# Acquisition, Interference, and Retention of Cucurbit Leaf Curl Viruses in Whiteflies

S. Cohen, J. E. Duffus, and H. Y. Liu

Visiting scientist, Volcani Institute of Agricultural Research, Bet-Dagan, Israel, and plant pathologists, USDA-ARS, 1636 E. Alisal St., Salinas, CA 93905.

The work was conducted at the U.S. Agricultural Research Station, Salinas, and was supported by BARD grant project I-589-83.
We thank Willy Wasik and Tamar Cohen for valuable technical assistance.
Accepted for publication 28 July 1988 (submitted for electronic processing).

## ABSTRACT

Cohen, S., Duffus, J. E., and Liu, H. Y. 1989. Acquisition, interference, and retention of cucurbit leaf curl viruses in whiteflies. Phytopathology 79:109-113.

Squash leaf curl virus (SLCV) antigens could be detected by enzyme-linked immunosorbent assay (ELISA) in *Bemisia tabaci* extracts when batches of at least 20 females previously fed for 48 hr or more on a SLCV source were tested. ELISA reaction intensity of extracts depended on the age of the squash source plants and the position of the leaves on which *B. tabaci* fed. ELISA detectable virus antigen and transmission rate were higher with a longer acquisition access period. SLCV antigen detected by ELISA decreased rapidly with time after acquisition feeding, but the insects remained inoculative for many more days. As long as SLCV antigen could be detected in *B. tabaci*, no significant decrease in transmission efficiency was observed. A reduction in transmission efficiency of melon leaf curl virus (MLCV; closely related to SLCV) by *B. tabaci* was demonstrated when insects were first allowed to acquire SLCV. A higher SLCV antigen titer per unit weight was found to accumulate in the nonvector whitefly, *Trialeurodes abutilonea*, than in *B. tabaci*. These findings are compatible with a model described for luteoviruses, in which the virus in the haemocoele serves as a reservoir for the salivary gland system where virus specific sites exist. It appears, however, that once the SLCV becomes attached to these sites, it remains infectious, but can no longer be detected by ELISA. In the case of *T. abutilonea*, the inability of the virus to pass through the salivary glands is possibly the reason for its failure to transmit SLCV.

Many of the whitefly-borne viruses are transmitted in a persistent circulative manner (2,3,7,21). So far, where the virus has been determined to be circulative in the whitefly vector, the viruses have been classified as geminiviruses. There is no direct evidence for the multiplication of the circulative viruses in the whitefly. However, there are some indications that the pathway of these viruses is not passive. For instance, tomato yellow leaf curl virus (TYLCV) (10) triggers an antiviral mechanism in *Bemisia tabaci* (Genn.). Two factors were found in homogenates of whiteflies carrying TYLCV. The introduction of these materials into the whiteflies before or after TYLCV acquisition resulted in reduced ability of the insects to acquire and transmit the virus. These factors apparently are responsible for the phenomenon of "periodic acquisition" of the virus (5,6,8,9,19,20). A similar phenomenon was observed with tomato yellow mosaic in India (24). Squash leaf curl virus (SLCV) has an apparent harmful effect on the vector (7).

This paper describes additional information on the behavior of SLCV in its vector, *B. tabaci*, in its nonvector, *Trialeurodes abutilonea* (Hald), and on the transmission interference in *B. tabaci* between two closely related geminivirus isolates responsible for the leaf curl disease complex of cucurbits in the desert of southwestern U.S. (11). These two viruses, distinguished by host range and serology, are SLCV and melon leaf curl virus (MLCV) (13). The SLCV infects *Cucurbita maxima* Dcne., *C. moschata* Dcne., *C. pepo* L., and *Phaseolus vulgaris* L., but not melons (*Cucumis melo* L.), cucumbers (*C. sativus* L.), or watermelons (*Citrullus vulgaris* Schrad.). MLCV infects all of the above mentioned species.

