VIRUS-VECTOR RELATIONSHIPS IN THE TRANSMISSION OF TOSPOVIRUSES

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 28 november 1995
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

The research described in this thesis was carried out at the Department of Virology, Agricultural University Wageningen, The Netherlands.
This research was supported by the EC Programme of Scientific and Technical Activities in the Plant Health Field, Contract 89/399003, and in part by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for Scientific Research (N.W.O.).
Financial support by the Stichting "Landbouw Export Bureau 1916/1918", Greiner B.V. (Alphen aan de Rijn, The Netherlands) and Selecta KLEMM (Stuttgart, Germany) is gratefully acknowledged.

S & G Seeds B.V.
(Enkhuizen, the Netherlands)

Cover design: Nanda Kaashoek

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Wijkamp, Ineke

Virus-vector relationships in the transmission of tospoviruses / Ineke Wijkamp,
- [S.l.:s.n.]. - Ill,
Thesis Wageningen, - With summary in Dutch
ISBN 90-5485-448-0
Subject headings: tospoviruses / plant diseases

NN02701, 2014

Stellingen

1. De efficiëntie van TSWV-acquisitie kan niet gemeten worden na een verwervingsperiode van 48 uur.

   Bautista *et al.*, 1995. Potential of tomato spotted wilt tospovirus plant hosts in Hawaii as virus reservoirs for transmission by *Frankliniella occidentalis* (Thysanoptera: Thripidae). Phytopathology 85: 953-958.

2. De aanwezigheid van "autophagic vacuoles" in middendarmepitheelcellen van met TSWV geïnfecteerde thripsen, bewijst niet dat TSWV pathogeen is voor de thrips vector.

   Ullman *et al.* 1995. Compartmentalization, intracellular transport, and autophagy of tomato spotted wilt virus proteins in infected thrips cells. Phytopathology 85: 644-654.

3. Er bestaat geen verband tussen de schade die thripsen op planten veroorzaken en de efficiëntie waarmee tospovirussen worden overgedragen.

4. Het gebruik van de begrippen persistent, semipersistent en nonpersistent in verband met overdracht van plantevirussen door insekten, dient beperkt te worden tot de overdracht door Homoptera en Thysanoptera.

5. Het evolutionaire succes van baculovirussen kan worden verklaard door hun vermogen tot efficiënte recombinatie.

6. De conclusie van Mayer *et al.*, dat een veranderde locatie van het MRP eiwit in hepatocyten verantwoordelijk is voor het canaliculaire transport defect in mutante TR^- ratten, is onjuist.

   Mayer *et al.*, 1995. Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. The Journal of Cell Biology 131: 137-150.

7. De hoge mate van genetische verwantschap tussen SVDV (blaasjesziekte) en Coxsackie B5 virus (humaan pathogeen) doet vermoeden dat de mens niet zo ver van het varken afstaat.

   Monlux *et al.*, 1975. Brain and spinal cord lesions in pigs inoculated with swine vesicular disease (UKG strain) virus and Coxsackie virus B5. The American Journal of Veterinary Research 36: 1745-1749.

8. Thripsen kennen geen onderscheid tussen werkdagen en weekeinden.

9. Dat dameswaterpolo niet voor vol aangezien wordt, blijkt uit het feit dat deze tak van sport, in tegenstelling tot herenwaterpolo, geen olympische sport is.

10. Bij de Nederlandse overheid dreigt de "gouden handdruk" een premie op incompetentie te worden.

11. Gezien het arbeidsperspectief en salaris van gepromoveerden betekent de promotie eerder een financiële aderlating dan de weg naar het grote geld.

12. Religieus extreem fundamentalisme misbruikt het geloof als middel om het doel te heiligen.

13. Ter ondersteuning van de anti-rook campagnes dient Sinterklaas zich te onthouden van het uitdelen van chocolade sigaretten aan jonge kinderen.

14. Het is een Voor-recht om uit de Achter-hoek te komen.

Stellingen behorend bij het proefschrift:

Virus-vector relationships in the transmission of tospoviruses

Wageningen, 28 november 1995 Ineke Wijkamp

Voorwoord

Ik zou graag de volgende personen willen bedanken voor hun bijdrage aan de totstandkoming van dit proefschrift. Allereerst Dick Peters en Rob Goldbach voor de prettige begeleiding en waardevolle kritiek tijdens het onderzoek. Verder bedank ik voor de fijne samenwerking de mensen van de Tospovirus groep, zoals daar in de loop der tijd waren en zijn: Antonio, Renato, Peter, Wies, Richard, Frank, Marcel, Cor, Axel, Kit, Marc, Erwin, Alice, Tatsuya en Claire én natuurlijk mijn mede-kamergenoten, de twee andere tospoezen, Fennet en Marjolein. Ook wil ik bedanken de (ex) studenten, Nuria Almarza, Guido Pennings, Frank Wiegman, Wim Reidt, Bernadette Duineveld en Karin de Jong.

Daarnaast gaat mijn dank uit naar Jan van Lent en Joop Groenewegen van de EM-afdeling, Wout Rozeboom, Annemieke van der Jagt en de mensen van de fotolocatie. Verder bedank ik Ine Derksen voor haar hulp bij de thripsen kweek en de mensen van het kassencomplex voor alle goede zorgen bij de toevoer van de grote hoeveelheid planten die ik in de loop der jaren gebruikt heb.

Special thanks also to Basil Arif en David Theilmann for critical reading of some of the manuscripts, and Basil for all the hours of swimming instructions during lunch breaks, I enjoyed it a lot.

En natuurlijk wil ik Nanda Kaashoek bedanken voor het ontwerpen van de omslag binnen record tijd en die mijn vage en zeer abstracte gedachten wist om te zetten naar een tastbare omslag.

Naast deze personen bedank ik ook alle niet genoemde (ex) medewerkers van de vakgroep Virologie, waaronder de mensen op het planteviruslab, het EM-lab en de zeer trouwe leden van de "RBBS", die samen met de tospo's naast alle werk ook voor de broodnodige ontspanning zorgden tijdens een partijtje squash, volleybal en voor hun nuttige (en soms minder nuttige) discussies tijdens sessies bij Loburg, het Gat of Onder de Linden. Door al deze mensen was mijn tijd op Virologie een erg fijne tijd, en het mag wel duidelijk zijn dat zonder hen allen dit proefschrift niet tot stand zou zijn gekomen.

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Chapter 1

Introduction

Tospoviruses

Tospoviruses are rated within the top ten of most devastating plant viruses, and because of their importance they have been the subject of many reviews (Best, 1968; Francki and Hatta, 1981; Francki *et al.*, 1984; Reddy *et al.*, 1991b; Peters, 1991; German *et al.*, 1992; Ullman *et al.*, 1992a; Goldbach and Peters, 1994). The impact of tospoviruses is enormous, mainly due to their extremely broad host range, which exceeds 650 different plant species in more than 70 distinct botanical families (Goldbach and Peters, 1994), and their worldwide distribution. In warmer climate zones many agricultural production areas on all continents are implicated in tospovirus epidemics, while in areas with a temperate climate the viruses often prevail in greenhouse cultivations.

The history of tospoviruses dates back to 1915 when the virus was found in tomatoes (Brittlebank, 1919). The onion thrips, *Thrips tabaci*, was the first vector species described and was for long believed to be the main vector. In the 1930s and 1940s the disease incidence of tomato spotted wilt tospovirus (TSWV) in Northern America and Western Europe faded away almost completely. This decline of the disease has been attributed to an effective control of *T. tabaci*. From 1980 on, however, a resurgence of TSWV occurred in these areas which was preceded with a rapid expansion of the Western flower thrips, *Frankliniella occidentalis*. The concealed way of life and short life cycle of this thrips species, their ability to colonize many weed and cultivated plant species, their increased tolerance to insecticides and the global trading of thrips-infested plant material are factors which have contributed to the success and worldwide dispersion of *F. occidentalis*.

Tospoviruses form a genus within the family of the *Bunyaviridae*, a large family of mainly arthropod-borne viruses (Elliot, 1990; Francki *et al.*, 1991). At least five species have been distinguished within the genus *Tospovirus*, based on serological properties and nucleotide sequence data (Table 1). The established species are TSWV (type species), impatiens necrotic spot virus (INSV), tomato chlorotic spot virus (TCSV), groundnut

Table 1 Geographical distribution, host range and vectors of tospoviruses.

Tospovirus	Geographical distribution	Host	Vector
Established species			
Tomato spotted wilt virus (TSWV)[1]	worldwide[10]	Monocots and Dicots, i.e. groundnut, tobacco, pea, tomato, potato, pepper, lettuce, chrysanthemum, cyclamen, *Impatiens*, dahlia, gerbera	*F. fusca* [16] *F. intonsa* [17] *F. occidentalis* [18] *F. schultzei* [17,19] *T. palmi* [20] *T. setosus* [20] *T. tabaci* [21]
Impatiens necrotic spot virus (INSV)[2,3]	U.S.A.[2], the Netherlands,[3] Italy[11], France[12]	Ornamentals, i.e. *Impatiens*, begonia, dahlia, exacum, gloxinia	*F. occidentalis* [22,23]
Tomato chlorotic spot virus (TCSV)[4]	Brazil[13]	Tomato	*F. occidentalis* [17] *F. schultzei* [17] *F. intonsa* [17]
Groundnut ringspot virus (GRSV)[4]	South Africa, Brazil[13], Argentine[14]	Groundnut, tomato	*F. occidentalis* [17] *F. schultzei* [17]
Watermelon silver mottle virus (WSMV)[5]	Japan[15], Taiwan[5]	Watermelon, other cucurbits	*T. palmi* [24]
Tentative species			
Groundnut* bud necrosis virus (GBNV)[6]	India[6]	Groundnut	*T. palmi* [25,26] *S. dorsalis* ?[25,26#] *F. schultzei* ?[25,26#]
Peanut yellow spot virus (PYSV)[7]	India, Thailand[7]	Groundnut	-
Tospovirus (Onion)[8]	U.S.A. (Idaho, Oregon)[8]	Onion	-
Tospovirus (*Verbesina alternifolia*)[9]	U.S.A.[9]	*Verbesina alternifolia*	-

-: no reported vector *: In literature GBNV is also named peanut bud necrosis (PBNV),#: *F. schultzei* later correctly identified as *T. palmi* (Palmer *et al.*, 1990), no transmission of GBNV by *F. schultzei* and *S. dorsalis* (Vijayalakshmi, 1994). References: [1] Francki *et al.*, 1991, [2] Law and Moyer, 1990, [3] De Ávila *et al.*, 1992, [4] De Ávila *et al.*, 1993a, [5] Yeh and Chang, 1995, [6] Reddy *et al.*, 1992, [7] Reddy *et al.*, 1991a, [8] Hall *et al.*, 1993, [9] Hayati *et al.*, 1990, [10] Goldbach and Peters, 1994, [11] Vaira *et al.*, 1993, [12] Marchoux *et al.*, 1991, [13] De Ávila *et al.*, 1990, [14] Dewey *et al.*, 1993, [15] Kameya-Iwaki *et al.*, 1984, [16] Sakimura, 1963, [17] Wijkamp *et al.*, 1995a, [18] Gardner *et al.*, 1935, [19] Samuel *et al.*, 1930, [20] Fujisawa *et al.*, 1988, [21] Pittman, 1927, [22] Wijkamp and Peters, 1993, [23] DeAngelis *et al.*, 1994, [24] Yeh *et al.*, 1992, [25] Palmer *et al.*, 1990, [26] Vijayalakshmi, 1994.

ringspot virus (GRSV) and watermelon silver mottle virus (WSMV). Tentative species include groundnut bud necrosis virus (GBNV), peanut yellow spot virus (PYSV), a tospovirus isolated from onion, which is serologically different from the previously mentioned species and a virus from *Verbesina alternifolia*. The present knowledge about the geographical distribution, host range and thrips vectors of the tospoviruses is summarized in Table 1.

Disease symptoms on plants vary from chlorosis, mottling, stunting and wilting to severe necrosis of leaf and stem tissue. Electron microscopical analysis of tospovirus infected plant tissue demonstrates the presence of spherical lipid-bound particles which are 80-120 nm in diameter. In addition, specific cytopathic structures associated with tospovirus infection are found. Depending on their structure and density they are denoted viroplasms, nucleocapsid aggregates or paracrystalline inclusions. (Kitajima *et al.*, 1992). Purified particles contain at least 4 structural proteins, the nucleocapsid protein (N) of 28.8 kilodalton (kD), the glycoproteins, G1 and G2 of 78 kD and 58 kD, respectively (Mohamed *et al.*, 1973; Tas *et al.*, 1977), and a large protein (L) of 331.5 kD which represents the putative viral RNA polymerase (van Poelwijk *et al.*, 1993). The genome consists of three single stranded linear RNA segments denoted the S (small) and M (medium) RNA, which have an ambisense arrangement, and the L (large) RNA, with a negative polarity. In addition to the four structural proteins, two non-structural proteins, NS_M and NS_S, are encoded. The NS_M protein is involved in cell-to-cell movement of the virus (Kormelink *et al.*, 1994; Storms *et al.*, 1995), however, the function of the NS_S protein sofar remains unknown. The particle morphology, genome structure and expression strategy are depicted in Figure 1.

Mechanisms of virus transmission

Tospoviruses are transmitted by a limited number of thrips (Thysanoptera; Thripidae) in a persistent manner (Sakimura, 1962b). While the virus can be acquired only by the larval stages, transmission is due almost exclusively to adult thrips. Larvae cannot transmit the virus immediately after acquisition, but after a latent period of approximately 3 to 10 days. In order to classify the mechanism by which tospoviruses are transmitted, a short description of the different modes of virus transmission by arthropods are given. To overcome the epidermal cell walls of their hosts, plant viruses have developed a wide range

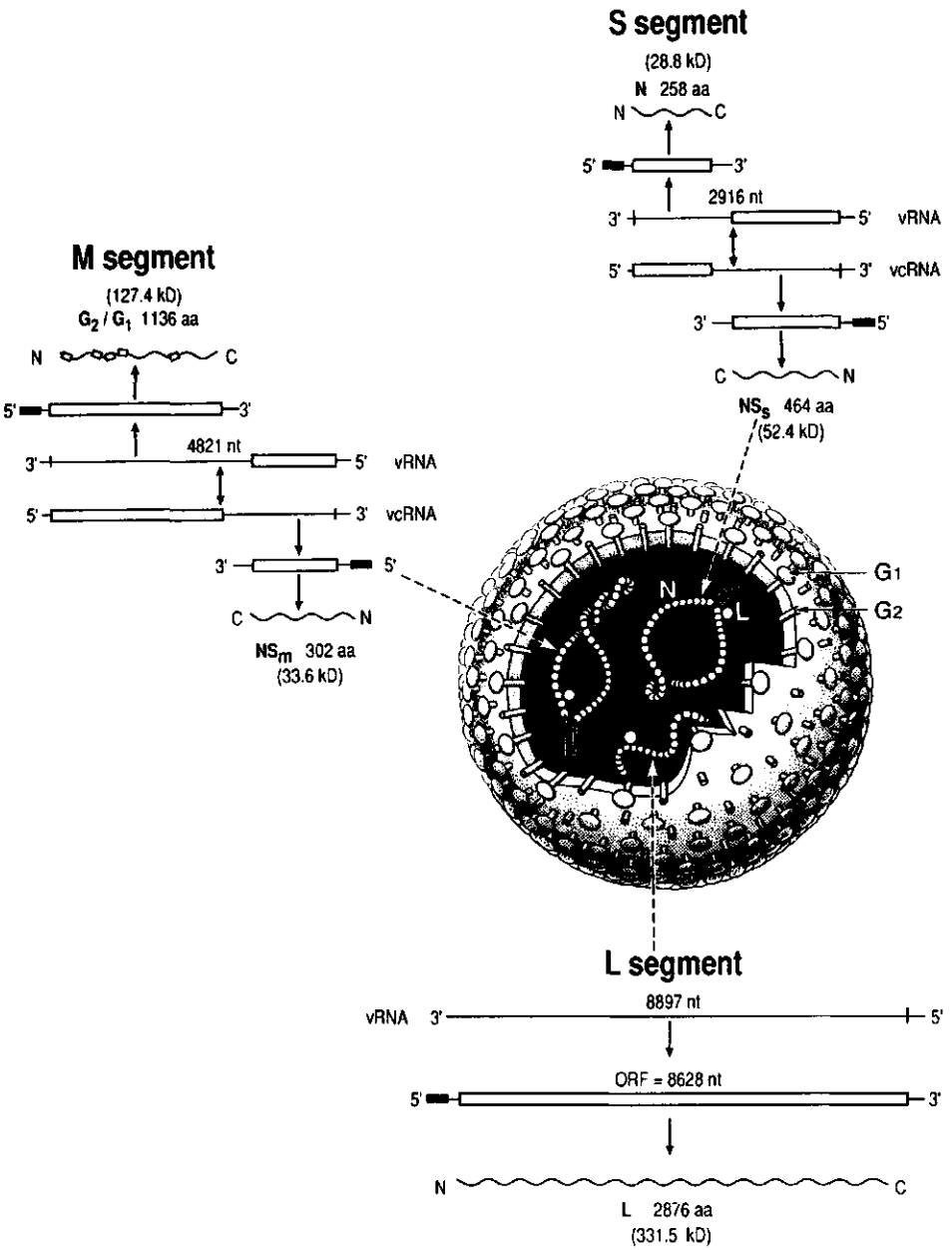


Figure 1. Structure and strategy of gene expression of tomato spotted wilt virus. Data are taken from Kormelink *et al.*, 1992.

of systems which involve many types of transmitting agents or vectors (Matthews, 1991; Hull, 1994), i.e. mechanical, vegetative propagation of plant tissue, seed, pollen, fungi, nematodes and arthropods.

Biological transmission involves various forms of specificity between the virus and its vector. Plant viruses are transmitted in either a non-circulative or circulative way by their arthropod vectors (Table 2). Non-circulative transmission can be further classified as nonpersistent or semipersistent based on retention of inoculativity and other characteristics. Circulative transmission can be divided in propagative or nonpropagative based on the multiplication or not of a particular virus in its vector (Lopez-Abella *et al.*, 1988).

Non-circulative viruses are acquired and inoculated via the maxillary food canal and carried in the foregut (or possibly as external stylet contaminants) during retention. An ingestion-egestion mechanism is used to describe the process in which virus, retained in the foregut lumen, is subsequently egested in the process of feeding and/or probing (Harris, 1977). Lopez-Abella and co-workers (1988) suggest that the basic difference between nonpersistent and semipersistent transmission may be the tenacity with which virus can be held in a transmissible state, and the titer in the foreguts of aphid and leafhopper vectors.

Circulative nonpropagative and propagative viruses are acquired via the food canal, passed through the cells of the gut, carried in the hemocoel, and inoculated with virus-laden saliva via the maxillary saliva canal to the plant; an ingestion-salivation mechanism. Nonpropagative viruses circulate in the vector, whereas propagative viruses multiply in both plants and insects and can therefore be considered as both plant and insect viruses.

Virus transmission by beetles appears more complex. In general, beetle transmission shares some characteristics with that of the semi-persistent transmission of plant viruses by leafhoppers (Wang *et al.*, 1992). However, circulation of virus in the hemocoel does occur in some beetle-virus combinations, but is not a prerequisite for transmission. Some beetle transmitted viruses, injected into the hemocoel, can be transmitted by beetles (Sanderlin, 1973). Furthermore, the specificity is determined by regurgitant which has an inhibitory effect on non-beetle transmitted viruses but not on beetle transmitted viruses (Gergerich and Scott, 1991). Plant viruses which are transmitted by beetles are members of four groups, i.e. the sobemo-, como-, tymo- and bromoviruses (Harris, 1981).

In another plant/virus/arthropod relation, the plant host is merely involved in the transmission cycle as a transitory (circulative) reservoir of virus. In contrast to the above-mentioned plant viruses, these viruses do not seem to replicate in plants but they propagate

Table 2 Virus-insect relationships.

	Non-circulative transmission	
	Nonpersistent	Semipersistent
Acquisition	20 seconds to 5 minutes[1]	Up to several hours[1]
Retention in vector	Few hours[2]	1-4 days[3]
Latent period	No[2]	No[3]
Transstadial passage of virus	Virus lost on moult[2]	Virus lost on moult[3]
Vector specificity	Often many vector species (>50)[2]	Often some vector species (<22)[2]
Barriers to cross in vector	No barrier[2]	No barrier[3]
Virus genes involved in vector interaction	Interaction via capsid protein (CP) only (cucumovirus) or both virus particle and virus-encoded helper component (HC) (potyvirus)[2,7,9]	Interaction via both virus particle and virus-encoded HC (Caulimovirus)[10]
Site of interaction with vector	Exoskeleton[2], cuticle of maxillary food canal and foregut (potyvirus)[7]	Exoskeleton[2], behind cibarium (PYFV)[13], cuticle (pre)-cibarium, pharynx, up to fore-oesophagus (MCDV)[14]
Viruses with helper-assisted transmission	Potyvirus (HC* separate)[2]	Caulimovirus (HC* separate)[2], waikavirus[3,15]
Insect vectors	Aphid, mealybug, whitefly,[2]	Aphid, leafhopper, whitefly, mealybug [2,3],
Viruses	Alfamovirus, carlavirus, cucumovirus, fabavirus, potyvirus[2]	Badnavirus[18], caulimovirus, closterovirus, waikavirus, (sequivirus), (trichovirus), cucumber vein yellowing virus[2,3]

*: Two forms of helper component (HC): 1. HC encoded separately from the capsid protein (HC separate), References: [1] Harrison and Murant, 1984, [2] Hull, 1994, [3] Raccah *et al.*, 1990, [4] Nault, 1994, [5] Wijkamp and [10] Schmidt *et al.*, 1994, [11] Briddon *et al.*, 1990, [12] Brault *et al.*, 1995 [13] Murant *et al.*, 1976, [14] Childress and

	Circulative transmission	
	Nonpropagative	Propagative
Acquisition	Several hours or longer[1,2]	Several hours or longer[1,2]
Retention in vector	Many days to weeks[1]	Weeks, throughout lifespan[1,4]
Latent period	Yes, mean LP range: 4-48 hours[4]	Yes, mean LP range: 98-744 hours[4,5]
Transstadial passage of virus	Virus not lost on moult[2]	Virus not lost on moult[2]
Vector specificity	Few vector species (< 10)[2]	Few vector species (< 10)[2]
Barriers to cross in vector	1. Gut wall barrier, 2. Salivary gland barrier[2,6]	1. Midgut infection barrier, 2. Midgut escape barrier, 3. Salivary gland infection barrier, 4. Salivary gland escape barrier, 5. Transovarial barrier[8]
Virus genes involved in vector interaction	Interaction via CP only (geminivirus)[2,11] or HC (readthrough protein of the capsid protein) is involved (luteovirus, enamovirus)[2,12]	Surface proteins interact with receptor sites at various locations (barriers). (Rhabdovirus, tospovirus; glycoproteins[2])
Site of interaction with vector	Acquisition in hindgut via receptor mediated endocytosis (luteovirus)[6]	Acquisition in gut, receptor-mediated?[8]
Viruses with helper-assisted transmission	Luteovirus and enamovirus (HC* integral)[2]	No HC known[2]
Insect vectors	Aphid, leafhopper, whitefly, mite[2,16]	Aphid, leaf- and planthopper, thrips, piesmid[2,17]
Viruses	Enamovirus, geminivirus, luteovirus, rymovirus[2,16]	Cyto/nucleorhabdovirus, fijivirus, marafivirus, oryzavirus, phytoreovirus, tenuivirus, tospovirus[2]

and 2. HC formed by readthrough of a weak stop codon on the coat protein (HC integral) (Hull, 94). Peters, 1993, [6] Gildow, 1987, [7] Ammar *et al.*, 1994a, [8] Ammar *et al.*, 1994b, [9] Chen and Francki, 1990, Harris, 1989, [15] Hunt, *et al.*, 1988, [16] Mandahar, 1990, [17] Proesler, 1980, [18] Lockhart *et al.*, 1995.

in the insect and are, therefore, true insect viruses. Some of these viruses are detrimental to the survival of their insect host (D'Arcy *et al.*, 1981) and consequently, vertical transmission alone cannot maintain the pathogen in the host population and some mechanism for horizontal transmission is necessary. Transmission to plants appears to be of primary importance for the survival of these viruses. Examples are the leafhopper A virus (LAV) (Ofori and Francki, 1985), Peregrinus-maidis reovirus (PgMV) (Falk *et al.*, 1988) and other reovirus-like viruses (Ammar *et al.*, 1994b; Nakashima and Noda, 1995), Rhopalosiphum padi virus (RhPV) (Williamson *et al.*, 1989; Gildow and D'Arcy, 1988) and Himetobi P virus (Guy *et al.*, 1992).

Thrips

Besides vectoring tospoviruses, thrips generally infest a wide range of agricultural and horticultural crops. In addition to causing direct feeding damage to many economically important crops, they cause gall formation or vector microbial pathogens such as bacterial, fungal and viral diseases, other than tospoviruses (Ananthakrishnan, 1980; Fermaud and Gaunt, 1995). Some ilarviruses which are transmitted by thrips are prune dwarf virus (PDV) (Greber *et al.*, 1992), prunus necrotic ringspot virus (PNRSV) (Greber *et al.*, 1991) and tobacco streak virus (TSV) (Sdoodee and Teakle, 1993). Furthermore, maize chlorotic mottle machlomovirus (MCMV) (Jiang *et al.*, 1992), pelargonium flower break carmovirus (PFBV) (Krczal *et al.*, 1993), sowbane mosaic sobemovirus (SoMV) (Hardy and Teakle, 1992), sweet clover necrotic mosaic dianthovirus (SCNMV) (Hiruki *et al.*, 1989) and tobacco ringspot nepovirus (TRSV) (Messieha, 1969) can also be transmitted by thrips. In most of these cases the thrips functions as a vehicle to carry virus-infected pollen on the outside of their bodies from one plant to another and virus is subsequently inoculated via wounds in leaf cells caused by the feeding habits of thrips (mechanical, non-specific transmission). However, transmission of TRSV and SoMV is demonstrated to occur also in the absence of virus-infected pollen. The high stability and high concentration of virus presumably contributes to its survival on the mouthparts of its vector and subsequent transmission.

The biological transmission of tospoviruses involves a more specific relation between the virus and its vector. Thus far eight thrips species have been reported as vectors of the tospoviruses (Table 3). Most species are highly polyphagous species with an extensive

Table 3 Geographical distribution and host range of reported tospovirus vectors.

Thrips species	Geographical distribution	Host
Frankliniella fusca Hinds[1] (tobacco thrips)	Widespread throughout North America and Mexico[1,10]	Common in grasslands, groundnut, tobacco, cotton[10]
Frankliniella intonsa Trybom[2,3] (flower thrips)	Palaearctic, widespread throughout, Europe, CIS, Asia. Also reported from U.S.A, India[2,10]	Polyphagous (flowers),many vegetables and ornamentals, clover, alfalfa[2]
Frankliniella occidentalis Pergande[4] (western flower thrips) (alfalfa thrips) (California thrips)	Widespread throughout North and Central America, Europe Hawaii and New Zealand. Also reported from Asia, CIS, Africa and Australia, Argentina[11,12]	Polyphagous (flowers), many fruits, vegetables, ornamentals and seed crops, cotton[10,11,18]
Frankliniella schultzei Trybom[5] (cotton bud thrips) (common blossom thrips)	Tropics, widespread throughout South America, Caribbean, Africa Australia, Pacific and Asia. Also reports from Florida, temperate zones (introduced); The Netherlands, Italy,Great Britain (incidentally)[13]	Polyphagous (flowers), cotton, sorghum, groundnut, pigeon pea, mung bean, chilli, onion tomato, composite crops[10]
Scirtothrips dorsalis Hood[6] (chilli thrips) (Assam thrips) (yellow tea thrips)	Tropics, widespread throughout Asia, Australia and Pacific[14]	Polyphagous, acacia, chilli, castor, rubber, tea, groundnut, soybean,tamarind,asparagus, fruit and fruit trees[2,10,14]
Thrips palmi Karny[7] (melon thrips)	Tropics, widespread throughout Asia, Northern Australia, Pacific, Carribean, and Central America. Also reported from Florida, Guyana, Venezuela, Sudan and Nigeria. Temperate zones (intr.); The Netherlands and Finland[15,16]	Polyphagous, cucurbits, Leguminosae and solanaceous hosts, cotton [10,15,19,20]
Thrips setosus Moulton[8]	Japan and Korea[2,10]	Polyphagous, tea, watermelon, cucumber, cowpea, soybean, tomato, sweet pepper, dahlia strawberry, narcissus[2]
Thrips tabaci Lindeman[9] (onion thrips)	Worldwide, widespread on all continents[17]	Polyphagous,onion,cabbage, tobacco, cotton, vegetables and ornamentals[10,20]

References: [1] Sakimura, 1963, [2] Umeya *et al.*, 1988, [3] Wijkamp *et al.*, 1995a, [4] Gardner *et al.*, 1935, [5] Samuel *et al.*, 1930, [6] Amin *et al.*, 1981, [7] Yeh *et al.*, 1992, [8] Kobatake *et al.*, 1984, [9] Pittman, 1927, [10] Palmer *et al.*, 1992, [11] CAB, 1993, [12] Dal Bó *et al.*, 1995, [13] Vierbergen and Mantel, 1991, [14] CAB, 1986, [15] CAB, 1992, [16] Cermeli and Montagne, 1993, [17] CAB, 1969, [18] Brødsgaard, 1989, [19] Walker, 1994, [20] Talekar, 1991.

geographical distribution.

The transmission of tospoviruses by thrips may be explained by the feeding habits and structure of the mouthparts of phytophagous thrips. Based on external morphology the stylets of leaf feeding thrips are classified as piercing-sucking structures (Chisholm and Lewis, 1984). During feeding, individual plant cells are punctured with the mandible and emptied by ingestion of cell contents through a feeding tube formed by the maxillary stylets (Hunter and Ullman, 1989). During this feeding process, tospovirus particles are ingested. When acquired by larvae, virus can be transmitted by second larval instars and adults after circulation and replication in the vector (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993; Wijkamp and Peters, 1993). Larvae cannot transmit the virus immediately, but after a latent (incubation) period of several days transmission occurs. Inoculation occurs during probes in which cells are not extensively damaged because successful inoculation of tospovirus requires intact cells for initiation of the infection process. Virus is transstadially passed in the thrips and retained for life. Adult thrips do not acquire the virus (Sakimura, 1962b; Ullman *et al.*, 1992b) and virus is not transmitted to the progeny (Wijkamp *et al.*, 1995b).

The general life cycle of phytophagous thrips is explained by that of *F. occidentalis*. The duration of the life cycle varies with abiotic factors and host plants. Eggs are inserted singly by the female into leaf or petal tissue in an incision made by the saw-like ovipositor. After approximately 3 days, the eggs hatch into larvae. The insect passes through two larval stages. The duration of the L1 stage is 1-2 days and that of the L2 stage 2-4 days. Old second stage larvae stop feeding and usually move into the soil or leaf litter to pupate. The prepupal stage requires 1-2 days and the pupal stage 2-3 days. Prepupae and pupae do not feed. The adults after emergence resume feeding and are readily dispersed by flying or wind currents (Brødsgaard, 1989).

Populations of most thrips species are bisexual but females often predominate. Female thrips are always diploid and males haploid (arrhenotoky). Virgin females produce only male offspring, whereas fertilized females produce mostly females and fewer males from non-inseminated eggs. In contrast, reproduction in species without males can only be by female to female parthenogenesis (thelotoky). Sometimes both reproduction mechanisms are found or the insect changes from thelotoky to arrhenotoky (Lewis, 1973).

Scope of investigation

At the onset of this research, information on virus-vector relations for tospoviruses and thrips, and the description of transmission parameters was limited. Experiments described in this thesis were carried out to elucidate these parameters. The process of tospovirus transmission by thrips can be divided in different phases. The period in which the virus is acquired by immature stages is followed by the period in which the virus can be inoculated onto host plants. The duration of the acquisition access period and inoculation access period was studied and related to the efficiency of virus transmission (Chapter 2). In addition, the susceptibility of different plant species to TSWV and the applicability of leaf disk assays in transmission studies were investigated (Chapter 3). Since tospoviruses circulate through the thrips body, virus transmission is characterized by a latent period (LP). In this period the vector is not able to infect a plant. The LP was measured quantitatively by serial transfers and expressed as the median latent period LP_{50} (Chapter 4). To gain further insight in the mechanism of virus transmission, the fate of virus in the vector was investigated. The titers of two viral proteins, the nucleocapsid (N) and a non-structural protein (NS_S), were analyzed in thrips which were sampled at several moments after acquisition. The results obtained showed convincingly that the virus replicated in thrips. This conclusion was supported by *in situ* localization studies to elucidate the sites where virus replicated in the vector (Chapter 5). Virus multiplication in the vector may possibly cause deleterious effects and consequently alter the bionomics of the thrips infected. Possible effects of virus infection were analyzed by comparing the developmental time, reproduction rate and survival of virus exposed and non-exposed thrips (Chapter 6). Vector competence and transmission efficiency may give more insight in epidemiology and spread of the tospoviruses. These parameters were investigated in the transmission of four different tospovirus species by four thrips species. Finally, the transmission of tospovirus mutants, which were generated upon virus maintainance by mechanical inoculation, was studied in order to analyze the intrinsic properties of the virus which may influence thrips transmission (Chapter 8).

Chapter 2

Transmission of tomato spotted wilt virus by *Frankliniella occidentalis*; median acquisition and inoculation access period

SUMMARY

To quantify the transmission of tomato spotted wilt virus (TSWV) by *F. occidentalis*, two parameters, i.e. the median acquisition access period (AAP_{50}) and median inoculation access period (IAP_{50}) were determined. The median values were established using transmission rates obtained after AAPs and in IAPs which both ranged from 5 to 2560 min. An AAP_{50} of 67 min was found when larvae acquired virus from TSWV-infected *Impatiens* plants. IAP_{50}s of 59 or 133 min, respectively, were calculated when petunia or *D. stramonium* leaf disks were used to test the inoculation efficiency of viruliferous thrips. The virus could successfully be acquired and inoculated in periods of 5 min. Transmission reached an optimum after an AAP of 21.3 h (AAP_{opt}) and in an IAP of 42.7 h (IAP_{opt}). These results show that TSWV can efficiently be acquired and transmitted by *F. occidentalis* in short feeding periods.

This chapter will be published in a slightly modified version as: Ineke Wijkamp, Fennet van de Wetering, Rob Goldbach and Dick Peters. Transmission of tomato spotted wilt virus by *Frankliniella occidentalis*; median acquisition and inoculation access period. Ann. Appl. Biol., submitted.

INTRODUCTION

Tospoviruses are transmitted by several species of thrips (Thysanoptera: Thripidae) and cause worldwide serious diseases in various economically important crops. Significant yield losses in agricultural crops and ornamentals such as tomato, pepper, lettuce, chrysanthemum and impatiens are the result of infections by tomato spotted wilt virus (TSWV), the type species of the *Tospovirus* genus and other tospoviruses (Goldbach and Peters, 1994). The worldwide expansion of *Frankliniella occidentalis* Pergande, an efficient vector of TSWV (Wijkamp *et al.*, 1995a), the resistance of this vector to pesticides and its ability to colonize many plant species are the main reasons for outbreaks of TSWV and other tospoviruses.

