(*11*). We proposed that the conformations of the A ring of vitamin D steroids might be of critical importance to their biological activity. Thus, the predominant chair conformation of 3-D-1α-OH-D_3 present in solution would be predicted to have most of its 1α-hydroxyls in the equatorial conformer, while 1α-OH-D_3 would be anticipated to have its 1α-hydroxyl in a 1 : 1 equilibrium mixture of axial and equatorial orientations.

These results emphasize both the complexity of a precise determination of structure-activity relationships for analogs of 1α,25-$(OH)_2$-D_3 and the possibility of designing new molecular species that enhance or diminish facets of its activity. Not only is the presence or absence of hydroxyls on a putative analog of importance, but also consideration of their precise orientation or conformation must be respected.

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8 July 1974; revised 4 October 1974

Corn Stunt Spiroplasma: Isolation, Cultivation, and Proof of Pathogenicity

Abstract. *A spiroplasma isolated from corn infected with corn stunt disease has been successfully cultivated in vitro. Acid formation was associated with its growth in liquid medium, and fried egg–shaped or granulated colonies developed on solid agar medium. Healthy leafhopper vectors injected with or allowed to feed on isolates from various serial passages through liquid medium were able to transmit the corn stunt agent to healthy corn plants, inducing typical corn stunt disease in the plants. The spiroplasma was reisolated and cultivated from such diseased plants and was indistinguishable morphologically from the original isolates. These results provide the first definite proof that the corn stunt disease is caused by the corn stunt spiroplasma.*

Corn stunt (CS) is a serious disorder of corn (*Zea mays* L.) occurring in the southern United States and in Central and South America. It is one of the many yellows-type plant diseases suspected to be caused by mycoplasma-like organisms. Numerous attempts have been made to cultivate the causal agents of these diseases in cell-free media. Chen and Granados (*1*) achieved a long-term maintenance of CS infectivity in a liquid medium. Although no colonies were obtained on agar, evidence from negative staining and ultrathin-section electron microscopy of mycoplasma-like organisms maintained in vitro, as well as infectivity tests, suggested that limited growth did take place in the primary cultures and that the organism in culture was the CS agent (*2*).

Davis *et al.* (*3*) reported that, unlike mycoplasmas, the presumed CS agent possesses a helical-filamentous morphology and exhibits a definite contractile motility. The trivial term "spiroplasma" was proposed for the microorganism (*4*). Subsequently, another spiroplasma was dis-

Table 1. Transmission of CS spiroplasmas from various successive subcultures in C-3 medium by *D. elimatus* either injected with or fed on the cultures; the primary culture was derived from corn plants infected with CS disease. Reisolations of CS spiroplasmas were made from inoculated corn plants showing typical CS symptoms. In experiments 1 to 4 insects were injected with cultured spiroplasma; in experiment 5 insects were fed with cultured spiroplasma in 6 percent sucrose solution. In all experiments the control insects (50 leafhoppers injected with sterile C-3 medium or allowed to feed on pure sucrose solution) were not infective.

Experiment	Isolate	Passage at bioassay	Insects injected (*N*)	Incubation period + inoculation test feed (days)	Insects surviving at end of test feed (*N*)	Plants infected/ plants inoculated	Positive reisolations/ diseased plants used
1	746	3	85	14+7	10	9/10	3/3
2	746	4	89	14+7	32	26/32	2/2
3	747	3	120	14+7	62	35/62	3/3
4	747	8	106	0+10	61	0/61	0/2
				10+5	46	28/46	2/2
				15+5	37	21/37	
				20+5	26	26/26	
5	746	5	30	0+7	23	0/23	0/1
				7+7	17	10/17	2/2
				14+7	11	8/11	
				21+7	8	6/8	

covered to be associated with citrus stubborn disease (*5*). The stubborn disease spiroplasma, *Spiroplasma citri*, has recently been cultivated in vitro (*6*). Stubborn disease has since been induced in healthy citrus plants by transmission of *S. citri* through nonvector leafhoppers in the laboratory and the leafhoppers found in citrus orchards (*7*). Thus, the motile, helical spiroplasmas represent an entirely new type of plant pathogen.

