Recent Studies on Citrus Greening in India

S. P. Raychaudhuri, T. K. Nariani, S. K. Ghosh, S. M. Viswanath, and Dinesh Kumar

Greening, one of the most serious diseases of citrus in India, is also a major factor in citrus die-back (*9, 10*). The disease was first reported in 1966 (*2, 7*) and is transmitted by *Diaphorina citri* Kuwayama (*1*). Recently, mycoplasma-like bodies have been found to be associated with the disease (*5*). The pathogen has been successfully cultured on artificial media (*3*), and found to resemble a T-strain of mycoplasma isolated from nongonococcal urethritis (*13*). The present paper reports investigations conducted on culture of the pathogen, pathogenicity studies, immunofluorescent detection, and therapeutic control of the disease and the vector in India.

EXPERIMENTS AND RESULTS

Culture on artificial media. Young, greening-affected leaves from sweet orange plants in the glasshouse were thoroughly washed, and small portions containing phloem cells were removed. These were surface-sterilized with 0.1 per cent mercuric chloride, and homogenized in a tissue grinder with a small quantity of double-distilled water. The homogenate was streaked on a medium containing 3.4 per cent dehydrated PPLO agar or PPLO broth with crystal violet (Difco Laboratories, USA) and 20 per cent rabbit serum. The pH was adjusted to 7.8, and the plates or tubes were incubated at 30° C. Typical colonies with burrowing centers, 100 to 225 μ in diameter, developed on agar medium in 24 to 48 hours. In broth cultures, turbidity developed in 24 to 48 hours. Colonies were successfully transferred from agar to broth and vice versa, and were treated with Dienes' stain as described by Hayflick (*4*). Colonies containing pleomorphic bodies acquired a dense blue stain, confirming the mycoplasmal nature of the colonies.

Reproduction of greening by cultured organism. A colony of healthy psylla was maintained in an insectary. Batches of 10 to 30 psylla were inoculated by injecting them with the liquid culture of the pathogen, by means of a microsyringe. Insects were anesthetized either by CO_2 or ether, in petri plates, and were then placed on a plastic insect holder for injection. Surviving treated insects were transferred to sweet orange test plants and placed under a glass chimney fitted with a muslin top. Test plants were kept covered until the last insect died.

Greening disease symptoms were successfully reproduced in seven out of nine sweet orange plants inoculated with infective psylla. Symptoms of greening disease were observed in two to three months after inoculation feeding of the test plants.

Preparation of antiserum. Antigen was prepared by differential ultracentrifugation. The culture was centrifuged at 10,000 rpm for 20 minutes in a Spinco Model L. centrifuge, with phosphate buffer (pH 7.0) to separate the suspended impurities, followed by high-speed centrifugation at 25,000 rpm for 2 hours. The final pellet was dissolved in a small quantity of 0.033 M phosphate buffer and finally centrifuged at 5,000 rpm for 15 minutes. Rabbits were given three weekly intramuscular injections with Freund's adjuvant followed by one intravenous injection without adjuvant. Rabbits were bled 10 days after the last injection, and the serum was separated by low-speed centrifugation (7,000 rpm for 15 minutes).

The antiserum reacted specifically with the mycoplasma culture, giving a

granular precipitate of the somatic type after 1 hour of incubation in a hot-water bath at 37°C, in twofold dilutions. The antiserum had a titer of 1:512. No precipitation was obtained with the unseeded medium that served as a control. The antiserum did not react with the juice of greening-affected plants, possibly because of low concentration of the antigen.

Immunofluorescent detection of greening. Gamma-globulins of the mycoplasma antiserum were separated by precipitation with equal volume of 3.2 M ammonium sulfate. The precipitate was dissolved in 2 ml Tris-HCl buffer (pH 7.2) and repeatedly precipitated until it was absolutely white. Dissolved precipitate was dialyzed against the buffer until all sulfate was removed. The pH was raised to 9.5 with carbonate-bicarbonate buffer and the dye, fluorescein isothiocyanate (FITC), added at the rate of 50 ml per gm protein. The dye-protein mixture was stirred for 4 to 6 hours in the cold (4°C) until conjugation was complete. The preparation was passed through a column of Sephadex (G-25) and eluted with Tris-HCl buffer (pH 7.2) to remove unconjugated dye.

Sections of infected as well as healthy leaves were cut by hand and flooded with Tris-HCl buffer (pH 7.2). They were then transferred to slides and covered with 1 to 2 drops of FITC conjugated antiserum. Slides were kept in a moist chamber and left at room temperature for 8 to 12 hours. Excessive antiserum was drained off, and the sections were washed with Tris-HCl buffer, mounted in Tris-HCl buffered glycerine, and examined under the fluorescent microscope. Tissues of uninfected leaf sections showed autofluorescence in a dull blue shade except for the thick-walled cells (xylem), which showed a green fluorescence. Infected sections also showed brilliant apple-green fluorescent dots in the phloem cells, indicating the presence of mycoplasma.