## MATERIALS AND METHODS

**Virus source and whitefly maintenance.** SLCV was maintained in squash (*Cucurbita pepo*) and MLCV in watermelon (*Citrullus vulgaris*). The viruses were transferred from plant to plant with the whitefly vector, *B. tabaci*. Colonies were reared on sweet potato (*Ipomoea batatas* (L.) Lam.) grown in muslin-covered cages. The cages were kept in an insectary greenhouse. Sweet potato is immune to the virus isolates under study. *T. abutilonea* colonies were reared on *Physalis alkekengi* L. grown in muslin-covered cages. The cages were maintained in a growth room at temperatures that ranged from 21 to 27 C. Unless otherwise stated, young leaves (3–4 cm in length) from squash plants that had been inoculated 2–3 mo earlier were used as the SLCV source. Squash plants in the first true leaf stage of growth were used as test plants. Young leaves of watermelon plants 15–20 days after inoculation were used as the MLCV source. Watermelon plants in the first true leaf stage were used as test plants. Following inoculation by whiteflies, plants were sprayed with resmethrin before they were returned to greenhouses. All plants were grown in screened greenhouses fumigated at weekly intervals with vapona and resmethrin.

**Virus transmission tests.** Transmission tests were made by using a leaf-cage method described previously (8). Virus retention tests were performed by using adult females collected from the same colony. The whiteflies were allowed different acquisition feedings and were then transferred individually at 2-day intervals onto healthy squash plants.

**Enzyme-linked immunosorbent assay (ELISA) tests.** Leaf extracts were prepared in a micromortar by grinding a 5-mm-diameter disk of tissue in 0.2 ml of phosphate-buffered saline (0.02 M, pH 7.4) with 0.5 ml of Tween-20 per liter, 20 g of polyvinylpyrrolidone ($M_r$ 44,000) per liter, and 2 g of egg albumin per liter. Whitefly homogenates were prepared in a micromortar by grinding in 0.2 ml of the same buffer. The antiserum to SLCV was prepared in the Salinas laboratory (7).

The double-antibody sandwich method described by Clark and Adams (4) was used, except that the coating globulin was used at 1 $\mu$g/ml and enzyme-conjugated globulin was 1:400. The same bleeding of immunoglobulin and enzyme-conjugated globulin were used in all the tests. $A_{405}$ values were recorded 30 min after adding the substrate. During that period the plate was left in an incubator at 37 C.

In each of the microtiter plates, the following control samples were routinely used: six to nine samples of 20 whiteflies previously fed on sweet potato, two samples from SLCV-infected squash, and two from healthy squash.

©1989 The American Phytopathological Society

Where homogenates from more than 20 whiteflies were used, an additional six to nine control samples were used with the corresponding numbers of virus-free whiteflies. When *T. abutilonea* was used, additional controls were prepared from whiteflies fed on *P. alkekengi*. Data were expressed as a percentage of the appropriate negative (nonviruliferous whitefly) control or in $A_{405}$ values.

**Interference between SLCV and MLCV in *B. tabaci*.** *B. tabaci* females collected from young colonies were divided into two or three groups. One group was given a 48-hr acquisition access feed on SLCV-infected squash leaves, followed by a 24-hr acquisition feed on MLCV-infected watermelon leaves. The second group was fed on healthy squash for 48 hr, followed by a 24-hr acquisition access feed simultaneously with the first group on the same MLCV-infected watermelon leaves. The third group was given a 48-hr acquisition feed on SLCV-infected squash leaves, followed by a 24-hr feeding on healthy watermelon. The whiteflies of the first and second groups were placed individually on watermelon seedlings for a 48-hr inoculation feed on watermelon seedlings. Those of the third group wers placed individually on healthy squash plants for 48 hr. Results of the experiments were analyzed by the sign test or the Mann-Whitney U test (22) at $P \leq 0.05$.

## RESULTS

**Effect of source plant on the uptake of SLCV by *B. tabaci*.** The relative concentration of SLCV antigen was determined by ELISA in young and old leaves, collected from plants infected for 15–20 days and from plants infected for 2–3 mo. The results indicate a higher antigen content in young leaves compared with older leaves of the same plant and a higher content in older plants compared with younger ones (Table 1). Also, a higher SLCV antigen content was found in whiteflies fed on old plants than in those fed on young plants (Fig. 1).

