Spiroplasma: Motile, Helical Microorganism Associated with Corn Stunt Disease

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ABSTRACT

Helical morphology and contractile movements of filaments produced by the organism associated with corn stunt disease distinguish it from described species of mycoplasmas. The filaments are 0.2 to 0.25 μ by 3 to ca. 15 μ, usually with regular wave (gyre) length and amplitude in a given coil, rarely loosely or irregularly coiled, and often with spherical bodies (0.4 to 0.6 μ in diam) attached. Three-dimensionality of the filament helix was observed by phase contrast light microscopy and by stereo electron microscopy. Size and apparent lack of cell wall, axial fibrils, flagellar structure, and envelope suggest affinities with mycoplasmas, but in juice expressed from infected plants, the filaments whirled or spun rapidly about the long axis of the helix and exhibited flexional (flexing, bending, and curling) motions reminiscent of movements by some spirochaetes. In order to reflect important differences from previously described species, we propose use of the trivial term spiroplasma for the organism, until sufficient data are available to assign the organism a Latin binomial and a taxonomic position.

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Additional key words: Zea mays, spirochaete, mycoplasmalike organism.

Recently, Davis et al. (7) reported the first occurrence of a plant-inhabiting microorganism with a helical morphology. Because of the association of this unusual organism with development of corn stunt (CS) disease in plants, they suggested that the organism may be the corn stunt agent itself. Isolation and purification or cultivation of the organism in vitro and fulfillment of Koch's postulates, however, are necessary to prove this hypothesis.

Even though a causal role in CS disease cannot yet be assigned this organism, its association with CS disease and its unusual morphology are significant. Because the microorganism as described by others (3, 9, 10, 13) resembles mycoplasmas in size, pleomorphism, and ultrastructure, it has been referred to as "mycoplasmalike", as have the presumed agents of other yellows diseases (5, 12, 19). Prior to work by Davis et al. (7), however, helical morphology was overlooked. In our view, helical morphology of filaments is an important feature of the organism associated with CS disease. Yet, helical morphology has not been described for any member of the Mollicutes (mycoplasmas and acholeplasmas). A question of appropriateness of the term "mycoplasmalike" thus arises when it is applied to the presumed CS agent. In addition, the helical organism in CS infections exhibits a motility that is undescribed, to our knowledge, for a filamentous mollicute (member of class Mollicutes) from any source.

This paper describes the contractile movements of helical filaments produced by the organism associated with CS disease, and documents in three-dimension its helical shape. We also discuss some of the implications of the findings for ultrastructure and classification, and propose use of a trivial name, spiroplasma, for the motile, helical organism in CS disease. A brief report on motility has appeared (6).

MATERIALS AND METHODS.—The Rio Grande strain of the corn stunt disease agent was employed throughout this work. The isolate was kindly provided by T. S. Chen (Rutgers University, New Brunswick, N.J.), and it descends from that reported on by Chen & Granados (3), Granados (9), Granados et al. (10), and Maramorosch et al. (13). The agent was maintained in our laboratory in corn (*Zea mays* L. 'Golden Bantam') and was transmitted from plant to plant by the leafhopper vector, *Dalbulus elimatus* Ball.

We prepared specimens for observation by expressing a drop of juice from tissue removed from the midrib of a leaf exhibiting corn stunt symptoms, or from the tassel of infected plants onto a glass slide. The drop was overlaid with a glass cover slip and observed under a high contrast phase, oil immersion objective on a Zeiss photomicroscope. Motility of the helical filaments was recorded by cinematography (Kodak Tri X, 16 mm, 18 frames/sec).

For treatment with glutaraldehyde fumes, drops of plant juice on glass slides were inverted over vials containing 3% glutaraldehyde in 0.15 M phosphate buffer, pH 7. For heat treatments, slides bearing drops of juice were placed on slide warming plates set at 40 or 56 C.

