

Uptake and Persistence of Oxytetracycline in Aster Plants and Vector Leafhoppers in Relation to Inhibition of Clover Phyllody Agent

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ABSTRACT

Oxytetracycline-HCl (terramycin) was shown, by microbiologic assay, to be absorbed from solution by roots of aster plants (root treatment) and translocated to stems, petioles, and leaves. Extending the root treatment from 1 to 4 days increased the relative concentration of antibiotic in the extracts of aerial tissues. The concentration gradually declined thereafter, but antibiotic could still be detected 19 days after initiation of treatment, irrespective of the original concentration. Antibiotic was not detected in plants after a single application of terramycin to the leaves (by spraying) or to the soil. Leafhoppers, *Macrostelus fascifrons*, that were caged on asters whose roots were immersed in terramycin solution (100 ppm), also accumulated active antibiotic in their bodies, the concentration being dependent upon the length of the feeding period.

Root treatment (terramycin, 100 ppm) of clover phyllody-affected asters resulted in remission of symptoms in most plants. Fewer leafhoppers were able to acquire and transmit clover phyllody agent (CPA) when fed on antibiotic-treated infected plants than when fed on

infected but untreated plants. Healthy aster plants did not become infected when subjected to the root treatment either immediately before, or soon after, inoculation by infective leafhoppers. As the interval between inoculation and antibiotic treatment increased, the number of plants that eventually became infected also increased. After an acquisition access period of 7 days, CPA in leafhoppers was inactivated, as determined subsequently by their ability to transmit, when they were caged on aster plants maintained in terramycin solution (100 ppm). The degree of inactivation was dependent upon the length of time the leafhoppers were allowed to ingest the antibiotic from plants. Healthy leafhoppers that ingested the antibiotic lived much longer than did untreated insects.

Ultrathin sections of infected plants and vector tissues treated with the antibiotic showed that the mycoplasma bodies (found associated with the disease) were often broken or devoid of their internal structure.

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The occurrence of an organism resembling a *Mycoplasma* species, in plants affected with a "yellows" type of disease, was first demonstrated by Doi et al. (7). Since then, the association of such organisms with several other plant diseases has been demonstrated, and the subject has been discussed in two recent reviews (15, 28). Symptoms of such diseases have been suppressed in plants (5, 6, 8, 9, 11, 25), and the agents adversely affected in vector insects (8, 9, 27) by antibiotics of the tetracycline group known to be effective against diseases caused by mycoplasma in mammals and avian species (10).

Recently, the association, development, and localization of mycoplasma in plants (21) and in vector leafhoppers (22) carrying clover phyllody agent (CPA) were reported. The present investigation was undertaken to study the effects of oxytetracycline-HCl (terramycin) on CPA infection in aster plants (*Callistephus chinensis* Nees) and vector leafhoppers, *Macrostelus fascifrons* (Stål.). This paper reports on (i) detection and persistence of terramycin in aster plants treated variously with the antibiotic; (ii) detection and persistence of terramycin in leafhoppers fed on plants maintained in the antibiotic solution; (iii) susceptibility of plants to CPA infection either before or after the antibiotic treatment; (iv) the effects on transmission of CPA by leafhoppers fed on antibiotic-treated plants; and (v) effect of the antibiotic on the mycoplasma associated with infected plants and leafhoppers.

MATERIALS AND METHODS.—Disease and leafhoppers.—An isolate of the clover phyllody agent originally collected from diseased strawberry plants in Nova Scotia (2) and subsequently maintained in aster plants through inoculation by infective leafhoppers, *M. fascifrons*, was used in the present studies. Adult female leafhoppers were used in all experiments because transmission of CPA is greater with females than with males (3). Rearing of noninfective leafhoppers and maintenance of plants in the greenhouse was as described earlier (19). To obtain infective leafhoppers, adult females were caged for 1 week on infected plants, then maintained in groups on healthy aster plants for 5 weeks (1 week each on five successive plants) for completion of the incubation period. These leafhoppers have been referred to as "exposed". It has been shown earlier that only 50% of leafhoppers exposed to infected plants for 7 days are subsequently capable of transmitting CPA to aster plants (20). Although the maximum incubation period of CPA in aster plants is about 6 weeks (as in leafhoppers), the inoculated plants treated with terramycin were observed for symptom development for at least 90 days. The extra time was allowed to determine whether symptom development was delayed in the antibiotic-treated plants.

