Transmission of Tomato Spotted Wilt Virus by *Frankliniella occidentalis* After Viral Acquisition During the First Larval Stage

F van de Wetering, R. Goldbach, and D. Peters

Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6700 EM, Wageningen, The Netherlands

The efficiency at which tomato spotted wilt virus (TSWV) was transmitted by adult Frankliniella occidentalis *that ingested the virus at different larval ages was determined by a petunia leaf-disk assay and enzyme-linked immunosorbent assay (ELISA). The results show that 0- to 2-day-old larvae (mostly first instars) can acquire TSWV, which suggests that after ingestion and accumulation of virus they were subsequently converted into transmitters in the adult stage. Older larvae (second instars) failed to acquire TSWV, because as adults they did not transmit TSWV. The relation between age at which virus acquisition took place and the amount of virus ingested was analyzed by ELISA. Ingestion of viral antigen increased with age of larvae, and the amount of virus ingested could not be correlated with the development of infectivity in the thrips. The finding that only first-stage larvae might be able to acquire TSWV is crucial for understanding the epidemiology of tomato spotted wilt disease.*

It is generally accepted that transmission of TSWV requires virus ingestion during the larval stages. The minimum acquisition access period (AAP) reported for *F. occidentalis*, the most efficient vector for TSWV (Wijkamp et al. 1995), is 5 min (Anonymous 1988; Wijkamp et al. unpub. results). After ingestion, the virus replicates during a latent period before it can be transmitted by late second instars and adults (Ullman et al. 1993; Wijkamp et al. 1993). However, a minimum inoculation access period (IAP) of 5 min was found to be sufficient to transmit the virus (Anonymous 1988; Wijkamp et al. unpub. results). Once the vector is viruliferous, it remains infective throughout its life span, but the rate at which each individual transmits may vary considerably (Sakimura 1962).

To control the spread of TSWV and to study the epidemiology of the disease, it is necessary to elucidate the mechanisms by which TSWV is acquired, and to understand how ingestion and accumulation of the virus in the larvae leads to adults that can transmit the virus. This study reports on the development of infectivity in thrips after virus acquisition by different larval stages. The age of larvae at which the ingestion of virus results in transmission in the adult stage, and the amount of

virus present in these adult, was followed. The influence of ingestion on the development of infectivity within thrips was also analyzed.

Materials and Methods

Thrips

F. occidentalis were obtained from a greenhouse infestation in The Netherlands and reared as virus-free colonies on pods of common bean (*Phaseolus vulgaris*) at 27 ± 0.5°C and 16/8 h L/D.

Virus isolate and plant material

In all experiments, the Brazilian TSWV isolate BR-01 (de Ávila et al. 1992) was used. This isolate was maintained by thrips inoculation on *Datura stramonium*. The plants used were mechanically inoculated on the first two leaves of *D. stramonium* seedlings with extracts from thrips-infected plants. These plants were grown from seed in greenhouses at about 22°C (16/8 h L/D) and used as the virus source.

Antiserum production and purification

Polyclonal antiserum raised against the nucleocapsid (N) protein of TSWV isolate BR-01 was used in ELISA (de Ávila et al. 1992). The immunoglobulin (IgG) fraction was partially purified by ammonium sulfate precipitation (Clark and Adams 1977). IgG was conjugated at a concentration of 1 mg/mL with 2,000 U alkaline phosphatase (Boehringer-Mannheim) in phosphate-buffered saline (PBS), pH 7.4 (Avrameas 1969). The IgG and conjugate were stored with 0.05% sodium azide at 4°C.

Virus detection

Double-antibody sandwich ELISA (DAS-ELISA) was used to detect TSWV in plant material (Clark and Adams 1977; Resende et al. 1991). The presence of N protein in individual thrips was detected using cocktail ELISA with enzyme amplification (Van den Heuvel and Peters 1989). The threshold value was calculated as the mean of the readings obtained from virus-free material plus three times the standard deviation. All the readings above the threshold value were considered positive. As control, virus-free thrips and a dilution series of virus purified from *Nicotiana rustica* plants were used.

