Determination of the Median Latent Period of Two Tospoviruses in *Frankliniella occidentalis*, Using a Novel Leaf Disk Assay

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ABSTRACT

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A novel assay system, based on the use of the local lesion host *Petunia* × *hybrida*, was developed for studying the transmission of tospoviruses by the thrips *Frankliniella occidentalis*. Efficient transmission was obtained for two different tospoviruses, tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INSV), reaching rates up to 55.1 and 92.5%, respectively. Because the majority of the thrips became viruliferous in the second larval stage, the median latent period (LP_{50}) was determined for this stage. The LP_{50} values decreased with increasing temperatures. For INSV, the LP_{50} values were 157, 103, and 82 h at 20, 24, and 27C, respectively, and for TSWV, they were 171, 109, and 84 h at the respective temperatures.

Additional keywords: virus-vector relations.

Tospoviruses cause diseases in many economically important crops in the field and in greenhouses throughout the tropical, subtropical, and temperate climate zones. More than 550 plant species in 75 families have been reported as susceptible to these viruses (D. Peters, *personal communication*). The renewed incidence of the virus in the Northern Hemisphere in the 1980s has been attributed to the spread of the western flower thrips *Frankliniella occidentalis* (Pergande) from the western United States throughout North America and Europe (5,10,19,36).

Tospoviruses are transmitted by thrips (Thysanoptera: Thripidae) in a persistent manner (26). The virus is passed transstadially and replicates in the vector (39). Seven species have been reported as possible vectors (6,15,26). Thrips feed by piercing

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a cell and sucking its contents (16). However, mechanisms of viral transmission are not clearly understood, and most of the parameters describing the virus-vector relationships have not been thoroughly analyzed. It has generally been accepted that only larvae can acquire the virus (27,35). The minimal acquisition period is 15–30 min (26). Between acquisition and transmission, a period occurs in which the thrips is not infectious. The reported latent periods of tomato spotted wilt virus (TSWV) ranged from 4 to 18 days in *Thrips tabaci* and from 4 to 12 days in *Frankliniella fusca* (27). Although some larvae can transmit the virus before pupation, adults are more efficient in transmitting the virus. Individuals may retain infectivity for life, but they transmit the virus irregularly (26).

Here we report studies on the efficiency of transmission of two distinct tospoviruses, impatiens necrotic spot virus (INSV) (9,17) and TSWV as well as their median latent periods (LP_{50}). Temperature has been shown to affect transmission and length of latent period (LP) of circulatively transmitted (29,31,38) viruses as well as propagative viruses (12). Therefore, the LP_{50} was studied at three different temperatures. This study was performed using a newly developed local lesion assay that efficiently measures tospovirus transmission.

MATERIALS AND METHODS

Thrips. Virus-free *F. occidentalis* was reared on bean pods (*Phaseolus vulgaris* L. 'Prelude') at 27 ± 0.5 C with a 16-h photoperiod (light/dark, 16:8 h). The colony was started with adults collected from a greenhouse infestation in the Netherlands.

Virus isolates. The Brazilian TSWV isolate BR-01 (10) and the Dutch INSV isolate NL-07 (9) were used in these experiments. TSWV is the type species of the newly established *Tospovirus* genus within the family of the Bunyaviridae (11,14). INSV has been recently characterized and found to be a serologically distinct tospovirus (9,17). It occurs in ornamental plant species and was isolated from *Impatiens* sp.

Acquisition feeding. Impatiens plants were inoculated 2–3 wk after sowing by single viruliferous *F. occidentalis* adults harboring either TSWV isolate BR-01 or INSV isolate NL-07. The plants were grown in a greenhouse at approximately 22 C (light/dark, 16:8 h). Systemically infected leaves (two leaves from three plants) were used for acquisition feeding. Each leaf was cut into three pieces and divided between three different Tashiro (33) cages. Each of the cages contained three leaf pieces. Leaves were kept fresh with a piece of wet filter paper. First instar larvae of *F. occidentalis* larvae (0- to 4-h old) of the same cohort were confined to these pieces in the cages. The thrips were given an acquisition access period (AAP) of 24 h in a controlled environment at 20 (±0.5), 24 (±0.5), and 27 (±0.5) C. First instar larvae, caged on virus-free impatiens plants, were used as controls.