Treatment of plants with the antibiotic.—Aster plants (4-5 weeks old) were treated with the desired

concentration of terramycin (dissolved in 0.001 M phosphate buffer, pH 7.0) by three methods: (i) leaves were sprayed, using an atomizer, with the antibiotic solution supplemented with 100 ppm of sodium lauryl sulfate, a chemical shown to enhance the activity and systemicity of certain antifungal antibiotics (26); (ii) plants were removed from the soil, their roots washed with water, and then immersed in the antibiotic solution (referred to hereafter as "root treatment") in a flask for the desired period of time; after treatment, roots were washed, and plants were repotted in sterilized soil and kept shaded for 24 hr before transfer to the greenhouse; (iii) antibiotic solution (100 ml/500 g soil) was applied to the surface of the soil (referred to hereafter as "soil treatment") in pots containing aster plants, and were kept in saucers filled with water. The day on which plants were treated with the antibiotic was numbered 0, the next day 1, and so on throughout the experiment. In all experiments, unless stated otherwise, 100 ppm of terramycin and 0.001 M phosphate buffer (pH 7.0) were used.

Ingestion of the antibiotic by leafhoppers.—Uptake of terramycin by leafhoppers was accomplished by our allowing them to feed on aster plants, the roots of which were immersed in the antibiotic solution in a flask. The flasks, plugged with cotton so that plant tops were exposed, were placed in pots and surrounded with soil. Leafhoppers were caged on such plants and, whenever required, flasks were supplemented with freshly prepared antibiotic solution. When the ingestion period exceeded 7 days, the leafhoppers were transferred at weekly intervals to fresh plants maintained in antibiotic solution as described above.

Preparation of plant and leafhopper extracts for antibiotic assay.—To determine the relative terramycin concentration in plants, either the whole plant (without roots) or different parts of the plant (stem, petiole, leaves, and roots) were separately ground in a mortar. The crude extract was squeezed through a double layer of cheesecloth and centrifuged for 10 min at 6,500 g, and the supernatant stored at -20 C. Leafhoppers were ground in 0.85% NaCl (dilution 1/5, w/v) in a tissue grinder (19), the extracts centrifuged, and the supernatants stored as described for plants. The various samples thus collected during the course of any experiment were assayed for antibiotic simultaneously by the method described below. Preliminary tests had shown that freezing of samples did not cause any loss in antibiotic activity.

Antibiotic assay.—The antibiotic content of clarified extracts from plants or leafhoppers was determined by means of a paper disc assay method (14). The test organism, *Arthrobacter globiformis* (Conn) Conn & Dimmick was maintained on Difco penassay base agar supplemented with soil extract (13). Seed inoculum consisted of a cell suspension prepared from a 5-day-old agar slant culture of *A. globiformis*; turbidity was adjusted by dilution with sterile water to 60 units (Klett-Summerson colorimeter, 540-m μ filter). For each assay plate, 1

ml inoculum was mixed into 3 ml Difco penassay seed agar (at 45 C) and flooded over the surface of a base layer of Difco plate count agar previously poured and solidified. The extracts were applied to sterile filter paper discs (5 mm diam), each at two rates, 0.02 and 0.01 ml/disc, and air-dried for 1 hr at 20 C before the discs were planted on the seeded medium. Similar discs were also treated with a 100-ppm solution of terramycin to serve as controls. Plates were incubated at 25 C for 24 hr, and inhibition zone diameters were measured. The antibiotic content of the extracts was subsequently estimated from these data by reference to a standard curve (Fig. 1) established for the test organism. The resulting estimates, expressed as μ g/ml, represent the mean for the two rates of application from duplicate assay plates.

Bioassays of extracts of untreated plants and leafhoppers, included as controls concurrently with each experiment, were negative.

Electron microscopy.—Samples of plant and leafhopper tissues were processed, sectioned, and stained following the procedure described earlier (21, 22), and examined in a Siemens Elmiskop I.

