Tobacco and Tomato Ringspot Viruses and Their Relationships with Tetranychus urticae

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ABSTRACT

The two-spotted spider mite *Tetranychus urticae* failed to act as a vector of tobacco ringspot virus (TRSV) and tomato ringspot virus (TomRSV). A total of 4,210 mites did not transmit TRSV and 1,800 mites failed to transmit TomRSV. Tobacco ringspot virus, but not TomRSV was detected in mites, when homogenates of acquisition-fed mites were used for mechanical inoculations. In 10 trials, an average of 4.9 local lesions per cotyledon was obtained when 1.0 ml of each homogenate was rubbed on 10 cotyledons. Exposing mites for 10 min, 15 min, or 1 h to 2% formaldehyde; and to a 1:16 dilution of antiserum for 30 min, 1 h, or 24 h prior to homogenizing did not reduce the number of local lesions. The virus remained infective in the mites for at least 24 h. No virus could be recovered from mite feces. Tobacco ringspot virus was not acquired by mites from purified preparations, nor was it released by mites carrying TRSV.

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Seed transmission is characteristic of nematode-transmitted viruses (3). TomRSV and TRSV are seed-transmitted (1, 2, 6, 12) but this is not considered of importance in field spread (1).

The nematode *Xiphinema americanum* is a vector of TRSV (9, 10, 14) and TomRSV (10, 29). These viruses are considered to be primarily soil-borne due to the transmission efficiency of the nematode. TomRSV is apparently limited to nematode transmission, but TRSV has several reported arthropod vectors: the aphids, *Myzus persicae* (13, 21, 24) and *Aphis gossypii* (21); the grasshoppers, *Melanoplus differentialis, M. mexicanus,* and *M. femur-rubrum* (7); the tobacco flea beetle, *Epitrix hirtipennis* (23); the thrips, *Thrips tabaci* (15) and probably *Frankliniella* sp. (4); and the spider mites, *Tetranychus* sp. (30). In all cases percentage of transmission is low when compared to that by the nematode, *X. americanum*.

Among the arthropod vectors of TRSV, the two-spotted spider mite, *Tetranychus urticae* Koch, is the most questionable vector. Mites have not received as much attention as other vectors of plant viruses (16). Eriophyid mites are the only proven vectors of plant viruses. Recently, transmission of potato virus Y by the two-spotted spider mite *Tetranychus telarius* (*T. urticae*) (22) and TRSV by *Tetranychus* sp. (3) has been claimed. Other workers report that *T. urticae* failed to transmit some plant viruses (17), including a strain of TRSV (8, 18). Lack of transmission of TRSV by *Tetranychus* sp. has also been reported (23).

In these studies (8, 18, 30) several species have been used as source and test plants, but the concn of the virus in the source plants, which is of critical importance in both mechanical transmission and purification of TRSV, was not considered. Approximately 6 days after inoculation of 10-day-old cucumber seedlings, TRSV attains a relatively high concn. This peak is maintained for only a few days and after this critical period the concn of the virus is greatly reduced (20). Virus concn in the source plant may influence mite transmission of TRSV.

Since the *T. urticae*-TRSV relationship was unclear and the *T. urticae*-TomRSV unknown, two series of experiments were carried out: (i) acquisition of these viruses by the two-spotted spider mite and their persistence in the mite body; and (ii) transmission of TRSV and TomRSV by *T. urticae*.

MATERIALS AND METHODS.—*Plant material.*—All experiments utilized cucumber (*Cucumis sativus* L. 'National Pickling'), lima bean (*Phaseolus lunatus* L. 'Henderson Bush'), and cowpea [*Vigna sinensis* (Torner) Savi 'Black Crowder']. Seeds were planted in a steamed soil mixture contained in 8.75-cm diam styrofoam pots. Young plants were maintained in a greenhouse at ca. 22-25 C, until suitable for use.

