Tomato Spotted Wilt Tospovirus Ingestion by First Instar Larvae of Frankliniella occidentalis Is a Prerequisite for Transmission

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ABSTRACT


Tomato spotted wilt tospovirus (TSWV) is, after ingestion by first instar larvae, efficiently transmitted by second instar larvae and adults of the thrips Frankliniella occidentalis in a propagative manner. The developmental stage at which thrips larva acquire an infectious dose, resulting in adults that can transmit the virus, is further defined. TSWV accumulation and transmission occurred after ingestion by first instar larvae. Second instar larvae failed to acquire and retain TSWV upon ingestion and did not develop into transmitters. No correlation was found between the quantity of TSWV ingested by thrips and their ability to acquire TSWV. Instead, first instar larvae gradually lost their acquisition ability with increasing age, suggesting the development of a barrier preventing TSWV acquisition and, consequently, replication needed for transmission. The finding that TSWV is acquired exclusively by first instar larvae of F. occidentalis is of crucial importance for understanding the epidemiology of this devastating virus.

Additional keywords: Thysanoptera, virus-vector relations, western flower thrips.

Tomato spotted wilt tospovirus (TSWV) ranks among the most devastating plant viruses worldwide (19). The recent resurgence of TSWV, and other tospoviruses (27), is due, in part, to two major factors. First, TSWV has a very broad host range, spanning 650 plant species, including many important vegetable and horticultural crops, as well as numerous weeds, which provides a broad array of ecological niches (9,17,19). Second, the different thrips species that transmit TSWV infest a range of host plants. Currently, seven thrips species have been reported to transmit TSWV (6,29,45), of which Frankliniella occidentalis (Pergande) (Thysanoptera:Thripidae), the western flower thrips, is a very efficient vector (45). This species has expanded its geographic range to almost all of the subtropical and temperate climate zones of most of the continents (7,13,17,24,28,30,41). During the past few years, control of F. occidentalis became more difficult in part to its resistance to many chemical insecticides. In addition, incidence of TSWV cannot be controlled by insecticides applied to incoming thrips (20,37). Host-plant resistance and biological control could possibly provide alternatives to control thrips and satisfy consumers’ demands for a decrease in the use of pesticides (4,14,25). To develop sustainable management strategies for control of thrips and TSWV epidemics, a more detailed understanding of the TSWV-infection cycle and virus-vector interactions are essential (9).

It is generally accepted that only thrips larvae acquire TSWV (33). After ingestion and subsequent virus multiplication (39,48), TSWV can be transmitted by second instar (L2) larvae as well as adults (47). Once the vector becomes viruliferous, it remains so throughout its lifespan, but the rate at which each individual transmits may vary considerably (33,46).

In this report, the developmental stages at which thrips larvae acquire and transmit TSWV were analyzed and defined. The results show that only first instar larvae of F. occidentalis can acquire TSWV after ingestion and that these thrips become transmitters later in their development. Possible explanations for the exclusive capacity of first instar larvae to acquire TSWV are discussed.

MATERIALS AND METHODS

Thrips. The F. occidentalis used in our studies were obtained from infested bean plants in a greenhouse in the Netherlands and were reared as virus-free colonies on bean pods (Phaseolus vulgaris L.) at 27 ± 0.5°C with a 16-h photoperiod each day.

TSWV isolate and plant material. In all experiments, the Brazilian TSWV isolate BR-01 (13) was used. The isolate was maintained by thrips inoculation on Datura stramonium L. plants. Plants used in acquisition experiments were mechanically inoculated on the first two leaves with extracts from thrips-inoculated plants. These plants were kept in a greenhouse at approximately 22°C (16/8 h of light/dark).