Thrips are piercing-sucking insects which feed by emptying cells (Ullman *et al.*, 1992a). Sensory structures on the mouthcone and within the alimentary canal may determine feeding activity and behavior which will subsequently determine virus acquisition or transmission. Only larval thrips can acquire tospoviruses (Ullman *et al.*, 1992b), while second stage larvae and adult thrips can transmit the virus after propagation (Wijkamp *et al.*, 1993, Wijkamp and Peters, 1993). Quantitative analysis of transmission characteristics of TSWV enables better understanding of virus epidemiology. Both tenure time on host plant and the period required by the vector to acquire or inoculate the virus are factors determining the spread of these viruses. The acquisition access period (AAP) and inoculation access period (IAP) are parameters which can be used to quantify transmission. Varying minimum acquisition and inoculation thresholds for tospoviruses have been reported, which range from 5 min to longer periods (Razvyazkina, 1953; Sakimura, 1962b; Reddy *et al.*, 1983, Vijayalakshmi, 1994). However, longer access periods are usually required for more efficient transmission. Contrary to minimum access periods which represent extremes, median values for AAP and IAP are ecologically more significant and can statistically be analyzed more reliably than minimum values.

In this report, we describe the influence of access periods on the acquisition and inoculation efficiency of TSWV by *F. occidentalis* and present the determination of the median acquisition access period (AAP_{50}) and inoculation access period (IAP_{50}) from these transmission data.

MATERIALS AND METHODS

Virus isolate, test plants and thrips

In all transmission studies, the Brazilian TSWV isolate BR-01 (De Ávila *et al.*, 1993a) was used. *Impatiens* sp. plants, used as virus source plants for acquisition of TSWV by thrips larvae, were infected 2 to 3 weeks after sowing by single viruliferous adults of *F. occidentalis*. The plants were grown in a greenhouse at approximately 22 °C (light/dark: 16/8 h) for symptom development. Leaf disks were cut from 3 to 6 weeks old healthy *Petunia x hybrida* Hort. Vilm.-Andr. 'Blue Magic' and *Datura stramonium* L. plants.

Cultures of virus-free *F. occidentalis* were reared on bean pods (*Phaseolus vulgaris* L. 'Prelude') at 27 (±0.5) °C and 16 h photoperiod (light/dark: 16/8 h). The colony was started with adults collected from a greenhouse infestation in The Netherlands. To obtain uniformly aged larvae, fresh bean pods were placed in the thrips colonies for egg oviposition. After 24 h beans were taken from the colonies after removal of infesting thrips, cleaned, and placed separately at 25 °C. Larvae of 0 to 12 h old emerging from bean pods were used for experiments.

Virus detection by enzyme-linked immunosorbent assay (ELISA)

The antigen titer was determined by ELISA in leaf extracts from *Impatiens* sp. plants used as virus source for acquisition. This assay was also used to confirm the infection of leaf disks of petunia and *D. stramonium* after the inoculation by *F. occidentalis* in the AAP and IAP experiments. The extracts were prepared by grinding leaf tissue at a ratio of 15 mg per ml of 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). Leaf disks from healthy plants were used as controls. Polyclonal antiserum, raised against the nucleocapsid (N) protein (anti-N serum) of the TSWV isolate BR-01 was used in a double antibody sandwich ELISA (DAS-ELISA) format as described previously (Wijkamp and Peters, 1993).

Individual thrips were analyzed by cocktail-ELISA, with amplification of the enzyme reaction, for their N protein content as described previously (Wijkamp *et al.*, 1993) with one modification; individual thrips were ground in 80 µl of sample buffer (2% polyvinylpyrrolidone (M_r about 44000) and 0.2% ovalbumin in PBS-T) and mixed with 20 µl of 2.5 µg/ml anti-N conjugate of the TSWV isolate BR-01 isolate in the same buffer.

The extract was incubated overnight at 4 °C and assayed as described. Absorbance values were read on a EL 312 ELISA-reader (Bio-Tek Instruments Greiner BV, Alphen aan de Rijn, the Netherlands) at 492 nm. The absorbance values were corrected for blank values read for wells that contained only sample buffer in the sample incubation step.

Determination of the median acquisition access period (AAP_{50})

Systemically infected leaves of *Impatiens* sp. which showed comparable high virus titers in a dilution series in ELISA were used for acquisition feeding. The leaves were cut in 4 pieces which were divided at random in leaf cages (Tashiro, 1967) as to minimize effect of different virus titers in the leaves. First instar larvae of *F. occidentalis*, 0-12 h old, were confined to the surface of these leaf pieces and allowed to feed for 5, 10, 20, 40, 80, 160, 320, 640 or 1280 min. In addition, one group of larvae spent their whole larval development on virus-infected material to determine the maximum transmission rate. First instar larvae, caged on virus-free *Impatiens* leaves, served as blanks. After the AAPs, the larvae were transferred to healthy *D. stramonium* leaves to complete their development. After emergence, the adults were tested individually for virus transmission on petunia leaf disks (diameter: 13 mm) in 1.5 ml Eppendorf tubes in 3 successive IAPs of 48 h as described previously (Wijkamp and Peters, 1993). All experiments with thrips were performed at 25 ($\pm$0.5) °C with a 16 h photoperiod (light/dark: 16/8 h). After each IAP, the leaf disks were incubated at 27 °C in 24 well plates (Costar Europe Ltd., Badhoevedorp, the Netherlands), while floating on water for the development of local lesions (Wijkamp and Peters, 1993). The percentage of leaf disks that developed virus symptoms was regarded as a measure for the transmission efficiency. For each acquisition period an average of 20 adults were used and the experiment was repeated 5 times. The transmission percentages were transformed by adjusting the maximum transmission rate at 100% in order to correct for virus-exposed thrips which did not transmit virus. The period at which maximum transmission occurred was denoted the optimum acquisition period (AAP_{opt}). The median acquisition access period (AAP_{50}) was estimated by log-probit analysis of transmission percentages (Sylvester, 1965). The AAP_{50} and its 95% fiducial limits were calculated by the method of Finney (1962). Data were processed and analyzed with the POLO-PC computer program (LeOra Software, 1987).

At the end of the IAPs on petunia, the adults were collected and stored at -70 °C to be assayed for their N protein content in ELISA in order to quantitatively establish the relation

between virus transmission and N protein content of each individual. ELISA values for single thrips were classified as either positive or negative, i.e. thrips which gave readings higher than the readings from average healthy control thrips plus 3 times standard deviation were considered to be positive, those with lower readings were negative.

Determination of the median inoculation access period (IAP_{50})

To obtain viruliferous thrips for studies on the IAP_{50}, first instar larvae of *F. occidentalis* (0-12 h old) were confined to the surface of systemically infected *Impatiens* sp. leaves in cages for 72 h. After acquisition, larvae were transferred to healthy *D. stramonium* leaves to complete their development. After emergence, adults were tested individually for virus transmission on petunia leaf disks as described in the AAP experiments. Thrips transmitting virus to petunia leaf disks were selected to determine the IAP_{50}.

The viruliferous adults were allowed to inoculate either leaf disks (diameter: 6 mm) of petunia or *D. stramonium* in IAPs of 5, 10, 20, 40, 80, 160, 320, 640, 1280 or 2560 min. Thrips were starved for 2 h prior to the inoculation periods. This preliminary fasting may increase and equalize the feeding activity especially during short inoculation feedings. After the IAPs, the leaf disks were incubated for 6 days at 27 °C in 24 well plates, while floating on water for the development of either local lesions on petunia or the infection in *D. stramonium* disks, which remain symptomless. ELISA was employed to confirm infection in leaf disks of both plant species. The percentage of disks which scored positive in ELISA was used to calculate the transmission efficiency. An average of 22 adults were tested per IAP and the total experiment was repeated 3 times. The median inoculation access period (IAP_{50}) was estimated by log-probit analysis of transmission percentages as described for the determination of the AAP_{50}.

RESULTS

Determination of the median acquisition access period (AAP_{50})

To determine the relation between acquisition access period and transmission efficiency, *F. occidentalis* larvae, confined for different AAPs to virus-infected *Impatiens* leaf pieces, were transferred to healthy *D. stramonium* leaves to complete their development. After

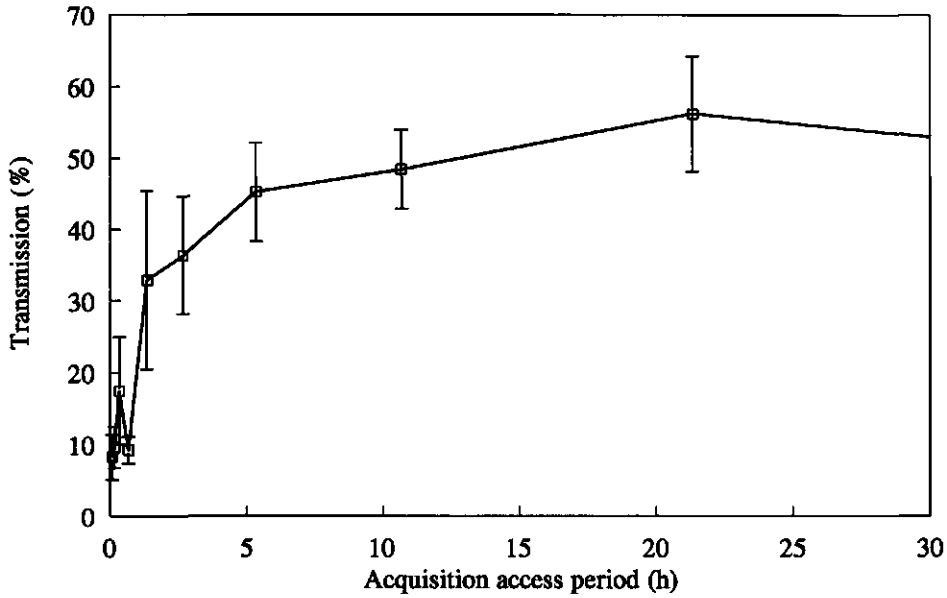


Figure 1. The mean efficiency and associated standard errors in the transmission of tomato spotted wilt virus (TSWV) by *Frankliniella occidentalis* as a function of the acquisition access period (AAP). Virus was acquired from *Impatiens* sp. and the transmission by adults was tested on petunia leaf disks.

emergence, the adults were tested individually for virus transmission to petunia leaf disks. The percentage infected leaf disks was plotted as a function of the length of the AAP (Figure 1). An average of 8.2% of the thrips, which were given an AAP of 5 min, was already able to transmit virus; this period was denoted the minimum AAP (AAP_{min}). The number of petunia disks that became infected increased with the length of the AAP given to the larvae, the highest rate (56.2%) was found when the larvae fed for 21.3 h (1280 min) on infected leaves. This AAP can therefore be considered as the optimum acquisition access period (AAP_{opt}). In comparison, a transmission rate of 50.1% was obtained when the thrips spent their whole larval life period on virus-infected material. The highest transmission rate was transformed to 100% and the AAP_{50} and the 95% fiducial limit were estimated by log-probit analysis. The average AAP_{50} was found to be 67 min with 95% fiducial limits of 39-113 min (Table 1).

Antigen titer in viruliferous and non-viruliferous thrips

To establish the relationship between transmission and antigen titer in the thrips, the

Table 1. Transmission of tomato spotted wilt virus (TSWV) by *F. occidentalis*.

	Access period (min)	Transmission (%)[a]
Impatiens sp.[b]		
AAP_{min}[c]	5	8.2 ± 3.2
AAP_{opt}	1280	56.2 ± 8.1
AAP_{50}	67 (39-113)[d]	
Petunia[d]		
IAP_{min}[c]	5	6.3 ± 2.3
IAP_{opt}	2560	100.0
IAP_{50}	59 (44-77)	
D. stramonium[c]		
IAP_{min}[c]	5	16.7 ± 0.0
IAP_{opt}	2560	100.0
IAP_{50}	133 (82-232)	

[a] Transmission efficiency represents the mean percentage ± standard error. AAP experiments were performed 5 times, IAP experiments 3 times.
[b] Thrips larvae acquired TSWV from *Impatiens* sp.
[c] The shortest AAP and IAP tested were 5 min, these periods were denoted AAP_{min} and IAP_{min}, respectively.
[d] TSWV was transmitted by adult thrips to leaf disks of petunia or *D. stramonium*, respectively. Only thrips which were able to transmit virus were selected for inoculation studies.
[d] In parenthesis; fiducial limits of the AAP_{50} or IAP_{50}.

adults which were tested on petunia leaf disks for virus transmission, were individually ground in sample buffer to assay the amount of N protein by ELISA. Thrips could be divided into three groups on basis of transmission data and ELISA readings (Table 2). The first group of thrips consisted of individuals which transmitted virus to petunia leaf disks and in which viral antigen could readily be detected by ELISA (petunia positive; ELISA positive). These results showed that the length of the acquisition period had no effect on the N protein content of viruliferous thrips; hence transmitting thrips which acquired virus in AAPs of different lengths exhibited similar ELISA readings. The second group of thrips did not transmit virus, however, each individual contained viral antigen as was concluded from the positive ELISA readings (petunia negative; ELISA positive). Several ELISA positive thrips were found, especially after AAPs of 80 min and longer, demonstrating that virus was acquired and virus replication may have occurred, though resulting in lower titers

Table 2. Virus content of adult thrips expressed as average ELISA values plus standard deviations. Virus was acquired by larvae in acquisition access periods (AAP) of different lengths on virus-infected *Impatiens* leaves. Transmission by adults was tested on petunia leaf disks.

AAP (min)	Transmitters[a,b]	Non-transmitters[a,c]	
		ELISA-positive	ELISA-negative
5	0.435 ± 0.179	0.137 (2)	0.011 ± 0.005 (98)
10	0.552 ± 0.128	0.185 (3)	0.005 ± 0.004 (97)
20	0.489 ± 0.187	0.037 (3)	0.003 ± 0.004 (97)
40	0.439 ± 0.128	0.184 ± 0.034 (6)	0.007 ± 0.006 (94)
80	0.456 ± 0.110	0.160 ± 0.098 (37)	0.018 ± 0.006 (63)
160	0.483 ± 0.104	0.173 ± 0.127 (42)	0.021 ± 0.004 (58)
320	0.434 ± 0.195	0.118 ± 0.027 (14)	0.019 ± 0.006 (86)
640	0.490 ± 0.121	0.186 ± 0.123 (72)	0.011 ± 0.003 (28)
1280	0.477 ± 0.186	0.261 ± 0.157 (100)	- (0)
2560	0.446 ± 0.160	0.079 ± 0.075 (64)	0.018 ± 0.006 (36)

[a] The minimum threshold values for ELISA-positive thrips in ELISA consisted of average ELISA values of healthy thrips plus 3 times standard deviation; all readings above this threshold were considered positive in ELISA, readings below this value were considered negative.

[b] All ELISA readings for transmitting adults were positive.

[c] Non-transmitting thrips were divided in ELISA-positive or ELISA-negative individuals. Values in parenthesis represent the percentage of non-transmitting thrips which are positive or negative in ELISA, respectively.

than those of transmitting thrips. The third group of thrips did not transmit virus either and ELISA values were comparable to those found for healthy thrips (petunia negative; ELISA negative). The majority of thrips after AAPs from 5 to 40 min scored negative in ELISA indicating that no virus was acquired.

Determination of the median inoculation access period (IAP_{50})

To determine the relation between the inoculation access period and transmission efficiency, viruliferous adults of *F. occidentalis* were allowed to feed for different IAPs on either petunia or *D. stramonium* leaf disks. The percentage of infected disks was plotted as a function of the length of the IAP (Figure 2). An IAP of 5 min already resulted in the infection of 6.3% or 16.7% of the petunia or *D. stramonium* leaf disks, respectively. This period was denoted the minimum IAP (IAP_{min}). Transmission increased with increasing lengths of the IAPs. A transmission rate of 100% was reached when viruliferous thrips had access to leaf disks for 42.7 h (2560 min) which can be considered as the IAP_{opt}. The IAP_{50}

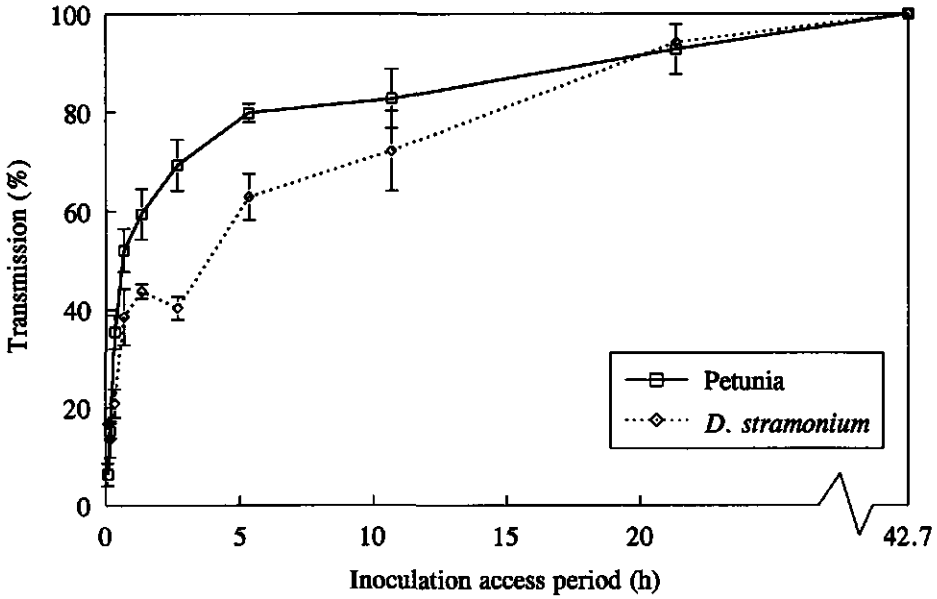


Figure 2. The mean efficiency and associated standard errors in the transmission of tomato spotted wilt virus (TSWV) by *Frankliniella occidentalis* as a function of the inoculation access period (IAP). The IAP was either on petunia or *D. stramonium*.

and the 95% fiducial limits (FLs) for viruliferous adults were estimated by log-probit analysis and were found to be 59 min (FLs: 44-77 min) on petunia leaf disks and 133 min (FLs: 82-232 min) on *D. stramonium*, respectively (Table 1). The IAP_{50} values on the two test plants differed significantly ($P<0.05$).

Use of ELISA to verify TSWV transmission to leaf disks

Infection of leaf disks of petunia resulted in the formation of local lesions within 3 days, allowing easy screening of transmission. Leaf disks of *D. stramonium*, however, remained symptomless and therefore ELISA was used to demonstrate infection of these disks 6 days after the start of the inoculation feeding periods. Leaf disks which gave readings higher than the average healthy control plus 3 times standard deviation were considered to be positive and those with lower readings to be negative. The average ELISA readings and the standard deviations for leaf disks of petunia and *D. stramonium* are presented in Figure 3. A 100% correlation was found between petunia disks showing local lesions and positive ELISA readings. A few petunia disks exhibited less definite lesions, but 95% of those leaf

disks scored positive in ELISA. The efficient transmission and the development of high virus titers in leaf disks from both the local lesion host (petunia) and the systemic host (*D. stramonium*) showed that leaf disks formed suitable substrate in transmission studies of tospoviruses by thrips.

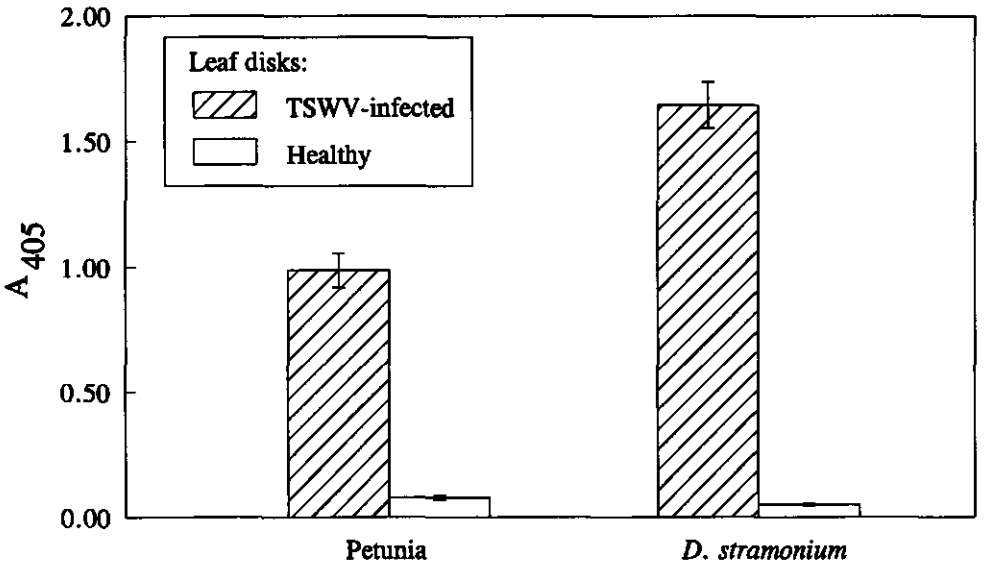


Figure 3. Average ELISA values and standard errors for leaf disks of petunia and *Datura stramonium*, using antiserum to the nucleocapsid protein of tomato spotted wilt virus (TSWV). Extracts of leaf disks after inoculation by thrips which scored higher than the average healthy control plus 3 times standard deviation were considered to be positive, those with lower readings were considered negative.

DISCUSSION

In the present study, two parameters, i.e. the AAP_{50} and the IAP_{50}, were established in the transmission of TSWV by *F. occidentalis*. Although quantitative studies on virus acquisition and transmission have been performed for several propagative viruses, this is the first report of median acquisition and inoculation access periods for a propagative virus. Since median access periods have been established for circulative, non-propagative viruses, these parameters can be compared to median values of these viruses. The values found for

the AAP_{50} and IAP_{50} for potato leafroll luteovirus (PLRV) and *Myzus persicae* were 12 h and 45-105 min (Peters, 1986), respectively. For chickpea chlorotic dwarf geminivirus (CCDV), transmitted by the leafhopper *Orosius orientalis*, an AAP_{50} and IAP_{50} of 8.0 h and 2.3 h were found. A comparison demonstrates that the IAP_{50}s for TSWV transmission were in the same range, whereas the AAP_{50} was considerably shorter as those for circulative-nonpropagative viruses. The relatively short AAP_{50} for TSWV may be explained by smaller amounts of virus required to convert the vectors into transmitters. It can be expected that replication of virus in the vector results in a decreased dose-dependency for transmission compared to circulative-nonpropagative viruses where transmission increases linearly with the acquired dose, which either may be obtained by an increased acquisition access period (Paliwal and Sinha, 1970; Sylvester, 1980, Fargette *et al.*, 1982) or by increased virus concentrations during a finite acquisition period (Van den Heuvel *et al.*, 1991).

Minimum values for the acquisition and inoculation of tospoviruses have been published before, however, these data vary widely. In the present study with *F. occidentalis* acquiring TSWV from *Impatiens* a minimum AAP of 5 min was found, the same threshold was also found for acquisition of groundnut bud necrosis virus (GBNV) by *Thrips palmi* from groundnut (Vijayalakshmi, 1994). Minimum values of 30 min were observed for *Thrips tabaci* on *Nicotiana rustica* (Razvyazkina, 1953) and *Frankliniella schultzei* on groundnut (Reddy *et al.*, 1983) transmitting TSWV and GBNV, respectively. For circulative virus transmission by insects, the probability of transmission generally increases with the length of the AAP until all insects that are able to do so have acquired the virus. Sakimura (1962b) found a minimum AAP for *T. tabaci* of 15 min and the percentage of plants that became infected increased with the length of the feeding period, being 4% with 15 min, 33% with 1 hour, 50% with 1 day and 77% with 4 days feeding. In the present experiments an increasing transmission rate is also evident but the AAP_{opt} is noticeably shorter and is reached within 21.3 h. Our results are in good agreement with those reported by Vijayalakshmi (1994) who found an AAP_{opt} of 24 h for the transmission of GBNV by *T. palmi*.

The present study shows that high transmission rates can be reached after short AAPs. *F. occidentalis* acquired virus from *Impatiens* very efficiently as was concluded from previous experiments which showed that immediately after an AAP of 12 h high amounts of viral antigen were present in the thrips larvae (Wijkamp *et al.*, 1995a), which resulted in high transmission rates (Wijkamp and Peters, 1993). Several factors govern the

acquisition of virus from infected plant tissue (German *et al.*, 1992). Host suitability for the thrips and distribution of infected cells in the leaf tissues determine the amount of virus ingested from a particular plant host. Uniform distribution of the virus in leaves is of major importance for efficient acquisition as the possibility to ingest virus will increase, especially when short AAPs are employed. Importance of a regular distribution of tospovirus in leaves has been shown by Ullman and co-workers (1992a) who demonstrated that more ELISA positive larvae were found with higher antigen titers when *D. stramonium* was used as acquisition host as compared to *Arctium lappa*. Although both plants species exhibited similar high virus titers, *D. stramonium* leaves displayed an uniform virus distribution whereas a more patchy distribution was found in *A. lappa* leaves as shown by direct tissue immunoblotting.

When adults were assayed for their content of N protein in ELISA, some non-transmitting *F. occidentalis* were found to exhibit higher titers than healthy thrips (Table 2), especially at longer acquisition periods, suggesting that after acquisition some replication of virus occurred, though not sufficiently to pass the threshold at which virus could be transmitted. Alternatively, the virus may not have reached the proper tissues (e.g. the salivary glands) for successful transmission. Since transmitting thrips exhibited comparable virus titers irrespective of the length of the AAP, a dose dependency, as found for circulative non-propagative viruses (Van den Heuvel *et al.*, 1991) appeared not to occur.

Inoculation of TSWV will occur during short probes in which little or no cell contents will be ingested and the cells are not extensively damaged. It can safely be assumed that inoculation of tospovirus requires intact cells for initiation of the infection process. In the present study, 5 min is sufficient to establish virus infection at a low transmission rate. A similar threshold has also been found for *T. tabaci* on *N. rustica* (Razvyazkina, 1953). However, Vijayalakshmi (1994) found a considerably longer minimum IAP (1 h) in the transmission of GBNV by *T. palmi* to groundnut. Longer IAPs are usually required for more efficient transmission (Amin *et al.*, 1981; Allen and Broadbent, 1986; Mau *et al.*, 1991; Sakimura, 1962b, 1963). As can be expected, the transmission increased with the length of IAP, reaching an optimum after approximately 42 h. This was also observed for GBNV transmission by *T. palmi* to groundnut (Vijayalakshmi, 1994) where maximum transmission rates were obtained with an IAP of 2 days.

The rate of transmission may also be influenced by feeding responses of the thrips to the plant host and susceptibility of this plant species to the virus. The difference in the

IAP_{50} on petunia or *D. stramonium* may be explained by different feeding preferences of *F. occidentalis*. Both plant species seem to be equally susceptible to virus infection as can be concluded from Figure 2 which shows equal percentage of infected leaf disks for both plant species at longer IAPs. At shorter IAPs, however, transmission to *D. stramonium* is less efficient which may be explained by a difference in feeding preference. Observations on feeding behavior indicate that adults feed more readily on *D. stramonium* than on petunia. Efficient feeding will result in ingestion of cell contents and fewer shallow probes, whereas on petunia increased shallow probing may increase virus transmission.

Study of parameters involved in the host-virus-vector relationship are essential in understanding the epidemiology and spread of tospoviruses. Transmission characteristics of TSWV by *F. occidentalis* show that acquisition and inoculation occurs in short periods, which has implications for virus epidemiology. The larvae have to acquire small virus doses to become viruliferous and dispersing adult thrips may only need short inoculation periods to spread the virus. Moreover, a decreased host suitability for thrips may result in increased movement of thrips thereby causing increased virus spread.

Chapter 3

Differential susceptibilities between leaf disks and plants in the transmission of tomato spotted wilt virus by *Frankliniella occidentalis* to TSWV hosts and transgenic plants

SUMMARY

The efficiency by which tomato spotted wilt virus (TSWV) was transmitted to plants and leaf disks by the vector *Frankliniella occidentalis*, was analyzed. The virus was efficiently transmitted to *Datura stramonium*, *Impatiens* sp. and tobacco plants, i.e. 60-100% of the plants became infected when 1-3 viruliferous thrips were confined per plant for a period of 3 days. However, lettuce exhibited a lower susceptibility since only 25% of the test plants were infected when challenged by 10 viruliferous thrips per plant for 3 days. In contrast, complete resistance was found when transgenic tobacco plants, expressing the nucleocapsid protein of TSWV, were challenged with up to 10 viruliferous thrips per plant, whereas all non-transgenic control plants were infected when 5 viruliferous thrips per plant were used. To improve and accelerate the transmission studies, the applicability of leaf disks in these studies was tested. Leaf disks of 16 different plant species appeared to be highly susceptible. Infection rates ranging from 51.6 to 95.0% were obtained when one viruliferous adult was placed singly on these leaf disks for a period of 24 h. The leaf disk assay was also employed to screen resistance of transgenic plants expressing the nucleocapsid protein of TSWV. One transgenic tomato line displayed complete immunity whereas a second line appeared to be susceptible. For the transgenic tobacco line, positive ELISA reactions were found for a few leaf disks (7.5%) suggesting that some virus replication did occur. However, the ELISA readings for these disks were significantly lower than those for leaf disks of non-transgenic controls. Finally, the significance of the use of leaf disks and test plants in virus-vector studies is discussed.

This chapter will be published in a slightly modified version as: Ineke Wijkamp, Rob Goldbach and Dick Peters. Differential susceptibilities between leaf disks and plants in the transmission of tomato spotted wilt virus by *Frankliniella occidentalis* to TSWV hosts and transgenic plants. J. Phytopath., submitted.

INTRODUCTION

Tospoviruses cause diseases in many economically important crops in the field and in greenhouses throughout the tropical, subtropical, and temperate climate zones. More than 650 plant species in at least 70 families have been reported as susceptible to these viruses (Goldbach and Peters, 1994). The type species of the tospoviruses is tomato spotted wilt virus (TSWV). Significant yield losses caused by TSWV are found in crops such as tomato, tobacco, pepper, lettuce, chrysanthemum and *Impatiens* sp. Furthermore, many weed species, which can serve as virus reservoirs, are implied in the disease cycle of TSWV under field conditions (Kobatake *et al.*, 1984; Cho *et al.*, 1986; Johnson *et al.*, 1995).

The western flower thrips *Frankliniella occidentalis* Pergande, which has at present, a worldwide distribution, is a major pest on a wide range of greenhouse and field crops. Besides its pest status it is the main vector of TSWV (Wijkamp *et al.*, 1995a). Mainly due to its resistance to insecticides, routine sanitary measures are not adequate to limit TSWV incidence which emphasizes the need for other control strategies. One option is breeding for resistance against TSWV. High levels of TSWV resistance in tobacco and tomato have recently been obtained by transforming plants with the nucleoprotein gene of TSWV (Gielen *et al.*, 1991; De Haan *et al.*, 1992; Prins *et al.*, 1995; Ultzen *et al.*, 1995).

Tospoviruses are transmitted by thrips (Thysanoptera: Thripidae) in a propagative manner (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993). Currently, eight different thrips species are known to transmit these viruses (Wijkamp *et al.*, 1995a). When virus is acquired by young larvae it can be transmitted by second larval instars and adults (Wijkamp and Peters, 1993). Quantitative studies on virus acquisition and transmission have shown that virus can be transmitted when thrips were allowed short acquisition and inoculation feeding periods on TSWV-infected leaves. The median acquisition and inoculation access periods in the transmission of TSWV by *F. occidentalis* were 67 min and, depending on the host used, 59 or 133 min (Wijkamp *et al.*, 1995c).

TSWV hosts display differences in susceptibility to infection upon mechanical or thrips inoculation (Allen and Broadbent, 1986; Allen and Matteoni, 1991). Studies with test plants which display a low susceptibility will result in low transmission rates, and consequently, an underestimation of vector capacity of thrips. Cho *et al.*, (1988) found a poor correlation between the TSWV titers in individual thrips and the ability of these thrips to transmit; the

majority of thrips that were TSWV-positive did not transmit. To obtain better correlations, it is favorable to use highly susceptible plant species in virus-vector studies by which an optimal analysis of vector capacity will be achieved. Recent studies on the transmission of several tospoviruses by thrips have shown that leaf disks of *Petunia x hybrida* 'Blue Magic' provide an excellent tool in thrips transmission studies (Wijkamp and Peters, 1993; Wijkamp *et al.*, 1995a,b,c).

Here, we report studies on the differential susceptibility of test plants and leaf disks in transmission studies with viruliferous *F. occidentalis*. Leaf disks of petunia 'Blue Magic' were used to select viruliferous thrips. Subsequently, selected thrips were confined in different numbers to several TSWV hosts and transgenic plants, expressing the nucleocapsid protein gene of TSWV, in order to evaluate the susceptibility of these plants to TSWV transmission by *F. occidentalis*. The results were correlated with those obtained with leaf disks. On basis of the results presented in this study, the application of leaf disks or test plants in virus-vector studies and in screening methods for host resistance against tospoviruses is discussed.

MATERIALS AND METHODS

Thrips, virus isolate and test plants

Virus-free *F. occidentalis* were reared on bean pods (*Phaseolus vulgaris* L. 'Prelude') at 27 ($\pm$0.5) °C and 16 h photoperiod (light/dark: 16/8 h). The colony was started with adults collected from a greenhouse infestation in the Netherlands. To obtain cohorts of uniformly aged larvae, fresh bean pods were placed in thrips colonies for egg oviposition. After 24 h, the pods were collected from the colonies after removal of infesting thrips and placed at 25 °C. Larvae, 0 to 12 h old, which emerged from the bean pods were used in the experiments.

In all transmission studies, the Brazilian TSWV isolate BR-01 was used (De Ávila *et al.*, 1993a). *Datura stramonium* L. plants were used as virus source plants for acquisition of TSWV by thrips larvae. These plants were infected by single viruliferous adults of *F. occidentalis* 2 to 3 weeks after sowing.

Test plants of *D. stramonium*, *Impatiens* sp., *Lactuca sativa* 'Isabel', *Nicotiana tabacum*, 'SR1' and transgenic line 'SR1-12' which were challenged by viruliferous thrips,

were 2 to 3 weeks old at the start of the experiment. Transgenic tobacco line 'SR1-12', expressing the TSWV nucleoprotein gene (Gielen *et al.*, 1991), is resistant to TSWV when inoculated mechanically or by thrips (De Haan *et al.*, 1992).

The leaf disks used in the transmission studies were cut from 3 to 7 weeks old host plants. The following plants species were used: *Arachis hypogaea* L. (groundnut), *Chenopodium quinoa* Wild, *Capsicum annuum* L. 'Westlandse Zoet' (sweet pepper), *Cucumis sativus* L. (cucumber), *D. stramonium*, *Dendranthema grandiflora* Tzvelev 'Majoor Bosshardt' (chrysanthemum), *Impatiens* sp., *L. sativa* 'Isabel' (lettuce), *Lycopersicon esculentum* Mill. 'Radja' (tomato), *Nicotiana benthamiana* Domin., *Nicotiana rustica* L., *N. tabacum* 'SR1' (tobacco), *Petunia x hybrida* Vilm. 'Blue Flash', *Petunia x hybrida* 'Blue Magic', *P. vulgaris* 'Prelude' (french bean) and *Vicia faba* L. (broad bean).