In previous work, a high percentage of the whiteflies fed on young leaves of young plants became infective (7). Based on the results of the ELISA tests (Table 1 and Fig. 1), it was important to compare virus acquisition from young and old plants. In this experiment, whitefly females were given a 48-hr acquisition access on five different young leaves from young and old plants, portions of which were tested by ELISA. At least 10 insects were selected from each leaf and placed individually on healthy squash for a 48-hr inoculation feed. Sixty percent (30 out of 50) transmission was achieved by the whiteflies fed on young plants, compared with 60.3% (41 out of 68) by those fed on old plants. These results indicate no apparent difference in efficiency of transmission between whiteflies fed on virus sources with antigen content within the range of 0.375–0.630 $A_{405}$ values.

**Detection of SLCV antigen in *B. tabaci* by ELISA.** ELISA detected SLCV antigen in homogenates prepared from 10 or more whiteflies previously fed on SLCV-infected plants for 48 hr, but not from whiteflies fed on sweet potato or healthy squash (Fig. 2). These differences were found in all tests made with 10 or 20 whiteflies. No differences were observed between whiteflies fed on two different control plants, i.e., healthy squash and sweet potato.

SLCV could also be detected in homogenates when groups of 20 inoculative whiteflies were mixed with 80 virus-free ones; in three experiments with a total of 21 replications, ELISA readings averaged 32, 23, and 48% greater than the controls.

An increase in SLCV antigen was detected in *B. tabaci* extracts by ELISA when the acquisition access period was increased from 1 to 6 days (Fig. 1).

**Retention of SLCV antigen by *B. tabaci*.** In these experiments the whiteflies were given an acquisition access period of 48 hr on young leaves from old or young plants and then transferred to sweet potato. After removal from the virus source, SLCV antigen decreased in whiteflies with time (Fig. 3). Viral antigen could not be detected by ELISA in insects fed on the young leaves of young plants 2 days (Fig. 3B) after leaving the source. When the whiteflies fed on young leaves of older plants (Fig. 3A), the SLCV antigen was still detectable 6 days after removal from the source, but the antigen concentration was one-third of its initial level.

A significantly higher antigen concentration was observed in homogenates of whiteflies 1 day after being removed from high virus content sources (Fig. 3A), compared with that found in those tested immediately after the end of acquisition access. This was thought to result from weaker insects dying in the transfer process from the acquisition source to the sweet potato holding plants.

**Retention of SLCV antigen by *T. abutilonea*.** No transmission of SLCV was achieved by *T. abutilonea* in tests involving several thousand insects. The experiments on the retention of SLCV and/or SLCV antigen in these insects were conducted by employing the same techniques used for *Bemisia* experiments. However, extracts from 15 *T. abutilonea* were used in each sample. This number of *Trialeurodes* whiteflies weighs approximately the same as 20 *B. tabaci*. The results summarized in Figures 3 and 4 show that the initial amount of SLCV antigen acquired by *T. abutilonea* during a 48-hr acquisition access is roughly six to nine times higher than that acquired by *B. tabaci*. Also, *T. abutilonea*

TABLE 1. ELISA absorbance readings for squash leaf curl virus in squash (mean $A_{405}$ values of 10–20 samples of indicated tissue)[a]

| Test no. | 2–3 mo after inoculation: Youngest leaf | 2–3 mo after inoculation: 3rd youngest leaf | 10–20 days after inoculation: Youngest leaf | 10–20 days after inoculation: 3rd youngest leaf | Healthy squash control |
|---|---|---|---|---|---|
| 1 | 0.612 a | 0.434 bc | 0.363 c | 0.248 d | 0.036 |
| 2 | 0.627 w | 0.249 y | 0.382 x | 0.174 z | 0.043 |

[a] Numbers marked with different letters differ significantly at $P \leq 0.05$ with the Mann-Whitney U test.