Preparation of antiserum and conjugate for enzyme-linked immunosorbent assay (ELISA). Polyclonal antiserum raised against the nucleocapsid protein (N protein) of TSWV isolate BR-01 was used in ELISA (12). The immunoglobulin G (IgG) fraction was partially purified by ammonium sulfate precipitation (10). IgG was conjugated at a concentration of 1 mg/ml with 2,000 units of alkaline phosphatase (grade I, Boehringer GmbH, Mannheim, Germany) in phosphate-buffered saline (PBS; 0.14 M NaCl, 1 mM KH2PO4, 8 mM Na2HPO4, 2.5 mM KCl), pH 7.4 (3). The IgG and conjugate were stored with 0.05% sodium azide at 4°C.
TSWV-N protein detection by ELISA. The presence of TSWV-N protein in individual thrips was detected by a cocktail ELISA with enzyme amplification (31,42). Wells of Nunc-Immunoplate Maxisorb F96 plates (Nunc, Roskilde, Denmark) were coated with 150 μl of 0.5 μg of IgG per ml in coating buffer (0.05 M sodium carbonate, pH 9.6). After incubation overnight at 4°C, the plates were rinsed three times with PBS-Tween (PBS with 0.05% Tween 20). Individual thrips were placed in Eppendorf tubes and ground with a micropore in 100 μl of sample buffer (2% polyvinylpyrrolidone, 0.02% ovalbumin in PBS-Tween). The samples were added to the wells and mixed with 25 μl of 1 μg of TSWV-N protein conjugate per ml in sample buffer. Virus-free thrips and a dilution series of a nucleocapsid preparation purified from TSWV-infected Nicotiana rustica plants were used as the control and standard, respectively. The cocktail was incubated overnight at 4°C and, afterwars, was rinsed three times with PBS-Tween.

The enzyme amplification reaction was performed as described by Van den Heuvel and Peters (42). Initially, 100 μl of 0.2 mM NADP monosodium salt in 0.05 M diethanolamine buffer, pH 9.5, was added to each well. After incubation at room temperature for 45 min, 140 μl of the amplification mixture was added per well. The stock amplification mixture consisted of 700 units of alcohol dehydrogenase and 100 μl of 1 M p-iodonitrotetrazolium violet in 1 ml of 0.025 M phosphate buffer, pH 7.0. The reaction was allowed to proceed at room temperature. Absorbance values were determined with a EL 312 ELISA-reader (Bio-Teck Instruments, Greiner BV, Alphen aan de Rijn, the Netherlands) at 490 nm. The values were corrected for blank values read for wells that contained only sample buffer in the sample incubation step. The threshold value was calculated as the average of at least six virus-free thrips plus three times the standard deviation. All the readings above the threshold value were considered positive. The amount of TSWV-N protein found in each thrips was calculated by the values of the dilution series of nucleocapsid preparations.

Handling thrips in transmission experiments. Systemically infected leaves of D. stramonium plants that showed equally high TSWV-N protein titers in a dilution series, using double-antibody sandwich-ELISA (10,31), were used as sources for acquisition. The infected leaves were cut in pieces and placed randomly in Tashiro cages (36) in which F. occidentalis larvae were confined. Separate groups of instar larvae were caged on virus-free plant material as control treatments. After the acquisition access period (AAP), the thrips were transferred to virus-free D. stramonium plant material and allowed to complete their development until adult emergence. All the experiments were carried out at 25 ± 0.5°C. Adult-stage thrips were individually tested for TSWV transmission on leaf disks (13 mm in diameter) of Petunia × hybrida ‘Polo Blauw,’ as previously described (47), for three successive inoculation access periods (IAP) of 48 h. After each IAP, the leaf disks were floated on water for 2 days at 27 ± 0.5°C in 24-well plates (Costar Europe Ltd., Badhoevedorp, the Netherlands) for symptom development. Transmission efficiency was calculated as the percentage of leaf disks that developed local lesions (43).

TSWV transmission experiments. Newly hatched and 1-, 2-, 3-, 4-, and 5-day-old thrips were placed on TSWV-infected material for 24 h, using in total 58, 71, 50, 40, 51, and 50 insects, respectively, in two replicates. Before and after AAPs, the thrips were kept on virus-free D. stramonium plant material. Transmission efficiency was expressed as the number of adults that transmitted TSWV, using the petunia local lesion assay. Adult thrips were subsequently stored at −70°C prior to ELISA. Samples of thrips that fed on noninfected plant material were used as controls. The ELISA values for thrips were analyzed by Duncan’s multiple range test with a STATGRAPHICS 6.0 PLUS computer program (32,34).