In studies on the susceptibility of leaf disks of transgenic plants, transgenic and non-transgenic tobacco and tomato plants of 2-4 weeks old served as sources for leaf disks. For tobacco, *N. tabacum* 'SR1' and the transgenic line 'SR1-12' were used. For tomato, *L. esculentum* hybrid 'Radja' and the two transgenic 'Radja' hybrids, '698/Radja' and '815/Radja', were selected. Hybrid '815/Radja' displays complete immunity, whereas '698/Radja' demonstrates intermediate resistance upon mechanical inoculation of test plants (Ultzen *et al.*, 1995). All plants were grown in a greenhouse at approximately 22 °C (light/dark: 16/8 h).

Virus detection by enzyme-linked immunosorbent assay (ELISA)

ELISA was employed to determine the antigen titer in extracts from plants, leaf disks and thrips. Extracts of plants and leaf disks were prepared by grinding leaf tissue at a ratio of 15 mg per ml of 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). Extracts from healthy plants were used as controls. Polyclonal antiserum raised against the nucleocapsid (N) protein (anti-N serum) of the TSWV isolate BR-01 and conjugate (anti-N conjugate) were used in a double antibody sandwich ELISA (DAS-ELISA) format as described previously (Wijkamp and Peters, 1993).

Plants and leaf disks from transgenic plants expressing the N protein of TSWV were screened for virus infection using antisera raised against the membrane glycoproteins (G) and non-structural protein (NS_S) of TSWV. The monoclonal antibody G1 to the glycoproteins of TSWV isolate BR-01 (formerly CNPH1) (Huguenot *et al.*, 1990) was used in a triple antibody sandwich ELISA (TAS-ELISA) as described by De Ávila *et al.*, (1990).

Polyclonal antiserum against the NS_S protein (anti-NS_S serum) (Kormelink *et al.*, 1991) was used in an antigen coated plates ELISA (ACP-ELISA) format. Wells were coated with 50 µl of the leaf extracts mixed with an equal volume of 2 X coating buffer (0.05 M sodium carbonate, pH 9.6) and incubated overnight at 4 °C. Coating was followed by blocking the plates with 150 µl 1% BSA in PBS-T for 0.5 h at 37 °C. Next steps involved incubating the wells with 100 µl of 1 µg/ml of anti-NS_S serum in PBS-T for 2 h at 37 °C followed by incubation with 0.5 µg/ml goat anti-rabbit IgG alkaline phosphatase conjugate in PBS-T for 2 h at 37 °C. Between incubation steps, except after blocking, the plates were washed with PBS-T. The antigens were detected by adding 100 µl of 1 mg/ml *p*-nitrophenyl phosphate in 0.01 M diethanolamine buffer, pH 9.6. Absorbance values were read on a EL 312 ELISA-reader (Bio-Tek Instruments Greiner BV, Alphen aan de Rijn, the Netherlands) at 405 nm. Samples which gave ELISA readings higher than the average of healthy control readings plus 3 times standard deviation were considered to be positive, those with lower readings negative.

Adult thrips were analyzed in a cocktail-ELISA for their N protein content after the last given inoculation access period (IAP) on leaf disks. The reaction in this ELISA was followed by an amplification of the enzyme reaction as described previously (Wijkamp *et al.*, 1993) with one modification; individual thrips were ground in 80 µl of sample buffer (2% polyvinylpyrrolidone (M_r about 44,000) and 0.2% ovalbumin in PBS-T) and mixed with 20 µl of 2.5 µg/ml anti-N conjugate in sample buffer. The suspension was incubated overnight at 4 °C. Absorbance values were read at 492 nm. Absorbance values were corrected for blank values read for wells that contained only sample buffer in the sample incubation step.

TSWV transmission to different host plants

To obtain viruliferous thrips to inoculate the various test plants, 0-12 h old first instar larvae of *F. occidentalis* were confined to systemically infected *D. stramonium* leaves in cages (Tashiro, 1967) for 72 h. After this period, the larvae were transferred to healthy *D. stramonium* leaves to complete their development. After emergence, adults were individually tested on petunia 'Blue Magic' leaf disks for two successive IAPs of 24 h as described previously, for their ability to transmit virus (Wijkamp and Peters, 1993). Thrips transmitting virus to these disks were used in inoculation experiments. The viruliferous thrips were placed on single plants of *D. stramonium*, *Impatiens* sp., lettuce, tobacco 'SR1'

and transgenic line 'SR1-12' for IAPs of 3 days. A minimum of 15 plants were tested with either 1, 3 or 5 thrips/plant, for 10 thrips/plant a minimum of 8 plants was tested. Three healthy thrips per plant served as negative control. After the IAP, thrips were killed by an insecticide treatment. The plants were monitored for the development of infection which was confirmed by ELISA.

Additionally, in stead of confining thrips to single test plants, an experiment was performed which simulated a field situation more closely. In two of these experiments 10 lettuce test plants were placed in one cage with either 16 or 25 viruliferous thrips. After an IAP of 3 days the thrips were killed and plant were monitored for disease development.

TSWV transmission to leaf disks of different hosts and transgenic plants

To study the transmission of TSWV by *F. occidentalis* to leaf disks of 16 plant species, 0-12 h old first instar larvae were confined to the surface of infected *D. stramonium* leaves in cages for 72 h. After this period, larvae were transferred to healthy *D. stramonium* leaves to complete their development. After emergence, adults were used for inoculation studies. All experiments with thrips were performed at 25 ($\pm$0.5) °C with a 16 h photoperiod (light/dark: 16/8 h).

Individual adults were confined for inoculation access periods (IAPs) of 20 h to leaf disks (diameter: 13 mm) of the 16 plant species in 1.5 ml Eppendorf tubes as described by Wijkamp and Peters (1993). To randomize the effect of aging of the thrips, these experiments were setup in a Latin square design in such a way that individual thrips were tested on 9 different plant species for 9 successive days and each plant species was tested on every day. Prior to each IAP, thrips were confined to petunia 'Blue Magic' leaf disks for a period of 4 h in order to reduce possible food preferences developed on the plant host in the previous IAP. As controls, healthy thrips were confined to leaf disks of the 16 plant species. At the end of the last IAP, the adults were collected and stored at -70 °C to be assayed for their N protein content in ELISA in order to quantitatively establish the relation between virus transmission and N protein content of each individual. ELISA values for single thrips were classified as either positive or negative, i.e. thrips which gave readings higher than the average readings from healthy control thrips plus 3 times standard deviation were considered to be positive, those with lower readings were negative.

To study the infection of leaf disks of transgenic lines and non-transgenic control plants, tobacco ('SR1' and 'SR1-12') and tomato ('Radja', '698/Radja' and '815/Radja') were used

in a similar experimental setup. Individual adults were tested in a Latin square design for 6 successive days and Petunia 'Blue Magic' was included as a reference.

After the inoculation, the leaf disks were incubated for 5 days at 27 °C in 24 well plates (Costar Europe Ltd., Badhoevedorp, the Netherlands) for virus infection. After this incubation, the disks were tested in ELISA to confirm infection and determine virus titers. The disks that exhibited higher ELISA values than the average ELISA value of healthy control readings plus 3 times standard deviation were considered to be infected.

RESULTS

Thrips inoculation of different host plants

Susceptibility of test plants for infection with TSWV by viruliferous *F. occidentalis* was studied by confining either 1, 3, 5 or 10 viruliferous adults to *D. stramonium*, *Impatiens* sp., lettuce, tobacco 'SR1' and transgenic line 'SR1-12' for IAPs of 3 days. After this access period, thrips were removed and plants were monitored for the development of symptoms. Infection of the plants was verified by ELISA. The transmission efficiency of TSWV by *F. occidentalis* to *D. stramonium*, *Impatiens* sp., lettuce, and tobacco, is depicted in Figure 1. When 1 thrips per plant was used, efficient transmission of TSWV to *D. stramonium*, *Impatiens* sp. and tobacco was observed. Using 5 thrips/plant, all plants of these species became infected. Transmission of virus to lettuce, however, was inefficient. Even with 10 thrips/plant only 25% of the plants became infected. Figure 2 shows the results of the transmission of TSWV to tobacco plants of the transgenic line 'SR1-12' and non-transgenic line 'SR1'. None of the 'SR1-12' plants became infected, even when 10 viruliferous thrips/plant were used, whereas the use of 5 thrips per plant resulted in 100% infection of the non-transgenic control. All plants which were challenged with healthy thrips remained symptomless.

Since only a few lettuce plants became infected another experiment was performed which simulated a field situation more closely. In two experiments 10 lettuce test plants were placed in one cage with either 16 or 25 viruliferous thrips. After an IAP of 3 days the thrips were killed. In both experiments 40% of the lettuce plants became infected, demonstrating again the low susceptibility of lettuce. The use of test plants like lettuce with a low susceptibility, will underestimate the vector capacity of viruliferous thrips. Moreover,

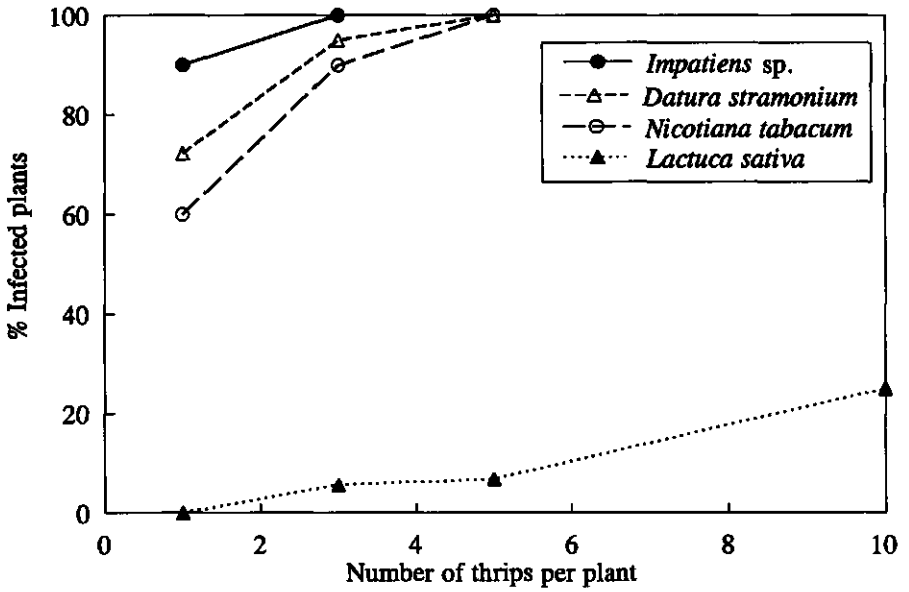


Figure 1. Percentage of infected plants of *Datura stramonium*, *Impatiens* sp., *Lactuca sativa* and *Nicotiana tabacum* plotted as a function of the number of viruliferous *Frankliniella occidentalis* confined per plant. Inoculation access period was 3 days.

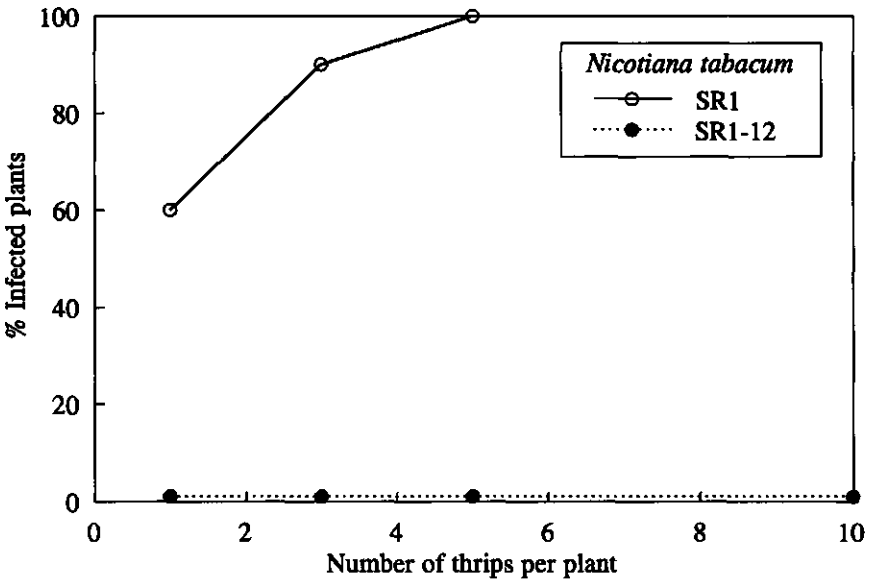


Figure 2. Percentage of infected plants of *Nicotiana tabacum* transgenic line 'SR1-12' and non-transgenic control 'SR1', plotted as a function of the number of viruliferous *Frankliniella occidentalis* confined per plant. Inoculation access period was 3 days.

Table 1. Transmission of tomato spotted wilt virus (TSWV) to leaf disks of 16 plant species by *Frankliniella occidentalis*. Virus was acquired by larvae from *Datura stramonium* and transmission by viruliferous adults was tested in inoculation access periods of 20 h.

Plant species	% infected leaf disks[a]
Arachis hypogaea	56.9 (58)
Chenopodium quinoa	73.3 (60)
Capsicum annuum 'Westlandse Zoet'	79.5 (44)
Cucumis sativus	81.8 (44)
Datura stramonium	81.4 (43)
Dendranthema grandiflora 'Majoor Bosshardt'	69.8(106)
Impatiens sp.	86.0 (43)
Lactuca sativa 'Isabel'	81.7 (60)
Lycopersicon esculentum 'Radja'	70.7 (60)
Nicotiana benthamiana	64.3 (42)
Nicotiana rustica	51.6 (62)
Nicotiana tabacum 'SR1'	53.2 (47)
Petunia x hybrida 'Blue Flash'	70.5 (61)
Petunia x hybrida 'Blue Magic'	72.3(101)
Phaseolus vulgaris	76.2 (42)
Vicia faba	95.0 (60)

[a] In parenthesis: the number of leaf disks tested

using test plants in virus transmission studies is cumbersome, especially due to difficulty of recovering the thrips. To avoid the use of test plants we studied the potential of leaf disks as substrate in transmission studies.

Thrips inoculation of leaf disks of different host plants

Leaf disks of 16 different plant species were compared for their susceptibility to TSWV inoculation by thrips. *F. occidentalis*, which were confined to virus-infected leaves of *D. stramonium* in the larval stage, were placed on the leaf disks during IAPs of 20 h. In between the access periods, thrips were transferred to control petunia 'Blue Magic' leaf disks for a period of 4 h. After the IAPs, the leaf disks were incubated for 5 days and subsequently tested in ELISA to observe whether they were infected.

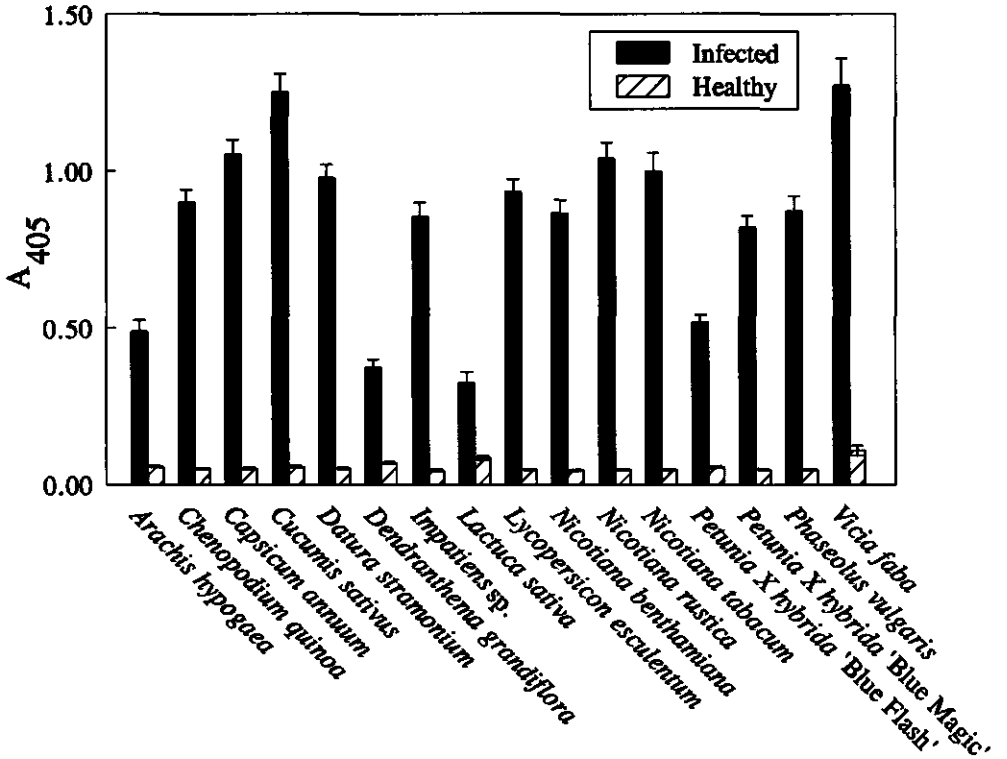


Figure 3. Mean ELISA values and associated standard errors of infected and non-infected leaf disks of 16 plant species, using antiserum to the N protein of TSWV.

Of all the adults, 25.3% never transmitted virus to leaf disks in the 9 successive days the thrips were tested. They, therefore, were excluded from the calculations. For the other 74.7% of thrips, which transmitted virus, the number of days at which transmission occurred was recorded and the relationship between inoculative capacity and antigen titer in the thrips established.

TSWV transmission to petunia 'Blue Magic' and 'Blue Flash' disks resulted in the appearance of local lesions which became visible within 2-3 days. All leaf disks with lesions were positive in ELISA. Infected leaf disks of *C. quinoa* and cucumber, which were detected by ELISA, did not show symptoms. Usually, *C. quinoa* plants display pin-point local lesions upon TSWV infection, whereas cucumber reacts with chlorotic lesions on the inoculated leaves. Both species are not systemically infected by TSWV. The other 12 plant

Table 2. Transmission of tomato spotted wilt virus (TSWV) to leaf disks of transgenic and non-transgenic tomato and tobacco by *Frankliniella occidentalis*. Virus was acquired by larvae from *Datura stramonium* and transmission by viruliferous adults was tested in inoculation access periods of 20 h. Leaf disks of petunia served as positive controls.

Plant species	% infected leaf disks[a]
Lycopersicon esculentum	
non-transgenic control 'Radja'	83.3 (36)
transgenic hybrid '698/Radja'	75.7 (37)
transgenic hybrid '815/Radja'	0.0 (37)
Nicotiana tabacum	
non-transgenic control 'SR1'	71.1 (38)
transgenic line 'SR1-12'	7.5 (40)
Petunia x hybrida 'Blue Magic'	79.5 (39)

[a] In parenthesis: the number of leaf disks tested

species tested show systemic symptoms after infection. Infected leaf disks of these species, as detected by ELISA, did not develop any symptoms. Upon challenge by healthy thrips, ELISA values of leaf disks of all species tested exhibited readings similar to healthy control disks.

For each plant species the percentage infected disks was determined. This value varied from 51.6 to 95.0% (Table 1). Lowest infection rates were obtained for *Nicotiana* species and groundnut. Lower values for *Nicotiana* species may be explained by feeding preferences of the thrips; these plant species are less preferred hosts for *F. occidentalis*. For the other species approximately 70% or more of the leaf disks were infected indicating that virus transmission by thrips to the leaf disks was efficient. The average ELISA values of infected and non-infected leaf disks and their standard errors are shown in Figure 3. The virus titer in the infected leaf disks varied considerably, high average ELISA values were recorded for all species but chrysanthemum, groundnut, lettuce and petunia 'Blue Flash'.

Thrips inoculation of leaf disks of transgenic tobacco and tomato plants

Resistance of transgenic tobacco and tomato to TSWV has been demonstrated by

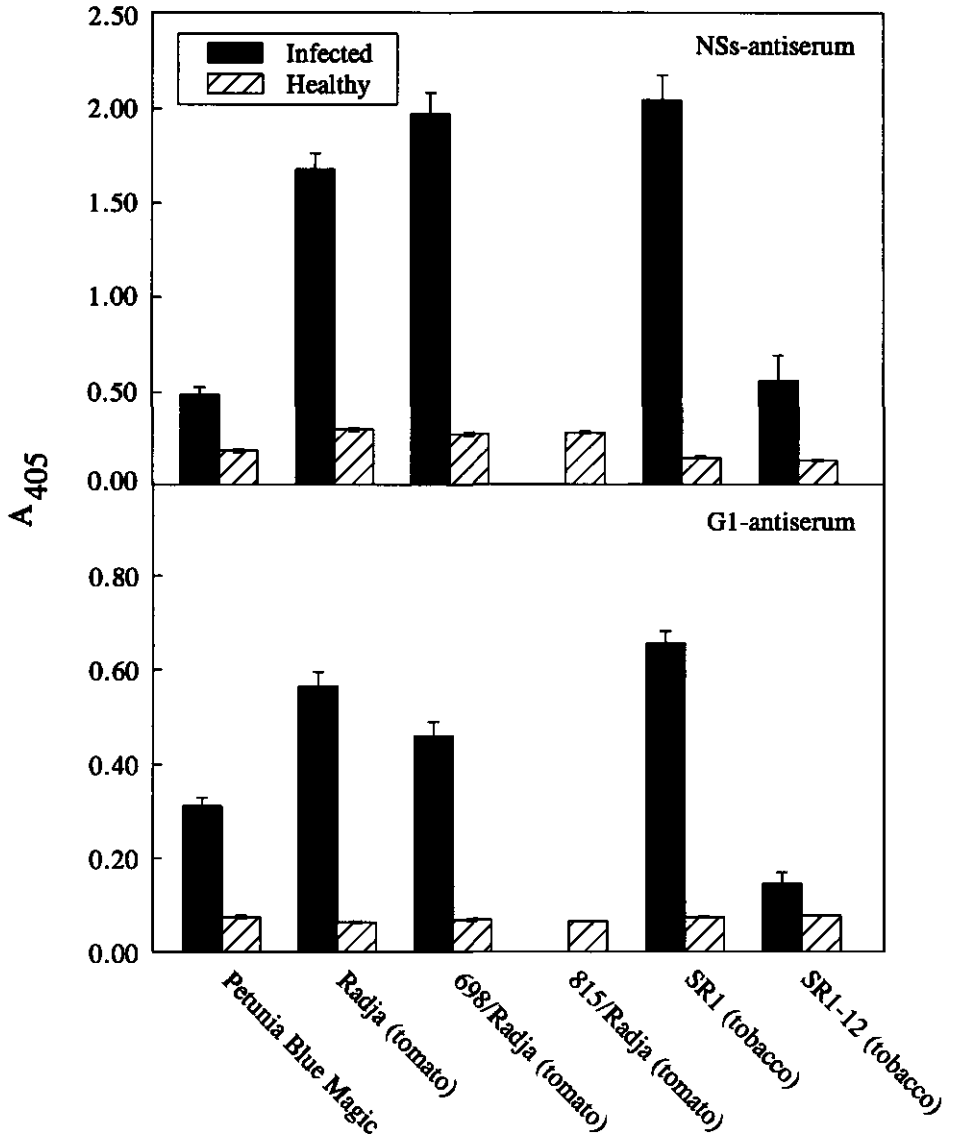


Figure 4. Mean ELISA values and associated standard errors of infected and non-infected leaf disks using polyclonal antiserum to the NS_S protein or monoclonal antibody G1 of TSWV. Leaf disks of transgenic and non-transgenic tomato and tobacco and petunia were tested.

Table 3. Inoculation capacity and nucleocapsid protein content of adults of *Frankliniella occidentalis*. The virus was acquired from *Datura stramonium* and transmitted by adults to leaf disks of 16 different hosts for 9 successive days.

Virus transmission (days)[a]	% of adults	ELISA-values[c]
Transmitters		
9	20.8	0.528 ± 0.025
8	18.5	0.449 ± 0.038
7	10.0	0.512 ± 0.041
6	7.7	0.503 ± 0.038
5	3.1	0.443 ± 0.084
4	2.3	0.421 ± 0.018
3	4.6	0.213 ± 0.082
2	6.2	0.313 ± 0.092
1	1.5	0.130 ± 0.056
Non-transmitters[b]		
0	14.9	0.213 ± 0.031
0	10.4	0.008 ± 0.001

[a] Inoculation capacity is expressed as the number of days on which transmission of TSWV by viruliferous individuals occurred out of a total of 9 days.
[b] Non-transmitters were divided in ELISA-positive thrips and ELISA-negative thrips.
[c] Average ELISA-value ± standard error of mean

mechanical inoculation of plants (De Haan *et al.*, 1992; Ultzen *et al.*, 1995). In the present experiments, this resistance was challenged by inoculating leaf disks using viruliferous thrips. Leaf disks of petunia 'Blue Magic' were included in the experiment as reference to evaluate infection rates. The percentage of infected leaf disks for non-transgenic and transgenic tobacco and tomato and petunia, was determined by ELISA (Table 2). These values were 71.1, 83.3 and 79.5% for non-transgenic tobacco 'SR1', tomato hybrid 'Radja' and petunia, respectively. High ELISA readings were found for leaf disks of non-transgenic controls when G1 or NS_S antisera were used (Figure 4). The results show that TSWV could efficiently be transmitted to leaf disks of these 3 species and, hence, the disks are a valuable tool in transmission studies. The higher rate of positive leaf disks for 'SR1' and 'Radja' in this experiment as compared to transmission in the previous experiment (Table

1) might be explained by the age of source plants from which the leaf disks were cut; the plants in this experiments were 3 weeks younger. Transgenic tobacco line 'SR1-12' which is resistant upon mechanical and thrips inoculation (De Haan *et al.*, 1992) also showed significant resistance when leaf disks were challenged by viruliferous thrips, as compared to the control 'SR1'. Some virus accumulated in 3 out of 40 leaf disks (7.5%) of 'SR1-12'. However, the ELISA values for these leaf disks were significantly lower than those found for non-transgenic 'SR1' (Figure 4).

Transgenic tomato hybrid '698/Radja', which demonstrates intermediate resistance upon mechanical inoculation (Ultzen *et al.*, 1995), appeared to be susceptible when leaf disks were tested. A similar percentage of leaf disks (75.7% and 83.3% for '698/Radja' and non-transgenic 'Radja') became infected, while the amount of virus that accumulated in the disks from both sources reached the same levels. The transgenic hybrid '815/Radja', which displays complete immunity upon mechanical inoculation, proved to be resistant since leaf disks did not become infected when challenged by viruliferous thrips.

Inoculative capacity of thrips

In order to establish whether the inoculation capacity of *F. occidentalis* could be correlated to the amount of viral antigen in the thrips, the virus transmission of individual thrips was monitored. For the 74.7% transmitting thrips, the number of days, out of the total of 9 days at which virus transmission occurred, was recorded (Table 3). The majority of the viruliferous thrips were efficient transmitters; 57% out of a total of 74.7% the viruliferous thrips did transmit virus at 6 or more days out of a total of 9 days, while 20.8% of the thrips transmitted virus at each day. At the end of the experiment, adults were sampled by ELISA for their N protein content (Table 3). Efficient transmitters exhibited high ELISA values whereas thrips which transmitted virus on 1, 2 or 3 out of 9 days displayed lower ELISA values. Of all the adults, 25.3% never transmitted virus to leaf disks in the 9 successive days that they were tested. Non-transmitters were divided in ELISA-positive or negative thrips. Virus was detected in 14.9% of the thrips, which failed to transmit, demonstrating that virus was acquired and replication may have occurred. The average titers were lower than in the efficient transmitters. Virus could not be detected in 10.4% of the thrips indicating that the thrips either failed to ingest or to replicate the virus.

DISCUSSION

In this chapter, the use of either leaf disks or plants as substrate in TSWV transmission studies with the vector *F. occidentalis* was evaluated. Transmission of TSWV by viruliferous thrips to test plants of *D. stramonium*, *Impatiens* sp. and 'SR1' tobacco during access periods of 3 days was highly efficient; 1 to 3 viruliferous thrips per plant resulted in 60 to 100% infection. As can be concluded, these plant species are highly susceptible to TSWV infection. Confirming earlier reports (De Haan *et al.*, 1992) none of the transgenic 'SR1-12' became infected; not even when single plants were challenged with 10 thrips. For lettuce, virus transmission to leaf disks was more efficient than to lettuce plants, even when 10 viruliferous thrips were placed on a plant. This observation demonstrates that in virus-vector studies leaf disks of some species are preferred as substrate over plants. When thrips are tested on less susceptible hosts, the capacity of the viruliferous thrips to transmit will be underestimated; in these cases the susceptibility of plants is measured rather than vector capacity of the thrips. The high percentages of lettuce leaf disks which become infected suggest that the virus accumulates readily in the leaf disks whereas the low infection rates of lettuce plants can be explained by a failure of virus transport from the inoculation sites to locations in the plant where the virus may replicate. The mechanism which prevents infection of lettuce plants to become infected is currently not known.

Inefficient transmission of TSWV to lettuce plants has also been observed by Cho and co-workers (1988) who found that only 7% of the *F. occidentalis* transmits TSWV to the lettuce cultivar 'Parris Island Cos', although the virus was detected in more than 50% of the adults suggesting a low susceptibility of lettuce plants. Since only a small percentage of the lettuce plants became infected when they were challenged individually by 1 to 10 thrips, another experiment was performed simulating the field situation more closely. Increasing the infection pressure, i.e. when 16 or 25 thrips could move freely between 10 test plants of lettuce resulted in an infection percentage of 40%, showing that mobility of viruliferous thrips is also a factor in the rate at which infections occur. In field situations in Hawaii losses of 50-90% in lettuce are reported (Yudin *et al.*, 1990). In these occasions lettuce is known to support large populations of thrips. Moreover, cultivation and harvesting activities will disrupt and agitate thrips which causes high intercrop movement (Cho *et al.*, 1989). These factors may partly explain the high infection percentages in lettuce under natural conditions.

Besides the use of plants in transmission studies, the suitability of leaf disks as substrate for TSWV was analyzed. In these experiments, the susceptibility of 16 different plant species was compared in a leaf disks assay. Parameters involved were the percentage infection and the accumulation of virus as analyzed by ELISA. Results show that leaf disks of all 16 species were susceptible to TSWV. Included in the comparisons were leaf disks of plant species which are local lesion hosts for TSWV, i.e. petunia, which previously proved to be a suitable host in transmission studies with the vector *F. occidentalis* (Wijkamp and Peters, 1993), *C. quinoa* and cucumber. Leaf disks of the latter two species were also susceptible and virus titers in ELISA were comparable to those of systemic hosts. The remaining 12 species are all systemic hosts of TSWV. Leaf disks of these species were also infected after challenge by viruliferous thrips. The differences in transmission efficiencies (Table 1) may be explained by a close link of virus transmission to both susceptibility of plant host and thrips feeding behavior.

All leaf disks did not become infected, although only viruliferous thrips were used in the calculation of transmission rates. The length of the IAP apparently was too short to obtain a 100% transmission rate. This conclusion is supported by earlier findings in which the effect of the length of inoculation access periods on transmission efficiency is investigated. These previous studies demonstrate that an IAP of 42 h is necessary to infect all leaf disks of petunia and *D. stramonium* (Wijkamp *et al*, 1995c).

The use of leaf disks was also evaluated in screening for resistance of transgenic tobacco and tomato lines. Using leaf disks of transgenic plants 3 out of 40 disks exhibited slightly higher ELISA values than healthy disks indicating that some viral antigen accumulated. Our results confirmed the data previously found by De Haan and co-workers (1992) which demonstrate that homozygous S2 progeny plants of line 'SR1-12 'are completely protected either by mechanical or thrips inoculation. In experiments presented here, leaf disk of 'SR1-12' showed significant resistance as compared to the control 'SR1' line. For tomato, complete immunity is found in the transgenic line '815/Radja' and moderate resistance in '698/Radja' upon mechanical inoculation (Ultzen *et al.*, 1995). Results of field experiments, demonstrate the resistance of '815/Radja'. However, the previously found resistance in '698/Radja' does not exist under field conditions (T. Ultzen, pers. comm.). Our experiments showed that, when challenged by viruliferous thrips, leaf disks of '815/Radja' supported the previous observations of total immunity. Transgenic '698/Radja' leaf disks were as susceptible as the hybrid 'Radja' which confirmed the results of the field trials.

Challenge of leaf disks of transgenic plants with viruliferous thrips show that this assay is a useful tool to study virus resistance of transgenic plants expressing TSWV sequences.

In conclusion, current experiments employing leaf disks as substrate for transmission of TSWV by *F. occidentalis* demonstrate the high value of this assay for studies in plant-TSWV-thrips relationships. This assay may be employed for other combinations of tospoviruses, thrips species and plant hosts as well. Moreover, infection of disks can readily be confirmed by ELISA. In order to investigate levels of resistance, the leaf disk assay employed in this chapter and mechanical inoculation may be applied to elucidate resistance in plants.

Chapter 4

Determination of the median latent period of two tospoviruses in *Frankliniella occidentalis*, using a novel leaf disk assay

SUMMARY

A novel assay system, based on the use of the local lesion host *Petunia x 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 27 °C, respectively, and for TSWV, they were 171, 109 and, 84 h at the respective temperatures.

This chapter has been published in a slightly modified version as: Ineke Wijkamp and Dick Peters (1993). Determination of the median latent period of two tospoviruses in *Frankliniella occidentalis*, using a novel leaf disk assay. Phytopathology 83: 986-991.

INTRODUCTION

Tospoviruses cause diseases in many economically important crops in the field and in greenhouses throughout the tropical, subtropical, and temperate climate zones. More than 650 plant species in at least 70 families have been reported as susceptible to these viruses (Goldbach and Peters, 1994). 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 (Barker, 1989; De Ávila *et al.*, 1990; Marchoux *et al.*, 1991; Vaira, *et al.*, 1992).

Tospoviruses are transmitted by thrips (Thysanoptera; Thripidae) in a persistent manner (Sakimura, 1962b). The virus is passed transstadially and replicates in the vector (Wijkamp *et al.*, 1993). Seven species have been reported as possible vectors (Sakimura, 1962b; Best, 1968; German *et al.*, 1992). Thrips feed by piercing a cell and sucking its contents (Hunter and Ullman, 1992). 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 (Sakimura, 1963; Ullman *et al.*, 1992b). The minimal acquisition period is 15-30 min (Sakimura, 1962b). 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* (Sakimura, 1963). 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 (Sakimura, 1962b).

Here we report studies on the efficiency of transmission of two distinct tospoviruses, impatiens necrotic spot virus (INSV) (De Ávila *et al.*, 1992; Law *et al.*, 1992) 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 circulative transmitted viruses (Sylvester, 1965; Sylvester and Richardson, 1966; Van der Broek and Gill, 1980) as well as propagative viruses (Duffus, 1963). 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 (De Ávila *et al.*, 1990) and the Dutch INSV isolate NL-07 (De Ávila *et al.*, 1992) were used in these experiments. TSWV is the type species of the newly established *Tospovirus* genus within the family of the *Bunyaviridae* (De Haan *et al.*, 1989; Francki *et al.*, 1991). INSV has been recently characterized and found to be a serologically distinct tospovirus (De Ávila *et al.*, 1992; Law *et al.*, 1992). It occurs in ornamental plant species and was isolated from *Impatiens* sp.