Impressive indirect evidence (*1, 2, 8*) supports the hypothesis that CS disease is induced by a spiroplasma, but direct definitive proof of this hypothesis has not previously been provided. We report here (i) repeated isolation of spiroplasmas from CS-infected corn plants, (ii) consistent cultivation and serial passage of the isolated organism in both liquid and solid media, (iii) successful inoculation of healthy corn with the cultivated spiroplasma through leafhopper vectors, and (iv) reisolation and cultivation of the spiroplasma from diseased plants inoculated by vectors with cultured spiroplasma.

Table 2. Separation of CS spiroplasma and *S. citri* by four different serological tests. Antiserums were produced in rabbits by intravenous injections of the respective antigens. Results of metabolic inhibition (*13*) and organism immobilization tests (*14*) are shown as the titer of antiserum. Ring interface precipitation test (*15*) results are expressed as the titer of antigen. Growth inhibition test (*16*) results are given as the diameter of the zone of growth inhibition in agar cultures. Experiments 1 and 2 were performed using antiserum from different immunized rabbits. Abbreviations: CS, corn stunt spiroplasma; SC, *S. citri*.

Antiserum/ antigen	Metabolic inhibition (titer)		Organism immobolization (titer)		Ring precipitation (titer)		Growth inhibition (mm)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
CS/CS	768	448	6144	1792	64	64	13	14
CS/CS	24	56	768	448	8	2	5	4
SC/SC	384	896	6144	3584	64	32	7	7
SC/SC	48	56	384	28	8	2	1	1

