Movement and Multiplication of *Spiroplasma kunkelii* in Corn

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ABSTRACT

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After the introduction of *Spiroplasma kunkelii* into the leaves of corn (*Zea mays*) seedlings by inoculative corn leafhoppers (*Dalbulus maidis*), the pathogen was detected by enzyme-linked immunosorbent assay. Spiroplasmas were detected in leaves 14 days after exposure to inoculative leafhoppers (as much as 2 weeks before symptoms appeared) and in roots after 20 days. The probabilities of detecting spiroplasmas in different plant organs, determined by logistic regression, generally increased over time; the probability was greater in roots than in leaves at most testing dates. Spiroplasmas moved into tassels as they developed. The age and/or size of the plant at the time of infection affected pathogen movement; spiroplasmas were detected earlier in roots of plants inoculated at the one-leaf seedling stage than in those of plants inoculated at the four-leaf stage. Spiroplasma titers remained relatively low in all the plants until the third or fourth week after inoculation. After that time, populations in different plant samples varied within a large range, and the upper limit of the range generally increased with time. The maximum titers were reached earlier in plants inoculated at the four-leaf stage than in plants inoculated at the one-leaf stage. Because *S. kunkelii* may be present in various plant organs well before symptoms appear, spiroplasmas might be acquired by leafhoppers before disease is obvious in the field.

Spiroplasma kunkelii, a wall-less prokaryote of the class Mollicutes, is among several pathogens that may cause corn stunt disease (3,8,15,18,19), considered to be one of the most important diseases of maize (*Zea mays* L.) in the United States, Mexico, and Central and South America (3,19). The mollicute is introduced into the sieve tubes of host plants by phloem-feeding leafhoppers, primarily the corn leafhopper, *Dalbulus maidis* (Delong and Wolcott) (24). Studies with other phytopathogenic mollicutes (*S. citri* and the phytoplasmas associated with western X-disease, aster yellows, and potato witches'-broom) indicate that these organisms multiply within their plant hosts (12,13,15,16) and are translocated toward meristems, storage organs, fruits, and other photosynthate "sink" regions of the plant, which exhibit the most intense symptoms. Although quantitative studies of *S. kunkelii* have not been reported, dark-field microscopy was used in earlier work to detect the presence of the spiroplasma in sap expressed from diseased plants, showing that *S. kunkelii* could be detected a week prior to the onset of disease symptoms (7,9).

In this research, our objectives were to document changes in the location of *S. kunkelii* in corn and quantitate spiroplasma titer changes in the plant at time intervals during the development of disease. In addition, we examined the effect of plant age at the time of infection on the multiplication and movement of the spiroplasma within the host plant. To accomplish these objectives, corn plants at the one- and four-leaf growth stages were inoculated with *S. kunkelii,* and the movement and titer of the spiroplasma were documented by quantitative enzyme-linked immunosorbent assay (ELISA), a rapid and reliable method for detecting *S. kunkelii* in corn (10,21). The information is important in the evaluation of pathogen transmission potentials and disease control strategies in the field.

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MATERIALS AND METHODS

Spiroplasma source and cultivation. *S. kunkelii*-infected corn leaves were provided by L. R. Nault and W. Styer (Ohio Agricultural Research and Development Center, Ohio State University, Wooster), who have maintained this spiroplasma isolate in the greenhouse via leafhopper transmission (18). The isolate has been deposited with the American Type Culture Collection (ATCC 29051); however, for this study, spiroplasmas were isolated directly from the infected corn leaves and triply cloned. The spiroplasma was cultured in liquid C3-G medium (4) to log phase (approximately 10^8 CFU/ml, achieved after 6 to 8 days at 31°C under aerobic conditions). Titer of the culture was determined by direct counts with a dark-field BH-2 microscope (Olympus, Lake Success, NY).

