Occurrence of Corn Stunt Spiroplasma at Different Elevations in Mexico

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ABSTRACT

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A survey of *Spiroplasma kunkelii*, or corn stunt spiroplasma (CSS), was conducted in Mexico from October 1985 to March 1988 using phase contrast or dark field light microscopy (DFM) and enzyme-linked immunosorbent assay (ELISA). Three types of symptoms were observed: consistently stunted plants whose leaves had well-defined broad chlorotic streaking and that were usually observed at 60–940 m above sea level, plants that were not always stunted but whose leaf margins showed red to purple streaks, and plants that usually were not stunted but whose leaves showed either a diffuse yellow or a chlorotic stripe condition with or without red margins. Both the second and third symptom types were observed at all elevations surveyed and usually appeared around 7 days before or after anthesis. ELISA was better than DFM at detecting CSS, but both methods demonstrated that all samples with the first type of symptom, 51–70% of those with the second type, 43–46% of those with the third type, and 3–11% of those without symptoms were infected with CSS. The disease was more prevalent at lower than at higher elevations. These results indicate high prevalence and wide distribution of CSS in Mexico and also confirm that maize plants having reddish or purplish leaves are often infected with CSS.

Additional keywords: incidence, mollicute, resistance

Two distinct types of corn stunting disease that are attributed to mycoplasmas occur in Mexico (1,7,21,23). These are: 1) the Rio Grande corn stunt, now termed simply corn stunt (CS), which is caused by *Spiroplasma kunkelii* Whitcomb et al (29), or corn stunt spiroplasma (CSS) (4,30), a helical, motile mycoplasma (12,13), and 2) the Mesa Central corn stunt, now referred to as maize bushy stunt, which is caused by the maize bushy stunt mycoplasma (MBSM), a nonhelical or pleiomorphic mycoplasma (1,2,22,23).

Corn stunt is one of the most important biological stresses affecting the productivity of maize in Latin America, including Mexico (23). To address this problem, the International Maize and Wheat Improvement Center (CIMMYT), based in Mexico, has assigned high priority to the development and improvement of maize germ plasm with resistance to this disease. Little is known, however, about CSS in Mexico, and the information available about its distribution and incidence is inconclusive. A 1955 report stated that based on symptoms, Rio Grande CS "was not found in the same fields or even in the same geographic region where the Mesa Central corn stunt or MBSM occurs" (21). Eighteen years later, Davis (7) reported the presence of CSS in maize plants with reddened and purplish leaves, a condition previously described for Mesa Central corn stunt (21).

More precise information about the incidence and distribution of CSS in Mexico is needed so that locations can be identified for screening maize germ plasm for resistance to this disease. The need is especially urgent because a method of artificial field inoculation has not been developed. Here we report the detection of CSS in maize plants grown at various elevations in Mexico, particularly at CIMMYT's stations, using phase contrast (PCM) or dark field (DFM) light microscopy and enzyme-linked immunosorbent assay (ELISA).

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MATERIALS AND METHODS

Location and collection of samples. Samples were collected at random from open-pollinated cultivars grown at CIMMYT experiment stations in Mexico: Toluca, state of Mexico, with an elevation of 2,640 m above sea level (masl); Texcoco, state of Mexico, 2,249 masl; Tlaltizapan, Morelos, 940 masl; and Poza Rica, Veracruz, 60 masl. Other samples came from CIMMYT trials conducted in San Andres, Jalisco, 1,100 masl; Texin, Veracruz, 1,200 masl; and Xicotepec de Juarez, Puebla, 1,200 masl. One leaf, usually the first or second leaf above the lowest symptomatic leaf, was obtained per plant. The samples were put inside plastic bags containing a moist paper towel or sprinkled with water and then transported to the laboratory in Texcoco. They were examined or tested for spiroplasmas upon reaching the laboratory or within 24 hr after collection. The samples tested by ELISA did not represent all these locations, however.

