Effects of Tetracycline on the Transmission and Pathogenicity of the Western X Disease Agent in its Insect and Plant Hosts

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ABSTRACT

Treatment of diseased celery plants with tetracycline resulted in a remission of Western X disease (WX) symptoms. Fewer *Colladonus montanus* leafhoppers acquired the WX agent when fed on treated plants than when fed on diseased controls. Tetracycline, acquired by feeding or injection into inoculative leafhoppers, reduced WX transmission by vectors in proportion to numbers of injections or feeding time on the antibiotic. Tetracycline also extended the incubation period of WX in the leafhopper vectors, and such insects lived longer than did untreated, infective controls. After feeding on tetracycline, diseased leafhoppers laid more eggs, on the average, than did untreated diseased controls. Phytopathology 61:696-702.

Additional key words: mycoplasma, virus.

Forty or more plant diseases, affecting a wide variety of food, forage, and horticultural plants, are grouped under a category of "yellows" diseases. Western X disease (WX) of peaches and cherries is one such disease.

The WX disease agent was transmitted from peach to celery (*Apium graveolens* L.) by means of two of its leafhopper vectors, *Colladonus geminatus* (Van Duzee) (11) and *C. montanus* (Van Duzee) (12). Infected celery showed general stunting, reduced leaf lamina, pale yellowish-green margins on older leaves, and brown, necrotic tips on newly emerged leaves. In the late stages of the disease, the older leaves became white.

The causal agent of WX was long thought to be a virus because symptoms induced resembled those caused by leafhopper-borne viruses. However, all efforts to find a virus in infected leafhopper or celery tissues, by electron microscopy, or to purify a virus from infectious extracts have been unsuccessful.

In 1967, Doi et al. (4) reported the presence of microorganisms resembling mycoplasma (PPLO or pleuropneumonialike organisms) or psittacosis-lymphogranuloma-trachoma (PLT) in the phloem cells of plants infected with mulberry dwarf, potato witches' broom, and aster yellows diseases. Nasu et al. (17) also reported mycoplasmalike organisms in rice and in leafhopper vectors infected with rice yellow dwarf disease. Ishii et al. (10) found that tetracycline antibiotics suppressed the symptoms of mulberry dwarf disease. On the basis of these observations, Doi et al. (4) suggested that a number of leafhopper-transmitted yellows and witches' broom diseases were possibly due to such microorganisms rather than to viruses.

These important developments were followed by studies that revealed the presence of mycoplasmalike organisms in the plant hosts and leafhopper vectors of aster yellows (9, 16), corn stunt (2, 6, 7), and Western X-disease (18). Similar microorganisms were reported from plants infected with stolbur, parastolbur, Crimean yellows, clover dwarf, and European aster yellows (19); legume little leaf, tomato big bud, and lucerne witches' broom (1); sugarcane white-leaf disease (20); pear decline (8); and greening disease of citrus (15).

Tetracycline antibiotics suppressed disease symptoms of aster yellows (3, 5), and sugarcane white-leaf (20). Freitag & Smith (5) also reported that fewer individuals of the leafhopper vector, *Macrosteles fascifrons*, acquired aster yellows from diseased plants treated with tetracyclines than from untreated controls.

MATERIALS AND METHODS.—Inoculative leafhopper vectors were obtained by caging young nymphs of *C. montanus* on WX-diseased celery plants and allowing them to feed for 30-40 days or by injecting healthy last instar nymphs with infectious insect extracts as described previously (2). Extracts containing WX inoculum were prepared by grinding infective adult insects in either 0.85% NaCl or in tissue culture medium, using 100 adults for each 1.5 ml of fluid. Prior to injection, the extracts were clarified by centrifugation and then passed through a Millipore filter of 0.45 µ to remove any contaminating bacteria. Control insects were injected with extracts from noninfectious leafhoppers. Approximately 40-60% of the leafhoppers became infective after feeding on diseased celery for 1 week; after 4 weeks, 90-95% acquired infectivity.

The effect of tetracycline (95%, Lederle Laboratories, Pearl River, N. Y.) on the transmission of WX by *C. montanus* was tested by injecting the antibiotic into the vectors or by allowing them to feed through a Parafilm membrane (Marathon Corp., Menasha, Wis.) into a solution containing tetracycline in distilled water.