Transmission of greening by dodder. Strands of dodder (*Cuscuta reflexa*) collected from lime were established on greening-affected sweet orange in the glasshouse. When well established, actively growing tips of the strands were trained to healthy test plants of sweet orange and allowed to establish on them. Greening symptoms appeared on the test plants in 6 to 9 months after establishment of the dodder.

Effects of antibiotics and other chemicals. Aureomycin, achromycin, and ledermycin (500 ppm) were sprayed on separate batches of four greening-affected sweet orange plants at weekly intervals for 10 weeks, in the glasshouse. A set of four infected plants was sprayed with water at the same intervals, to serve as controls. Spraying with achromycin and ledermycin resulted in recovery of plants from greening symptoms. Those sprayed with aureomycin and water (control) remained unaffected (*8*).

An experiment was conducted with field-grown plants the following season. Three citrus trees were sprayed with achromycin (500 ppm) and three with ledermycin (500 ppm) for 8 weeks. Trees showed temporary recovery of varying degrees, which lasted for about 3 months, but later the symptoms of greening reappeared.

Experiments were also conducted with B.P.-101, a chemical supplied by Hindustan Antibiotics, Pimpri, Poona. The chemical, at 500 ppm, was injected daily into trunks of four young and two old infected trees. Holes were bored into the trunks, and the chemical was applied through a plastic funnel fitted to the hole. About 1 gm of the chemical was absorbed by each tree. All young trees showed recovery from the greening symptoms. The two older trees, however, showed only partial recovery. Even though the effect of B.P.-101 was comparatively more lasting than that of the other treatments, symptoms of greening disease started reappearing after about

TABLE 1
EFFECT OF HEAT TREATMENT ON GREENING-AFFECTED BUDWOOD OF CITRUS

Treatment (°C)	Treatment time	No. plants budded	No. plants with viable buds	No. plants remaining free of greening symptoms
Hot water				
40	5 hrs.	8	5	0
45	5 hrs.	8	5	0
50	30 min.	6	6	6
55	15 min.	6	6	2
Control (no treatment)	.....	8	7	0
Hot (moist) air				
45	6 hrs.	8	8	5
47	4 hrs.	8	8	5
49	2 hrs.	8	8	0
51	1 hr.	8	8	0
Control (no treatment)	.....	8	8	0

6 months from the last application of the chemical.

Effect of heat treatment. Budsticks from greening-affected trees were subjected to various temperatures ranging from 40 to 55°C by immersing them in a water bath (moist heat) or suspending them above the water level (moist air) for different periods of time. Untreated pieces from the budsticks were retained as controls. Sweet orange plants were budded with the treated and untreated budwood in an insect-proof glasshouse. Results of heat treatments of greening-affected budwood are given in table 1.

Hot-water treatment of greening-affected budwood at 40°C and 45°C for 5 hours, 50°C for 30 minutes, and 55°C for 15 minutes was not effective in inactivating the greening pathogen. Treating the infected budwood to hot (moist) air at 51°C for 1 hour or 49°C for 2 hours also had no effect. Subjecting the infected budwood to 47°C for 4 hours or 45°C for 6 hours freed some of the budsticks of the pathogen.

In another experiment, 1- to 1½-year-old, greening-affected potted seedlings of sweet orange, Kagzi lime, and Rangpur lime were subjected to 38° to 40°C, ± 1°C, in a fluorescent-lighted hot chamber for 21 days. Plants were kept in the glasshouse after treatment. Three of 4 plants of sweet orange, the 2 Kagzi lime plants, and 1 or 2 Rangpur lime plants treated at 38°C, and 5 of 8 sweet orange plants treated at 40°C showed recovery from greening.

Effect of a systemic insecticide on psylla populations. Three citrus trees in an orchard were sprayed with Rogor (Dimethoate) (Tate Fison Ltd.) at 0.2 per cent, at weekly intervals for 5 weeks. Three trees were kept unsprayed. Psylla incidence in Rogor-sprayed trees declined to almost nil. One month after the last spray, however, a small population of psylla was observed to appear. Possibly they had migrated from the unsprayed trees, or perhaps they survived the effect of Rogor.