For electron microscopy, 2 mm^2 pieces of tissue of various plant parts from infected plants with typical CS symptoms and similar pieces from uninfected plants were fixed in 3% glutaraldehyde in 0.15 M phosphate buffer, pH 7, postfixed in 2% osmium tetroxide in the same buffer, dehydrated in an ethanol series, and embedded in araldite resin. Thin (silver to gold) and thick (purple to green) ultramicrotome sections were stained with uranyl acetate and lead citrate. For stereo pairs, sections cut at the maximum setting of the thermal advance of a Reichert OmU2 ultramicrotome were most suitable.

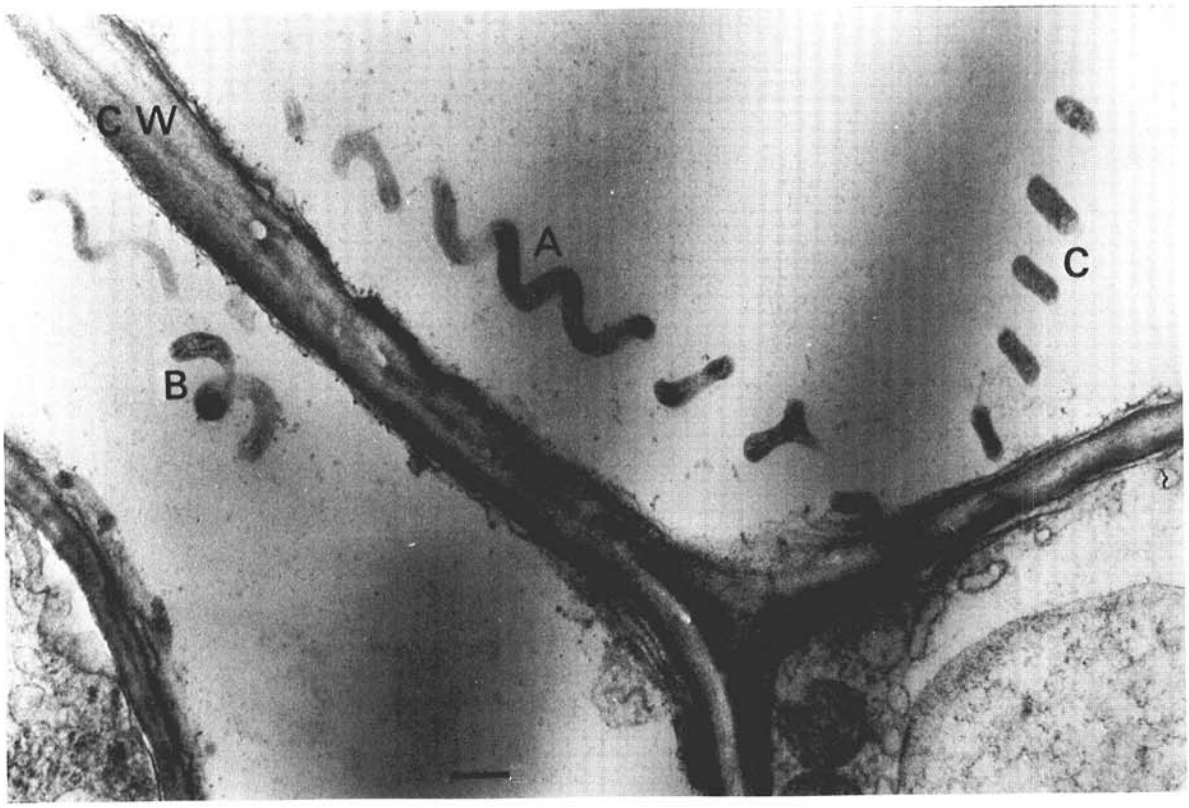

Fig. 1. Electron micrograph of a thick ultramicrotome section (cut at about 0.3 μ) of phloem tissue from a corn stunt (CS)-infected corn plant. CW = cell wall. A = helically coiled filament of the microorganism consistently associated with CS disease. Note the regularity of wavelength and amplitude of the coil. B = portion of a helical filament. C = portions of a single filament cut in a plane that included only the crests of adjacent gyres. Bar represents about 0.5 μ.