Detection of corn stunt spiroplasma by light microscopy. Initial studies (October 1985 to April 1986) for detecting spiroplasmas were done by PCM and later studies (November 1986 to April 1987) were done by DFM as described by Davis (7) and Davis and Worley (12). The leaf lamina were removed from a 10-cm portion of the leaf sample and the midvein was used for examination, since this part of the infected plant appears to have a higher population of spiroplasmas (18). The midvein was rinsed with tap water to remove surface debris. A fresh cut was made about 1 cm from the original cut, and the freshly cut portion was pressed to force sap onto a glass slide containing a drop of 10% sucrose solution; the slide was then covered with a coverslip. Another cut was made from the opposite end of the sample, and sap was squeezed onto another drop of the sucrose solution on the other end of the glass slide to serve as a duplicate. The preparation was examined under a compound microscope at 1,000–1,250× (Zeiss) using the oil immersion objective. A sample was considered free from spiroplasmas (negative) when these organisms were not observed in the extract from either end of a midvein of a sample in five microscopic fields of view and was rated positive when spiroplasmas were seen. To familiarize ourselves with the spiroplasmas under the microscope, we followed the same procedure with samples showing Rio Grande symptoms (Fig. 1A).

Detection of corn stunt spiroplasma by ELISA. The indirect ELISA procedure as described for $F(ab')_2$ ELISA (6), with minor modifications, was used to detect CSS in the samples starting in April 1987. Antisera against CSS were provided by R. E. Davis (USDA, Beltsville, MD) and J. Fletcher (Oklahoma State University, Stillwater). The immunoglobulin (IgG) fraction was precipitated from the antisera, and subsequent preparation of $F(ab')_2$ fragments from the IgG with pepsin was done by S. Haber (Agriculture Canada, Winnipeg), using the protocols described by Clark et al (6). The IgG and $F(ab')_2$ fragments were lyophilized and stored at 4 C until they were used. To coat (sensitize) the Immulon II plates (Dynatech Laboratories, Alexandria, VA),

$F(ab')_2$ fragments at 5 μg/ml carbonate buffer, pH 9.6, were added and incubated for 3 hr at 22–25 C. The plate was washed with phosphate buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 (PBST), with at least 2 min between each wash. Plant extracts were prepared by cutting 4–6 cm of the midvein into small pieces and grinding them with mortar and pestle in 2 ml PBST containing 0.02% polyvinylpyrrolidone (M_r = 40,000) (PBSTP); 100-μl aliquots were