Handling thrips in transmission experiments

Systemically infected leaves of *D. stramonium*, which showed comparably high virus titers in a dilution series in DAS-ELISA, were used as the source on which the larvae were given AAPs. The infected leaves were cut in pieces and distributed randomly over Tashiro cages (Tashiro 1967) containing *F. occidentalis* larvae. Separate groups of instars were caged on virus-free leaves as a control treatment. After AAP, the thrips were transferred to TSWV-free *D. stramonium* leaves, where

they completed their development until adult emergence. All experiments were held at 25 ± 0.5°C. Adults were individually tested for virus transmission on leaf disks (13-mm diam.) of *Petunia x hybrida* cv. Polo Blauw as described by Wijkamp and Peters (1993) for three successive IAPs of 48 h. After each IAP, the leaf disks were incubated for 2 days at 27 ± 0.5°C in 24 well plates (Costar Europe), while floating on water for development of local lesions. Extracts of the disks were tested in a DAS-ELISA format, to confirm that local lesions on disks were caused by TSWV infection and to determine if the absence of lesions indicated no virus infection. To study the correlation between the infectivity of the thrips and symptom development, the area covered with lesions of each leaf disk was correlated with the amount of virus present within a disk. The surface covered with symptoms was divided into four categories, i.e., without symptoms and 0–33 %, 33–67 %, and 67–100 % covered with lesions. The percentage of leaf disks that developed virus symptoms was regarded as the transmission efficiency.

Virus transmission experiments

Cohorts of 0 to 4-h-old larvae were collected and offered virus-infected material for 24 h (Figure 1). Before and after the AAP, the thrips were placed on virus-free *D. stramonium* leaves. In the adult stage, the transmission efficiency, expressed as the number of adults transmitting, was determined using leaf disks of petunia. Subsequently, the adults were stored at -70°C, to measure the amount of virus present by ELISA. The experiments were executed twice with an average of 30 insects per treatment. Thrips on noninfected leaves were used as controls.

The previous experiment was repeated using larvae that were 0 h, 12 h, 20 h, 28 h, 36 h, 44 h, 52 h, and 56 h old for acquisition feeding. At the start of acquisition feeding, the instar 1 to instar 2 (L1/L2) ratio of the larvae was determined by examining exuviae at subsequent ages. In the adult stage, the transmission efficiency was determined using the local lesion assay. The experiment was repeated twice with an average of 30 insects per treatment.

Determination of the amount of virus ingested

Larvae were offered virus-infected material at age 0- to 5-days. After 8 h feeding, about 25 larvae of each age group were collected and stored at -70°C. The amount of virus in each thrips was measured by cocktail ELISA with enzyme-amplification. At the same acquisition feeding times, samples of thrips, confined for 8 h on virus-free *D. stramonium* leaves, were collected to be used as (negative) controls.

Results

Transmission studies using a leaf-disk lesion assay

Black local lesions appear on petunia leaf disks 2–3 days after inoculation access of thrips. Our results confirm that disks without visible symptoms are not infected

Larvae			Time (days)					
	Stage	Age	1	2	3	4	5	6
Treatment	L1	0d	i	n	n	n	n	n
		1d	n	i	n	n	n	n
		2d	n	n	i	n	n	n
	L2	3d	n	n	n	i	n	n
		4d	n	n	n	n	i	n
		5d	n	n	n	n	n	i

Figure 1. The experimental set-up in which the larvae were allowed to ingest virus after emergence from eggs. Larvae 0- to 5-days-old were placed for 24 h on infected material (i) and spent the other days on noninfected material (n). TSWV-infected *D. stramonium* plants were used as the virus source.

with TSWV (Figure 2). Also, there was a correlation between the area covered with symptoms and the amount of virus in the leaf disk.

Relation between ingestion of TSWV by larvae and transmission by adults
Acquisition by larvae that were 0- to 2-days-old resulted in adults that successfully transmitted the virus; whereas, larvae that ingested virus in later stages (3- to 5-days-old) developed into adults that failed to transmit (Figure 3). Transmission efficiency sharply decreased among the 0- and 2-day-old larvae. The number of adults in which virus could be detected was higher than the number of transmitting adults, but decreased as the instars aged. The highest rate was found when larvae fed at the age of 0 days on infected material; the transmission efficiency of the resulting adults was 37%, and 90% of the adults contained virus. Virus could neither be transmitted nor detected in adults that were given AAPs when they were 3- to 5-days-old.

