Isolation and Cultivation of, and Inoculation with, a Mycoplasma Causing White Leaf Disease of Sugarcane

Shu-chen Lin, Ching-shiou Lee, and Ren-jong Chiu

Plant Pathologist and Assistant Plant Pathologist, Taiwan Sugar Experiment Station, Taiwan; and Plant Pathologist, Plant Industry Division, Joint Commission on Rural Reconstruction, Taipei, Taiwan, respectively.

Published with approval of the Director, Taiwan Sugar Experiment Station, as Technical Paper No. R-4-88.

The authors are grateful to K. C. Liu for encouragement and to Y. P. Lee and W. S. Teng for technical assistance.

Accepted for publication 2 December 1969.

ABSTRACT

Treatment by immersing, for 48 or 72 hr, cuttings from white leaf-diseased sugarcane in solutions of tetracycline antibiotics markedly delayed the development of disease symptoms in young shoots subsequently arising from the treated cuttings. A *Mycoplasma* was obtained in Morton's PPLO culture media from infected cane buds. When inoculated to susceptible sugarcane cuttings by repeated pin-pricking of the root bands and buds, while being immersed in inoculum, it induced white leaf symptoms, to a rather low percentage, in young plants which grew from the inoculated cuttings. Because no plants from comparable but noninoculated cuttings developed the disease, the *Mycoplasma* is considered to be the etiological agent of the white leaf disease of sugarcane. Phytopathology 60:795-797.

The white leaf disease of sugarcane first found in 1958 in southern Taiwan was once confused with the sugarcane leaf scald caused by *Xanthomonas albilineans* Ashby because of similarity of initial symptoms. The disease was later considered to be different from leaf scald, and the name "leaf scaldlike disease of sugarcane" suggested (5). Based on the field pattern of disease spread and the results of heat-curing experiments, Ling (3) believed that the disease might be caused by a virus and proposed the name white leaf disease. In experiments in which buds of diseased cuttings were either heat-treated or nontreated, transmission of the white leaf disease agent from the nontreated to the heat-treated buds was observed. Liu (4) simulated this phenomenon with virus transmission by grafting, and hypothesized the virus nature of the disease. Recently a leafhopper, *Epitettix hiroglyphicus* Mats., was found to be the vector responsible for disease spread (6). In ultra-thin sections of diseased leaves, pleomorphic bodies suggestive of *Mycoplasma* could be seen in sieve tubes (2, 7).

Upon the assumption that *Mycoplasma* may be the etiological agent of the disease, experiments were performed to determine whether suppressing the multiplication of *Mycoplasma* with tetracycline antibiotics would bring about restrained symptom development. The experiments employed sugarcane of a susceptible selection, 56-2080, infected in the field. One-bud cuttings taken from the diseased stalks were either treated in solutions of the test antibiotics of the tetracycline group, or immersed in distilled water as controls. The antibiotics used were: tetracycline; oxytetracycline; chlortetracycline; agrimycin 100 (containing oxytetracycline 1.5% and streptomycin 15%); methacycline (from Pfizer & Co. Inc.); achromycin HCl; aureomycin HCl; and ledermycin HCl (from Taiwan Cyanamid Co.). Each compound was tested at one selected concentration, but with treating times varied at 24, 48, or 72 hr. The solutions were renewed at 24-hr intervals wherever desirable. Suppression, to different degrees, of white leaf symptoms was obtained by immersing cane cuttings for 48 and 72 hr with all test antibiotics except agrimycin 100, which contained nontetracyclines in greater proportion (Fig. 1, Table 1). Young shoots arising from treated cuttings remained normal for as much as 10 months or longer in several cases before symptoms developed. The delayed symptoms took the form of narrow stripes parallel to the leaf veins, in contrast to complete whiteness of the whole leaves often observed when the latter first emerged from the control cuttings. Of the test antibiotics, aureomycin HCl, achromycin HCl, chlortetracycline, and tetracycline were most effective. With a treating time of 24 hr, these antibiotics produced a slight delay of white leaf symptoms while the remaining compounds showed little suppressing effect.

In attempts to obtain cultures of *Mycoplasma*, Morton's PPLO media as appearing in Difco Manual, 9th edition, were used. The liquid medium used was prepared by dissolving 21 g of dehydrated Bacto-PPLO Enrichment Broth (Difco) in 1,000 ml distilled water and sterilizing at 121 C for 15 min. To the broth was added 2.85 ml Bacto-Chapman tellurite solution containing 1% potassium tellurite. A volume of 750 ml was then mixed with 250 ml undiluted Bacto-ascitic fluid. The liquid medium had a pH of 7.8. For preparing agar medium, 34 g of dehydrated Bacto-PPLO agar (Difco) was first thoroughly dissolved in 1,000 ml distilled water. After sterilization at 121 C for 15 min, the agar solution was cooled to 45-60 C, mixed with 10 ml Bacto-PPLO serum fraction, and distributed in petri dishes, 5 ml/60-mm dish. The agar medium had a pH of 7.8 when nonenriched. Buds taken from a stalk showing typical symptoms were cut into a size of about 5×7 mm^2 and, after washing in 5% calcium hypochlorite for 5 min, were transferred to the liquid medium in culture tubes. Usually, one bud was placed in each tube containing about 10 ml liquid medium. They were then incubated for 36-72 hr at 37-38 C. The liquid culture, turned slightly turbid by then, was inoculated to the solid media by streaking and again incubated at the same temperature. After 36- to 48-hr incubation, fried-egglike colonies of *Mycoplasma* developed on the agar plates (Fig. 2). In six attempts using a total of 30 buds, we succeeded in obtaining *Mycoplasma* cultures in two cases. The *Mycoplasma*