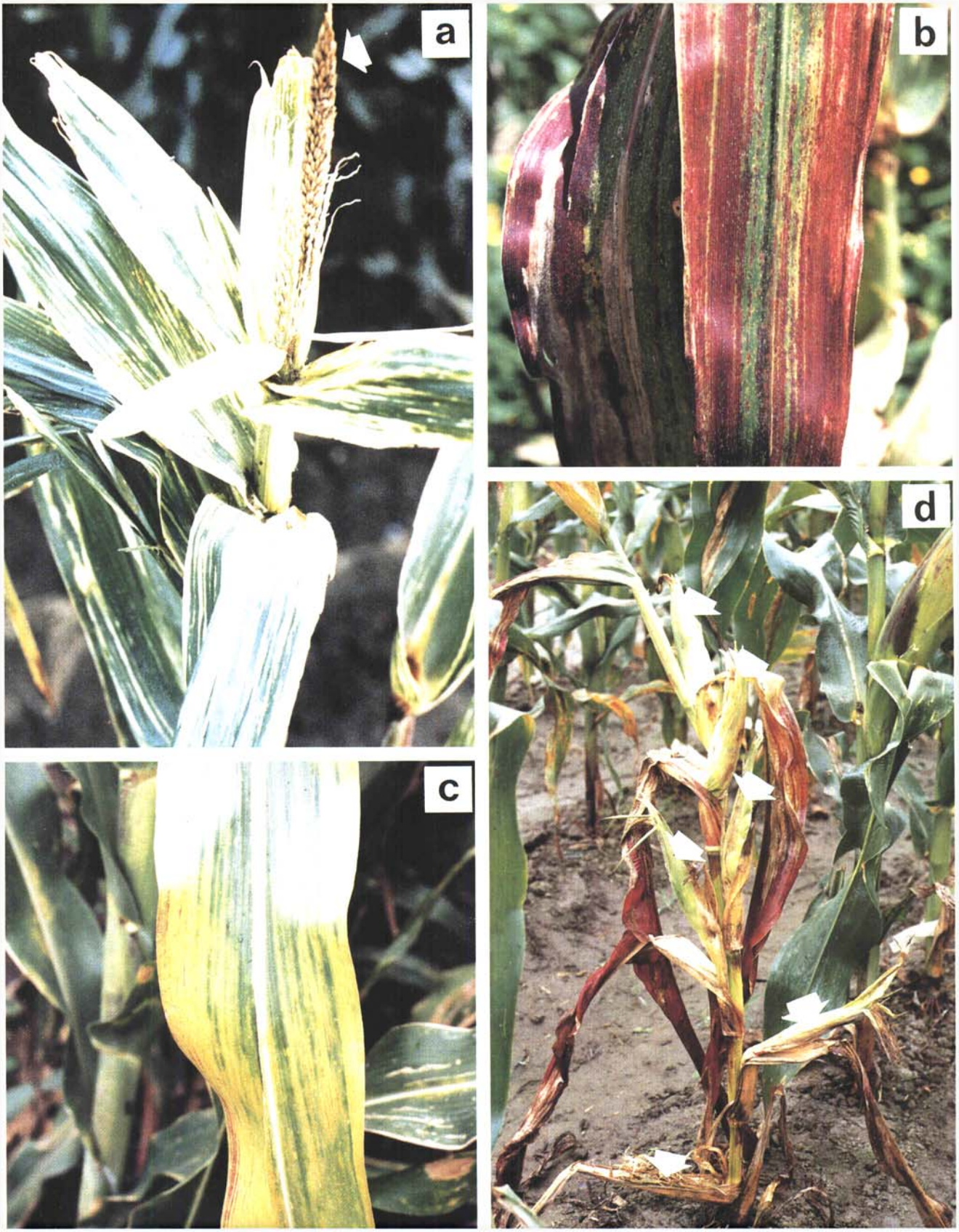

Fig. 1. Symptoms observed in maize plants from different elevations in Mexico where *Spiroplasma kunkelii*, or corn stunt spiroplasma (CSS), was detected by phase contrast or dark field light microscopy or enzyme-linked immunosorbent assay. **(A)** Plant with typical corn stunt symptoms; the tassel is not fully emerged (arrow). **(B)** Leaves with reddish or purplish streaking. **(C)** Leaf with general yellowing or chlorotic striping and reddish streaking. **(D)** Multiple thin, tapering ears (arrows) on symptomatic plant.

placed in each well and incubated for 10–14 hr at 4 C. After the plate was washed as described above, the homologous IgG at 5 μg/ml PBS was used as the trapping IgG and incubated for 2–3 hr at 22–25 C, followed by washing as described above. Alkaline phosphatase conjugated protein A (Sigma Chemicals, St. Louis, MO) at 5 μg/ml PBS was then added, followed by incubation and washing as described above. The alkaline phosphatase substrate at 1 mg/ml diethanolamine buffer, pH 9.8, was added, and the reaction was allowed to proceed for 45–60 min and then stopped by adding 50 μl of 3 N NaOH per well. Each sample was tested in duplicate wells. Results were evaluated using the Minireader II (Dynatech). Any absorbance value greater than the mean absorbance value, plus four standard deviations of the healthy control (noninfected, greenhouse-grown seedlings), was considered positive for CSS (27). A positive control consisting of extracts of plants having the Rio Grande CS symptoms (Fig. 1A) was also included in all tests. Regression analysis (15) was used to determine the relationship between elevation and the proportion of symptomatic plants where CSS was detected (Tables 1 and 2).

RESULTS

Field observations. Symptoms observed in maize at the different elevations where spiroplasmas were detected were grouped into three types (Fig. 1). The symptomatic plants were observed mostly at the edges of fields but were also randomly distributed within them. Type I plants showed broad, well-defined chlorotic streaks emanating from the base of the leaf (Fig. 1A); this symptom is typical of and associated with CSS infection. Type II plants had leaves showing relatively broad, chlorotic, and usually reddish and slightly purplish streaking generally manifested at the margins and extending toward the midveins (Fig. 1B). Type III plants had pale green to yellowish leaves with diffuse chlorosis or chlorotic striping delimited by the secondary veins; some leaves had thin, reddish to purplish borders (Fig. 1C). Few plants showed the diagnostic symptoms of CSS (Fig. 1A) and were found only in Poza Rica and Tlaltizapan. These plants were almost always stunted, with a pronounced shortening of the internodes above the ear-bearing nodes. Among the three types, type II (Fig. 1B) predominated in all sites and within a site, followed by type III (Fig. 1C), although it was not uncommon to see a combination of these two on the same plant. Most plants showing those two types of symptoms did not show obvious stunting. The symptomatic leaves were those directly around and near the ear, and in general, symptoms appeared at about anthesis or thereafter. As the season progressed, however, more of the upper leaves developed symptoms, and leaves that developed early symptoms produced more intense discoloration. Ears formed by these plants usually appeared normal in size, with well-developed grains. In a few instances, plants with severe type II symptoms accompanied by stunt developed multiple, long, thin, tapering ears, with one ear emanating from a single internode and the uppermost ear much more fully developed (Fig. 1D). The husk leaves produced leaflike appendages that often displayed symptoms similar to those observed on the true leaves. The tassels of plants having very severe foliar symptoms and stunting were usually not fully emerged, with their branches remaining in the sheath (Fig. 1A, arrow). None of the plants with either symptom type showed excessive tillering.