LP experiments. After the AAP, each larva was individually transferred at 24-h intervals to fresh leaf disks of *Petunia* × *hybrida* Hort. Vilm.-Andr. 'Blue Magic' (2). Young, fully expanded leaves were used as sources for the leaf disks. One leaf disk (13 mm in diameter) was placed in a 1.5-ml Eppendorf tube with a small piece of Kleenex tissue paper to absorb excess water evaporating from the disk. The cap of the Eppendorf tube was punctured three times with holes through which larvae could not escape. Thrips were carefully picked up with a small brush and transferred daily to new leaf disks. Individuals were tested until day 4 of adult development or until they died. The thrips acquired the virus and were tested for their infectivity at the same temperature. The transmission of INSV was studied using 40, 41, and 49 larvae, and transmission of TSWV was studied using 87, 58, and 79 larvae incubated at 20, 24, and 27 C, respectively. Ten healthy control thrips were used at each temperature. After each inoculation access period (IAP), the leaf disks were incubated at 27 C in 24 well plates (Costar Europe Ltd., Badhoevedorp, the Netherlands) while floating on water to develop local lesions. Symptoms were visually scored and infection was confirmed by DAS-ELISA (double antibody sandwich enzyme-linked immunosorbent assay).

The LP was defined as the time interval from the start of the AAP to the end of the IAP, during which the first transmission occurred. Two LP_{50}s were established: one for viruliferous thrips in the larval stages and one for the cumulative number of thrips transmitting in the larval or the adult stages for the first time. The LP_{50}s were estimated by log-probit analysis of the time-series of cumulative percentages of thrips transmitting the virus for the first time (29). The LP_{50}s and their 95% fiducial limit (FL) were calculated by the method of Finney (13). Data were processed and analyzed with the POLO-PC program (18).

Virus transmission to petunia leaf disks and a systemic host. The transmission of INSV and TSWV to petunia leaf disks was compared to the rate at which systemic hosts (*Impatiens* sp.) were infected, by giving the larvae an AAP of 24 h at 27 C as previously described. The larvae were transferred to healthy *Datura* leaves in Tashiro (33) cages at 27 C to complete their development. On day 2 after adult emergence, 30 thrips per isolate were tested individually on petunia leaf disks, and 30 were tested on impatiens seedlings for an IAP of 24 h. Leaf disks were incubated in 24 well plates, and impatiens seedlings were transferred to the greenhouse for symptom development.

Antiserum production and purification. Polyclonal antiserum raised against the nucleocapsid protein (anti-N-serum) of isolates NL-07 and BR-01 was used in ELISA. Production of the antiserum has been described previously (9). The immunoglobulin (IgG) fraction was partially purified by ammonium sulphate precipitation (8). IgG was conjugated at a concentration of 1 mg/ml with 2,000 U of alkaline phosphatase (Grade I, Boehringer, Mannheim, Germany) in phosphate-buffered saline (PBS), pH 7.4 (4). The IgG and conjugate were stored with 0.05% sodium azide at 4 C.

Virus detection by ELISA. To confirm that the local lesions on the leaf disks were caused by INSV or TSWV infection, extracts of the disks were tested in a DAS-ELISA format (8,23). Wells of Nunc Maxisorp F96 immuno plates (Life Technologies BV, Breda, the Netherlands) were coated with 150 μl of 1 μg/ml IgG in coating buffer (0.05 M sodium carbonate, pH 9.6) overnight at 4 C. The plate was rinsed three times with PBS-T (0.14 M NaCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.5 mM KCl, and 0.05% Tween 20), and 100 μl of sample extracts was added and incubated for 2 h at 37 C. The extracts were prepared by grinding one leaf disk in PBS-T at a ratio of 15 mg/ml of PBS-T. Leaf disks from healthy plants were used as controls. After incubation, the plates were rinsed, 100 μl of conjugate at 1 μg/ml in PBS-T was added to each well and incubated for 2 h at 37 C. After washing, 100 μl of substrate was added to each well, and color development was allowed to proceed at room temperature. Absorbance values were read on an EL 312 ELISA-reader (Bio-Tek Instruments, Greiner BV, Alphen aan de Rijn, the Netherlands) at 405 nm. Wells containing only PBS-T in the sample incubation step were used as blanks.