The feeding cage used for this purpose (Fig. 1) was made by cutting a small plastic tube (15 mm, internal diam) into sections 15 mm long. The ends were covered with nylon screen, and a hole was made in the side wall for introducing leafhoppers into the cage. Two layers of membrane were stretched over one end of a separate ring of equal size. By means of a syringe, about 1 ml of a solution of tetracycline in distilled water was injected between the two membranes. After the membranes were stretched fully, the syringe was removed
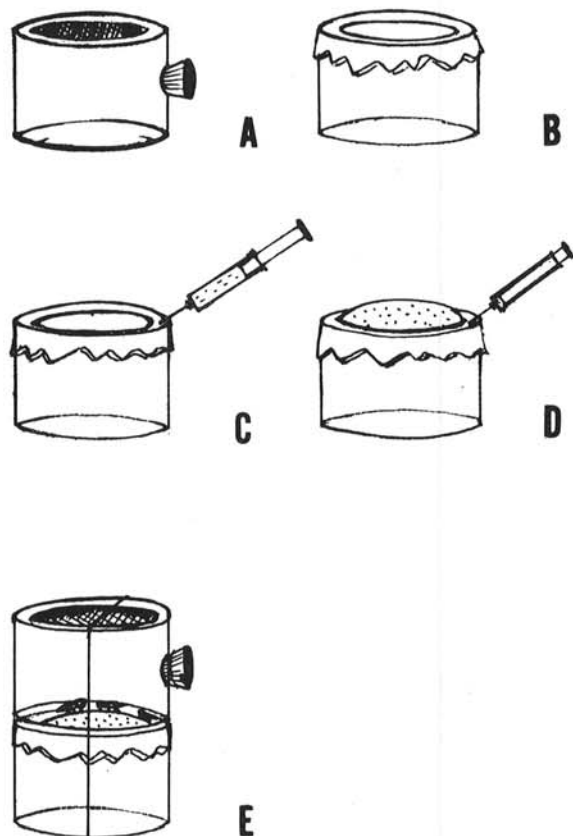
Fig. 1. A) Plastic leafhopper feeding cage with nylon screen over both ends and a corked hole in the side for introducing leafhoppers. **B)** Second cage with two layers of Parafilm membrane stretched over one end. **C)** Syringe needle inserted between two layers of membrane. **D)** Stretched membranes resulting from introduction of tetracycline solution. **E)** Two cages held together by rubber band while leafhoppers feed through membrane into tetracycline solution.

and the puncture sealed by applying pressure. The cage was inverted over the feeding cage containing the leafhoppers. The two cages were held in position together by means of a rubber band. The test leafhoppers fed through the membrane into the tetracycline solution. Control leafhoppers were fed on distilled water.

Celery, *Apium graveolens,* was used as test plants and for WX disease source plants. The test plants were usually in the three-leaf stage at the time of the experimental insect feedings. All test plants were held in the greenhouse for possible WX symptom development.

The effect of tetracycline on the remission of Western X-disease symptoms in celery was tested by dipping the diseased plants, growing in small plastic pots, for 2 min in solutions of tetracycline at 100, 200, or 400 ppm in distilled water. Diseased controls were dipped in distilled water only. The treatments were repeated every 3rd day for a period of 1 month. The treatments were started 5-6 weeks after the plants had been infected and when early symptoms were appear-

ing in all plants. Ten plants were used in each of the four treatments.

RESULTS.—*Remission of WX symptoms in celery after treatment with tetracycline.*—Repeated dippings of diseased celery plants in 100, 200, and 400 ppm tetracycline solutions resulted in complete remission of WX symptoms. Diseased plants first began to show evidence of recovery from symptoms 10-12 days after treatment began. The effect appeared first in the plants receiving 400 ppm. As the treatments progressed, the celery plants receiving 100 and 200 ppm grew somewhat taller than did those receiving 400 ppm. At the end of 1 month, plants treated with 400 ppm were the healthiest in appearance, with those receiving 100 and 200 ppm being almost normal. In contrast, all untreated, diseased control plants were dwarfed and most of them were dying. When the root systems of the various treated plants were examined, it was found that there was a close parallel between the apparent health of the foliage and the vigor of the root system. Tetracycline-treated plants retained good root systems, whereas the roots of the diseased controls were collapsing and dying.

