Evaluation of thrips resistance in pepper to control *Tomato spotted wilt virus* infection

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Paul Maris

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Voor Cees en Plona

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Chapter 1

Introduction

Tospoviruses.

Tospoviruses are enveloped RNA-viruses transmitted by thrips which cause devastating diseases in a wide variety of food and ornamental crops throughout the world. They represent the plant-infecting members (genus *Tospovirus*) within the large family of *Buynaviridae*, which is further restricted to animals (Murphy *et al.*, 1995). Many field crops grown in warmer climate zones are threatened by tospovirus epidemics, while in the more temperate climate zones these viruses infect mostly greenhouse crops. The number of tospovirus species has increased over recent years, partly due to improved recognition, partly due to emergence of new species (de Ávila *et al.*, 1992a, b and 1993; Law *et al.*, 1992; Law & Moyer, 1990; Moyer *et al.*, 1991; Reddy *et al.*, 1991; Sreenivasulu *et al.*, 1992). To date, at least 14 tospoviruses have been recognised or proposed (Table 1.1) of which *Tomato spotted wilt virus* (TSWV) is the most devastating member due to its world-wide distribution (Goldbach & Peters, 1994) and its wide host range of more than 1100 plant species (Chatzivassiliou *et al.*, 2001; Peters, 2004). A wide variety of (mostly severe) symptoms can be observed on TSWV-infected plants. Systemic symptoms include (in order of severity) necrosis leading to partial or complete plant death, wilting, fruit abortion, stunting, leaf deformation, mosaic, chlorosis, mottling, and ring pattern formation, depending on host plant, season and environment (German *et al.*, 1992). Besides this variation in systemic infection, TSWV has a number of local lesion hosts, the lesions induced often being necrotic (Black *et al.*, 1991). Yield losses of up to 50 to 90% in lettuce have been reported due to TSWV-infestations (Cho *et al.*, 1987), while in groundnut losses between 50 and 100% caused by another tospovirus *Groundnut bud necrosis virus* (GBNV) have been reported in India (Reddy *et al.*, 1983). Another tospovirus of growing economical impact is *Impatiens necrotic spot virus* (INSV), which mainly infects ornamentals (Daughtrey *et al.*, 1997).

With respect to molecular studies on tospoviruses so far, most attention has been given to TSWV. Like all bunyaviruses, TSWV has membrane bound, spherical particles of approximately 80-110 nm in diameter (Fig 1.1A). Various specific cytopathic structures can be distinguished in TSWV-infected tissue such as

viroplasms, nucleocapsid aggregates and paracrystalline inclusions (Kitajima *et al.*, 1992). The TSWV-particle contains a tripartite RNA-genome and at least four structural proteins (Fig. 1.1B). The single stranded RNA-segments are denoted S (small), M (medium) and L (large) RNA. A marked distinction with the animal-infecting members of the *Bunyaviridae* is that in TSWV, like all tospoviruses, both the S- and M-RNA are ambisense (de Haan, 1991; de Haan *et al.*, 1990; Kormelink *et al.*, 1991). The L-RNA is of a negative polarity and encodes the putative RNA-dependent RNA polymerase (also referred to as the L-protein; size 331.5 kDa), which is thought to encompass several enzymatic activities, i.e. transcriptase, replicase and endonuclease (Adkins *et al.*, 1995; Kormelink *et al.*, 1992b; van Poelwijk *et al.*, 1996). The M-RNA encodes the cell-to-cell movement protein (NSm; 33.6 kDa) and the precursor to the envelope glycoproteins G1 (78 kDa) and G2 (58 kDa) (Kormelink *et al.*, 1992a). The S-RNA segment encodes a non-structural protein (NSs; 52.4 kDa), involved in the suppression of gene-silencing (Bucher *et al.*, 2003), and the nucleoprotein (N; 29 kDa) (de Haan *et al.*, 1990).

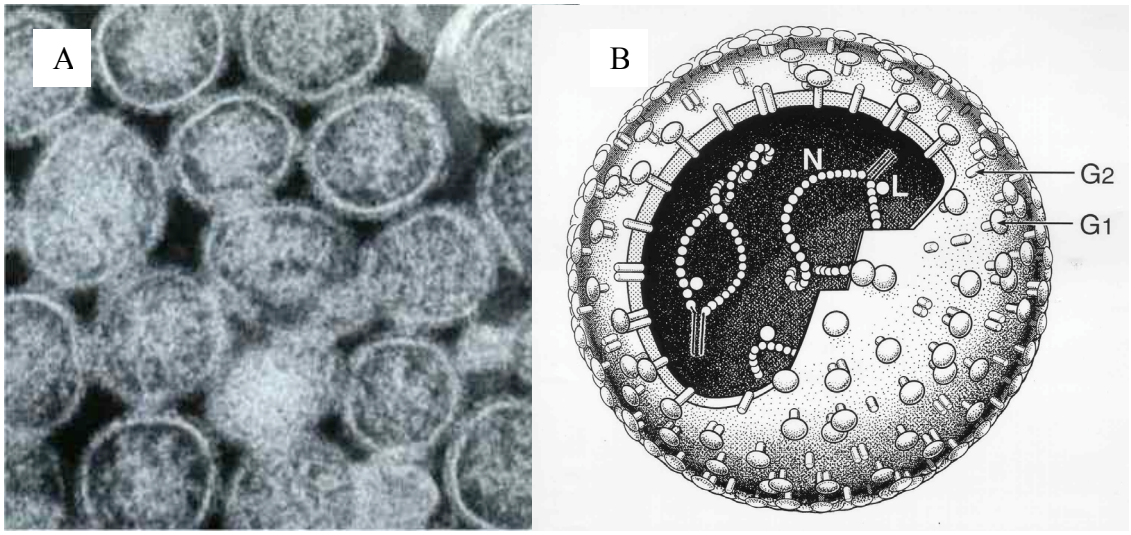

Figure 1.1. Electron micrograph (A) and schematic presentation (B) of TSWV-particles.

Transmission of TSWV

TSWV is exclusively transmitted by a limited number of thrips species belonging to two genera (*Thrips* and *Frankliniella*) within the family *Thripidae* (Table 1.1). Thrips are minute insects, usually up to 1-2 mm long. They acquire the virus in the larval stages and transmit actively the virus in the adult stage. Their life-cycle from egg to adult encompasses two larval and two pupal stages. The females lay eggs in plant tissue, mainly leaves, from which the first larval stage hatches within 4-6 days dependent on the temperature (Fig. 1.2). The larval stages L1 and L2 take almost 2 and 4 days, respectively. The second larval stage pupates into prepupae, which become pupae after 1-2 days. The pupal stages occur in litter or soil, while the larval and adult stages can mainly be found on the top parts of a plant. Larvae and adults

Table 1.1. Geographical distribution, host range and vector species of recognised and proposed tospoviruses.