RESULTS

Infectivity assay. Because thrips are not readily recovered in transfers between plants, a leaf disk assay was developed in which the thrips were allowed to feed on leaf disks from *P.* × *hybrida* plants. This species reacts by forming small black or brown local lesions within 2–3 days after inoculation with either virus (2,28). Thrips that acquired virus were placed on these disks to test their infectivity. Each viruliferous thrips differed considerably in its inoculation activity during an IAP of 24 h (Fig. 1). The number of local lesions produced on one leaf disk varied from one lesion to approximately 30. ELISAs were used to verify the presence of virus in leaf disks. Based on the number of lesions, disks were divided into five categories: disks without symptoms (0), disks with one local lesion (1), disks with two to three local lesions (2–3), disks with four to 10 lesions (4–10), and disks with more than 10 (>10) lesions. These categories contained 11, six, 14, 12, and 11 leaf disks, respectively. The average ELISA readings and their standard deviations are presented in Figure 2. All disks showing local lesions gave positive reactions in ELISA. However,

no correlation could be observed between the number of lesions and the ELISA values, which explains the high standard deviation (Fig. 2).

Efficiency of INSV and TSWV transmission. For a reliable comparison of the transmission efficiency, experiments had to be performed with a common systemic host for acquisition feeding. We found impatiens a suitable host that replicated both viruses to comparable high titers. The applicability of the disk assay was first tested by comparing transmission of INSV and TSWV to impatiens seedlings. The results (Table 1) showed that the transmission rate of each virus was similar in the two assay systems.

In the LP_{50} experiments, virus was acquired from impatiens leaves systemically infected with either INSV or TSWV during an AAP of 24 h at 20, 24, or 27 C. The results obtained showed that INSV was transmitted more efficiently than was TSWV. Assaying each thrips on leaf disks as larva and adult revealed that 92.5, 85.0, and 81.6% transmitted INSV to the leaf disks at 20, 24, and 27 C, respectively. These percentages were 55.1, 45.8, and 43.0 for TSWV (Table 2). It was noteworthy that most of the thrips already transmitted the virus when they were still in their second larval stage. At the temperatures applied, the percentages of the thrips that transmitted during the larval stage were 80.0, 70.0, and 63.3 for INSV and 52.8, 40.7, and 32.9 for TSWV at 20, 24, and 27 C, respectively. Thrips do not feed in their prepupal and pupal stages and, therefore, are not expected to transmit virus during these stages; the emerging adults resume feeding. Only a few thrips that did not transmit as larvae, transmitted the virus as adults. The values for those adults were 12.5, 15.0, and 18.3% for INSV, and 2.3, 5.1, and 10.1% for TSWV when the thrips were kept at 20, 24, and 27 C, respectively (Table 2). These results show that at higher temperatures a relatively larger part of the thrips starts to transmit virus as adults.

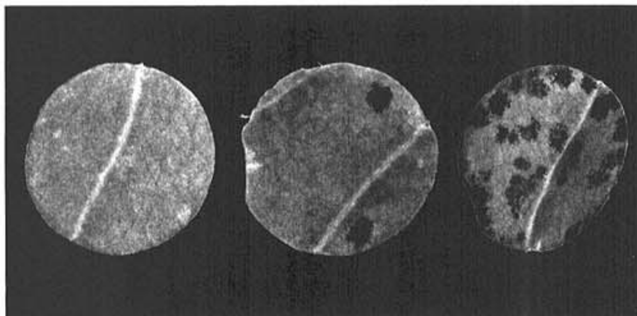

Fig. 1. Local lesions caused by the impatiens necrotic spot virus isolate NL-07 on leaf disks of *Petunia* × *hybrida* cv. Blue Magic 3 days after the start of the inoculation access period (IAP). Healthy thrips fed on the left disk; infected *Frankliniella occidentalis* larvae were given an IAP of 24 h on the two remaining leaf disks.

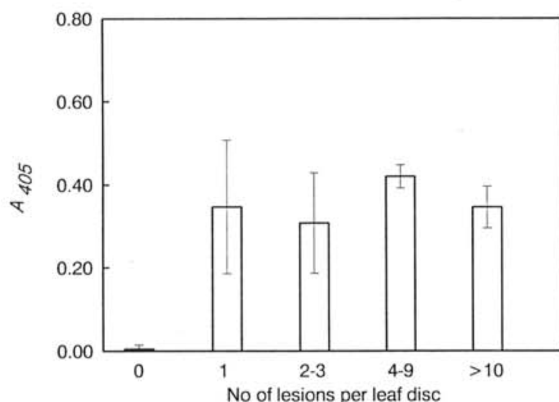

Fig. 2. Average enzyme-linked immunosorbent assay values and standard deviations of leaf disks, using antiserum to the N protein of impatiens necrotic spot virus isolate NL-07. Leaf disks were divided into five categories: disks without symptoms (0), one lesion per disk (1), one to three lesions per disk (1–3), four to 10 lesions per disk (4–10), and more than 10 lesions per disk (10).

