Chemotherapy of Aster Yellows: Tetracycline-Hydrochloride Uptake by Healthy and Diseased Plants

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ABSTRACT

Measurable amounts of tetracycline-hydrochloride were translocated to leaves of aster yellows-affected aster plants when roots were immersed in a solution of the antibiotic. Pronounced remission of symptoms occurred, but phytotoxicity, especially in healthy controls, often resulted in death. Application by soil drenching was ineffective. Foliage spraying did not induce recovery of diseased asters, and caused chlorosis of young leaves on healthy plants. Tetracycline-hydrochloride could not be detected in plants after undamaged leaves were immersed in high concentrations of the antibiotic. A considerable amount of antibiotic was acquired through cut leaves subsequently immersed in the test solutions. Healthy plants treated in this fashion developed severe chlorosis. Glycerol and Tween 20 (polyoxyethylene sorbitan monolaurate), used as adjuvants, had no significant effect on antibiotic uptake through roots or through wounded (cut) leaves.

Additional key word: mycoplasma.

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RESUMEN

Después de sumergir en solución de hidrocloruro de tetraciclina las raíces de plantas de áster atacadas por la amarillez se descubrió la presencia del antibiótico en cantidades apreciables en las hojas. Aún cuando los síntomas desaparecieron casi por completo, la acción fitotóxica del antibiótico fue tan intensa que muchas plantas sucumbieron, especialmente las plantas sanas que servían de control. Fue muy leve el efecto de la tetraciclina aplicada directamente al suelo en solución acuosa. En aspersiones al follaje, el antibiótico no sólo tuvo resultados muy poco satisfactorios sino que, además, provocó la manifestación de síntomas de clorosis en las hojas tiernas de plantas sanas. En plantas cuyas hojas no habían sido lesionadas no fue posible discernir la presencia de la tetraciclina después de sumergir aquellas en soluciones muy concentradas del antibiótico. En cambio, después de lesionar las hojas y sumergir las plantas en la solución, se descubrió el antibiótico en cantidades apreciables. En las plantas sanas tratadas de esta manera se observaron pronunciados síntomas de clorosis. El uso de glicerol y Tween 20 no facilitó de manera significativa la absorción de la tetraciclina a través de las raíces o de hojas lesionadas.

Tetracycline antibiotics received increased attention from plant pathologists after the discovery by Doi et al. (4) in Japan that some yellows-diseased plants contain bodies assumed to be mycoplasma. Application of these antibiotics to plants caused temporary suppression of disease, and the results supported the assumption of a mycoplasma etiology (8). In further studies during the past 3 years, tetracyclines were used in treatments of several yellows diseases of plants and on some of the respective insect vectors in attempts to verify the assumed mycoplasma etiology (3, 14, 19). Tetracycline chemotherapy of yellows diseases was recently reviewed by Maramorosch et al. (11) and by Whitcomb & Davis (19).

Differences in uptake and translocation of tetracycline-hydrochloride by healthy and aster yellows-affected plants as influenced by several methods of application were determined by bioassay. The main points studied were uptake and distribution of the antibiotic in the treated plant.

MATERIALS AND METHODS.—Young seedlings of China aster (*Callistephus chinensis* Nees) plants were transplanted to nonsterilized garden soil in 6.5-cm disposable peat pots and held in a greenhouse for 45 days before transfer to a chamber with artificial illumination. Individual plants were covered with plastic cages, so as to confine individual leafhoppers (*Macrosteles fascifrons* Stål.) that had previously fed on healthy or yellows-infected asters. Cages were removed from the plants, and the plants were returned to the greenhouse after 7 days. Symptoms developed fully in two or more young leaves of the inoculated plants 3 to 4 weeks later.