Tospovirus species	Geograph. distribution	Hosts	Vector species
Capsicum chlorosis virus (CaCV) [1]	Australia[1]	Chilli, sweet pepper, tomato	?[#]
Chrysanthemum stem necrosis virus (CSNV) [2]	Brazil[2]	Chrysanthemum	*F. occidentalis*[24]
Groundnut bud necrosis virus (GBNV) [3,4]	India[4], South-east Asia	Watermelon	*T. palmi*[25,26,27] *F. schutzei*[25,28]
Groundnut ringspot virus (GRSV)[5]	South America[15], South Africa	Groundnut, tomato	*F. occidentalis*[29] *F. schultzei*[29]
Impatiens necrotic spot virus (INSV)[6,7]	USA[7], West and South Europe[6,16,17,18]	Ornamentals, i.e alstroemeria begonia, *Impatiens*, gerbera and several other asteraceous species	*F.occidentalis*[30,31]
Iris yellow spot virus (IYSV) [8]	Brazil[2], Israel[19], the Netherlands[8], USA	Iris, onion, leek	*T. tabaci*[32]
Melon yellow spot virus (MYSV) [9]	Taiwan[9], Japan[20]	Melon	*T. palmi* [32]
Peanut chlorotic fan-spot virus (PCFV) [10]	Taiwan	Groundnut	?[#]
Peanut yellow spot virus (PYSV) [11]	India[11], Thailand[11]	Groundnut	?[#]
Tomato chlorotic spot virus (TCSV) [5]	South America[12,21]	Tomato, sweet pepper	*F. occidentalis*[29] *F. intonsa*[29] *F. schultzei*[29]
Tomato spotted wilt virus (TSWV) [12,13]	World wide[22], but is not frequently recorded in African countries, except for South Africa	Monocots and dicots, i.e. chysanthemum, cyclamen, dahlia, gerbera, groundnut, impatiens, lerttuce, pea, sweet pepper, potato, tobacco, tomato	*F. bispinosa*[34] *F. fusca*[33] *F. intonsa*[29] *F. occidentalis*[35] *F. schultzei*[29,36] *T. setosus*[37,38] *T. tabaci*[39]
Watermelon bud necrosis virus (WBNV)	India	Watermelon	*T. palmi*[40]
Watermelon silver mottle virus (WMSoV) [14]	Japan[23], Taiwan[14]	Watermelon, other cucurbits, Tomato	*T. palmi*[40]
Zucchini lethal chlorotic virus (ZLCV) [2]	Brazil[2]	Zucchini (*Cucurbita pepo)*	*F. zucchini?*

[1]McMichael *et al.*, 2002; [2]Resende *et al.*, 1996; [3]Reddy *et al.*, 1992; [4]Satyanarayana *et al.*, 1996; [5]de Ávila *et al.*, 1993; [6]de Ávila *et al.*, 1992b; [7]Law & Moyer, 1990; [8]Cortês *et al.*, 1998; [9]Kato & Hanada, 2000; [10]Chen & Chiu, 1996; [11]Reddy *et al.*, 1991; [12]Francki *et al.*, 1991; [13]de Haan, 1991; [14]Yeh & Chang, 1995; [15]de Ávila *et al.*, 1990; [16]Vaira *et al.*, 1993; [17]Marchoux *et al.*, 1991; [18]Louro, 1996; [19]Gera *et al.*, 1998a; [20]Takeuchi *et al.*, 2001; [21]Granval de Millan *et al.*, 1998; [22]Goldbach & Peters, 1994; [23]Kameya-Iwaki *et al.*, 1984; [24]Nagata & de Ávila, 2000; [25]Lakshmi *et al.*, 1995; [26]Palmer *et al.*, 1990; [27]Vijayalakshmi, 1994; [28]Amin *et al.*, 1981; [29]Wijkamp *et al.*, 1995a; [30]DeAngelis *et al.*, 1993; [31]Wijkamp & Peters, 1993; [32]Gera *et al.*, 1998b; [33]Sakimura, 1963; [34]Webb *et al.*, 1998; [35]Gardner *et al.*, 1935; [36]Samuel *et al.*, 1930; [37]Fujisawa *et al.*, 1988; [38]Tsuda *et al.*, 1996; [39]Pittman, 1927; [40]Yeh *et al.*, 1992; [#]: unknown

are the feeding stages. Of an estimated 8000 different thrips species, over 5000 have been described and placed into two suborders *Tubulifera* and *Terebrantia*. The first suborder consists of one family and the *Terebrantia* of seven families (Mound *et al.*, 1980; Mount, 1997; Gaston & Mound, 1993). Approximately, half of them feed on fungi, a few are predators and relatively few species live on plants of which some can form serious pests on crops (Palmer *et al.*, 1989). Thrips may not only cause direct damage by feeding on different plant parts, but may also transmit viruses, bacteria and fungi, thus causing indirect damage (Ananthakrishnan, 1980; Fermaud & Gaunt, 1995).
Only eight or nine species are known to transmit tospoviruses (Table 1.1), of which *Frankliniella occidentalis*, the western flower thrips, is nowadays considered to be the most important vector. Indeed this thrips species has expanded worldwide during the last two decades, has a wide host range and is able to vector at least five different tospovirus species including TSWV (Table 1.1).

TSWV is propagatively transmitted, i.e. is able to multiply in the vectoring thrips (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993). While the first instar larvae can acquire the virus during feeding on infected plants, late second stage larvae, and in particular adults transmit the virus (Fig. 1.2) (van de Wetering *et al.*, 1996; Wijkamp *et al.*, 1993; Wijkamp & Peters, 1993). Being wingless, dispersal of larvae is of course rather limited and larvae will therefore mainly feed on the plant on which they hatch. This means that the ability to acquire virus depends on whether the plant selected by females for oviposition is infected or not. The translocation of ingested virus has been studied to some detail. Virus particles ingested with the contents of infected cells are first translocated to the midgut. The virus replicates in the midgut epithelium of the first midgut section (Mg1). Infections can subsequently be found in the viscernal and longitudinal muscular cells surrounding the midgut and next spread to Mg2 and Mg3 (Tsuda *et al.*, 1996; Ullman *et al.*, 1993; Nagata *et al.*, 1997; 1999). Three hypotheses exist concerning the pathway the virus has to follow to become transmissible by the insect. One hypothesis assumes that the virus has to pass the midgut epithelial cells (the so called ‘midgut-barrier’) to reach the hemocoel, and to be next translocated to the salivary glands (Nagata *et al.*, 1999; Ullman *et al.*, 1992b). However, thus far no virus particles have been found in the hemocoel, and intra-hemocoelar injection renders thrips not infective (Nagata *et al.*, 2002).

