Greening Disease of Citrus in the Deccan Trap Country and its Relationship with the Vector, *Diaphorina citri* Kuwayama

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Citrus greening was observed for the first time in India in 1960 (*3, 6, 7*). The disease was again observed at Modlimb, on Kagzi lime trees, in 1963. Thereafter it was commonly encountered in orchards in which trees were being indexed for virus-free budwood. The oriental citrus psyllid, *Diaphorina citri* Kuwayama, was established as the vector of greening pathogen in India (*4*). Laflèche and Bové (*10*) observed mycoplasmalike bodies in sieve tubes of greenhouse-grown sweet orange seedlings affected with greening, and concluded that it is most probably caused by a mycoplasmalike organism.

The data in this paper pertain to the Deccan Trap Country, and include studies of etiology, spread, host range, and relationship with the psyllid vector.

MATERIALS AND METHODS

Greening disease was maintained in seedlings of sweet orange previously infected by psylla in the glasshouse. The disease was identified from leaf and fruit symptoms, from the general growth of the trees (*11*), and by indexing budwood from affected trees on seedlings of sweet orange, Kagzi lime, and grapefruit (*4*). Later, only sweet orange was used because it consistently gave a typical reaction to the greening disease. Seedlings were raised inside a glasshouse, and selected nucellar seedlings were transplanted individually in 15-cm earthen pots. Testing was done by budding, shoot grafting, or by leaf-patch grafting (*15*).

Bark extracts of several plants were also examined for fluorescent-marker substance by the method described by Schwarz (*12, 13*).

For sap inoculation, young, diseased leaves of sweet orange were homogenized with 10 per cent sucrose in 0.1 M phosphate buffer at pH 7.6 (*5*). Leaves were frozen for four hours before grinding with 50 mg of activated charcoal to every 10 gm of leaves. Inoculum was kept cool in crushed ice until the inoculations were completed. Inoculations were made by the leaf-rubbing method, with 600-mesh carborundum. Test plants were predarkened before inoculation, and maintained at 22 to 24° C after inoculation.

Acquisition and transmission feeding of the insect species for insect transmission tests was the same as used previously (*4*). As a check, the psyllids were also used to transmit the greening pathogen from source plants used in these experiments. Test plants were sweet orange seedlings. Newly emerged adults from a colony of *Diaphorina citri* (mixed population) were used in the experiments unless stated otherwise. After test-feeding, psyllids were killed by spraying with 0.02 per cent Folidol-E 605.

In surveys for greening, particular attention was given to the nurseries from which the citrus trees in orchards had originated.

A survey for the disease in the Deccan Trap Country, carried out from 1965 to 1970, revealed that, but for a few areas, the greening disease was present in most of the localities of the region, and trees of sweet orange, mandarin, grapefruit, lime and lemon were

affected. Trees showing dieback symptoms invariably proved positive for greening. Several trees that were positive for greening were found free of tristeza virus, but mixed infections of tristeza and greening were prevalent.

EXPERIMENTS AND RESULTS

Symptomatology. Infected trees show stunted growth, sparsely foliated branches, unseasonal bloom, leaf and fruit drop, and twig dieback (fig. 1). Young leaves are chlorotic, with green banding along the major veins. Mature leaves have yellowish-green patches between veins, and midribs are yellow. In severe cases, leaves become almost chlorotic, with scattered spots of green (fig. 2).

Fruits on greened trees are small, generally lopsided, underdeveloped, and unevenly colored. Sides exposed to the sun develop prominent, deep orange spots, while the rest of the fruit remains a dull orange-green. Fruits are hard, and poor in juice. The columella was almost always curved in sweet orange fruits, and appears to be the most reliable diagnostic symptom of greening. Some seeds in diseased fruits develop normally, but most are small and dark colored.

Fig. 2. Chlorotic leaf of sweet orange with green patches on narrow lamina.

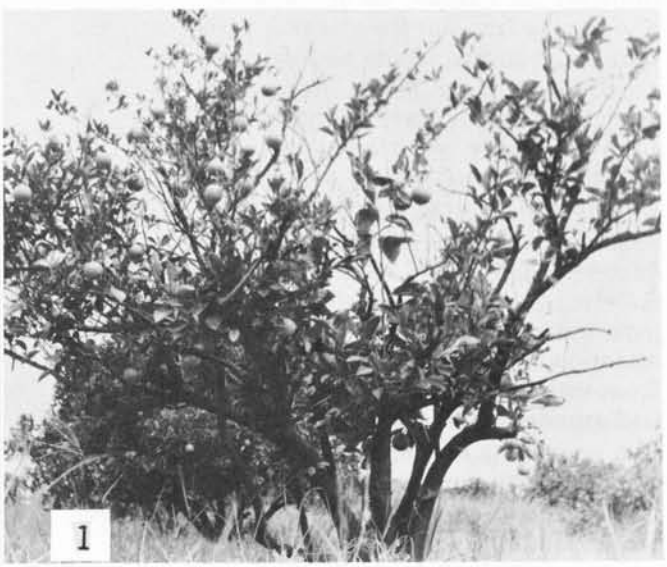

Fig. 1. Greening-affected Mosambi sweet orange tree showing sparsely foliated branches, twig dieback, and poor yield.