All thrips that transmitted INSV during their second larval stage continued to do so as adults, whereas 6% of the TSWV viruliferous larvae failed to transmit virus after adult emergence (results not shown).

The LP_{50} of INSV and TSWV. The larvae reached the prepupal stage in 12–13 days at 20 C, in 7–8 days at 24 C, and in 5–6 days at 27 C (results not shown). Thrips do not feed during the prepupal and pupal stages. Because only a low percentage of thrips developed into viruliferous thrips during these stages or in the first hours of being an adult, the LP_{50} was determined for the second larval stage. The cumulative percentages of larvae transmitting the virus for the first time were plotted against time (Fig. 3). The LP_{50} was estimated from these curves by log-probit analysis (Table 3). The LP_{50} decreased with increasing temperature. The time at which 50% of the larvae that eventually transmitted began transmitting, when increasing the temperature from 20 to 27 C, ranged from 157 to 82 h for INSV and from 171

TABLE 1. Comparison of impatiens seedlings and petunia leaf disks as bioassay hosts for transmission of impatiens necrotic spot virus (INSV) isolate NL-07 and tomato spotted wilt virus (TSWV) isolate BR-01 by *Frankliniella occidentalis*[a]

Isolate	Percentage of infected impatiens seedlings	Percentage of petunia leaf disks with local lesions
INSV	86.7	80.0
TSWV	36.7	43.3

[a] Acquisition access period was 24 h on systemically infected impatiens leaves. Adults were given an inoculation access period of 24 h at 27 C on petunia leaf disks and on impatiens seedlings.

TABLE 2. Efficiency of transmission of impatiens necrotic spot virus (INSV) isolate NL-07 and tomato spotted wilt virus (TSWV) isolate BR-01 by larvae and adults of *Frankliniella occidentalis* to petunia leaf disks at 20, 24, and 27 C[a]

Temp. (C)	Transmitting larvae (%)[b]		Transmitting adults (%)[c]	
	INSV	TSWV	INSV	TSWV
20	80.0	52.8	92.5	55.1
24	70.0	40.7	85.0	45.8
27	63.3	32.9	81.6	43.0

[a] Acquisition access period (AAP) was 24 h on systemically infected impatients leaves. After the AAP, thrips were transferred at 24-h intervals to fresh petunia leaf disks.
[b] Thrips that began to transmit virus in the second larval stage.
[c] Cumulative number of thrips that transmitted virus for the first time as larvae or as adults.

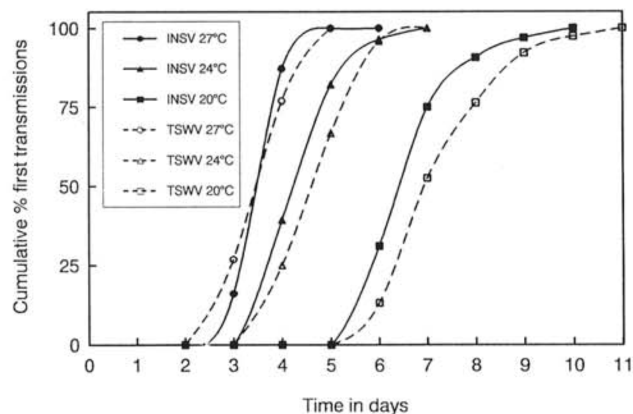

Fig. 3. The cumulative percentages of *Frankliniella occidentalis* larvae transmitting impatiens necrotic spot virus (INSV) isolate NL-07 and tomato spotted wilt virus (TSWV) isolate BR-01 for the first time are plotted as a function of time. Acquisition access period was 24 h on systemically infected impatiens leaves. The thrips were exposed during these experiments to temperatures of 20, 24, and 27 C.

to 84 h for TSWV. The LP_{50} values for both viruses differed significantly ($P < 0.05$) in the three temperature treatments with different intercepts but parallel slopes. At 20 C, the LP_{50} for INSV was slightly shorter than that for TSWV. However, at 24 and 27 C, the LP_{50}s for INSV and TSWV were comparable, indicating that the infectivity for both viruses developed at almost the same rate in the vector.