Acquisition feeding

Impatiens plants were inoculated 2-3 weeks 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 (2 leaves from 3 plants) were used for acquisition feeding. Each leaf was cut into 3 pieces and divided between 3 different leaf (Tashiro, 1967) cages. Each of the cages contained 3 leaf pieces. Leaves were kept fresh with a piece of wet filter paper. First instar larvae of *F. occidentalis* (0-4 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 x hybrida* Hort. Vilm.-Andr. 'Blue Magic' (Allen and Matteoni, 1991). 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 3 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 (Sylvester, 1965). The LP_{50}s and their 95% fiducial limit (FL) were calculated by the method of Finney (1962). Data were processed and analyzed with the POLO-PC program (LeOra Software, 1987).

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 leaf (Tashiro, 1967) cages at 27 °C to complete their development. On day 2 after adult emergence, 30 thrips per isolate were tested 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

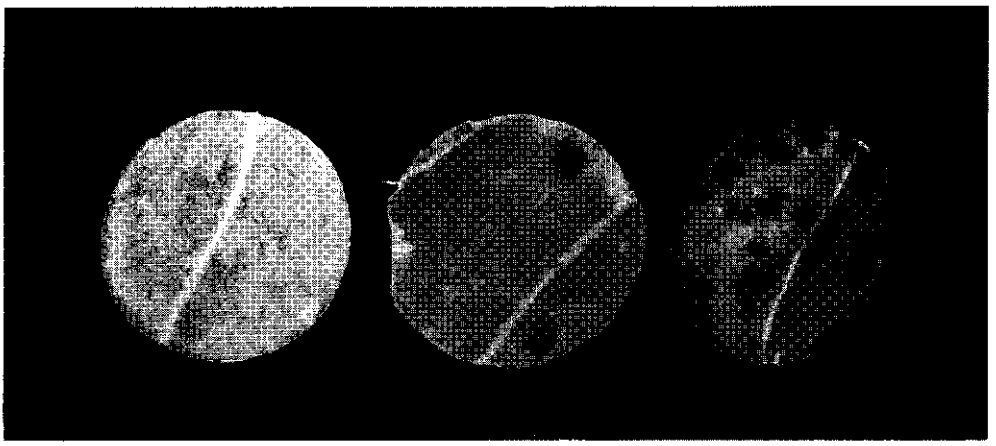


Figure 1. Local lesions caused by the impatiens necrotic spot virus isolate NL-07 on leaf disks of *Petunia x hybrida* 'Blue Magic' 3 days after the start of the inoculation access period (IAP). Healthy thrips fed on the left disk; infected *Frankliniella occidentalis* larvae were given an IAP of 24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

previously (De Ávila *et al.*, 1992). The immunoglobulin (IgG) fraction was partially purified by ammonium sulphate precipitation (Clark and Adams, 1977). 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 (Avrameas, 1969). 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 (Clark and Adams, 1977; Resende *et al.*, 1991a). 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. x hybrida* plants. This species reacts by forming small black or brown local lesions within 2-3 days after inoculation with either virus (Selman, 1964; Allen and Matteoni, 1991). 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 (Figure 1). The number of local lesions produced on one leaf disk varied from one lesion to approximately 30. ELISA was 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 2 or 3 local lesions (2-3), disks with 4 to 10 lesions (4-10), and disks with more than 10 (>10) lesions. These categories contained 11, 6, 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 (Figure 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.

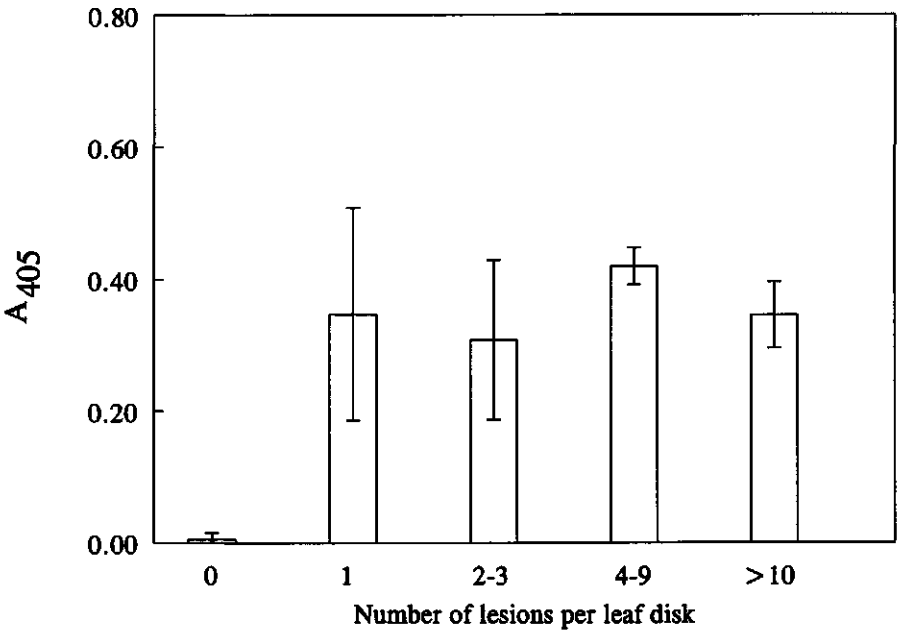


Figure 2. Average enzyme-linked immunosorbent assay values and standard deviations of leaf disks, using antiserum to the N protein of impatiens necrotic spot virus isolate NL-07. Leaf disks were divided into five categories: disks without symptoms (0), 1 lesion per disk (1), 1 to 3 lesions per disk (1-3),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

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 impatiens 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.

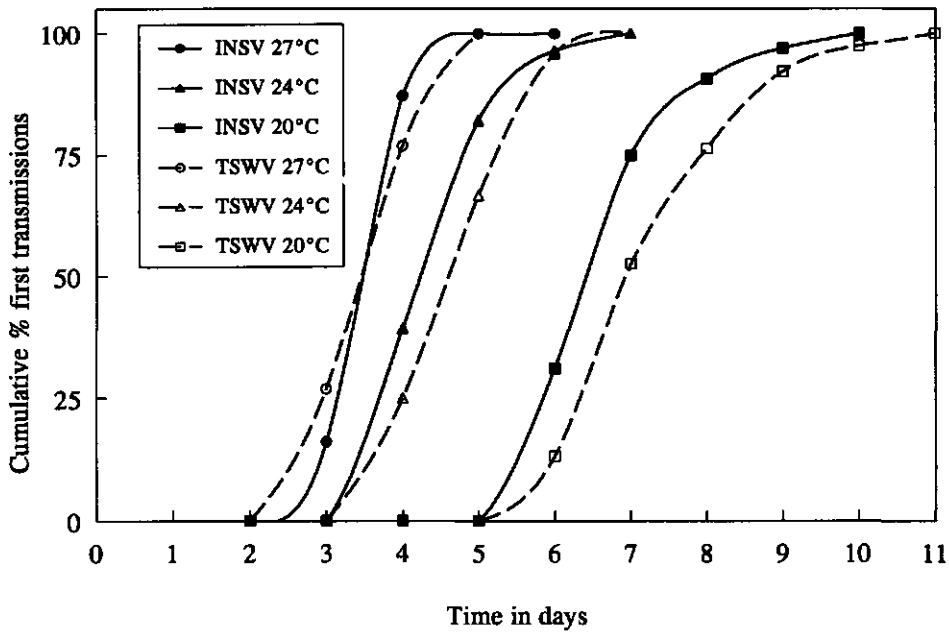


Figure 3. The cumulative percentages of *Frankliniella occidentalis* larvae transmitting impatiens necrotic spot virus (INSV) isolate NL-07 and tomato spotted wilt virus (TSWV) isolate BR-01 for the first time are plotted as a function of time. Acquisition access period was 24 h on systemically infected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

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.

All thrips that transmitted INSV during their second larval stage continued to do so as adult, 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 (Figure 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 h to 82 h for INSV and from 171 h 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

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]

Temp. (°C)	INSV LP_{50}[b] (h)	INSV 95% FL[c] (h)	TSWV LP_{50} (h)	TSWV 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 impatiens 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]

Temp. (°C)	INSV LP_{50}[b] (h)	INSV 95% FL[c] (h)	TSWV LP_{50} (h)	TSWV 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 impatiens 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.

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 and 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 chapter describes an efficient and novel assay system to study the transmission of tospoviruses by thrips larvae and adults, using leaf disks of *P. x hybrida* 'Blue Magic'. This cultivar was used by Allen and Matteoni (1991) 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 (Sakimura, 1962a). 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 (Anderson, 1981). California encephalitis virus (genus *Bunyavirus*) could be transmitted 13 days after acquisition when the adult mosquitoes were kept at 13 and 23 °C (McLean *et al.*, 1977). Northway virus (genus *Bunyavirus*) was transmitted after incubating mosquitoes for 27 days at 13 °C (McLean *et al.*, 1978). 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 (Sakimura, 1962a; Paliwal, 1976; Allen and Broadbent, 1986; Cho *et al.*, 1988). Efficiencies of approximately 20-30% have been reported for the transmission of TSWV by *F. occidentalis* (Sakimura, 1962a; Paliwal, 1976). Allen and Broadbent (1986) showed that 18 and 33% of the thrips transmitted TSWV to *Lycopersicum esculentum* and *Gomphrena globosa*, respectively, whereas Cho and co-workers (1988) observed that only 7% of the *F. occidentalis* transmitted TSWV to the lettuce cultivar '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 (Cho *et al.*, 1988). 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 (Robb, 1989).

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 for other circulative transmitted viruses, such as pea enation mosaic virus (PEMV) (Sylvester, 1965) and barley yellow dwarf virus (BYDV) (Van der Broek and Gill, 1980) and for propagative viruses, such as sowthistle yellow vein virus (SYVV) (Duffus, 1963). The temperature effect on the LP of viruses in their vector may be due to a higher virus-replication rate (Duffus, 1963) or an increased movement of virus through the vector at higher temperatures (Tamada and Harrison, 1981). 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 to 2.5 days for PEMV (Toros *et al.*, 1978), BYDV (Van der Broek and Gill, 1980) and potato leafroll virus (PLRV) (Van den Heuvel *et al.*, 1991). On the other hand, the long LPs (6 days or more) of the plant infecting reo- and rhabdoviruses are indicative for replication of these viruses in their vectors (Sakimura, 1962b). The LP_{50} found for the tospoviruses is of intermediate length, between those of reo- and rhabdoviruses, and those of BYDV, PLRV and PEMV. This observation may indicate that tospoviruses replicate in their vector. Experiments are in progress to verify this hypothesis.

Chapter 5

Multiplication of tomato spotted wilt virus in its insect vector, *Frankliniella occidentalis*

SUMMARY

The accumulation of two proteins, the nucleocapsid (N) protein and a non-structural (NS_S) protein both encoded by the S RNA of tomato spotted wilt virus (TSWV), was followed in larvae during development and in adults of *Frankliniella occidentalis* after ingesting the virus for short periods on infected plants. The amounts of both proteins increased, as shown by ELISA and Western blot analysis, within 2 days above the levels ingested, indicating multiplication of TSWV in these insects. Accumulation of these proteins and of virus particles was further confirmed by *in situ* immunolabelling of the salivary glands and other tissues of adult thrips. The accumulation of large amounts of N and NS_S protein, the occurrence of several vesicles with virus particles in the salivary glands and the massive numbers of virus particles in the salivary gland ducts demonstrate that the salivary glands are a major site of TSWV replication. The occurrence of virus particles in the salivary vesicles is indicative of the involvement of the Golgi apparatus in the maturation of the virus particles and its transport to the salivary ducts.

This chapter has been published in a slightly modified version as: Ineke Wijkamp, Jan van Lent, Richard Kormelink, Rob Goldbach and Dick Peters (1993). Multiplication of tomato spotted wilt virus in its insect vector, *Frankliniella occidentalis*. Journal of General Virology 74: 341-349.

INTRODUCTION

Tomato spotted wilt virus (TSWV), the type species of the *Tospovirus* genus of the family *Bunyaviridae* (Francki *et al.*, 1991), causes serious diseases world-wide in various crops. The genome of this plant-infecting bunyavirus consists of two ambisense RNA segments, S (small) and M (medium), and one negative-stranded, L (large) RNA segment. The L RNA encodes a protein which presumably represents the viral RNA polymerase (de Haan *et al.*, 1991). The M RNA encodes a non-structural (NS_M) protein and a precursor to the G1 and G2 glycoproteins which are associated with the lipid envelope of the virus particle (Kormelink *et al.*, 1992). The S RNA codes for an additional non-structural (NS_S) protein and for the nucleocapsid (N) protein (de Haan *et al.*, 1990).

The virus is exclusively transmitted by thrips (Thysanoptera) in a persistent way. Eight species have been reported to serve as vectors, of which the western flower thrips, *Frankliniella occidentalis* Pergande, is apparently one of the most important. Virus acquired by larvae renders the thrips infectious, and transmission of the virus is mainly ascribed to adults (Sakimura, 1962b). Recently, it has been shown that *F. occidentalis* larvae also transmit the virus efficiently. When virus was acquired by less-than-one-day-old first stage larvae, 80% of the larvae could transmit before pupation (Wijkamp and Peters, 1993). To gain more insight into the mechanism of virus transmission by larvae and adults, and factors affecting vector specificities, an investigation on the fate of TSWV in its vector was warranted.

Circumstantial evidence exists, showing that several animal-infecting bunyaviruses multiply in their insect vectors (Beaty and Calisher, 1991), but such evidence is lacking for the tospoviruses. This is probably because the vectors are diminutive insects in which the increase of virus titer is difficult to assay quantitatively and from which cell cultures have not been developed so far. Conclusive evidence for multiplication of tospoviruses in thrips may, alternatively, be obtained by detection of the non-structural proteins, NS_S or NS_M, which do not occur in the mature virus particles and whose presence depends on the replication of the viral genome (Kormelink *et al.*, 1991, 1992), and by demonstrating an increase of the N protein which may play an essential role in the switch from transcription to replication.

In the present study, TSWV replication was investigated in thrips after exposing newly

hatched first instar larvae to infected plants for only 2 h. Multiplication was monitored by ELISA and Western blot analysis using antisera to the N protein and NS_S protein. In addition, evidence was obtained by immunolabelling that the salivary glands are a major site of TSWV multiplication in thrips.

MATERIALS AND METHODS

Virus isolate and thrips

In all experiments described, the Brazilian isolate BR-01 (De Ávila *et al.*, 1992) of TSWV was used. Virus-free stock colonies of *F. occidentalis* were reared on bean pods (*Phaseolus vulgaris* L. 'Prelude') at 27 ($\pm$ 0.5) °C with a daily photoperiod of 16 h. The culture was started with adults collected from a greenhouse infestation in The Netherlands.

Infection of thrips

Impatiens sp. plants were used as virus sources. The plants were inoculated 2 to 3 weeks after sowing, by single viruliferous adults of *F. occidentalis* carrying the BR-01 isolate. The plants were grown in a greenhouse at approx. 22 °C (light/dark: 16/8 h) for symptom development. Systemically infected leaves of source plants were used for acquisition feeding. *F. occidentalis* first instar larvae, 0-2 h old, were confined to the surface of infected leaves using cages as described by Tashiro (1967). The thrips were given an acquisition access period (AAP) of 2 h at 27 ($\pm$0.5) °C. First instar larvae, caged on virus-free *Impatiens* plants, were used as controls.

After the AAP, the larvae were transferred to healthy leaves of *Datura stramonium* L. in leaf cages. For ELISA and Western blotting, a subgroup of thrips was collected and frozen at -70 °C directly after the AAP (2 h). To perform the ELISA, samples of approximately 30 larvae were frozen at 6 h intervals up to 48 h and at 60 and 72 h after the beginning of the AAP. At the same intervals samples of thrips, confined for 2 h on healthy *Impatiens* leaves, were collected to be used as controls.

In the Western blot analysis, thrips that had fed on infected plants for 2 h were sampled at 6, 24 and 72 h after the start of the AAP, late L2 stage (120 h), prepupal (PP) stage, pupal (P) stage, and as 1 and 8 day old adults. Leaves were replaced each day until larvae reached the prepupal stage. This was done to prevent re-ingestion of newly replicated virus

in *Datura* leaves inoculated by the viruliferous thrips. Healthy thrips of the same age served as controls.

Selection of viruliferous thrips

Adults grown from larvae exposed to infected plants were tested on leaf disks of *Petunia x hybrida* 'Blue Magic' as described previously (Wijkamp and Peters, 1993). Inoculation access periods (IAP) of 24 h at 27 (± 0.5) °C were allowed. After the IAP the leaf disks were incubated at 27 °C in 24-well microtiter plates while floating on water for the development of local lesions. Symptoms were scored visually. The viruliferous adults identified by this method and healthy adults, as controls, were used for light and electron microscopy studies.

Antiserum production and purification

Polyclonal antisera were raised against the N protein (anti-N serum) of isolate BR-01 (Huguenot *et al.*, 1990; De Ávila *et al.*, 1992). The production of polyclonal antiserum against the NS_S protein (anti-NS_S) has been described by Kormelink and co-workers (1991). The immunoglobulin (IgG) fractions of both antisera were partially purified by ammonium sulphate precipitation as described by Clark and Adams (1977). For the cocktail ELISA, IgGs of anti-N serum were conjugated at a concentration of 1 mg/ml with 2,000 units of alkaline phosphatase (Grade I, Boehringer) in phosphate-buffered saline, pH 7.4 (Avrameas, 1969). The IgG and conjugate were stored with 0.05% sodium azide at 4 °C. The NS_S antiserum was cross-absorbed with acetone-washed powder of healthy thrips (Harlow and Lane, 1988) to eliminate background in ELISA and Western blot analyses due to non-specific reactions.

ELISA of thrips

Thrips were analyzed both by cocktail ELISA (Resende *et al.*, 1991a) and by antigen-coated plate (ACP) ELISA. Cocktail ELISA was employed to detect the N protein of the virus in individual thrips. Wells of Nunc Maxisorp F96 immunoplates were coated with 150 µl of 0,5 µg/ml γ-globulins in coating buffer (0.05 M sodium carbonate, pH 9.6) and incubated overnight at 4 °C. After rinsing of the plates 3 times with PBS (0.14 M NaCl, 1 mM KH_2PO_4, 8 mM Na_2HPO4 and 2.5 mM KCl) containing 0.05% Tween-20 (PBS-T) they were incubated with 150 µl per well of 1% BSA in sample buffer (2%

polyvinylpyrrolidone, M_W about 44,000, and 0.2% ovalbumin in PBS-T) for 30 min at 37 °C to block non-specific binding sites. Individual thrips were placed in Eppendorf tubes and triturated with a micropestle in 100 µl of sample buffer. Each suspension was divided in two portions which were analyzed with anti-N and anti-NS_S sera, respectively. One portion of the sample was transferred to the ELISA wells and mixed with 50 µl of 1 µg/ml anti-N conjugate in sample buffer. As a control a dilution series of virus purified from *Nicotiana rustica* plants was used. The cocktail was incubated overnight at 4 °C. After incubation the plates were rinsed 3 times with PBS-T and the enzyme reaction was amplified as described by van den Heuvel and Peters (1989). Per well 100 µl of 0.2 mM NADP monosodium salt in 0.05 M diethanolamine buffer, pH 9.5, was added. After incubation at 20 °C for 30 min, remaining alkaline phosphatase activity was blocked by adding 15 µl of 0.05 M ortho-nitrophenyl phosphate disodium salt (o-NPP) in 0.05 M diethanolamine buffer per well. Subsequently, 150 µl of the amplification mixture was added per well. The stock amplification mixture consisted of 700 units of alcohol dehydrogenase, 100 units of lipoamide dehydrogenase (type VI), 3% (v/v) ethanol and 1 mM p-iodonitrotetrazolium violet in 15 ml of 0.025 M phosphate buffer pH 7.0. The reaction was allowed to proceed at room temperature. Absorbance values were read on a Titertek Multiskan colorimeter (Flow Laboratories Ltd., Irvine, Scotland) at 492 nm. Blank values were read for wells that contained only sample buffer in the sample incubation step.

For monitoring NS_S protein production in thrips, ACP ELISA was performed. Wells were coated with the second portion of the thrips sample in 50 µl sample buffer mixed with an equal volume of coating buffer (2X) and incubated overnight at 4 °C. As a control a dilution series of NS_S purified from *Spodoptera frugiperda* cells (Kormelink *et al.*, 1991) was used. Coating was followed by blocking of the plates with 150 µl 1% BSA in sample buffer for 0.5 h at 37 °C. Subsequent steps involved incubating the wells with 100 µl of 0.4 µg/ml of anti-NS_S serum in sample buffer for 2 h at 37 °C followed by incubation with 0.3 µg/ml goat anti-rabbit IgG-alkaline phosphatase conjugate in sample buffer for 2 h at 37 °C. Between incubation steps, except after blocking, the plates were washed with PBS-T. The procedure was continued as described for the cocktail-ELISA.

SDS-PAGE and immunoblot analyses of proteins in thrips

Samples of 15 thrips were triturated in 15 µl PBS-T with a micropestle in an Eppendorf tube. For SDS-PAGE analyses of proteins, the samples were mixed with an equal volume

of protein loading buffer (10 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 0.001% w/v bromophenol blue, 4% v/v 2-mercaptoethanol). Samples were divided into two portions and analyzed with each of the antisera. Each of these portions was applied to a 12.5% SDS-polyacrylamide gel (Laemmli, 1970). Virus preparations purified from plants and an NS_S protein preparation from *S. frugiperda* cells were used as controls. After SDS-PAGE, proteins were transferred to Immobilon membranes (Millipore) by semi-dry blotting in semi-dry transfer buffer (39 mM glycine, 48 mM Tris, 0.0375% w/v SDS, 20% methanol) using a semi-dry transfer cell (Trans-blot SD, Bio-Rad). Membranes were blocked overnight at room temperature in PBS containing 3% BSA. After several washing steps with PBS containing 0.3% BSA, membranes were incubated in the same buffer containing either 1 μg/ml cross-absorbed NS_S antiserum or 1 μg/ml BR-01 nucleocapsid antiserum. After washing, antigen-antibody complexes were detected using 0.6 μg/ml alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Tago Inc., Burlingame, CA), using 0.33 mg/ml nitroblue tetrazolium and 0.165 mg/ml bromochloroindolyl phosphate as a substrate (Leary *et al.*, 1983).

Electron microscopy

Viruliferous adult thrips, which fed as 1 day old larvae for 24 h on infected *Impatiens* leaves, and virus-free insects were immersed in 2% (w/v) paraformaldehyde and 3% (w/v) glutaraldehyde in PC buffer (0.1 M $Na_2HPO_4.2H_2O$, 9.7 mM citric acid, pH 7.2, 1.5 mM $CaCl_2$). A small piece of the anterior part of the head and posterior part of the abdomen was cut off to allow penetration of the fixative into the tissues. The fixative was infiltrated under vacuum (7000 Pa) for 2 h at room temperature (RT) and for another 16 h at 4 °C under atmospheric pressure. The thrips were then washed 6 times for 10 min in demineralized water and dehydrated in a series of ethanol concentrations of 30, 50, 70, 80, 90% for 10 min and twice for 30 min each in 100% ethanol at RT. Thrips were then infiltrated with a 1:1 mixture of ethanol and LR White (London Resin Company) for 5 h, and pure LR White for 40 h (refreshing the medium after 16 h). The specimens were transferred to gelatine capsules and polymerized at 50 °C for 48 h. Alternatively, after washing in demineralized water the thrips were dehydrated in a series of ethanol at low temperature; in 30% at 0 °C for 30 min, in 50% at 0 °C for 30 min and subsequently at -20 °C for 30 min, in 70, 80, 90% and twice in 100% at -20 °C for 1 h. Thrips were then infiltrated with a 1:1 mixture of ethanol and LR Gold (London Resin Company) for 16 h

and pure LR Gold for 48 h (refreshing the medium after 24 h) at -20 °C. The specimens were transferred to BEEM capsules and polymerized with ultraviolet light (wavelength 360 nm) for 24 h at -25 °C and 48 h at RT.

Semi-thin sections with a thickness of 1 μm for light microscopy or ultrathin sections of 70 nm for electron microscopy were cut with a histo-diamond knife (Diatome) using a Reichert Ultracut E microtome. For light microscopy semi-thin sections of LR Gold-embedded specimens were individually mounted in drops of distilled water with glycerin albumen (Gurr) (1:200 v/v) on glass slides pretreated with dimethyldichlorosilane. The slides were dried on a hot plate at 60 °C. The sections were stained for 3 min in 1% toluidine blue in distilled water, washed in water, dried and mounted in Eukitt. Ultrathin sections of LR Gold- and LR White-embedded specimens were collected on golden slot grids with a formvar film and stained for 5 min with an aqueous solution of 2% (w/v) uranyl acetate and for 1 min with lead citrate according to Reynolds (1963).

Immunogold labelling and silver enhancement

For light microscope detection of viral antigens by immunogold/silver staining, unstained semi-thin sections of LR Gold-embedded thrips were treated as described by van Lent and Verduin (1987). Sections were examined with phase-contrast or with epi-illumination in a Leitz Laborlux S light microscope equipped with a polarization filterblock (epipolarization microscopy). Immunogold labelling and silver enhancement of antigens in ultrathin sections were performed as described by van Lent and co-workers (1990). Sections were examined in a Philips CM12 transmission electron microscope.

Antisera against N and NS_S protein were used in a concentration of 1 μg/ml.

RESULTS

Accumulation of viral proteins after TSWV acquisition in F. occidentalis

To determine whether TSWV multiplies in its vector *F. occidentalis*, the accumulation of viral proteins was monitored by ELISA and Western blot analysis. As viral antigens the N protein (28.8 kD) and the NS_S protein (52.4 kD) were chosen. Monitoring of the N protein would give a measure of the accumulation of virus particles in thrips, whereas monitoring of the NS_S protein, which is not present in virus particles (Kormelink *et al.*,

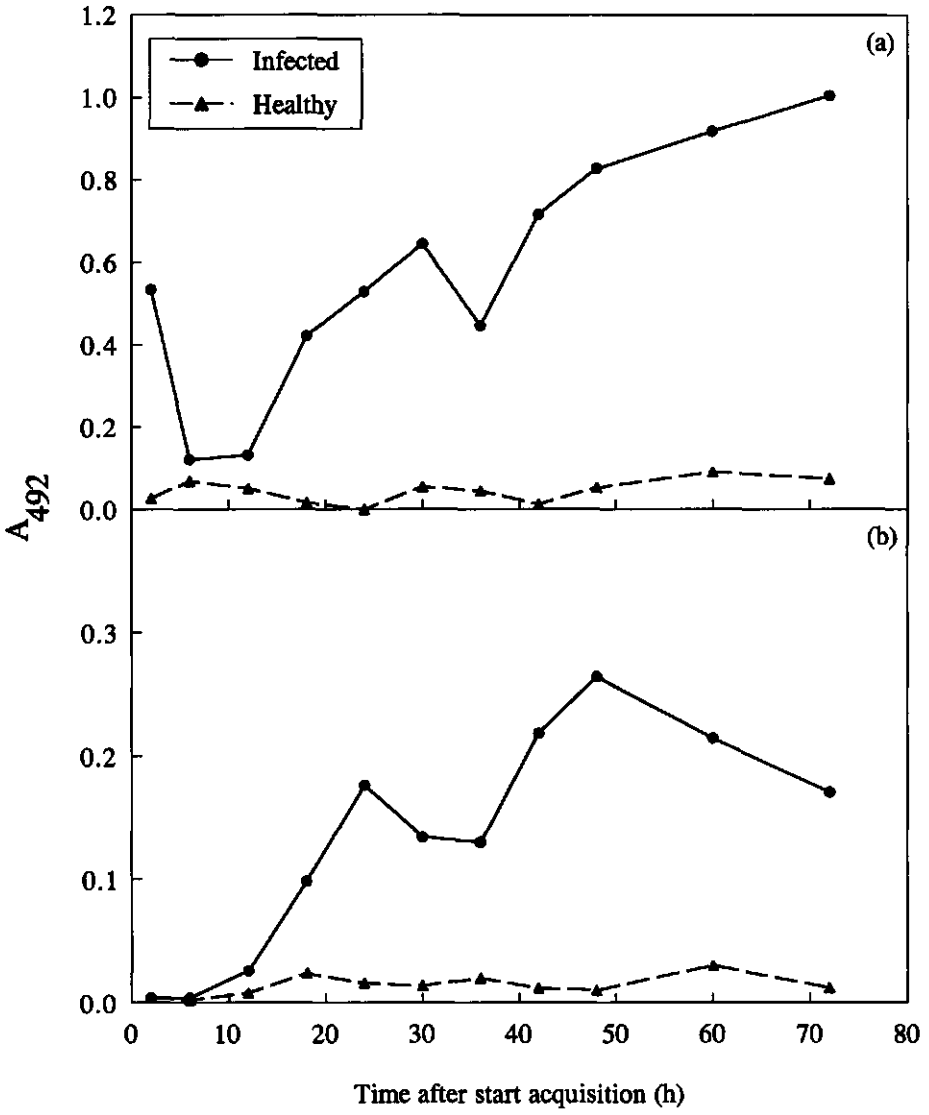


Figure 1. Production of N protein (a) and the NS_S protein (b) encoded by the S RNA segment of TSWV in infected and healthy larvae of *F. occidentalis* after a 2 h AAP. The mean ELISA values obtained at the various intervals from 30 thrips which were singly tested are given.

1991), would provide evidence for expression and replication of the viral genome. Using ELISA and polyclonal antibodies against the N protein, it was found that the titer of this protein dropped within the first few hours after acquisition but increased after 24 h to levels above the amount ingested, eventually achieving a plateau before the prepupal stage (Figure 1). Lower values for the N protein were consistently observed around 36 h after acquisition, a phenomenon which coincided with moulting of the larvae from the first to the second instar. Although the increase of N protein in the thrips larvae already indicated that TSWV multiplied in its vector, further evidence was obtained by demonstrating the *de novo* synthesis of the NS_S protein in thrips larvae. The amount of this protein detected in thrips immediately after acquisition was virtually zero but started to increase simultaneously, though at a dissimilar rate, with the N protein (Figure 1).

The production of the N and NS_S proteins in thrips was further substantiated by immunoblot analysis. Both proteins were consistently found in extracts prepared from all instars, except when the larvae were sampled 2 or 6 h after acquisition (Figure 2). Lower amounts of NS_S occurred in samples prepared from second stage larvae (just before pupation), prepupae and pupae, than in samples from larvae 72 h after acquisition and in 1 to 8 day old adults. The concentration of N protein is apparently slightly lower in the adults than in old larvae (Figure 2). The production of the N as well as the NS_S protein was further investigated in a more detailed analysis made during the development of the infection in 2 to 72 h old larvae. Both proteins could be detected by immunoblotting 18 h after acquisition and their amounts increased in the following 24 h (results not shown). From the increasing accumulation of N and NS_S protein, as determined by ELISA and immunoblot analysis, it can be concluded that TSWV replicates in its insect vector.