This indicates that larvae lose, as second instars, their ability to become transmitters. The results also showed that all viruliferous thrips contained a significantly higher amount of virus than nonviruliferous thrips (Figure 4). Viral antigens could also be detected in some individuals that did not transmit virus to petunia leaf disks, but the amount of virus detected was consistently lower than those of transmitters, indicating that a certain threshold amount of TSWV is necessary for transmission to occur.

The highest transmission efficiency (42%) was obtained when 0-h-old larvae were fed on infected material (Figure 5). Instars (56-h L2s) were not able to acquire virus and no adult transmission occurred. The ability to acquire virus decreases with the age of first-stage larvae, and this ability is completely lost in the second stage.

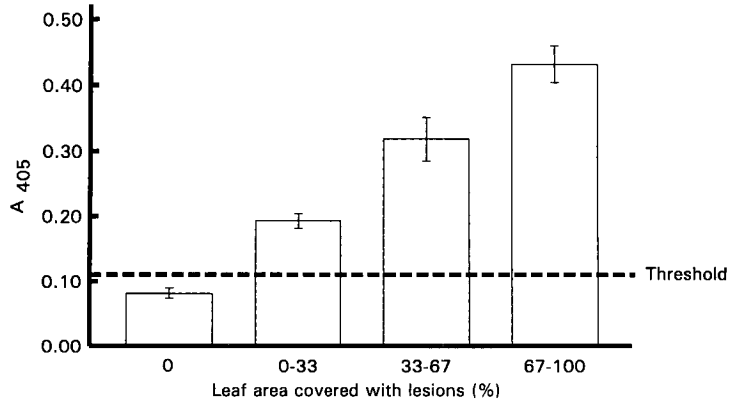

Figure 2. Average ELISA values of petunia leaf disks. Leaf disks were divided into four categories based on leaf area covered with symptoms. All readings above the threshold value (dotted line) were considered positive. Vertical bars indicate the standard errors.

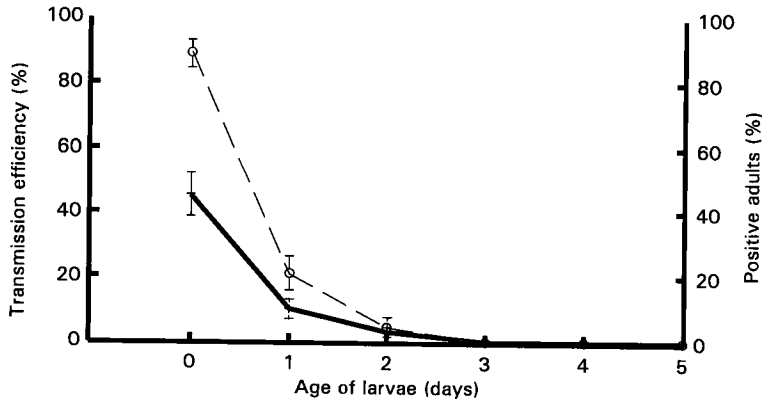

Figure 3. The transmission efficiency (———) and the percentage of adults that contained TSWV (– – – –) after ingestion of TSWV by 0- to 5-day-old larvae that were given an acquisition access period of 24 h. Vertical bars indicate the standard errors.

Influence of amount of virus ingested on the ability to acquire TSWV

The loss of transmission capacity with age might be explained by the amount of virus ingested during larval development. The amount of virus ingested increased with the age of the larvae (Figure 6A), which may reflect an increase in consumption rate. The amount of virus ingested by larvae; however, does not determine or influence the resulting infectivity of adults (Figure 6B). The failure of some larvae to ingest virus may be explained by the absence of TSWV in the cells on which these thrips fed.