Antiserum production and ELISA. A modified double antibody sandwich ELISA (5) was used to detect *S. kunkelii* in corn. For antigen production, *S. kunkelii* was grown to log phase in 500 ml of C3-G broth in which rabbit serum was substituted for half the horse serum. Cells were harvested at 22,100 × *g* for 45 min. The pellet was resuspended in 250 ml of phosphate-buffered saline (3 mM $K_2HPO_4 \cdot 3H_2O$, 3 mM KH_2PO_4, and 3 mM NaCl) containing 10% sucrose (PBS-S). Cells were washed twice, resuspended in 25 ml of PBS-S, and frozen (–20°C) in 3-ml aliquots. For use as immunogen, 750 µl of thawed cell suspension was emulsified with an equal volume of Freund's complete adjuvant (GIBCO Laboratories, Grand Island, NY). Volumes of 0.5 ml of this immunogen were injected intramuscularly into each hip and subcutaneously into the neck of a young female New Zealand white rabbit. The rabbit was inoculated six more times

by the same method over the next 3 months. Serum, collected 122 days after the first injection and clarified by low-speed centrifugation, had a titer of 1:6,400, determined by a spiroplasma deformation test (25). Immunoglobulin G (IgG) was purified by ammonium sulfate precipitation and separation on a DEAE-trisacyl M column (22). Conjugate was prepared by incubating IgG and alkaline phosphatase (Sigma, St. Louis, MO) at a ratio of 1:2 (wt/wt) and cross linking with glutaraldehyde (added to a final concentration of 1%). The same conjugate preparation was used throughout this study.

ELISA plates (Immulon I, Dynatech, Chantilly, VA) were coated with IgG at 2 μg/ml and incubated at 4°C overnight. Plant tissues were weighed and passed through an electric tissue squeezer (Piedmont Machine & Tool, Six Miles, SC). Expressed sap was diluted with phosphate buffer (140 mM NaCl, 1.5 mM KH_2PO_4, 20 mM Na_2HPO_4, 3 mM KCl, and 3 mM NaN_3, pH 7.4). After three washes of the plate with PBS containing 0.05% Tween 20 (PBS-T), diluted sap (200 μl) was added to the plate and incubated at 4°C overnight. Three consecutive plate washes were followed by the addition of conjugate (1:400 in PBS-T; 200 μl) at room temperature for 3 h. Substrate (*p*-nitrophenyl phosphate, 1 mg/ml in 10% diethanolamine) was incubated at room temperature for 3 h, and then reaction was stopped by the addition of 50 μl of 3 N sodium hydroxide per well. Absorbance of each well was measured at 405 nm with a model EL-307C ELISA reader (Biotek Instruments, Winooski, VT).

Test plant and insect maintenance. To evaluate the effect of plant age at the time of infection on the titer and movement patterns of *S. kunkelii,* corn plants were inoculated at two growth stages. Test plants (*Z. mays* cv. Early Golden Bantam) were at either the one-leaf stage (approximately 14 days old) or the four-leaf stage (approximately 28 days old) at the time of inoculation. In the following sections, references to one-leaf plants and four-leaf plants indicate plants inoculated at these growth stages.

Plants were grown in a growth chamber with 12 h of light at 27°C, 12 h of darkness at 22°C, and 70% relative humidity. Corn plants infected with *S. kunkelii* were used as source plants for vector acquisition. *D. maidis* from colonies established in 1989 were used as vectors of *S. kunkelii*. Late-instar nymphs were caged in groups on infected corn plants in the greenhouse for 7 days and then transferred to healthy corn plants for a 17-day incubation period, during which the nymphs eclosed to adults.

Movement of *S. kunkelii* in corn. Corn plants were grown and maintained in a growth chamber under conditions described above. Five (experiment 1) or seven (experiment 2) leafhoppers, previously caged for an acquisition access period of 4 days with *S. kunkelii*-infected corn plants, were caged for 5 days in a plastic and screen leaf cage (5 × 5 × 2 cm) on the youngest fully developed leaf on each of 35 (experiment 1) or 50 (experiment 2) corn plants of each age group. Control plants were not exposed to leafhoppers. Plants were sprayed with diazinon immediately after leafhopper removal and were subsequently sprayed three more times at weekly intervals to kill emerging nymphs. The day that spiroplasma-exposed leafhoppers were placed on the experimental plants was designated day 0. An ELISA test was conducted every 5 days beginning with day 15 (experiment 1) or every 7 days beginning with day 14 (experiment 2) up to day 35 or day 42, respectively. Symptoms were assessed visually at each test date.