Two months after treatment began and 1 month after it terminated, some plants treated with 400 ppm showed an apparent toxic effect of the tetracycline. Some of the older leaves on such plants became white with curled margins.

Recurrence of WX symptoms in celery after treatment with tetracycline.—Approximately 2-3 weeks after tetracycline treatments were stopped, disease symptoms began to reappear in celery plants treated with 100 ppm. First symptoms recurred in the plants treated at 200 and 400 ppm in 3-4 and 5-6 weeks, respectively, following the last treatment.

Recovery of WX infectivity from tetracycline-treated plants.—After the treatments were stopped, groups of noninfective leafhoppers were caged on the treated plants for 7-day acquisition feeding periods and subsequently tested singly for transmission. A new group of noninfective leafhoppers was caged on the treated plants every week over a 6-week period and tested for transmission, to measure the titer and availability of WX in the plants (Table 1).

Infectivity was acquired from the untreated controls throughout the test period, but recovery from the tetracycline-treated plants was a function of the concentration of the antibiotic. No WX infectivity was re-

TABLE 1. Recovery of Western X disease agent, by leafhoppers, from tetracycline-treated celery

| Treatment | Frequency of leafhopper transmission: weeks after treatment was stopped | | | | |
	0-1	1-2	2-3	3-4	5-6
Controls	4/8[ab]	8/8[b]	9/9[b]		
100 ppm	0/10	3/9	11/15[b]	9/9[b]	
200 ppm	0/30	2/13	2/15	4/7[b]	4/6
400 ppm	0/13	0/14	0/15	2/8	

[a] Numerator = the number of insects transmitting; denominator = the number of insects tested.
[b] Visible symptoms in plants.

covered from any of the tetracycline-treated plants during the 1st week after the treatments had stopped. Thereafter, progressively increasing numbers of leafhoppers acquired infectivity from plants treated at 100 and 200 ppm. No infectivity was recovered from plants receiving 400 ppm of tetracycline until the 4th week, at which time 25% of the vectors became infective.

The effect of injected tetracycline on the transmission of WX by C. montanus.—Effect of tetracycline on infectivity acquired by feeding.—In this experiment, the effect of tetracycline at 1,600 ppm was tested by injection into 250 adults of *C. montanus* that had acquired WX infection by feeding on diseased celery for 33 days previously. Three days after injection, 40 of these leafhoppers were caged singly for transmission tests and compared with a control group of 40 adults that received no tetracycline.

The results (Table 2) show that tetracycline, when injected into inoculative vectors, eliminated the infective agent from almost 50% of them. Moreover, in those that did transmit, the concentration of the infective agent was sharply reduced and an additional incubation period in the vector was required. Whereas 72% of the tetracycline-free controls transmitted during the 1st week on test plants, only 14% of the ultimate transmitters receiving tetracycline transmitted during the 1st week.

Simultaneous injections of WX and dilutions of tetracycline.—The tetracycline dilutions were made as follows: A 3,200-ppm solution of tetracycline in distilled water was made up on a w/v basis. Equal volumes of this solution and of the extract of leafhoppers containing WX were then mixed to provide inoculum in which the tetracycline was 1,600 ppm. Part of this was further diluted with WX extract to provide tetracycline solutions of 800 ppm, 400 ppm, and 100 ppm. Each of the above inocula was injected into last instar nymphs of *C. montanus*. Control nymphs were injected with WX extract only. The WX inocula used in all the treatments were more than adequate to infect a high percentage of the insects in the absence of tetracycline. Previous work (21) demonstrated that WX inoculum could be diluted 10^{-3} and still infect 95% of the injected leafhoppers.

In the present experiment, WX extract alone infected 24 of 30 leafhoppers. The results for insects receiving WX plus tetracycline at dilutions of 100, 400, 800, or 1,600 ppm were, respectively, 2, 9, 5, and 2 transmitters out of 30 tested at each dilution.

TABLE 2. Effect of one injection of tetracycline (1,600 ppm) on the transmission by *Colladonus montanus* of Western X disease acquired by feeding[a]

Treatment	Transmission results	
	1 week	10 weeks
Injected with tetracycline[b]	3/40[c]	22/40
Noninjected controls	28/40	39/40

[a] Prior feeding time on diseased celery = 33 days.