Ribbons were usually compressed, and required several passes of a cotton swab saturated in chloroform to flatten them. On the basis of preliminary trials where we varied the tilt angles from -30° to $+30^\circ$, an angle difference of 15° was chosen as providing the best third-dimensional effect. Most commonly, the stereo pairs were taken at 0° and $+15^\circ$ tilt.

RESULTS.– *Motility.*–The helical filaments described here and previously (7) were found only in CS-infected plants, and were not found in any CS-free plants. In thick ultramicrotome sections (Fig. 1) of phloem of CS-infected plants, the helically coiled filaments were 0.2 to 0.25 μ by 3 to about 15 μ, usually with regular wave (gyre) length and amplitude in a given coil, rarely loosely or irregularly coiled. Spherical bodies (0.4 to 0.6 μ in diam) were seen attached to some filaments; whether such bodies represent a stage in the growth of the organism is not known. As previously described (7), no cell wall, envelope, axial fibrils, or flagellae were seen. In stereo pairs of electron micrographs of thick ultramicrotome sections of phloem (Fig. 2), the spiral morphology of filaments was especially apparent. Steere & Davis (18) recently reported the helical morphology of filaments in electron micrographs of replicas of freeze-fractured phloem from CS-infected plants.

Juice expressed from all parts of plants with symptoms contained motile filaments. Most, but not all, filaments in a given sample were motile. Since juice from tassels of infected plants frequently contained higher numbers of filaments than did juice from other parts of the same plants, tassels were used as source material for most tests. Nevertheless, the same type of motility described for filaments from tassels could be seen in filaments from all other plant parts. Filaments exhibited the same movements whether or not spherical bodies were attached. None of the motility described here could adequately be explained by invoking external forces of Brownian motion.

Filaments floating free in the suspending medium or attached to the glass slide exhibited rapid spinning about the long axis of the spiral, as well as flexional movements (curling, flexing, and bending). These movements were reminiscent of those described for some spirochaetes (4, 11), but in the case of the presumed CS agent we have observed no translational motion. The movements were always readily apparent in fresh preparations, and often aided recognition of

the filaments, even though expressed juice contained abundant host plant subcellular organelles and debris. Because of the short depth of focus at the high magnification used, however, the movements of filaments were more easily documented by cinematography when one or several points of a filament were attached to the glass slide or cover slip.

Under phase contrast, filaments in expressed juice were also obviously helical, although this is not apparent from our phase contrast photographs. Figures 3 through 10 illustrate two groups (Fig. 3-6 and 7-10) of consecutive frames from a motion picture film of one filament in juice expressed from an infected plant. The writhing and "vermiform" movements of this filament are more easily illustrated in still figures than are the rapid spinning movements of filaments less thoroughly adhered to the glass slide. The position of the filament between any two consecutive frames occupies a time span of 1/18 sec. Thus, movement, for example, from a folded position in Fig. 8 to a straightened coil in Fig. 10 took place within 1/9 sec, and illustrates relatively rapid motion. In other cases, however, slower sinuous movements could be seen. Stationary round bodies (R in Fig. 3) near the filament serve as reference points.

Figure 5 shows development of a second "bend" in the filament. Often, such an apparent bend travelled along part of the length of a filament, then reversed direction if the filament adhered to the glass at both ends. At times, when a filament became adhered over much of its length, movements such as this were irregular and seemed "vermiform", or writhing. Occasionally, changes in position occurred following vigorous motion that led to partial detachment of the filament from the glass slide. Throughout the observations, the filament seemed most firmly attached to the glass at one point (A in Fig. 3).

Frequently, contractile waves appeared to travel from end to end of a filament. This was especially apparent in longer filaments, which may be 15 μ or more in length. So far, we have observed no swimming or gliding.

Adherence of many motile filaments to the glass slide or cover slip afforded the opportunity to replace the suspending fluid (a mixture of plant sap, xylem fluid, and various other cell contents) with media of known composition. Rotational and flexional movements of the coiled filaments were observed in the medium of Saglio et al. (16), Chen's medium (3), or 10.5% sucrose.