In situ *localization of virus and viral proteins in thrips*

Ultrathin sections of 3 to 4 day old adult thrips were analyzed by immunogold labelling to determine in which organs and tissues the virus replicates. Adults contained readily detectable amounts of NS_S protein in all adult stages sampled (Figure 2) and responded positively in infection tests (results not shown). These adults had fed as 1 day old larvae for 24 h on leaves of infected *Impatiens* plants and were selected for their infectivity as described. Immunogold/silver staining showed the abundant presence of the N and NS_S proteins in the salivary gland tissue (Figure 3). The salivary glands are positioned in the prothorax of the insect, partly surrounding the oesophagus (Figure 3b) (Ullman *et al.*,

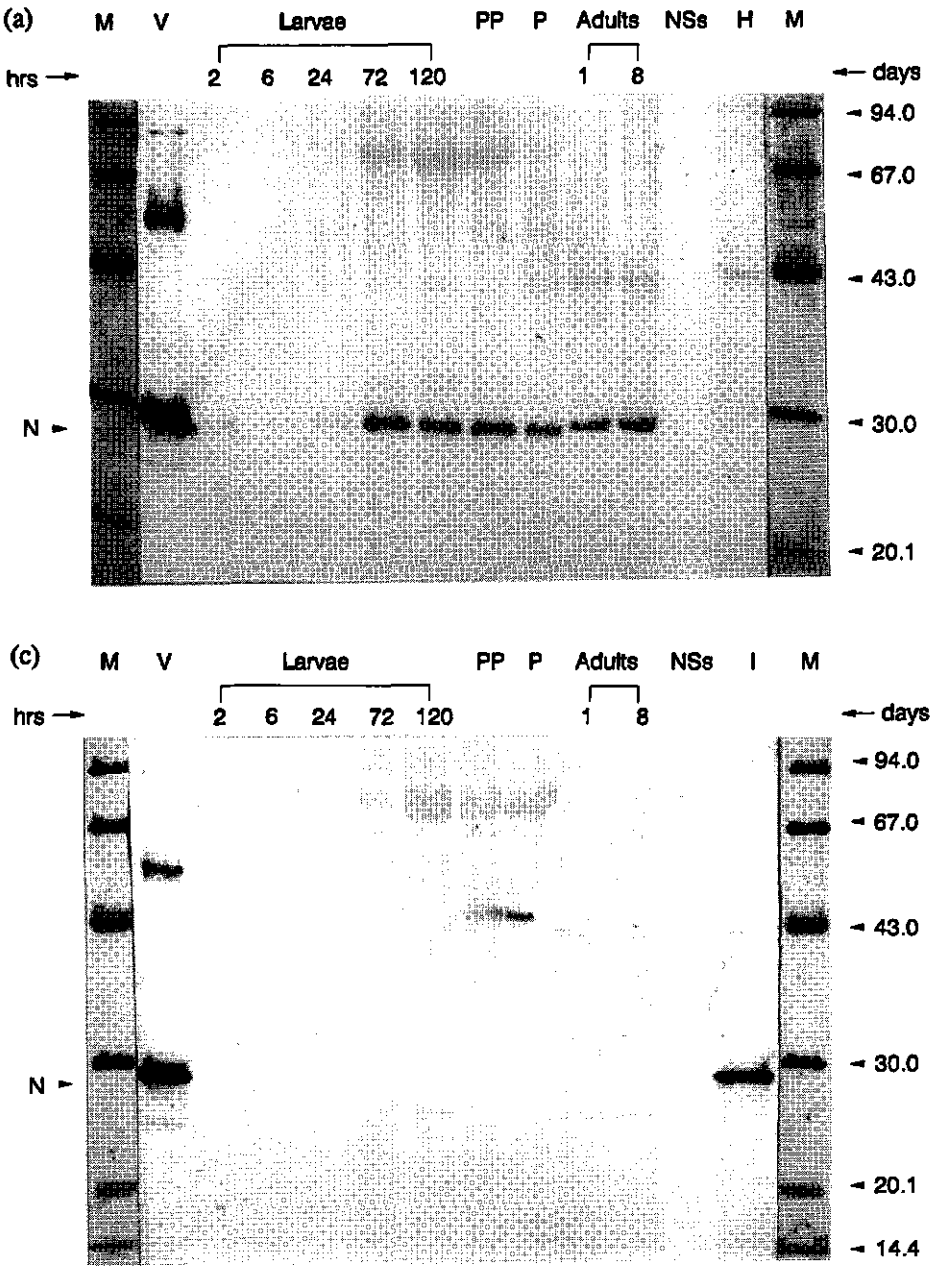
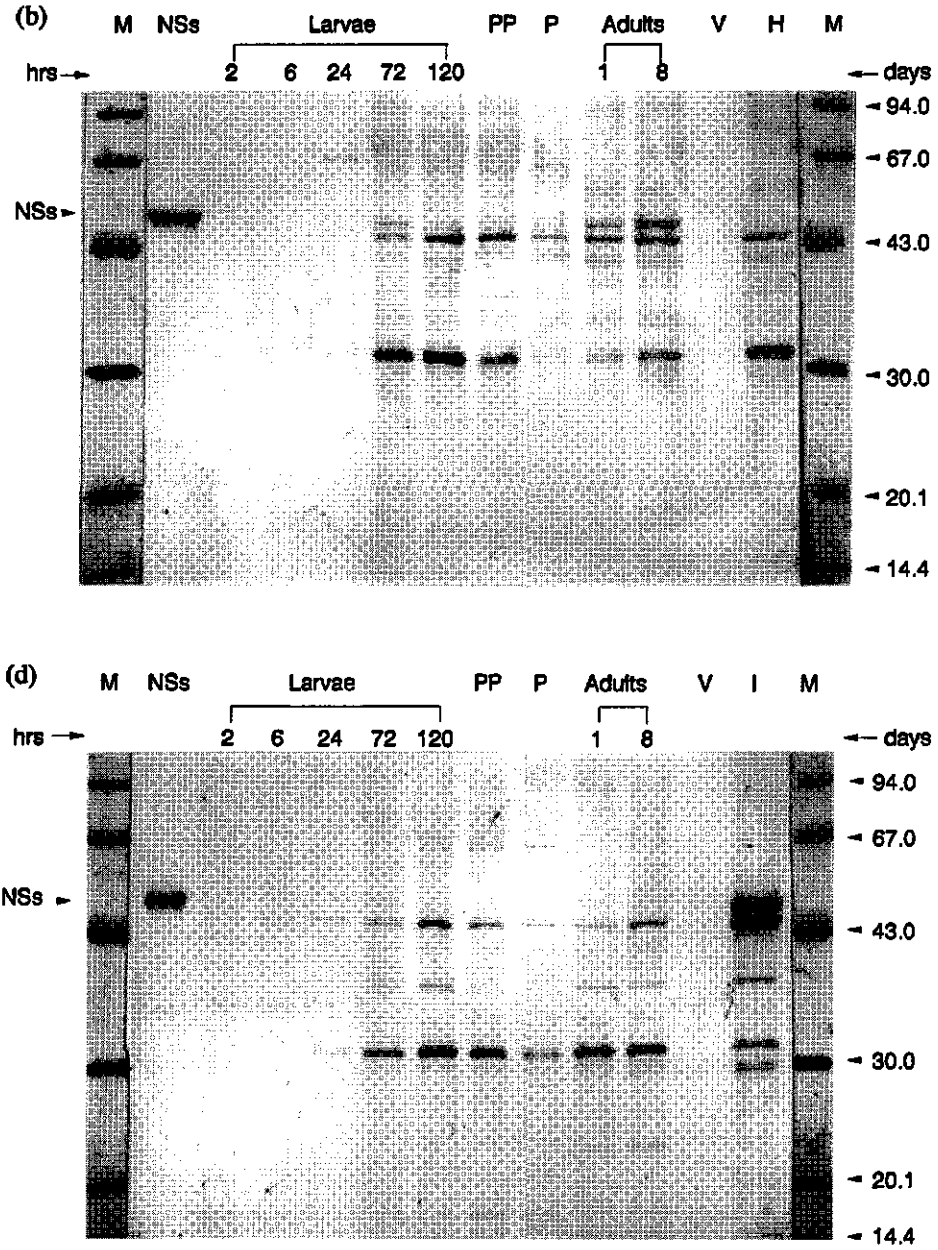


Figure 2. Western immunoblot analysis of extracts from 15 larvae and adults sampled at subsequent periods after virus-free thrips (c, anti-N serum; d, anti-NS_s serum). M: Markers. V: Purified virus. Larvae were sampled 2, at 1 or 8 days old were also tested. H: Sample of 15 virus-free adults. I: Sample of 15 viruliferous adults.



the onset of a 2 h AAP using (a) antiserum against the TSWV N protein or (b) NS_s protein, and (c,d) extracts of 6, 24, 72 and 120 h (harvested just before pupation) after the start of AAP. Prepupae (PP), pupae (P) and adults

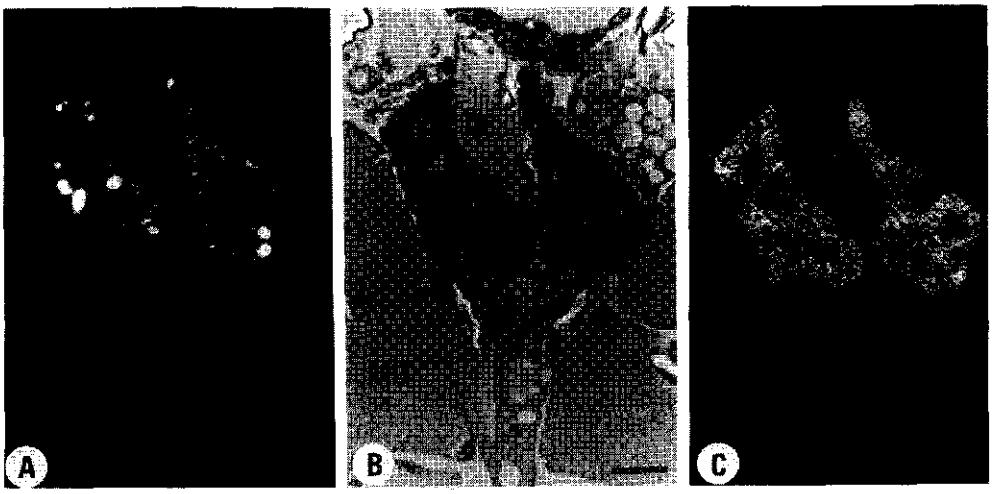


Figure 3. Serial sections of the salivary gland (arrows) of an adult thrips which acquired the virus in the first larval instar. (a) Epipolarization microscopy of the section after immunogold/silver staining with N antiserum. (b) Phase-contrast image of the same area. (c) Epipolarization of the section following immunogold/silver staining with NS_s antiserum. MU, muscle; OE, oesophagus. Bar marker represents 10 μm.

1989). Epipolarization studies showed the presence of the N protein in confined areas of the gland tissue (Figure 3a), and the NS_s protein appeared evenly spread throughout the cells (Figure 3c).

Thin sections of the salivary glands revealed the presence of many electron-dense aggregates embedded in the cytoplasm (Figure 4a). These aggregates (viroplasms) are specifically gold-labelled after treatment with N antiserum (Figure 4b). Numerous virus particles were observed in the salivary ducts, aligned with the duct membrane (Figure 4c). The nature of these particles could be deduced from immunogold labelling with N antiserum (Figure 4d) and their characteristic shape with a membrane. The saliva vesicles which accumulate in the salivary gland cells usually contained a limited number of virus particles (Figure 4e), compared to the number of virus particles in the salivary ducts. A few virus particles were also discerned in the cytoplasm.

The NS_s protein was not confined to specific compartments, but appeared to be distributed through the whole cytoplasm of the gland cells (Figure 4f). Another, prominent location of both N and NS_s proteins was the muscle cells associated with the midgut epithelium. Figure 5a shows two segments due to looping of the midgut (Ullman *et al.*,

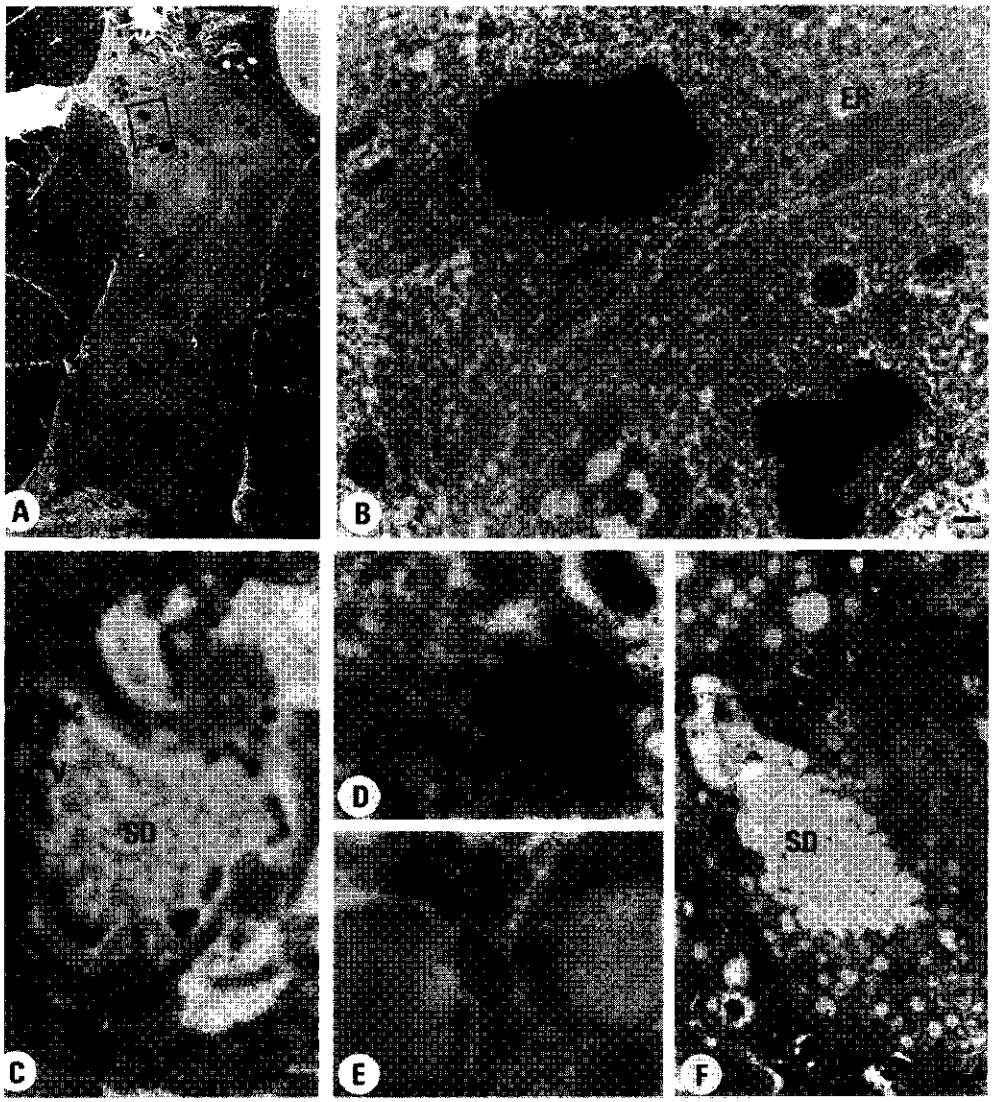


Figure 4. Electron micrographs of thin sections of the salivary gland. (a) View of the salivary gland (SG) showing electron-dense aggregates (viroplasms, arrows). (b) Enlargement of the boxed area in (a) showing dense gold labelling of the viroplasms (VP) with N antiserum. (c) Virus particles (V) in a cross-section of a salivary gland duct (SD). (d) N protein gold labelling of virus particles in the lumen of a salivary gland duct. (e) Virus particles in salivary gland vesicles (arrows). (f) Gold labelling of NS_5 protein in the cytoplasm of a salivary gland cell. N, nucleus; ER, endoplasmic reticulum; MU, muscle. Bars markers represent 10 μm (a), 1 μm (b, f), 0.3 μm (c) and 0.15 μm (d, e).

1989). Like the salivary gland cells, the muscle cells contain viroplasms, gold-labelled with N antiserum (Figure 5a and b) and the NS_S protein scattered through the cytoplasm (Figure 5c). The viroplasms, clearly shown in figure 5c, have an appearance similar to those found in TSWV-infected plant cells (Kitajima *et al.*, 1992).

DISCUSSION

Multiplication of plant viruses in their insect vectors has been demonstrated previously by serial passaging of the virus from one insect to another. This technique has not been used to demonstrate replication of tospoviruses in thrips in view of the size of this insect and the difficulties in handling them. Multiplication of plant viruses in their insect vectors can also be demonstrated by quantitatively assaying the increase of virus-specific antigens in their vectors as shown by Reddy and Black (1966). Applying this approach in the present study, an increase in concentration of two TSWV-encoded proteins, the N protein and the NS_S protein encoded by the S RNA, was demonstrated in *F. occidentalis* after short (2 h) acquisition of the virus on infected plants. After a lag phase, the amount of N protein increases above the level ingested during acquisition (Figure 1). The increased amounts of this protein and of the NS_S protein firmly demonstrate replication of TSWV in its vector. The accumulation of N protein is indicative of the production of virus particles, but the accumulation of the NS_S protein, which has not been found in virus particles (Kormelink *et al.*, 1991) can only occur after transcription of its mRNA from the complementary viral RNA strand which is formed during the replication of viral RNA. Hence, the presence and increase of this protein in thrips as well as that of the N protein both give conclusive proof that TSWV replicates in its vector. As expected, the development of the titer of proteins in the larvae coincides closely with the development of their infectivity (Wijkamp and Peters, 1993).

The *in situ* localization of the N and NS_S proteins in salivary glands and in midgut muscle cells presents further evidence for multiplication of TSWV in thrips and provides information on the sites of active replication. The N protein was abundantly present in electron-dense aggregates showing obvious similarity in structure to the viroplasms found in infected plant cells (Kitajima *et al.*, 1992). The NS_S protein was scattered in the cytoplasm, again similar to what is found in infected plant cells. The intracellular

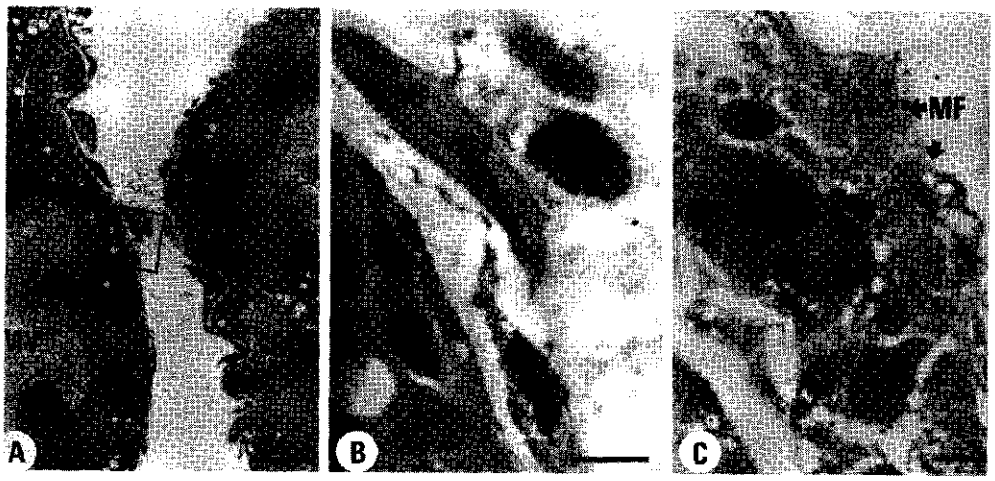


Figure 5. (a) Electron micrograph showing two basal parts of the midgut epithelium (ME) with several muscle cells (arrows) and a regenerative cell (RC). Immunogold/silver staining shows the location of N protein in viroplasms. (b) Enlargement of the boxed area in 5 (a) showing a muscle cell with viroplasms containing N protein. (c) Muscle cell treated with NS_S antiserum. A viroplasm with dense aggregates occurs in the cytoplasm which contains the NS_S protein. MV, microvilli; MF, muscle fibrils. Bars markers represent 3 μm (a), and 0.15 μm (b, c).

appearance of NS_S may depend on the TSWV isolate studied. Kitajima and co-workers (1992) reported the presence of NS_S exclusively in fibrous aggregates in plant cells infected with several TSWV isolates. The TSWV BR-01 isolate used in the present study does not induce such structures in plant cells (Kormelink *et al.*, 1991; Kitajima *et al.*, 1992) nor, as shown here, in insect cells.

Virus particles are efficiently released into the salivary ducts as evidenced by their abundant presence there. The observation of virus particles in the saliva secretory vesicles supports the idea that the virus particles are released during the secretion of saliva into the ducts. The occurrence of virus particles in these vesicles may be indicative of a direct involvement of the Golgi apparatus in the multiplication of TSWV, e.g. the assembly of the virus particles, and transport to the ducts by the vesicles, which bud from the Golgi apparatus. The saliva granules may be the equivalent of the vesicles formed in sandfly fever virus infections (Smith and Pifat, 1982) transporting the virus particles from the Golgi apparatus to the cell surface. It is generally accepted that the tospoviruses, while replicating in plants, accumulate in the cisternae of the endoplasmic reticulum (Kitajima *et al.*, 1992).

Some observations have been made which suggest that the Golgi apparatus is involved in the maturation of the tospoviruses in plants (Kitajima *et al.*, 1992), e.g. in a process to modify the membrane-bound glycoproteins. This does not exclude the possibility that tospoviruses mature in different cell compartments of plant and thrips cells.

Viroplasms in thrips cells are also interspersed with electron-dense aggregates as described by Ie (1982) and Kitajima and co-workers (1992), showing another similarity to infected plant cells. Those in plant cells are considered to be formed by nucleocapsids. The nature of the dense aggregates in thrips has yet to be elucidated.

Chapter 6

Virus-vector interaction of tomato spotted wilt virus in *Frankliniella occidentalis* is neither accompanied by pathological effects nor by transovarial transmission

SUMMARY

The effect of tomato spotted wilt virus (TSWV) on *Frankliniella occidentalis* following a 6 hours acquisition access period on infected plants was investigated. No differences were observed among viruliferous, non-viruliferous and control thrips with respect to developmental time, reproduction rate and survival. Thrips larvae, exposed or unexposed to TSWV, developed from egg to adult in 13.1 and 13.2 days, respectively. Exposed females produced an average of 28.3 larvae whereas control thrips produced 22.3 larvae and longevity was 13.4 and 12.5 days, respectively. Population reproductive statistics, net reproductive rate (R_0), mean generation time (T) and intrinsic rate of increase (r_m) were calculated from the life fertility tables. R_0 and r_m were higher for viruliferous thrips as compared to non-viruliferous and non-exposed thrips. Virus transmission studies revealed that viruliferous thrips were able to transmit virus until death. Virus was not transovarially transmitted to the offspring.

This chapter will be published in a slightly modified version as: Ineke Wijkamp, Rob Goldbach and Dick Peters. Virus-vector interaction of tomato spotted wilt virus in *Frankliniella occidentalis* is neither accompanied by pathological effects nor by transovarial transmission. Ent. Exp. Appl., submitted.

INTRODUCTION

The western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera; Thripidae), is an important insect pest of several greenhouse crops throughout Northern America and Western Europe, causing damage to at least 219 vegetables and ornamentals (Brødsgaard, 1989). In addition to its pest status, this thrips species is also the main vector of several tospoviruses (Wijkamp *et al.*, 1995a) which cause worldwide devastating diseases to many economically important crops (Goldbach and Peters, 1994). Tospoviruses are transmitted by thrips in a propagative way (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993). Virus, acquired by young larvae, can be transmitted by second instar larvae and adults (Wijkamp and Peters, 1993). Propagative virus transmission requires an intimate relationship between virus and vector. In TSWV-transmitting thrips, particles have been visualized within the larval midgut epithelium, fat body, brain, hemocoel and salivary glands (Ullman *et al.*, 1992a; Wijkamp *et al.*, 1993). Immunogold/silver staining showed the presence of N and NS_S protein in muscle cells associated with the midgut and salivary glands. Their abundant presence in the salivary glands indicates that this organ may be the major site for virus replication (Wijkamp *et al.*, 1993).

Virus replication in vector tissues may alter vector physiology resulting in a potential pathogenic effect. Physiological aberrations have been detected in vectors infected with some plant viruses. The propagative transmission of sowthistle yellow vein rhabdovirus (SYVV) by *Hyperomyzus lactucae* for instance, is associated with a decreased life span of the insect vector, and occasionally, a lower fecundity is observed (Sylvester, 1973). Also reoviruses can cause deleterious effects in their leafhopper vectors (Shinkai, 1962; Nakasuji and Kiritani, 1970). For tenuiviruses, reports do not agree on certain areas involving the effect of virus on the vector (Nault, 1994). Marafiviruses have been shown to be widespread in the organs of infected vector insects, but no evidence for cytopathological or other effects caused by the virus were found (Gamez and Léon, 1983).

Propagation of plant viruses in their vector occasionally results in transovarial transmission of virus to offspring. *Rhabdoviridae* (Sylvester, 1969) and some members of the *Reoviridae* (Conti, 1984) are transmitted through the egg, often with a low frequency, while efficient transovarial transmission has been reported for tenuiviruses (Gingery, 1988). By contrast, marafiviruses are not vertically transmitted (Gamez and Léon, 1983).

Conflicting results have been reported on effects of tospovirus infection on thrips survival, development and reproduction. An early study reports no deleterious effect of virus on the vector (Sakimura, 1963). However, negative effects were observed on the survival of thrips when exposed for long periods to virus-infected plants (Robb, 1989) and an adverse effect was demonstrated on survival, reproduction and development of thrips by DeAngelis and co-workers (1993). In the experiments presented in this report, we have compared life parameters for thrips that were exposed for a restricted period of 6 h to TSWV-infected plants with those for thrips that were not exposed to virus. A short acquisition period was chosen as to exclude possible deleterious effects of TSWV-infected plant tissue on vector physiology. The aspect of transovarial transmission does not appear to have received as much attention as other areas of virus-vector relationships. In order to study whether viruliferous females did transmit virus to their offspring, progeny thrips were tested for virus transmission and presence of viral antigen.

MATERIALS AND METHODS

Virus and source plants

In the experiments described, the Brazilian isolate BR-01 (De Ávila *et al.*, 1992) of tomato spotted wilt virus (TSWV) was used. TSWV is the type species of the *Tospovirus* genus within the family of the *Bunyaviridae* (De Haan *et al.*, 1989; Francki *et al.*, 1991). *Datura stramonium* L. plants were used as virus sources. Plants were inoculated 2 weeks after sowing, by single viruliferous adults of *F. occidentalis*. Infected and healthy control plants were grown in a greenhouse at approx. 22 °C (light/dark: 16/8 h). Systemically infected leaves of source plants and healthy control leaves of the same age were used for acquisition feeding.

Thrips

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

Development

To investigate the developmental time of thrips, female *F. occidentalis* from the stock colonies were allowed to oviposit on *D. stramonium* leaf disks in Eppendorf tubes for 24 h. After removal of the females, the leaf disks were incubated for 2 days in 24 well plates (Costar Europe Ltd.) while floating on water and subsequently transferred to 24 well plates on disks of wetted Whatman paper, to allow hatching of the eggs deposited by the adults in the leaf disks. The Whatman paper disks prevented wilting and drying of the leaf disks. Each well was sealed with parafilm, so that emerging larvae could not escape and, therefore, be counted reliably. The number of eggs oviposited was determined as the number of first instar larvae hatched successfully. Thus, egg mortality was not recorded. First instar larvae, 0-4 h after emergence, were confined to the surface of infected *D. stramonium* leaves in cages as described by Tashiro (1967). The larvae were given an acquisition access period (AAP) of 6 h. First instar larvae, caged on virus-free *D. stramonium* plants, were used as controls. After the AAP, the larvae were transferred to healthy leaves of *D. stramonium* in leaf cages. The condition and developmental stage of thrips were checked every 24 h and leaves were changed every 2 days. The experimental temperature was 25 ($\pm$0.5) °C with a daily photoperiod of 16 h (light/dark: 16/8 h).

Reproduction

To investigate bionomics of the thrips and virus transmission by adults, thrips which reached the adult stage were transferred daily to leaf disks of *Petunia x hybrida* Hort. Vilm.-Andr. 'Blue Magic' as described previously (Wijkamp and Peters, 1993). After each inoculation access period (IAP) of 24 h, the leaf disks were incubated for 2 days in 24 well plates while floating on water for the development of local lesions, to quantify virus transmission by thrips. Subsequently, the leaf disks on which females had fed and oviposited, were transferred to 24 well plates on disks of humid Whatman paper as described before. Emerging larvae per leaf disks were counted 5 to 6 days after the start of the IAP. Offspring numbers and virus transmission were recorded daily. Virus infection of the leaf disks was confirmed by DAS-ELISA (Wijkamp and Peters, 1993).

Life-fertility tables

Mortality and fertility data were summarized in the form of age-specific life-fertility tables for the cohorts of viruliferous, non-viruliferous and healthy thrips, according to the

Table 1. Development of *Frankliniella occidentalis* at 25 °C, exposed and not exposed to TSWV. Juvenile stages were confined to *Datura stramonium* leaves and adults were transferred daily to fresh petunia leaf disks.

	Days required to complete development[a]	
	TSWV-exposed[b]	Non-exposed[c]
n	(125)	(47)
Egg	3.5 ± 0.054	3.5 ± 0.054
L1 + L2[d]	6.0 ± 0.068a	6.0 ± 0.088a
Prepupa	1.0 ± 0.048a	1.1 ± 0.084a
Pupa	2.6 ± 0.043a	2.6 ± 0.070a
Egg to adult	13.1 ± 0.086a	13.2 ± 0.136a

[a] Means ± standard error of mean (SEM) ($P < 0.05$ *t* test); means followed by the same letter within a row are not significantly different.
[b] Acquisition access period for L1 (age 0-4 h) was 6 h on TSWV-infected *D. stramonium* plants.
[c] Acquisition access period for L1 (age 0-4 h) was 6 h on healthy *D. stramonium* plants.
[d] Development time of the 2 larval stages together.

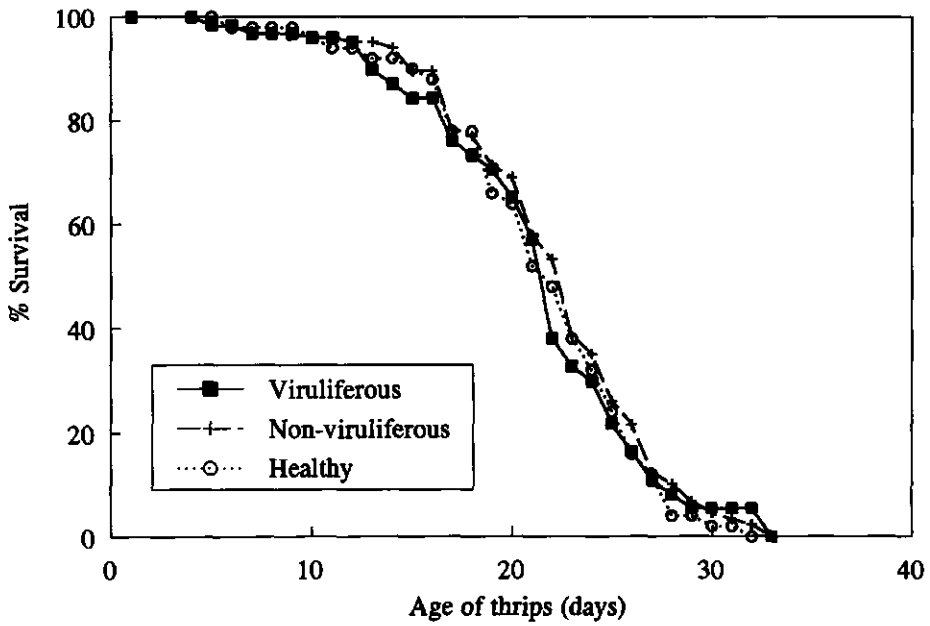


Figure 1. Survival curves of viruliferous, non-viruliferous and control (non-exposed) thrips. After the acquisition access period of 6 h on TSWV-infected *Datura stramonium* leaves (control; healthy *D. stramonium* leaves), juvenile stages were confined to healthy *D. stramonium* leaves and adults were transferred daily to fresh petunia leaf disks.

procedures described by Southwood (1978) and Price (1984). The columns included in the life-fertility tables were: x, pivotal age (number of days at beginning of each age class); l_x, number surviving at beginning of age class x; d_x, number dying in age interval x; q_x, the apparent mortality during age class x ($q_x = d_x/l_x$); r_x, the real mortality at age interval x ($r_x = d_x/l_0$); L_x, the survivorship of age class x ($L_x = l_x/l_0$); and m_x, the number of female offspring produced per female in each age class x. The proportion of females in the progeny population of mated females from the stock culture was found to be 0.57 (female/[female + male]) and was used in the calculation of m_x.

From the life-fertility tables, the following population reproductive statistics were calculated: net reproductive rate ($R_0 = \Sigma L_x m_x$); intrinsic rate of increase (r_m, $1 = \Sigma e^{(-r m^x)} L_x m_x$); and mean generation time ($T = (\ln R_0)/r_m$).

Vertical transmission

To study whether viruliferous females could transmit virus to their offspring, adult females were allowed to oviposit on parafilm as described by Murai and Ishii (1982) with slight alterations of the method. Females were placed for 2 days inside perspex cylinders (15 mm x 30 mm diameter). Pollen was placed inside the cylinder and a 10% sucrose solution as oviposition substrate was supplied between two layers of stretched parafilm at one end of the cylinder. The other end of the cylinder was sealed with parafilm. Eggs were collected after deposition and placed on a piece of humid Whatman paper (10 x 10 mm) which was transferred to a healthy *D. stramonium* leaf in a leaf cage (Tashiro, 1967). This allowed emerging larvae to have access to healthy leaf tissue. Egg mortality was recorded. Larvae were tested 4 days after emergence in an IAP of 24 h for virus transmission and again as adults in 3 subsequent IAPs of 24 h on petunia leaf disks as described before. After the final IAP, individual adults were collected and frozen at -70 °C until assayed by ELISA for the presence of viral antigen. The ELISA reaction was followed by an amplification of the enzyme reaction as described previously (Wijkamp *et al.*, 1993) with one modification where individual thrips were ground in 80 μl of sample buffer (2% polyvinylpyrrolidone, M_r about 44,000, and 0.2% ovalbumin in PBS-T) and mixed with 20 μl of 2.5 μg/ml anti-nucleocapsid (N) conjugate in sample buffer. This suspension was incubated overnight at 4 °C. Antiserum directed against the N protein of the TSWV isolate BR-01 was used to detect the N protein of TSWV in thrips.

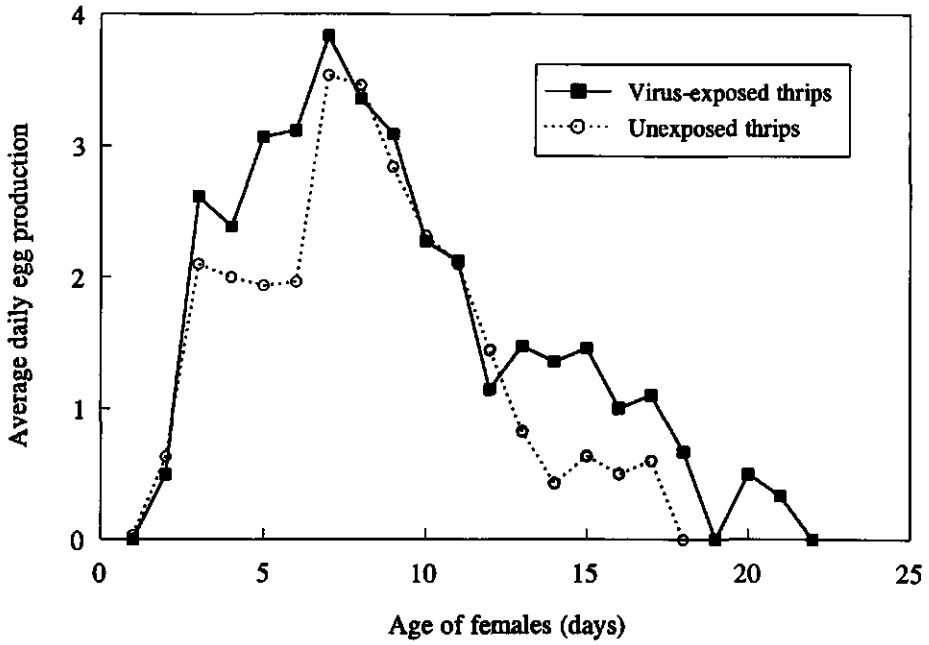


Figure 2. Mean number of larvae emerging from eggs laid by female *Frankliniella occidentalis* on petunia leaf disks.

Table 2. Reproductive parameters for *Frankliniella occidentalis* at 25 °C. Acquisition access period was 6 h on TSWV-infected *Datura stramonium* in the larval stage. Adult females were transferred daily to fresh petunia leaf disks.

	n	Fecundity[a] (larvae/female)	Female longevity (days)
Exposed to TSWV			
Total[b]	62	28.3 ± 1.8a[c]	13.4 ± 0.5a
viruliferous	36	30.9 ± 2.3	14.3 ± 0.6
non-viruliferous	26	24.7 ± 2.4	12.1 ± 0.7
Not exposed[d]	30	22.3 ± 2.3a	12.5 ± 0.7a

[a] Expressed as number of larvae eclosed per female.

[b] TSWV-exposed thrips (total) could be divided in two cohorts: females that transmitted virus during the experiment (viruliferous) and females that did not (non-viruliferous).

[c] Means ± standard error of mean (SEM) ($P < 0.05$ *t* test); means followed by the same letter within a column are not significantly different.

[d] Females exposed to healthy *D. stramonium* plants.

RESULTS

Development of thrips

The effect of exposure of larvae to TSWV on the development of thrips was compared to that of non-exposed thrips. Larvae, 0-4 h after emergence, were exposed to virus-infected leaves for 6 h and subsequently transferred to healthy leaves of *D. stramonium*. Observations were made every 24 h. The time required for each developmental stage was recorded. However, the period of development for the two larval stages was taken at the same time, because of difficulties to discriminate between old L1 and young L2 larvae. Mean development time from egg to adult did not differ between thrips exposed or unexposed to virus. Individuals became adults in 13.1 and 13.2 days, respectively (Table 1). Pre-adult mortality was also recorded and was low for both groups, with 4% and 6% found for exposed and non-exposed thrips, respectively.

After becoming adults, the thrips were transferred daily to petunia leaf disks to record mortality and virus transmission. Out of 121 thrips exposed to TSWV in the first 6 h of their larval life, 84 were able to transmit virus to petunia leaf disks. The almost identical developmental rates of the larval stages indicated that the replication of virus did not affect the development of the thrips. The TSWV-exposed thrips were further classified as either viruliferous or non-viruliferous. Survivor curves for viruliferous, non-viruliferous and healthy control thrips were constructed (Figure 1). The results demonstrated that the mortality and life span did not differ between infected and non-infected thrips.