DISCUSSION AND CONCLUSIONS

Although mycoplasmalike bodies were found associated with the greening disease in citrus, as observed in ultrathin sections of diseased plants (*5*),

there was no direct evidence that they caused the disease. Investigations reported herein have established that the pathogen can be isolated and cultured in the laboratory. Pathogenicity tests show that the greening disease can be reproduced by using cultured organisms. Like most yellows-disease pathogens, greening pathogen was also successfully transmitted by dodder. This dodder species was also reported to transmit tristeza virus (*6*). Although diagnosis of greening disease from the presence of fluorescent-marker substance has been reported (*11, 12*), immunofluorescent detection of the disease by means of the fluorescent-antibody technique is reported here for the first time, and provides a quicker method of detection once labelled antiserum is available. Immunofluorescence is used in the study of communicable diseases involving bacteria and viruses of animals. Steward (*14*) used this technique for specific identification of different animal mycoplasmas, but little work has been done with it in the field of plant pathology, and none dealing with plant mycoplasmas.

Control of greening disease remains a problem. Use of tetracycline antibiotic sprays is encouraging although the effect of tetracyclines seems to be temporary. Research on longevity of different dosages is needed before their economic use can be ascertained. Sprayed trees are always exposed to reinfection through psylla. Tetracycline sprays must be combined with sprays that control the vector. In our tests, Rogor (dimethoate) has shown some effect in controlling the vector for periods up to about four weeks.

Hot water does not hold much promise in checking greening disease. Moist hot air at lower temperatures for prolonged periods offers more promise.

ACKNOWLEDGMENTS

Grateful thanks are due the U. S. Department of Agriculture and the Indian National Science Academy for providing funds under projects "Epiphytology of Greening Disease of Citrus in India (PL 480)" and "Mycoplasma Diseases of Plants" for carrying out these investigations.

LITERATURE CITED

1. Capoor, S. P., D. G. Rao, and S. M. Viswanath
1967. *Diaphorina citri* Kuway., a vector of the greening disease of citrus in India. Indian Jour. Agr. Sci. **37:** 572–76.
2. Fraser, L. R., D. Singh, S. P. Capoor, and T. K. Nariani
1965. Greening virus, the likely cause of citrus die-back in India. FAO Plant Protect. Bul. **14:** 127–30.
3. Ghosh, S. K., S. P. Raychaudhuri, A. Varma, and T. K. Nariani
1971. Isolation and culture of mycoplasma associated with citrus greening disease. Current Sci. **40:** 299–300.
4. Hayflick, L.
1965. Tissue cultures and mycoplasma. Texas Rept. Biol. and Med. **23** (Suppl. 1): 285–303.
5. Laflèche, D., and J. M. Bové
1970. Structures de type mycoplasme dans les feuilles d'orangers atteints de la maladie du "greening." Compte rend. Acad. Sci. Paris **270:** 1915–17.
6. Nariani, T. K., and S. P. Raychaudhuri
1970. Transmission of citrus tristeza virus by dodder, *Cuscuta reflexa* Roxb. Ann. Phytopath. Soc. Japan **36:** 289–90.
7. Nariani, T. K., S. P. Raychaudhuri, and R. B. Bhalla
1967. Greening virus of citrus in India. Indian Phytopath. **20:**146–50.
8. Nariani, T. K., S. P. Raychaudhuri, and S. M. Viswanath
1971. Response of greening pathogen of citrus to certain tetracycline antibiotics. Current Sci **20:** 552.
9. Raychaudhuri, S. P., T. K. Nariani, and V. C. Lele
1969. Citrus die-back problem in India. *In:* Proc. 1st Intern. Citrus Symp. 3. (H. D. Chapman, ed.) Riverside: University of California, pp. 1433–37.

10. RAYCHAUDHURI, S. P., T. K. NARIANI, V. C. LELE, AND G. R. SINGH
1969. Greening and citrus decline in India. *In:* Proc. 5th Conf. Intern. Organ. Citrus Virol. (W. C. Price, ed.) Gainesville: Univ. Florida Press, pp. 35–37.
11. SCHWARZ, R. E.
1965. A fluorescent substance present in tissues of greening affected sweet orange. S. African Jour. Agr. Sci. **8:** 1177–80.
12. SCHWARZ, R. E.
1968. Indexing of greening and exocortis through fluorescent marker substances. *In:* Proc. 4th Conf. Intern. Organ. Citrus Virol. (J. F. L. Childs, ed.) Gainesville: Univ. Florida Press, pp. 118–24.
13. SODHI, S. S., S. S. DHILLON, R. JEYARAJAN, AND S. S. CHEMA
1973. Isolation and characterization of mycoplasma-like organisms associated with citrus greening disease. Indian Jour Microbiol. **13:** 13–16.
14. STEWARD, S. M.
1967. The use of fluorescent antibody technique for the identification of cultures of mycoplasma. Immunology **13:** 513–16.