In another experiment, 0-, 12-, 20-, 28-, 36-, 44-, 52-, and 56-day-old larvae were given a 24-h AAP, with 30 insects per treatment per replication. Prior to AAP, the ratio of first instar (L1) and second instar (L2) larvae was determined by counting the molting skins found in each treatment. Transmission efficiency was determined for adults. This experiment was repeated three times. Thrips grown on virus-free material were used as controls.

Ingestion and transmission by the L1 and L2 larval stages. Newly hatched, 0-h-old first instar larvae (n = 84), 60-h-old second instar larvae (n = 102), and 40-h-old (n = 138) larvae were placed for 4 h on infected material. Before and after the AAP, the larval stage of the 40-h-old larvae was determined based on the number of molting skins and gut color. The gut has a green color during feeding on D. stramonium and turns transparent during the molting stage. Using this qualitative technique together with the molting skins found, the larvae were divided into four categories, i.e., first instar larvae (n = 26), second instar larvae (n = 21), larvae in the molting stage (n = 45), and a mixture of first and second instar larvae (n = 46). After the AAP of 4 h, larvae from each group were collected randomly, frozen at −70°C, and later assayed by ELISA. Transmission efficiency was determined for adults by the petunia local lesion assay.

TSWV accumulation in maturing thrips. TSWV accumulation was analyzed in maturing thrips after they ingested virus as newly hatched larvae (L1) and 2.5-day-old larvae (L2). The thrips were given for 4-h AAP on TSWV-infected D. stramonium plant material. Respective groups of 25 individuals were collected randomly immediately after AAP every day in the larval, pupal, and the adult stages. These thrips were frozen immediately at −70°C and later tested individually for the presence of N protein. Thrips that were confined for 4 h on noninfected D. stramonium plant material were used as controls.

Determination of the amount of TSWV ingested. Larvae ranging in age from 0 to 5 days old were given an 8-h AAP on TSWV-infected D. stramonium plant material. Before the AAP, the thrips were kept on virus-free D. stramonium plant material. Immediately after the AAP, the larvae (approximately 25 per treatment) of each age group were collected and stored at −70°C until tested by ELISA. Samples of thrips confined for 8 h on virus-free D. stramonium plant material were used as controls.

RESULTS

Relationship between ingestion of TSWV by larvae and transmission by adults. We allowed different developmental stages of F. occidentalis larvae, ranging between 0 and 5 days old, to ingest TSWV to compare the ability of the adult-stage thrips to transmit TSWV. Our data showed that TSWV acquisition by 0- to 2-day-old larvae resulted in adults that were capable of transmitting TSWV, whereas when 3- to 5-day-old larvae fed on TSWV-infected plant material the adults failed to transmit TSWV (Fig. 1). The highest adult transmission rate was obtained when newly hatched larvae had ingested TSWV. Transmission efficiency decreased sharply with increase in the age at which first instar larvae fed on infected plant material. Also, when thrips were tested for TSWV-N protein by ELISA, the number of ELISA-positive adults decreased with increasing age at which TSWV was ingested by first instar larvae and paralleled the decrease in adult transmission efficiency.

Ninety percent of the adults that fed as newly hatched larvae were ELISA-positive, and 48% transmitted TSWV. No adult thrips that ingested TSWV as 3- to 5-day-old larvae were ELISA-positive. The results also showed that when ELISA values for adult thrips (TSWV transmitters versus nontransmitters) were compared, all thrips transmitters showed a significantly higher ELISA value than the nontransmitters (P < 0.05), hence a certain threshold amount of TSWV might need to be present for successful transmission (Table 1). Furthermore, thrips that were nontransmitters could be divided into adults that were ELISA-positive and -negative. ELISA-positive thrips were found only when TSWV was fed to 0- to 2-day-old larvae.
In the next experiment, we studied acquisition of TSWV, using groups of larvae ranging in age from 0 to 56 h to define more precisely the moment at which the larvae lost their ability to acquire TSWV. The L1/L2 ratio of larvae, age of larvae when fed on TSWV-infected plant material, and ability to transmit TSWV in the adult stage were determined. The highest transmission efficiency (42%), again, was observed for adults that were fed as newly hatched larvae on TSWV-infected plant material. Larvae by the age of 56 h (L2 larvae) could not acquire TSWV, because when they were adults they did not transmit the virus. These results, again, show that the ability to acquire TSWV decreased with the age of the L1 larvae and that the potential to acquire TSWV was significantly reduced for L2 larvae. The data, however, cannot exclude the possibility that some early L2 larvae acquired TSWV, because some were present in the 36- and 44-h-old populations analyzed (Fig. 2).