At each sampling date, seven (experiment 1) or ten (experiment 2) inoculated plants and six uninoculated plants were selected randomly for destructive sampling. Samples from each plant consisted of the youngest leaf, oldest leaf, roots, and tassel (when present). Initially, the youngest leaf of the one-leaf plants was also the oldest leaf, but by day 20, all the so-called one-leaf plants had at least two leaves and the inoculated leaf in the four-leaf plants had become the oldest leaf because of senescence and abscission of the previous oldest leaves.

Each sample was weighed and forced through a tissue squeezer. Extracted sap was diluted 3:1 (vol/wt) (experiment 1) or 2:1 (vol/wt) (experiment 2) in phosphate buffer. Diluted sap was placed randomly into wells of IgG-precoated ELISA plates. Each sample was tested in two wells, one on each of two plates. A sample was considered positive if the average of the absorbance values of the two wells was higher than four times the standard deviation plus the mean of the control wells (17).

Analysis of spiroplasma movement data. Because of the differences in numbers of leafhoppers per plant and numbers of plants sampled at each testing date, experiments 1 and 2 were evaluated individually rather than as replicates of one experiment. A logistic regression model was used because the response or dependent variable had only two possible values (i.e., spiroplasmas were either detected or not detected) and because the sample sizes (seven or ten corn plants at each day) were small enough that tests based on frequencies, such as chi-square, were inappropriate. The data were coded as $Y = 1$ when spiroplasmas were detected and $Y = 0$ when spiroplasmas were not detected. The logistic regression model was then used to model the probability of spiroplasma detection x days after inoculation (1,14). The general form of a logit model,

$$p_x = e^{(\alpha + \beta x)}/[1 + e^{(\alpha + \beta x)}], \quad (1)$$

was used, where p_x denotes $P(Y = 1)$ after x days. The linear probability model (LPM), resulting from the transformation of equation 1,

$$\log[p_x/(1 - p_x)] = \alpha + \beta x, \quad (2)$$

may be solved as a general linear regression by using least squares techniques. After the transformation, the regression coefficients α and β are no longer the intercept and slope, but rather the odds and odds ratio, respectively. The symbol e^{α} represents the odds that spiroplasmas will be detected at day 0 or the probability of detection divided by the probability of no detection, and e^{β} is the odds ratio for an increase of 1 day in the elapsed time after inoculation or the odds of detection at day $x + 1$ divided by the odds of detection at day x. In this application, $\alpha < 0$, since $P(Y = 1)$ should be 0 at day 0, and $\beta > 0$, since $P(Y = 1)$ should increase as x increases.

The simple linear regression model ($\alpha + \beta x$) indicated in equations 1 and 2 may be replaced with any multiple linear regression model as the LPM of interest; however, the coefficients may become more difficult to interpret as the complexity of the model increases. In any case, logistic regression models allow estimation of the probabilities of interest and assessment of the goodness of fit.

PROC LOGISTIC from SAS (23) was used to analyze data by specifying LOGIT transformation (the default selection) as the LINK function and STEPWISE as the method of SELECTing the variables in the model. This stepwise option was chosen in order to incorporate a standard indicator variable model so that the model for a particular organ of one-leaf plants could be compared with that of the corresponding organ of four-leaf plants. The LPM inserted into equation 2 then gives the equation

$$\log[p_x/(1 - p_x)] = \alpha + \alpha' I + \beta x + \beta' I x, \quad (3)$$

where the indicator variable I was assigned a value of 0 for any data point corresponding to a one-leaf plant and value of 1 for any data point corresponding to a four-leaf plant. The variable x is the day number, and the variable Ix is the product of I and x. If the estimates of α' and/or β' are significant, then one-leaf and four-leaf plants have different estimated LPMs; the LPM for the one-leaf plants is $\alpha + \beta x$, and for the four-leaf plants it is $(\alpha + \alpha') + (\beta + \beta')x$. This analysis was used to determine LPMs for data

from the youngest leaf, the oldest leaf, and the roots in each experiment. For any plant organ, the probabilities of detecting spiroplasmas on two different days were declared to be different if the respective 95% confidence intervals for the two probabilities did not overlap. The same type of comparison was used to compare probabilities of detection for organs of different one- and four-leaf plants x days after inoculation.

