A Novel Method for Tospovirus Acquisition by Thrips

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ABSTRACT

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An in vitro feeding method for the acquisition of impatiens necrotic spot tospovirus (INSV) and tomato spotted wilt tospovirus (TSWV) by western flower thrips (*Frankliniella occidentalis* (Pergande) was developed. This is the first system developed in which tospovirus viability was maintained in an in vitro technique specifically designed for thrips feeding. Three-day-old larvae of western flower thrips were caged in a cylindrical plastic tube with Parafilm membrane covering both ends. Leaf extracts from INSV- and TSWV-infected *Nicotiana benthamiana* or *Datura stramonium* were prepared. Following one cycle of differential centrifugation, the extract was placed on the membrane on one end of the cage. A second membrane was placed over the first membrane thus covering the extract. After a 24-h acquisition access feeding period, thrips were kept on green-bean pods in a container until they were 10-day-old adults. Enzyme-linked immunosorbent assays (ELISA) showed that acquisition feeding of virus suspensions prepared from infected plant hosts resulted in thrips testing positive for viruses. Cohorts from these ELISA-positive thrips were shown to be viruliferous when placed onto virus-susceptible host plants. This in vitro method is an important tool in the study of tospovirus/thrips interactions.

Tospoviruses transmitted by thrips cause severe economic losses in agricultural and ornamental crops worldwide (14,16). More than five distinct viruses are now grouped in the newly formed genus *Tospovirus,* of which tomato spotted wilt tospovirus (TSWV) and impatiens necrotic spot tospovirus (INSV) occur worldwide (16). Nine species of thrips have been reported as vectors of tospoviruses. These include the western flower thrips, *Frankliniella occidentalis* (Pergande), the common blossom thrips, *Frankliniella schultzei* (Trybom) (1,5,22), the tobacco thrips, *Frankliniella fusca* (Hinds) (21), *Frankliniella intonsa* (Trybom) (M. Kameya-Iwaki, *personal communication*), *Frankliniella tenuicornis* Uzel (11), the onion thrips, *Thrips tabaci* Lindeman (4,19), the melon thrips, *Thrips palmi* Karny (9,17), the chili thrips, *Scirtothrips dorsalis* Hood (1), and *Thrips setosus* Moulton (20). Of these, *Frankliniella occidentalis* (Pergande) has a worldwide distribution and is considered to be the primary vector (16).

TSWV replicates within the insect vector (25) and is transmitted in a persistent manner, although sporadically (20). Tospoviruses must be acquired by the larvae if the adults are to become inoculative (19,20). Adult thrips that feed on tospovirus-infected plants do not transmit the virus (7,24). To obtain viruliferous adults, young larvae are fed on infected plants. The lack of an in vitro system for virus acquisition by thrips has been an obstacle to studying how specific viral components interact with thrips and which components are required for virus acquisition. An artificial feeding technique that allows the manipulation of tospoviruses and acquisition by thrips vectors would benefit laboratory investigations. An in vitro method for tospovirus acquisition by *F. occidentalis* was developed to facilitate examination of virus/vector interactions.

MATERIAL AND METHODS

Virus and host. An isolate of TSWV from lettuce, *Lactuca sativa* L. was collected in Hawaii and an isolate of INSV from *Schizanthus* sp. was collected in California. Viruses were maintained in propagation hosts *Datura stramonium* L. and *Nicotiana benthamiana* Domin. by inoculation with extract from infected leaves ground in inoculation buffer (0.1 M $K_2HPO_4 \cdot KH2_PO_4$ and 0.01 M Na_2SO_3, pH 7.2) with approximately every tenth pass being vectored by *F. occidentalis* to *D. stramonium.* Crude virus concentrates were prepared from infected *D. stramonium* or *N. benthamiana.* These two host species were also used as source plants for acquisition by thrips.

