Effect of Tetracycline Antibiotics on Symptom Development of Stubborn Disease and Infectious Variegation of Citrus Seedlings

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Accepted for publication 10 February 1973.

ABSTRACT

Uptake, translocation, and effect of tetracycline compounds on development of symptoms of stubborn and on those of citrus infectious variegation were investigated. We demonstrated, using *Bacillus cereus* as a test organism, that shoot extract of healthy sweet orange seedlings grown in tetracycline-HCl (achromycin) solution contained higher antibacterial activity than did shoot extract of similar seedlings grown in chlortetracycline-HCl (aureomycin) solution. Thin-layer chromatography of shoot extract of treated plants revealed achromycin but not aureomycin, suggesting that the latter is not readily translocated upwards or is rapidly inactivated in plant shoots or their extracts. Tetracycline compounds applied to roots of citrus seedlings inoculated with citrus infectious variegation virus were ineffective in suppressing disease symptoms. Stubborn symptom development in infected seedlings was completely suppressed by tetracycline compounds applied to the roots as a dip or in hydroponic culture. Tetracycline compounds as quartz sand drenches were ineffective in suppressing stubborn symptom development. Achromycin, which appeared more stable than aureomycin, was more efficacious in suppressing stubborn symptom development. These results and the finding of mycoplasmalike bodies in the phloem of stubborn plants suggest that the stubborn pathogen is a mycoplasmalike organism and not a virus.

Phytopathology 63:1044-1048.

Additional key words: chromatography, citrus infectious variegation virus, mycoplasmalike organism, hydroponic culture, uptake, translocation.

Stubborn, a stunt and yellows disease of citrus, was believed for many years to be caused by a virus. After repeated failures, between October 1967 and December 1968, to transmit the stubborn pathogen by sap inoculations or to photograph it under the electron microscope (7) we investigated the possibilities that stubborn, like mulberry dwarf, might be associated with a mycoplasmalike organism and be sensitive to tetracycline antibiotics (5, 9). Subsequent studies (8, 11) revealed mycoplasmalike

bodies in sieve tubes of stubborn-infected citrus seedlings. This paper reports the results of studies on (i) the effect of certain antibiotics on the development of symptoms of stubborn and on those caused by citrus infectious variegation virus (CIVV), which has been purified and characterized (4), and (ii) the uptake and translocation of two tetracycline antibiotics by citrus seedlings.

MATERIALS AND METHODS.—*Plants, inoculations, and indexing.*—One- to 2-year-old 'Madame Vinous' sweet orange [*Citrus sinensis* (L.) Osb.] seedlings grown in pasteurized soil mix were cut back, leaving four to five leaves per plant, to force production of vigorous succulent shoots.

Inoculations were made, at the time of cutback, by two leaf-patch grafts (7) per plant from seedlings with severe stubborn (C-189) or from two buds of seedlings infected with CIVV. Healthy controls received leaf-patch grafts from healthy plants. New shoots, which emerged a week or more after cutback, were removed except for a single shoot directly above the upper graft on each plant. To avoid delayed infection of seedlings after antibiotic treatments ceased, stubborn-infected grafts were carefully removed 20-25 days after inoculation.

Roots were carefully washed free of soil immediately before antibiotic treatment, which began 4-5 days after cutback of plants inoculated with CIVV, and at least 7 days after cutback of stubborn-inoculated plants.

Plants grown in hydroponic solutions to determine antibiotic effects on symptom development of stubborn were indexed by the leaf-patch technique (7) at the end of treatment and plants in hydroponic experiment 1 were reindexed 35 days later.

Antibiotic treatments.—Chlorotetracycline-HCl (aureomycin) and tetracycline-HCl (achromycin), from American Cyanamid, New York, were used singly at several concentrations. Penicillin, 50 ppm, was used for comparison in some experiments but caused no visible effect on plant growth or disease symptoms. Plants were treated with antibiotics by: (i) growing them for 30 days in a hydroponic solution similar to that of Wallihan et al. (16) except that antibiotic was added and the entire solution was replaced and adjusted to pH 4.0 to 4.5 every 7-9 days; (ii) drenching the root zone with antibiotic at 100 ppm in nutrient solution which was applied slowly at 3- to 4-day intervals for 2 months to quartz sand in siphon-equipped crocks; (iii) immersing the roots for 14 hr in water containing 1,000 ppm antibiotic, 19 days after cutback, followed by rinsing them with tap water and planting them in pasteurized soil mix.