Titer of *S. kunkelii* in corn. The youngest fully developed leaves of test plants were inoculated with *S. kunkelii* in the manner described for the movement experiment except that 10 leafhoppers were placed in each cage. Symptoms were visually assessed and ELISA was conducted every 7 days beginning with day 14 and continuing until all the corn plants were dead. At each sampling date, eight inoculated plants and six uninoculated plants were selected randomly. The sample for each plant consisted of the entire youngest fully expanded leaf. Each sample was weighed and forced through a tissue squeezer, and the sap was diluted 2:1 (vol/wt) in ELISA phosphate buffer. A standard curve was established for evaluation of the spiroplasma titer in plant samples: spiroplasmas were pelleted (8,250 × g for 30 min) from a log-phase culture and resuspended in a volume of phosphate buffer equal to the original culture volume. Serial dilutions of this suspension ranging from approximately 1.25×10^4 to 1.13×10^9 CFU/ml were included on each plate. Each dilution was made by mixing *S. kunkelii*-buffer suspensions of known concentration with healthy plant sap (10% spiroplasma suspension to 90% plant sap). Sap samples were also diluted with a phosphate buffer (10% buffer to 90% plant sap), so that the composition of samples matched that of the standard dilution series. The spiroplasma detection limit of the ELISA was determined with a dilution series in plant sap. Volumes of 200 µl were placed randomly into wells of IgG-coated ELISA plates. Each sample was tested in three wells, and the three values were averaged. The experiment was conducted twice, and a polynomial regression model (20) was fitted to the data.

RESULTS

ELISA. The spiroplasma detection limit of the ELISA was 3.1×10^3 CFU/g of plant tissue, determined by assaying known quantities of washed, cultured spiroplasmas suspended in a healthy plant sap-buffer mixture as described above.

Movement of *S. kunkelii* in corn. Spiroplasmas were detected by ELISA as early as day 14 in the youngest and oldest leaves of both the one- and four-leaf corn plants (Table 1). At succeeding sampling dates, the percentage of samples that tested positive for spiroplasmas varied; the overall trend was an increasing number of positive samples over time. Spiroplasmas were detected in a larger percentage of roots than other tissue types at many of the test dates; in fact, 100% of the roots were positive at several times (especially in one-leaf plants), whereas none of the other plant organs ever reached 100% positive reactions. Spiroplasmas were detected in some samples of all tissue types by day 21 in the one-leaf corn plants and by day 25 in the four-leaf corn plants. Symptoms of stunting and leaf reddening appeared between days 28 and 35 in the one-leaf corn plants and between days 35 and 42 in the four-leaf plants.

Figure 1A–D shows the estimated probability of detection of spiroplasmas in a particular plant organ over time as determined by application of a logistic regression to the data. The probability of spiroplasma detection was significantly higher in roots than in leaves at several time points; that is, confidence intervals for the probability of detection in these two organs did not overlap in these instances. This observation was true for one-leaf plants at days 25 and 30 in experiment 1 and at days 35 and 42 in experiment 2 and for four-leaf plants at days 28, 35, and 42 in experiment 2. There was no significant difference in the probability of pathogen detection between the youngest and oldest leaf for either one- or four-leaf plants.

Plant age and/or size at the time of infection affected the movement of spiroplasmas within the corn plant. Spiroplasmas were detected in roots of one-leaf plants earlier (days 20 and 21 for experiments 1 and 2, respectively) than in those of four-leaf plants (days 25 and 35, respectively) (Table 1). In experiment 1, the logistic regression models for the probability of detecting spiroplasmas in roots were significantly different for one- and four-leaf plants ($P = 0.019$, and $R^2 = 0.276$), indicating that the patterns of movement of spiroplasmas into roots are different in plants inoculated at these two growth stages. For one-leaf plants, the estimated LPM is $-3.6760 + 0.1686x$. At day 0, the estimated odds of spiroplasma detection are $e^{-3.6760} = 0.0253$, so that the estimated probability of spiroplasma detection is 0.0253 times as large as the estimated probability that it will not be detected. The odds ratio associated with any additional day of exposure is $e^{0.1686} = 1.1836$, indicating that the estimated odds of detecting spiroplasmas after an additional day are 1.1836 times as great as they were the day before. Since the data were collected every 5 days in experiment 1, the estimated odds of detecting spiroplasmas after an additional 5 days are $e^{5(0.1686)} = 2.3233$ times greater than at the beginning of that extra 5 days.