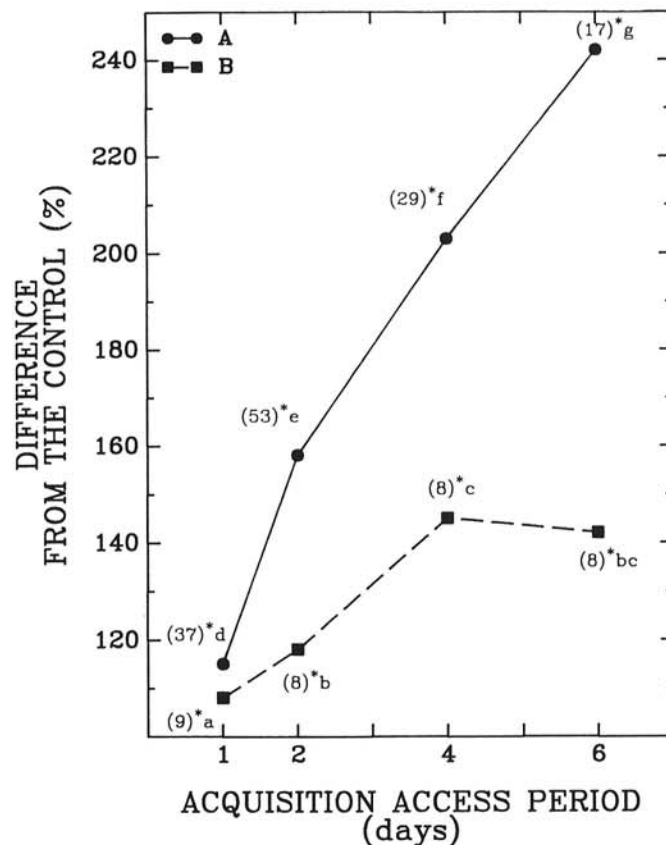


**Fig. 1.** Effect of acquisition access period on the squash leaf curl virus antigen content in *Bemisia tabaci*. Percentages represent homogenates of 20 insects per sample. Percentages with different superscripts differ from each other; those marked with an asterisk differ from the control. The number in parentheses equals the number of samples tested. **A** and **B** were analyzed independently from each other. Controls were homogenates of whiteflies fed on healthy squash. The mean $A_{405}$ values of the controls were between 0.025 and 0.035 in all tests. **A**, Virus source from young leaves of squash plants infected with SLCV for 2–3 mo. **B**, Virus source from young leaves of squash plants infected with SLCV for 15–20 days.

retained SLCV antigen for at least the same period as *B. tabaci*.

**Persistence of SLCV in *B. tabaci*.** The results of serial transfers of individual whiteflies show that 86% of the plants fed on were infected during the first 10 days following 48-hr acquisition feeding (group B) compared with 43% transmission during the days 11–34 (Fig. 5). The difference is significant ($P < 0.01$).

Following 5-day acquisition feedings (group A), 80% transmission was measured during the first 12 days, compared with 59% from days 13–34. The difference is significant ($P < 0.025$).

During the first 10 days, differences in *B. tabaci* transmission efficiency were not detected between the groups with 2-day and 5-day acquisition feedings (86% and 79%, respectively). But differences were found in the period from days 11–34 of the serial transfers (43% and 64%, respectively).

**Transmission interference between SLCV and MLCV in *B. tabaci*.** As previously mentioned, MLCV infects all the hosts that are susceptible to SLCV but not vice versa. Therefore, in the transmission interference tests, SLCV was used to test the interference of the transmission of MLCV. In all five experiments (Table 2), a reduction in transmission efficiency of MLCV occurred for whiteflies previously fed on SLCV, compared with those fed on MLCV only. The reductions were between 36–87% in the different tests. It should be noted that SLCV was acquired and transmitted by the whiteflies much more efficiently than by MLCV.

## DISCUSSION

The SLCV and MLCV have transmission characteristics typical of persistently transmitted circulative viruses (7,13, present work).

The virus antigen content in *B. tabaci* increased with an increase in the acquisition feeding period (Fig. 1). An increase in transmission efficiency accompanied the increase in the acquisition access period (7) (Fig. 5), suggesting an increase in virus content. However, during the first 10 days, no differences in the transmission efficiency were observed between whiteflies previously fed for 48 hr or 5 days on SLCV (Fig. 5). Thus, it seems that antigen and/or virus levels detectable by ELISA during the first 6 days after acquisition are not correlated with transmission efficiency during this early period but are in the later period. In all cases, *B. tabaci* females were inoculative many days after the virus could no longer be detected by ELISA in their bodies. The decrease in antigen detection with time may be due to the production of antiviral factors in the whiteflies, as previously reported for tomato yellow leaf curl virus (6,19,20), or to natural losses in the feeding process.