TABLE 1. Suppressing effects on sugarcane white leaf symptoms of tetracycline antibiotics as treatments for diseased cane cuttings

Test antibiotic and concn		Treating for 48 hr: Treated: Immediate symptoms[a]	Delayed symptoms[b] A	B	C	Control: Immediate symptoms	Treating for 72 hr: Treated: Immediate symptoms	Delayed symptoms A	B	C	Control: Immediate symptoms
	ppm										
Tetracycline	200	5 (20)[c]	13	2	0	25 (25)	0 (15)	2	4	9	15 (15)
Oxytetracycline	200	7 (19)	11	1	0	25 (25)	0 (14)	3	10	1	15 (15)
Chlortetracycline	200	7 (15)	6	2	0	25 (25)	0 (13)	2	9	2	15 (15)
Agrimycin 100	100	24 (24)	0	0	0	25 (25)	14 (14)	0	0	0	15 (15)
Methacycline	300	20 (23)	3	0	0	24 (24)	2 (9)	4	3	0	13 (13)
Achromycin HCl	200	1 (18)	12	2	3	25 (25)	0 (12)	0	2	10	15 (15)
Aureomycin HCl	200	2(23)	14	4	3	25 (25)	0 (15)	0	0	15	15 (15)
Ledermycin HCl	200	4 (15)	7	3	1	24 (24)	0 (8)	2	3	3	12 (12)

[a] White leaf symptoms developed in the first young leaves from cane cuttings or in less than 60 days after starting the treatments.

[b] The development of white leaf symptoms took longer than 60 days. A = symptoms were detected in 100 days following the treatment. B = symptoms were detected in 300 days. C = symptoms were detected after 300 days.

[c] The figures in parentheses represent the numbers of cuttings that received treatment and germinated, and from which cuttings killed of undetermined causes after germination have been subtracted.

was further purified and maintained by alternative passages through the liquid and agar media. However, some cultures were lost during these passages because of bacterial contamination.

Inoculation of cultured *Mycoplasma* to sugarcane was made by repeated pin-pricking of the root bands and buds of healthy one-bud cuttings while they were being immersed in the suspension of *Mycoplasma* taken from 24-hr-old colonies. A pure culture established 7 October 1968 was used in all inoculation experiments. For preparing inocula, the mycoplasma cells were always suspended in sterile water. Inoculated cane cut-

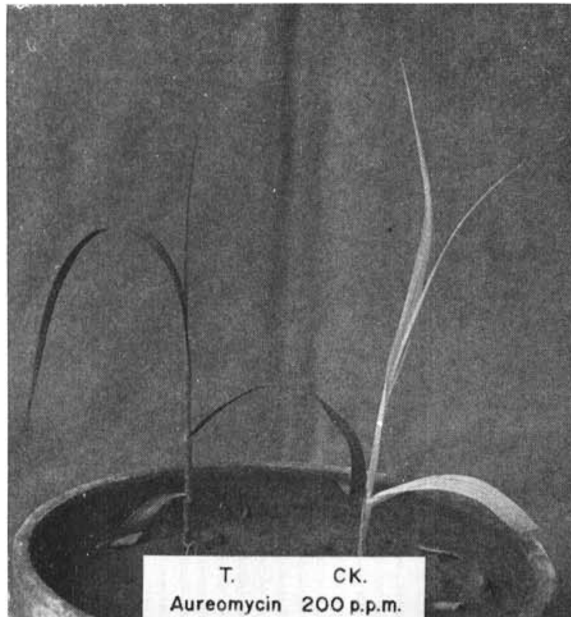

Fig. 1. Suppressing effect of aureomycin HCl on the development of sugarcane white leaf symptoms. The shoot on the left grew from a cutting which had been immersed for 72 hr in 200 ppm aureomycin HCl; shoot on the right was from the control (picture taken 60 days after treatment).