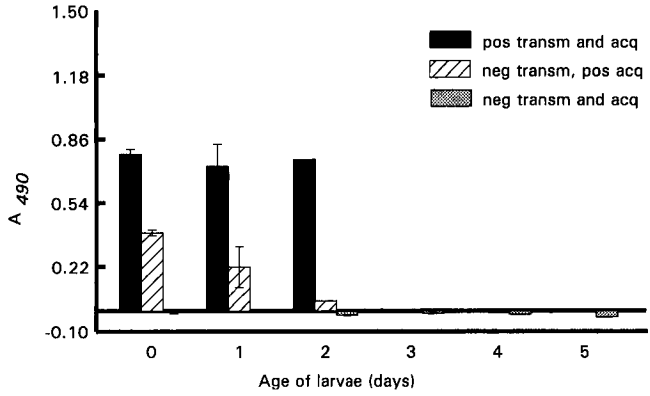

Figure 4. The average ELISA values, using antiserum against TSWV(N), in adult thrips after virus ingestion by larvae of different ages. Transmission by adults was tested on petunia leaf disks. Pos positive; neg negative; transm transmission; acq acquisition. Vertical bars indicate the standard errors.

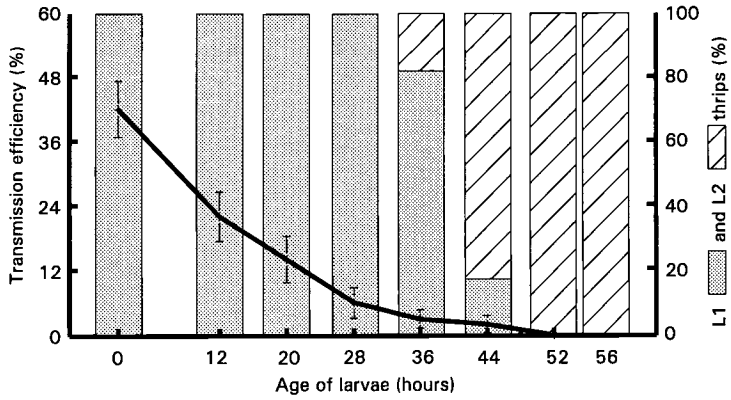

Figure 5. Relationship between L1/L2 ratio of larvae at acquisition and the transmission efficiency in their resulting adult stage. Vertical bars indicate the standard errors.

Discussion

Only first-stage larvae of the thrips *F. occidentalis* are able to acquire TSWV. This implies that only at this stage does ingestion of virus result in replication and subsequent transmission in later stages. The highest transmission efficiencies were obtained when acquisition feeding took place at the early first larval stage, and efficiencies decreased with instar age. No correlation was found between the amount of virus ingested and the ability to transmit TSWV.

The petunia leaf-disk assay can be used to monitor TSWV transmission by thrips. All leaf disks with lesions gave positive reactions in ELISA and disks without

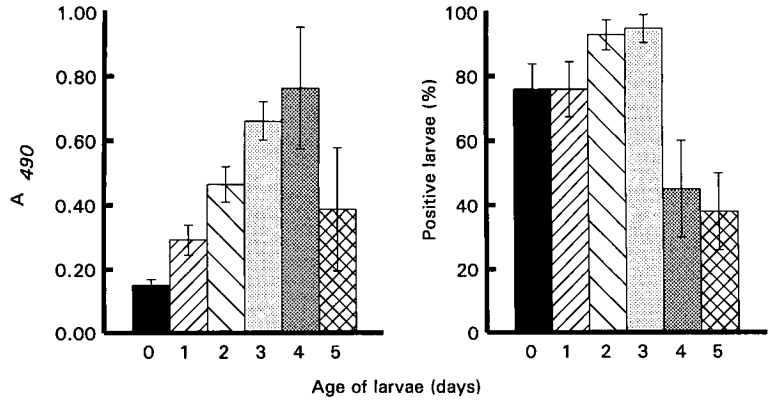

Figure 6. The average ELISA values, using antiserum against TSWV(N), of individual larvae (A) and the percentage of positive larvae (B) after virus acquisition when 0- to 5-days-old. Vertical bars indicate the standard errors.

lesions did not. This local lesion assay can, to a certain extent, also be used to study the feeding behavior of individual viruliferous thrips because viral concentration in the disk increased as the area covered with local lesions increased.

Not all adult thrips that contained virus were able to transmit virus. There are several possible explanations for the occurrence of thrips that contained virus but were not able to transmit. First, a certain threshold level of virus may be necessary to allow transmission. This would imply that a barrier exists, e.g., in the salivary glands, that prevents transmission, and that this barrier can only be passed if a certain threshold level of virus is present. A second explanation could be a host-plant effect, i.e., that a certain minimal inoculation titer is necessary to initiate an infection site (local lesion) in petunia.