Detection of corn stunt spiroplasma by light microscopy or indirect ELISA. With PCM and DFM, spiroplasmas

Table 1. Location and elevation in Mexico of maize plants collected and tested for the presence of *Spiroplasma kunkelii*, or corn stunt spiroplasma (CSS), by phase contrast or dark field light microscopy[a]

Location	Elevation (meters above sea level)	Plants with symptoms[b]						Plants without symptoms	
		Type I		Type II		Type III			
		Tested	Infected	Tested	Infected	Tested	Infected	Tested	Infected
Poza Rica, Veracruz	60	8	8 (100)[c]	40	37 (93)	3	3 (100)	20	2 (10)
Tlaltizapan, Morelos	940	1	1 (100)	40	33 (83)	10	4 (40)	10	0 (0)
Xicotepec, Puebla	1,200	...[d]	...	20	12 (60)	...	...	10	0 (0)
Texin, Veracruz	1,200	...	...	55	33 (60)	...	...	15	0 (0)
Texcoco, Mexico	2,249	...	...	73	53 (73)	24	10 (42)	30	1 (3)
Toluca, Mexico	2,640	...	...	40	19 (48)	...	...	10	0 (0)
	Total	9	9 (100)	268	187 (70)	37	17 (46)	95	3 (3)

[a]The freshly cut end of a midvein of a leaf sample was squeezed onto a glass slide containing a drop of 10% sucrose solution, then examined under the compound microscope at 1,000–1,250×. Regression analysis for the relationship between elevation and the proportion of symptomatic plants infected with CSS was significant ($r = 0.84$, $P = 0.05$).
[b]See Figure 1.
[c]Percent infection in parentheses.
[d]No plants tested.

Table 2. Location and elevation in Mexico of maize plants collected and tested for the presence of *Spiroplasma kunkelii*, or corn stunt spiroplasma (CSS), by indirect enzyme-linked immunosorbent assay[a]

Location	Elevation (meters above sea level)	Plants with symptoms[b]						Plants without symptoms	
		Type I		Type II		Type III			
		Tested	Infected	Tested	Infected	Tested	Infected	Tested	Infected
Poza Rica, Veracruz	60	6	6 (100)[c]	32	26 (81)	24	19 (80)	35	3 (9)
Tlaltizapan, Morelos	940	2	2 (100)	44	29 (66)	15	5 (33)	10	2 (20)
San Andres, Jalisco	1,100	...[d]	...	37	2 (5)	...	...	3	0 (0)
Xicotepec, Puebla	1,200	...	...	12	4 (33)	...	...	10	1 (10)
Texcoco, Mexico	2,249	...	...	70	39 (56)	88	31 (35)	15	2 (13)
	Total	8	8 (100)	195	100 (51)	127	55 (43)	73	8 (11)

[a]See text for details of test. Regression analysis for the relationship between elevation and the proportion of symptomatic plants infected with CSS was not significant ($r = 0.48$).
[b]See Figure 1.
[c]Percent infection in parentheses.
[d]No plants tested.

were observed in 100% of plants showing the classical CSS symptoms (type I, Fig. 1A), in 70% of those showing type II, in 46% of those showing type III, and in 3% of those without symptoms (Table 1). Helical and motile spiroplasmas (12,13) were generally more apparent and more clearly seen in extracts of plants with type I symptoms than in extracts of plants with the other two symptom types. With plants showing reddening or purpling and chorotic symptoms (Fig. 1B and 1C), spiroplasma detection was more difficult, especially in samples collected from the higher elevations (above 940 masl). The proportion of symptomatic plants infected with CSS was highest in Poza Rica, then in Tlaltizapan, and was found to be significantly correlated with elevation ($r = 0.84$, $P = 0.05$).