Detection of antibiotics in extracts.—Bioassays and paper- and thin-layer chromatography (TLC) were used to obtain data on the relative amounts of tetracycline antibiotic present in roots and shoots of experimental plants. Healthy seedlings were transferred 20-25 days after cutback from soil mix to hydroponic solutions that contained 50 ppm antibiotic. Two plants, harvested from each treatment after 2, 4, 6, and 9 days, were processed separately, assayed for antibacterial activity by the paper-disk plate method (13), and the results averaged. The solutions were also assayed on each of these days.

Roots were separated from the shoot of each plant, washed in running tap water and three changes of distilled water, and blotted dry with paper towels. Then the roots and shoot of each plant were wrapped separately in plastic bags, frozen overnight at −20 C, and thawed. The juice was pressed out in a hand garlic press, a method chosen after we found no antibacterial activity in extracts obtained by trituration with buffer in a mortar. Aliquots of 0.15 ml of juice, or hydroponic solution, were taken up with sterile paper disks (12.7-mm diam) and immediately placed on nutrient agar freshly seeded with *Bacillus cereus*. After 8-10 hr of incubation at 30 C, zones of inhibition were measured. The apparent concentration (μg/ml) of active antibiotic in each sample was determined from standard curves obtained by plotting the logs of known concentration of each antibiotic, in water, against the diameters of the inhibition zones.

Chromatographic tests were made, to investigate chemical alteration or adsorption of achromycin and aureomycin in plants, after we found that relatively high antibacterial activity was present in root extracts but not in shoot extracts of plants treated with aureomycin for several days. For these tests, healthy plants were grown for 16 hr in nutrient solution with or without 100 ppm achromycin or aureomycin.

Shoot extracts from the hand garlic press were then spotted on silica-H TLC plates 250-μ thick and on Whatman No. 1 chromatographic paper. Papers were impregnated with 0.1 M disodium ethylenediaminetetraacetic acid solution and air-dried before use by the descending method. The solvent was the upper phase of the *n*-butanol-acetic acid-water (4:1:5) system or the *n*-butanol-ammonium hydroxide-water (4:1:5) system (10). Spots on all chromatograms were exposed to ammonia vapor and viewed under ultraviolet light.

RESULTS.—*Effect of tetracyclines on symptom development of stubborn.—Hydroponic experiment 1.*—Plants were grown 30 days in nutrient solutions containing 0, 10, 20, or 50 ppm antibiotic, then were transferred for 35 days to antibiotic-free nutrient solution. All inoculated plants growing in antibiotic-free solutions, or in 10 ppm aureomycin or 50 ppm penicillin, and some of those in 20 ppm aureomycin or in 10 ppm achromycin, developed stubborn symptoms 20-30 days after inoculation. Inoculated plants treated in 50 ppm aureomycin, or in 20 or 50 ppm achromycin, remained symptomless (Table 1). At 50 ppm, achromycin, but not aureomycin, was slightly phytotoxic to plants grown in it 30 days. These plants resumed normal growth when antibiotic treatment ceased.

All symptomless inoculated plants that were grown in 20 or 50 ppm aureomycin, or in 10 ppm achromycin, developed stubborn symptoms in 2-5 weeks when they were transferred to antibiotic-free

TABLE 1. Effect of antibiotics in hydroponic solution on stubborn-infected sweet orange seedlings

Treatment & concn	Grown 30 days in solution		Treatment at left + 35 days in antibiotic-free solution[a]	
	Symptoms present[b]	Positive index[c]	Symptoms present[b]	Positive index[c]
Aureomycin				
10 ppm	25/25	25/25	25/25	25/25
20 ppm	6/25	25/25	25/25	25/25
50 ppm	0/25	0/25	20/25	25/25
Achromycin				
10 ppm	10/20	20/20	20/20	20/20
20 ppm	0/32	0/32	22/32	24/32
50 ppm	0/32	0/32	0/32	0/32
Penicillin				
50 ppm	25/25	25/25	25/25	25/25
Control	25/25	25/25	25/25	25/25

[a] Seedlings were grown in antibiotic or antibiotic-free solution for 30 days, then in antibiotic-free solution for 35 days.

[b] Numerator is number of seedlings showing stubborn symptoms; denominator is number of inoculated seedlings.

[c] Numerator is number of seedlings that indexed positive for stubborn; denominator is number of seedlings indexed for stubborn.