**Fig. 1.** Tomato spotted wilt virus (TSWV) transmission efficiency (±SE) and enzyme-linked immunosorbent assay (ELISA)-positive adult thrips after a 24-h acquisition access period (AAP) on TSWV-infected *Datura stramonium* plant material by 0- to 5-day-old larvae. Values below x-axis represent the number of thrips tested and are the sum of two replications.

**Fig. 2.** Relationship between L1 (first instar)/L2 (second instar) ratio of thrips larvae at acquisition and the subsequent transmission efficiency of the resulting adults. Larvae between 0 and 56 h old were given a 24-h acquisition access period (AAP) on tomato spotted wilt virus-infected *Datura stramonium* plant material. Averages ± SE are presented for three replications, with 30 insects per treatment per replication.

**TABLE 1.** Average enzyme-linked immunosorbent assay (ELISA) values (±SE), using antisera against tomato spotted wilt virus nucleocapsid protein (TSWV-N protein), for adult thrips after TSWV ingestion by larvae of different ages

<table>
<thead>
<tr>
<th>Age of acquisition (days)</th>
<th>ELISA value±</th>
<th>Nontransmitter ELISA-positive</th>
<th>ELISA-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.785 ± 0.024 (22)</td>
<td>0.390 ± 0.014 (21)</td>
<td>0.001 ± 0.005 (5)</td>
</tr>
<tr>
<td>1</td>
<td>0.723 ± 0.111 (2)</td>
<td>0.219 ± 0.103 (3)</td>
<td>0.002 ± 0.001 (6)</td>
</tr>
<tr>
<td>2</td>
<td>0.757 (5)</td>
<td>0.046 (1)</td>
<td>0.002 ± 0.002 (6)</td>
</tr>
<tr>
<td>3</td>
<td>...</td>
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<td>0.004 ± 0.002 (6)</td>
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<tr>
<td>4</td>
<td>...</td>
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<tr>
<td>5</td>
<td>...</td>
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</table>

*Transmitter: thrips that transmitted TSWV to petunia. All were ELISA-positive. Nontransmitter: nontransmitting thrips were divided into two categories based on their readings in ELISA. The threshold value was calculated as the average of virus-free thrips + 3 times the standard deviation. All readings above this threshold, ranging from 0.009 to 0.025, were considered positive, readings below this value were considered negative. Values followed by the same letter are not significantly different according to Duncan’s multiple range test (P < 0.05). The number of insects tested per combination is indicated in parentheses and is the sum of two replications.

**TABLE 2.** Tomato spotted wilt virus (TSWV) ingestion by 0-, 40-, and 60-h-old thrips larvae and subsequent transmission by the corresponding adults

<table>
<thead>
<tr>
<th>Age of larvae (h)</th>
<th>Larvae gut color before and after AAP</th>
<th>Larvae after AAP (ELISA)</th>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>0</td>
<td>Transparent</td>
<td>Green*</td>
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<tr>
<td>40</td>
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<td>Transparent*</td>
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<td></td>
<td>Transparent</td>
<td>Transparent*</td>
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<tr>
<td>60</td>
<td>Green</td>
<td>Green*</td>
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<tr>
<td></td>
<td>Transparent</td>
<td>Green</td>
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<tr>
<td></td>
<td>Green</td>
<td>Green*</td>
</tr>
</tbody>
</table>

*Gut color was used, in combination with counting molting, to identify larval-stage of larvae that had been given a 4-h acquisition access period (AAP).