RESULTS.—Uptake and persistence of antibiotic in aster seedlings.—Preliminary tests based upon bioassay with *Arthrobacter globiformis* indicated that terramycin was not only absorbed from solution by aster seedling roots, but was also rapidly translocated to stems, petioles, and leaves. More detailed study subsequently showed that plants treated by root immersion in terramycin solution for 24 hr accumulated a significant amount of antibiotic in their aerial tissues, and that the accumulation was increased ca. 3-fold by extending the immersion period from 1 to 4 days (Fig. 2). When plants were root-treated for various periods of time, then repotted in soil, the concentration of antibiotic in the aerial tissues gradually declined, but a detectable quantity still remained 19 days after initiation of treatment (Fig. 3). It is interesting to note that the higher initial quantity of antibiotic in the plants did not enhance its subsequent persistence in the tissues.

Detectable quantities of terramycin were not translocated either to stem or to root tissues of aster seedlings when a 1,000-ppm solution of the antibiotic was applied, either with or without sodium lauryl sulfate, to the foliage by spraying. Similarly, plants failed to accumulate detectable quantities of antibiotic in their aerial tissues after a single soil treatment with terramycin, either at a concentration of 100 or 400 ppm.

Uptake of antibiotic and persistence in leafhoppers.—Leafhoppers that were allowed to feed on aster seedlings maintained in the terramycin solution accumulated active antibiotic in their bodies, and the amount acquired increased gradually when the feeding period was prolonged from 1 to 7 days (Fig. 4). When leafhoppers were fed for 2 days on plants in antibiotic solution, then maintained on untreated plants, the ingested antibiotic persisted for at least 7 days (Fig. 5).