Reproduction

To analyze the reproduction of females, either exposed or non-exposed to TSWV, individuals were transferred daily to fresh leaf disks of petunia. Their longevity and the number of larvae produced by individuals per day were recorded. The mean daily offspring production, measured as the number of emerging larvae per female per day, is presented in Figure 2. A pre-oviposition period of one day was observed in which no eggs were deposited by the females. The highest daily egg production was reached 7 days after emergence, at which time each female from either the virus-exposed or non-exposed group, produced an average of 3.8 or 3.5 eggs per day, respectively. After day 7, egg production decreased gradually. Females exposed to TSWV produced an average of 28.3 larvae per

Table 3. Life-fertility tables for *Frankliniella occidentalis* at 25 °C exposed and non-exposed for 6 h to TSWV in the larval stage. Juvenile stages were confined to *Datura stramonium* leaves and adults were transferred daily to fresh petunia leaf disks. See materials and methods for explanation of codes.

a. Exposed to TSWV.

x	l_x	d_x	q_x	r_x	L_x	m_x
0	65	0	0.000	0.000	1.000	-
4	65	0	0.000	0.000	1.000	-
7	65	1	0.015	0.015	1.000	-
8	64	0	0.000	0.000	0.985	-
9	64	1	0.016	0.015	0.985	-
10	63	0	0.000	0.000	0.969	-
11	63	1	0.016	0.015	0.969	-
12	62	0	0.000	0.000	0.954	-
13	62	0	0.000	0.000	0.954	-
14	62	0	0.000	0.000	0.954	0.219
15	62	0	0.000	0.000	0.954	0.752
16	62	2	0.032	0.031	0.954	1.416
17	60	0	0.000	0.000	0.923	1.287
18	60	0	0.000	0.000	0.923	1.302
19	60	0	0.000	0.000	0.923	2.309
20	60	2	0.033	0.031	0.923	2.033
21	58	1	0.017	0.015	0.892	2.318
22	57	4	0.070	0.062	0.877	1.179
23	53	5	0.094	0.077	0.815	1.540
24	48	11	0.229	0.169	0.738	0.796
25	37	2	0.054	0.031	0.569	0.439
26	35	4	0.114	0.062	0.538	0.724
27	31	9	0.290	0.138	0.477	1.010
28	22	6	0.273	0.092	0.338	0.294
29	16	4	0.250	0.062	0.246	0.570
30	12	3	0.250	0.046	0.185	0.392
31	9	4	0.444	0.062	0.138	0.190
32	5	2	0.400	0.031	0.077	0.127
33	3	0	0.000	0.000	0.046	0.228
34	3	1	0.333	0.015	0.046	0.190
35	2	2	1.000	0.031	0.031	0.000

Table 3. Continued.

b. Not exposed to TSWV.

x	l_x	d_x	q_x	r_x	L_x	m_x
0	32	0	0.000	0.000	1.000	-
4	32	0	0.000	0.000	1.000	-
7	32	0	0.000	0.000	1.000	-
8	32	0	0.000	0.000	1.000	-
9	32	1	0.031	0.031	1.000	-
10	31	0	0.000	0.000	0.969	-
11	31	0	0.000	0.000	0.969	-
12	31	1	0.032	0.031	0.969	-
13	30	0	0.000	0.000	0.938	-
14	30	0	0.000	0.000	0.938	0.190
15	30	0	0.000	0.000	0.938	0.432
16	30	0	0.000	0.000	0.938	0.893
17	30	0	0.000	0.000	0.938	0.798
18	30	1	0.033	0.031	0.938	1.311
19	29	2	0.069	0.063	0.906	1.592
20	27	0	0.000	0.000	0.844	2.174
21	27	2	0.074	0.063	0.844	2.132
22	25	1	0.040	0.031	0.781	1.368
23	24	3	0.125	0.094	0.750	1.520
24	21	2	0.095	0.063	0.656	0.760
25	19	3	0.158	0.094	0.594	0.720
26	16	3	0.188	0.094	0.500	0.356
27	13	2	0.154	0.063	0.406	0.351
28	11	4	0.364	0.125	0.344	0.155
29	7	2	0.286	0.063	0.219	0.000
30	5	4	0.800	0.125	0.156	0.000
31	1	0	0.000	0.000	0.031	0.000
32	1	1	1.000	0.031	0.031	0.000

female (Table 2). A subdivision of TSWV-exposed thrips in viruliferous and non-viruliferous thrips showed that viruliferous thrips produced an average of 30.9 larvae per female, whereas non-viruliferous and non-exposed thrips produced 24.7 and 22.3 larvae, respectively. These results showed that exposed thrips produced more offspring than non-

Table 4. Population reproductive statistics for *Frankliniella occidentalis* at 25 °C exposed and non-exposed to TSWV. Acquisition access period in the first larval stage was 6 h on TSWV-infected *Datura stramonium*. Juvenile stages were confined to *Datura stramonium* leaves and adults were transferred daily to fresh petunia leaf disks.

Thrips	*n*	R_0	r_m	T
Exposed to TSWV				
Total[a]	65	15.11	0.140	19.44
viruliferous	38	16.72	0.142	19.88
non-viruliferous	27	13.63	0.135	19.37
Not exposed[b]	32	11.98	0.127	19.63

R_0 = net reproductive rate; r_m = intrinsic rate of increase; T = mean generation time

[a] TSWV-exposed thrips (total) were divided in two cohorts: females that transmitted virus during the experiment (viruliferous) and females that did not (non-viruliferous).

[b] Females exposed to healthy *D. stramonium*.

exposed ones. However, these values were not significantly different ($P < 0.05$). No significant difference in longevity of females could be observed either (Table 2).

Life-fertility tables and population reproductive statistics

The population reproductive statistics (Table 4) were calculated from the life-fertility data (Table 3). Mortality of eggs which were oviposited in *D. stramonium* leaf disk could not be recorded and was, therefore, assumed to be 0. This assumption seemed valid since results from the experiments on vertical transmission of virus to offspring showed that eggs deposited in 10% sucrose solution between parafilm membranes displayed a low mortality between 0 and 5%. The life table parameters revealed that the net reproductive rate (R_0) and the intrinsic rate of increase (r_m) were 15.11 and 0.140, respectively, for thrips exposed to virus. These values were higher than those for thrips not exposed to virus, being 11.98 for R_0 and 0.127 for r_m. Mean generation time (T) was identical for exposed and non-exposed thrips.

Virus retention in adults

Besides life parameters of the thrips, the retention of the virus in the thrips was studied. Adults were serially transferred to leaf disks of petunia for IAPs of 24 h until they died. The virus transmission rate obtained in each subsequent IAP and the number of surviving

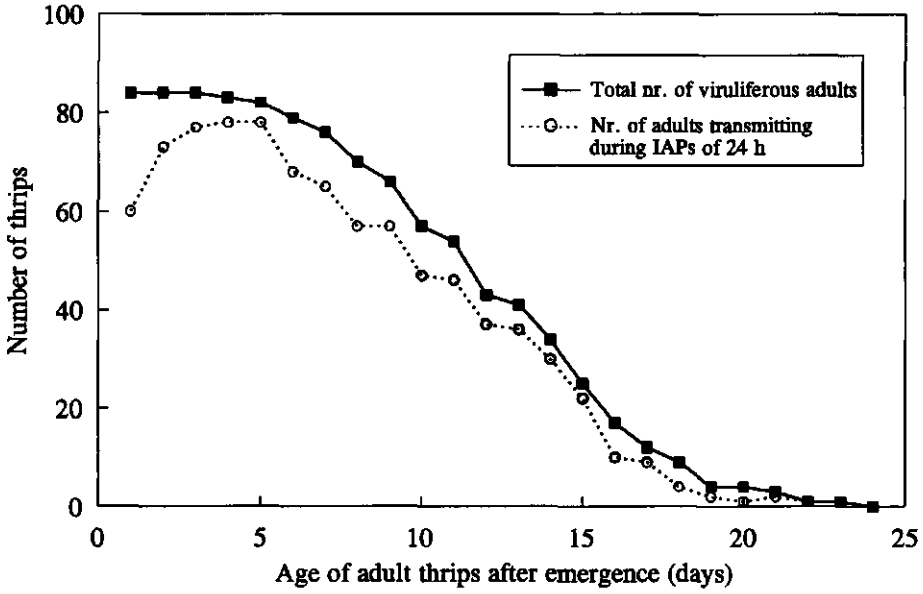


Figure 3. Number of viruliferous adult *Frankliniella occidentalis* transmitting virus during subsequent inoculation access periods of 24 h to petunia leaf disks.

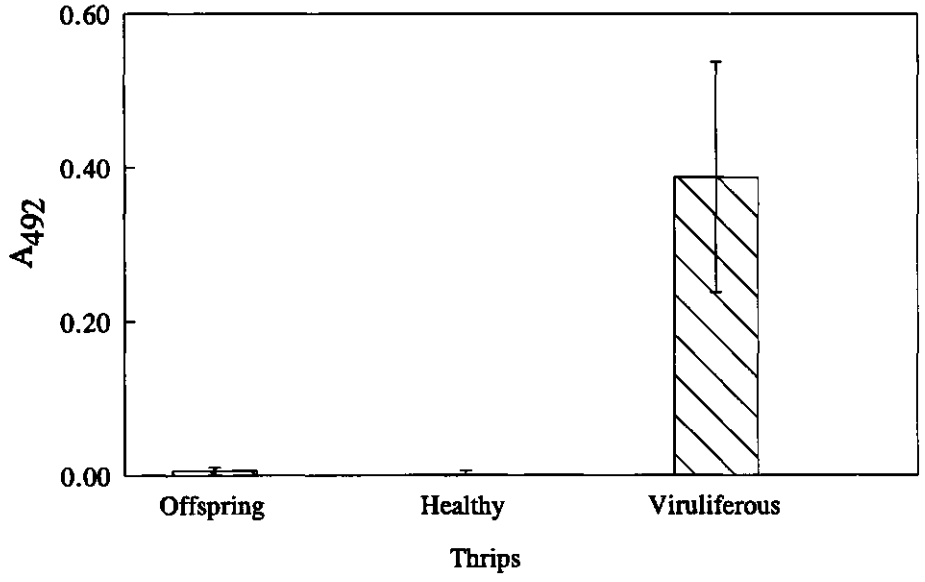


Figure 4. Average ELISA values plus standard deviation for the offspring from viruliferous *Frankliniella occidentalis* females. This progeny was tested on petunia leaf disks to which no virus was transmitted. Each individual thrips was subsequently assayed for antigen content by ELISA. As a control, readings for viruliferous thrips are also given.

viruliferous thrips were recorded as a function of time (Figure 3). Except for the first day after emergence, the percentage of thrips which transmitted virus remained constant at an average of 79% while the thrips increased in age. At the first day after emergence, 60 out of 84 viruliferous adults (71%) transmitted virus. This lower percentage may be attributed to a lower feeding activity of newly emerged adults.

Vertical transmission of virus

To study transovarial transmission of virus to offspring, viruliferous females were allowed to oviposit on a 10% sucrose solution between two layers of parafilm. Eggs were collected on humid Whatman paper and the number of emerging larvae were counted. Egg mortality ranged from 0 to 5%. The progeny thrips were tested for virus transmission to petunia leaf disks. They were tested in one IAP of 24 h when they were second stage larvae and in three IAPs as adults. A total of 350 larvae and 325 adults were tested, but none of them transmitted virus.

These thrips were subsequently assayed individually for the presence of viral antigen by ELISA. Mean absorbance values were similar to those for healthy control thrips, indicating the absence of any detectable amount of viral antigen in offspring thrips (Figure 4). As a reference, viral antigen could be readily detected in viruliferous thrips.

DISCUSSION

The results presented in this chapter demonstrate that survival, developmental time and reproduction of *F. occidentalis* are not significantly affected by replication of TSWV in thrips. In previous publications in which the total juvenile life span was spent on virus-infected material and in which negative effects of the virus were reported (Robb, 1989; DeAngelis *et al.*, 1993), a deleterious effect of nutritional quality on vector physiology could not be ruled out. By exposing thrips larvae for a short acquisition access period to virus-infected leaves possible deleterious effects of TSWV-infected plant tissue on the physiology of the vector were reduced as much as possible. Applying an acquisition access period of 6 h the effect of virus multiplication in thrips could be analyzed, while limiting the possible detrimental effect of food from infected plants.

Since *F. occidentalis* is a polyphagous species, demographical parameters of this species

on various food sources have been established. Comparison of developmental times for this thrips species at 25 °C on peanut (Lowry *et al.*, 1992), green bean (Brødsgaard, 1994), chrysanthemum (Robb, 1989), radish (Bryan and Smith, 1956) and cucumber (Gaum *et al.*, 1994) revealed the influence of different plant hosts on developmental rate of *F. occidentalis*. Values found in this study using *D. stramonium* as host plant for immature stages and petunia for adults indicated that leaves of the two latter plant species are a suitable substrate for *F. occidentalis* development.

Data on *F. occidentalis* pre-adult mortality on different food sources show large variability. On cucumber leaves and green beans mortalities of 25% and 67% have been reported, respectively. These high mortality rates, however, were most likely due to the combined effect of high air humidity and infections by fungal pathogens (Teulon, 1992; Brødsgaard, 1994). In host preference studies using 5 different plant hosts of TSWV, including *D. stramonium*, a high immature mortality was found on all 5 plant species tested and reached a value of 44.5% at 25 °C for *D. stramonium* (Bautista and Mau, 1994). Despite this high mortality, which might have been the result of the experimental setup, the authors concluded that *D. stramonium* was the most suitable plant species as substrate for *F. occidentalis* development compared to the other 4 hosts included in their study. The low pre-mortality rates ranging from 4 to 6% found in the present study are comparable to the rates of less then 10% observed by Robb (1989) and Trichilo and Leigh (1988) using chrysanthemum and cotton, respectively.

Developmental rates and life-fertility budgets provide quantitative parameters to assist with describing and predicting thrips population dynamics. The intrinsic rate of increase (Table 4) for virus-exposed and non-exposed *F. occidentalis* corresponded well with values found by Trichilo and Leigh (1988), Mollema (1990) and Brødsgaard (1994). However, significantly higher values for R_0 and r_m are found when thrips are supplied with pollen which has a major impact on fertility (Trichilo and Leigh, 1988; Robb, 1989).

A high percentage of thrips (70%) exposed to TSWV-infected plants were able to transmit virus to petunia leaf disks demonstrating that *D. stramonium* is an excellent plant species for efficient virus acquisition by thrips. This weed species is a known TSWV reservoir host (Bautista and Mau, 1994). Present results suggest that this species may play an important role in the disease cycle of TSWV, acting as alternate host and primary infection source for both the virus and the thrips.

Virus transmission data obtained in present experiments show that thrips are able to

transmit virus at the same high level during their adult life until they die. Earlier studies, reviewed by Best (1968), demonstrate that the virus is retained for long periods in the adults of the vector *Thrips tabaci*, but that the ability to transmit can be lost several days before the insect dies. Results of Paliwal (1976) indicate that TSWV titers in *Frankliniella fusca* adults decrease after the thrips have been exposed for two weeks to a susceptible host. The ability to transmit virus until the thrips died, as found in the present experiments, may be due to the high sensitivity of our test system, but discrepancies with earlier studies could also be explained by differences in vector or virus isolates used.

Several studies indicate that transovarial transmission appears to be related to the virus taxonomic classification. Since the majority of the bunyaviruses tested are efficiently transovarially transmitted (Ammar, 1994; Turell, 1988), TSWV might also be transmitted to the offspring of infected thrips. Our present data, however, unequivocally demonstrated that TSWV could not be transovarially transmitted to the eggs.

In conclusion, TSWV infection did not to have any significant effect on the bionomics of *F. occidentalis*. The previously reported negative effects (Robb, 1989; DeAngelis *et al.*, 1993) may therefore be explained by the fact that in those studies the total larval life span was spent on virus-infected plant material. Since infected plant material may have a lower nutritional quality, the detrimental effects could therefore be due to physiological changes induced by food rather than by virus replication. However, under natural field or greenhouse conditions, a situation may arise were adverse effects of infected leaf material may play a role in development and fecundity of the vector and thereby influencing virus epidemiology. Furthermore, less favorable conditions like suboptimal environmental conditions or reduced nutritional quality of food substrate may reduce fitness of *F. occidentalis* resulting in more pronounced effects of TSWV infection upon thrips development and reproduction. Also we can not rule out the possibility that exposure of thrips to another tospovirus species, i.e. impatiens necrotic spot virus, may have a deleterious effect on life parameters (DeAngelis *et al.*, 1993). Significant pathogenicity of TSWV to its thrips vectors would have been of considerable interest. Such an effect could influence the abundance of viruliferous thrips in nature, thus, negatively affect the spread of the virus. A pathogenic effect of TSWV, however, would be a new phenomenon in the relation between *Bunyaviridae* and their vectors since pathological effects in their vectors have not been described for animal-infecting bunyaviruses.

Chapter 7

Distinct levels of specificity in thrips transmission of tospoviruses

SUMMARY

Various thrips species were tested for their capacity to transmit different tospovirus species using a petunia leaf disk assay system. Transmission efficiencies were determined for four species of thrips and four tospovirus species, i.e. tomato spotted wilt virus (TSWV), Impatiens necrotic spot virus (INSV), tomato chlorotic spot virus (TCSV) and groundnut ringspot virus (GRSV). *Frankliniella occidentalis* appeared to be the most efficient vector for the four tospovirus species tested. A dark form of *Frankliniella schultzei* transmitted three (TSWV, TCSV and GRSV) of the four tospoviruses, whereas a light form of this species transmitted TSWV and TCSV rather poorly. *Frankliniella intonsa*, which has been documented as vector of TSWV although transmission data were not presented, transmitted TSWV efficiently and TCSV at a very low frequency. Strikingly, only one out of four different populations of *Thrips tabaci* from different geographic origins was able to transmit solely TSWV and this at a low efficiency. ELISA analysis showed that virus could be readily detected in transmitting adult thrips. Viral antigen could also be detected in some individuals which did not transmit virus to petunia leaf disks but the amount of virus detected was consistently lower than those of transmitters. Positive ELISA values were only found for thrips-tospovirus combinations in which virus transmission could occur, whereas negative ELISA scores were observed for all individuals from thrips-virus combinations in which no virus transmission took place, indicating that acquisition of the virus did not result in replication and accumulation of these viruses in thrips.

This chapter will be published in a slightly modified version as: Ineke Wijkamp, Nuria Almarza, Rob Goldbach and Dick Peters (1995). Distinct levels of specificity in thrips transmission of tospoviruses. Phytopathology, in press.

INTRODUCTION

The genus *Tospovirus* within the family *Bunyaviridae* encompasses a group of viruses that cause devastating diseases of many economically important crops worldwide (Peters *et al.*, 1991; German *et al.*, 1992). These viruses are exclusively transmitted by thrips (Thysanoptera: Thripidae). Thus far seven thrips species have been reported as vectors of the tospoviruses. They are *Frankliniella occidentalis* Pergande (Gardner *et al.*, 1935), *F. fusca* Hinds (Sakimura, 1963), *F. schultzei* Trybom (Samuel *et al.*, 1930), *Thrips tabaci* Lindeman (Pittman, 1927), *T. setosus* Moulton (Kobatake *et al.*, 1984), *T. palmi* Karny (Yeh *et al.*, 1992) and *Scirtothrips dorsalis* Hood (Amin *et al.*, 1981). Tospoviruses, following ingestion by larvae, are transmitted by second larval instars and adults after circulation and replication in the vector (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993; Wijkamp and Peters, 1993).

Based on serological properties and nucleotide sequence data at least five tospovirus species have been distinguished. The established species include tomato spotted wilt virus (TSWV), Impatiens necrotic spot virus (INSV) (Law and Moyer, 1990; De Ávila *et al.*, 1992), tomato chlorotic spot virus (TCSV), groundnut ringspot virus (GRSV) (De Ávila *et al.*, 1993a) and watermelon silver mottle virus (Yeh and Chang, 1995). The S RNA of the latter shares an almost identical nucleotide sequence with the S RNA of an isolate referred to as Tospo-to (Adam *et al.*, 1993). Tospo-to is serologically related to groundnut bud necrosis virus (GBNV), a virus severely affecting the groundnut cultures in India and South-East Asia (Reddy *et al.*, 1992). The taxonomic status of other reported isolates such as peanut yellow spot virus (PYSV) (Reddy *et al.*, 1991a) and a virus from *Verbesina alternifolia* (Hayati *et al.*, 1990) have not been established thus far.

Diseases of varying magnitude have been recognized in different geographic regions and are believed to be caused by different tospoviruses and spread by different thrips species. *T. tabaci* is the main vector of TSWV in tobacco in eastern Europe, whereas this thrips species does not play a principal role in the epidemiology of TSWV in other areas (German *et al.*, 1992). The spread of INSV appears to be highly correlated with the worldwide expansion and occurrence of *F. occidentalis*. These and other observed correlations are suggestive of the existence of specific and favored relations between certain thrips and virus species.

To understand these relationships more thoroughly and to describe the epidemiology and spread of the tospoviruses quantitatively, factors such as vector competence, and transmission efficiency have to be known in more detail. Vector competence which lies at the base of the question whether a species can transmit has been the subject of some limited studies (Sakimura, 1962a; Paliwal, 1976; Fujisawa *et al.*, 1988). This chapter reports the results of a study on the competence of several populations of thrips species to transmit four different tospoviruses and the efficiencies by which these viruses are transmitted.

MATERIALS AND METHODS

Thrips

Virus-free *F. occidentalis, F. schultzei* and *F. intonsa* Trybom were reared on bean pods (*Phaseolus vulgaris* L. 'Prelude'). The *F. occidentalis* colony was started with adults collected from a greenhouse infestation in the Netherlands in 1990. A dark form of *F. schultzei* was obtained from a nursery which imported Cactaceae from Brazil (Vierbergen and Mantel, 1991). A light form of *F. schultzei* was collected from beans imported from Northern Africa. The *F. intonsa* culture used originated in Japan (Murai, 1990). Four different *T. tabaci* populations were included in the study; two of which were collected in the Netherlands from natural field populations and consisted of females only. A third, thelotokous, culture originated in Japan. These three cultures were reared on leek. A fourth, arrhenotokous, *T. tabaci* population also was obtained from Japan and produced males and females (Murai, 1990). This culture was reared on bean pods. All thrips were reared at 27 ($\pm$0.5) °C with a 16 h photoperiod (light/dark: 16/8 h).

Virus isolates

The TSWV isolate BR-01 and TCSV isolate BR-03, isolated from tomato in Brazil, the GRSV isolate SA-05 from groundnut in South Africa, and the INSV isolate NL-07 from an *Impatiens* sp. plant in the Netherlands, described before by De Ávila and co-workers (De Ávila *et al.*, 1993a, b) were used in the present study.

Virus detection by enzyme-linked immunosorbent assay (ELISA)

The antigen titer in leaf extracts from plants used as virus source for acquisition was

determined by ELISA. This assay was also used to confirm the infection of petunia leaf disks showing local lesions after inoculation. The extracts were prepared by grinding leaf tissue at a ratio of 15 mg per ml of 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). Leaf disks from healthy plants were used as controls.

A panel of two polyclonal and two monoclonal antisera was used to differentiate the four virus species. Polyclonal antisera, raised against the nucleocapsid (N) protein (anti-N serum) of the INSV isolate NL-07 and TCSV isolate BR-03 were used in a double antibody sandwich ELISA (DAS-ELISA) format as described previously (De Ávila *et al.*, 1990; Huguenot *et al.*, 1990). Monoclonal antibodies N1 and N2, prepared against the N protein of TSWV isolate BR-01 (formerly CNPH1) (Huguenot *et al.*, 1990), were used in a triple antibody sandwich ELISA (TAS-ELISA) as described by De Ávila and co-workers (1990). The discriminative reactions of the four tospovirus species are summarized in Table 1.

Individual thrips were analyzed by cocktail-ELISA, with amplification of the enzyme reaction, for their N protein content as described previously (Wijkamp *et al.*, 1993) with one modification; individual thrips were ground in 80 μl of sample buffer (2% polyvinylpyrrolidone (M_r about 44000) and 0.2% ovalbumin in PBS-T) and mixed with 20 μl of 2.5 μg/ml anti-N conjugate in sample buffer. The suspension was incubated overnight at 4 °C. Anti-N sera of TSWV isolate BR-01, INSV isolate NL-07, TCSV isolate BR-03 and GRSV isolate SA-05 were used to detect the N protein of the respective viruses in thrips. Absorbance values were read on a Titertek Multiskan colorimeter (Flow Laboratories) at 492 nm. Absorbance values were corrected for blank values read for wells that contained only sample buffer in the sample incubation step.

Selection of plant species to be used in virus acquisition studies

To quantify and compare transmission efficiencies of all tospoviruses tested it is desirable to use the same host plant for acquisition feeding by thrips. Source plants should both display high virus titers and be suitable for thrips feeding. Host plants which react with systemic symptoms upon virus infection and display high virus titers for the four tospoviruses tested are *Nicotiana benthamiana* Domin., *N. clevelandii* A. Gray, *Emilia sonchifolia* (L.) DC. and *Impatiens* sp. (De Ávila *et al.*, 1993b). The suitability of these plant species to support thrips development was tested by monitoring the performance of thrips. Larvae of *F. occidentalis*, *F. schultzei* (dark and light form) *F. intonsa* and *T. tabaci*

Table 1. Reaction of tospovirus species with a panel of antisera consisting of two monoclonal antibodies, directed to the nucleocapsid protein of TSWV (N1 and N2) and two polyclonal antisera directed to INSV and TCSV nucleocapsids, respectively.

Antisera	Tospovirus			
	TSWV	INSV	GRSV	TCSV
TSWV-N1	+	-	+	-
TSWV-N2	+	-	-	-
INSV	-	+	-	-
TCSV	±	-	±	+

-: no reaction (0-0.05), ±: weak to moderate reaction (0.05-0.5) and +: strong reaction (0.5-3.00) in ELISA. In parenthesis the absorbance values at 405 nm 1 h after substrate addition.

(4 populations), 0 to 12 h old, were confined to healthy leaves of these host plants. Their survival and developmental stage were checked every 24 h. *Datura stramonium* L. was included as a control since this species is an adequate host for thrips feeding and development.

Selection of plant species to be used in transmission experiments

To compare the suitability of different host plants in virus transmission tests, systemically infected leaves of *D. stramonium*, *Impatiens* and *N. benthamiana* were used for the acquisition of TSWV and *Impatiens* and *N. benthamiana* for INSV. *N. benthamiana* was included as a control for both viruses, because it displays high virus titers after inoculation by TSWV and INSV and the transmission efficiencies are high when *F. occidentalis* acquires virus from this host. *D. stramonium* and *N. benthamiana* plants were mechanically inoculated 2 to 3 weeks and *Impatiens* sp. 4 to 6 weeks prior to the experiments. The plants were kept in a greenhouse at approximately 22 °C with a 16 h photoperiod (light/dark: 16:8 h). Systemically infected leaves which showed high virus titers in a dilution series in ELISA were used for acquisition feeding.

F. occidentalis larvae 0 to 12 h old were given an acquisition access period (AAP) of 24 h on TSWV, or INSV, infected *D. stramonium*, *Impatiens* and *N. benthamiana* leaves in leaf cages (Tashiro, 1967). Larvae confined to healthy leaves served as controls. After the AAP, larvae were transferred to healthy *D. stramonium* leaves to complete their development. Adults, 1 day after emergence, were transferred to fresh leaf disks (diameter:

13 mm) of *Petunia x hybrida* 'Blue Magic' in a 1.5 ml Eppendorf tube for testing their infectivity in 3 successive inoculation access periods (IAPs) of 48 h. All experiments with thrips were performed at 25 (±0.5) °C with a 16 h photoperiod (light/dark: 16:8 h). After each IAP, the leaf disks were incubated at 27 °C in 24 well plates (Costar Europe Ltd., Badhoevedorp, the Netherlands), while floating on water for the development of local lesions as described previously (Wijkamp and Peters, 1993).

Comparison of acquisition by larvae of different thrips

A prerequisite for successful virus transmission is the availability of virus that can be acquired by the thrips larvae. In addition, as feeding preference or behavior of different thrips species may vary, it was verified whether larvae of all the species tested were able to ingest virus from infected plant tissue. Virus uptake by larvae of *F. occidentalis, F. schultzei* (light and dark forms), *F. intonsa* and *T. tabaci* (4 different populations) was recorded by establishing the virus titers in larvae after they had access to virus-infected leaves. Larvae (0-12 h old) were given an AAP of 12 h on TSWV-infected *D. stramonium* and *Impatiens* leaves in cages, collected directly after the AAP and stored at -70 °C for later testing in ELISA. An average of 20 larvae was tested for each thrips species. Larvae confined to healthy *D. stramonium* leaves served as controls.

Testing of transmission frequencies

Virus isolates were maintained in stock plants of *D. stramonium* (TSWV, TCSV and GRSV) and *Impatiens* (INSV) by thrips transmission using *F. occidentalis* as vector. Prior to transmission tests, each isolate was mechanically inoculated from the stock plants onto either *D. stramonium* (TSWV, TCSV and GRSV) or *Impatiens* sp. (INSV) as described previously. Systemically infected leaves with comparable high virus titers in a dilution series in ELISA were used for acquisition feeding. First instars (0-12 h old) were given an AAP of 72 h. First instars caged on virus-free leaves were used as controls. After the AAP, larvae were transferred to healthy *D. stramonium* leaves to complete their development. Adults, 1 day after emergence, were tested individually on petunia leaf disks as described before. Each virus-vector combination was tested 3 times, with a minimum of 30 adults per repeat. The numbers of infected leaf disks were converted to transmission rates. Adult thrips, after the IAPs on petunia, were collected and stored at -70 °C to be tested in ELISA in order to quantitatively compare virus transmission and virus content of

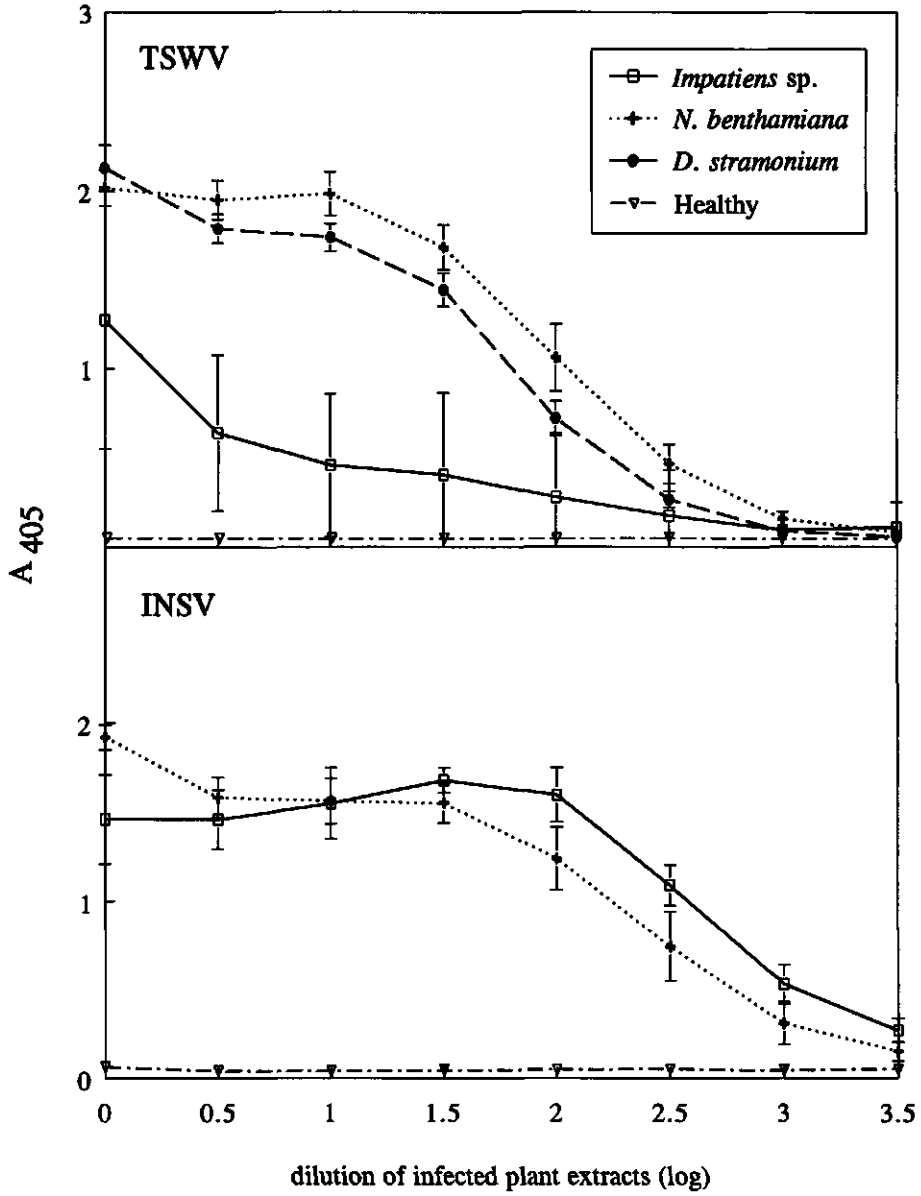


Figure 1. Average ELISA readings plus standard deviations of extracts from plant species used for acquisition feeding by *F. occidentalis*. Plants were infected with either TSWV or INSV and polyclonal antiserum against the nucleocapsid proteins of TSWV and INSV were used to detect the respective viruses. Leaf tissue of healthy *Impatiens*, *D. stramonium* and *N. benthamiana* (healthy) served as controls. Leaf tissue was ground in a ratio of 15 mg per ml PBS-T (starting dilution). The mean ELISA values and standard deviations are indicated in each bar.

adults. ELISA values for individual thrips were categorized in two classes based on their ELISA readings. Thrips which gave readings higher than the average readings from healthy control thrips plus 3 times standard deviation were considered to be positive, those with lower readings were negative.

RESULTS

Selection of plant species for virus acquisition

Distinct tospoviruses differ in symptom expression and host range and each thrips species shows different feeding preferences. Therefore, preliminary experiments were conducted to find an adequate host plant species on which the thrips could acquire the viruses to be tested. Healthy plants of *N. benthamiana*, *N. clevelandii*, *E. sonchifolia*, *Impatiens* sp. and *D. stramonium* were tested for suitability to support thrips development. All larvae of *F. occidentalis* and the dark form of *F. schultzei* survived on *N. benthamiana* until the adult stage. The mortality of juvenile stages of the other thrips species reached levels of 90 to 100% on this plant species. Similar results were obtained when thrips were kept on *N. clevelandii* and *E. sonchifolia*. *Impatiens* and *D. stramonium* were the only hosts on which no significant mortality of larvae of all thrips species was observed. These species were chosen as hosts for the acquisition of tospoviruses by thrips.