Our results show that in late (second) instars, a barrier exists that prevents acquisition and virus replication needed for transmission. The differences in the ability to acquire TSWV between adult and instar thrips were attributed to the fact that a midgut escape barrier to TSWV existed in the adults but not in the larvae (Ullman et al. 1992). This midgut barrier would prevent virus dissemination to the hemocoel (Ullman et al. 1995).

A clear difference between larvae versus adults or even between larval stages, in the ability to acquire virus, has also been described for Fiji disease virus (FDV, Reoviridae), which is transmitted by the planthoppers *Perkinsiella* spp. (Ammar 1994). This propagative virus can be acquired by first, second, and third instars, but not by the fourth and fifth larval and adult stage. Transmission

efficiency also decreases as the instars age (Francki et al. 1986; Egan et al. 1989; Murphy et al. 1995). Ammar (1994) suggested the occurrence of midgut (infection or escape) barrier or other dissemination barriers for FDV in the later instar and adult stages.

The finding that thrips larvae aged of 0- to 2-days (mostly first instars) have the exclusive capacity to acquire TSWV, which suggests that only in this stage does virus ingestion result in replication and subsequently transmission of virus, is crucial to understanding the epidemiology and spread of the virus. The dynamics of the infection cycle of TSWV by thrips can now be described more accurately. The virus must be acquired by early aged larvae, and after a latent period in which virus replication takes place, TSWV can be transmitted by late second instars and adults (Ullman et al. 1993; Wijkamp and Peters 1993; Wijkamp et al. 1993). This finding is relevant to the design of optimal control measures because control of TSWV is currently focussed on the transmitters; whereas, control might be more effective if aimed at the beginning of the transmission cycle, the first-stage larvae.

This research was supported by the Foundation of Dutch Technology of the Netherlands Organization for Scientific Research, and the Graduate School Production Ecology, Wageningen, The Netherlands. We wish to thank S&G Seeds, Enkhuizen, The Netherlands, for supplying seeds of Petunia x hybrida *cv. Polo Blauw.*

Allen, W.R. and Matteoni, J.A. 1991. Petunia as an indicator plant for use by growers to monitor for thrips carrying the tomato spotted wilt virus in greenhouses. Plant Dis. 75, 78-82.

Anonymous. 1988. *F. occidentalis*, Biology and Control. EPPO Publ. Ser. B, 91.

Ammar, E.D. 1994. Propagative transmission of plant and animal viruses by insects: factors affecting vector specifity and competence. Adv. Dis. Vector Res. 10, 289-331.

Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Immunochemistry, 6, 43-52.

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.

Daniels, J., Husain, A.A., Hutchinson, P.B., and Wismer, C.A. 1969. An insectary method for testing sugar cane varieties for resistance to Fiji Disease. Proc. Int. Soc. Sugar Cane Tech. 13, 1100-1106.

de Ávila, A.C., de Haan, P., Kitajima, E.W., Kormelink, R., Resende, R. de O., Goldbach, R.W., and Peters, P. 1992. Characterization of a distinct isolate of tomato spotted wilt virus (TSWV) from *Impatiens* sp. in the Netherlands. J. Phytopath. 134, 133-151.

Egan, B.T., Ryan, C.C., and Francki, R.I.B. 1989. Fiji Disease. In: Diseases of

subsequent work took place in a sterile fume hood. After sterilization, the eggs were transferred to a sterile 15-mL centrifuge tube with 10 mL of distilled water containing 0.1 mL of Nystatin and centrifuged (125 *g*, 3 min) in an IEC clinical centrifuge. The eggs were then transferred to a new sterile tube, with 10 mL of filtered distilled water and recentrifuged.

The disinfected eggs were covered with 1 mL of Tyrode's salt solution and centrifuged. The Tyrode's solution was removed; the eggs were homogenized in the 15 mL vial by tapping with a round-tip glass rod; and 0.5 mL of 0.05 % trypsin was added to the homogenized tissue and let sit for 15 min. Trypsinization was stopped by adding 6 mL of medium for thrips tissue culture (Hunter and Hsu 1995a,b). The tissue fragments and medium were then centrifuged at about 600 *g* for 5 min. The medium and trypsin solution was then removed, leaving the tissue fragments and cells at the bottom of the tube.