Establishment of colonies.—Approximately 4 wk after planting, host plants were placed in cages screened with dacron ninon. Old plants with severe feeding damage were discarded and replaced with new plants. Newly introduced plants were interspersed among infested plants so that the foliage was in close contact. The temp was maintained at ca. 26 C. Mite populations were easily perpetuated by this method.

Mites of similar age were obtained by transferring adult females, by use of a small brush, to detached lima bean leaves. Approximately 50 females were placed on each leaf and allowed a 24 h oviposition period, after which the adults were removed. These leaves were maintained under continuous fluorescent illumination with the adaxial side on wet cotton pads in petri dishes. When mites reached the protonymph stage, leaves were attached to the host plant with paper clips and observed until eggs were seen, at which time the group was considered to have reached the adult stage.

Survival of T. urticae on cucumber cotyledons and on

lima bean leaves.—Survival of adult mites of the same age was measured in cages similar to those of Colburn (5), shown in Fig. 1. The cage was constructed from a 5-×5-×3-cm plastic box. A hole, 1.5 cm in diam, was drilled in each of four sides of the box and three of these holes were covered with 200-mesh brass screen to provide ventilation. Screen was glued to the exterior surface of the box. A rubber stopper with a 4-mm diam opening was placed in the bottom hole to hold the stem of a cucumber seedling or the petiole of a lima bean leaf. Eight-day-old cucumber seedlings and the first true leaves of 12-day-old lima beans were obtained as previously described. Seedlings were removed from the soil, and the roots gently washed with tap water. Cucumber stems were then placed through the hole in the stopper with the roots remaining outside. Lima bean leaves were trimmed to 3.8 cm^2 and the petioles were placed through the stoppers. Cucumber stems and lima bean petioles were wrapped with cotton to hold them securely and to prevent mite escape.

Ten adult mites were placed in each cage; six replications were used per treatment. Mites in cages without host plants served as controls. Cages were sealed with cellophane tape to prevent mites from escaping. Cages were placed on a 1.5-cm-thick styrofoam base through which the root or petiole protruded to allow for uptake of water from a pan located beneath the styrofoam. Throughout the experiment, cages were kept under continuous fluorescent illumination. Mites were counted daily with a dissecting microscope and the number of surviving mites was recorded. To avoid overlapping of generations, mites were transferred to new hosts every third day.

Transmission of TRSV and TomRSV.—The TRSV isolate used was obtained from soil collected around peach trees which showed stem pitting symptoms (26). Infected cucumbers, lima beans, cowpeas, and purified preparations of TRSV were used as sources of inocula. Ten-day-old Carborundum-dusted seedlings were mechanically inoculated with TRSV. Leaves of rearing hosts that showed high mite infestations were detached and placed on the acquisition hosts 5 days after inoculation. Mites reared on lima beans were transferred to lima bean and cowpea and those reared on cucumber were transferred to cucumber. The acquisition period (the length of time the mites remained on the acquisition host) varied from 5 min to 7 days. Ten mites were transferred to each test plant for a feeding period of 1 to 8 days, and were killed with Kelthane 35 WP [4, 4′dichloro-alpha-(trichloromethyl) benzhydrol, 3.5 g per liter] to terminate the test feeding period. Control plants were brushed gently with the same brush used to transfer the mites to ascertain whether virus was being transmitted mechanically.

Purification procedures for TRSV utilized butanol and chloroform as clarifying agents as previously described by Steere (28). Infected cucumber cotyledons that showed severe symptoms 6 days after inoculation were placed in a chilled blender. For each gram of tissue, 1.0 ml of chloroform and 1.0 ml of n-butanol was added. The mixture was homogenized for 2-3 min and strained through four layers of cheesecloth to remove the fibrous plant material. The filtrate was placed in a 1.0-liter separatory funnel until the organic phase separated from the aqueous fraction. The organic phase was drawn off and discarded. The remaining suspension was then clarified by centrifuging for 10 min at 10,000 rpm in a Sorvall centrifuge model RC-2 utilizing an SS-34 rotor. The pellet was discarded and the supernatant centrifuged for 2 h at 29,000 rpm using a No. 30 rotor in a Spinco centrifuge model L. The supernatant was discarded, and the pellet resuspended overnight in 0.05 M phosphate buffer, pH 7.1. The resuspended pellet was centrifuged at 10,000 rpm for 10 min in the SS-34 rotor, and the resulting supernatant was saved.