Effect of glutaraldehyde and heat treatments.—The contractile movements exhibited by the helical filaments appeared to be nonrandom. Nevertheless, it seemed advisable to attempt to determine whether Brownian motion might be

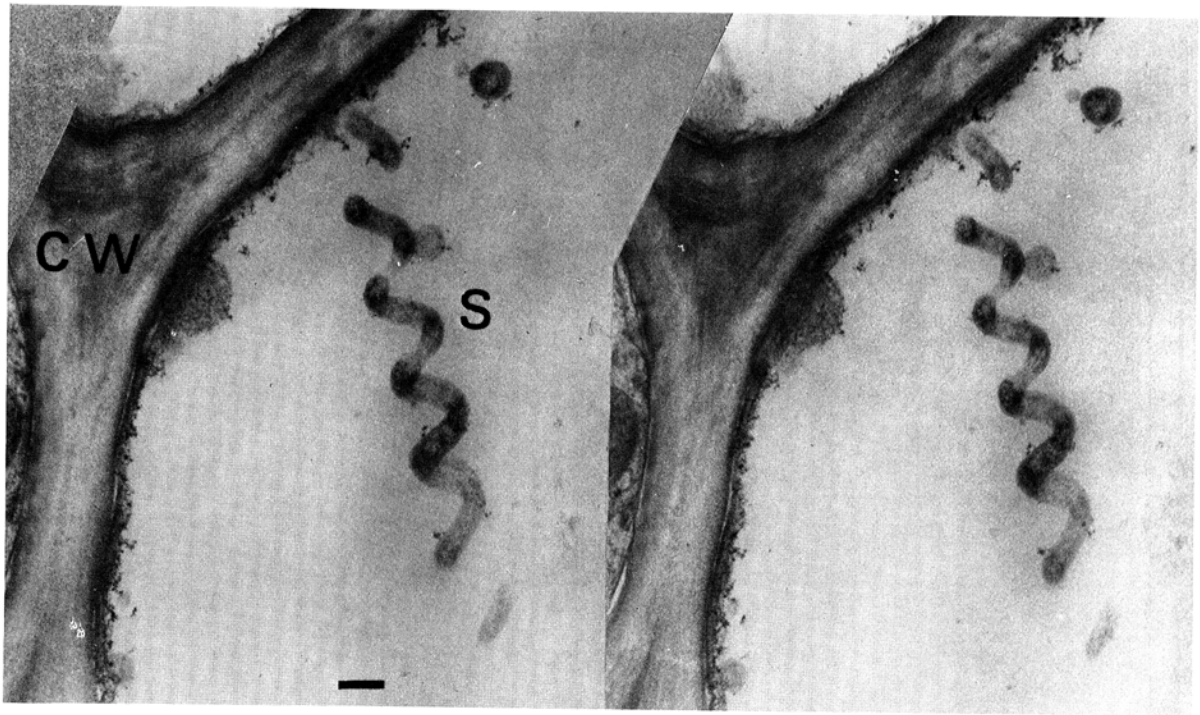

Fig. 2. Stereo pair of electron micrographs of a thick ultramicrotome section (cut at about 0.3 μ) of phloem tissue from a corn stunt (CS)-infected plant. CW = cell wall. S = helically coiled filament of the microorganism (spiroplasma) associated with CS disease. Note three-dimensionality and right-handed sense of the spiral. Stereo pair tilt angles on X and Y coordinates, respectively; (0°, 0°) and (0°, + 15°). Bar represents about 0.5 μ.