Fresh medium (2 mL) was added to resuspend the tissue and cells, and the mixture was dispensed into a 25-mL flask with an additional 2 mL of medium. Primary cultures were kept at 24°C in an incubator and fresh medium added or changed thereafter at 4–7 day intervals. During the first change of medium, 3-day-old medium was drawn off the cultures, centrifuged to remove debris, and mixed with fresh medium (ratio 1:1). In this manner, cultures could be maintained for 6–7 months. In these cultures, most explants from embryonic fragments attached to the substrate within 24–48 h if kept on a stationary surface. Cell growth around the explants or from single cells could be observed after 48 h of incubation.

Primary tissue cultures were from tissues of unknown origin because they stemmed from seeded embryonic fragments. At 24–48 h after cultivation, fibroblast-like cells were observed on the plate (Figure 1). After 10–20 days, monolayers of epithelial-like cells were observed (Figure 1). The presence of fibroblast-like and monolayers of epithelial-like cells were similar to descriptions of cells from other established insect cell lines (Kuroda and Shimada 1989).

Thrips cells were grown on round, quartz, coverslips for 10–15 days. Cells were inoculated for 1 h with plant sap from *Nicotiana benthamiana* infected with impatiens necrotic spot virus (INSV). Approximately 1 g of leaf material was sterilized with 70% ethanol, rinsed once in sterile deionized, distilled water (ddH_2O), once with a histidine solution (L-histidine·HCl monohydrate 0.8 g/100 mL + L-histidine freebase 1 g/100 mL), pH 6.4, then ground with a mortar and pestle. The sap was poured into a 15 mL centrifuge tube, brought up to 10 mL with the the histidine solution, and placed in an IEC centrifuge at 800 *g* for 10 min. About 3 mL of the sap was drawn off and placed on ice until needed. The media covering the cells was removed, the cells rinsed once with the histidine solution for few seconds, and the virus-infected plant sap placed on the cells (coverslips) for 1 h. At the end

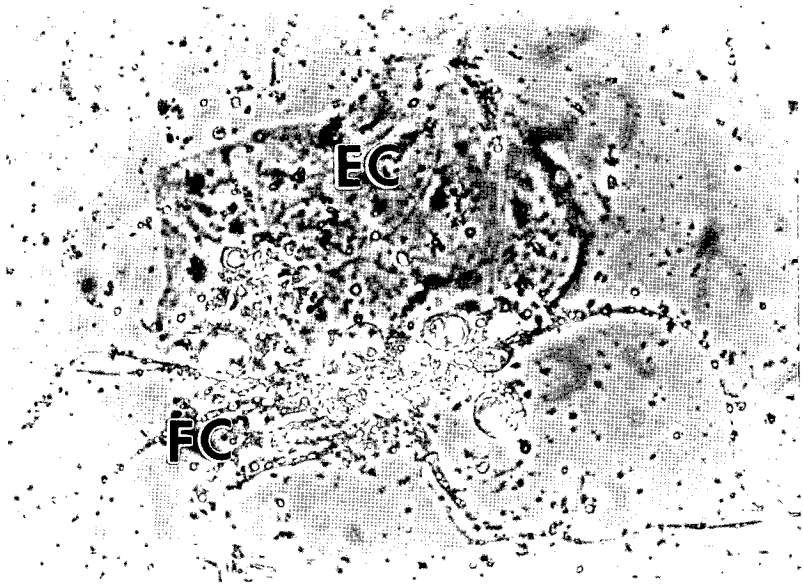

Figure 1. Light micrograph of fragment of thrips tissue showing outward growth of fibroblastoid-like cells (FC) and monolayer of epithelial-like cells (EC) at 20 days.

of the inoculation period, the virus sap was removed, the coverslips were rinsed twice with fresh medium, and fresh medium was placed on the cells. Cells were harvested at 24 h, 48 h, and 72 h.