The evidence on the interference of SLCV with MLCV transmission in *B. tabaci* (Table 2) leads to the possibility that the mechanism involved in the transmission of SLCV is similar to that described in the case of luteoviruses in aphids (14,15,16,17,18,23).

If mechanisms similar to those suggested by Gildow and Rochow (16,17,18) are operative in these whitefly-transmitted viruses, this may explain the failure to detect the virus by ELISA after a relatively short period (Fig. 3). Thus, while attaching to the membrane of the accessory salivary glands, at least part of the capsid surface is blocked, which may lower the probability to react with the antiserum. Preliminary investigations (F. Gildow, personal communication) indicate whitefly salivary gland structure may be quite similar to that of aphids. However, Al Musa

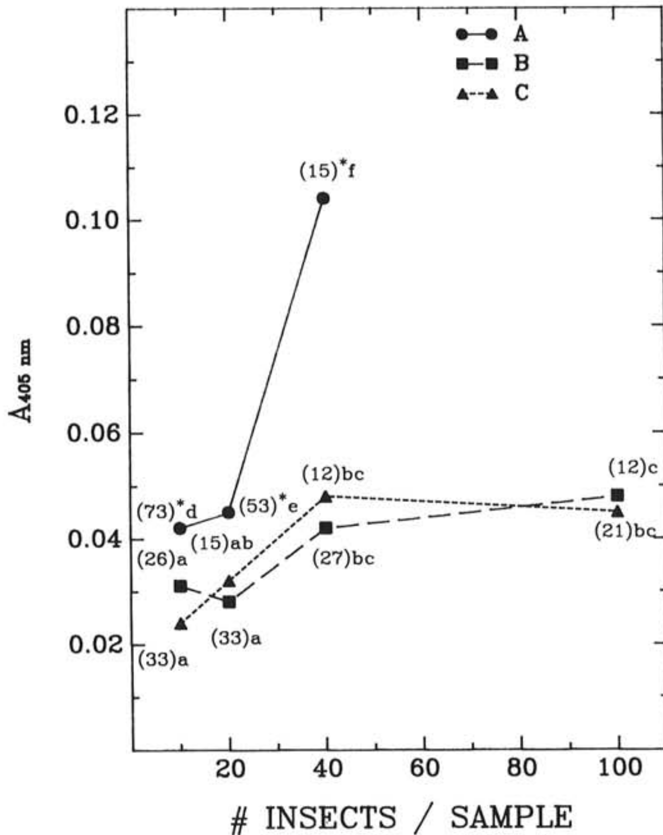


**Fig. 2.** Detection of squash leaf curl virus antigen in samples containing different numbers of *Bemisia tabaci*. $A_{405}$ values with different superscripts in **A**, and in **B** and **C** are different from each other. **A** was analyzed independently from **B** and **C**. The control for **A** was homogenates of whiteflies fed on healthy squash, and all values in **A** were different from the controls as indicated by asterisks. The number in parentheses equals the number of samples tested. **A**, *B. tabaci* fed for 48 hr on young leaves of squash plants infected with SLCV for 2–3 mo. **B**, *B. tabaci* fed on sweet potato. The standard deviation is 0.006–0.007. **C**, *B. tabaci* fed for 48 hr on healthy squash. The standard deviation is 0.006–0.010.