[b] Injected 3 days prior to beginning of transmission feedings.

[c] Numerator = the number of insects transmitting; denominator = the number of insects tested.

Delayed injection of tetracycline.—Of the insects whose first injection had consisted of WX extract only, 100 were injected 12 days later with tetracycline (1,600 ppm in distilled water). When 30 of these were tested singly for WX transmission later, 13 were inoculative, as compared to 2/30 for those receiving WX and tetracycline simultaneously, and 24/30 for those receiving WX extract only.

Effect of multiple injections of tetracycline.—In this experiment, the effects of 1, 2, and 3 injections of tetracycline at 1,600 ppm were compared. A group of 450 last-instar nymphs was injected with WX leafhopper extract. One week later, 300 of these were injected with tetracycline. One week later, 200 of the group that previously received tetracycline were given a second injection of tetracycline. One week later, 100 of this group received a third injection of tetracycline.

Three weeks after the first injection with WX extract, insects from each treatment were caged singly on celery and transferred weekly for transmission tests. Of the insects injected with WX extract only, 55 out
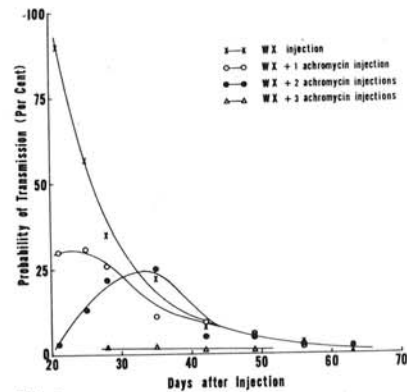

FIG. 2

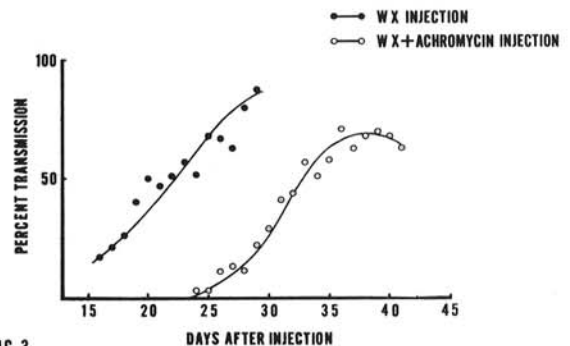EFFECT OF ACHROMYCIN ON THE INCUBATION PERIOD OF WX IN C. MONTANUS

FIG. 3

Fig. 2-3. Effect of tetracycline on Western X disease. 2) Effect of multiple injections of 1,600 ppm tetracycline on transmission of WX by *Colladonus montanus*, expressed as the probability of transmission in per cent. Formula: LX × TX = transmission probability, where LX = survival probability (number of insects surviving on any given week divided by the original number tested) and where TX = transmission rate (number of transmitters divided by the number of surviving insects at that interval). 3) Effect of tetracycline on the incubation of WX in *C. montanus* and on the disease transmission pattern.

of 60 transmitted. The results for those receiving WX extract plus 1, 2, or 3 injections of tetracycline were 26/54, 16/60, and 3/80, respectively.

Figure 2 presents curves for the four treatments depicting the probability of transmission in per cent. This is based on the formula: $LX \times TX =$ transmission probability, where $LX =$ survival probability (number of insects surviving on any given week divided by the original number tested) and where $TX =$ transmission rate (number of transmitters divided by the number of surviving insects at that interval).

As is apparent from Fig. 2, the transmission probability for insects receiving only WX extract was 90% during the 1st week of the test period, as compared to 30% for those receiving one injection of tetracycline and 2.5% for those receiving two. It is also evident that two injections of tetracycline resulted in a second incubation period of the WX agent in the vector following an initial great reduction in WX titer. Three injections of tetracycline eliminated the infectious WX from all but three of the injected insects.