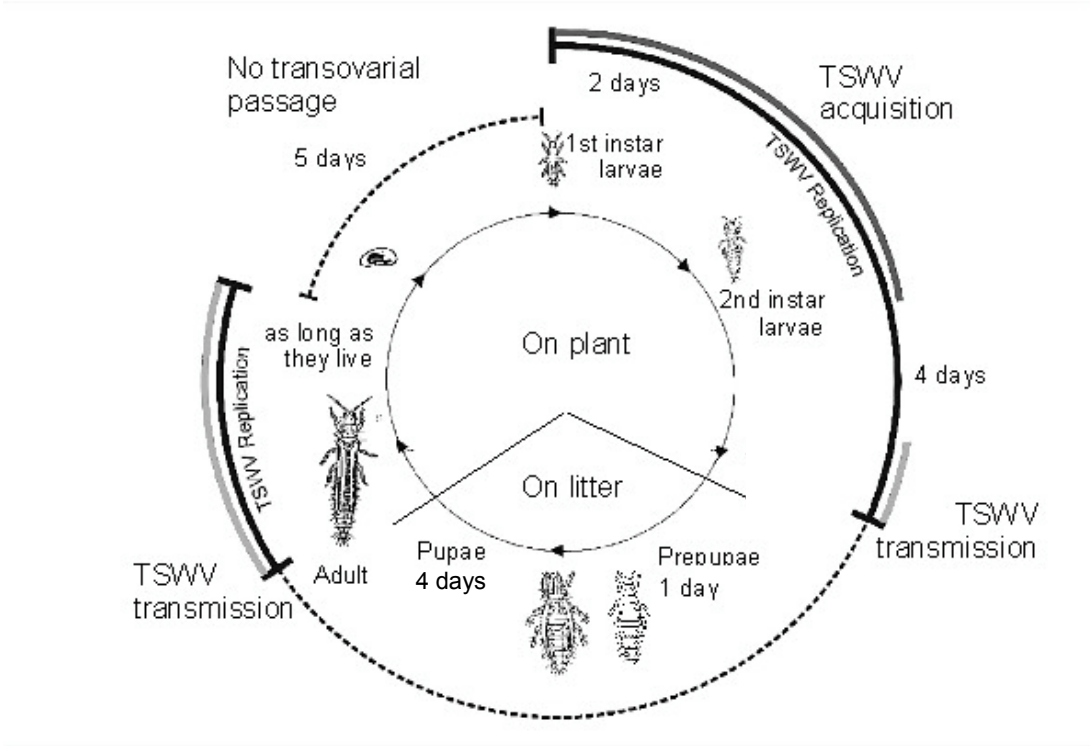

Figure 1.2. Life-cycle of the western flower thrips, *Frankliniella occidentalis*, showing the periods at which the thrips acquire (dark grey circle segment), replicate (black circle segment) and transmit *Tomato spotted wilt virus* (TSWV) (light grey circle segment).

Alternatively, virus may be translocated to the salivary glands along specific thread-like structures, called ligaments (Nagata, 1999). A third hypothesis assumes that - during larval development- a transposition of the thrips' brain into the thoracic region results in a temporary tight contact between the visceral muscle cells of the midgut and the salivary glands. This contact could enable direct translocation of virus particles to the salivary glands (Moritz, 2001). Loss of direct contact after a few days would then prevent translocation of virus particles later in the second instar larvae and adults (Moritz, 2001). Evidence is accumulating that the contact of the salivary glands and the midgut is indeed a critical event for thrips to become tranmitters (van de Wetering *et al.*, 1996; Kritzman *et al.*, 2002; Nagata, 1999). When the virus is acquired in the first larval stage a high percentage of larvae becomes transmitters before pupation, whereas one day old second stage larvae or adults remain uninfectious after acquisition (Kritzman *et al.*, 2002; Nagata *et al.*, 1999; van de Wetering *et al*., 1996; Wijkamp & Peters, 1993).

Both acquisition and transmission of tospoviruses can occur in relatively short periods. Minimal acquisition access periods (AAP's) of 5 min have been reported for TSWV-uptake by *F. occidentalis* from *Impatiens* (Wijkamp *et al.*, 1996b) and GBNV by *T. palmi* from groundnut (Vijayalakshmi, 1994). After a temperature dependent latent period (varying between 84 and 171 h for TSWV and 82 and 157 h for INSV;

Wijkamp & Peters, 1993), the virus can be transmitted by the meantime matured adult within 5 to 10 min (Wijkamp *et al.*, 1996b). A median acquisition access period of 67 min was found for TSWV when larvae had fed on infected *Impatiens* plants, whereas median inoculation access periods were 59 and 133 min when *Petunia* or *D. stramonium* plants were used in inoculation tests (Wijkamp *et al.*, 1996b).

Control of TSWV-infections.

Control of TSWV-disease is difficult, due to the wide host range of the virus and its hard to control vector. Several cultural practices have been tested for effective control of TSWV. Host plant resistance is probably the most effective way to control this virus. Natural resistance to TSWV has been reported for different crops, including chrysanthemum (Daughtrey *et al.*, 1997), lettuce (Cho *et al.*, 1996), pepper (Black *et al.*, 1991; Boiteux & de Ávila, 1994; Boiteux *et al.*, 1993) and tomato (Stevens *et al.*, 1992). Currently, two single dominant resistance genes against TSWV, *Sw-5* in tomato (Stevens *et al.*, 1992) and *Tsw* in pepper (Boiteux & de Ávila, 1994), are known and both provide “HR” resistance. This means that the resistant plants react with a hypersensitivity reaction (HR) against virus-infection resulting in necrotic local lesions. Both resistance genes have been introduced in commercial tomato and pepper cultivars, respectively. Although resistance-breaking TSWV-isolates have been reported (Hobbs *et al.*, 1994; Roggero *et al.*, 2002) and are shown to be relatively stable (Thomas-Carroll & Jones, 2003) and fully competitive with non-breaking isolates (Latham & Jones, 1998), they fortunately seem not to have spread yet over large areas.

TSWV-resistant crops can also be obtained by transformation of plants with viral cDNA-sequences. It has been demonstrated that transgenic expression of viral sequences (especially derived form the viral N gene) provokes a natural defence mechanism of the plant, which is commonly referred to as “post-transcriptional gene silencing” (PTGS) or simply “RNA-silencing” (Kim *et al.*, 1994; Prins *et al.*, 1995; Sherman *et al.*, 1998; Ultzen *et al.*, 1995). Nevertheless, despite their excellent performance, cultivars with transgenic resistance to TSWV have not yet been used on a large scale due to current public concerns with respect to transgenic crops.

Using virus-free stock and planting material is a prerequisite to control TSWV in non-resistant cultivars. Additionally, growing crops in periods that infection pressure is low (Brown *et al.*, 1998) and using optimum plant density (Brown *et al.*, 1996) and fertilisation (Brodbeck *et al.*, 1998) might reduce virus infections, although optimum levels might differ per crop and field. It has been reported for tomato, pepper, and tobacco that application of UV-reflective mulch barriers can decrease thrips and virus-incidence significantly (Greenhough & Black, 1990; Kring & Schuster, 1992; Reitz *et al.*, 2003; Scott *et al.*, 1989). An additional important measure for TSWV-control is of course early virus detection in the field or greenhouse. The use of