Similar results were obtained with ELISA. Extracts of samples with any of the three types of symptoms collected from all locations surveyed reacted positively to the anti-CSS IgG in ELISA (Table 2). Plants with type I symptoms (Fig. 1A) reacted very quickly and strongly, with most registering absorbance values above the limit of the ELISA reader (OD = 2.5) in 45 min of reaction (*data not shown*). On the other hand, 51% of the samples with type II and 43% of those with type III symptoms (Fig. 1B and 1C) reacted positively to the IgG in ELISA. About 11% of the plants without symptoms reacted with the anti-CSS IgG in ELISA. As with the results obtained by light microscopy, the proportion of samples in which CSS was detected varied among locations, with the lower elevations (Poza Rica and Tlaltizapan) having a higher incidence of the disease than the higher elevations. The correlation coefficient ($r = 0.48$) between elevation and disease incidence was not significant, however.

Scoring of symptomatic plants by DFM or ELISA. Samples with type II (Fig. 1B) symptoms were collected, brought to Texcoco, and processed (only sample 7 had type I symptoms). A section about 20 cm long was removed from each sample and numbered appropriately on both ends. Half of each leaf sample was checked by DFM for the presence of spiroplasmas, and the other half was processed for ELISA testing. Of 22 samples tested, nine were scored positive and three negative for CSS by both DFM and ELISA (Table 3). The nine positive samples had absorbance values of 0.70 to more than 2.50 in ELISA. Three samples scored positive for CSS by DFM but had absorbance values of 0.01 or 0.02 in ELISA and were lower than the threshold value of 0.04. On the other hand, seven samples with absorbance values ranging from 0.05 to 0.50 were scored negative by DFM. Chi-square analysis of the data (15) to test the independence between DFM and ELISA showed that one method did not affect the other in scoring for CSS.

DISCUSSION

The two mycoplasmas that affect maize in Mexico are CSS and MBSM (1,7,21,23). The distribution of these diseases has not been extensively studied, although it has been reported, on the basis of symptoms, that CSS was not present in the area where MBSM occurs, and vice versa, in Mexico (21). Nault (22) studied the symptomatology of these two diseases under greenhouse conditions with varying temperature regimes, using a single cultivar of maize. However, the symptoms expressed under those conditions may not be the same as those expressed in the field, where the strain of the pathogen, genetic background of the host, environment, and interaction of those factors play a very important role in the expression of the disease (3,8,11).

We have found CSS in exudates of maize plants grown at all elevations sampled in Mexico, with the highest incidence in the lowlands. Our results confirm and extend those results obtained by Davis (7) indicating that spiroplasmas may be present in plants showing reddening or purpling with slight chlorosis ascribed to MBSM infection in the high plateaus of Mexico (1,2,21–23). Similar results were obtained with maize plants showing reddened and purplish leaves in Florida (3,11), the Dominican Republic and El Salvador (*unpublished*), and Costa Rica (R. Gamez, *personal communication*). There have also been reports of outbreaks of what seemed to be a new disease of maize in Florida (3,5) and California (17), but evidence obtained through serology and cultural or electrophoretic patterns of cellular proteins showed that the spiroplasmas isolated were CSS (5,9,11). Plants from this study without symptoms that were shown positive for CSS were also infected, but in all likelihood the disease had not completed its incubation period. Davis et al (13) reported that CSS was found in extracts of infected plants as early as 7 days before symptoms appeared. Our results demonstrate that the maize plants with symptoms ascribed to MBSM infection are often infected with CSS. However, mixed infection was possible, as we were not able to check for the presence of MBSM in the samples because of the lack of appropriate assay procedures.

The presence of this pathogen in maize grown at different elevations in Mexico (Tables 1 and 2) indicates that CSS is not confined solely to the warm and humid tropics. Because it has a wider distribution in Mexico than was previously implied (21), it may also occur in much of the neotropic highlands. Our results from field-collected samples support those obtained in the greenhouse by Nault (22), which showed that this spiroplasma is able to infect and tolerate wide temperature regimes (18–31 C). We expected to find this pathogen at higher elevations in Mexico because CSS had been detected earlier by ELISA in *Dalbulus* spp. collected from Texcoco, Mexico (16). Several *Dalbulus* leafhopper species are known to be vectors of CSS and are widely distributed in Mexico (20,28).