Differences for the other organ and experiment combinations were not statistically significant; the indicator variables I and Ix in

TABLE 1. Number of one-leaf or four-leaf[a] corn plants in which *Spiroplasma kunkelii* was detected by enzyme-linked immunosorbent assay at various time intervals after exposure of the youngest fully expanded leaf to inoculative leafhoppers, *Dalbulus maidis*

Days after inoculation	One-leaf plants			Four-leaf plants			
	Youngest leaf	Roots	Oldest leaf	Youngest leaf	Roots	Oldest leaf	Tassel
Experiment 1							
15	0/7[b]	0/7	0/7	0/7	0/7	0/7	NT[c]
20	0/7	7/7	4/7	0/7	0/7	0/7	NT
25	3/7	4/7	4/7	1/7	4/7	3/7	NT
30	3/7	4/7	1/7	5/7	2/7	5/7	NT
35	3/6	7/7	3/6	3/5	2/5	2/6	9/10
Experiment 2							
14	1/10	0/10	1/10	2/10	0/10	1/10	NT
21	1/10	3/10	1/10	2/10	0/10	0/10	0/7
28	4/10	10/10	2/10	1/10	0/10	4/10	1/10
35	2/7	8/9	5/7	5/7	8/9	1/7	NT
42	7/10	10/10	5/10	7/10	10/10	3/10	10/10

[a] Designations one-leaf and four-leaf indicate corn plants exposed to inoculative *S. kunkelii* at these growth stages.
[b] Number of samples positive/total number of samples; total numbers fewer than seven (experiment 1) or 10 (experiment 2) occur where samples were lost during processing.
[c] No tassels present.

equation 3 did not result in different logistic regression models for one- and four-leaf plants, even at $\alpha = 0.10$. R^2 values for the youngest leaf were 0.224 in experiment 1 and 0.208 in experiment 2; for the oldest leaf, 0.076 in experiment 1 and 0.093 in experiment 2; and for the roots, 0.513 in experiment 2.

Tassels were present only after 21 days in experiment 2 and not at all in experiment 1, probably because test plants died earlier in the first experiment than in the second. Thus, the data were insufficient for statistical analysis. However, spiroplasmas were detected in tassels soon after their development (Table 1).