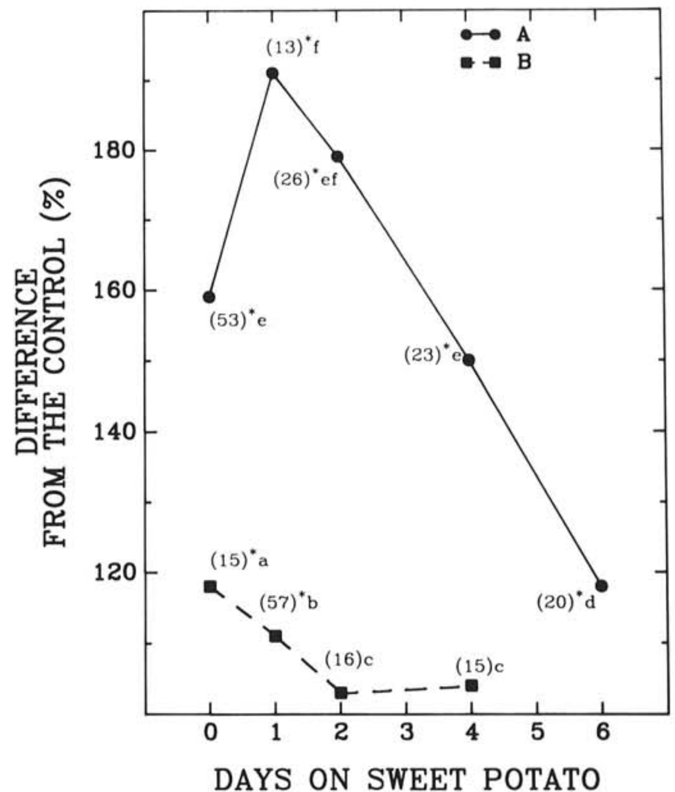


**Fig. 3.** Retention of squash leaf curl virus antigen in *Bemisia tabaci*. Percentages represent homogenates of 20 insects per sample previously given an acquisition access period of 48 hr on SLVC-infected squash. Percentages with different superscripts differ from each other; those marked with an asterisk differ significantly from the control. The number in parentheses equals the number of samples tested. **A** and **B** were analyzed independently from each other. Controls were homogenates of whiteflies fed on healthy squash. The mean $A_{405}$ values of the controls were between 0.025 and 0.035 in all tests. **A**, Virus source from young leaves of squash plants infected with SLCV for 2–3 mo. **B**, Virus source from young leaves of squash plants infected with SLCV for 15–20 days.

et al (1) failed to detect viral nucleic acid in insect extracts in as short a period as 72 hr after acquisition. Thus, the possibility exists that the amount of virus required to achieve transmission is much lower than the amount that can be detected by ELISA or spot hybridization.

The ELISA technique cannot be used as the only tool in epidemiological studies to evaluate the rate of inoculative vectors in the population, because inoculativity survives by many days the ability to detect virus by ELISA. However, *T. abutilonea*, which is not a vector, but is common in the area of SLCV (12), acquired markedly more virus than *B. tabaci* and therefore may be used in epidemiological studies as a marker for the presence of SLCV in the region.