Bioassay.—The bioassay method for the antibiotic has been described in detail (6). Plants were incubated at 25 C for 3 days after treatment and then used for the bioassay. Blank paper discs (BBL, Division of BioQuest, Cockeysville, Md.) were immersed into the supernatant fluid extracted from the leaves and allowed to partially dry at room temperature before assaying. These were placed on an

agar plate seeded with *Bacillus cereus* var. *mycoides* (Flugge) Smith et al. (ATCC-11778). The zones of inhibition of the extracted material were compared with those produced by known amounts of tetracycline-hydrochloride. This served as the basis for determining the relative amounts of antibiotic associated with the leaves either by absorption or translocation.

In most cases, healthy and infected plants chosen for the antibiotic treatment were 75 days old. Healthy ones had at least four fully developed leaves, whereas infected ones had only three fully expanded and one partially expanded leaf. In the spraying experiments and in the immersion experiments, where indicated, 105-day-old healthy plants were used. These plants had 10 to 16 leaves, providing a large area of exposure to the antibiotic. Aster yellows-affected plants of the same age had stopped growing and were not suitable for bioassay. At least three plants were included in each treatment.

Tetracycline-hydrochloride (Achromycin; Lederle Inc., Pearl River, N.Y.) was used in all experiments. Solutions were prepared in 0.05 M citrate buffer at pH 5.2-5.3. A concentration of 100 μg/ml was used in root-immersion experiments, and 1,000 μg/ml in the other tests. The volumes used for each treatment were as follows: root and cut leaf immersion, 35 ml; full leaf immersion, 100 ml; and foliage spray, 125 ml. Treated tissues were usually in contact with the antibiotic solution for 3 hr at 25 C. The antibiotic application was usually terminated by removing the treated part from the solution and by carefully washing with tap water to prevent contamination of the untreated leaves with the antibiotic.

Samples were usually taken from leaves or parts of the leaves that had had no direct contact with the antibiotic. In two of the leaf-immersion experiments, treated leaves were also sampled. Two of the largest leaves from the lower portion of the stem were chosen. In the leaf-immersion experiment (105-day-old plants) one of the leaves was from between, and one from above, the treated leaves.

RESULTS.—*Root immersion.*—After removal of the plants from the soil, the roots were carefully rinsed so as to minimize damage. They were then dried with blotting paper, placed in the tetracycline-hydrochloride solution, and repotted in fresh garden soil. Healthy plants contained significantly ($P > .99$) higher amounts of tetracycline-hydrochloride in the samples checked than did the infected plants (mean healthy, 1.61 μg/ml; mean infected, 0.48 μg/ml). In two infected plants, the antibiotic was not detectable.

Soil drenching.—The soil in each of the small pots was drenched slowly with the tetracycline-hydrochloride solution. The pots were kept in a tray and watered only from below, 24 hr after treatment. No activity was detected in leaf samples taken from healthy and aster yellows-infected plants. In additional tests in which the drenching was repeated several times, no suppression of the yellows symptoms was achieved.