Fig. 1. Stubborn-infected sweet orange seedlings repotted after 2 weeks' growth in hydroponic solution, beginning 1 week after cutback. (Left) seedling grown in antibiotic-free solution and showing typical stubborn symptoms, small leaves and stunting. (Right) recovered seedling grown in 20 ppm achromycin.

solution. Some inoculated plants formerly grown in 20 ppm achromycin, and all of those grown in 50 ppm achromycin, remained symptomless for 35 days in antibiotic-free solutions; these were transplanted into soil mix. Eight months later, 28 of 32 inoculated plants previously grown in 50 ppm achromycin remained symptomless. The portions of this experiment involving treatment with 0, 20, or 50 ppm achromycin were repeated twice, on 10 plants per treatment, with similar results.

Reindexing results (Table 1) indicated the presence of the stubborn pathogen in all symptomless plants treated with aureomycin but failed to detect the pathogen in any plants treated with 50 ppm achromycin.

Achromycin, at 50 ppm, may have eliminated the stubborn pathogen from some plants and was more effective than aureomycin in suppressing stubborn symptoms.

Hydroponic experiment 2.—Twenty plants that 2 months after inoculation were in an advanced stage of stubborn and 20 noninoculated controls were cut back and divided into two groups of 20 plants each (10 infected and 10 control). One week later, plants in groups 1 and 2 were transferred to antibiotic-free nutrient solution and to 20 ppm achromycin, respectively.

Three weeks after transfer, the infected plants in 20 ppm achromycin were vigorous and indexed negative for stubborn but infected plants in antibiotic-free solution were very stunted (Fig. 1), had typical stubborn symptoms, and indexed positive for stubborn. The experiment was repeated with identical results.

We conclude that 20 ppm achromycin in nutrient solution may suppress stubborn symptoms in new growth of diseased plants but, in view of the results of hydroponic experiment 1, it usually does not eliminate the pathogen.

Drench treatments of roots in quartz sand.—Achromycin, aureomycin, and penicillin, applied at 100 ppm in nutrient solution as drenches at 3- to 4-day intervals had no apparent effect on the development of stubborn symptoms. Symptoms developed on all 12 inoculated plants within 1 month after treatment; then plants were cut back and symptoms appeared in the new growth. Noninoculated plants and those treated with antibiotic-free solution remained symptomless. No phytotoxicity was detected but a brownish deposit appeared on sand grains in crocks receiving the tetracycline antibiotics. This experiment was repeated with identical results. We conclude that periodic drenching is a very inefficient method of applying tetracycline antibiotics.

Root immersion treatment.—Achromycin and aureomycin applied as root dips at 1,000 ppm for 14 hr on the 19th day after cutback completely suppressed development of stubborn symptoms in new growth of infected plants for 6 months in two tests, one using plants inoculated when cut back, and the other using previously infected plants that had developed strong symptoms before cutback. Plants

treated with 1,000 ppm penicillin or water, developed stubborn symptoms 20-30 days after cutback and retained them until the experiment ended 6 months later. Achromycin was slightly phytotoxic in both tests as indicated by minor scorching of the edges of a few leaves and some defoliation.

Effect of tetracyclines on symptom development of citrus infectious variegation.—Achromycin at 20 and 50 ppm, 50 ppm aureomycin, and 50 ppm penicillin, applied singly in hydroponic solutions for 30 days had no apparent effect on development of citrus infectious variegation symptoms. All 16 inoculated plants developed severe symptoms 13-18 days after inoculation, and all 16 noninoculated controls remained symptomless. The experiment was repeated twice with identical results.

Uptake and translocation of tetracyclines.—Bioassay.—Root extracts of healthy plants grown in 50 ppm achromycin or 50 ppm aureomycin nutrient solutions were active against *Bacillus cereus* at all sampling dates. Antibacterial activity was also detected in all shoot extracts of plants grown in achromycin but shoot extracts of plants treated with aureomycin had no apparent antibacterial activity after day 2 (Fig. 2).

With the exception of root extracts from plants growing in achromycin, all extracts of treated plants and the antibiotic solutions had higher antibacterial activity at day 2 than at day 9; this decrease was greater for aureomycin than for achromycin (Fig. 2), indicating greater stability for the latter inside and outside the plant. Antibiotic-free nutrient solutions and extracts of plants grown therein had no apparent activity against *B. cereus*. The bioassay results indicate that large amounts of both antibiotics were readily absorbed by roots and that some achromycin, but little or no aureomycin, moved into and remained active in the shoots.

Chromatography.—TLC plates and paper were spotted with shoot extracts of healthy plants grown in 100 ppm achromycin or aureomycin for 16 hr and with pure solutions of the pure chemicals and developed. One spot on chromatograms of shoot extracts of plants grown in achromycin had an R_F value identical to that of pure achromycin. No spot on chromatograms from shoot extract of plants grown in aureomycin had an R_F value comparable to that of pure aureomycin and no spots on chromatograms of shoot extracts of plants grown in antibiotic-free solution had R_F values identical to those of achromycin or aureomycin. The results supplemented those from the bioassays since they indicated prompt translocation of achromycin, though not of aureomycin, to the shoots.