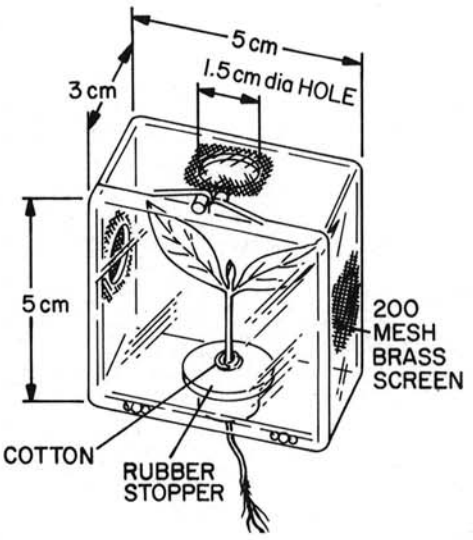

Fig. 1. Cage utilized for survival test of *Tetranychus urticae* (after Colburn).

Approximately 0.3 ml of this partially purified preparation containing ca. 1.29 mg/ml of TRSV, calculated according to Stace-Smith (27), was placed on the top of a feeding membrane. These membranes were similar to that designed for toxicological studies with two-spotted spider mites (11) and shown in Fig. 2.

Plastic tubing 1.5-mm thick and 2 cm in diam was cut into sections 2-cm long. Four holes ca. 6 mm in diam were drilled in each section and covered with 200-mesh brass screen. Parafilm was stretched over one end of each tube. A depression was formed in the parafilm to contain the TRSV preparation. The virus solution was placed in the depression and covered with another piece of parafilm. A lima bean leaf disk 2 cm in diam was placed on top of the second layer of parafilm, covered with a circular cover glass and secured with a 4- to 5-mm-wide parafilm strip wrapped around the tube. The leaf disk was used to stimulate the mites to feed through the parafilm membrane. About 50 mites were placed in each feeding cage through the open end, which was then closed with a layer of parafilm. The cages were kept under continuous fluorescent light with the leaf disk side on top. After 24 h, 10 mites were transferred to each of the cucumbers for a 24-h test feeding period. Infectivity of the purified preparation was checked by mechanical inoculation of Carborundum-dusted cucumber and cowpea seedlings before it was placed on the feeding membrane and after exposure to the mites.