Petunia plants has been advocated in the early detection of this virus in greenhouses (Allen & Matteoni, 1991). This indicator plant produces local lesions within two days on the inoculated leaf. TSWV-infections can be determined serologically (by ELISA) even prior to the appearance of the first symptoms (Cho *et al.*, 1988), enabling very early roguing of infected plants. Besides early and sensitive detection, also vector thrips can be monitored using yellow or blue sticky traps (Brødsgaard, 1993a and b; Robb & Parella, 1989) or bait plants such as *D. stramonium* or *Phaseolus vulgaris*.
Insecticides might keep thrips numbers low. A complete eradication, though, is an utopia, due to the hidden life-style of larvae in buds, pupae in the soil, and larvae and adults in flowers by which these stages might escape from the insecticides applied (Heyler & Brobyn, 1992). Furthermore, the use of insecticides should always remain restricted to prevent development of resistance in thrips populations (Brødsgaard, 1994; Robb, *et al.*, 1995; Zhao, *et al.*, 1995). There are further drawbacks of insecticide use (apart from environmental concerns). For instance, application of insecticides in outdoor grown crops does not kill incoming thrips rapidly enough to prevent infection as TSWV is transmitted within short access periods (Wijkamp *et al.*, 1996b). Besides, biological control of thrips by predatory insects in greenhouses will be counteracted by the use of insecticides. To date, predatory mites and flower bugs (*Orius*) (Cook *et al.*, 1996; Tavella *et al.*, 1996) are mainly used in biological control programs against thrips. Application of parasitic nematodes seems to be one of the most recent promises for future control of thrips (Arthurs & Heinz, 2002; Ebbsa *et al.*, 2001; Lim *et al.*, 2001). For greenhouse grown crops, use of insect-proof screens over greenhouse vents and openings, and air locks should keep thrips out of the greenhouse, and roguing weeds and/or virus infected plants around the greenhouse crop may also reduce potential virus and thrips infection pressure.

Against the background of inefficiency of the different control methods alone, there is increasing pressure and interest for developing other (combined) methods to control the spread of TSWV. Combined strategies in integrated pest management (IPM) programs might reduce virus spread. Each crop usually requires its own program, which might even differ on accession level, as host plant susceptibility and growing conditions affect introduction and spread of the virus. Given this variation, a “TSWV Risk Index” has been developed for groundnut crops in Georgia (USA) as a tool for evaluating the risk of TSWV-infection for each parcel or farm. This index contains different factors that can influence the incidence of the virus, like the peanut variety, planting date, plant population density, use of insecticides, row pattern and type of tillage (Brown *et al.*, 2003). Risk index points are allocated to each factor, and summation of the points may show whether groundnut growing will have a low risk, a moderate risk or a high risk to become infected with TSWV. Developing risk indexes for other crops as to help forecasting TSWV infections could be of great value.

Insect-resistant crops.

Another trait which may be useful IPM-programs for controlling insect-transmitted plant viruses is vector resistance, especially for virus vectors like thrips that are also pests themselves (Jones, 1987). In this section, different types of plant resistance to insects as well as their potential for virus control will be discussed. In view of the limited data on thrips resistance in relation to TSWV-transmission available in literature, mainly information on other insect/virus systems will be discussed here.

Different types of insect resistance have been distinguished, i.e. antixenosis (low preference of insect for the crop), antibiosis (reduced reproduction of insect) and tolerance (crop withstands insect feeding) (Painter, 1951). Insect tolerance may be an important plant property from the point of view of breeding for pest resistance, but does not restrict virus spread and therefore is no option to be used for IPM programmes aiming virus control. Tolerant accessions will still be visited by large insect populations whose behaviour and biology is hardly affected, and might therefore not prevent virus acquisition and inoculation but even enhance spread (Kennedy, 1976).

Antixenosis will only affect virus transmission when the insects are prevented from landing on a host by plant colours and/or physical and chemical stimuli. Cucurbits with silvery leaves reflect UV-light much more than green plants. Indeed it has been reported that aphid infestation was lower on the former plants resulting in significant delay of the incidence of (the non-persistently transmitted) *Cucumber mosaic virus* (CMV) and *Clover yellow vein virus* (CYVV) (Davis & Shifriss, 1983). It was also observed that brown coloured lettuce accessions were less frequently infected with *Lettuce mosaic virus* and were infested by less aphids than green accessions (Müller, 1964). Spread of TSWV by its vector *Frankliniella schultzei* was also lower on dark green coloured groundnut plants than on lighter coloured individuals of the same susceptible cultivar (Amin, 1985). Antixenosis might also alter the behaviour such that the insect makes more interplant movements. This higher restlessness does not always result in a greater spread of a virus. Resistance to the green leafhopper *Nephotettix virescens* has been associated with a greater restlessness (Chowdhurry *et al.*, 1996) but this did not lead to higher incidences of *Rice tungro virus*. Also higher inter-plant movement of *Aphis gossypii* did not co-incide with enhanced spread of non-persistently transmitted viruses (Kennedy & Kishaba, 1976; 1977; Lecoq *et al.*, 1979, 1980).

When resistance to the insect is based on antibiosis, the biology of the insect is affected and therefore population size might be lower on resistant plants. This type of resistance might merely restrict secondary virus spread, e.g. spread within the crop, rather than primary spread. Antixenosis resistance might also affect the vector's feeding behaviour and hence the transmission of viruses positively or negatively. Altered probing behaviour of insects has for instance been reported for *A. gossypii*, *F.*

occidentalis and *T. tabaci* on muskmelon, cucumber and leek, respectively (Kennedy *et al.*, 1978; Harrewijn, 1996) (Table 1.2).

Table 1.2. Examples of insect feeding behaviour which differed on plants of a resistant accession from that on plants of a susceptible accession.

Insect vector	Crop	Resistance type
Aphis craccivora	Cowpea[1]	Antibiosis
A. gossypii	Muskmelon[2]	Antixenosis
A. gossypii	Melon[3]	Antixenosis and antibiosis
Frankliniella occidentalis	Cucumber[4]	?
Macrosiphum euphorbiae	Tomato[5]	Antibiosis
Myzus persicae	Sugar beet[6]	Antixenosis and antibiosis
Nasonovia ribisnigri	Lettuce[7]	Antibiosis
Nephotettix virescens	Rice[8,9,10]	Antixenosis and antibiosis
Thrips tabaci	Leek[4]	?

[1]Atiri *et al.*, 1984; [2]Kennedy *et al.*, 1978; [3]Garzo *et al.*, 2001; [4]Harrewijn *et al.*, 1996; [5]Kaloshian *et al.*, 2000; [6]Lowe & Russell, 1974; [7]van Helden & Tjallingii, 1993; [8]Cheng & Pathak, 1972; [9]Khan & Saxena, 1985; [10]Heinrichs & Rapusas, 1984; ?: unknown.

Virus transmission in insect-resistant crops.

Despite available examples where insect resistance has successfully led to restricted virus spread (Table 1.3), there are also examples indicating the opposite, i.e. increased virus spread in insect-resistant crops. In cowpea, a higher incidence of *Cowpea aphid-borne mosaic virus* was found in aphid-resistant lines than in an aphid-susceptible or aphid-tolerant line (Atiri *et al.*, 1984). Also for TSWV, the inoculation efficiency of *F. occidentalis* was found to be significantly higher on thrips-resistant than on thrips-susceptible chrysanthemum (van de Wetering, 1999). These examples demonstrate that insect resistance might either reduce or enhance the spread of plant viruses, depending on the virus/vector/plant system studied, and –moreover- that the underlying mechanisms often remained obscure. A general principle of non-persistently transmitted viruses can not be found, although different examples are available. Little is known for persistently transmitted viruses, probably due to the more complex interaction between virus and its vector. Indeed, there is only little information how insect resistance affects the various features of virus transmission, e.g. virus acquisition and inoculation (Jones, 1987; Zitter & Simons, 1980).