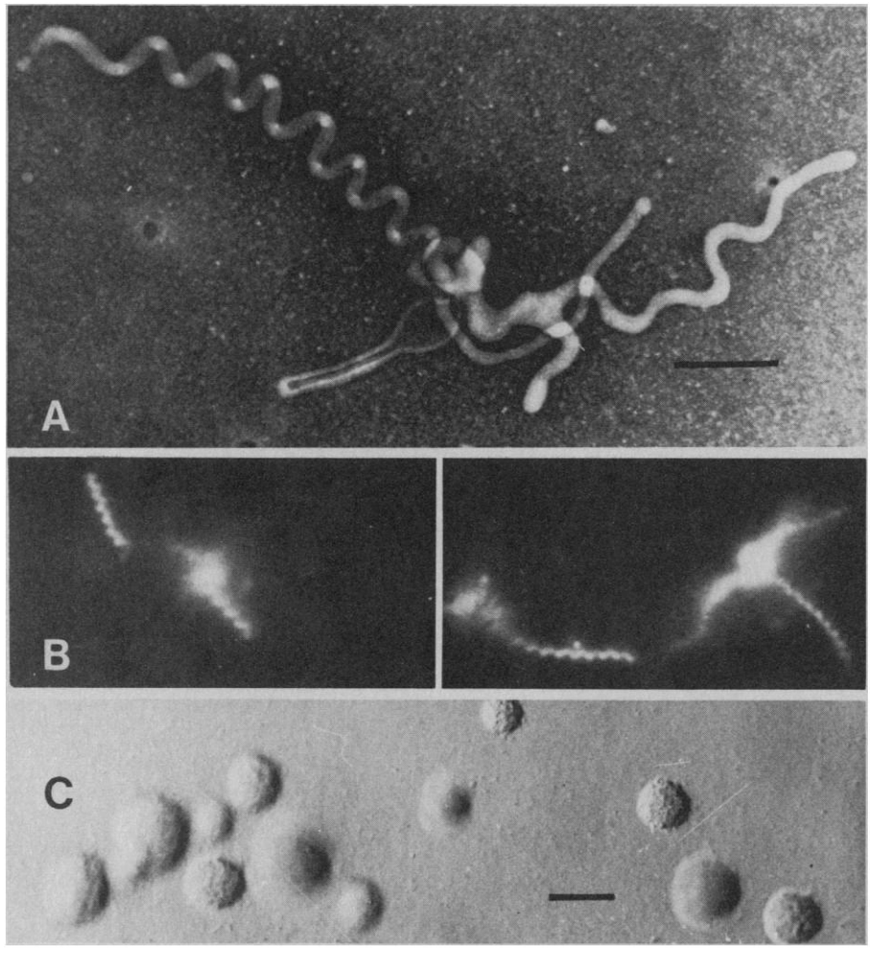

Fig. 1. Cultured CS spiroplasmas isolated from corn (*Zea mays* L.) plants infected with Rio Grande strain of CS disease. (A) Helical morphology of a spiroplasma cell negatively stained with 2 percent phosphotungstic acid (scale bar, 0.5 μm). (B) Living cells from liquid cultures observed by dark-field microscopy. (C) Colonies of CS spiroplasmas observed on agar plate 14 days after inoculation (scale bar, 0.05 mm).

The Rio Grande strain of CS disease and the leafhopper vector *Dalbulus elimatus* (Ball) were used for this study. Healthy plants, diseased plants, and plants inoculated for infective assay studies were kept isolated from one another in insect-proof screened sections in three separate greenhouses. Helical organisms were found only in CS-infected plants and were absent in disease-free plants. The procedure for rearing insects and tissue treatments for isolating CS agent were as previously described (*1, 9*).

Several media were tried for the isolation and cultivation of CS agent. We found that the best result was obtained in a medium (C-3) modified slightly from our maintenance medium (*1*). The C-3 medium consists of 199 medium (1×), 1 ml; Schneider's drosophila medium, 0.5 ml; CMRL-1066 medium, 0.5 ml; Difco PPLO broth base, 1.5 g; sucrose, 16 g; horse serum, 20 ml; fresh yeast extract, 10 ml; and water to 100 ml. This liquid medium, containing 10 μg of phenol red per milliliter, has a final *p*H of 7.4 and osmolality of 735 ± 15 mosm.

Before we succeeded in cultivating the CS spiroplasma, we encountered many failures. While waiting for the primary cultures to show evidence of growth, we observed only an occasional culture from hundreds of attempted isolations in which the *p*H indicator in the medium changed from red to yellow. However, attempts to subculture from cultures that showed the *p*H change were not successful. Such primary cultures thus seemed to contain live organisms, judging by morphology and motility under dark-field microscopy, that for some reason could not multiply when transferred to fresh medium. We then suspected that the crude extract inoculum prepared from diseased corn tissues contained factors which became inhibitory during incubation of primary cultures. To test this hypothesis, we checked the morphology and motility of the spiroplasmas in new primary cultures daily with a dark-field microscope to make certain that the helical organisms had survived the isolation. At the same time we transferred 0.2 ml of the primary culture daily to 3.5 ml of fresh C-3 medium. We discovered that subcultures could be achieved only when the helical form of the spiroplasmas showed no distortion in the primary culture and only within 1 to 3 days of the primary isolation. Consequently all subculturing of primary

cultures was performed within this 3-day period, during which the primary cultures showed no color change of the phenol red indicator.

Using C-3 medium, we have attempted 24 isolations of the CS spiroplasma from CS-infected plants. All attempts were successful, and 18 of the isolates thus obtained have been subcultured repeatedly. The same number of attempts at isolation were made with healthy plants as the source. In no case were spiroplasmas obtained from healthy control plants or from unseeded sterile medium. Of the 18 isolates retained, four cultures were randomly selected, maintained by serial passage in C-3 medium, and used for other studies, including infectivity assays.

The first subculture, in which both the suspected inhibitory factors and the isolated spiroplasmas would be greatly diluted, usually showed signs of color changes or growth of the organisms only after 14 days or more. Subsequent serial subcultures, however, were carried out by transferring 0.1 ml of the liquid culture into 3.5 ml of fresh C-3 medium at 6-day intervals (Fig. 1, A and B). The cultures were kept at 29°C. It was estimated by counting several 5-μl samples of the subcultures daily that the isolated spiroplasmas in C-3 medium had a 150-fold increase during the 6-day culturing period. The medium became acidified after 6 days, and the organisms in the medium showed a quick deterioration of the helical form and a drastic reduction in viability.