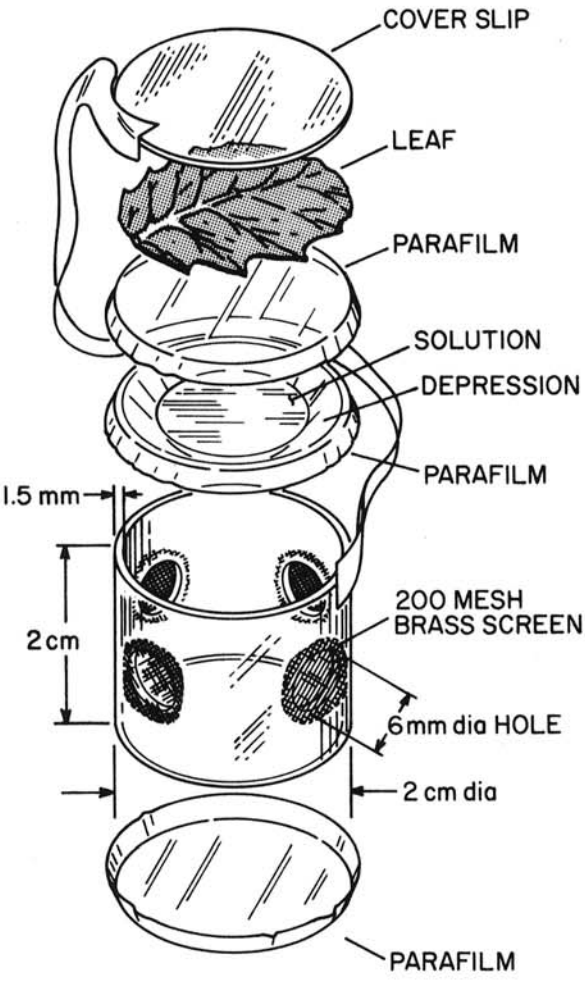

Fig. 2. Feeding cage utilized for acquisition of purified preparation of tobacco ringspot virus by *Tetranychus urticae* (after Hanna and Hibbs).

In the transmission experiments with TomRSV, the sources of inocula were infected cucumber and cowpea seedlings. The test plants included cucumber, cowpea, and lima bean. A 2-day acquisition period and a 1-day test feeding period were given. The method of mite transfer from the rearing hosts to acquisition and test hosts was as previously described for TRSV.

The test plants for both the TRSV and TomRSV were observed for symptoms over a 30-day period, after which recovery of the virus was attempted by grinding the inoculated plants in a mortar containing 0.05 M phosphate buffer, pH 7.1, and mechanically inoculating the crude sap to Carborundum-dusted cucumber and cowpea seedlings. Plants inoculated during the attempted recovery were again used for subinoculations 15 days later as a further check for the presence of virus. This was done because it has been demonstrated that visible symptoms can be obtained by subinoculation of virus-infected plants that did not show symptoms following insect inoculation (25).

Serology.—Homogenates prepared from mites that had fed 24 h on cucumber seedlings inoculated with TRSV, were used as antigen and homogenates of mites unexposed to the virus served as controls. Homogenates were prepared by grinding 400 mites in 1 ml of 0.05 M phosphate buffer, pH 7.1. Agar-gel diffusion tests were performed in 9-cm diam plastic petri dishes containing a layer of 0.75% Ionagar No. 1 3-mm deep, dissolved in an aqueous solution of 0.85% sodium chloride and 0.01% sodium azide. The antiserum was placed in the central well, which was 8 mm in diam and the antigen was placed in the four peripheral wells, which were 6 mm in diam and 15 mm apart. The antigen wells were 8 mm from the antiserum well. A 2-fold dilution series in 0.85% saline solution was established for the antiserum. The mite homogenates were not diluted. The petri dishes were incubated at ca. 21 to 25 C.

Recovery of TRSV and TomRSV from mites.—Inoculation of acquisition hosts and transfer of mites from rearing plants to the source of virus was done as previously described for the transmission experiments. The sources of virus were either inoculated cucumbers or a purified preparation of TRSV. Four hundred adults were transferred from the virus source to a mortar in which they were ground in 1.0 ml of 0.05 M phosphate buffer, pH 7.1. The homogenate was rubbed on five Carborundum-dusted cucumber seedlings.

To inactivate any TRSV carried externally, mites were treated with either formaldehyde or TRSV antiserum. Prior to being homogenized, 400 mites were placed in 1.5-ml vials which contained either 2% formaldehyde for a 10-min, 15-min, 60-min, and 24-h period or a 1:16 (v/v) dilution of antiserum in 0.9% NaCl for a 30-min, 1-h, and 24-h period. Vial tubes were sealed with a cork and shaken several times to assure that the mites were completely immersed. Solutions containining the mites were then placed on filter paper and the mites were washed with distilled water. Washing was accomplished by attaching a 500 ml suction flask to a 10-cm diam funnel containing a piece of 12.5-cm diam Whatman No. 1 filter paper. This washing was intended to remove any excess formaldehyde or antibodies. After being washed, the mites were transferred from the filter paper to a mortar by washing with distilled water. The water in the mortar was removed with a pipette. The homogenate was prepared by grinding 400 mites per 1.0 ml of buffer solution and the homogenate was assayed on cucumber seedlings as previously described.