Also, the LP_{50}s were established for all thrips either transmitting virus for the first time as larvae or as adults. These LP_{50}s were slightly higher. They ranged from 169 to 98 h for INSV and from 176 to 103 h for TSWV at temperatures from 20 to 27 C (Table 4).

DISCUSSION

This paper describes an efficient and novel assay system to study the transmission of tospoviruses by thrips larvae and adults, using leaf disks of *P.* × *hybrida* cv. Blue Magic. This cultivar was used by Allen and Matteoni (2) in a study monitoring the presence of viruliferous thrips in greenhouses. An assay in which local lesions are formed on petunia leaf disks has a number of advantages over the use of systemic hosts as test plants. First, successful transmission can be reliably scored within 2–3 days. Feeding scars caused by the thrips can be distinguished readily from the lesions caused by INSV and TSWV. Second, the number of infectious piercings can be determined, and the infection can be precisely located. Third, because the larvae are very active and tend to move to the ground before pupation, assaying the larvae on a leaf disk provides better biological containment than does the use of whole test plants. Finally, the thrips transmit the virus as efficiently to petunia leaf disks as to plants, which respond with a systemic infection (Table 1).

We showed that 80–85% of the thrips that transmitted virus did so for the first time when they were in their second larval stage (Table 2). The LP_{50} was 5–19 h longer when the first transmissions of the larvae and adults were cumulated than was the LP_{50} for only larvae (Tables 3 and 4). These results demonstrate that the first transmissions of the adults contribute only slightly to the LP_{50}.

The moment at which the thrips, which transmitted only as adults, became infectious cannot be elucidated. These adults may have become infectious at the end of their second larval stage, during the prepupal and pupal stage, or during the early moments of their adult stage. Development of infectivity in the late second larval stage larvae and the prepupae and pupae cannot be demonstrated, because the former exhibits progressively decreasing feeding activity and the latter shows no feeding activity at all. These considerations suggest that the LP_{50} for the larvae will underestimate the real LP_{50}, whereas the LP_{50} that includes the first transmissions of the adults results in an overestimated LP_{50}.

LP_{50} values for INSV and TSWV obtained with this novel test system were considerably shorter and showed less variation than did previously reported values for TSWV (25). This difference may be due to the high sensitivity and efficiency of this test system. The latent periods of both INSV and TSWV also are shorter than are the extrinsic incubation periods of animal-infecting bunyaviruses, which are often as long as the life expectancy of the adult vectors (3). California encephalitis virus (genus *Bunyavirus*) could be transmitted 13 days after acquisition when the adult mosquitoes were kept at 13 and 23 C (20). Northway virus (genus *Bunyavirus*) was transmitted after incubating mosquitoes for 27 days at 13 C (21). Because a small number of mosquitoes was used and not tested for viral transmission at regular intervals after acquisition, conclusive comparisons cannot be made between the incubation periods of plant and animal bunyaviruses in their vectors.

Considerable variation was observed in the efficiency of tospovirus transmission. High efficiency of INSV transmission (up to 92.5%) was found in our local lesion system. Values for TSWV were consistently lower (43.0–55.1%), though still higher than the results previously reported (1,7,22,25). Efficiencies of approximately 20–30% have been reported for the transmission of TSWV by *F. occidentalis* (22,25). Allen and Broadbent (1) showed that 18 and 33% of the thrips transmitted TSWV to *Lycopersicum esculentum* and *Gomphrena globosa*, respectively, whereas Cho et al (7) observed that only 7% of the *F. occidentalis* transmitted TSWV to the lettuce cv. Parris Island Cos, although the virus was detected in more than 50% of the adults that fed on infected plants when they were larvae. This low level of transmission probably reflects differences in host suitability for virus transmission and infection, as indicated by low levels of TSWV infection after mechanical inoculation (7). Variation in transmission efficiencies by *F. occidentalis* between our study and those in previous reports might be due to differences in the populations of *F. occidentalis* used, their host preferences, the isolates of the virus, the susceptibility of the test plants, and the various methods applied. In view of the higher efficiencies reported here, we conclude that systemically infected impatiens (virus source) and petunia (local lesion host) are suitable plant species for performing transmission studies.

When the individual thrips were tested for their ability to transmit virus to petunia leaf disks and impatiens seedlings, the mortalities ranged between 5 and 10% for both virus-free and viruliferous thrips. A slightly higher mortality (16–20%) was found in the LP experiments when the larvae were transferred daily. It is plausible that this mortality is due to handling of the animals and not to a pathogenic effect of the virus on the thrips. In contrast, high mortalities (86.9%) of immature thrips have been reported when they were fed on infected chrysanthemum plants. In view of our results, this high mortality has to be explained by factors other than a pathogenic effect (24).

