DETECTION AND POSSIBILITIES OF THERAPEUTIC CONTROL OF THE GREENING DISEASE OF CITRUS CAUSED BY MYCOPLASMA

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The greening mycoplasma has been cultured on PPLO broth and the disease reproduced by feeding Sweet Orange plants with the psyllid vector, *Diaphorina citri* Kuway, artificially injected with the culture. The pathogen was successfully detected in the diseased plants using fluorescent antibody technique. Spraying with tetracycline antibiotics, achromycin and ledermycin at 500 ppm, suppressed the symptom expression in diseased plants. More lasting effect was obtained by injecting trees with B.P., a chemical produced by Hindustan Antibiotics Factory, Pimpri, Poona. Heating of infected budwood by hot (moist) air at 47°C for four hours or 45°C for 6 hr inactivated the greening mycoplasma. Also, subjecting potted greening affected budded plants to 38°C or 40°C in a heat therapy chamber for three weeks freed the plants from greening symptoms.

The citrus psylla, *Diaphorina citri*, Kuway, the vector of the greening disease, could be controlled by spraying with a systemic insecticide, Rogor (Dimethoate) a Tata Fison Ltd. product.

INTRODUCTION

Greening is one of the most serious problems of the citrus industry in India. The disease was first reported to be due to virus from South Africa in 1965 where it is transmitted by the psylla, *Trioza erytreae* (McLean and Oberholzer, 1965*a*, 1965*b*). In India the disease was reported in 1966 (Frazer *et al.* 1966, Nariani *et al.* 1967) and is transmitted by *Diaphorina citri* Kuway (Capoor *et al.* 1967). The disease has also been reported from the Philippines where also the same vector, *D. citri* is responsible for its spread (Martinez and Wallace, 1967). Recently, however, mycoplasma like bodies have been found to be associated with the disease (Lafleche and Bove 1970) and the mycoplasma has been cultured on artificial synthetic media in the laboratory (Ghosh *et al.* 1971). The present paper reports the investigations conducted on the reproduction of the disease using the mycoplasma culture, immunofluorescent detection and therapeutic control of the disease as well as the vector control in India.

MATERIALS AND METHODS

Greening mycoplasma was cultured on PPLO broth by the method described earlier (Ghosh *et al.* 1971).

For reproduction of the disease, batches of 10–30 psylla, *Diaphorina citri* were inoculated by injecting them with the liquid mycoplasma culture using a microsyringe. The insects were anaesthetized by using CO_2 or ether in Petri plates and held on a plastic insect holder at the time of injection. The surviving insects were

transferred to sweet orange test plants under a glass chimney fitted with muslin cloth at the top. The insects were allowed to feed on the test plants till the last psylla was alive.

The antigen for antiserum preparation was prepared by concentrating the mycoplasma culture by one cycle of differential centrifugation which was achieved by centrifuging it at 10,000 rpm for 20 min in a Spinco Ultracentrifuge Model L using phosphate buffer (*p*H 7.0) to separate the suspended impurities, followed by high speed centrifugation at 25,000 rpm for 120 min and the pellet dissolved in a small quantity of M/30 phosphate buffer and finally centrifuged at 5000 rpm for 15 min. Antiserum was prepared by injecting the rabbits with the antigen, giving three weekly intramuscular injections using Freund's adjuvant (Bacto) followed by one intravenous injection without the adjuvant. The rabbits were bled after 10 days following the last injection and the serum separated by low speed centrifugation (7000 rpm for 15 min).

For labelling of mycoplasma antiserum gamma globulins were separated from the antiserum by precipitation with equal volume of 3.2 M ammonium sulphate. The precipitate was dissolved in 2 ml tris-HCI buffer (*p*H 7.2) and reprecipitated repeatedly till the precipitate was absolutely white. The dissolved precipitate was dialysed against the buffer till all sulphate was removed. The *p*H was raised to 9.5 with carbonate-bicarbonate buffer and the dye Fluorescein isothiocyanate (FITC) added at the rate of 50 mg per gram of protein. The dye protein mixture was stirred for 4–6 hours in cold (4°C) till the conjugation was complete. The preparation was passed through a column of Sephadex (G-25) and eluted with tris-HCI buffer (*p*H 7.2) to remove unconjugated dye. The conjugated gamma globulin was used to detect mycoplasma in the affected citrus tissues.