Persistence of TRSV in mites.—After a 24-h acquisition period on TRSV-infected cucumbers, ca. 50 mites were transferred to individual detached lima bean leaves. The leaves were kept in petri dishes as previously described for obtaining adults of the same age. After a 24-h, 48-h, or 72-h period of feeding on these lima bean leaves, the mites were transferred to a mortar. Homogenates were prepared by grinding 400 mites in 1.0 ml of 0.05 M phosphate buffer, pH 7.1, and rubbed on Carborundum-dusted cucumber seedlings. Homogenates of mites reared on TRSV-infected cucumbers served as a control. The number of local lesions produced on cucumber cotyledons were counted and recorded. Virus identification was confirmed by recovery from the local lesion hosts and serological reaction with TRSV antiserum. To determine whether TRSV was present in the feces of the mites, 400 fecal pellets were collected with a dissecting needle (from lima

bean leaves after mites had fed for 24-h) and transferred to a mortar containing 1.0 ml of phosphate buffer. Fecal pellets were ground and the homogenate assayed on cucumber seedlings. Plants were observed for a 30-day period and recovery of the virus was attempted as in the transmission experiments.

Release of TRSV through feeding membrane.—Approximately 0.15 ml of 1.5% sucrose in 0.05 M phosphate buffer, pH 7.1, was placed on the top of the feeding membrane. The feeding cages were the same as those used in the transmission experiments. The mites were transferred to the cages after a 24-h acquisition period on TRSV-infected cucumber plants. Fifty mites were placed on each cage, maintained under continuous fluorescent illumination, and allowed to feed for 6, 12, 24, 48, or 72 h. Recovery of TRSV from the buffer solution and the mites was attempted after each feeding period. The buffer solution was removed from each membrane and rubbed on a Carborundum-dusted cucumber seedling. Homogenates were prepared by grinding 400 mites in 1.0 ml of the same phosphate buffer, and assayed on five cucumber seedlings. A control was established by rubbing cucumber plants with homogenates prepared from mites maintained on TRSV-infected cucumbers. The plants were observed for a 30-day period, and recovery of the virus was attempted by mechanical subinoculations, as previously described. When recovery was positive, the virus was further identified by its serological reaction with TRSV antisera in an agar-gel diffusion test.

Survival in feeding cages.—Survival of mites on phosphate buffer and 1.5% sucrose solution was tested in feeding cages. Ten adults of the same age were placed in each feeding cage and four replications were used per treatment. Mites without food served as a control. The feeding cages were maintained under continuous fluorescent illumination.

RESULTS.—*Mite survival on cucumber cotyledons and on lima bean leaves.*—For starved mites the 50% mortality time (MT50) was 2.7 days and 100% mortality time (MT100) was 4 days. Mites fed on cucumber cotyledons had a MT50 of 9.8 days and a MT100 of 22 days. In mites placed on lima bean leaves, MT50 and MT100 were 14 days and 32 days, respectively.

Transmission of TRSV and TomRSV.—When infected cucumber, cowpea and lima bean seedlings served as source of virus, a total of 4,110 two-spotted spider mites failed to transmit TRSV to 300 cucumbers, 81 cowpeas, and 30 lima beans (Table 1). In a preliminary trial, 100 mites starved for 1 h failed to transmit TRSV to 10 cucumber cotyledons after a 5-min acquisition and test feeding period. TRSV was not transmitted to 20 cucumber seedlings by 200 mites after a 24-h acquisition period on a feeding membrane containing a purified preparation of the virus. The purified virus preparation was still infective after exposure to the mites. Transmission of TomRSV was also unsuccessful when 60 cucumber, 60 cowpea and 60 lima bean test plants were exposed for 48 h to 1,800 mites that had fed for 24 h on TomRSV inoculated cucumber and cowpea seedlings. No symptoms of virus infection were observed after a 30-day period in any of the test plants following attempted inoculation with TRSV or TomRSV using mites, and no virus could be recovered by mechanical subinoculations.