Preparation of viruses. Infected *D. stramonium* or *N. benthamiana* tissues (20–30 g) were homogenized in a Waring blender with inoculation buffer containing 0.01 M EDTA (1 part tissue to 3 parts buffer, w/v), filtered through cheesecloth, and centrifuged in an SS-34 rotor at 9,000 rpm at 4 C for 10 min in a Sorvall RC-5B centrifuge. The supernatant was further centrifuged on a 25% sucrose cushion (sucrose + distilled H_20, w/v) in a Beckman Type 50.2 Ti rotor at 41,000 rpm at 4 C for 90 min in a Beckman L8-70 M centrifuge. Pellets were then resuspended in 1.0–1.5 ml, 7% sucrose-histidine buffer solution (0.1 M L-histidine and 0.01 M $MgCl_2$, pH 6.5) using an Omni 1000 hand-held homogenizer (8,10). The resuspended pellets were then pooled and the virus/sucrose solution then used in acquisition studies.

Serological assay. Standard double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using Nunc Maxisorb microtiter plates (A/S Nunc, Roskilde, Denmark) was used for detection of viral antigens. TSWV- or INSV-specific immunoglobulins and alkaline phosphatase-labeled virus-specific immunoglobulins were used as recommended by the supplier (Agdia, Inc., Elkhart, IN). Individual thrips were ground in 70 μl of thrips extraction buffer (20 g PVP-40 into 1 L PBS-T) (PBS-T = 100 ml of phosphate-buffered saline (PBS) + 0.5 ml of Tween 20; PBS= 8 g NaCl, 0.2 g KH_2PO_4, 1.2 g Na_2HPO_4, 0.2 g KCl). Thrips samples were left in ELISA plates overnight, washed twice with PBS-T, then 70 μl of conjugate was added to each well and kept at room temperature for 3 h, washed 5 times, then 140 μl of substrate added to each well and kept at room temperature for color development. Plates were read with an M700 Dynatech ELISA-reader. ELISA A_{405} readings greater than 3 times the mean value of the negative control healthy thrips were considered positive (Fig. 1). Frequencies were compared by chi-square test. Cohorts of thrips from ELISA-positive groups were placed on

healthy virus-susceptible plants to determine if viruses acquired under these conditions remained thrips transmissible.

Maintenance of thrips colonies. Healthy thrips were continuously reared on virus-free green-bean pods (*Phaseolus vulgaris* L.) as verified by ELISA, in clear plastic containers at room temperature. When 3 days old, larvae were collected from bean pods with a small brush.

Acquisition access feeding. Three-day-old larvae were starved 2 h prior to 24-h acquisition access feeding on systemically infected *D. stramonium* or *N. benthamiana*. Larvae were then transferred to green-bean pods in plastic cups and reared to adults. Ten-day-old adults were tested using DAS-ELISA to detect viral antigens.

For acquisition access feeding on extracted virus preparations, starved 3-day-old thrips larvae were caged in a 10-cm-long plexiglass cylindrical tube (10 cm diameter) covered with Parafilm (Fig. 2). The virus preparation was placed above the surface of a membrane on the cage and covered with a second membrane to prevent spillage or evaporation (15). After a 24-h acquisition access period at room temperature (approximately 20 C), thrips were placed on green-bean pods in a cup until they became adults. Ten-day-old adults were tested for the presence of viral antigen by DAS-ELISA. Before and after thrips feeding, samples of the virus preparation were tested by DAS-ELISA and infectivity assay on *N. benthamiana*. Thrips were said to have acquired virus if the larvae ingested enough virions to become viruliferous adults.

Transmission, inoculation access period. Cohorts from groups of ELISA-positive thrips that had been given acquisition access on TSWV or INSV preparations were given access to healthy plants. The inoculation access period occurred on either *Petunia hybrida* leaf disks, 1 cm diameter, var. Yellow Magic (Park Seed Co., Greenwood, SC), using 5 thrips per disk, or on *D. stramonium* seedlings, 25 days old, 2 plants with 10 thrips each, and 2 plants with 25 thrips each.