Temperature had a considerable influence on the LP. With increasing temperatures, both the LP and the time required for larval development decreased. The same pattern has been found

TABLE 3. Transmission of impatiens necrotic spot virus (INSV) isolate NL-07 and tomato spotted wilt virus (TSWV) isolate BR-01 to petunia leaf disks during the larval stages of *Frankliniella occidentalis* at 20, 24, and 27 C[a]

	INSV		TSWV	
Temp. (C)	LP_{50}[b] (h)	95% FL[c] (h)	LP_{50} (h)	95% FL (h)
20	157	150–163	171	165–176
24	103	96–108	109	103–116
27	82	78–87	84	77–89

[a] Acquisition access period (AAP) was 24 h on systemically infected impatients leaves. After the AAP, larvae were transferred at 24-h intervals to fresh petunia leaf disks.
[b] Median latent period (LP_{50}): the time interval at which 50% of the larvae completed their latent period. The LP was defined as the period of time between the start of the AAP and the end of the inoculation access period in which the first virus transmission was accomplished.
[c] Fiducial limits (FL) of the LP_{50}.

TABLE 4. Transmission of impatiens necrotic spot virus (INSV) isolate NL-07 and tomato spotted wilt virus (TSWV) isolate BR-01 to petunia leaf disks during the larval and adult stages of *Frankliniella occidentalis* at 20, 24, and 27 C[a]

	INSV		TSWV	
Temp. (C)	LP_{50}[b] (h)	95% FL[c] (h)	LP_{50} (h)	95% FL (h)
20	169	137–194	176	162–189
24	118	82–148	119	98–138
27	98	ND[d]	103	71–137

[a] Acquisition access period (AAP) was 24 h on systemically infected impatients leaves. After the AAP, thrips were transferred at 24-h intervals to fresh petunia leaf disks.
[b] Median latent period (LP_{50}): the time interval at which 50% of the thrips completed their latent period. The LP was defined as the period of time between the start of the AAP and the end of the inoculation access period in which the first virus transmission was accomplished.
[c] Fiducial limits (FL) of the LP_{50}.
[d] Not determined.

for other circulatively transmitted viruses, such as pea enation mosaic virus (PEMV) (29) and barley yellow dwarf virus (BYDV) (38), and for propagative viruses, such as sowthistle yellow vein virus (SYVV) (12). The temperature effect on the LP of viruses in their vector may be due to a higher virus-replication rate (12) or an increased movement of virus through the vector at higher temperatures (32). Our results show that with increasing temperature the percentage of transmitting larvae decreased while the percentage of thrips transmitting virus for the first time as adults increased. This might indicate that the development of thrips is relatively faster than the progress of infectivity in these thrips.

The length of the LP_{50} may provide an indication of whether plant viruses multiply in their vector. The LP_{50} values of both tospoviruses are higher than those found for plant viruses circulating, but not replicating, in their vector. The LP_{50} values of such circulating viruses vary between 0.5 and 2.5 days for PEMV (34), BYDV (38), and potato leafroll virus (PLRV) (37). On the other hand, the long LPs (6 days or more) of the plant-infecting reo- and rhabdoviruses are indicative of the replication of these viruses in their vectors (30). The LP_{50} found for the tospoviruses is intermediate in length between those of reo- and rhabdoviruses and those of BYDV, PLRV, and PEMV. Recently, it has been shown that tospoviruses do indeed replicate in their vector (39). The accumulation of two viral proteins, the nucleocapsid protein and the nonstructural (NS) protein, reached a maximum at the end of the second larval stage, coinciding closely with the development of infectivity of the larvae in the experiments described in this paper.

Limited attention has been given to the role of larvae in the epidemiology of tospoviruses. Because the larvae do not fly, they will not be trapped on yellow sticky traps (40). In addition, the limited infectivity of larvae observed thus far is a further argument against emphasizing a possible role of the larvae in the epidemiology. Because our results indicate that the larvae are more efficient transmitters than was supposed, their role in the epidemiology has to be reconsidered. Although they will not spread the virus as winged vectors, they will migrate from one plant to another when these plants make leaf contact. In this case, secondary spread caused by larvae may occur more rapidly than spread caused by adults, which have to molt two extra times before becoming active as vectors.

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