Suppression of WX transmission by infective vectors fed on tetracycline.—Fifth instar nymphs were caged on diseased celery for 29 days to acquire WX infection. Groups of these leafhoppers were then given feeding access, through a membrane, to a 400-ppm tetracycline solution for 4, 8, or 24 hr. Untreated, infective control leafhoppers were fed on distilled water. At the end of the membrane feeding periods, the leafhoppers were caged individually on young celery plants for transmission feeding tests. Transfers were made daily for 15 days after the first membrane feeding. This was followed by two transfers at 3-day intervals, then by weekly transfers until a total of 86 days had elapsed. On the 7th day after the first membrane feedings had occurred, the leafhoppers were given a second tetracycline or water access feeding period of 4, 8, or 24 hr (Fig. 4). Their test feedings and transfers were then continued as described above. The number of test insects used after 4-, 8-, and 24-hr tetracycline feedings and the 4-hr water feeding were 15, 15, 25, and 15, respectively.

The transmission results (Fig. 4) indicate that, the longer the access feeding period on tetracycline, the more profound the effect on the elimination or suppression of WX transmission. It is of interest that there is a delay of 1 day after tetracycline feedings before the leafhoppers show a reduction in WX transmission. In fact, transmission actually increases immediately after the leafhoppers feed on tetracycline. It then drops to zero for 3-4 days before rising to a maximum within 7-10 days. Two 24-hr feedings on tetracycline rendered infective leafhoppers noninoculative for almost 30 days before even a small amount of transmission occurred. Only 3 of 21 such leafhoppers transmitted WX as compared to 15 of 15 untreated controls. The results for the groups that fed 4 or 8 hr on tetracycline were 10/15 and 4/15, respectively. These results suggest that tetracycline, taken in during access feeding periods of 8 and 24 hr, completely eliminated WX infection from most of the previously infective vectors. Moreover, in the case of the 24-hr feeding experiment, the

infectious agent was reduced to such a low level in the three insects that did transmit that a second incubation period of approx 25 days was required in these vectors.

Effect of tetracycline on the incubation period of Western X disease in Colladonus montanus.—The effect of tetracycline in extending the incubation period of WX in leafhoppers has been evident in some of the experiments reported in previous sections. Separate experiments on the incubation period were also carried out. In one test, the leafhoppers were made infective by injection with WX leafhopper extract. The vectors were then fed on a 400-ppm tetracycline solution for 4 hr at 0, 5, or 10 days after injection with WX. Controls were infective insects that fed on distilled water. After feeding on tetracycline solutions or distilled water, the test insects were caged singly on young celery plants, which were changed every 3 days, for transmission feedings. The effect of tetracycline on the incubation and transmission of WX by the vector was considerable when its application was delayed for 10 days. No transmission occurred during the first 20 days, and only 7% on the 23rd day. In contrast, on the 23rd day after injection with WX, 100% of the diseased controls transmitted, as did 75% and 81%, respectively, of the insects fed on tetracycline at 0 and 5 days.

In a separate experiment, 150 healthy, fifth-instar nymphs were injected with WX extract from infective insects. An equal number of healthy nymphs were injected with WX extract plus 500 ppm of tetracycline. Both of these groups were then held on healthy celery for 15 days. Ninety adults from each of the two groups were then caged singly on young celery plants for transmission feedings and daily transfer to new celery plants. Figure 3 illustrates the transmission (in per cent) for the two groups, and reveals a longer incubation period of WX in the insects receiving tetracycline.

On the basis of the LP-50 (latent period-50, or the time required for 50% o fthe infective individuals to transmit WX), tetracycline extended the incubation period from 20 days, in the insects injected with WX only, to 31 days in those injected with WX plus 500 ppm tetracycline.

Effect of tetracycline on the longevity of infective vectors.—Western X disease infection in the leafhopper vector, *C. montanus*, was shown previously to cause the premature death of this insect (13). We found that tetracycline not only reduced the transmission of WX, but also increased the mean longevity of infective vectors over that of infective controls when administered either by feeding or by injection. More leafhoppers died during or immediately after feeding on tetracycline solutions than after feeding on water. But the tetracycline survivors consistently lived longer than did the surviving controls.

The data in Fig. 4 show that all untreated, infective control insects died within 80 days after the beginning of the acquisition feeding period on diseased celery; 50% of them died within 54 days. In contrast, 50% or more of the insects that fed on a 400-ppm tetracycline solution for 4 hr were still alive after 80 days.