**TABLE 3.** Average enzyme-linked immunosorbent assay (ELISA) values (±SE), using antisera against tomato spotted wilt virus nucleocapsid protein (TSWV-N protein), for adult thrips after TSWV ingestion by larvae of different ages

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**Fig. 3.** Relationship between L1 (first instar)/L2 (second instar) ratio of thrips larvae at acquisition and the subsequent transmission efficiency of the resulting adults. Larvae between 0 and 56 h old were given a 24-h acquisition access period (AAP) on tomato spotted wilt virus-infected *Datura stramonium* plant material. Averages ± SE are presented for three replications, with 30 insects per treatment per replication.

**Fig. 4.** Tomato spotted wilt virus (TSWV) transmission efficiency (±SE) and enzyme-linked immunosorbent assay (ELISA)-positive adult thrips after a 24-h acquisition access period (AAP) on TSWV-infected *Datura stramonium* plant material by 0- to 5-day-old larvae. Values below x-axis represent the number of thrips tested and are the sum of two replications.

**Fig. 5.** Relationship between L1 (first instar)/L2 (second instar) ratio of thrips larvae at acquisition and the subsequent transmission efficiency of the resulting adults. Larvae between 0 and 56 h old were given a 24-h acquisition access period (AAP) on tomato spotted wilt virus-infected *Datura stramonium* plant material. Averages ± SE are presented for three replications, with 30 insects per treatment per replication.

**Fig. 6.** Tomato spotted wilt virus (TSWV) transmission efficiency (±SE) and enzyme-linked immunosorbent assay (ELISA)-positive adult thrips after a 24-h acquisition access period (AAP) on TSWV-infected *Datura stramonium* plant material by 0- to 5-day-old larvae. Values below x-axis represent the number of thrips tested and are the sum of two replications.

**Fig. 7.** Relationship between L1 (first instar)/L2 (second instar) ratio of thrips larvae at acquisition and the subsequent transmission efficiency of the resulting adults. Larvae between 0 and 56 h old were given a 24-h acquisition access period (AAP) on tomato spotted wilt virus-infected *Datura stramonium* plant material. Averages ± SE are presented for three replications, with 30 insects per treatment per replication.

**Fig. 8.** Tomato spotted wilt virus (TSWV) transmission efficiency (±SE) and enzyme-linked immunosorbent assay (ELISA)-positive adult thrips after a 24-h acquisition access period (AAP) on TSWV-infected *Datura stramonium* plant material by 0- to 5-day-old larvae. Values below x-axis represent the number of thrips tested and are the sum of two replications.
thrips in this population transmitted TSWV after becoming adults. Second instar larvae that were 60 h old contained, on average, 10.8 ng of N protein immediately after the AAP but failed to transmit TSWV in the adult stage. At the age of 40 h, the late first instar larva after AAP contained, on average, less TSWV-N protein than newly hatched larvae, and only one adult was able to transmit TSWV. After AAP, no N protein was detected in larvae that were in the process of molting. Adults emerging from these larvae failed to transmit TSWV. The thrips from the third group, consisting of early second instar larvae, contained, on average, lower amounts of N protein (1.3 ng) than 60-h-old larvae (10.8 ng) but more than late first instar larvae (0.6 ng) (Table 2). Although the early second instar larvae did ingest virus, the adults that developed from these larvae did not become transmitters. An average of 5.4 ng of N protein was detected in the larvae from the fourth group. It is possible that they might represent a mixture of first and second instar larvae. The level of TSWV-N protein was higher in these larvae than in 40-h-old first instar larvae. Therefore, it seems likely that this group of 40-h-old larvae consisted mainly of second instar larvae. The results obtained in these analyses show that only first instar larvae acquire TSWV.