Table 1.3. Examples of plant resistance to insects that were associated with restricted virus spread in some crops.

Insect / vector	Virus	Mode of Transmission	Crop	R- gene	R- type
Amphorophora rubi	*Raspberry leaf mottle virus* *Raspberry leaf spot virus*	Non-persistent	Raspberry[1]	A1, A10	Antixenosis
Aphis craccivora	*Groundnut rosette assitor virus* *Groundnut rosette virus*, satellite RNA	Persistent	Groundnut[2,3]	?	Antibiosis
A. gossypii	*Cucumber mosaic virus*	Non-persistent	Melon[4,5,6]	Vat	Antixenosis and antibiosis
A. gossypii	*Watermelon mosaic virus* 1 *Watermelon mosaic virus* 2	Non-persistent	Melon[7]	Vat	Antixenosis and antibiosis
Bemisia tabaci	*Tomato leaf curl virus*	Persistent	Tomato[8]	?	Antixenosis
Frankliniella occidentalis	*Tomato spotted wilt virus*	Persistent	Tomato[9]	?	Antixenosis
F. schultzei	*Groundnut bud necrosis virus*	Persistent	Groundnut[10]	?	Antixenosis
Macrosiphum pisi	*Pea stunt virus* *Pea mosaic virus*	Non-persistent	Pea[11]	?	Antixenosis and antibiosis
Myzus persicae	*Potato leaf roll virus*	Persistent	Potato[12]	?	Antixenosis
M. persicae	*Beet yellows virus*	Semi-persistent	Sugar beet[13]	?	?
Nephotettix virescens	*Rice tungro bacilliform virus* *Rice tungro spherical virus*	Semi-persistent	Rice[14,15]	Glh1,2,3,5,6,7 glh4, 8	Antixenosis and antibiosis

[1]Jones, 1976; [2]van de Merwe *et al.*, 2001; [3]Padgham *et al.*, 1990; [4]Lecoq *et al.*, 1979; [5]Pitrat & Lecoq, 1982; [6]Martin *et al.*, 1998; [7]Lecoq *et al.*, 1980; [8]Channarayappa *et al.*, 1992; [9]Kumar *et al.*, 1993; [10]Amin, 1985; [11]Wilcoxon & Peterson, 1960; [12]Rizvi, 1982; [13]Haniotakis & Lange, 1974; [14]Dahal *et al.*, 1990; [15]Hibino *et al.*, 1987;?: unknown.

Scope of the investigation.

The research described in this thesis was part of a larger STW-funded project entitled "Antagonistic and synergistic effects of resistance in pepper on the spread of *Tomato spotted wilt virus* and the western flower thrips" (STW-project WBI.4827). Within the framework of this project two PhD studies were simultaneously executed, aiming different, but complementary, goals and which as a whole would provide better insight in the spread of TSWV in thrips-resistant pepper. The aim of the project executed at the Laboratory of Entomology (Wageningen University) was to obtain insight in the (altered) feeding behaviour of thrips on resistant plants in relation to virus transmission. The second project, of which the results are described in this thesis, deals with the spread of TSWV and its main vector *F. occidentalis* in thrips-resistant pepper.

At the onset of the research presented in this thesis, information on the effects of thrips resistance on the spread of TSWV was limited. Preliminary experiments performed with a thrips-resistant chrysanthemum accession indicated that this accession becomes more readily infected with TSWV than a susceptible accession, possibly by altered feeding behaviour of the vector (van de Wetering, 1999). Given this seemingly adverse effect, the aim of the research described in this thesis was to determine potential positive and adverse effects of thrips resistance on TSWV-spread using pepper (*Capsicum*) as model crop. Pepper was chosen as it represents an economically important crop grown in the field in the tropic and subtropic regions and in greenhouses in temperate climate zones including the Netherlands. Both culturing conditions support thrips and TSWV infestations. Furthermore, since in some accessions a single dominant TSWV resistance gene was introgressed, the chocie of pepper allowed a comparative study of possible synergistic and/or antagonistic effects of thrips resistance and virus resistance.

First of all, the levels of both thrips resistance and TSWV resistance were determined in a series of pepper accessions. Subsequently, the impact of thrips resistance on acquisition and inoculation of TSWV by *F .occidentalis* was investigated using a leaf-disk assay (Chapter 2). Primary infections and secondary spread of TSWV in a thrips-resistant accession was studied first in small cage experiments, and next in a large-scale greenhouse experiment (Chapters 3 and 4). Also, the development of local lesions on two virus-resistant pepper accessions differing in vector resistance was studied to determine possible synergistic or antagonistic effects of vector resistance and TSWV resistance (Chapter 4). Thrips resistance was defined by low host preference and subsequent impeded population built-up (Chapter 2). To unravel which of these two aspects contributes most to the resistant phenotype, thrips development from egg to adult on these plants and migration of thrips among resistant plants were studied (Chapter 5).

Since TSWV-infections of the plants might affect the behaviour and life-history parameters of the vector, the possible effect of virus infection on host plant attractiveness, thrips oviposition and larval development was investigated (Chapter 6). Finally, differences between volatiles released from infected and non-infected plants were analysed as well as their potential involvement in attraction thrips to TSWV-infected plants (Chapter 7).

Chapter 2

Thrips resistance in pepper and its consequences for the acquisition and inoculation of *Tomato spotted wilt virus* by the Western flower thrips

Different levels of thrips resistance were found in seven *Capsicum* accessions. Based on the level of feeding damage, host preference, and host suitability for reproduction, a thrips-susceptible and a resistant accession were selected to study their performance as *Tomato spotted wilt virus* (TSWV) sources and targets during thrips-mediated virus transmission. Vector resistance did neither affect the virus acquisition efficiency nor the amount of virus ingested in a broad range of acquisition access periods. Inoculation efficiency was also not affected in short inoculation periods, but was significantly lower on plants of the thrips-resistant accession during longer inoculation access periods. Under the experimental conditions used, the results obtained show that the transmission of TSWV is little affected by vector resistance. However, due to the lower reproduction rate on resistant plants and a lower preference of thrips for these plants, beneficial effects of vector resistance might be expected under field conditions.

This chapter is an extended version of: Maris, P.C., Joosten, N.N., Peters, D., and Goldbach, R.W. 2003. Thrips resistance in pepper and its consequences for the acquisition and inoculation of *Tomato spotted wilt virus* (TSWV) by the Western flower thrips. Phytopathology 93:96-101. The extension presents the results and discussion of a TaqMan RT-PCR analysis of the amount of virus ingested by *F. occidentalis* larvae fed on infected thrips-resistant or susceptible plants.