Transmission by tissue grafts. A high percentage of transmission was obtained by bark or leaf-patch grafting. Shoot grafts almost always transmitted greening. Budding was less successful because of failure of bud-take. Symptoms appeared within eight weeks in summer (March to June) and 10 weeks in other months, in Poona. Sometimes test seedlings developed symptoms after two years or more.

Transmission by mechanical means. Fifteen plants each of *Phaseolus vulgaris, Vigna sinensis, Nicotiana tabacum* cvs White Burley and Xanthi-nc, *N. sylvestris, Chenopodium quinoa, C.*

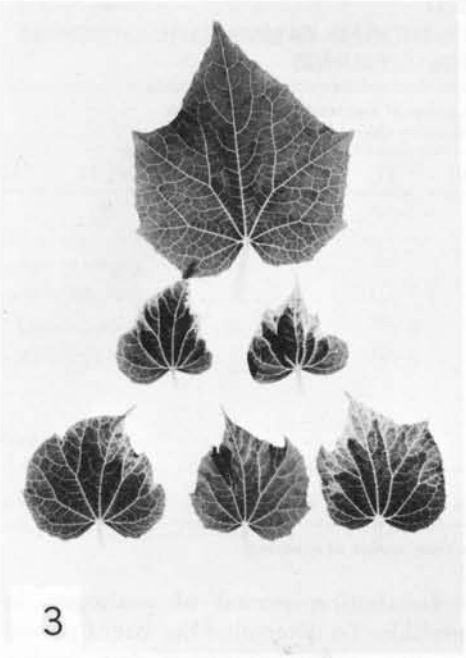

Fig. 3. Leaves of *Cucumis sativus* cv. Long Green, showing characteristic symptoms following mechanical inoculation with extract from leaves of greened sweet orange. Healthy leaf at top.

amaranticolor, Carica papaya, Cucumis sativus cvs. Long Green and Khira Local, *C. melo,* and *Citrullus vulgaris* cv. *fistulosus* were inoculated mechanically. Four plants of each species were kept as uninoculated controls. Yellowing, puckering, and marginal distortion at the apical region of the first and the second true leaves appeared in *C. sativus* cv. Long Green (fig. 3) and *C. vulgaris* cv. *fistulosus.* Subsequent leaves, however, appeared normal. Back inoculation from cucumber to citrus was unsuccessful. Vigor of cucumber plants was greatly reduced, and leaf size was about half that of normal leaves. Schwarz (*14*) reported similar results with the National Pickling variety of cucumber infected with South African greening disease.

Seed transmission. Twenty-four hundred seeds of Mosambi sweet orange and 850 seeds of grapefruit extracted from greened fruits were sown for a test of seed transmission. Normal-looking seeds and brown and abortive seeds were planted. Of these, 2,025 seeds of sweet orange and 704 of grapefruit germinated and produced normal plants. No evidence was found of seed transmission of greening in seeds of sweet orange and grapefruit.

Transmission by insects. Although *Diaphorina citri* was established as the vector of citrus greening in India (*4*), attempts were made to transmit greening by means of other insects collected on greened sweet orange shoots in orchards in Poona.

Insect species tested were: *Toxoptera citricidus* (Kirk.); *T. aurantii* (B. de F.); *Phyllocnistis citrella* (Staint); *Dialeurodes citri* (Ash.); *Papilio demoleus* L.; and an unidentified leafhopper, *Eupocretis* sp. Greening appeared only in test seedlings exposed to psyllids.

RELATIONSHIPS OF THE GREENING PATHOGEN WITH ITS VECTOR

In preliminary experiments on transmission of greening, adult female psyllids were collected from greened trees, and liberated directly on test seedlings. Results were erratic, and percentage of transmission was low. Subsequently, psyllids were fed on a diseased plant in the insectary for 24 hours and then liberated on to the test plants for the life of the insect. Percentage of transmission of disease in these tests was uniformly high. These observations indicated a biological relationship between greening and the psyllids.