Antibiotic treatment of diseased plants.—Root

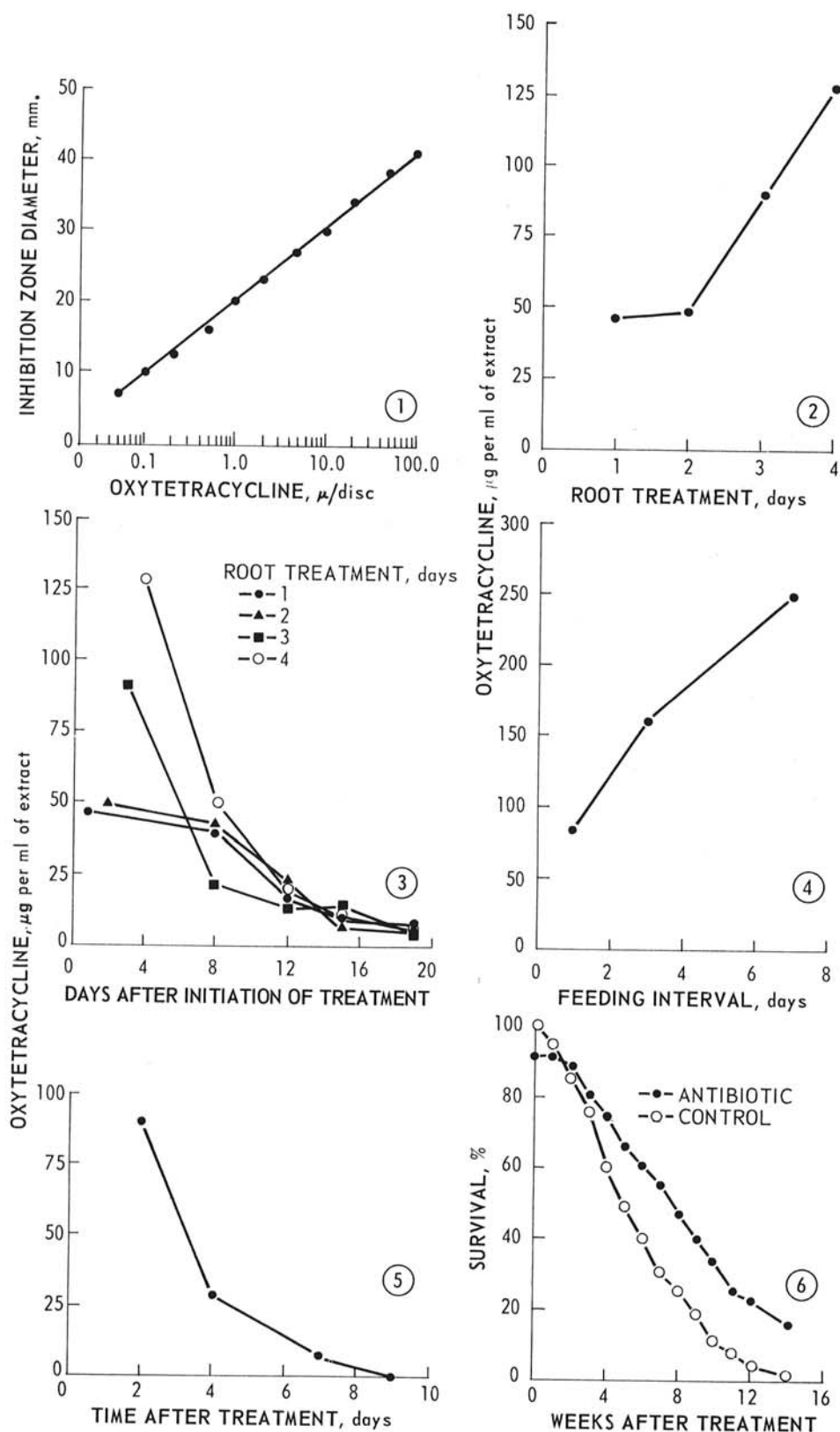


Fig. 1-6. 1) Standard curve depicting the sensitivity of *Arthrobacter globiformis* to oxytetracycline-HCl. 2) The relative concentration of oxytetracycline in extracts of aerial tissues of aster plants whose roots were immersed for different periods in the antibiotic solution (100 ppm). 3) Persistence of oxytetracycline in aerial tissues of aster plants after root treatment with the antibiotic solution (100 ppm). 4) The relative concentration of oxytetracycline in extracts of leafhoppers, *Macrostelus fascifrons*, that had fed for different periods on aster plants maintained in the antibiotic solution (100 ppm). 5) Persistence of oxytetracycline in leafhoppers which had fed for 2 days on plants in the antibiotic solution (100 ppm). 6) Survival of leafhoppers after feeding for 7 days on aster plants maintained in the antibiotic solution (100 ppm).

treatment of infected aster plants for 24 hr with terramycin solution resulted in remission of symptoms in most plants in 2-3 weeks. Eighteen of 20 treated plants, inoculated with CPA by means of infective leafhoppers 6 weeks earlier, developed new shoots with normal green leaves that showed no external disease symptoms. Also, flowers with normal pink-colored petals eventually developed on these treated plants, although they were slightly smaller than those of healthy plants. Five to 6 weeks after the treatment, symptoms reappeared on the leaves, but were milder than those on untreated plants. The 20 infected control plants, roots of which had been left 24 hr in the phosphate buffer without terramycin, continued to show marked symptoms with no signs of recovery, and eventually developed phyllod flowers, a response typical of CPA infection in aster plants.

Effect of the antibiotic on leafhopper survival.—Healthy adult female leafhoppers were caged for 7 days on two aster plants, one maintained in the antibiotic solution and the other in the phosphate buffer to serve as control. Both groups of leafhoppers were then maintained separately for 10 weeks (1 week each on 10 successive plants) on aster plants potted in sterilized soil, and at each transfer the number of surviving insects was recorded. The combined results of two experiments showed that 9 of 100 leafhoppers died during the period when they fed on plants in the antibiotic solution, but no mortality occurred with control insects during the same period. Subsequent survival, however, was higher for leafhoppers that had ingested antibiotic than for control insects (Fig. 6). For example, the 50% survival time for antibiotic-treated and control leafhoppers was 53 and 34 days, respectively.

Effect of antibiotic on mycoplasma in infected plants and leafhopper tissues.—Aster plants showing well-developed symptoms of the disease were root-treated with terramycin for 7 days. Similar plants left in the phosphate buffer alone for the same period served as controls. Ultrathin sections of roots and leaves of both antibiotic-treated and control plants were then examined in the electron microscope. Although numerous mycoplasma bodies were found in the control plants, they occurred very infrequently in the antibiotic-treated plants, and of those present, many were incomplete or broken (Fig. 7). Such disrupted bodies were not observed in the controls (Fig. 8).