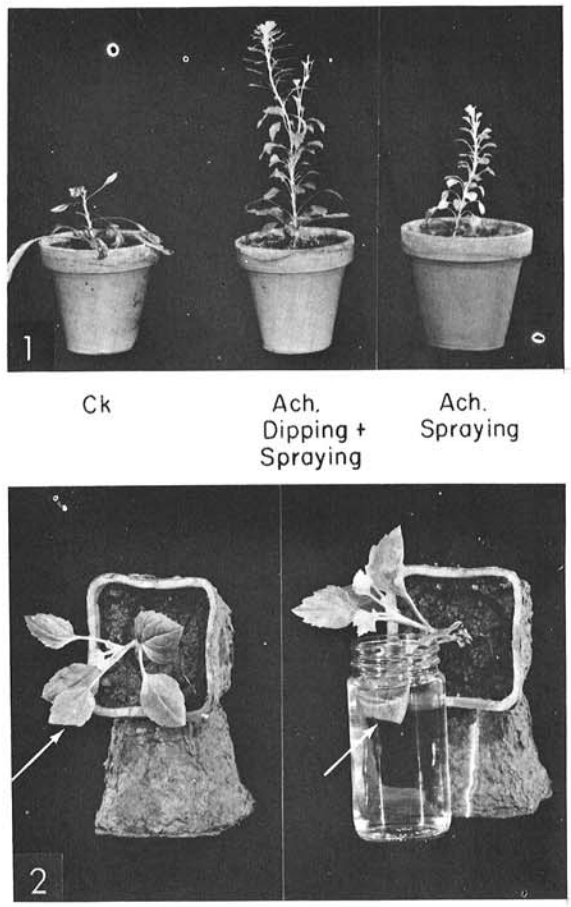

Fig. 1-2. 1) Comparison between the effect of buffer (Ck), of tetracycline-hydrochloride sprayed alone (Ach. spraying), and tetracycline-hydrochloride sprayed as well as applied by root immersion on aster yellows-diseased plants. **2)** Tetracycline-hydrochloride application (left) through the cut-leaf method, and (right) through immersion. Note cut leaf in plant on left. Plant on right is treated by immersion.

Spraying.—Plants were sprayed to runoff with a Model 2Z 365 spray gun (Speedaire, Dayton Electric Mfg. Co., Chicago, Ill.) according to the method described by McCallan & Wellman (13) and McCallan (12). Two leaves of each of three plants were covered by enclosing them in polyethylene bags so that no residue would drip on them. One leaf was chosen from the lower part of the plant and one from the upper part. The bags were removed 3 hr after spraying, when the antibiotic solution had dried completely. Tetracycline-hydrochloride could not be detected in either healthy or infected plants. In six tests, more than 50% of the infected plants partially recovered within 70 days, during which they were sprayed 20 times. The effects of spraying on growth after 72 days are shown in Fig. 1. Recovery was more pronounced if the first treatment was by root immersion, as seen in the same figure. The decrease with time of relative antibiotic levels in the leaf disc preparations that followed root immersion could not be prevented by frequent leaf spraying or soil

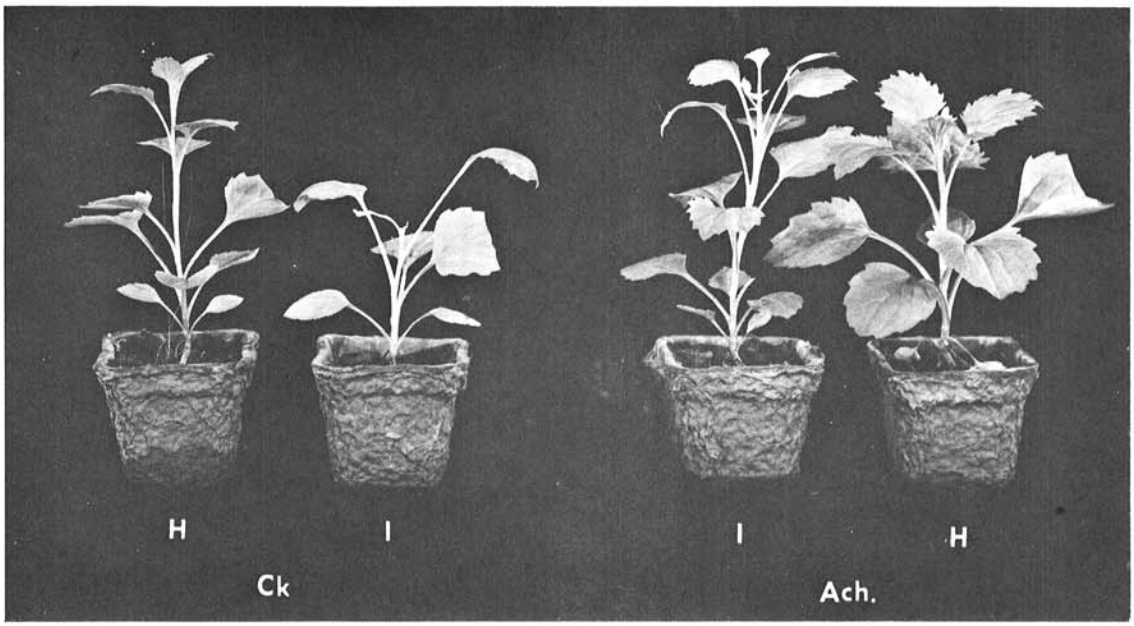

Fig. 3. Reaction of plants to tetracycline-hydrochloride treatment by the cut-leaf method. H = healthy plant; I = infected plant; Ck = control plant dipped in citrate buffer 0.005 M, at pH 5.3; Ach. = plant treated with 1,000 ppm of tetracycline-hydrochloride in the same buffer as control Ck.

drenching with 1,000 ppm concentrations of tetracycline-hydrochloride. Healthy plants suffered a slight phytotoxic effect after repeated sprayings.