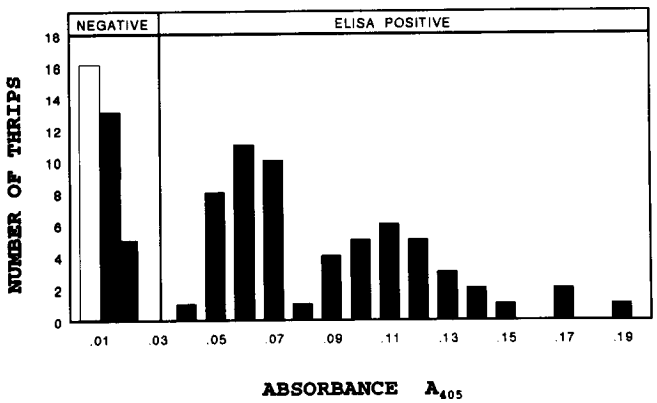

Fig. 1. Distribution of enzyme-linked immunosorbent assay (ELISA) absorbance, A_{405}, of healthy control thrips (empty bar) and for thrips given access to virus suspension from INSV-infected *Nicotiana benthamiana* (solid bar). Range of thrips considered negative or positive by ELISA is indicated. (Greater than 3 times the mean of the healthy controls was considered positive for presence of viral antigen. Mean = 0.008, 3× = 0.024).

TABLE 1. Range and mean of enzyme-linked immunosorbent assay (ELISA) A_{405} values for thrips that were given an acquisition access feeding period on impatiens necrotic spot tospovirus (INSV) suspensions from *Nicotiana benthamiana* or *Datura stramonium*

Healthy range	Mean	N	Positive range	Mean	N	Total tested[a]
INSV prepared from *N. benthamiana*						
0.002–0.004	0.004	4	0.017–0.228	0.117	11	15
0.001–0.010	0.005	4	0.039–0.273	0.130	14	20
0.006–0.009	0.007	7	0.039–0.314	0.202	19	24
0.001–0.009	0.004	12	0.025–1.460	0.107	27	35
0.007–0.009	0.008	16	0.040–0.187	0.089	62	84
Total					133	178
INSV prepared from *D. stramonium*						
0.006–0.014	0.009	8	0.053–0.319	0.220	19	40
0.002–0.018	0.008	8	0.097–0.642	0.428	15	32
Total					34	72

[a]Values include thrips that gave ELISA A_{405} readings of less than (3× mean) of the healthy negative controls.

RESULTS

Thrips larvae acquired either INSV or TSWV when fed on virus suspensions through membranes and resulted in viruliferous adults.

Thrips acquired INSV from virus suspensions from two host plants, *N. benthamiana* and *D. stramonium,* with a significantly higher proportion of ELISA-positive thrips obtained from acquisition feeding on virus suspensions from *N. benthamiana* (Table 1, 75 to 47% respectively, $X^2 = 6.2$, $P < 0.05$). Thrips were considered positive if they had a value that was greater than three times the mean value of the negative healthy control thrips. Acquisition access feeding on leaves of *D. stramonium* infected with either INSV or TSWV showed a higher proportion of virus acquisition from INSV than from TSWV-infected plants (Table 2, 58.3, 18.8% respectively).

When comparing INSV with TSWV, membrane acquisition feeding from *N. benthamiana* extracts was significantly greater for INSV, with 75% (Table 1, 133/178) of adult thrips testing ELISA-positive, while 44% (Table 3, 46/104) of thrips tested positive for TSWV antigen ($X^2 = 10.8$, $P < 0.05$). Thrips fed on virus suspensions from *D. stramonium* showed no significant differences between the percentage of adult thrips testing positive for either virus, with 47% ELISA-positive adult thrips for INSV (Table 1, 34/72) and 35% ELISA-positive for TSWV (Table 3, 25/71) ($X^2 = 1.9$, $P > 0.05$). Acquisition feeding on INSV-infected *Datura* leaves versus virus suspension showed no significant difference between the numbers of INSV ELISA-positive thrips, with

TABLE 2. Range and mean of enzyme-linked immunosorbent assay (ELISA) A_{405} values for thrips that were given an acquisition access feeding period on *Datura stramonium* leaves infected with either impatiens necrotic spot tospovirus (INSV), or tomato spotted wilt tospovirus (TSWV)