INTRODUCTION

Thrips (Thysanoptera: Thripidae) cause serious problems in the cultivation of a wide range of greenhouse and field crops. They create major damage on plants by causing reduction in plant growth, deformation of plant organs, and cosmetic damage in the form of silver scars on leaves and flowers. Moreover, during feeding, thrips may transmit tospoviruses (*Bunyaviridae*) (Sakimura, 1963; Ullman *et al.*, 1993; Wijkamp *et al.*, 1995a), of which *Tomato spotted wilt virus* (TSWV) is economically the most important representative (German *et al*., 1992). Several thrips species belonging to the genera *Thrips* and *Frankliniella* transmit TSWV. The western flower thrips (*Frankliniella occidentalis)* is thought to be the most predominant and effective vector in many parts of the world (Daughtrey *et al.*, 1997; Ullman *et al.*, 1997; Wijkamp *et al.*, 1996b).

Attempts to impede tospovirus spread by chemical control of its vectors has proven to be difficult, due to the development of resistance of the vectors to insecticides (Brødsgaard, 1994; Robb *et al.*, 1995; Zhao *et al.*, 1995), the polyphagous behaviour of thrips, and their often hidden way of life inside flowers where they can escape from insecticides sprayed. In addition, TSWV cannot efficiently be controlled in some crops by application of insecticides as the virus often spreads by incoming adults. These thrips may transmit the virus before they acquire a deadly dose of insecticides (Laviña *et al.*, 1993; Todd *et al.*, 1996). Moreover, there is increasing public demand to develop alternative control measures, as the practical use of most of the potentially effective pesticides is no longer allowed in an increasing number of countries.

The use of virus and/or vector-resistant cultivars is another option to control TSWV. Virus resistance has been reported in accessions of pepper and tomato (Black *et al.*, 1991; Jahn *et al.*, 2000; Kumar *et al.*, 1993; Roselló *et al.*, 1997; Stevens *et al.*, 1992). A single dominant TSWV resistance trait has been described for pepper (Boiteux & de Ávila, 1994). This gene, designated *Tsw*, has been introduced in a number of cultivated *Capsicum* hybrids (Boiteux *et al.*, 1993). An operational level of thrips resistance (towards *F. schultzei*) has been reported in the groundnut cultivar Robut 33-1 (Amin, 1985). The incidence of *Groundnut bud necrosis virus* (GBNV) was 30–60% lower in this cultivar than in the widely used cultivar TMV2, although both cultivars were equally susceptible to this tospovirus by mechanical inoculation. Since the acquisition and transmission rate by thrips did not differ between these cultivars, the lower incidence in the field was explained by a lower preference of *F. schultzei* for ‘Robut 33-1’. This type of resistance, restricting the spread of a given virus and often designated ‘field resistance’ was also observed for TSWV in tomato (Kumar *et al.*, 1993; 1995). Significantly fewer plants of four *Lycopersicon* cultivars became infected with TSWV by thrips inoculations than by mechanical inoculation (Kumar *et al.*, 1993), indicating that vector-mediated components were involved.

Partial resistance against *F. occidentalis* has also been reported for five different pepper accessions (Fery & Schalk, 1991). However, this resistance was not evaluated with respect to its effect on the spread of TSWV in the field or on the virus acquisition and inoculation efficiencies of the thrips. Hence, despite several reports, the effect of plant resistance to thrips on transmission of TSWV is not fully understood and requires more investigation.

The objective of this study was to identify and define thrips resistance among a number of pepper accessions, and to analyse the potential effects of this type of resistance on both the acquisition and inoculation efficiency of *F. occidentalis* for TSWV.

MATERIAL AND METHODS

Pepper accessions, thrips population and virus isolate.

Seven *Capsicum* accessions (Pikante Reuzen, Perla RZ, Mazurka RZ, CPRO-1, CPRO-2, PI 152225 and PI 159236) were used in this study. Accessions PI 159236, PI 152225 (Black *et al.*, 1991; Boiteux & de Ávila, 1994) and Perla RZ (J. Haanstra, *personal communication*, Rijk Zwaan, de Lier, the Netherlands) were known to be resistant to TSWV.

A population of *F. occidentalis* 'IS2' (van de Wetering *et al.*, 1999), which originated from an infestation on mango in Israel, was used in the transmission studies. This virus-free population was reared on *Phaseolus vulgaris* cv. Prelude pods in glass jars at 25±0.5°C under a 16-h light and 8-h dark cycle and supplied with *Pinus* pollen.

The TSWV isolate BR01 (de Ávila *et al.*, 1990) was utilised for mechanical inoculation of the plants and in the virus transmission studies. To preserve its virulence this isolate was maintained by thrips-mediated passages on *Datura stramonium* L. plants.

Assessing thrips resistance levels in pepper accessions.

To select a thrips-resistant pepper accession, seven accessions were assessed and tested for preference by the thrips, for development of feeding damage and for supporting the reproduction of a *F. occidentalis* population. The levels of thrips resistance of the pepper accessions were evaluated in "choice" and "non-choice" tests. In the choice tests, the preference of the thrips and the feeding damage produced were determined in a batch using plants of all accessions. One three-weeks-old seedling of each accession was randomly placed in a circle in a thrips-proof cage (0.75x0.75x1m). This test was made in four replicates. Plants were grown at 26±4°C under a 16-h light and 8-h dark cycle.

Before releasing 75 *F. occidentalis* females, some *Pinus* pollen was dusted on the primary leaves of all plants to promote the development of the thrips population in the first two weeks after their release. Three times a week, larvae and adults were

visually counted on the individual plants by inspecting and turning leaves carefully by which adults did not disperse. The feeding damage was also rated, using a relative scale ranging from zero (no damage) up to three (severe damage).
Thrips population development was determined on four accessions in a non-choice test. Six seedlings were placed in a separate thrips-proof cage for each accession. Primary leaves were dusted with *Pinus* pollen and 30 female *F. occidentalis* were released in each cage. Thrips counts were monitored weekly for each individual plant. This study was repeated two times for each accession.

Determining TSWV resistance levels in pepper accessions.
For a correct assessment of the effect of thrips resistance on the acquisition and inoculation of TSWV, pepper accessions differing in this property had to be equally susceptible to virus infection. Ten seedlings of each accession in their six-leaf stage were mechanically inoculated with the TSWV-BR01 isolate. Inocula were prepared from the first systemically infected leaves of thrips inoculated *D. stramonium* plants by triturating 1 g of leaf material in 10 ml inoculation buffer (0.01 M NaH_2PO_4 and 0.01 M Na_2HPO_4, pH 7.0). Plants were reinoculated 4 days later to ensure infection. Another group of 10 seedlings of each accession was inoculated with a 100-fold diluted extract. Plants were grown in a greenhouse at approximately 22°C under a 16-h light and 8-h dark cycle. Symptom development was monitored for 4 weeks postinoculation. Two leaf disks (1.3 cm i.d.) were taken from two top leaves of each plant, 2 weeks postinoculation. Extracts of these disks were prepared by grinding leaf disks with a Polähne press in phosphate-buffered saline with Tween (PBST) and assayed by enzyme-linked immunosorbent assay (ELISA) for their virus titer.