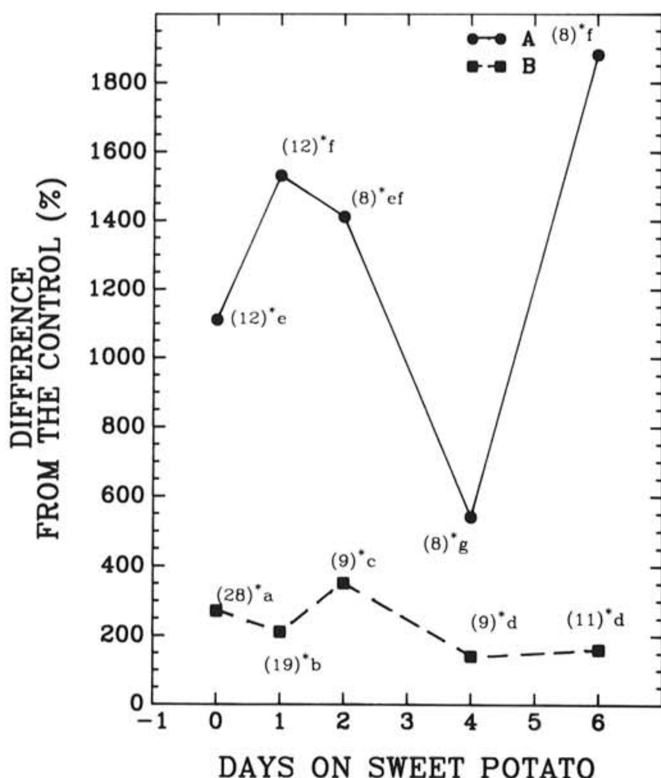


**Fig. 4.** Retention of squash leaf curl virus antigen in *Trialeurodes abutilonea*. Percentages represent homogenates of 20 insects per sample previously given an acquisition access period of 48 hr on SLCV-infected squash. Percentages with different superscripts differ from each other; those marked with an asterisk differ from the control. The number in parentheses equals the number of samples tested. **A** and **B** were analyzed independently from each other. Controls were homogenates of whiteflies fed on healthy squash. The mean $A_{405}$ values of the controls were between 0.025 and 0.035 in all tests. **A**, Virus source from young leaves of squash plants infected with SLCV for 2–3 mo. **B**, Virus source from young leaves of squash plants infected with SLCV for 15–20 days.

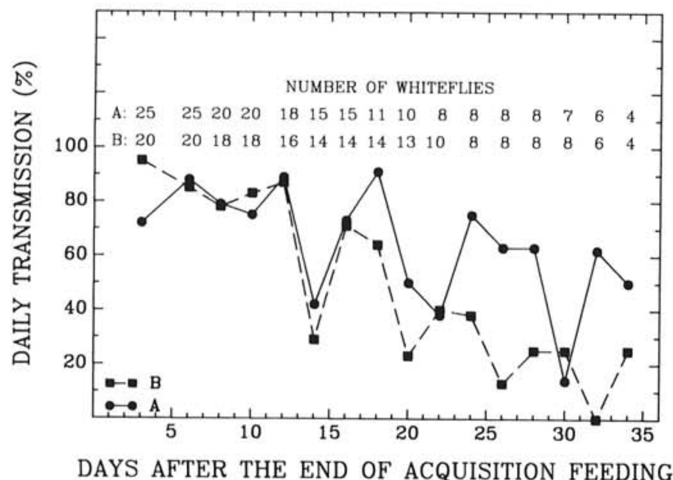


**Fig. 5.** Persistence of squash leaf curl virus in *Bemisia tabaci* females. **A**, 5-day acquisition feeding. **B**, 2-day acquisition feeding.

TABLE 2. Effect of squash leaf curl virus on transmission of melon leaf curl virus by *Bemisia tabaci*

| Test no. | Transmission of SLCV (%)[a] | Transmission of MLCV (%)[b] | Transmission of MLCV (%)[c] after acquisition of SLCV |
|---|---|---|---|
| 1 | 89.4 | 22.2 | 14.2 |
| 2 | ··· | 50.0 | 27.7 |
| 3 | 94.4 | 77.7 | 27.7 |
| 4 | ··· | 37.5 | 5.5 |
| 5 | ··· | 57.1 | 7.6 |
| Total | 91.8 (34/37)[d] | 48.9 (47/96) | 17.3 (14.81) |

[a]Transmission percentages of individual whiteflies fed in sequences for 48 hr on SLCV-infected squash, 24 hr on healthy watermelon, and 48 hr on healthy squash. ··· = test not done.

[b]Transmission percentages of individual whiteflies fed in sequence for 48 hr on healthy squash, 24 hr on MLCV-infected watermelon, and 48 hr on healthy watermelon.

[c]Transmission percentages of individual whiteflies fed in sequence for 48 hr on SLCV-infected squash, 24 hr on MLCV-infected watermelon, and 48 hr on healthy watermelon.

[d]Numerator indicates number of plants infected, and denominator indicates the number of plants inoculated. The transmission efficiency differences are significant at $P \leq 0.05$.

