four ingredients: PPLO broth, sucrose, horse serum, and distilled water. The high concentration of sucrose (about 0.25 M) was mainly to adjust the osmolality of culture medium. Whether sucrose is utilized as a carbon source is not clear. The osmolality of C-3G medium falls within the optimum range, which had been determined to be 610-840 mosm (6). The CS spiroplasma failed to grow in horse-serum-free medium (not shown in Results), the addition of horse serum in culture media was essential. At present, the role of horse serum in the nutrition of CS spiroplasma is not understood. Sterols have been reported to be indispensable nutrients for *S. citri* (9). Whether horse-serum is only a source of sterols or contains some other growth factors for CS spiroplasma remains to be proven. The ingredients in the PPLO broth must provide certain important nutrients which also may act as a detoxicant to neutralize surface-active agents in culture medium. Certain species of sterol-requiring *Mycoplasma* had been shown to be lysed by surface-active agents such as bile salts, unesterified long-chain fatty acid in horse serum (7).

Efforts were made to chemically define the culture media for mycoplasmas and spiroplasmas. The difficulties encountered were primarily due to the highly fastidious nature of the spiroplasma as well as the plasticity and the limited metabolic pathways of this group of fragile microorganisms. The only two defined synthetic media so far available for *Acholeplasma*

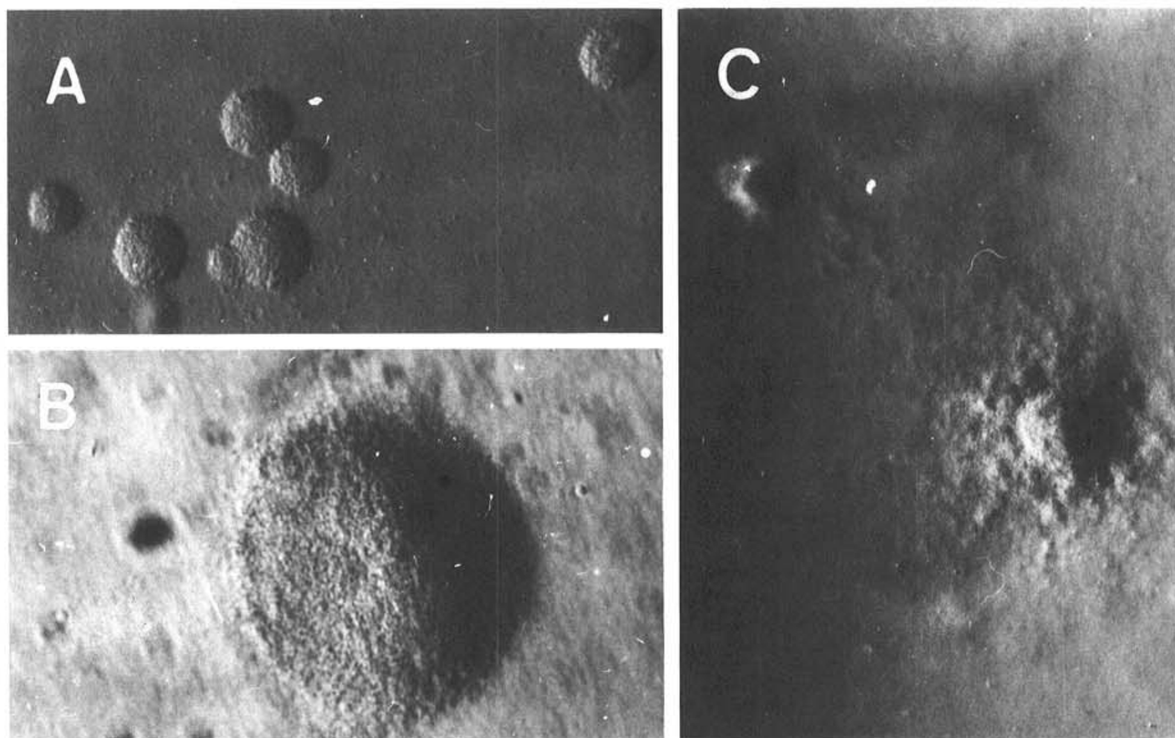

Fig. 3-(A to C). The colony formation of corn stunt spiroplasma in agar medium. **A)** Small, granulated colonies formed in C-3G medium containing 0.8% of Oxoid Ionagar (phase-contrast microscopy, ×450): **B)** During the late stage of growth, the heaping of the organism in agar medium (phase-contrast microscopy, × 1,850): **C)** In C-3G medium containing Oxoid Ionagar less than 0.5%, the colony margins were diffused, lacking a clearly defined edge (phase-contrast microscopy, × 450). Medium C-3G contained 12% sucrose, 1.5% PPLO (pleuropneumonialike organism) broth, 20% agamma horse serum, and distilled water.