Effect of numbers of psyllids on transmission. Adult psyllids were maintained on diseased sweet orange seedlings and liberated on test seedlings for

TABLE 1
INCUBATION PERIOD OF GREENING PATHOGEN IN DIAPHORINA CITRI ON SWEET ORANGE SEEDLINGS

Groups of psyllids	Greening symptoms in a series of test seedlings exposed to feeding of psyllids on successive days after access feeding*											
	2	4	6	8	9	10	11	12	13	14	15	16
First.....												
Second...	–	–	–	–	+	+	+	+	+	+	+	+
Third.....												
Fourth....												
Fifth.....	–	–	–	–	–	–	–	–	+	+	+	+
Sixth.....	–	–	–	+	+	+	+	+	+	+	+	+
Seventh..												
Eighth....	–	–	–	–	–	–	–	–	+	+	+	+
Ninth.....	–	–	–	–	–	–	+	+	+	+	+	+
Tenth.....	–	–	–	–	–	+	+	+	+	+	+	+

* + = positive transmission of greening pathogen; – = no transmission of greening.

a period of 24 hours. Sixty test seedlings were exposed to varying populations of psyllids. Forty-one of 60 plants exposed to a single psylla were infected with greening. Groups of five or more psyllids infected all 60 plants.

Minimum-acquisition feeding period. Adult psyllids maintained on healthy sweet orange seedlings were fasted for 2 hours before access feeding on diseased plants. The psyllids were then transferred, in groups of five, to test plants and allowed to feed for a minimum period of 12 days (*4*). The psyllids transmitted the disease to 45 of 50 test plants after access feeding of 15 minutes, and to all the plants after access feeding of 30 minutes or longer.

Minimum-infection feeding period. Freshly emerged adult psyllids were caged on diseased sweet orange seedlings for a period of 12 days. Psyllids from these colonies were transferred, in groups of five, to each of the test seedlings for periods ranging from 5 minutes to 24 hours. Psyllids transmitted greening in a minimum infection feeding of 15 minutes, but the percentage of transmission was low. One hundred per cent infection was obtained when the psyllids fed for 1 hour or more.

Incubation period of pathogen in psyllids. To determine the latent period in the insect, groups of five freshly emerged, pathogen-free female psyllids were fed on diseased plants for 24 hours, and transferred serially to healthy test seedlings of sweet orange every second day for the first 8 days and every day for the second 8 days. Data in table 1 show that a waiting period of from 8 to 12 days is required before the psyllid can transmit the pathogen following acquisition feeding. Only 10 per cent of the psyllids could transmit the disease on the eighth day, while all transmitted after 12 days.

In similar experiments with 4th- and 5th-instar nymphs, it was observed that the incubation period in the adult was reduced by several days.

Retention by psylla. Freshly emerged female psyllids were fed on diseased sweet orange seedlings for 24 hours, and transferred to a series of test seedlings. The first transfer was made at 2 days and the others at intervals of 8 days until the insects died. Under the conditions of the experiment, the psyllids lived for 70 to 86 days, although the life span of disease-free psyllids is about 150 days at Poona. The test was re-

TABLE 2
RETENTION OF GREENING PATHOGEN BY ADULTS OF DIAPHORINA CITRI ON SWEET ORANGE SEEDLINGS

Experiment no.	Test plants serially exposed to psyllids on successive transfer at intervals (days)*												
	2	10	18	26	34	42	50	58	66	74	76	78	86
1†.....	–	–	+	+	+	+	+	+	+	+	+	+‡	
2.....	–	+	+	+	+	+	+	+	+	+	+	+	+‡
3.....	–	–	+	+	+	+	+	+	+‡				
4.....	–	–	+	+	+	+	+	+	+	+	+	+	+‡
5.....	–	–	+	+	+	+	+	+	+	+	+	+‡	
6.....	–	+	+	–	+	+	+	+	+	+	+	+	+‡
7.....	–	–	+	+	+	+	+	+	+	+	+	+	+‡
8.....	–	–	+	+	+	+	+	+	+	+‡			
9†.....	–	–	+	+	+	+	+	+	+	+	+‡		
10†.....	–	–	+	+	+	+	+	+	+	+	+‡		

* + = positive transmission of greening pathogen; – = no transmission of greening.
† Psyllids collected from orchard trees.
‡ Psyllids died.

peated 10 times, starting with a group of five adults for each test. Three of the 10 tests in table 2 were carried out with freshly emerged adult psyllids collected from orchard trees of sweet orange. Results of these experiments (table 2) clearly show that the psyllids retained infectivity throughout their life span.