Exposed leafhoppers were caged for 7 days on two sets of healthy aster plants, one set maintained in the antibiotic solution, the other, in phosphate buffer (control). The alimentary canals and salivary glands of 10 leafhoppers from each set were removed and sectioned. At least 50 sections from each sample were examined. Mycoplasma bodies were not found in the alimentary canal of the antibiotic-treated leafhoppers, but were frequently observed in the salivary glands, and many of these bodies appeared to be devoid of ribosomelike structures and DNA-like strands (Fig. 9). By contrast, typical mycoplasma bodies were

commonly observed in both the alimentary canal and salivary glands (Fig. 10) of control insects.

DISCUSSION.—The association of mycoplasma with clover phyllody-infected plants and their presence in vector tissues was demonstrated earlier (21, 22). The present study shows that the causal agent of clover phyllody is sensitive to terramycin, thus providing further evidence that this disease is caused by a *Mycoplasma* species, rather than a virus. The proof of mycoplasma etiology, however, awaits the isolation and culturing of the organism in order to satisfy Koch's postulates, and has been done for corn stunt and white leaf disease of sugarcane (1, 12).

The causal agents of several other plant diseases, suspected to be mycoplasmas, have been shown to be suppressed, both in plants and in vector insects by tetracyclines (9, 11, 27). Our results clearly show that terramycin can be absorbed from solutions by aster roots and translocated to stems, petioles, and leaves; thus, they confirm the findings of Cousin & Staron (4). Also, the antibiotic persists in the plants for more than 2 weeks, although its concentration gradually declines. The susceptibility of healthy plants to CPA-infection, either before or after root treatment with the antibiotic, was dependent upon the concentration of the antibiotic in the plants at the time of inoculation by the leafhoppers. As the concentration of the antibiotic in plants decreased, their susceptibility to infection increased (Fig. 3, Table 1).

TABLE 1. Effect of terramycin on susceptibility of aster plants to clover phyllody infection

Interval between antibiotic treatment and inoculation ^a	Plants infected ^b
Days	%
1	0
3	15
5	20
7	50
12	80
16	90
Untreated plants	90

^a Plants were treated by root immersion in the antibiotic solution (100 ppm) for 1 day. At intervals following treatment, plants were inoculated by caging five exposed leafhoppers/plant for 2 days.

^b Percentage is based on 20 plants for each figure. Combined results of two experiments.

The number of leafhoppers that could acquire CPA from antibiotic-treated (root treatment 4 weeks earlier) and untreated infected plants was determined. Groups of noninfective leafhoppers were caged on such plants for 1 week, then maintained on healthy aster plants for 5 weeks, and finally tested singly for their transmissibility by maintaining them for 2 weeks on aster seedlings. The combined results of two such experiments showed that 5 of 27 (16%) and 10 of 20 (50%) leafhoppers that had fed on treated and untreated infected plants, respectively, transmitted

CPA to aster seedlings. These results, based only on those leafhoppers that had survived the entire test period, indicate a reduction of CPA titer in the treated plants over that in the untreated infected plants.

When infected plants were subjected twice weekly for 4 weeks to the soil treatment with the antibiotic, remission of symptoms occurred in four of five treated plants.

Susceptibility of plants to infection when inoculated either before or after antibiotic treatment.—To study the susceptibility of plants to CPA infection after the antibiotic treatment, healthy aster seedlings were subjected for 24 hr to root treatment with terramycin. At various intervals, the treated plants were inoculated with CPA by caging five exposed leafhoppers/plant for 2 days. The combined results of two such experiments (Table 1) showed that none of the plants inoculated 1 day after the treatment developed symptoms of the disease. As the interval between treatment and inoculation of plants increased, the incidence of infection also increased, and by the 16th day, 90% of both treated and untreated plants became infected. However, plants which had been inoculated up to 7 days after the treatment and subsequently became infected showed milder symptoms than did the untreated plants. Also, the incubation period of CPA in these infected plants was about 1 week longer than in untreated infected plants. Similar results were obtained when plants were inoculated first with CPA by means of leafhoppers and then root-treated with the antibiotic at various times after inoculation.

In two experiments, healthy aster plants were sprayed with the antibiotic solution (1,000 ppm) either immediately before or after inoculation with CPA by means of leafhoppers as described above. The results showed that all 60 plants, irrespective of the time when they were sprayed, eventually became infected. However, the development of symptoms in sprayed plants was delayed about 1 week longer than in unsprayed plants.