Effect of TSWV infection on the level of thrips resistance.
To elucidate whether TSWV infection did affect the thrips-resistant phenotype, the effect of virus infection on thrips resistance was assessed using a leaf disk assay. Two virus-free or virus-infected leaf disks (2.5 cm i.d.) of a thrips-susceptible and resistant accession were placed in petri dishes (7.5 cm i.d.) on 1.5% agar. Each dish was infested with four *F. occidentalis* females. The specimens were counted during daytime at 30-min intervals. Those present on the two susceptible and resistant disks were separately counted, and those on the agar, wall and lid were placed in one group, designated “off” disk. This monitoring was repeated the next day after transferring the thrips to fresh disks. Each treatment contained six replicates and the experiment was repeated twice. The data were analysed by Kruskal-Wallis one-way analysis of variance (ANOVA) on the ranks using Genstat (Payne *et al.*, 1993).

Effect of plant resistance to thrips on TSWV acquisition.

To determine the effect of thrips resistance on the acquisition efficiency by thrips, first instar larvae of *F. occidentalis*, 0-4 h old, were confined to leaf disks (0.6 cm i.d.) from systemically infected thrips-susceptible or resistant plants with comparable virus titers as shown by ELISA (A_{450} = 1.10 ± 0.08). The disks were placed on 1.2 ml 1.5% agar in 1.5 ml Eppendorf tubes and covered with transparent foil. Larvae had access to these disks for periods of 0.5, 1, 2, 8, 24 or 48 h, and were transferred to non-infected leaf disks (2.5 cm i.d.) of *D. stramonium* (1.5% agar in a petri dish; 3.4 cm i.d.) after the acquisition access periods (AAP) until adult emergence. The resulting adults were individually tested for their ability to transmit TSWV on *Petunia x hybrida* cv. Blue Magic leaf disks (1.3 cm i.d.) during two successive inoculation access periods (IAPs) of 48 h (Wijkamp & Peters, 1993). After these IAPs, the disks were incubated in a 24-well plate on water at 27°C for 72 h for development of local lesions. Approximately 20 thrips were used for each AAP and the experiments were repeated three times. The thrips' sex ratio was similar in each treatment (female/male = 6:5). Virus acquisition efficiencies were analysed as binomially distributed variables using Genstat.

Quantitative determination of TSWV acquisition.

The amount of virus that larvae acquire during feeding on virus infected thrips-resistant or susceptible plants was determined by a quantitative TaqMan reverse transcriptase polymerase chain reaction (RT-PCR). *F. occidentalis* larvae, 0-4 h old, were allowed to feed on systemically infected leaf disks of plants of either accession for 1, 2, 4, 8, 24 or 48 h. After the AAP the larvae were individually transferred to a 1.5 ml Eppendorf tube and stored at -80ºC.

For RT-PCR, the larvae were squashed under a binocular in the tube in 30µl sterile MilliQ using a pestle. First strand cDNA was synthesised using 10µl of thrips extract, primer TSWV-CP-100R (TCT CAA AGC TAT CAA CTG AAG CAA TAA) (Boonham *et al.*, 2001) and superscript reverse transcriptase (Invitrogen) according to manufacturer's instructions. TaqMan PCR amplification was performed on one eight of the cDNA reaction product using 100 nM reverse primer, 200 nM forward primer TSWV-CP-17F (CTC TTG ATG ATG CAA AGT CTG TGA), 200 nM FAM labelled probe TSWV-CP-73T (AGG TAA GCT ACC TCC CAG CAT TAT GGC AAG) (Boonham *et al.*, 2001) and AmpliTaq Gold polymerase (0.05 U/µl) according to manufacturer's instructions. Samples were incubated in a ABI PRISM 7700 sequence detector for 10 min at 95ºC after which 45 cycles of 95ºC for 15 sec, 60ºC for 30 sec and 72ºC for 45 sec were made.

For each AAP, six individual thrips were tested for their relative virus load. The average threshold cycle for each AAP after which a significant increase of fluorescence occurred in the amplification plot (C_T value) was compared for thrips that fed on infected disks of either thrips-resistant or susceptible leaves.

Effect of plant resistance to thrips on TSWV inoculation.

First instar larvae of *F. occidentalis*, 0-4 h old, were given an AAP of 48 h on leaf disks from systemically infected *D. stramonium* leaves to acquire TSWV. The disks (2.5 cm i.d.) were placed on 1.5% agar in a petri dish (3.4 cm i.d.) covered with transparent foil. After the AAP, the larvae were transferred to virus-free leaf disks of *D. stramonium* until they became adult. The ability of each adult to transmit virus on leaf disks (1.3 cm i.d.) of thrips-susceptible and resistant plants was tested using IAPs of 0.5, 1, 2, 8, 16, 24 or 48 h. Approximately 35 thrips were used for each IAP and the experiment was repeated twice. The thrips' sex ratio was similar in each treatment (female/male = 6:5). Fifty adults, given an IAP of 48 h on *Petunia* leaf disks were used to determine the percentage of viruliferous thrips in the population used for this experiment. Inoculation efficiencies were compared and analysed as binomially distributed variables using Genstat.

ELISA.

Virus titers in extracts of leaf disks from mechanically inoculated plants or thrips inoculated leaf disks were tested by targeting the nucleocapsid protein of TSWV using ELISA with antiserum produced in rabbits (Resende *et al.*, 1991). This assay was also used to determine the virus titer in leaves used as virus source for acquisition feeding.

Wells of Nunc Maxisorp F96 immunoplates (Greiner, Alphen aan de Rijn, the Netherlands) were incubated with 150 µl of 1 µg IgG per ml of coating buffer (0.05 M sodium carbonate, pH 9.6) for 2 h at 37°C. After rinsing the plates two times with tap water and once with demineralised water, leaf disk extracts were added to the wells. The extracts were prepared by grinding leaf disks in PBST (0.14 M NaCl, 2 mM KCl, 2 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 2H_2O$ and 0.05% Tween-20). After incubation of the extracts for 2 h at 37°C and rinsing the plates, 150 µl of 1 µg conjugate per ml of PBST was added to each well and incubated overnight at 4°C. After rinsing the plates, nucleocapsid proteins were detected by adding 150 µl of 1 mg p-nitrophenyl phosphate disodium per ml of diethanolamine buffer (0.01 M, pH 9.6). Absorbance values were read on an EL 312 ELISA-reader (Bio-Tek Instruments Greiner BV, Alphen aan de Rijn, the Netherlands) at 405 nm. Samples giving an ELISA reading higher than the average of six healthy control readings plus 3 times the standard deviation were considered positive and those with lower readings negative.

RESULTS

Thrips resistance levels in seven pepper accessions.