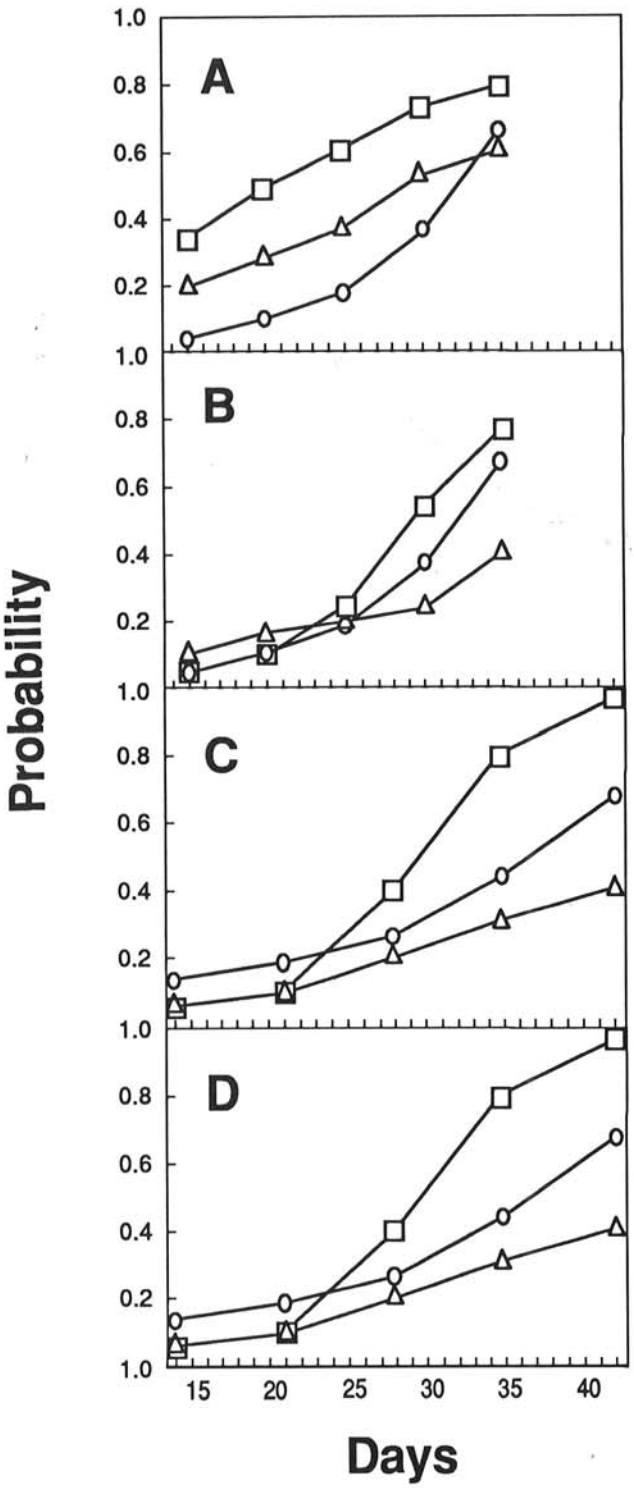

Fig. 1. Probability, calculated by application of a logistic regression to the data, of detection of *Spiroplasma kunkelii* in different plant organs at various time intervals after inoculation of corn seedlings at the one-leaf or four-leaf growth stage. **A,** Experiment 1, one-leaf plants; **B,** experiment 1, four-leaf plants; **C,** experiment 2, one-leaf plants; and **D,** experiment 2, four-leaf plants. O = youngest leaves; Δ = oldest leaves; and □ = roots.

Titer of *S. kunkelii* in corn. Results are presented graphically in Figure 2. Spiroplasmas were first detected as early as day 14 (Fig. 2A and D). Numbers of spiroplasmas varied somewhat but remained relatively low (near the detection limit) in all samples until days 35 to 49. From day 49 to the end of the experiment, the range of spiroplasma titers began to broaden; titers remained low in some plants but reached much higher levels in others. The maximum reliable titer, approximately 2.0×10^9 CFU/g, was measured in a one-leaf plant in experiment 2 (Fig. 2C) on day 56. Four estimated sample titers, positioned above the axis breaks in Figure 2C and D, could not be reliably quantitated since they were more than four times greater than the highest standard used for titer estimation. Corn stunt symptoms were more pronounced in corn plants with higher titers of *S kunkelii* than in plants with lower titers (data not shown).

Although there were no significant differences in maximum spiroplasma titer between one- and four-leaf corn plants, the maximum was reached earlier in four-leaf plants (days 49 and 42 for the two experiments) than in one-leaf plants (days 63 and 49).

DISCUSSION

In a study in which phase contrast microscopy was used to detect spiroplasmas in corn, Davis et al (7) detected the pathogens first in the roots and then in the youngest leaves. In our work, however, *S. kunkelii* was detected first in the youngest and oldest leaves, although on most later test days the pathogen was present in a larger proportion of root samples than in other organ samples. These observations are consistent with the hypothesis (6,16) that spiroplasmas, which are phloem limited, move with the flow of photosynthate to actively growing regions such as roots, growing points, and flowers or fruits. Plant tissues that have high levels of metabolic activity serve as sinks for sugars and other energy-rich compounds moving in the phloem sap. Alternatively, the spiroplasmas could have moved simultaneously into all plant organs, but higher spiroplasma multiplication rates in the actively growing plant regions could have led to their early detection in these organs. The fact that the first detection of spiroplasmas in youngest and oldest leaves was later in experiment 1 than in experiment 2 may have resulted from the greater dilution of the sap prior to ELISA in the first experiment.