For detection of mycoplasma in citrus tissues, sections of infected as well as healthy leaves were cut and flooded with tris-HCI buffer (*p*H 7.2). They were then transferred to slides and covered with 1-2 drops of FITC conjugated antiserum. The slides were kept in moist chamber and left at room temperature for 8-12 hr. The excessive antiserum was drained off and the sections were washed with tris-HCI buffer. They were then mounted in tris-HCI buffered glycerine and examined under fluorescent microscope.

The effect of tetracycline and other chemicals on the greening disease was tested by spraying known concentrations of the chemicals on affected seedlings in the glasshouse or trees in the orchards till dripping stage or injecting solutions in the main trunk through plastic funnels fitted in holes bored in them.

For heat therapy greening affected budwood was subjected to required temperatures (40–55°C) by immersing in water bath (moist heat) or suspending it above the water level (moist air) for required period of time.

The experiments on the control of psylla vector were conducted by using Rogor (Active ingredient dimethoate) a Tata Fison Product and a Four Oaks pressuresprayer.

RESULTS

Reproduction of the greening disease using mycoplasma culture

The greening disease symptoms were successfully reproduced in sweet orange (*Citrus sinensis* Osbeck) plants inoculated with psylla, *Diaphorina citri* Kuway

injected and made infective with mycoplasma culture in PPLO broth. The symptoms of greening disease were observed in 2–3 months after feeding of the infective psylla on test plants.

Immunofluorescent detection of greening disease

Antiserum of the mycoplasma culture was prepared and tested with the greening mycoplasma culture as well as the juice of greening affected plants clarified by low speed centrifugation (5000 rpm for 15 min). The antiserum reacted specifically with the mycoplasma culture giving a granular precipitate of somatic type after one hour of incubation in hot water bath at 37°C in two fold dilutions. The antiserum had a titre of 1:512. No precipitation was obtained with the culture medium which served as control. The antiserum did not react with the juice of greening affected plants.

The antiserum was labelled with the fluorescent dye (FITC) and was tested on hand sections of greening affected leaves. Under the Fluorescent microscope the tissues of uninfected leaf sections showed autofluorescence in dull blue shade except in thick walled cells (Xylem) which showed green fluorescence. The infected sections showed in addition brilliant apple green fluorescence in the phloem cells in the form of dots indicating the presence of mycoplasma.

Effect of tetracycline and other chemicals on greening disease

Three tetracycline antibiotics namely, aureomycin, achromycin and ledermycin at 500 ppm were sprayed on batches of four greening affected Sweet Orange plants in the glasshouse at weekly intervals for ten weeks. A set of four infected plants were sprayed with water at the same intervals to serve as control. It was observed that spraying with achromycin and ledermycin resulted in recovery of plants from greening symptoms whereas those sprayed with aureomycin and water (Control) remained unaffected (Nariani *et al.* 1971).

The experiment was repeated on field plants in the following season and three citrus trees were sprayed with achromycin (500 ppm) and three with ledermycin (500 ppm) for 8 weeks and the trees kept under observation. The trees showed temporary recovery to varying extent, but later the symptoms of greening reappeared.

Experiments were also conducted with B.P. a chemical supplied by Hindustan antibiotics, Pimpri, Poona, by injecting at 500 ppm into trunks of young infected leaves through holes bored in them and fitted with plastic funnels daily for three months. All the four young trees showed recovery from the greening symptoms. The two older trees injected with the chemical, however, showed partial recovery. It was, however, observed that although the effect of this chemical was comparatively more lasting, ultimately the symptoms of the disease started appearing after about six months from the last application of the chemical.

Effect of heat treatment on greening disease

The results of heat treatment of greening affected budwood are given in Table I.