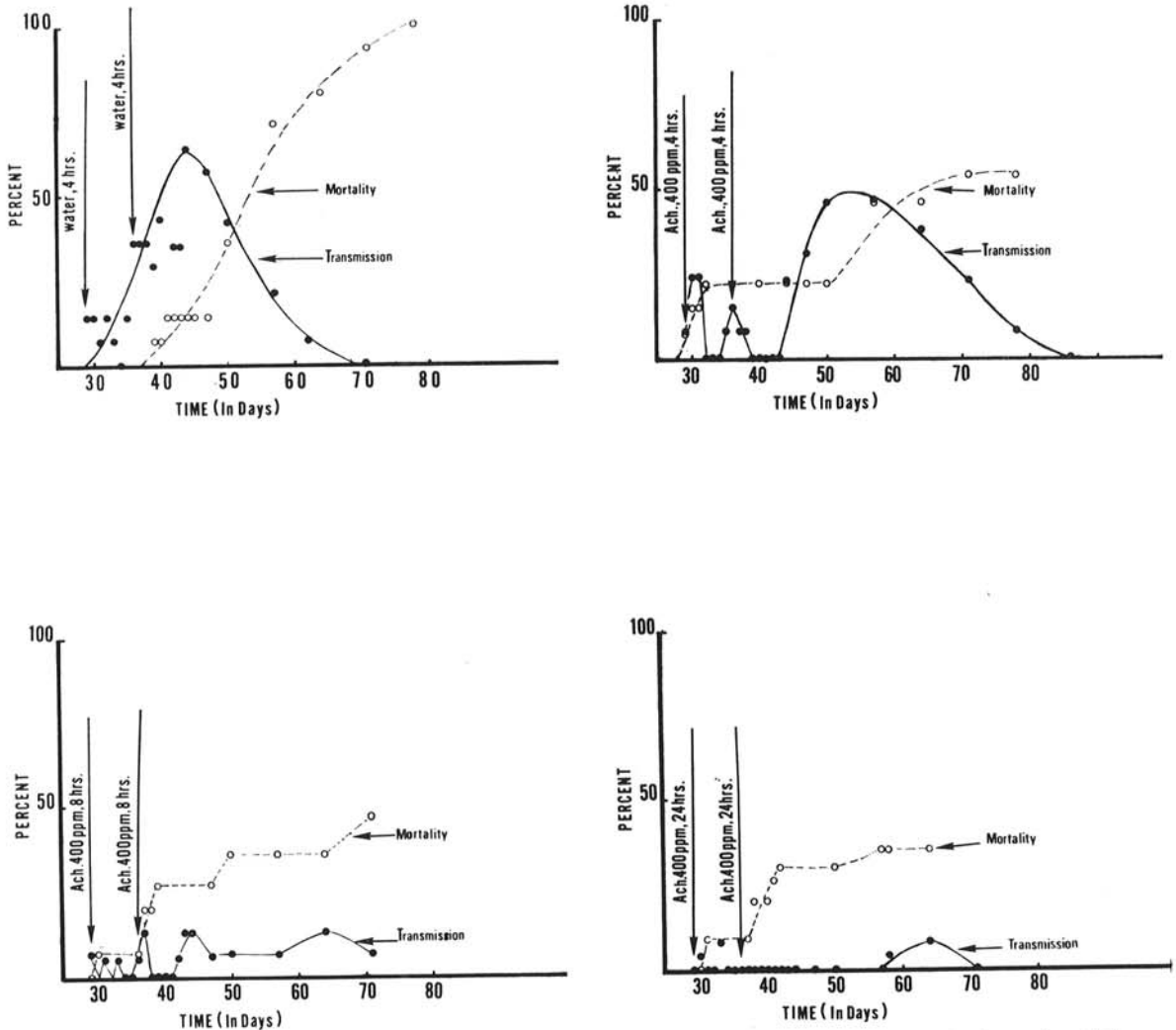In a similar WX acquisition feeding experiment,

Fig. 4. Effect of tetracycline on the transmission of WX by *Colladonus montanus* and on the longevity of the vector. Time = number of days since the beginning of the acquisition feeding period on diseased plants. Vertical arrows indicate the dates on which test insects fed on tetracycline solutions or on water.

those leafhoppers that fed for 4 hr on a 400 ppm tetracycline solution had a mean longevity of 67 days as compared to 55 for the controls that fed on distilled water.