Transmission by nymphs. The vector *Diaphorina citri* has five nymphal instars in addition to the egg and the adult (*8*). Nymphs of all stages readily settle down to feed if young and succulent flush is available. Nymphs of each instar were collected and fed for 24 hours on diseased sweet orange in the insectary and then transferred, in groups of 20, to each test plant and allowed to feed for 15 to 20 days until they emerged into adults. The freshly emerged adults were transferred to another test plant for the rest of their life span. In each series, 50 test plants were exposed to nymphs of each instar. The data in table 3 show that the 1st-, 2nd-, and 3rd-instar nymphs were unable to acquire the pathogen. The nymphs of the 4th- and 5th-instars, however, did acquire it and transmitted it to a high percentage of test seedlings of both series (193/200 plants). Symptoms appeared in 110 to 150 days.

Effect of number of nymphs on transmission. Fourth- and 5th-instar nymphs

TABLE 3
TRANSMISSION OF GREENING PATHOGEN BY NYMPHS OF DIAPHORINA CITRI ON SWEET ORANGE SEEDLINGS

Nymphs	Number of test plants infected/number exposed	
	1st stage*	2nd stage†
First instar	0/50	0/50
Second instar ..	0/50	0/50
Third instar.....	0/50	0/50
Fourth instar	43/50	50/50
Fifth instar	50/50	50/50

* Indicates first series of test plants on which nymphs were liberated for test feeding for first 15 days.

† Indicates series of test plants on which nymphs were transferred for life after 15 days of feeding on first series of test plants.

fed for 24 hours on diseased sweet orange were transferred, singly and in groups of 3, 5, 10, 25, and 50, to each of the 20 test seedlings. Twenty-five or more nymphs per test plant gave 100 per cent transmission, whereas only 15 per cent of the plants became infected when only 1 nymph per plant was used.

Absence of transovarial transmission. Freshly emerged adults of *Diaphorina citri* were colonized on diseased plants of sweet orange. Close watch was maintained in order to pick up females that had copulated. These were then transferred to healthy, luxuriantly growing seedlings of Mosambi sweet orange, on which they laid eggs. Nymphs that hatched were carefully transferred, in groups of 5, to test seedlings, where they spent their entire lives. None of the 304 test seedlings that were colonized with 1,520 nymphs over a period of 3 years developed greening. These data prove that greening is not carried over to the next generation through the eggs of infective females of *D. citri.*

DISCUSSION AND CONCLUSIONS

The greening disease of citrus in the Deccan Trap Country in India appears similar to the South African greening and Philippines leaf mottling diseases since all three induce identical symptoms in certain citrus species, and are transmitted by psyllids. Since mycoplasmalike organisms are found associated with these diseases of citrus (*10*), and are not found in healthy citrus tissues, it has been concluded that they are the probable cause of greening or citrus decline in India, greening in South Africa, and leaf mottling in the Philippines.

The relationship existing between the greening disease pathogen and the vector, *Diaphorina citri,* is clearly shown to be a circulative type (*2*). The psyllid requires an incubation period of about 21 days in which to transmit the pathogen, which it retains for life following a short access feeding on a diseased plant. Apparently most of the psyllids in orchards usually acquire the pathogen as 4th- and 5th-instar nymphs, and become vectors for life after going through a latent period of 5 to 9 days. It is unnecessary for adult psyllids arising from infectious nymphs to have access feeding on diseased shoots in order to become vectors. Nor is it essential that the psyllids have access feeding as nymphs in order to become infectious in the adult stage—a requirement with thrips, the vector of tomato spotted-wilt virus, which must feed on infected plants in the larval stage in order to become viruliferous (*1*).

The significance of these findings is that psyllids continue to serially infect healthy test plants over a long period, and remain infective after molting. This strongly indicates that the causal agents of greening multiply in the body of the psyllid. The greening pathogen may be classed as propagative as well as circulative (*2*). Since there is no transovarial transmission, however, it would be necessary to demonstrate multiplication of greening pathogen in psyllids in order to class it finally as propagative. The only other disease known to be transmitted by a psyllid, pear decline, vectored by *Psylla pyricola* Foerster, is caused by a circulative pathogen (*9*).

In South Africa the vector of the greening pathogen, *Trioza erytreae* (Del Guercio), does not require an incubation period, and becomes infective after 24 hours on diseased tissues. The greening pathogen persists for at least 2 to 3 weeks in adults, but nymphs do not appear to transmit it, and there is no evidence for transovarial transmission (Dr. H. D. Catling, personal communication). How Philippines leaf mottling disease pathogen is related to its vector, *Diaphorina citri,* is not known.