Leaf immersion.—Tetracycline-hydrochloride absorption, systemic translocation, and local adsorption were also checked by means of leaf immersion in six groups of plants. When only half the leaf (the tip portion) was immersed, then carefully washed with distilled water in order to prevent any contact of the untreated halves with the antibiotic solution, no activity could be detected in the untreated portion. Similarly, when a full leaf was completely immersed, no tetracycline-hydrochloride was found in untreated leaves, and phytotoxic effects were not apparent in healthy plants. The results were also negative when two large leaves from each of 105-day-old plants were immersed in the same solution and when the immersion time was extended to 3 days, changing the antibiotic solution daily.

Samples were taken from the treated leaves 3 days after immersion with and without washing the leaves with distilled water prior to sampling. Treated unwashed leaves possessed large amounts (more than 10 ppm) of tetracycline-hydrochloride in both healthy and diseased plants. The results from experiments with immersed, washed leaves indicated that the antibiotic could be washed off treated leaves of diseased plants with distilled water. Approximately 10% of the amount adsorbed by treated leaves of healthy plants was retained when leaves were washed in the same manner. Each of the six samples which were examined from washed leaves still had considerable amounts of tetracycline-hydrochloride [mean of 1.27 ppm $\pm$ 0.47 (SD)]. Treated leaves from the healthy and infected plants were not damaged by the large quantity of tetracycline-hydrochloride and no discoloration was observed, whereas death of the whole plant sometimes resulted when smaller amounts were absorbed through the roots.

Absorption of tetracycline-hydrochloride through wounds made in leaves.—A straight cut was made across the leaf in the upper area just above the third marginal tooth (Fig. 2) of one leaf of each plant. The cut leaf was then immersed in the tetracycline-hydrochloride solution. The antibiotic was taken up in measurable quantities by leaves from both healthy [mean 1.08 μg/ml $\pm$ 0.96 (SD)] and infected [mean 1.56 $\pm$ 0.86 (SD)] plants. The difference was not significant ($P > .95$). The lower leaves contained, in general, more tetracycline-hydrochloride activity than the upper ones. The infected plants began to show signs of recovery (Fig. 3) at about the same time and in the same percentage of treated plants as did those that had been treated by root immersion. Healthy plants were not killed, but the color of the upper leaves changed from dark green to yellow green.

Effect of Tween 20 and glycerol on the uptake of tetracycline-hydrochloride by plants.—The effects of Tween 20 (polyoxyethylene sorbitan monolaurate) (Nutritional Biochemical Corp., Cleveland, Ohio) and glycerol (Mallinckrodt, St. Louis, Mo.) on tetracycline-hydrochloride penetration into aster plants were studied, using three different modes of application: the root immersion; the leaf immersion; and the cut-leaf method. A concentration of 0.5% of each of the two compounds was used throughout all tests. Statistical analysis of the data indicated that no significant difference occurred between

tetracycline-hydrochloride treatment alone and the Tween 20 or glycerol addenda in healthy and diseased plants. The cut-leaf method resulted in significantly higher uptake ($P > 0.99$), and a significantly greater uptake has been found in the healthy samples ($P > 0.99$).