TABLE 1. Transmission of tobacco ringspot virus to cucumber, cowpea, and lima bean, by *Tetranychus urticae*, after an acquisition feeding period of 1 to 7 days on cucumber, cowpea, and lima bean

Source of inoculum	Acquisition period (days)	Test feeding period (days)	Test plants		
			Cucumber	Cowpea	Lima Bean
Cucumber	1-7	3-7	0/230[a]		
Cowpea	1	5	0/40	0/51	
Lima Bean	1	7	0/30	0/30	0/30

[a]Numerator = plant infected; denominator = plant tested.

Serology.—Tobacco ringspot virus was detected serologically in the two-spotted spider mites. Precipitation lines were formed with the virus and the plant material in agar-gel diffusion test. For antisera dilutions lower than 1:8, and higher than 1:16, precipitation lines to the virus were not obtained. Host plant material, but not virus, was detected in the nonviruliferous mites. Straight coalescent precipitation lines were formed with infected and healthy cucumber extracts and were considered to be reactions to host material. Another precipitation line was present only in extracts of TRSV infected material and was considered to be a reaction to the virus.

Recovery of TRSV and TomRSV from mites.—Tobacco ringspot virus, but not TomRSV, was recovered from mite homogenate. In 10 trials, TRSV was detected in the mites after a 24-h acquisition period when homogenates were rubbed on Carborundum-dusted cucumber cotyledons. An average of 4.9 local lesions per cotyledon was obtained when 1.0 ml of homogenate was rubbed on 10 cucumber cotyledons per trial.

Tobacco ringspot virus was detected in mites after 10 min, 15 min, and 1 h but not after a 24-h treatment with 2% formaldehyde prior to homogenizing. The virus was also recovered from mites treated for 30 min, 1 h, and 24 h with a 1:16 (v/v) dilution of TRSV antiserum prior to being homogenized (Table 2). The number of local lesions was not reduced. The virus identification was confirmed by recovery from infected test plants, and serological reactions with TRSV antiserum.

In two trials, TomRSV was not detected in the mites after a 24-h acquisition period, when 1.0 ml of mite homogenate was rubbed on 10 Carborundum-dusted cucumber cotyledons. No symptoms were observed after a 30-day period, nor could the virus be recovered by mechanical subinoculations to cucumber seedlings.

TABLE 2. Number of tobacco ringspot-induced local lesions per cucumber cotyledon inoculated with homogenate of mites which had received a 24-h acquisition feeding prior to treatment with 2% formaldehyde or tobacco ringspot virus antiserum

Treatment	Exposure time					Control
	10 min	15 min	30 min	1 h	24 h	
Formaldehyde[a]	9.8	11.7			0	6.0
Formaldehyde[b]		2.4		2.1	0	2.2
Antiserum			4.2	4.6	5.4	4.1

[a]Experiment 1.
[b]Experiment 2.

Persistence of TRSV in mites.—TRSV was recovered from mite homogenates after 24 h but not after 48 and 72 h of feeding on the lima bean leaves. After 24 h, nine local lesions were produced on 10 cucumber cotyledons, while 28 local lesions were obtained on 10 cotyledons for the control. Identity of the virus was confirmed by recovery from the infected plants and serological reactions with TRSV antisera. In three trials, no virus could be detected in fecal pellets, when homogenates were assayed on Carborundum-dusted cucumber cotyledons, nor was the virus recovered after mechanical subinoculations.

Release of TRSV through feeding membrane.—TRSV was not recovered from buffer solutions placed on the top of the feeding membranes when two-spotted spider mites that had a 24-h acquisition period on TRSV infected cucumber seedlings were transferred to feeding cages for 6-, 12-, 24-, 48-, or 72-h feeding periods. The buffer solution contained on each membrane was assayed on one Carborundum-dusted cucumber seedling. A total of 10 cucumber cotyledons were inoculated upon completion of each feeding period. The virus was recovered from the mites after 6-, 12-, and 24-h, but not after 48-h and 72-h feeding periods, when homogenates were assayed on cucumber cotyledons; the number of local lesions produced on 10 cotyledons were 39, 40, and 38, respectively; whereas 43 local lesions were produced in the control. Recovery and identification of the virus was done as previously described. After the 72-h feeding period, however, many of the mites were dead.