Healthy range	Mean	N	Positive range	Mean	N	Total tested[a]
INSV-infected *D. stramonium* leaves						
0.004–0.022	0.014	8	0.044–0.160	0.081	50	86
0.002–0.009	0.007	8	0.025–0.062	0.043	13	22
Total					63	108
TSWV-infected *D. stramonium* leaves						
0.000–0.005	0.003	8	0.034–0.074	0.050	7	32
0.000–0.011	0.007	8	0.027–0.209	0.083	27	149
Total					34	181

[a]Values include thrips that gave ELISA A_{405} readings of less than (3× mean) of the healthy negative controls.

TABLE 3. Range and mean of enzyme-linked immunosorbent assay (ELISA) A_{405} values for thrips that were given an acquisition access feeding period on tomato spotted wilt tospovirus (TSWV) suspensions from infected *Nicotiana benthamiana* or *Datura stramonium*

Healthy range	Mean	N	Positive range	Mean	N	Total tested[a]
TSWV prepared from *N. benthamiana*						
0.003–0.019	0.009	8	0.028–0.075	0.043	28	60
0.001–0.018	0.009	8	0.030–0.059	0.040	18	44
Total					46	104
TSWV prepared from *D. stramonium*						
0.001–0.018	0.009	8	0.031–0.079	0.043	8	51
0.000–0.008	0.002	8	0.011–0.027	0.020	17	20
Total					25	71

[a]Values include thrips that gave ELISA A_{405} readings of less than (3× mean) of the healthy negative controls.

TABLE 4. Thrips transmission of impatiens necrotic spot tospovirus (INSV) and tomato spotted wilt tospovirus (TSWV) by cohorts from enzyme-linked immunosorbent assay–positive groups after acquisition access on virus suspensions

INSV			TSWV		
	Acquisition virus preparation			Acquisition virus preparation	
Transmission	*Nicotiana*	*Datura*	Transmission	*Nicotiana*	*Datura*
5 Thrips/petunia leaf disk	4 or 4[a]	4 of 4[a]	5 Thrips/petunia leaf disk	4 of 4[a]	4 of 4[a]
10 Thrips/*Datura*	2 of 2	1 of 2	10 Thrips/*Datura*	2 of 2	2 of 2
25 Thrips/*Datura*	2 of 2	2 of 2	25 Thrips/*Datura*	2 of 2	2 of 2

[a]Number of leaf disks or plants infected out of total.

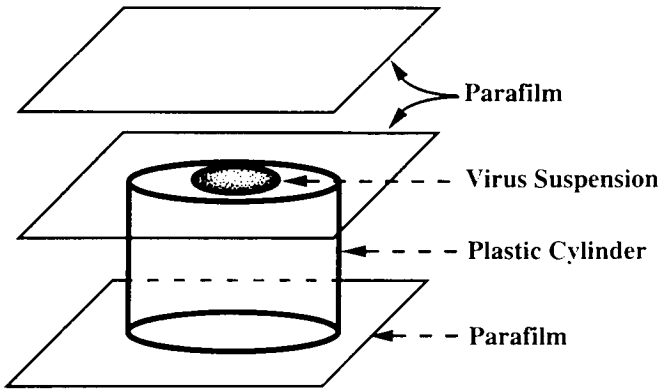

Fig. 2. Acquisition access feeding cage. Leaf extracts from infected plants are contained between two stretched pieces of Parafilm through which thrips larvae feed.

58% on detached leaves (Table 2, 63/108) and 47% from virus suspensions (Table 1, 34/72) ($X^2 = 2.2$, $P > 0.05$).

On TSWV-infected *D. stramonium,* there was a significantly greater number of TSWV ELISA-positive thrips from virus suspension feeding, 35%, than from detached leaves, 18% (Tables 2 and 3, $X^2 = 19.2$, $P < 0.05$). Thrips that tested positive for TSWV, given acquisition access feeding on suspensions from either TSWV-infected *N. benthamiana* (44%) or *D. stramonium* (35%), showed no significant difference (Table 3, 46/104, 25/71) ($X^2 = 1.4$, $P > 0.05$).