Impatiens is susceptible to all four virus species, but TCSV and GRSV do not spread uniformly throughout the plant. *D. stramonium* is infected systemically by TSWV, TCSV and GRSV, but not by INSV. Based on these findings *Impatiens* sp. was chosen as acquisition host for INSV; in this plant INSV is evenly distributed throughout the plant. *D. stramonium* was selected for the acquisition of TSWV, TCSV and GRSV as these three viruses display high virus titers, as can be concluded from dilution endpoints in ELISA, and the development of nonnecrotic symptoms in this plant species.

Comparison of different host plants in transmission experiments

The performance of *D. stramonium*, *Impatiens* sp. and *N. benthamiana* as hosts for virus acquisition by *F. occidentalis* was studied in a preliminary transmission experiment. TSWV was acquired from *D. stramonium*, *Impatiens* and *N. benthamiana*; INSV from the latter two species. To compare the amount of viral antigen present in leaves for acquisition

Table 2. Comparison of transmission efficiency of TSWV and INSV by *F. occidentalis* using different plant species as acquisition hosts. Acquisition access period was 24 h on systemically infected leaves (Figure 1). Transmission by adults was tested on leaf disks of petunia.

Tospovirus	Acquisition host	n[a]	Transmission (%)
TSWV	*Impatiens* sp.	20	35.0
	D. stramonium	24	33.3
	N. benthamiana	23	30.4
INSV	*Impatiens* sp.	25	84.0
	N. benthamiana	24	91.7

[a] Number of thrips tested per combination in one experiment. Thrips that were confined to non-infected leaves of *Impatiens* sp., *D. stramonium* and *N. benthamiana* as larvae, did not transmit virus.

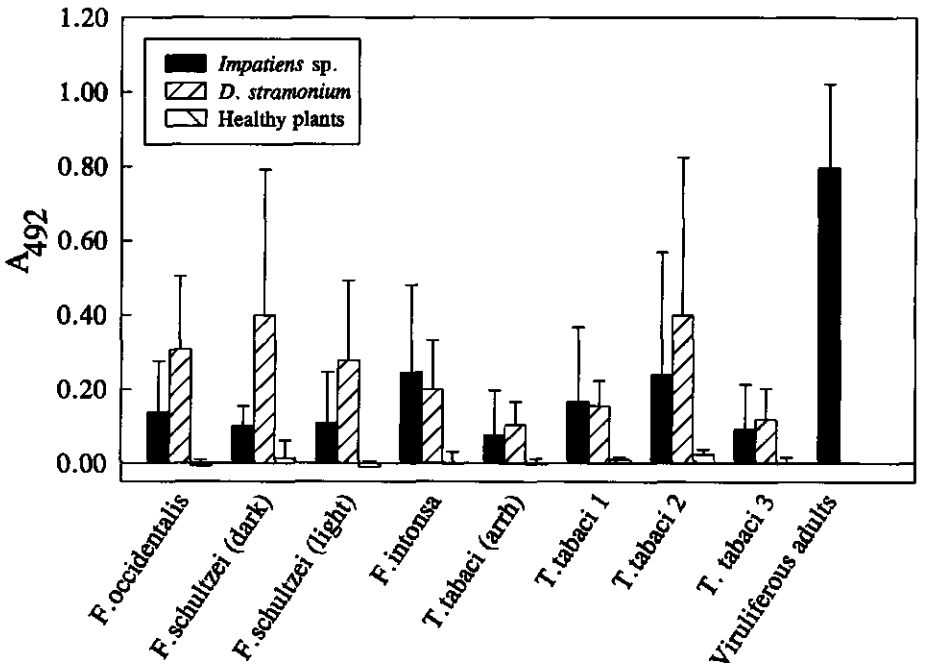


Figure 2. Acquisition of TSWV by larvae of different thrips species after an acquisition access period on leaves of infected *Impatiens* sp. or *D. stramonium*. Larvae of each species were also confined to healthy leaves of *D. stramonium* (healthy plants). *T. tabaci* (arrh) represents an arrhenotokous population whereas *T. tabaci* 1, 2 and 3 represent 3 different thelotokous populations; one thrips culture from Japan (1) and two from the Netherlands (2 and 3). Larvae were sampled 12 h after the start of AAP. The mean ELISA values and standard deviations obtained for larvae which were singly tested are given.

feeding, dilution series of plant tissue in PBS-T were analyzed by ELISA (Figure 1). At lower dilutions, ELISA values for TSWV in *N. benthamiana* and *D. stramonium* were higher than in *Impatiens*, but virus titers were comparable as can be concluded from dilution endpoints which were very similar at around 3 log. INSV titers in *N. benthamiana* and *Impatiens* sp. were comparable, dilution endpoints were approximately 3.5 log.

F. occidentalis larvae were confined for 24 h on infected leaves and subsequently adults were tested for virus transmission to petunia leaf disks (Table 2). Transmission efficiencies ranged from 30.4 to 35.0% for TSWV and from 84.0 to 91.7% for INSV, demonstrating that *D. stramonium*, *Impatiens* and *N. benthamiana* were equally suited for virus acquisition by *F. occidentalis*.

Acquisition of viral antigen by larvae of different thrips

The quantity of virus available for acquisition by thrips larvae was measured by allowing larvae an AAP of 12 h on leaves of *D. stramonium* and *Impatiens* sp. The amount of viral antigen present in larvae directly after the AAP was monitored by ELISA. The average ELISA values and standard deviations calculated for each thrips species which had fed on *Impatiens* sp. or *D. stramonium* are presented in Figure 2. The results show that one day old larvae of all thrips species acquired viral antigen from both *Impatiens* sp. and *D. stramonium*, although the amount of virus ingested per individual varies dramatically as indicated by the high standard deviations.

Testing of transmission frequencies

Larvae (0-12 h old) which were confined for an acquisition access period of 72 h on infected leaves in cages, were tested in the adult stage on petunia leaf disks for the efficiency by which the virus was transmitted (Table 3). The percentage of viruliferous thrips for each thrips-virus combination was calculated after three IAPs of 48 h. *F. occidentalis* was the only species which could transmit all four virus species tested. TSWV and INSV were very efficiently transmitted to petunia leaf disks reaching efficiencies of 66.0 and 85.4%, respectively. TCSV and GRSV were less efficiently transmitted by *F. occidentalis*, in ratios of 27.6% and 10.2%, respectively. The dark form of *F. schultzei* could transmit three (TSWV, TCSV and GRSV) out of the four tospoviruses tested. This vector transmitted TCSV at the highest rate (37.5%), while GRSV and TSWV transmission was lower (15.7% and 13.7%, respectively). The light form of *F. schultzei* appeared to be

Table 3. Efficiency of tospovirus transmission by several thrips species. Acquisition access period was 72 h on systemically infected *D. stramonium* (TSWV, TCSV and GRSV) and *Impatiens* sp. (INSV) leaves. Adult were tested individually on leaf disks of petunia for virus transmission.

Thrips species[a]	Tospovirus species			
	TSWV	TCSV	GRSV	INSV
F. occidentalis[b]	66.0 ± 0.8 (140)	27.6 ± 2.7 (109)	10.2 ± 5.8 (99)	85.4 ± 5.8 (83)
F. schultzei (dark)[c]	13.7 ± 2.3 (168)	37.5 ± 0.4 (157)	15.7 ± 2.0 (174)	0 (179)
F. schultzei (light)[d]	2.3 ± 1.3 (161)	5.9 ± 4.8 (123)	0 (95)	0 (>200)
F. intonsa	31.8 ± 8.1 (103)	0.7 ± 0.5 (130)	0 (115)	0 (>200)
T. tabaci (arrhenotokous)[e]	9.8 ± 3.3 (178)	0 (132)	0 (117)	0 (119)
T. tabaci (thelotokous)[f]	0 (>200)	0 (>200)	0 (>200)	0 (>200)

[a] Transmission efficiency represents the mean percentage ± standard error of infected petunia leaf disks for three experiments. The number of insects tested per combination are indicated in parenthesis; sum of three replications. Thrips that were confined to non-infected leaves of *Impatiens* sp. and *D. stramonium* as larvae did not transmit virus.

[b] The combination BR-01 and *F. occidentalis* was tested 4 times.

[c] A dark form of *F. schultzei*, consisting of males and females.

[d] A light form of *F. schultzei*, consisting of females only.

[e] *T. tabaci* colony producing males and females (arrhenotoky).

[f] *T. tabaci* colony producing females only (thelytoky); results of 3 different populations consisting of females only.

a rather inefficient transmitter of TSWV (2.3%) and TCSV (5.9%), while transmission experiments with GRSV and INSV were negative. *F. intonsa* efficiently transmitted TSWV (31.8%), whereas TCSV transmission was poor (0.7%). For *T. tabaci*, only the arrhenotokous form of *T. tabaci* could transmit TSWV (9.8%), while the three different thelotokous populations of this species were not able to transmit the four tospoviruses tested.

Antigen titer in viruliferous and non-viruliferous thrips

The adults which were tested on petunia leaf disks for virus transmission, were individually ground in sample buffer to assay the amount of antigen by ELISA. Results of virus transmission to leaf disks were correlated to readings in ELISA (Figure 3). Thrips could be categorized in three classes on basis of transmission and ELISA readings. Thrips which did not transmit virus to petunia leaf disks, were divided in two classes based on their ELISA readings. The first class of thrips did not transmit virus and ELISA values were comparable to those found for healthy thrips (petunia negative; ELISA negative). In

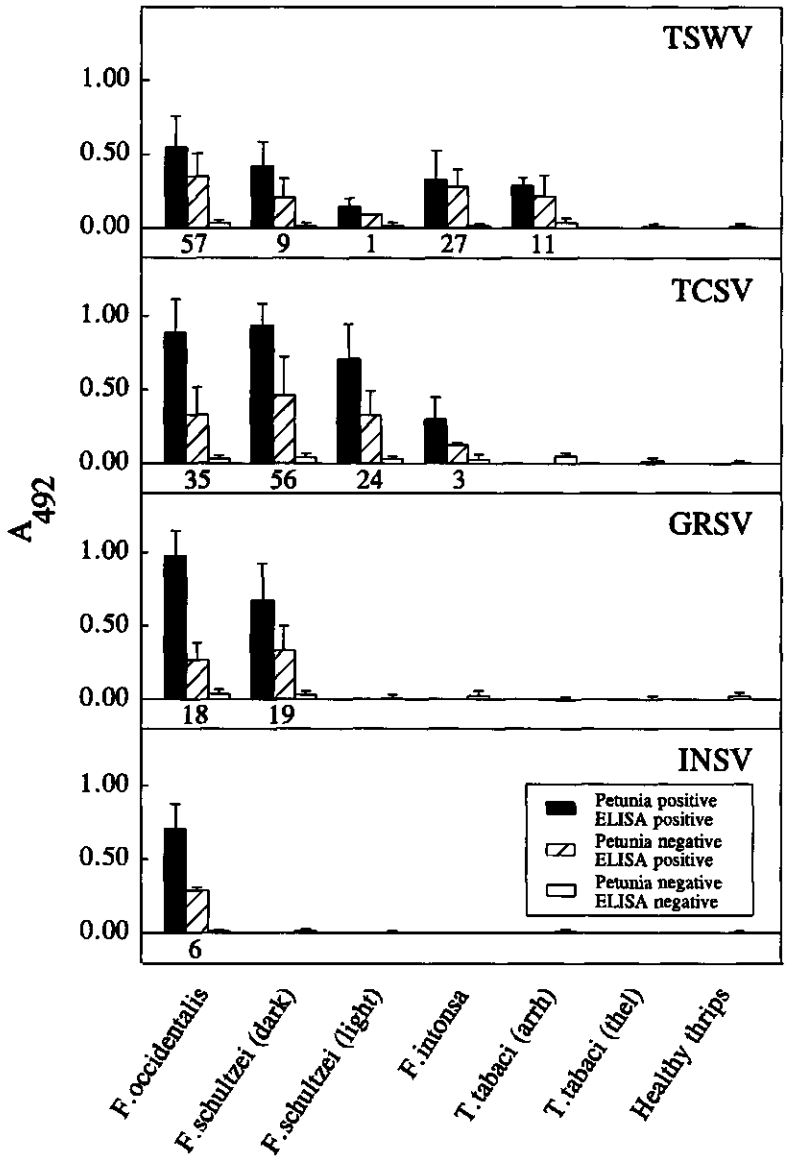


Figure 3. Average ELISA values plus standard deviation for different tospovirus-thrips combinations. Larvae were given an AAP of 72 h on virus-infected leaves. Transmission by adults was tested on petunia leaf disks. Individual adults were assayed for antigen content by ELISA. Thrips were categorized in three classes based on their ability to transmit virus to leaf disks of petunia and their readings in ELISA. The minimum threshold values for positive thrips in ELISA consisted of healthy mean plus 3 times standard deviation; all readings above this threshold were considered positive in ELISA, readings below this value were considered negative. Values below bars represent the percentage of non-transmitter thrips which scored positive in ELISA.

the second class, viral antigen was detected in individuals which did not transmit virus to petunia after they were given access to virus-infected leaf tissue in the larval stages (petunia negative; ELISA positive), indicating that virus replication occurred, but virus titers were lower than those of transmitting thrips. Values below bars in Figure 3 represent the percentage of non-transmitter thrips which scored positive in ELISA. The third class consisted of thrips which transmitted virus to petunia leaf disks and viral antigen could readily be detected by ELISA (petunia positive; ELISA positive).

The experiments with 4 virus species and 6 thrips cultures resulted in 24 possible combinations of tospovirus-thrips species. In each of the 24 combinations in which virus transmission occurred, ELISA values of thrips were either positive or negative, whereas in combinations in which no virus transmission took place, ELISA reading for individual adults were always negative which shows that acquisition of virus from infected plants by these species does not result in replication and accumulation of virus in thrips.

DISCUSSION

For a reliable comparison of the transmission efficiency of different tospovirus isolates, experiments have to be performed using a plant species which will react systemically upon virus infection with all virus isolates, and support development and feeding of all thrips species tested. No single plant species was found which could meet all requirements, we therefore chose *D. stramonium* as a host from which the thrips could acquire TSWV, TCSV and GRSV, while *Impatiens* sp. was used for INSV.

It was found that *F. occidentalis* was the only species able to transmit the four tospoviruses included in this study. Efficient transmission of TSWV by *F. occidentalis* has been reported previously (Sakimura, 1962a; Paliwal, 1976; Allen and Broadbent, 1986; Wijkamp and Peters, 1993). *F. occidentalis* is the only thrips species that so far has been tested for INSV transmission (Wijkamp and Peters, 1993; DeAngelis *et al.*, 1994). The current results demonstrate that the other thrips species tested are not able to transmit INSV and apparently are unable to acquire and replicate virus as can be concluded from the absence of detectable amounts of virus in adults as shown by ELISA. The high efficiency of INSV transmission by *F. occidentalis* explains the rapid spread of INSV in the early 90s in greenhouses in North America and Europe, and in the open in more subtropical climate

regions, where this thrips species is presently the predominant tospovirus vector.

So far, the spread of TCSV and GRSV seems to be restricted to the (sub)tropics as they have only been found in Brazil, South Africa (De Ávila *et al.*, 1990) and Argentine (Dewey *et al.*, 1993). The natural thrips vectors for TCSV and GRSV are not known. Our results show that *F. occidentalis* is a vector for these two tospoviruses, but the transmission by the dark form of *F. schultzei* is the most efficient. TCSV as well as some GRSV isolates originate in Brazil where these viruses are frequently found in tomato crops (Nagata *et al.*, 1995). *F. schultzei* is a common pest in the tropics (Vierbergen and Mantel, 1991) and might play an important role in the epidemiology of these viruses in Brazil. *F. schultzei* and *F. occidentalis* are both reported from South Africa and might contribute to the epidemiology of GRSV in that region.

The light form of *F. schultzei* appears to be an inefficient vector of TSWV and TCSV (Table 3). This conclusion confirms previous results of Sakimura (1969) who could not demonstrate virus transmission by a pale form of *F. schultzei*. Some non-transmitting *F. schultzei* were found to exhibit higher titers in ELISA than healthy thrips (Figure 3), suggesting that after acquisition some replication of virus may occur, though not resulting in sufficient amount to achieve virus transmission. Alternatively, the virus may not reach the proper tissues for successful transmission (e.g. the salivary gland).

Several earlier reports indicate that *T. tabaci* has been one of the most important TSWV vectors (Linford, 1932; Sakimura, 1940; Sakimura, 1962a, b; Sakimura, 1963). Efficient transmission by this vector has also been reported for some specific cases like transmission of a tospovirus from dahlia in Japan (Fujisawa *et al.*, 1988) and TSWV in Finland (Lemmetty and Lindqvist, 1993). However, other studies failed to show transmission of this virus by *T. tabaci* (Paliwal, 1974, 1976; Mau *et al.*, 1991; German *et al.*, 1992). A reason for the failure of *T. tabaci* populations to transmit virus might have been that the adults were tested before the latent period (LP) was completed. Sakimura (1963) reported a LP of 18 days for *T. tabaci*. In the present experiments however, individuals of non-transmitting *T. tabaci* populations, which were tested 18 to 20 days after start of the AAP, exhibited ELISA values comparable to those found in healthy thrips, indicating that no virus accumulation or replication had taken place.

Incompatibility between virus isolate and thrips species, each originating in different locations, could be another reason for the observed non-transmissibility. This phenomenon was previously suggested by Paliwal (1976). A third possibility is that races or ecotypes of

vector species exist, which can not transmit the virus. In Poland some races of *T. tabaci* were able to transmit TSWV while others did not (Zawirska, 1976). Failure to transmit the virus in Poland has been correlated with the absence of males in local *T. tabaci* populations. Populations consisting of both males and females, apparently did transmit TSWV, whereas populations consisting of only females did not (Zawirska, 1976; Peters *et al.*, 1991). Furthermore, it has been claimed that the mediterranean ecotypes of *T. tabaci* are unable to vector TSWV isolates (Nikouka, 1977; Lacasa, 1990). Our results seem to confirm the presence of different types of *T. tabaci* which exhibit different transmission characteristics. Of the four different populations tested, only one was able to transmit virus. Strikingly, this population consisted of females and males, while the other, non-transmitting populations did not produce males. However, further testing is required to correlate virus transmission with the presence of males in *T. tabaci* populations.

The results in this chapter indicate that *F. intonsa* can potentially act as a vector of tospoviruses. This species is restricted to the Northern hemisphere, from eastern Asia to Europe and in one region of North America. In one report this species was documented as a vector but transmission data were not yet available (Umeya *et al.*, 1988). The results presented here definitely indicate that *F. intonsa* can transmit tospoviruses and thus may contribute to the natural spread of TSWV. In Europe *F. intonsa* is a flower thrips, mainly feeding on pollen, and hence may play only a limited role in the epidemiology of TSWV. In Japan, however, *F. intonsa* is a pest on vegetables and ornamentals and may be important as a vector of tospovirus species present in Japan, in addition to *T. setosus*, *T. tabaci* and *T. palmi* whose status as vectors has been previously confirmed (Kobatake *et al.*, 1984; Fujisawa *et al.*, 1988).

From the results presented it is evident that specificity in transmission, which has been found for other plant viruses and their vectors, does also exist between tospoviruses and their thrips vectors. In different geographical areas the three components of virus epidemiology, i.e. the plant species, the virus isolate, and the virus vector species, should therefore be well characterized to establish the relative importance of each vector species in a particular area. New variants of tospoviruses are usually characterized rapidly but information concerning the vector's role in the epidemiology of the virus is usually less clear or even lacking.

Chapter 8

Studies on thrips transmission of defective tospovirus mutants

SUMMARY

The ability of the Western flower thrips, *Frankliniella occidentalis*, to transmit two types of tospovirus mutants, i.e. envelope-deficient (*env*$^-$) mutants and defective interfering RNA (*di*) mutants, was investigated. After larvae were given access for three days to infected plants, the transmission by adults of the mutants and the wild type (*wt*) virus to *Petunia x hybrida* leaf disks was tested. *F. occidentalis* was unable to transmit *env*$^-$ lines from impatiens necrotic spot virus (INSV) and tomato spotted wilt virus (TSWV). Time course analysis by ELISA of larvae which were placed on plants infected with either a *wt* or an *env*$^-$ line of INSV showed that the *env*$^-$ line was not retained and acquired by larvae, whereas the *wt* virus persisted and subsequently replicated in thrips tissues. In contrast, two different *di* lines of TSWV were successfully transmitted though at a rate 10 to 20 times lower than that of *wt* virus. The efficiencies by which *di* mutants were transmitted could be increased by prior acquisition of *wt* virus, acting as helper.

INTRODUCTION

In nature, tospoviruses are exclusively transmitted by a limited number of thrips species (Thysanoptera; Thripidae). Propagation of these viruses in the thrips vector seems to be a prerequisite for transmission (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993; Wijkamp *et al.*, 1995a). Experimentally, it has been common practice to maintain tospoviruses by mechanical inoculation. These inoculations, however, can result in the generation of two types of mutants (Ie, 1982; Resende *et al.*, 1991b; 1992; 1993). Firstly, envelope-deficient (*env*$^-$) mutants arise most likely by accumulation of point mutations or small deletions in the M RNA (Resende *et al.*, 1991b; 1993). Electron microscopic analysis of these mutants in infected plant tissue show the absence of the characteristic, enveloped tospovirus particles but the presence of non-enveloped nucleocapsid aggregates. Secondly, mutants are found that accumulate an extra RNA molecule which consists of a deleted form of the L RNA segment. These defective L RNA fragments have been shown to interfere with the replication of wild type viral RNA and, therefore, referred to as defective interfering (DI) RNAs (Resende *et al.*, 1991b; 1992). The DI L RNA molecules are packaged into virus particles and cause an attenuation of symptom expression in TSWV-infected plants.

A decrease or loss of transmissibility of tospovirus isolates after successive mechanical transfers has been observed by Paliwal (1976) and Vijayalakshmi (1994), however, the nature of these isolates has not been analyzed. Sofar no data have been published on the transmissibility of *env*$^-$ or DI RNA containing mutants, further referred to in this chapter as *di* mutants. Here, we report studies aimed to establish whether the two afore-mentioned distinct tospovirus mutants can potentially be transmitted by thrips.

MATERIAL AND METHODS

Virus isolates and thrips.

Two TSWV isolates, the Brazilian BR-01 isolate originating from tomato and the Dutch isolate NL-04 from chrysanthemum, as well as their *env*$^-$ and DI-containing (*di*) mutant lines, were used. For INSV, a Dutch isolate NL-07 from *Impatiens* sp. (De Ávila *et al.*, 1992) and the American isolate TSWV-I, in these experiments referred to as US-01/*env*$^-$,

were employed. US-01/*env*$^-$ was kindly provided by J.W. Moyer (North Carolina State University). The wild type BR-01 (BR-01/*wt*), NL-04 (NL-04/*wt*) and NL-07 (NL-07/*wt*) were maintained by thrips inoculation to either *Datura stramonium* L (TSWV) or *Nicotiana benthamiana* Domin. (INSV). The *di* and *env*$^-$ lines of TSWV and INSV were maintained by mechanical inoculation of infected leaf tissue in a ratio of 1 g in 5 ml inoculum buffer (0.01 M phosphate buffer, pH 7.0, containing 0.01 M Na_2SO_3) onto *D. stramonium* (TSWV) or *N. benthamiana* (INSV) plants.

The *di* mutant lines, referred to as BR-01/*di* and NL-04/*di*, contain stable DI L RNAs of 3.2 kb or 3.7 kb, respectively (Resende *et al.*, 1991b). Line NL-04/*env*$^-$ does not produce any enveloped virus particles as shown by electron microscopic studies on thin sections from infected plants, whereas the US-01/*env*$^-$ isolate is characterized by the almost complete absence of such particles in infected cells (Law and Moyer, 1990; Urban *et al.*, 1991).

To study the mutant and wild type virus transmission by thrips, *D. stramonium* and *N. benthamiana* plants, 2 to 3 weeks after sowing, were inoculated mechanically with the TSWV and INSV isolates, respectively. The plants were grown in a greenhouse at approx. 22 °C (light/dark: 16/8 h) for symptom development. Systemically infected leaves of these plants served as sources for acquisition feeding by *F. occidentalis* larvae.

Virus-free stock colonies of *F. occidentalis* were reared on bean pods (*Phaseolus vulgaris* L. 'Prelude') at 27 ($\pm$0.5) °C (light/dark: 16/8 h). The culture was started with adults collected from a greenhouse infestation in the Netherlands.

Elimination of DI RNA

The *di* tospovirus lines were maintained by mechanical inoculation of infected leaf tissue in a ratio of 1 g in 5 ml inoculum buffer. In attempts to eliminate the DIs from the NL-04/*di* isolate by mechanical inoculation of diluted inoculum, leaf tissue was ground in inoculum buffer and serially diluted from 5 to 10,000 times and mechanically inoculated onto *Nicotiana rustica* L. plants. These plants were screened 14 days post-infection for the presence of virus by ELISA and analyzed for wild type and DI L RNA molecules by total RNA extraction and Northern blot analysis.

Total RNA extraction and Northern blot analysis of plants

Total RNA was extracted from healthy and infected plants according to De Vries and

co-workers (1982). Total RNA samples of 5 μg were analyzed by electrophoresis in 1% agarose gels under denaturing conditions (Bailey and Davidson, 1976). After transfer to nitrocellulose membranes the RNA of TSWV isolates was hybridized to ^{32}P-labelled TSWV-70 cDNA directed to the L RNA of isolate BR-01 (De Haan *et al.*, 1991; Resende *et al.*, 1991b). For INSV isolates the ^{32}P-labelled NL-07.1L cDNA (De Ávila *et al.*, 1992) of NL-07 was used as a probe. Both probes reveal the presence of smaller L RNA molecules (Resende *et al.*, 1991b).

Virus detection by enzyme-linked immunosorbent assay (ELISA)

A double antibody sandwich ELISA (DAS-ELISA) was employed to verify virus infection in plants and leaf disks. A DAS-ELISA with amplification of the enzyme reaction was used to analyze antigen titers in thrips. For samples containing TSWV or INSV isolates, antisera raised against the nucleocapsid (N) protein of the BR-01 isolate of TSWV or the NL-07 isolate of INSV were used, respectively. All ELISA procedures have been described in detail before by Wijkamp and co-workers (1993, 1995a).

Time course analysis of replication of INSV in larvae of F. occidentalis

F. occidentalis first instar larvae, 0-4 h old, were confined to either NL-07/*wt*, US-01/*env*$^-$ or healthy *N. benthamiana* leaves. The thrips were given an acquisition access period (AAP) of 2 h and subsequently transferred to healthy leaves of *D. stramonium*. For ELISA, a subgroup of thrips was collected and frozen at -70 °C directly after the AAP and at 10, 20, 30 and 40 h after the start of the AAP.

Transmission studies

F. occidentalis first instar larvae, 0-12 h old, were confined to the surface of infected leaves of *D. stramonium* (TSWV isolates) or *N. benthamiana* (INSV isolates) using cages as described by Tashiro (1967). The larvae were given an AAP of 3 days. As control, first instar larvae were caged on virus-free plants. After the AAP, the larvae were transferred to healthy leaves of *D. stramonium* in leaf cages. After emergence, the adults were tested on *Petunia x hybrida* Hort. Vilm.-Andr. 'Blue Magic' leaf disks (diameter: 13 mm) in 1.5 ml Eppendorf tubes for virus transmission as described by Wijkamp and Peters (1993) for 3 successive inoculation access periods (IAPs) of 48 h. After the IAPs, leaf disks were incubated at 27 °C in 24 well plates (Costar Europe Ltd., Badhoevedorp, the Netherlands)

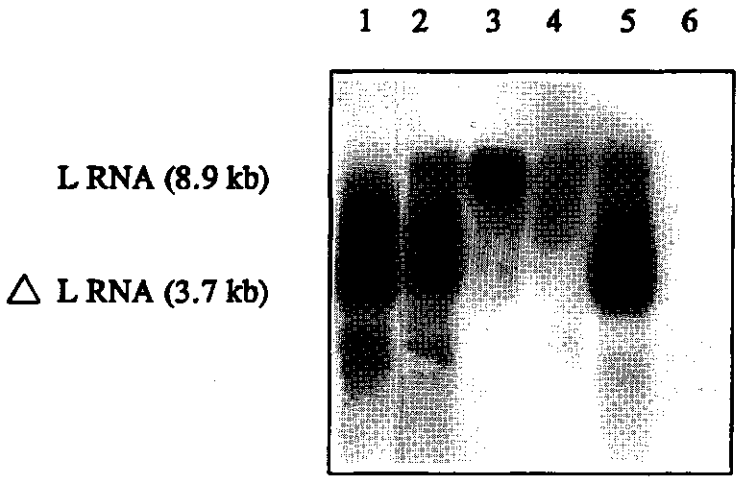


Figure 1. Northern blot analysis of total RNA extracts from *N. rustica* plants mechanically inoculated with different dilutions of inoculum of TSWV isolate NL-04/*di*. ^{32}P-labelled TSWV70 cDNA was used as a probe specific for L RNA. Inoculum was diluted 5 X (lane 1), 5,000 X (lane 2) and 10,000 X (lanes 3-5). Total RNA from a healthy plant was included as a control (lane 6).

while floating on water to develop local lesions.

After inoculation of the petunia leaf disks, the thrips were transferred to 2-3 weeks old *N. rustica* (TSWV isolates) or *N. benthamiana* plants (INSV isolates); viruliferous thrips were placed individually on test plants for an IAP of 5 days, and thrips that did not transmit virus to leaf disks were confined to test plants in groups of 5. All thrips transmission studies were performed at 25 (±0.5) °C. Plants were grown in the greenhouse for symptom development.

Since preliminary experiments showed that the *di* lines were transmitted very inefficiently, an experiment was performed in which 0-12 h old larvae were first confined for an AAP of 4 h to BR-01/*di* and subsequently for an AAP of 3 days to BR-01/*wt* infected plants. Thrips were also allowed to acquire virus in the reciprocal order, i.e. 4 h on BR-01/*wt* and 3 days on BR-01/*di* infected plants. Control larvae were either confined for 3 days to BR-01/*di* infected, for 4 h to BR-01/*wt* infected or to healthy *D. stramonium* plants. After acquisition of the virus the experiment was continued as described before.

After being challenged by thrips the *N. rustica* or *N. benthamiana* test plants were screened for virus infection by ELISA and analyzed for the presence of wild type and DI

Table 1. Transmission of mutants and wild type isolates of tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INSV) by *Frankliniella occidentalis*. Larvae were confined to *Datura stramonium* for an acquisition access period of 3 days.

Virus isolate	% infected petunia leaf disks[a]
TSWV:	
BR-01/*wt*[b]	60 (50)
BR-01/*di*[c]	0-4 (<100)
NL-04/*wt*	52 (90)
NL-04/*di*	0-6 (<100)
NL-04/*env*$^-$[d]	0 (<300)
INSV:	
NL-07/*wt*	85 (50)
US-01/*env*$^-$	0 (<200)

[a] In parenthesis: the number of thrips tested.
[b] *wt*: Wild type isolates not containing detectable amounts of DI L RNA.
[c] *di*: DI L RNA containing isolates.
[d] *env*$^-$: Envelope-deficient isolates, lacking viral envelopes.

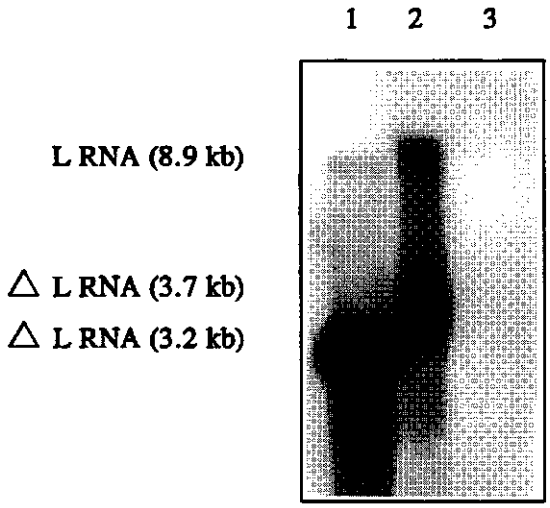


Figure 2. Northern blot analysis of total RNA extracts from infected *N. rustica* plants. ^{32}P-labelled TSWV70 cDNA was used as a probe specific for L RNA. Plants were challenged by viruliferous thrips carrying either TSWV isolate BR-01/*di* (lane 1) or NL-04/*di* (lane 2). Total RNA from a healthy plant was included as a control (lane 3).

L RNA molecules by total RNA extraction an Northern blot analysis employing the TSWV-70 or NL-07.1L cDNA probes as described previously. All plants which became infected by thrips, which had acquired virus from BR-01/*di*, NL/04 *di* or combinations of *di*/*wt* infected plants, were analyzed. A random selection of plants was analyzed, when they were infected with the BR-01/*wt*, NL-04/*wt* and NL-07/*wt* isolates.

RESULTS

Elimination of DI L RNAs during mechanical inoculation

At the onset of the experiments, a TSWV NL-04 isolate free of DI RNAs (NL-04/*wt*) was not available due to its maintenance by successive mechanical inoculations. In order to eliminate the DI RNA molecules from the NL-04/*di* isolate, plants were inoculated with a serially diluted extract of infected leaf material. After symptom expression and verification of infection by ELISA, total RNA was extracted from systemically infected leaves and analyzed on Northern blots for the presence of L-specific DI RNA molecules. All *N. rustica* plants inoculated with 5, 50, 100, 500 and 1000 times diluted inocula from NL-04/*di* plants became infected. Northern blot analysis of total RNA extracts showed that all these plants contained DI L RNAs of a size similar as illustrated in lanes 1 and 2 of Figure 1. However, when plants were inoculated with higher dilutions, i.e. 5,000 and 10,000 times, only 11% and 25% of the test plants became infected. In two plants inoculated with a 10,000 times diluted inoculum, DI L RNAs were (visually) absent when total RNA extracts were analyzed on Northern blots (lanes 3 and 4, Figure 1). In all other cases the plants contained high levels of DI L RNA (lane 5, Figure 1). Inocula from the 2 plants, apparently lacking DI RNAs, were mechanically transferred to *D. stramonium*. Total RNA extracts from the systemically infected *D. stramonium* were analyzed on Northern blots to verify the absence of DIs. In one line, DIs were found again, whereas in the other no DI RNAs were detected. In the latter case, the DI L RNAs were apparently eliminated from the original NL-04/*di* inoculum, rendering a DI RNA free NL-04 isolate. This observation was supported by the drastic increase of the rate by which this isolate was transmitted. The NL-04 line in which DIs could still be detected was transmitted with an efficiency of 13% whereas the transmission of the recovered DI free NL-04 line was

Table 2. Transmission of mutants and wild type isolates of tomato spotted wilt virus (TSWV) by *Frankliniella occidentalis*. Larvae were confined to *Datura stramonium* for acquisition of virus.