Effect of the antibiotic on transmission of clover phyllody agent by leafhoppers.—The effect of terramycin on CPA transmission by leafhoppers was studied in two different ways: (i) Leafhoppers were allowed to acquire CPA and the antibiotic simultaneously by feeding for 7 days on two infected plants, one of which was kept with its roots immersed in the antibiotic solution, the other with its roots in the phosphate buffer to serve as control. Leafhoppers thus exposed to CPA were then maintained on healthy plants for 5 weeks before being tested singly for their transmissibility by maintaining them for 4 weeks (2 weeks each on two successive plants) on aster seedlings. The extra 2 weeks were allowed in case the incubation period of CPA was prolonged in insects that had ingested the antibiotic. The combined results of two experiments showed that the number of leafhoppers that transmitted CPA after feeding on antibiotic-treated and untreated infected plants was 0/57 (0%) and 22/44 (50%), respectively. (ii) Leafhoppers were given an acquisition access feed

of 7 days, then caged for various periods on healthy plants maintained in 100 ppm terramycin solution. Leafhoppers thus exposed to the antibiotic were then maintained on untreated healthy plants for an additional 4 weeks before being tested singly on aster seedlings (for 4 weeks, 2 weeks each on two successive plants) for their ability to transmit CPA. The combined results of two such experiments (Table 2) showed that most of the leafhoppers which had fed for 1 or 2 weeks on plants maintained in the antibiotic solution failed to transmit CPA, and none transmitted after feeding for 3 weeks on the antibiotic-treated plants.

TABLE 2. Effect of terramycin on transmission of clover phyllody by leafhoppers^a

No. days leafhoppers were allowed to ingest the antibiotic through plants ^b	Leafhopper transmission ^c
0	25/50 (50)
7	2/49 (4)
14	1/49 (2)
21	0/48 (0)

^a All leafhoppers were given an acquisition access feed of 7 days.

^b Leafhoppers after acquisition access feed were caged on healthy aster plants maintained in the solution containing 100 ppm terramycin.

^c Five weeks after the leafhoppers were removed from plants in the antibiotic solution, the insects were tested singly for their ability to transmit by maintaining them for 4 weeks on aster seedlings. Numerator is the number of infective leafhoppers, denominator, the number tested. The percentage is given in parentheses.

The leafhoppers also were able to acquire detectable amounts of the antibiotic when they were allowed to feed on plants maintained in the antibiotic solution. When exposed leafhoppers thusly acquired the antibiotic, their ability to transmit CPA was either decreased or completely lost, depending upon the amount of antibiotic ingested (Fig. 4, Table 2), which suggests that terramycin inhibits the multiplication of CPA in leafhoppers. Our knowledge of the transmission of agents causing "yellows" type of diseases, including that of clover phyllody, suggests that after these agents are sucked from the infected plants into the vector's alimentary canal, they are released into the hemolymph, carried to the salivary glands, and then are injected into the healthy plants (17, 18). Our results on the inactivation of CPA in leafhoppers indicate that the ingested antibiotic also goes into the alimentary canal, is able to penetrate the gut wall, and presumably reaches the salivary glands through the hemolymph. This indication is supported by the occurrence of disintegrated and disrupted mycoplasma cells brought about, we believe, by the action of terramycin.

Freitag & Smith (8) reported a high mortality of leafhoppers, *M. fascifrons*, after feeding on achromycin solution through a membrane. In our experiments, when healthy leafhoppers were allowed to feed on terramycin-treated plants, their survival