While the spiroplasma was at the peak of growth (5 or 6 days) 0.1-ml portions of dilutions of the culture were transferred to an agar plate. The solid culture medium, containing 0.8 percent Oxoid ionagar (by weight), was made with the same composition as the C-3 medium. The cultures were kept in a CO_2 container (BBL Gas Pak) and incubated at 29°C. Small, fried egg–shaped or granulated colonies (Fig. 1C) developed about 10 to 14 days after inoculation. When such colonies were examined, typical spiral-shaped organisms were always found, in agreement with findings on *S. citri* by Davis (*10*).

Two methods were employed to test the pathogenicity of the cultivated CS spiroplasma. In one, 6-day-old cultures of various passages were used to inject healthy *D. elimatus*. In the other, the leafhoppers were allowed to feed, through a thin parafilm membrane, on spiroplasmas suspended in 6 percent sucrose solution. The control insects were injected with sterile C-3 medium or were allowed to feed on pure sucrose solution.

The surviving insects were caged on healthy corn plants for 14 days, transferred singly to individual young corn seedlings grown in sterilized vermiculite in test tubes (3.5 by 25 cm) and left to feed for 7 days, and then discarded. Using an alternate method, a single insect was placed on a young corn seedling in a test tube immediately after injection. Each insect was transferred to a healthy young seedling every 7 days. After 21 days the insects were discarded. The inoculated plants were first treated with insecticide and then transferred to a 10-cm pot and kept in an isolated greenhouse.

The results of the infectivity assay for CS isolates and of tests for CS spiroplasmas in inoculated plants showing CS symptoms are shown in Table 1. A high percentage of inoculated corn seedlings became diseased and showed typical CS symptoms. The cultured organism was not only pathogenic to the plants but was detrimental to the leafhopper vectors. When inocula were diluted in later experiments, the rate of insect mortality also decreased. Nevertheless, the concentration of spiroplasma in the diluted subculture remained much higher than that in the expressed juice from diseased plant tissue or from the primary culture (based on estimated number with dark-field microscopy). The observed shortening of incubation time for the insects to become infective was probably due to the increased concentration of inoculum that was injected.

Insects injected with the eighth successive subculture of isolate 747 (*11*) in C-3 medium successfully transmitted the disease. This subculture represents a dilution of 61×10^{-12} of the primary culture and therefore far surpasses the theoretical dilution end point for the original organisms isolated from the diseased corn and placed in the primary culture medium. Pathogenicity was retained even though many generations of CS spiroplasma, through multiplication, must have occurred in the C-3 medium. In all experiments control insects were not infective.

The organism was reisolated, as described above, from inoculated plants showing symptoms of CS disease. Invariably, we obtained a spiroplasma that was indistinguishable morphologically from our original isolates. We believe, therefore, that the role of CS spiroplasma in the etiology of CS disease has been firmly established, since Koch's postulates have been fulfilled.

The CS organisms in our culture are morphologically identical to *S. citri*. Previous tests have shown that *S. citri* cannot infect corn and produce the CS syndrome (*12*). We have found that *S. citri* achieves excellent growth in C-3 medium as well as in complete sorbital medium (SMC) (*6*), whereas the CS organism does not survive in SMC. To further characterize the CS spiroplasma, we prepared antiserums by injecting rabbits for serological studies. Four different tests (*13–16*) were employed; the results are summarized in Table 2. The findings suggest that the two organisms are distantly related since they share certain common antigens. This is in agreement with previous interpretations of serological results (*17*) and DNA-DNA hybridization and base composition studies (*18*). However, the CS spiroplasma cannot be confused with *S. citri* because of the high titers obtained in serological tests for each organism against its homologous antiserum and the relatively low titers obtained for reciprocally heterologous immunoreactions. More biochemical and biological studies are required to determine whether the CS spiroplasma actually represents a species distinct and separate from *S. citri*.

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23 December 1974; revised 19 February 1975