Cells were placed into cooled acetone for 10 min, rinsed three times in 0.5% PBS, once in ddH_2O, and then air dried. Cells were then incubated with tospovirus antibody for 1 h, rinsed again, and air dried. Conjugated fluorescein-isothiocyanate antibody was then applied for 1 h, after which the samples were rinsed. Healthy *N. benthamiana* was used to prepare control inocula. The air-dried cells (coverslips) were then mounted in 50% glycerol/ddH_2O and observed with an inverted microscope using ultraviolet light. Negative healthy controls lacked glowing cells, while positive samples glowed strongly.

This is the first successful inoculation of thrips-cell monolayers with a tospovirus (INSV). However, the condition of cells deteriorated rapidly 72 h after inoculation, which may indicate that the virus had a detrimental effect on cell viability, or that the conditions of inoculation were not optimal. More work is needed to determine the best conditions for inoculation of thrips cells. At any rate, the successful inoculation of thrips cell monolayers provides evidence that the development and research of thrips cell cultures may provide an important system with which information about virus–vector interactions may be gathered. Further studies on the

development of continuous thrips cell lines and the cycle of tospovirus replication and infection of these primary cell cultures, should yield valuable information about virus–vector cellular interactions.

The development of a vector-cell monolayer system from thrips may also provide a way to develop more sensitive assay systems for the detection of tospoviruses. Such systems will increase our understanding of the replication cycle of tospoviruses in thrips vectors, and possibly explain why there is no replication in nonvector species. This knowledge may provide us with important information that might allow the creation of more innovative and efficacious management strategies.

Adam, G. and Sander, E. 1976. Isolation and culture of aphid cells for assay of insect-transmitted plant viruses. Virology, 70, 502-508.

Chiu, R.J. and Black, L.M. 1967. Monolayer cultures of insect cell lines and their inoculation with a plant virus. Nature, 215, 1076-1078.

German, T.L., Hu, Y., Cho, J.J., and Ullman, D.E. 1991. Detection of tomato spotted wilt virus RNA in plant and thrips using strand- specific probes. In: Virus-thrips-plant interaction of tomato spotted wilt virus. H.T. Hsu and R. H. Lawson, eds., US Dept. Agr., Agr. Res. Serv., ARS-87, 137-143.

Hunter, W.B. and Hsu, H.T. 1995a. Establishing thrips cell cultures to study tospoviruses. In: Thrips biology and management. B.L. Parker, M. Skinner, and T. Lewis, eds., Plenum Press, NY, USA, 163-166.

Hunter, W.B. and Hsu, H.T. 1995b. Formulation of an insect medium for thrips monolayer cell cultures (Thysanoptera: Thripidae: *Frankliniella occidentalis*). J. Invertebrate Path. (in press).

Kuroda, Y. and Shimada, Y. 1989. Electron microscopic studies on in vitro differentiated cells from Drosophila embryos. In: Invertebrate cell system applications. J. Mitsuhashi, ed., CRC Press, FL, USA, 77-89.

Mitsuhashi, J. 1989. Invertebrate Cell System Applications.CRC Press, FL, USA.

Murai, T. 1982. Simple rearing method of thrips. Plant Protection Bull. 36, 34-37.

Ullman, D.E., Cho, J.J., Mau, R.F.L., Hunter, W.B., Westcot, D.M., and Custer, D.M. 1992. Thrips-tomato spotted wilt virus interactions: morphological, behavioral and cellular components influencing thrips transmission. In: Advances in disease vector research, K.F. Harris, ed., Springer-Verlag, NY, USA.

Ullman, D.E., German, T.L., Sherwood, J.L., Westcot, D.M., and Cantone, F.A. 1993. Tospovirus replication in insect vector cells: Immunocytochemical evidence that the nonstructural protein encoded by the S RNA of tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology, 83, 456-463.

Ullman, D.E., Westcot, D.M., Chenault, K.D., Sherwood, J.L., German, T.L., Bandla, M.D., Cantone, F.A., and Duer, H.L. 1995. Compartmentalization, intercellular transport, and autophagy of tomato spotted wilt tospovirus proteins in infected thrips cells. Phytopathology, 85, 644-654.

Wijkamp, I., van Lent, J., Kormelink, R., Goldbach, R., and Peters, D. 1993 Multiplication of tomato spotted wilt virus in its insect vector, *Frankliniella occidentalis*. J. Gen. Virol. 74, 341-349.