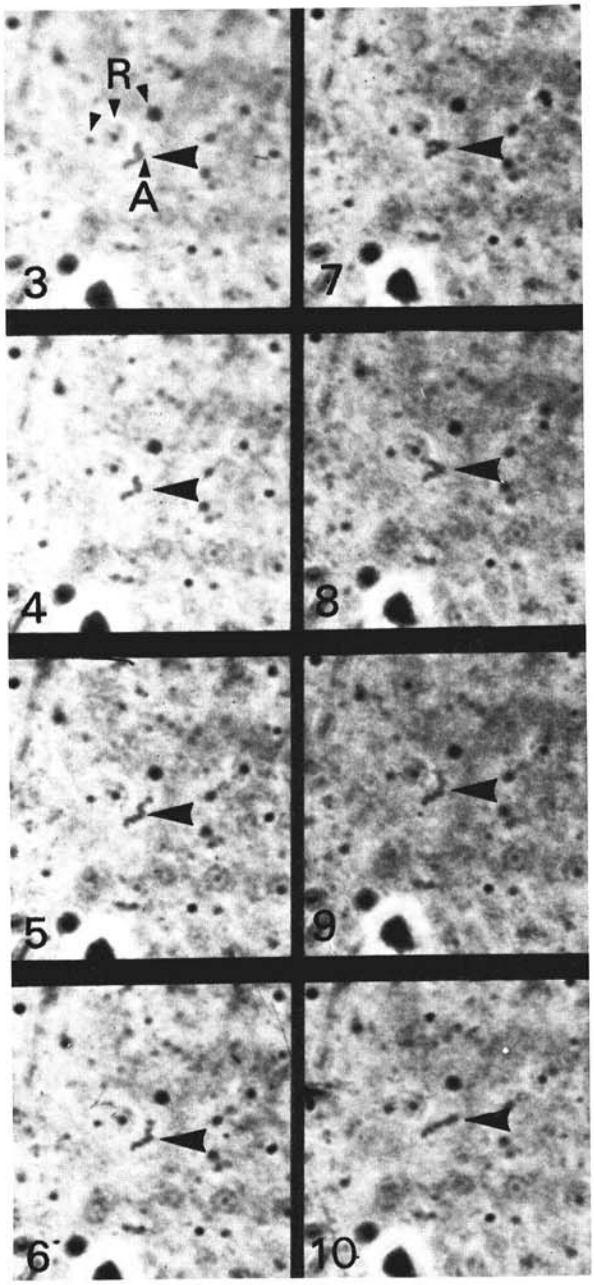

Fig. 3-10. Two series of portions (3-6, 7-10) of consecutive frames from a 16-mm motion picture of a helical filament (arrow) observed by phase contrast light microscopy in juice expressed from a corn stunt-infected corn plant. R = stationary particles that may serve as reference points. A = area of filament firmly adhered to the glass slide (approximate magnification × 2,500).

responsible for these movements. For this purpose, we examined filaments after fixation with glutaraldehyde. For treatment, one drop of juice expressed from the tassel of a CS-infected plant was placed on each of three slides. One slide was examined immediately. Of the remaining two, one was inverted and suspended over 3% glutaraldehyde, and the other (control) was inverted over distilled water for 10 min. Motility of helical filaments in the control samples was very vigorous, even after 2 hr at 22 C. After exposure to glutaraldehyde, however, no change in the morphology of the filaments was discerned by phase contrast, but the helical filaments were immobile, except for Brownian motion, and no contractile movements of the type described under *Motility* were observed. Although these results do not completely rule out a possible role of Brownian motion or of electrostatic charges on glass slide or cover slip, they are consistent with the concept that the spinning, curling, and bending of filaments are contractile movements.

Several tests were conducted to determine whether motility might be inhibited by brief heat treatments. Drops of juice were expressed onto glass slides and covered with cover slips, and the cover slips left unsealed or sealed with paraffin wax. These slides were then placed on slide warming plates at 40 or 56 C for various time intervals. Less than 1-min exposure at 56 C resulted in disruption of all spiral filaments in preparations with several hundred filaments/oil immersion phase contrast field at 0 time. No helical filaments could be seen by phase contrast following the treatment. Filaments pretreated with glutaraldehyde, however, were intact (but not motile) even after 30 min at 56 C. At 40 C, filaments were disrupted in less than 10 min, but were similarly protected by fixation with glutaraldehyde. The fragility of the filaments is consistent with the apparent absence of rigid cell wall.

DISCUSSION.—The contractile motility we describe here implies the existence of a structure necessary to accomplish this function. Preliminary data indicate that the contractile motility is inhibited by 2, 4-dinitrophenol, suggesting that the movements are energy-dependent. Although some of our unpublished electron micrographs suggest a possible internal structure, we have not discerned cell wall, envelope, flagellae, or axial fibril-like structures, nor reliably demonstrated internal ultrastructure, to which we can ascribe the function of motility. Yet, we postulate the existence of such a structure and are continuing our search.