We failed to show CSS in 30–49% of plants with type II symptoms and about 55% of those with type III symptoms, even with ELISA (Table 2), and in 14–27% of the samples tested by either or both DFM and ELISA (Table 3). This phenomenon could be explained by one or both of the following possibilities. First, the symptoms could have been the result of infections solely by MBSM (1,2,22) or other pathogens that were not detected by the assays used (3,11,26). In fact, since the assay for MBSM was not performed in those plants, including samples that reacted to CSS, double infection by MBSM cannot be ruled out. It is not known what types of symptoms develop or predominate in maize infected by both mollicutes. The second possibility is that the symptoms were the result of infection with CSS, but the sections processed for testing may not have been the portions of the plants that contained

Table 3. Scoring for the presence of *Spiroplasma kunkelii*, or corn stunt spiroplasma (CSS), in symptomatic plants by dark field light microscopy (DFM) and indirect enzyme-linked immunosorbent assay (ELISA)[a]

Sample number	DFM[b]	ELISA absorbance value
1	+	0.72 (infected)
2	+	>2.50 (infected)
3	+	0.09 (infected)
4	−	0.09 (infected)
5	−	0.19 (infected)
6	−	0.07 (infected)
7	+	>2.50 (infected)
8	?	0.02 (not infected)
9	?	0.02 (not infected)
10	−	0.50 (infected)
11	−	0.01 (not infected)
12	−	0.11 (infected)
13	−	0.05 (infected)
14	−	0.08 (infected)
15	+	>2.50 (infected)
16	+	>2.50 (infected)
17	+	0.72 (infected)
18	?	0.01 (not infected)
19	−	0.00 (not infected)
20	−	0.00 (not infected)
21	+	0.71 (infected)
22	+	>2.50 (infected)
Control	−	0.04 ···
Total	12	16

[a]One portion of the midvein of a leaf sample was used for DFM and an equal portion was used for ELISA. All samples except no. 7 had type II symptoms. Chi-square analysis for independence between DFM and ELISA was significantly different ($\chi^2 = 0.07$, $P = 0.01$).

[b]+ Detection, ? = questionable detection, and − = no detection of CSS.

the CSS. This pathogen has been demonstrated to induce reddening in maize infected and incubated at lower temperature regimes (22). This pathogen or strains of it (5,19) may induce other symptoms under field conditions or in different genetic backgrounds of the host, or both (3,11). Although CSS induces systemic infection in maize, its distribution in certain tissues is uneven (8). The latent period of CSS in plants is 15–45 days (22), but the appearance of symptoms in a leaf may not always indicate the presence of a high pathogen titer in that same leaf for the ELISA test to detect. Kloepper et al (18) reported that the titers of CSS differ among various parts of experimentally inoculated plants maintained in the greenhouse. The absorbance values of extracts from the tassel and the flag leaf of symptomatic plants collected from Texcoco were higher than those of the ear and bottom leaves. In some cases, positive reactions were obtained from extracts of the tassel and flag leaves but not from extracts of the ear or lower leaves of a symptomatic plant (*data not shown*). In addition, the titers were probably still very low as a result of late infection. Almost all the samples we examined came from experiment stations, where maize seeds were treated with carbofuran before planting and plants were treated regularly until the silks and tassels emerged. The concentration of CSS in infected maize inoculated at different stages of growth and incubated at different temperature regimes and light intensity has not been determined.

Three samples scored positive for spiroplasmas by DFM for which the extracts failed to react to the anti-CSS IgG in ELISA (Table 3). These could have been scored erroneously. In these studies, PCM or DFM and ELISA were used to detect CSS in symptomatic maize plants. DFM has been used in other studies (3,24), but no one has reported the use of ELISA to detect CSS in plants grown in the field. ELISA has been used to detect CSS in greenhouse-grown plants (10,25) and in artificially reared *Dalbulus* (14) spp. as well as those collected from the field (16). We demonstrate here that ELISA is better than DFM for detecting CSS (Table 3). Within the same group of samples, CSS was detected in more samples by ELISA (i.e., the seven samples with absorbance values of 0.05–0.50) than by DFM. This assay not only detected the whole or intact spiroplasma cells per se but also parts or components of the cells (cell breakdown products and building blocks) recognized by the antibodies. In addition, there may have been some instances in which CSS was present in the extracts but did not assume the helical morphology and was not noticed under the microscope. Strains of CSS that are defective in helicity and are nonmotile have been reported (19).

Our results provide evidence that CSS occurs at higher incidence in the more humid tropical lowlands of Mexico than in the highlands and may continue to be an important factor in the tropical maize-producing areas in Latin America. In addition, since the disease has the ability to infect and adapt to maize grown at higher elevations or more temperate climates in Mexico, it is evident that control measures, such as incorporation of resistance to this disease, should be a part of any maize germ plasm development programs for those and similar areas in Latin America. ELISA may prove to be a very valuable tool for more extensive surveys and epidemiology studies and for ascertaining and characterizing the reactions of breeding materials to the pathogen/disease, eliminating the need to rely on visual/phenotypic evaluation.

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