	% infected petunia leaf disks[a]
combination of *di*[b] and *wt*[c] acquisition:	
4 h BR-01/*di*, 3 days BR-01/*wt*[d]	16 (51)
4 h BR-01/*wt*, 3 days BR-01/*di*[e]	11 (45)
controls:	
3 days BR-01/*di*	0 (96)
4 h BR-01/*wt*	45 (30)
Healthy	0 (30)

[a] In parenthesis: the number of thrips tested.
[b] *di*: DI L RNA containing isolates.
[c] *wt*: Wild type isolates not containing detectable amounts of DI L RNA.
[d] Larvae were first placed for an AAP of 4 h on BR-01/*di* and subsequently on BR-01/*wt* infected plants for an AAP of 3 days.
[e] Larvae were first placed for an AAP of 4 h on BR-01/*wt* and subsequently on BR-01/*di* infected plants for an AAP of 3 days.

transmitted at a rate of 52%. The line cured from DIs was used in further thrips transmission studies and designated NL-04/*wt*.

Thrips transmission studies using defective mutants

To analyze whether thrips were able to transmit the two types of defective tospovirus mutants (*env*$^-$ and *di*), studies were performed in which their possible transmission was compared to that of their parental isolates. After acquisition by larvae in the first 3 days after emergence, the wild type isolates BR-01/*wt*, NL-04/*wt* and NL-07/*wt* were transmitted efficiently by adult thrips, i.e. to 60%, 52% and 85% of the petunia leaf disks, respectively (Table 1). However, the thrips failed completely to transmit the envelope-deficient mutants NL-04/*env*$^-$ and US-01/*env*$^-$. Compared to the wild type viruses, the *di* lines BR-01/*di* and NL-04/*di* were transmitted inefficiently, e.g. at a maximum rate of 4% and 6%, respectively.

After the tests on petunia leaf disks, the thrips were transferred to *N. rustica* (TSWV isolates) or *N. benthamiana* (INSV isolates) plants for IAPs of 5 days. Plant infected with the *wt* isolates did not show any detectable amounts of DI L RNAs, whereas plants which

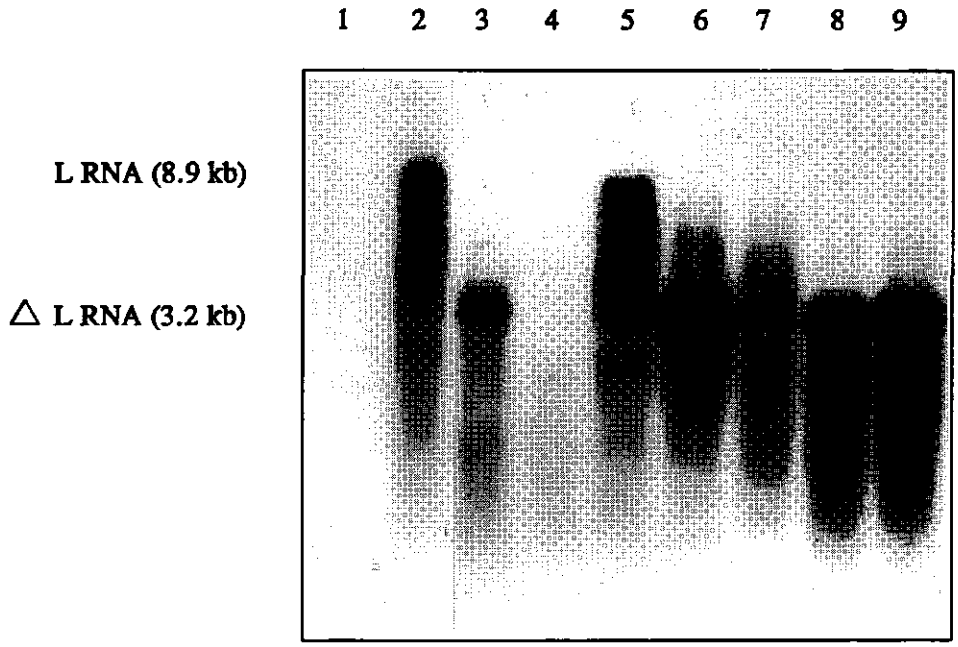


Figure 3. Northern blot analysis of total RNA extracts from infected *N. rustica* plants. ^{32}P-labelled TSWV70 cDNA was used as a probe specific for L RNA. Plants were challenged by viruliferous thrips which were placed as larvae for an acquisition access period of 4 h on BR-01/*wt* and subsequently for 3 days on BR-01/*di* infected plants (lanes 2-4) and vice versa (lane 5-9). Total RNA from a healthy plant was included as a control (lane 1).

became infected after thrips transmission of the BR-01/*di* and NL-04/*di* lines did contain the DI L RNAs (Figure 2). Since all plants infected with BR-01/*di* or NL-04/*di* contained DI RNAs, it is evident that thrips are capable to transmit DI L RNA containing particles. However, the *di* lines of TSWV are transmitted at a significantly lower rate as compared to that of wild type TSWV isolates.

Since the *di* lines were transmitted very inefficiently, another thrips transmission experiment was performed in order to analyze whether the presence of *wt* virus could function as helper virus, and hence, whether it would increase the transmission efficiency. To study this possibility, larvae were allowed to acquire virus from BR-01/*di* infected plants for 4 h and subsequently for 3 days on BR-01/*wt* infected plants and *vice versa* (Table 2). Control thrips that were confined for an AAP of 3 days in the larval stage on BR-01/*di* infected plants only, did not transmit virus. However, thrips which first acquired virus from

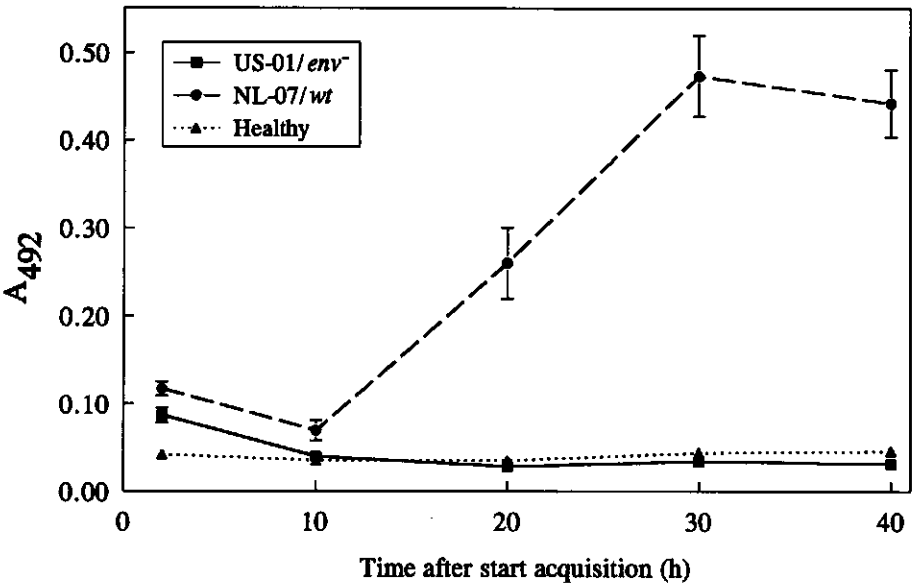


Figure 4. Production of nucleocapsid protein in thrips after an acquisition access period of 2 h on either US-01/*env*⁻, NL-07/*wt* or healthy leaf tissue. The mean ELISA values plus standard error of mean are given, which were obtained at the various intervals, of 30 thrips which were singly tested.

BR-01/*di* and subsequently from BR-01/*wt* infected plants transmitted virus to 16% of the leaf disks, and for the reciprocal treatment this rate was 11%. Total RNA extracts of infected plants after challenge by these thrips, were analyzed on Northern blots for the presence of DI L RNAs (Figure 3). The data showed that in 2 plants only the wild type L RNA was present (Figure 3, lane 2) whereas in the other plants different levels and ratios of wild type and DI L RNAs were observed (lanes 3-9). The increased transmission rates after combined acquisition of virus from *di* and *wt* plants as compared to acquisition on BR-01/*di* infected plants only, suggested that the transmission of the DI L RNA molecules was promoted by providing wild type helper virus. Possibly due to interference, however, the efficiency of transmission for *di*/*wt* combinations (11-16%) was never restored to wild type levels (45% after an AAP of 4 h) anymore.

Time course of INSV multiplication in F. occidentalis larvae

To determine whether the US-01/*env*⁻ isolate was acquired by *F. occidentalis*, the accumulation of the N protein in larvae was monitored in time by ELISA (Figure 4). The

N protein could be detected directly after the 2 h AAP in larvae which had fed on NL-07/*wt* or US-01/*env*$^-$ infected leaves. These results showed that viral material was ingested. However, for both isolates, the titer dropped within the first few hours after acquisition. At 10 h post-acquisition, the N protein level of thrips which acquired NL-07/*wt* remained slightly higher than background levels, indicating that either some virus was retained or that some replication already had occurred. However, at this time the titer of US-01/*env*$^-$ in larvae dropped to background levels, suggesting that all the virus was eliminated from the gut. From this moment on, up to 40 h post acquisition, titers of US-01/*env*$^-$ remained low at background levels as was found for thrips which were confined to healthy plant tissue, suggesting that larvae were unable to retain and acquire virus from US-01/*env*$^-$ infected leaf tissue. In contrast, when thrips acquired the NL-07/*wt* isolate, the amount of N protein increased again after 10 h post-acquisition, resulting in a peak 30 h after the start of the AAP. At 40 h post acquisition the titer of N protein was slightly lower than at 30 h. This coincided with molting of larvae from the first to the second instar shown previously by Wijkamp and co-workers (1993).

DISCUSSION

The results presented in this chapter demonstrate that tospovirus isolates containing DI RNA molecules can successfully be transmitted by thrips, albeit, at a lower efficiency as wild type isolates. On the contrary, transmission of the *env*$^-$ mutants seemed to be completely abolished. Time course analysis experiments of thrips larvae, which were confined to virus-infected leaves for a short acquisition period, showed that an *env*$^-$ INSV isolate was not retained and acquired by the larvae whereas a *wt* isolate persisted and subsequently replicated in thrips tissues. During acquisition of virus by insects, the gut represents the first of a series of barriers that the virus must overcome. For the animal-infecting *Bunyaviridae* it has been shown that the glycoproteins are imperative in the process of attachment of virus to mosquito midgut cells. (Ludwig *et al.*, 1989, 1991). Computer analysis of the TSWV glycoprotein precursor sequence demonstrates the presence of a potential cell attachment site (RGD-motif), which may be involved in binding of virus during receptor-mediated endocytosis. Altogether these data suggest that the glycoproteins are essential for the acquisition and subsequent transmission of tospoviruses by thrips, in

contrast to the infection of plants in which these *env*$^-$ lines can be maintained by mechanical inoculation (Resende *et al.*, 1991b).

Since defective interfering L RNAs are also enveloped, it was expected that these molecules could potentially be transmitted by thrips. The results presented here, indeed showed that TSWV mutants containing DI L RNA molecules were transmitted by *F. occidentalis*, but at a lower efficiency than the wild type TSWV isolates. Furthermore, the transmission efficiency of these mutants was increased again by providing the thrips with wild type helper virus. The inhibitory effect of the DI L RNAs on the transmission of the wild type by thrips is probably twofold. Firstly, DI RNA containing particles which rely on the helper virus for replication will dilute the amount of wild type virus that can be acquired by larvae and/or inoculated to plants by adults, i.e. because of the presence of major amounts of DI L RNA containing particles a relatively low concentration of functional virus is present which can initiate an infection. Secondly, interference may occur during replication in the thrips. Tospoviruses replicate in their thrips vector (Wijkamp *et al.*, 1993) and most likely the enveloped DI L RNAs which are acquired by larvae are also replicated in the thrips. If DI L RNAs are present these molecules will be preferentially replicated during infection, a process which is known to occur in plants that are infected with DI L RNA containing tospoviruses (Resende *et al.*, 1991b). This process will result in a low number of infectious virus particles to be transmitted.

Decreased transmissibility of mechanically maintained tospoviruses has been observed before (Paliwal, 1976; Vijayalakshmi, 1994). Two mutants were described by Paliwal (1976), the first one was transferred 6 times in 18 months after which a 10 to 20 fold decrease in transmissibility by thrips was found. A second TSWV isolate was not transmitted by thrips any more, however, thin sections of infected leaves revealed TSWV particles. Vijayalakshmi (1994) maintained an isolate of groundnut bud necrosis virus (GBNV) by mechanical sap inoculations for 2 years which resulted in a total loss of transmissibility by the vector *Thrips palmi*. No conclusions were drawn from these data because these mutants have not been analyzed. However, these observations emphasize the importance of maintenance of tospoviruses by thrips transmission under experimental conditions, avoiding undesired generation of mutants.

During implementation of genetically engineered protection, an interesting aspect of DI RNAs is their negative impact on virus transmission by thrips. Hypothetically, when conferring DI-mediated resistance by transforming host plants with DI sequences, tolerant

plants can be obtained in which transgenically expressed DI RNA molecules co-replicate and interfere with the replication of the wild type viral genome. Under natural field conditions, viruliferous thrips can infect DI RNA-transgenic plants. The progeny thrips which develop on these plants will create a lower infection potential since transmission will be drastically decreased or even abolished, and as a result the tospovirus-infected DI-transgenic plants will act as dead end hosts in the field and thereby, theoretically, eradicating tospovirus epidemics.

Defective mutants of tospoviruses have not been found under natural conditions. The loss of transmissibility by thrips of mutants that lack virus envelopes may explain why such mutants in nature have not been detected. However, the results in this chapter do not exclude the possibility that defective L RNAs potentially may be present in field isolates. Because of their inefficient transmission, the impact on the ecology of tospoviruses will, however, be limited.

Chapter 9

General discussion and concluding remarks

Tospoviruses have become of particular economic importance during the past ten years due to their resurgence in North America and Europe. In these regions the disease was preceded with a rapid expansion of the Western flower thrips, *Frankliniella occidentalis*. At the onset of this research, knowledge of tospovirus-vector relations was limited. To gain more insight in the epidemiology of tospoviruses, the parameters involved in transmission, the feeding behavior and the relationships between virus and vector have to be analyzed. The present study of tomato spotted wilt virus (TSWV) transmission by *F. occidentalis* demonstrates that this virus-vector interaction exhibits the characteristics of a circulative propagative association. Between acquisition and inoculation a latency period exists before the virus can be transmitted. In this period the virus circulates through the vector, while being propagated. Circulation can already be deduced from the observation that the insects can transmit after a molt. TSWV could efficiently be acquired and transmitted by *F. occidentalis* in short feeding periods (Chapter 2). Moreover, when acquired by first stage larvae, TSWV and impatiens necrotic spot virus (INSV) were transmitted by older second stage larvae or adults after a latent period which varied from 82 h to 176 h depending on the temperature at which the thrips were held (Chapter 4). These results demonstrated the circulative nature of tospovirus in its vector.

A long latent period is considered a strong indication for propagation of virus in its vector. A comparison of the mean latent periods of both propagative and non-propagative plant viruses demonstrates that the mean latent period for 13 propagative viruses from 4 virus groups is 368 h whereas this value is considerably shorter, i.e. 23 h, for 10 non-propagative viruses from 3 virus groups (Nault, 1994). Compared to these values a median latent period (LP_{50}) for tospoviruses of 98-176 h was significantly longer than those for non propagative viruses, while compared to the latent period of propagative viruses it was rather short. Environmental conditions, such as ambient temperatures affect the time between acquisition of virus and transmission of arboviruses which is known as the "extrinsic incubation period" (EIP) (Kay, 1989; Turell, 1985). The ambient temperature also affected

the latent period of tospoviruses in *F. occidentalis*. For TSWV or INSV the LP_{50} values decreased from around 170 h to around 100 h, respectively, with increasing temperatures from 20 to 27 °C (Chapter 2).

Conclusive evidence for multiplication of plant viruses in their vector can come from different observations. Firstly, virus may be transferred between insects either by continuous transovarial passage through a number of generations, or experimentally, by serial passage, by means of injecting insects. This is done until the dilution attained in the final inoculative insects, exceeds the dilution end point of virus in the initial inoculum (Whitcomb, 1972; Okuyama *et al.*, 1968). Secondly, the increase of viral protein titers in the vector is monitored using serological techniques, like fluorescent antibody techniques or ELISA (Reddy and Black, 1966; Falk *et al.*, 1987; Nault and Gordon, 1988). A third approach which proves multiplication, are ultrastructural observations of the sites of viral assembly, and the maturation and/or accumulation of viruses in infected vector cells (Shikata, 1979; Ammar and Nault, 1985; Hardy, 1988). For tospoviruses evidence of multiplication is formed by the "de novo" production of the non-structural (NS_S) protein which is present only after transcription of its mRNA from the complementary viral S RNA strand, during the replication of the virus. In the present study three different approaches were employed. Replication of TSWV in *F. occidentalis* was verified by (1) time course analysis, showing the increase in viral nucleocapsid protein titer to levels higher than initially acquired, (2) the presence and accumulation of the NS_S protein and (3) ultrastructural localization of viral products, i.e. the nucleocapsid and NS_S proteins, viroplasms and virus particles (Chapter 5).

The propagative transmission of tospoviruses by thrips vectors may follow the sequence of events that take place in the infection process of arboviruses in their arthropod vectors as reviewed by Hardy (1988) and Ammar (1994). In the arbovirus infection model movement of virus in infected mosquito females can be divided into three phases. Firstly, virus infection and multiplication in mesenteronal (midgut) epithelial cells and subsequent transfer of virus to the hemocoel is observed. Secondly, virus disseminates through the body of the insect via hemolymph with or without secondary amplification in tissues other than the midgut and salivary glands. Thirdly, virus infection and multiplication in salivary gland cells, and subsequent addition of the virus to the saliva will ultimately result in virus transmission during the feeding process. The infection process occurs in a step-wise fashion during which five barriers have been described for various virus-vector systems, i.e. the

midgut infection-, the midgut escape-, the salivary gland infection-, the salivary gland escape- and the transovarial barrier. The barriers may be dose (Kramer *et al.*, 1981) and possibly temperature dependent (Hardy *et al.*, 1983), however, little is known about the basic biochemical and/or biophysical mechanisms involved.

The gut represents the first barrier that the virus must overcome. Studies with La Crosse bunyavirus (LACV), indicate that attachment of virus to mosquito midgut cells depends on proteolytic cleavage of the G1 viral glycoprotein and consequent exposure of the G2 glycoprotein on the surface of the virion (Ludwig, 1989; 1991). The G2 protein may then fuse to midgut cells or bind to specific receptors on the cells. For tospoviruses, the relevance of the glycoproteins in the infection process in thrips was demonstrated by the inability of thrips to transmit an envelope-deficient virus isolate. Although able to infect plants after mechanical inoculation (Resende *et al.*, 1991b), these isolates were not acquired by larvae and consequently not transmitted by thrips (Chapter 8). Moreover, sequence and computer analysis of the G2-G1 precursor of TSWV, reveal the presence of a putative cellular attachment domain (Kormelink *et al.*, 1992) which may be involved in binding of the virus to the gut cells.

After entering the gut epithelial cells and a possible primary replication cycle in these cells, virus has to cross the basal lamina of the midgut into the hemocoel. This barrier is referred to as the midgut escape barrier. Following release into the hemocoel, the virus may infect other organs. Studies with LACV in *Aedes triseriatus* demonstrate the existence of this barrier; in a significant proportion of the mosquitoes with infected midguts the virus does not disseminate to the hemocoel, hence, these females are incapable to transmit virus (Paulson, 1989). A similar phenomenon is observed in a study on TSWV acquisition by adult thrips (Ullman *et al*, 1992b). Although some virus particles accumulate in the midgut epithelial cells, none are observed beyond these cells, indicating that dissemination to the hemocoel is blocked, suggesting the existence of a midgut escape barrier in adults. On the other hand, when virus was acquired by thrips larvae, the occurrence of N and NS_S proteins in muscle cells associated with the midgut epithelium, the presence of these proteins and virus particles in the salivary glands demonstrated that virus was disseminated from the midgut cells successfully (Chapter 5).

The existence of a salivary gland infection barrier which inhibits the transport of virus into the salivary glands, is demonstrated for Western equine encephalomyelitis virus (WEEV) in some individuals of *Culex tarsalis*. (Kramer *et al.*, 1981). Virus is detected

readily in the midgut of incompetent females. Lower amounts of virus (as compared to competent females) are found in hemolymph and other tissues, but no virus is detected in the salivary glands. A salivary gland escape barrier, preventing the release of virus in the salivary ducts, is implied in the transmission of LACV by *Aedes hendersoni* (Grimstad *et al.*, 1985). Although the salivary glands of 65% of this mosquito population are infected only 5% of these females transmits LACV.

One of the above-mentioned barriers may be involved in the inability of some thrips to transmit tospovirus. Several studies in which virus transmission was correlated to antigen titers of individual adults demonstrated that some thrips were incapable of virus transmission whereas N protein was detected in these individuals, indicating that virus acquisition and some virus replication had occurred. However, the N titers were consistently lower than those of competent thrips (Chapter 2, 3 and 7). This phenomenon is also observed for some arbovirus-vector combinations, where inability of infected female mosquitoes to transmit virus is correlated to lower virus titers (Hardy *et al.*, 1983). Virus is always readily detected in viruliferous thrips. Analysis of these adult thrips, demonstrated that both N and NS_S proteins were located in the muscle cells associated with the midgut epithelium. The N protein was abundantly present in viroplasms, whereas the NS_S protein was scattered throughout the cytoplasm. The accumulation of large amounts of N and NS_S protein, the occurrence of several vesicles with virus particles in the salivary glands, and the massive numbers of virus particles in the salivary gland ducts strongly indicated that the salivary glands are a major site of TSWV replication (Chapter 5).

For a virus to be transmitted transovarially, it must cross barriers additional to those encountered in oral transmission; i.e. before it will reach the oocytes, the ovarian and ovariole sheaths and the follicular epithelium have to be crossed. Several studies indicate that transovarial transmission appears to be related to the virus taxonomic classification (Ammar, 1994). Transovarial transmission is demonstrated for the animal-infecting counterparts within the *Bunyaviridae* (Turell, 1988). Present data however, demonstrate that transovarial transmission of TSWV to the offspring does not occur, supported by the observations that progeny thrips did not transmit virus and that viral antigen was absent in these thrips (Chapter 6).

In general, bunyaviruses require 1 to 7 days to establish detectable infections in gut epithelium and 4 to 14 days for virus to reach the salivary glands (Scott, 1989). In tospovirus-infected thrips, the levels of N and NS_S protein start increasing 12 h after

acquisition. At approximately 36 h after acquisition a drop in N and NS_S titers was observed which coincided with molting of the larvae from the first to the second instar. The decrease in virus titer just prior to metamorphosis, followed by an increase in virus titer after ecdysis, is reported for several tick-virus systems as well, since ticks, unlike mosquitoes, usually molt before taking their next bloodmeal. The lower virus titers probably reflect the physiological and histological changes that occur while molting (Nuttall *et al.*, 1991). After molting a new increase of N and NS_S protein was observed until the amount of N protein reached a plateau at the end of the second larval stage. This period coincided with the development of infectivity in larvae which were able to transmit after an LP_{50} of approximately 83 h. At this moment sufficient high levels of virus may have accumulated in salivary glands for successful transmission (Chapter 4 and 5).

In order to be maintained in nature tospoviruses must alternately be transmitted between plants and vectors and replicate well in the two hosts. Consequently evolution and adaptation of tospoviruses will occur in both the host plant and the thrips vector. In contrast to the deleterious effects of tospoviruses on their plant hosts, no pathological effects of TSWV on the thrips vector were apparent. Survival, developmental time and reproduction of infected *F. occidentalis* were not significantly affected by replication of the virus (Chapter 6). Because propagative plant viruses have two distinct life cycles, one in the insect and one in the plant, the question can be asked whether they originated as plant or insect viruses. Observations that the virus-insect interactions appear not severe (Madden and Nault, 1983; Madden *et al.*, 1984; Nault, 1985) as in contrast to virus-plant relations, suggests a better adaptation (= longer evolutionary relationship) between virus and insect. Insects supposedly have played a major role in RNA virus evolution and may have acted as sources of ancestral viruses as the host ranges of plant and animal viruses overlap in insects (Goldbach and De Haan, 1994).

Finally, within the *Tospovirus* genus distinct virus species have evolved which may be the result of the occurrence of different niches in which the various vector species and plant hosts prevail. It is evident that specificity in transmission does exist between tospoviruses and their thrips vectors (Chapter 7). For instance in the USA, South Africa and India groundnut is infected by TSWV, (Mitchell and Smith, 1991), GRSV (De Ávila *et al.*, 1990) and GBNV (Reddy *et al.*, 1992), respectively. This phenomenon may be explained by the prevalence of different vector species in the respective geographic regions, which results in unique host plant-tospovirus-thrips relations.

Summary

Tomato spotted wilt virus (TSWV), member of the genus *Tospovirus* within the family *Bunyaviridae*, ranks among the top ten of economically most important plant viruses. Tospoviruses cause significant yield losses in agricultural crops such as tomato, lettuce, pepper, tobacco, potato and groundnut, but also in ornamentals like chrysanthemum, alstroemeria, gloxinia and impatiens. Currently, more than 650 different plant species belonging to more than 70 distinct botanical families are known to be susceptible to tospoviruses. Following the introduction of the vector *Frankliniella occidentalis* from the United states in Europe, TSWV and impatiens necrotic spot virus (INSV), a tospovirus mainly occurring in ornamentals, are prevailing in the Netherlands, especially in greenhouse cultivations.

To gain more insight in the epidemiology of tospoviruses, information is needed on the kinetics of their transmission. The experiments described in this thesis were done to analyze some of the parameters involved.

An efficient local lesion assay was developed to facilitate transmission studies using leaf disks of *Petunia x hybrida* "Blue Magic". In this assay, transmission could be scored reliably within 2 to 3 days on the basis of local lesion formation. This assay also enabled easy handling of large numbers of thrips. Besides petunia, leaf disks of other plant hosts were found to be suitable as substrate in transmission studies as well.

Using this assay it was established that *F. occidentalis* could acquire and transmit the virus in periods of 5 min. Longer periods were required for more efficient transmission. To quantify virus transmission the median acquisition (AAP_{50}) and inoculation access periods (IAP_{50}), i.e. the periods needed for 50% of the thrips to respectively acquire or inoculate the virus were determined. The value for the AAP_{50} was 67 min whereas an IAP_{50} of 59 min was found. The leaf disk assay was also employed to determine the efficiency of transmission and the latent period (LP), the period between acquisition and inoculation of the virus, for both TSWV and INSV. The majority of thrips already transmitted virus at the end of the second larval stage at transmission rates for TSWV or INSV of 52.8% or 80.0%, respectively.

The fate of the virus in the thrips after ingestion was studied in different developmental stages of *F. occidentalis*. The accumulation of two viral proteins, the nucleocapsid (N) and a non-structural (NS_S) protein, as monitored by ELISA en Western blot analyses, increased

within two days above the levels initially ingested. Immunocytology of infected adults confirmed that viral products were present in high amounts, especially, in the salivary glands. Electron microscopic studies revealed the presence of many virus particles in the salivary ducts. These results unequivocally demonstrated that TSWV replicated in its insect vector and that the salivary glands were a major site of multiplication. This multiplication was not accompanied by pathological effects on the vector. No effect of virus infection was found on the developmental time, reproduction rate and survival. This study also revealed that no transovarial transmission of virus to the progeny took place.

The vector competence was determined for four thrips and four tospovirus species. The results showed that specificity of transmission, as occurring for other insect-transmitted plant viruses, also exists for tospovirus transmission by thrips. *F. occidentalis* appeared to be the most efficient vector for all 4 viruses tested, i.e. TSWV, INSV, tomato chlorotic spot virus (TCSV) and groundnut ringspot virus (GRSV). *Frankliniella schultzei* transmitted three (TSWV, TCSV and GRSV) of the four tospoviruses, whereas *Frankliniella intonsa*, not yet reported as tospovirus vector, transmitted TSWV and TCSV. Of *Thrips tabaci*, previously known as an important vector for TSWV, only one out of four populations tested was able to transmit TSWV at, moreover, a low efficiency.

Finally, the transmission of mutant tospoviruses, which were generated after successive mechanical transfers of virus, was studied. Defective interfering mutants were found to be transmittable by thrips, albeit at a frequency 10 to 20 times lower than the wild type virus. In contrast, envelope deficient mutants were not transmitted by thrips, indicating the importance of the envelope glycoproteins in virus-vector relationships.

Samenvatting

Het tomatebronsvlekkenvirus, in het Engels "tomato spotted wilt virus" (TSWV), heeft sinds een aantal jaren een plaats in de top tien van de economisch meest belangrijke plantevirussen ingenomen. Het virus behoort tot het genus *Tospovirus* binnen de familie *Bunyaviridae*, waarvan de meeste leden vertebraten infecteren en die voor het merendeel door arthropoden worden overgedragen. Tospovirussen veroorzaken grote schade in tal van belangrijke gewassen zoals tomaat, sla, paprika, tabak, aardappel en pinda, maar ook in siergewassen zoals chrysant, alstroemeria, gloxinia en impatiens. Inmiddels zijn meer dan 650 verschillende planten uit meer dan 70 verschillende families bekend als waardplant voor deze virussen. Sinds de introductie van de Californische thrips, *Frankliniella occidentalis*, vanuit Noord-Amerika in Europa, manifesteren TSWV en impatiens necrotic spot virus (INSV), een ander tospovirus dat voornamelijk van belang is in de sierteelt, zich in Nederland, met name in de kasteelten.

Om een beter inzicht te krijgen in de epidemiologie van tospovirussen, is meer informatie vereist omtrent de parameters die de virusoverdracht door thrips kwantitatief beschrijven. Daartoe is een aantal jaren geleden besloten om op individu niveau deze overdracht te bestuderen.

Daartoe is allereerst een nieuwe lokale lesie test ontwikkeld waarbij overdracht door thripsen op een eenvoudige wijze getoetst kan worden op bladponsjes van *Petunia x hybrida* cultivar "Blue Magic". Hierbij kunnen thripsen snel beoordeeld worden op hun infectie vermogen doordat de ponsjes, na overdracht van virus, reeds 2 tot 3 dagen later lokale lesies vertonen. Bovendien leent deze toets zich zeer goed voor het werken met grote aantallen thripsen. Verder blijkt dat bladponsen van andere waardplanten van tospovirussen ook zeer geschikt zijn om als substraat te dienen in overdrachtsexperimenten. Gebruikmakend van deze toets is ondermeer geconstateerd dat *F. occidentalis* het virus binnen 5 minuten kan opnemen en afgeven, hoewel langere perioden nodig zijn voor efficiënte overdracht. Om virusoverdracht te kunnen kwantificeren zijn mediane waarden berekend voor virusverwerving en -afgifte, d.w.z de periode die nodig is voor 50% van de thripsen om virus op te nemen, respectievelijk af te geven. Na een verwervingsperiode van 67 minuten is 50% van de thripsen, die uiteindelijk virus overdragen, infectieus, terwijl in een periode van 59 minuten 50% van de thripsen in staat is om virus af te geven. De bladponsentoets is ook gebruikt om de overdrachtsefficiëntie en de latente periode (LP)

te bepalen voor TSWV en INSV, dit is de periode tussen opname en afgifte van het virus. Uit de experimenten blijkt dat het merendeel van de thripsen reeds virus overdraagt in het larvale stadium; de percentages bedragen 52.8% en 80.0% voor respectievelijk TSWV en INSV.

Verder is, na opname, het lot van het virus in de verschillende ontwikkelingsstadia van de vector *F. occidentalis* bestudeerd. De accumulatie van twee virale eiwitten, het structurele nucleocapside eiwit en het niet-structurele NS_S eiwit, is geanalyseerd met behulp van ELISA en Western-blot-analyses. Deze eiwitten bereiken binnen twee dagen een niveau dat hoger ligt dan de aanvankelijk opgenomen hoeveelheid. Immunocytologie van geïnfecteerde adulten laat zien dat deze virale produkten in grote hoeveelheden in de speekselkliercellen worden aangetroffen en dat tevens een groot aantal virusdeeltjes in de speekselklierkanalen wordt gevonden. Uit deze resultaten blijkt dat het virus zich in de vector vermeerdert en dat de replicatie voornamelijk optreedt in de speekselklieren. Deze virusvermeerdering lijkt geen pathologische effecten in *F. occidentalis* te veroorzaken, zo wordt geen effect gevonden op de ontwikkelingstijd, reproduktie en levensduur van de thrips. Bovendien blijkt uit deze proeven dat het virus niet verticaal overgedragen wordt op het nageslacht.

Vervolgens is de overdrachtsefficiëntie van 4 verschillende tospovirussen door 4 verschillende thripssoorten onderzocht. De verzamelde resultaten laten zien dat specificiteit in transmissie, zoals bij andere insektenoverdraagbare plantevirussen, ook bestaat in de overdracht van tospovirussen door thripsen. *F. occidentalis* is de meest efficiënte vector van TSWV, INSV, tomato chlorotic spot virus (TCSV) en groundnut ringspot virus (GRSV). *Frankliniella schultzei* kan TSWV, TCSV en GRSV overbrengen, terwijl *Frankliniella intonsa*, een thrips die voorheen niet beschreven is als een tospovirus vector, in staat blijkt tot overdracht van TSWV en TCSV. *Thrips tabaci,* bekend uit vroegere literatuur als een belangrijke vector van TSWV, blijkt een minder efficiënte vector van dit virus te zijn; een van de vier populaties die getest is draagt virus over met een lage efficiëntie.

In het laatste gedeelte van het proefschrift is de overdracht van mutanten van tospovirussen behandeld. Deze mutanten ontstaan wanneer het virus herhaaldelijk mechanisch geïnoculeerd wordt. "Defective interfering" mutanten worden overgedragen door thripsen, maar de frequentie is een factor 10 tot 20 maal lager dan van het "wild type" virus. De mutanten zonder lipidemembraan daarentegen, zijn niet overdraagbaar, wat het belang aangeeft van de virale glycoproteinen in de virus-vector relatie.

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Curriculum vitae

Ineke Wijkamp werd geboren op 12 januari 1963 te Ruurlo. Zij behaalde in 1981 het diploma V.W.O. aan het Rijksscholengemeenschap te Lochem en begon in hetzelfde jaar een studie aan de Landbouwuniversiteit te Wageningen. In 1983 behaalde zij de propadeuse en vervolgde haar studie met een ecologische specialisatie binnen de Planteziektenkunde. In 1987 werd het kandidaatsexamen behaald en de doctoraalfase werd afgerond met afstudeeropdrachten in de Virologie en Entomologie. Tussentijds werd in de periode maart-september 1988 in het kader van een praktijktijd Entomologie onderzoek verricht op het "Insects Affecting Man and Animals Research laboratory USDA-ARS in Gainesville, Florida" onder leiding van Dr. D. Kline. In september 1989 behaalde zij het ingenieursdiploma in de Planteziektenkunde.
Vanaf februari 1990 was zij als assistent in opleiding verbonden aan de vakgroep Virologie van de Landbouwuniversiteit. Het onderzoek aan virus-vector relaties van tospovirussen werd onder supervisie van Dr. Ir. D. Peters en Prof. Dr. R.W. Goldbach verricht, waarvan de resultaten beschreven zijn in dit proefschrift.

Na de promotie zal zij een Post-Doc baan beginnen bij het Department of Plant Pathology and Microbiology, Texas A&M University te College Station, Texas, Verenigde Staten.