As indicated previously (5), the presumed plant yellows disease agents may comprise a taxon entirely new to plant pathology. The members of this taxon bear similarities in size, pleomorphism, and apparent lack of cell wall to organisms presently in the Mollicutes, but there appear to be some important fundamental differences. Intracellular habitat, vectorship, and multiplication in both plants and insects are intriguing differences supporting the contention (5) that the plant-inhabiting mycoplasmalike organisms will probably give rise to new genera and possibly new higher taxa as well. We now know that the organism associated with CS disease bears other intriguing differences from organisms presently in the class Mollicutes. One of these is the ability of the organism to form helically

twisted filaments. Helical morphology of filaments suggests important structural differences from the filaments formed by known mycoplasmas and acholeplasmas. Although formation of helical filaments possibly may be influenced by environment and stage of growth, we know of no information indicating that either mycoplasmas or acholeplasmas are capable of forming helical filaments under any circumstances. Nonseptate helical cells are produced by members of the Spirochaetales (4), of course, but these organisms are generally larger than the organism in CS, and possess cell wall and axial filaments or cristae. The spiral organisms produced by members of the genera *Spirillum* and *Bdellovibrio* possess cell walls and flagellae (17, 20).

The movements exhibited by the helical filaments is yet another characteristic divergent from members of the class Mollicutes. In spite of a large amount of research on mycoplasmas, especially over the past 30 years, only a few reports of motility have appeared. In 1946, Andrewes & Welch (1) reported motility of a mycoplasma isolated from mice. Spherical bodies, with a "stalk" attached to each, were observed to move against a liquid current. A wave of thickening passed along the stalk preceding a moving body. Motility was lost after several subcultures in vitro. In 1965, Nelson & Lyons (14) reported gliding of rods and spinning of spherical forms of *Mycoplasma pulmonis*. Motility was lost, however, during subculturing. Bredt (2) reported spinning and translational movement by spherical bodies of *M. pneumoniae*. In our judgment, the motility recorded in these papers differs greatly from the contractile motility we report here. Filaments are formed by a number of mycoplasmas in culture (15), but motility of mycoplasma filaments has not been reported. Indeed, the mycoplasmas and acholeplasmas have been characterized as nonmotile (8). Motility thus suggests the possibility of important differences in structure and function of filaments, and makes increasingly apparent the divergence of the organism in corn stunt from present known members of the Mollicutes.

For the present, it seems adequate to refer to most of the presumed yellows disease agents as "mycoplasmalike", but in view of our newer information, it seems preferable to refer to the organism associated with corn stunt disease by a specific name to reflect important differences from previously described organisms. It seems premature, however, to assign the organism a Latin binomial based on the available data; especially since the taxonomic position of the organism is uncertain at present. Moreover, inability to cultivate the organism hinders characterization and designation of a type specimen. We do feel, however, that this organism is a new species that probably will eventually be placed in a new genus. In order to distinguish the organism from previously described species and to avoid possible inappropriate use of the term "mycoplasmalike", therefore, we are coining the term spiroplasma. The etymology of the term is as follows: spiro, Greek noun "speira" meaning coil; plasma, Greek noun "plasma" meaning something formed or molded, to denote shape, form. We visualize that the term spiroplasma would be applied to the helical organism in corn stunt, and to other, similar organisms when described, until sufficient data are available to assign a Latin binomial and a taxonomic position. Morphology and contractile motility of filaments clearly separate such organisms from genera currently in the order Mycoplasmatales, and apparent lack of cell wall and axial fibrils or flagellae precludes inclusion in the Spirochaetales or in genera of spiral bacteria such as *Spirillum* and *Bdellovibrio*. Based on the fundamental importance of morphology in taxonomy, organisms to be termed spiroplasmas may necessitate the recognition of a new taxon that may or may not lie within the class Mollicutes.

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