Symptom expression and pathogen movement patterns were different in corn seedlings inoculated at an earlier plant growth stage than in seedlings inoculated at a later stage. Symptoms appeared 1 to 2 weeks earlier in one-leaf plants than in four-leaf plants. In experiment 1, there were greater probabilities of infection of roots and oldest leaves of one-leaf than of four-leaf plants at every test date, although these differences were not always significant. The probability of the youngest leaves being infected was similar for one- and four-leaf plants. The greater probabilities of detecting *S. kunkelii* in the oldest leaves of one-leaf plants compared with four-leaf plants is not surprising since that was the inoculated leaf for each one-leaf plant.

Spiroplasmas were first detected 2 to 3 weeks after inoculation of both one- and four-leaf plants, although symptoms were not visible until the sixth week. These data extend by 1 to 2 weeks the previously reported interval between pathogen detection and symptom appearance and are consistent with the conclusion of Davis et al (9) that the pathogen could be acquired by leafhoppers before the disease is obvious in the field. After approximately 1 month, the pathogen had moved and multiplied throughout the corn plant, resulting in detection in almost all the tissues tested, including the tassel as it developed.

The maximum spiroplasma titer was reached earlier in four-leaf plants than in one-leaf plants, suggesting that the pathogen may

multiply more quickly in the former. However, the fact that spiroplasmas were often detected in higher percentages of one-leaf plant organs (especially roots) than in corresponding four-leaf plant organs may indicate that they colonize younger corn plants more readily or that systemic colonization of the plant phloem proceeds at similar rates in small and large plants and is therefore completed more rapidly in small plants. These data suggest that, in a field situation, corn plants inoculated at an earlier growth stage may provide a greater opportunity for subsequent acquisition and dissemination by feeding leafhoppers.

The overall patterns of titer change observed in these experiments are similar to those reported for other phytopathogenic mollicutes in their plant hosts. For example, populations of *S. citri* in turnip seedlings remained constant or declined slightly, rose sharply to a peak, and then dropped (11). Archer et al (2) also noticed a decline in *S. citri* in plant hosts after a peak in exponential growth. The patterns also resemble those reported for the aster yellows phytoplasma in some tissues of infected periwinkle (*Catharanthus roseus*) (13). Corn stunt symptoms in our experiments were more pronounced in corn plants with high titers of *S. kunkelii* than in plants with lower titers (data not shown). This relationship was also reported for the aster yellows phytoplasma in periwinkle by Kuske and Kirkpatrick (16), who showed phytoplasma concentrations to be higher in symptomatic and actively growing plant parts than in asymptomatic or older plant parts.

Large variations in the percentage of samples positive for spiroplasma presence and in spiroplasma titer among the seven to ten test plants sacrificed for each treatment at each test date reflect plant-to-plant variation and/or unequal inoculum introduction by leafhoppers. Although the variation limited our ability to establish statistical significance in the differences in spiroplasma occurrence and titers at different test dates and in plants inoculated at different ages, the variation is itself of interest; earlier studies of a similar nature often did not quantitate titers (7,9) or sampled only single plants at each time point (16). Even greater variation would be expected in the field, where differences in individual plant microclimate and condition and time of inoculation would vary. Relatively low R^2 values in many of our comparisons suggest that the data do not closely fit the logistic model.