DISCUSSION.—Our data show that the stubborn pathogen, like pathogens causing certain other yellows-type diseases (2, 3, 9, 12, 15), is sensitive to tetracycline antibiotics and that concentrations of achromycin or aureomycin which completely suppress stubborn symptoms have no effect on symptoms caused by CIVV. Our results and those of others (1, 3, 14) indicating that tetracyclines have no effect on development of symptoms of virus diseases led us to conclude that the stubborn pathogen is not a virus but may be a mycoplasma as indicated by ultrastructural studies (8, 11).

The evidence that achromycin is more phytotoxic and more efficient than aureomycin for suppression of stubborn symptoms in sweet orange is similar to that reported by Cousin & Staron (2) for aster yellows and stolbur and by Freitag & Smith (6) for aster yellows. The superiority of achromycin over aureomycin in suppressing symptoms might be indirectly due to the greater phytotoxic effect of achromycin but this seems improbable because either achromycin or aureomycin, slightly below the phytotoxic level, effectively suppressed stubborn symptoms. The bioassays show that achromycin and aureomycin are both readily absorbed by sweet orange roots and that achromycin is translocated in substantial amounts to the shoots, yet neither the bioassays nor the chromatograms indicated much translocation of active aureomycin into shoots. Because stubborn symptoms were absent in new growth, and indexing showed the pathogen to be temporarily suppressed in leaves of plants grown in 50 ppm aureomycin (Table 1), we assume that aureomycin was taken up and modified or adsorbed in the plants or shoot extracts. The favorable results

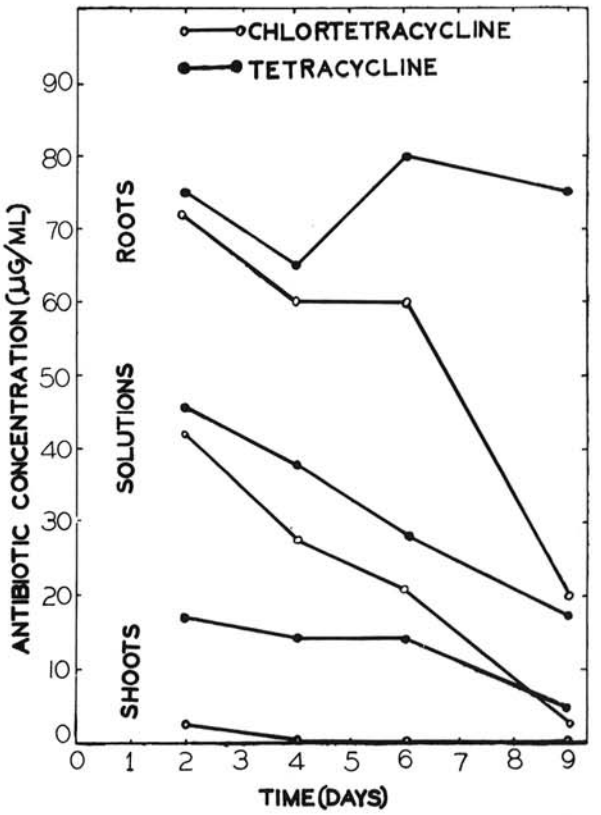

Fig. 2. Apparent concn of nonadsorbed antibiotics in hydroponic solutions and in extracts of shoots and roots of sweet orange seedlings grown therein. Solutions had 50 ppm antibiotics at day 0.

from root immersion in 1,000 ppm aureomycin indicate that aureomycin or a biologically active derivative is effectively translocated into the shoots from roots immersed in that concentration.

Our negative results from 100 ppm achromycin or aureomycin drenching of quartz sand over the roots of seedlings with stubborn disease are similar to those obtained by Ishiie et al. (9) on mulberry dwarf disease but they contrast sharply with others (15). Brown discoloration of the quartz sand treated with these solutions suggests that adsorption or degradation of the antibiotics limited their availability to the roots.

The discovery that some achromycin-treated stubborn plants remained symptomless for over 8 months is significant and possibly of practical importance. We attribute this prolonged suppression of stubborn symptoms to strong inhibition, or complete inactivation, of the pathogen. Possibly, stubborn-free citrus budlines can be obtained by treating infected plants with tetracycline antibiotics. Experiments to test this objective are in progress.

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