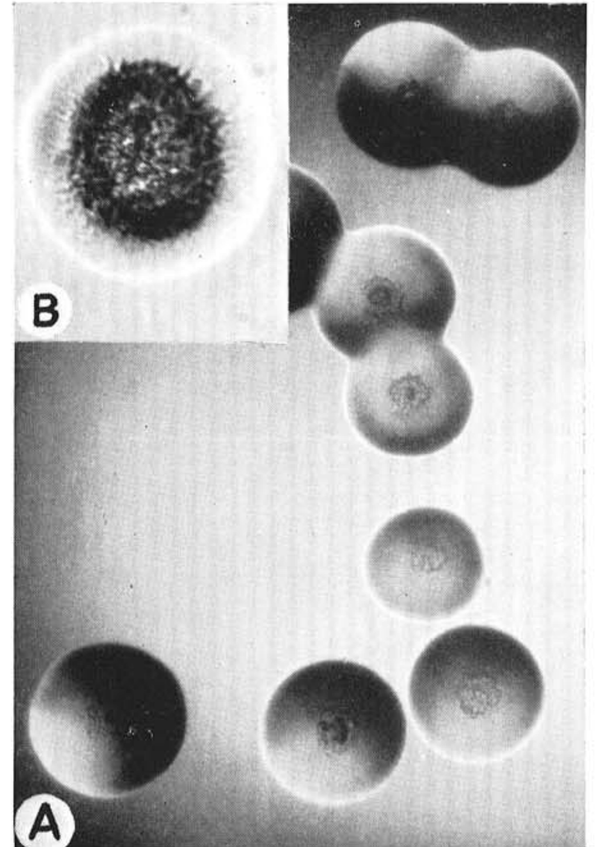

Fig. 2. Colonies of *Mycoplasma* on PPLO agar medium. **A)** Two-day-old colonies (approximately $\times$200). **B)** Four-day-old colonies (approximately $\times$300).

tings were left in the suspension for 18 hr at 37-38 C, before planting in pots in the greenhouse. Six buds were taken from a healthy stalk, and four of them were inoculated. Two controls were provided by pin-pricking one cutting as above but in distilled water, and by directly planting the remaining one. All the inoculation experiments employed the same susceptible sugarcane selection, 56-2080, propagated in areas where natural spread of white leaf had not occurred.

Pin-pricking caused damage to and germination failure of some buds in both the inoculated and control cutting groups. In a series of experiments, each employing 24 cuttings for inoculation and half this number as control, one out of 12 surviving plants inoculated on 31 October 1968 was seen with the initial white leaf symptoms on 23 January 1969; two out of 12 surviving plants inoculated on 11 November 1968 developed symptoms on 23 January 1969; and two out of 24 plants receiving inoculation on 18 March 1969 started to show symptoms on 25 April 1969, respectively. The white leaf infection, though occurring in low percentages in these experiments, is believed to have resulted from inoculation, for no symptoms appeared on any of the 32 plants which grew from control cuttings.

Although further experiments along a similar line and attempted isolation of *Mycoplasma* from the insect vector are still in progress, the results obtained so far seem to provide sufficient evidence that *Mycoplasma* is the causal agent of white leaf disease of sugarcane. It is probably a new species with proven pathogenicity to plants. The only other plant *Mycoplasma* previously obtained in culture was discovered in association with alfalfa mosaic virus from pea plant which exhibited necrotic streak symptoms. This *Mycoplasma* induced faint chlorotic mottle when inoculated alone by mechanical means. Its transmissibility by aphids was also suggested (1).

LITERATURE CITED

1. HAMPTON, R. O., J. O. STEVENS, & T. C. ALLEN. 1969. Mechanically transmissible *Mycoplasma* from naturally infected peas. Plant Dis. Reptr. 53:499-503.
2. LIN, S. C., & C. S. LEE. 1968. *Mycoplasma* or mycoplasma-like microorganism in white leaf disease of sugarcane, p. 17-22. Annu. Rep. Taiwan Sugar Exp. Sta. 1967-1968.
3. LING, K. C., & C. CHUANG-YANG. 1962. A preliminary study on the white leaf disease of sugarcane. [in Chinese, English summary] Annu. Rep. Taiwan Sugar Exp. Sta. 28:139-172.
4. LIU, H. P. 1963. The nature of the causal agent of white leaf disease of sugarcane. Virology 21:593-600.
5. LIU, H. P., & S. M. LEE. 1961. Studies on the sugarcane leaf scald. [in Chinese] Annu. Rep. Taiwan Sugar Exp. Sta. 1960-1961. p. 155-156.
6. MATSUMOTO, T., C. S. LEE, & W. S. TENG. 1968. Studies on sugarcane white leaf disease of Taiwan with special reference to the transmission by a leafhopper, *Epitettix hiroglyphicus* Mats. Int. Soc. Sugar Cane Technol. Proc. 13:1090-1099.
7. SHIKATA, E., K. MARAMOROSCH, K. C. LING, & T. MATSUMOTO. 1968. Detection of mycoplasma-like organisms in the sieve tubes of china aster infected with yellows, maize with corn stunt, rice with yellow dwarf and sugarcane with white leaf disease. [Abstr. in Japanese] Ann. Phytopathol. Soc. Japan 34:208-209.