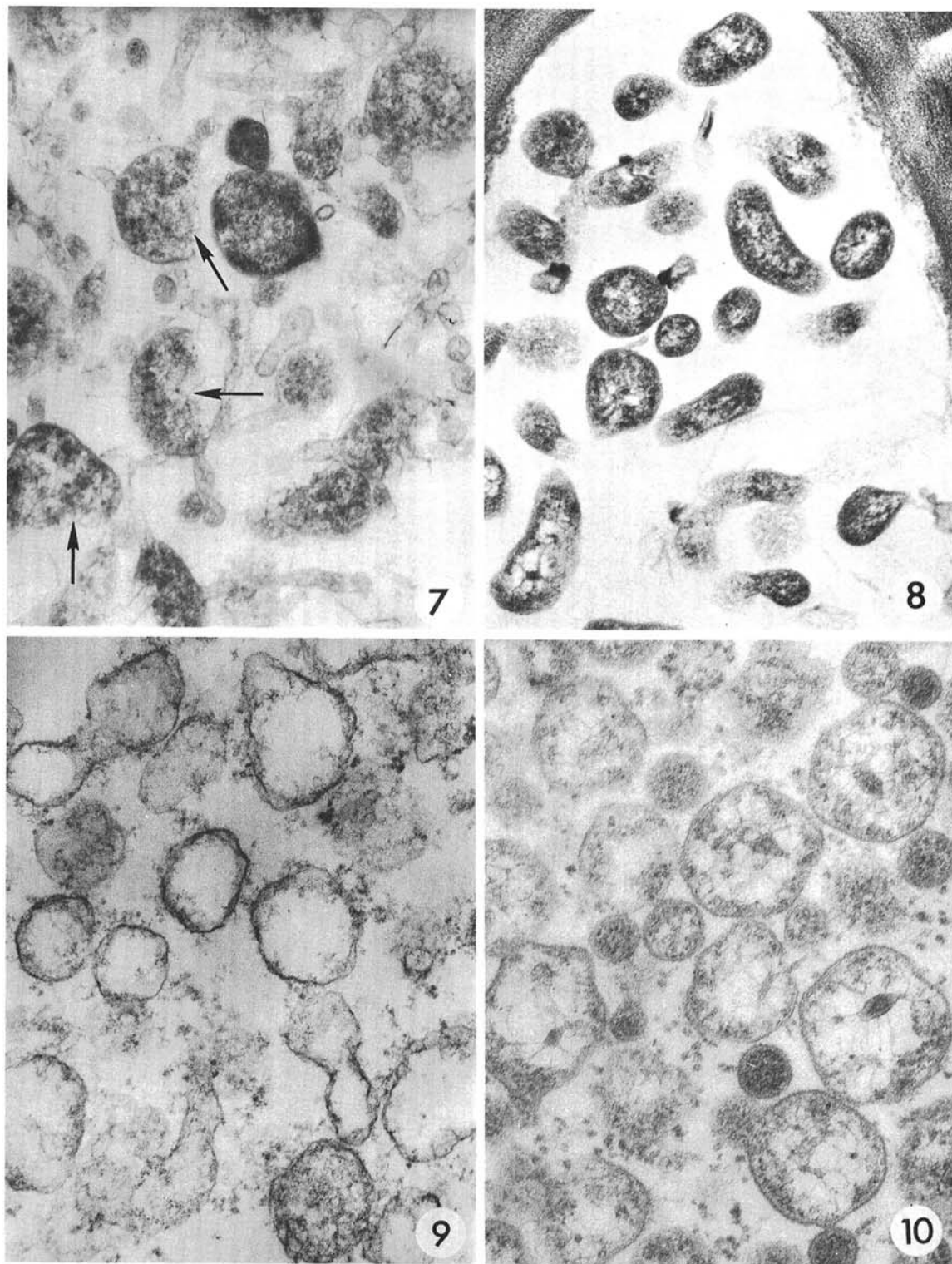


Fig. 7-10. 7) Part of a root sieve cell of a clover phyllody-affected aster plant after treatment with oxytetracycline (100 ppm) for 7 days showing (arrows) several broken mycoplasma. 8) Root section (sieve cell) of an untreated clover phyllody-affected aster plant showing mycoplasma. 9) Mycoplasma in an acinus of salivary glands of a leafhopper carrying clover phyllody agent. The infective leafhoppers were caged for 7 days on aster plants maintained in the antibiotic solution (100 ppm). Note lack of internal structures in the organisms. 10) Mycoplasma in the acini of salivary glands of an untreated infective leafhopper showing internal structures (X 51,000).

was increased significantly over those fed on untreated plants (Fig. 6). This increased survival could be due to inactivation of microorganisms which may be associated with leafhoppers in nature (29).

Terramycin could not be detected in plants after a single application of the antibiotic to leaves or to the soil. It has been demonstrated earlier that tetracyclines are adsorbed and often tenaciously held by clay complexes of the soil (16, 23), but may be released by treatment with phosphate or citrate buffers (24). Work along these lines is currently in progress to determine a more practical method for controlling the clover phyllody disease.

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