Survival in feeding cages.—For starved mites, the MT50 was 2.3 days and the MT100 was 3 days. Mites fed on buffer solution had a MT50 of 3.18 days and a MT100 of 5 days. For mites with 1.5% sucrose solution, MT50 and MT100 were 7.25 and 11 days, respectively.

DISCUSSION.—The two two-spotted spider mite, *T. urticae*, failed to transmit TRSV and TomRSV. However, after a 24-h accession feeding period on infected cucumber seedlings, the two-spotted spider mites acquired TRSV, but not TomRSV.

A high concn of extractable TRSV or TomRSV in cucumber cotyledons did not result in mite transmission of these two viruses. The cucumber cotyledons can be used as a source of virus before the first true leaves are formed. Migration of the spider mites from the cotyledons to the first true leaves as soon as they are formed, seems to indicate that as the plants become older, the cotyledons are less acceptable as a food source.

The lack of transmission of TRSV by the two-spotted spider mites reported in the present investigations is in accord with the results obtained by Dysart and Chamberlain (8), Orlob (17), and probably Schuster (23). We were unable to confirm the report of transmission of TRSV by Thomas (30), who utilized a mixed colony of *T. telarius*(=*T. urticae*) and *T. desertorum*, a different strain of TRSV, and several additional acquisition and test plants.

TRSV is acquired by the two-spotted spider mites after a 24-h acquisition period on infected cucumber seedlings as was demonstrated by recovery of the virus from mite homogenates. No reduction in the number of local lesions produced when mites were treated for 1.0 h with 2% formaldehyde, and 24 h with 1:16 dilution of TRSV antiserum, demonstrates that the virus is probably carried internally. The concn of the virus seems to be relatively high, because we were able to detect it in agar-gel diffusion tests. Orlob and Takahashi (19) in studies with the electron microscope observed that TRSV-like particles occurred in higher concn inside the digestive tracts of two-spotted spider mites fed on infected plant material than in those fed on noninoculated plants. Therefore, although there is good evidence that TRSV is present inside of the mites, this does not assure transmission.

The food source may influence retention of TRSV by the two-spotted spider mites. Mites survived for longer periods of time when fed on lima bean leaves, than on membranes containing buffer solutions. This, coupled with the fact that TRSV was not recovered from feces, that local lesions were reduced when viruliferous mites were fed for 24 h on lima bean leaves prior to being homogenized, and that there was an apparent reduction of local lesions after 24 h of feeding on membranes containing buffer solutions, would seem to indicate that TRSV is inactivated more rapidly when mites are feeding on a host plant. However, this cannot be stated unequivocally, since no attempts were made to determine presence of the virus in starving mites.

Tobacco mosaic virus, potato virus X, onion yellow dwarf virus and tomato bushy stunt virus are acquired by the two-spotted spider mite, but they are not transmitted (17). Therefore, the acquisition, but not the transmission, of plant viruses by *T. urticae* is not uncommon. Failure of *T. urticae* to transmit acquired plant viruses has been the subject of speculation before (19). Probably the lack of transmission of TRSV is either due to the fact that insufficient infective virus is retained on or returned to the mouth parts. The inability to demonstrate release of TRSV through feeding membranes containing buffer solutions supports this but it is likely there are other factors involved. The uptake and passage of plant viruses through the alimentary canal of *T. urticae* has been proposed (19).

The failure to detect TomRSV by bioassay of the mite homogenates after access to the virus source does not mean that the virus was not acquired. Virus inhibitors may be present in the mite that could inactivate the virus. Inhibitors to TMV have been found in mite homogenates (17) and there is no reason to believe that they do not inactivate other viruses. From our results, and those of others (8, 18), we suggest that *T. urticae* is not a vector of TRSV and TomRSV. Whether the two-spotted spider mite can act as vector of other plant viruses remains to be elucidated.

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