**TSBV accumulation in maturing thrips.** TSBV accumulation was analyzed in thrips after they ingested TSBV as newly hatched (L1) and 2.5-day-old larvae (L2) to explain the exclusive capacity of L1 larvae to acquire TSBV. The L1 and L2 larvae were given a 4-h AAP on TSBV-infected plant material. Samples of 25 individuals were collected randomly at intervals during their development into adults and analyzed individually in ELISA for the presence of TSBV-N protein (Fig. 3). On average, 3.6 ng of N protein was detected in L1 larvae when sampled directly after the AAP. Four-day-old thrips contained an average of 13.1 ng of N protein. A decrease in the level of N protein was detected in the pupal stage (1.5 ng), and again, an increase of N protein was detected in the adults (16.3 ng). The L2 larvae, however, ingested more virus (10.8 ng of N protein on average), but no increase in the amount of this protein could be detected during their development (Fig. 3).

**Influence of amount of virus ingested on ability to acquire TSBV.** The loss of acquisition capacity with age may be explained by the relative amount of TSBV-N protein ingested by larvae during development. To study TSBV ingestion, larvae ranging in age from 0 to 5 days were placed on TSBV-infected plant material for 8 h. Immediately after AAP, the thrips were frozen at -70°C and later tested for their N protein content (Fig. 4A). The results show that the amount of N protein after AAP increases with the age of the larvae, and, hence, reflects an increasing consumption rate with age. The amount of N protein ingested by larvae, therefore, does not appear to determine or significantly influence the resulting potential of adults to become transmitters. Finally, at all ages, some individuals showed no evidence of having ingested TSBV, as determined by ELISA done immediately after AAP (Fig. 4B).

**DISCUSSION**

The results presented in this paper clearly show that TSBV ingestion by first instar larvae of *F. occidentalis* is a prerequisite for transmission by thrips at later stages (Figs. 1 and 2; Tables 1 and 2). The percentage of adults that transmitted TSBV decreased sharply when older first instar larvae were used in TSBV-acquisition experiments (Fig. 2). The exclusive capacity of first instar larvae to acquire TSBV was explained by the ability to accumulate TSBV in later stages after ingestion, whereas second instar larvae failed to do so (Fig. 3). No correlation was found between the amount of TSBV ingested and the ability of thrips to acquire TSBV (Fig. 4).

A novel technique, in combination with counting molting skins, was used to discriminate between late first instar and early second instar larvae, because they are difficult to differentiate by any mor-

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[Fig. 3. Detection of tomato spotted wilt virus-nucleocapsid protein (TSBV-N protein) in maturing thrips (*n = 25* per treatment; newly hatched larvae [L1] and 2.5-day-old larvae [L2]) after a 4-h acquisition access period on TSBV-infected *Datura stramonium* plant material. Amounts of TSBV-N protein higher than the threshold value, ranging from 0.005 to 0.050 ng, were considered positive. The average amount of N protein, determined by enzyme-linked immunosorbant assay values ± SE, is shown.]
transovarial and venereal barriers has been demonstrated already, because female thrips cannot transmit TSVV vertically (46).

Larval instars of aphids, leafhoppers, and planthoppers acquire circulative and propagative viruses more efficiently than adults (1, 35). However, a principal difference between larvae and adults and even between larval stages in the ability to acquire virus has been described only for Fiji disease reovirus (FDRV), which is transmitted by the planthoppers Perkinsiella saccharicida, P. vititennis, and P. vastatrix. This virus can be acquired only by the L1, L2, and L3 larvae, not by L4 and L5 larvae and adults. As with TSVV, the ability to acquire an infectious dose of FDRV also decreases with increasing larval age (2,11,15,16,26,27). Ammar (1) suggested the occurrence of a midgut (infection or escape) barrier or other dissemination barriers for FDRV in the later instar and adult stages.

The finding that only L1 larvae of F. occidentalis can acquire and retain TSVV and that only these individuals develop into transmitters is crucial for understanding TSVV epidemiology. Our data allow us to describe the TSVV-thrips transmission cycle more accurately. TSVV must be ingested by L1 larvae, and after a latent period and virus replication, TSVV subsequently can be transmitted by late L2 larvae and adults (39,47,48). In defining the TSVV-thrips transmission relationship more accurately, our findings are relevant for designing optimal TSVV control measures. Control of TSVV at the moment, is focused on the transmitters, whereas control possibly could be more effective if aimed at the beginning of the transmission cycle, i.e., targeting L1 larvae.

LITERATURE CITED