## LITERATURE CITED

1. Al Musa, A., Polston, J. E., Perring, T. M., and Dodds, J. A. 1986. Use of spot hybridization with a cloned probe for squash leaf curl virus to investigate vector relationships. Pages X.14-X.16 in: Proc. Workshop Epidemiol. Plant Virus Dis. Orlando, Florida.
2. Anno-Nyako, F. O., Vetten, H. J., Allen, D. J., and Thottapilly, G. 1983. The relation between cowpea golden mosaic and its vectors, *Bemisia tabaci*. Ann. Appl. Biol. 102:319-323.
3. Bird, J., and Maramorosch, K. 1978. Viruses and virus diseases associated with whiteflies. Adv. Virus Res. 22:55-109.
4. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
5. Cohen, S. 1967. The occurrence in the body of *Bemisia tabaci* of a factor apparently related to the phenomenon of "periodic acquisition" of tomato yellow leaf curl virus. Virology 31:180-183.
6. Cohen, S. 1969. In vivo effects in whiteflies of a possible antiviral factor. Virology 37:448-454.
7. Cohen, S., Duffus, J. E., Larsen, R. C., Liu, H. Y., and Flock, R. A. 1983. Purification, serology, and vector relationships of squash leaf curl virus, a whitefly-transmitted geminivirus. Phytopathology 73:1669-1673.
8. Cohen, S., and Harpaz, I. 1964. Periodic, rather than continual, acquisition of a new tomato virus by its vector, the tobacco whitefly (*Bemisia tabaci* Genn.). Entomol. Exp. Appl. 7:155-166.
9. Cohen, S., and Marco, S. 1970. Periodic occurrence of an anti-TMV factor in the body of whiteflies carrying the tomato yellow leaf curl virus (TYLCV). Virology 40:363-368.
10. Cohen, S., and Nitzany, F. E. 1966. Transmission and host range of the tomato yellow leaf curl virus. Phytopathology 56:1127-1131.
11. Duffus, J. E., and Flock, R. A. 1982. Whitefly-transmitted disease complex of desert southwest. Calif. Agric. 36:4-6.
12. Duffus, J. E., Larsen, R. C., and Liu, H. Y. 1986. Lettuce infectious yellows virus—A new type of whitefly-transmitted virus. Phytopathology 76:97-100.
13. Duffus, J. E., Liu, H. Y., and Johns, M. R. 1985. Melon leaf curl virus—A new gemini virus with host and serological variations from squash leaf curl virus. Phytopathology 75:1312.
14. Gildow, F. E. 1982. Coated-vesicle transport of luteoviruses through salivary glands of *Myzus persicae*. Phytopathology 72:1289-1296.
15. Gildow, F. E. 1985. Transcellular transport of barley yellow dwarf virus into the hemocoel of the aphid vector, *Rhopalosiphum padi*. Phytopathology 75:292-297.

16. Gildow, F. E., and Rochow, W. F. 1980. Role of accessory salivary glands in aphid transmission of barley yellow dwarf virus. Virology 104:97-105.
17. Gildow, F. E., and Rochow, W. F. 1980. Transmission interference between two isolates of barley yellow dwarf virus in *Macrosiphum avenae*. Phytopathology 70:122-126.
18. Gildow, F. E., and Rochow, W. F. 1980. Importance of capsid integrity for interference between two isolates of barley yellow dwarf virus in an aphid. Phytopathology 70:1013-1015.
19. Marco, S., Cohen, S., Harpaz, I., and Birk, Y. 1972. In vivo suppression of plant virus transmissibility by an anti-TMV factor occurring in an inoculative vector's body. Virology 47:761-766.
20. Marco, S., Cohen, S., Harpaz, I., and Birk, Y. 1975. An anti-TMV factor in the tobacco whitefly after acquisition of tomato yellow leaf curl virus. J. Insect Physiol. 21:1821-1826.
21. Russo, M., Cohen, S., and Martelli, G. P. 1980. Virus-like particles in tomato plants affected by the yellow leaf curl disease. J. Gen Virol. 49:209-213.
22. Siegel, S. 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York. 312 pp.
23. Tamada, T., and Harrison, B. D. 1981. Quantitative studies on the uptake and retention of potato leafroll virus by aphids in laboratory and field conditions. Ann. Appl. Biol. 98:261-276.
24. Verma, H. N., Srivastava, K. M., and Mathur, A. K. 1975. A whitefly-transmitted yellow mosaic disease of tomato from India. Plant Dis. Rep. 59:494-498.