Acquisition access feeding from infected *N. benthamiana* leaves (75 thrips) resulted in high thrips mortality: all the thrips collected from leaves were dead. Therefore, ELISA readings from leaf acquisition trials on this plant were misleading.

Cohorts from ELISA-positive groups of thrips ranging from 5, 10, or 25 adults were given an inoculation access feeding period of 24 h on either healthy *D. stramonium* plants or *P. hybrida* leaf disks, var. Yellow Magic. These thrips transmitted infectious virions as judged by symptom expression and confirmed by ELISA (Table 4).

DISCUSSION

Membrane-feeding sachets and extracts from virus-infected plants have been utilized in assays of leafhopper-borne and aphid-borne viruses (12,18), but have not been reported for any thrips-transmitted viruses. The technique allows the acquisition of viruses by vectors, requiring less handling of insects and less space, under a more controlled environment. Tospoviruses are considered among the most labile plant viruses when extracted from infected tissues (3). An important factor in extracting and preparing virus samples for acquisition may be maintaining the correct pH, for when it was not maintained virus suspensions became noninfectious when attempting sap inoculations. Incorporation of histidine in the virus preparation probably helped maintain the infectivity of the membrane-bound tospoviruses (8,10). Maintaining infectivity, which is a very important property, makes a membrane-feeding sachet system applicable for tospovirus-thrips studies.

An in vitro membrane-feeding sachet system successfully applied to acquisition access feeding of tospoviruses by thrips vectors may provide a means to address questions about virus acquisition and interactions of tospoviruses with thrips. Both INSV and TSWV were acquired by thrips feeding through a membrane containing virus suspension. Successful acquisition of these viruses by insect vectors from infected plant tissues depends in part on whether the virus is systemically distributed through plant tissues, or restricted to local lesions. Studies using tissue blotting indicate that tospovirus distribution in different host plants varied widely (23). Patterns of tospovirus infections in different host plants may affect the efficiency of virus acquisition by thrips (2,6). The plant may also present barriers to insect feeding. The high mortality of larvae given an acquisition access feeding period on infected *N. benthamiana* leaves seemed to be due to the plant oil exuded by glandular trichomes that the larvae contacted during attempts to feed. Acquisition of virus from leaf extracts through a membrane bypasses external plant defenses, and allows the thrips larvae to feed on a uniform virus source that provides greater opportunity for thrips to ingest virions (Fig. 2). Although thrips were able to acquire and transmit viruses, we did not evaluate individual thrips transmission efficiencies in this study. Cohorts of five or more thrips were used to show that by using this in vitro method, viruliferous adult thrips could be obtained. Interestingly, there was a higher rate of INSV acquisition by thrips than of TSWV, which may suggest that this virus is acquired more easily. Past work has shown that *F. occidentalis* is an efficient vector of both viruses (26).

Although thrips acquired both tospoviruses, not all did. There are a number of factors that limit the success of virus acquisition from a host plant as well as from an artificial feeding system. Insect vectors may prefer a specific tissue type, such as phloem feeding insects, or on a specific location on the plant, such as next to larger veins of a leaf. Feeding next to leaf veins is common among several thrips species (13). Feeding preferences may lead insect vectors to feed more on areas of the plant that contain few virions, resulting in decreased virus acquisition.

Although the use of a membrane-feeding sachet system greatly increases the amount of time thrips may spend feeding directly on a virus source, there is no guarantee that all thrips will feed. The cage design used was completely enclosed so some condensation occurred. Condensation would provide an alternative source of moisture for the thrips. Furthermore, some thrips may feed only slightly, and as a result the ingested virus does not reach the level needed to induce infection. Finally, some thrips may have fed sufficiently prior to being caged so that the motivation to feed was low or absent. Steps to correct these conditions would probably result in higher rates of virus acquisition. The method described herein provides for the first time an in vitro method for further studies on tospovirus/thrips interactions.

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