DISCUSSION.—Spraying and leaf-immersion treatments commonly and successfully used in the application of various chemicals to plants (7) were inefficient with tetracycline-hydrochloride even though adjuvants and high concentrations of the antibiotic were used. It is not yet known how the antibiotic succeeded in penetrating the plants. Penetration may have occurred in the small young leaves of healthy plants close to the main bud, causing chlorosis in these leaves. In infected plants in which no such new leaves developed, undetectable amounts of tetracycline-hydrochloride might yet be acquired by the latest developed, and partially brown, leaves. Such undetectable amounts might be effective in very small areas, thereby requiring frequent leaf sprayings to achieve partial recovery. Tetracycline-hydrochloride might also be acquired through wounds caused by moving the plants during repeated treatments. Very delicate wounds may also be caused by the striking of the fine drops on the leaf surface during spraying. This is reminiscent of the effectiveness of spraying used in mechanical transmission of plant viruses (21). Dripping of the excess solution into the soil of the pots following the intensive spraying would not explain plant recovery, as soil drenching by itself was inefficient. The fact that soil drenching with tetracyclines has no curative effect has already been reported for several other yellows diseases (5, 8, 17, 18).

Root immersion in tetracycline-hydrochloride solution, first used in 1967 (8), was superior to other treatments of yellows diseases. Many plants recovered after such treatment, new growth started soon, recurring disease symptoms were mild, and the recovery lasted for ca. 3 weeks. Measurable amounts of tetracycline-hydrochloride could be detected for at least 10 days following treatment (6). Healthy aster plants suffered severe damage from this treatment, and many plants died.

Results of preliminary tests with the cut-leaf method indicated that it may be a more promising mode of application of the antibiotic than root immersion, especially since it prevented damage to the roots. The cut-leaf method avoids removal of plants from the soil and permits frequent repetition of treatments. Plants acquired high amounts of tetracycline-hydrochloride, and recovery started as fast as with root immersion. In leaves of healthy plants which had been previously immersed in the tetracycline-hydrochloride solution, the antibiotic seemed to be absorbed only on the external area of the leaves. It could not be washed off completely as from infected leaves. This could be due to changes in the constituent of the waxy surface in healthy and infected leaves. The great variability in bioassay, between leaves of the same plant or among different plants, prevented more definitive conclusions than those stated in the results.

Investigations on the systemic action, phytotoxicity, and lasting effect of some antibiotics, including tetracycline, have been carried out in France (1, 15) on five different plant varieties of three plant families. The effect of these antibiotics was also studied on aster yellows and Stolbur-infected plants of the same varieties. These studies seem preliminary and lacking in information from controls. It is essential in such bioassay studies to check the response of the plant extract by itself, but such data were not reported. The accuracy of the system was not ascertained by the authors. For aster plants, such data are now available (6). It is not advisable to use pieces of stems as a routine method in antibiotic studies, because this excludes the reaction of roots or leaves. There is no lasting effect of tetracycline antibiotics on the stems, compared to the continuous effect of the tetracycline-hydrochloride on whole aster plants (6). Large amounts of intact antibiotics may be lost during dipping of stems in distilled water following the treatment. Finally, the evaluation of the results may be misleading if infected, grafted plants are used shortly after grafting; how the yellows agent passes from the scion to the stem has not been ascertained as yet. In our own experience in infecting *Vinca* plants with aster yellows by grafting, a variety of responses was obtained. This lack of uniformity becomes even more complicated when an additional factor, such as an antibiotic, is introduced.

The absorption of antibiotics in general, and of tetracyclines in particular, depends upon many factors most of which are undetermined. The exact mode of the in vitro action of tetracyclines on microorganisms is still unknown (10). Therefore, it seems unwarranted to interpret results of yellows disease chemotherapy as indicating a specific antibiotic effect on, or spectrum of, plant mycoplasma, as has been suggested by Davis & Whitcomb (2, 3), Whitcomb & Davis (20), and Staron et al. (16). Results of in vivo experiments with antibiotics are not necessarily comparable to in vitro tests, as has already been pointed out in connection with tetracycline-hydrochloride and sulfadiazine treatments (9). Tetracycline treatments can still be used, however, to support evidence obtained from electron microscopic studies of mycoplasmalike bodies, presumed to be agents of the yellows diseases.

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