laidlawii (13) and *Mycoplasma* strain Y (8), respectively, were unable to support the growth of CS spiroplasma (C. H. Liao and T. A. Chen, *unpublished*). Although the chemical composition of C-3G medium is still quite complicated, the elimination of fresh yeast extract and tissue culture media such as TC 199 medium, Schneider's *Drosophila* medium, and CMRL 1066 medium from C-3 and M-1 media has provided a step forward to define a minimal medium for the culture of both CS spiroplasma and *S. citri*.

The members of *Mollicutes* are characterized by their particular form of colony. The typical colonies formed by mycoplasmas are normally round with a sharply defined edge. Their average diameter is about 100 μm but may vary from 10 to 600 μm, depending upon the species or growth condition. They usually exhibit a dense center and a translucent periphery, giving the so-called "fried-egg" shaped appearance. The greater density of the central area is primarily characteristic of growth of the mycoplasma in agar, but in some instances is due to heaping of the organism on the surface of medium. The formation of fried-egg shaped colonies has been reported with *S. citri* (9). The small, granulated colonies (about 30 μm in diameter) were normally observed with CS spiroplasma. The diffusable and granulated formation of colonies of CS spiroplasma likely resulted from the relatively slow growth rate and actively flexing motility of the organisms at the periphery of colonies.

The determination of turbidity (11) has frequently been used to measure the growth of *Mycoplasma* spp. such as *M. mycoides*. This method was not applicable for CS spiroplasma because CS spiroplasma produced negligible turbidity even under optimum conditions. Besides, the precipitate formed by the organisms in the culture medium after a long period of incubation interferes with the turbidity reading. Measurement of dry-weight (12), DNA, or protein nitrogen (5) has been used by other investigators. The small volume of CS spiroplasma and the residue from salts, etc., in culture media would make dry-weight or chemical analysis inaccurate. The most common, and probably the most accurate and simple means of assay is the measurement of the colony-forming unit (CFU). Unfortunately, many colonies formed by CS spiroplasma lack a sharply defined edge. As the organism moved in the solid medium it became very difficult to count individual colonies. The helicity and relatively large size of CS spiroplasma, however, makes it possible to count the single helix under the dark-field microscope. The only difficulty encountered was the formation of aggregates in old cultures. To ensure that the helices in aggregates dispersed in the culture medium, we shook old cultures on a Vortex mixer for 2 min and serially diluted them before counting.

We observed that the growth pattern of the helices involved four phases. A sigmoid type of increase resembled the growth cycle of bacteria and mycoplasmas, even though a comparatively longer lag phase and shorter stationary phase were repeatedly found. The length of the helices varied from 3 to 15 μm depending upon the age and medium. The helices were shorter in young cultures and increased their length as the culture became older. The rapid decrease of helices (7×10^6 helices/hr) in the last phase presumably resulted from the active production of acid in culture medium. The helices lost their helicity and motility as soon as the pH of culture medium dropped to 5.4 or lower. The pH of culture medium, therefore, plays an important role in the maintenance of helicity and motility of CS spiroplasmas.

Since the reproduction cycle of CS spiroplasma has not yet been elucidated, it remains questionable if the helices observed under the dark-field microscope are the only cellular shape of this organism throughout its life cycle. In this study we surveyed the growth pattern of the helices by direct counting under dark-field microscopy. Under these conditions it was not possible to distinguish coccoid forms, if they indeed exist in the life cycle of CS spiroplasma, from other artifacts in the medium. Recently, Whitcomb and Williamson (15) applied the color-change units (CCU) method to evaluate the viable cell concentration of CS spiroplasma in culture medium. Saglio et al. (10) employed the railisotope (^{32}p) incorporation technique to measure the growth of *S. citri*. Both methods might be useful to quantitate spiroplasma growth in the absence of suitable colony formation on agar.

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