In a separate injection experiment, the tetracycline (500 ppm) and the WX inoculum (insect extract) were administered to the insects simultaneously by injection. Controls were injected with WX extract and distilled water. In this trial, the mean longevity of the 60 controls was 50 days as compared to 64 days for the 60 leafhoppers injected with tetracycline.

Our results show that tetracycline antibiotic is active against the mycoplasmalike organisms associated with WX disease, and either eliminates them from the insect vector's system or substantially reduces their titer. This is inferred from the transmission tests in which the reduction in transmission is proportional to the amount of tetracycline the insects receive by injection or by feeding. The mean longevity of infective vectors

treated with tetracycline is somewhat longer than that of untreated infective controls. It seems probable, therefore, that the greater longevity results from the reduction or elimination of the mycoplasmalike organisms that are pathogenic in the vector.

Effect of tetracycline on the fecundity of infective leafhoppers.—The fecundity of *C. montanus* was previously shown to be reduced by infection with WX (14). In our experiments, leafhoppers were made infective by injection of WX extract into fifth instar nymphs. Controls were injected with extract from healthy leafhoppers. The nymphs were then caged on healthy celery plants. Adult females that had emerged 2 days after injection were used in membrane feeding trials. Of the females injected with WX, 20 were fed on 400-ppm tetracycline solution for 4 hr, and 20 diseased controls were fed on water for the same period. Healthy controls were also fed on water for 4 hr.

Each female adult was then caged singly on healthy

TABLE 3. Effect of tetracycline (acquired by feeding) on the fecundity of leafhoppers injected with Western X disease[a]

Treatment	No. of eggs per week						Total
	1st[b]	2nd	3rd	4th	5th	6th	
Infective females fed on water	449/18[c] 25/♀[d]	338/18 19/♀	360/18 20/♀	70/8 9/♀	20/8 3/♀		76
Infective females fed on tetracycline	528/16 33/♀	398/15 27/♀	411/15 27/♀	237/12 20/♀	87/10 9/♀	49/8 6/♀	122
Healthy females fed on water	383/13 30/♀	236/10 24/♀	246/10 25/♀	207/10 21/♀	72/4 18/♀	30/5 6/♀	124

[a] Adults fed on a 400-ppm tetracycline solution, for 4 hr, 2 days after injection with WX extract.
[b] The 1st week followed a preoviposition period of 9 days (11 days after injection).
[c] Number of eggs laid per total females.
[d] Number of eggs laid per female.

celery with five males to assure mating. At the end of a preoviposition period of 9 days, females were transferred singly to celery plants with one healthy male in each cage. The leafhoppers were transferred to a new celery plant each week for oviposition, and counts were made of the eggs laid by the individual females each week.

The data (Table 3) show that the average oviposition by WX-infected females is reduced to 61% of that of healthy females. Females fed on tetracycline laid 122 eggs/female as compared to an average of 76 eggs laid by diseased untreated controls. Females injected with extract from healthy insects averaged 124 eggs/female. There is a marked reduction in fecundity of diseased vectors about 5 weeks after injection with WX. Similar females, treated with tetracycline, showed a corresponding reduction in oviposition 1 week later; i.e., 6 weeks after injection with WX. Thus, tetracycline prolonged normal egg laying in diseased leafhoppers for approximately 1 week.

DISCUSSION.—The causal agent of WX disease is now thought to be a mycoplasmalike organism rather than a virus. Nasu et al. (18) found such bodies consistently associated with the disease both in the host plant and in infective vectors. The same mycoplasma were not found in healthy plants or in leafhoppers free of WX infectivity.

Our findings that (i) repeated dippings of WX plants in tetracycline solutions resulted in remission of symptoms but not cure of the disease; (ii) recurrence of symptoms after treatment was earlier when lower concentrations of antibiotics were used; (iii) noninfective leafhoppers could not recover infectivity from plants recently treated with tetracycline; (iv) tetracycline, acquired by feeding or by injection, eliminated or reduced infectivity in leafhoppers and resulted in a new or extended incubation period of WX in the vectors; and (v) tetracycline partially nullifed the deleterious effects that WX causes in the life functions of the vector, *C. montanus,* as indicated by greater longevity and higher fecundity, support the view that WX disease is caused by mycoplasma and not a virus.

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