TABLE I

Effect of heat treatment on greening affected budwood

Treatment and temperature	Time of exposure	No of plants budded	No. of plants with viable buds	No. of plants remaining free from greening symptoms
Hot water treatment :				
40°C	5 hr	8	5	0
45°C	5 hr	8	5	0
50°C	30 mt	6	6	1
55°C	15 mt	6	6	2
Control (no treatment)		8	7	0
Hot (moist) Air treatment				
45°C	6 hr	8	8	5
47°C	4 hr	8	8	5
49°C	2 hr	8	8	0
51°C	1 hr	8	8	0
Control (no treatment)		8	8	0

It was observed that hot water treatment of the greening affected budwood at 40°C and 45°C for five 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 one hour or 49°C for two hours also had no effect. However, subjecting the infected budwood to 47°C for 4 hours or 45°C for six hours helped in inactivating the pathogen in majority of the cases.

In another experiment 12 to 18 months old greening affected potted seedlings of Sweet Orange, Kagzi lime and Rangpur lime were subjected to 38°C or 40°C±1 in a hot chamber having fluorescent tubelight arrangement for a period of 21 days. The plants were kept in the glasshouse after the treatment. It was observed that three out of four plants of Sweet Orange, both the Kagzi lime plants and one out of the two Rangpur lime plants treated at 38°C were free from greening symptoms, whereas five out of eight Sweet Orange plants treated at 40°C showed recovery from greening symptoms.

Effect of spraying with a systemic insecticide, Rogor (Dimethoate) on psylla populations

Three citrus trees were sprayed with Rogor (Dimethoate) at 0.2 per cent (a Tata Fison Ltd. Product) at weekly intervals for five weeks. Three trees were kept unsprayed. The psylla incidence in Rogor sprayed trees declined to almost nil (Table II). However, one month after the last spray a small population of psylla was observed. This might be due to migration of psylla from the unsprayed trees. These could perhaps survive after the effect of Rogor had gone.

TABLE II

Effect of spraying with Rogor (Dimethoate) concentration 0.2% on psylla population

Spray schedule (weekly)	Number of psylla/100 leaves		Unsprayed control
	Before spray	After spray	
4-4-72	1,340	787	1,280
11-4-72	730	432	1,226
18-4-72	430	180	1,235
25-4-72	147	36	1,210
2-5-72	30	Nil	1,205
Observations			
2-5-72	—	Nil	1,147
16-5-72	—	Nil	932
23-5-72	—	Nil	860
30-5-72	—	Nil	778
6-6-72	—	13	491
13-6-72	—	18	427
20-6-72	—	25	420

DISCUSSION

The evidence so far obtained leads to the conclusion that the greening disease of citrus is caused by mycoplasma. Although mycoplasma like bodies were earlier found associated with the disease in ultra-thin sections (Lafleche and Bove, 1971) the evidence was only circumstantial. The investigations conducted have now clearly established that the greening mycoplasma can be isolated and cultured in the Laboratory. The pathogenicity tests have conclusively proved that the greening disease could be reproduced using mycoplasma culture. Although detection of greening disease has been reported by the presence of fluorescent marker substance (Schwarz 1965, 1968), the immunofluorescent detection of the disease using the fluorescent antibody technique has been used for the first time and has provided a quicker method of detection once the labelled antiserum is available. Most of the immunofluorescence work has been done in the field of communicable diseases dealing with bacteria and viruses. Steward (1967) used this technique for specific identification of different animal mycoplasmas. Very little work has been done in the field of plant pathology and none dealing with plant mycoplasma.

The control of greening disease is a great problem. The tetracycline antibiotic sprays have given encouraging results although the effect of tetracyclines and B.P., seems to be temporary. Research on how long the effect of spraying or injecting different doses of the chemicals will last can provide a possible economic use of the sprays. However, the sprayed trees are always exposed to re-infection through psylla. Hence, the tetracycline sprays must be combined with sprays that control the vector. Rogor (Dimethoate) has proved to be quite effective in controlling the vector.

Experiments on heat therapy have shown that hot water treatment of bud sticks does not hold much promise in checking the greening disease. Moist hot air at lower temperatures for prolonged periods offers more scope. Exposure of seedlings to hot air treatment also has given good but inconsistent results since the success varies with individual plants.

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