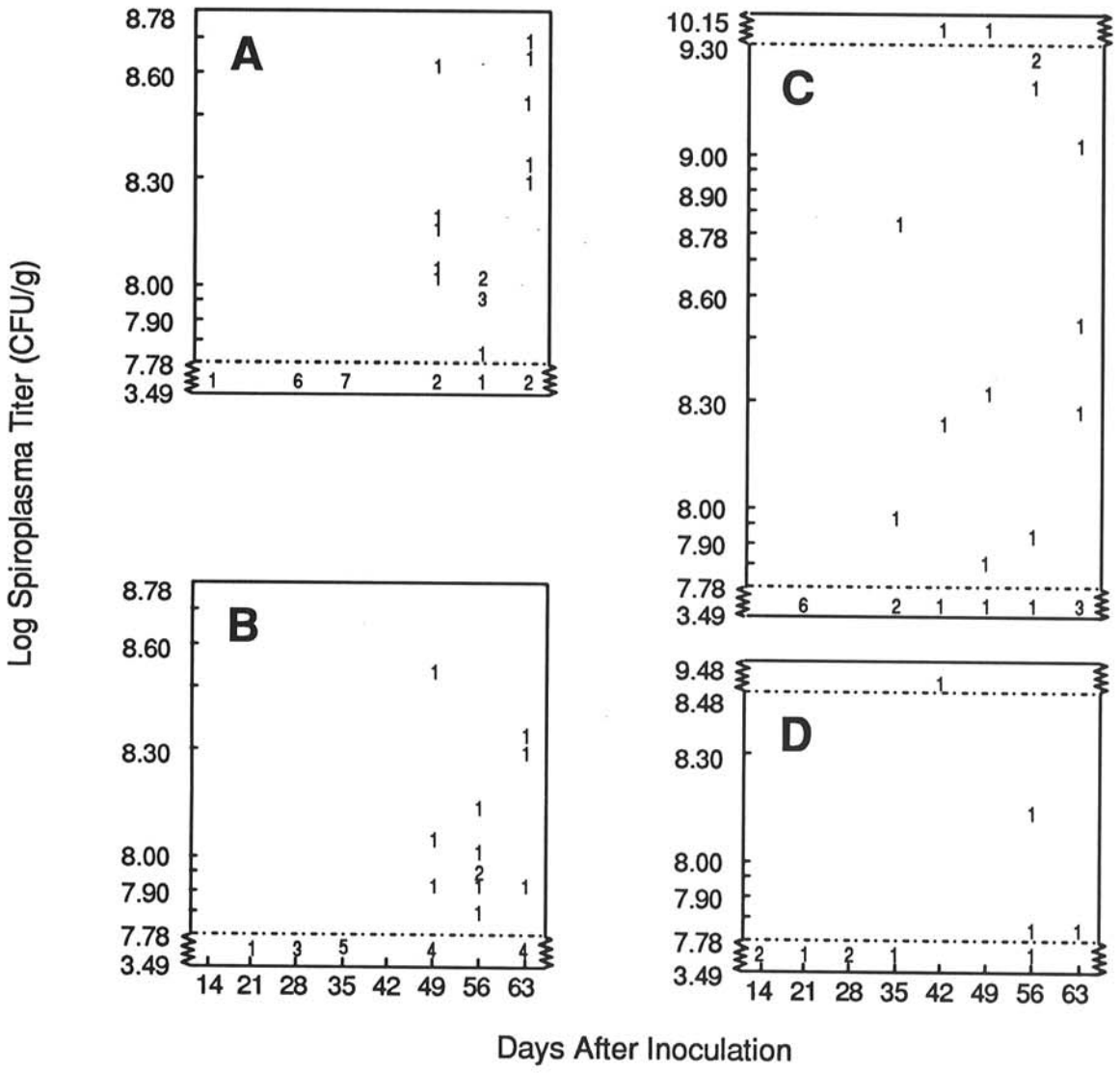

Fig. 2. Titers of *Spiroplasma kunkelii*, determined by quantitative enzyme-linked immunosorbent assay (ELISA), in the youngest fully expanded leaf of corn plants inoculated by leafhoppers at either the one-leaf or four-leaf growth stage. **A,** Experiment 1, one-leaf plants; **B,** experiment 1, four-leaf plants; **C,** experiment 2, one-leaf plants; and **D,** experiment 2, four-leaf plants. Integers placed as points on the graphs represent the number of test samples for which a particular titer was recorded on each date. Only plants in which spiroplasmas were detected are shown. The ELISA detection limit was 3.1×10^3 CFU/g (log = 3.49). Calculated titers above 2×10^9 CFU/g (log = 9.30) (**C and D**) are unreliable since they are more than four times higher than the highest standard titer used to calculate the sample titers. These, along with titers below 6×10^7 CFU/g (log values below 7.78), are placed out of scale on the graphs so that a scale maximizing the distance between the other points could be used.

However, in the absence of other published quantitative data of similar experiments for comparison, these R^2 values may be the best one can expect when constrained by physical and temporal limitations to a relatively small sample size. Thus, they do not invalidate the logit model and may serve as a point of reference for future studies of this type. The application of a logistic regression model is a useful strategy for analysis of data characterized by limited sample numbers and only two possible values for the dependent variable.

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