The seven pepper accessions tested showed considerable differences in resistance to thrips (Table 2.1). When plants of all accessions were batchwise exposed to thrips, i.e. in a choice test, feeding damage was observed on all accessions irrespective of whether or not they were preferred by thrips (Figs. 2.1 and 2.2; Table 2.1). Severe feeding damage was recorded on the accessions Pikante Reuzen and PI 159236, moderate damage on Mazurka RZ and Perla RZ, and only mild damage on CPRO-1, CPRO-2 and PI 152225 (Fig. 2.1; Table 2.1). Damage on the accessions on which only mild damage was recorded did not further increase 2 weeks after the release of thrips.

Table 2.1. Ranking of the thrips resistance levels of seven pepper accessions by the product of feeding damage index and cumulative number of thrips on plants in a choice test.

Accession	Feeding damage Index	Cumulative number of thrips	Ranking[z]
Pikante Reuzen	3.0	221 (±23)	1
PI 159236	2.5	178 (±32)	2
Perla RZ	1.5	118 (±9)	3
Mazurka RZ	1.5	105 (±18)	4
PI 152225	1.0	36 (±4)	5
CPRO-2	0.5	37 (±13)	6
CPRO-1	0.5	35 (±9)	7

[z] 1 = lowest level of resistance and 7 = highest level of resistance.

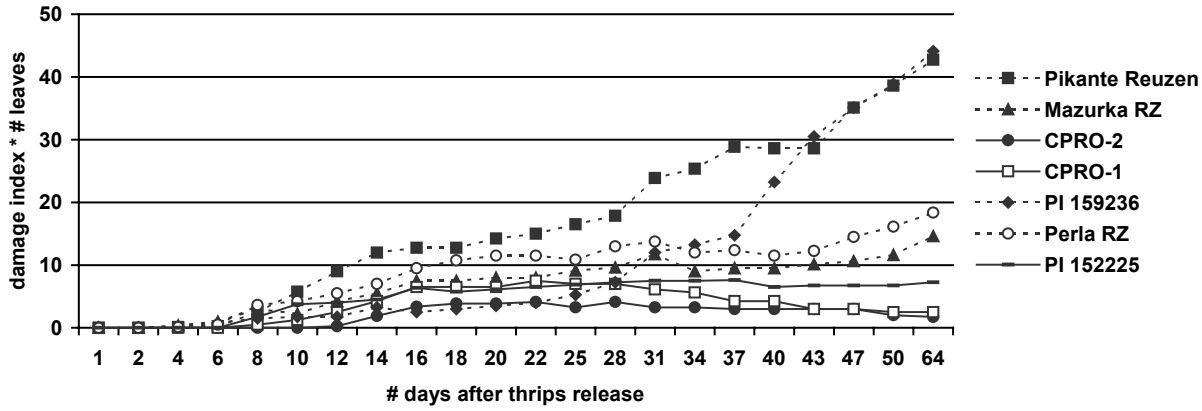

Figure 2.1. Relative feeding damage on seven pepper (*Capsicum*) accessions batchwise exposed to a thrips (*F. occidentalis*) population (choice test), expressed as the multiplication of the damage index and the number of leaves exhibiting symptoms (damage index * # leaves).

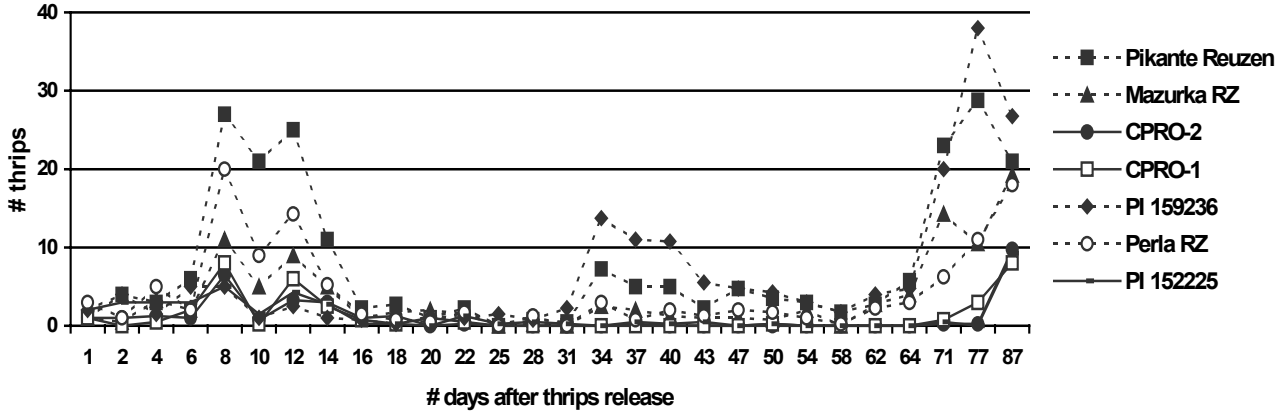

Figure 2.2. Mean thrips counts on plants of seven different pepper accessions batchwise exposed to a thrips (*F. occidentalis*) population (choice test).

The numbers of thrips monitored on the plants of each accession fluctuated with time, with three prominent peaks in weeks 2, 5 and 10 after release of the adults (Fig. 2.2). The sharp increase of the thrips numbers after week 10 coincided with the start of the flowering of the plants.

Thrips resistance levels of two of the most promising accessions, CPRO-1 and PI 152225, were further analysed in a non-choice test, along with the most thrips-susceptible accessions Pikante Reuzen and PI 159236 in a manner preventing any dispersal of thrips between the accessions. *F. occidentalis* reproduced most efficiently on plants of Pikante Reuzen, while only a few thrips accumulated on plants of the accessions CPRO-1, PI 152225 and PI 159236 during the entire test period of 8 weeks (Fig. 2.3). The high numbers of thrips found on PI 159236 in the choice test (Fig. 2.2; Table 2.1) might be explained either by dispersal of thrips from the more preferred accessions or by reproduction of these dispersing adults on this accession. Results obtained enabled us to rank the accessions from 1 through 7 (Table 2.1) for their level of thrips resistance as based on feeding damage and host preference. Results from the non-choice experiments in which population development was measured support this ranking (Fig. 2.3).

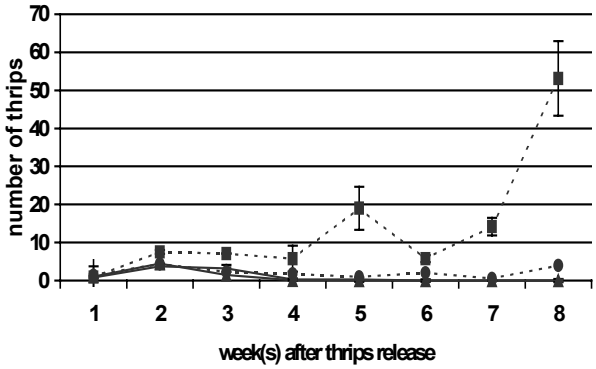

Figure 2.3. Mean thrips counts on plants of four pepper accessions (Pikante Reuzen: dashed line with square; CPRO-1: solid line with triangle; PI 159236: dashed line with dot; PI 152225: solid line with dash) separately enclosed in thrips-proof cages (non-choice test). Thirty thrips were released on all accessions. Bars indicate the standard error of the mean.

Determining TSWV resistance levels in pepper accessions.

The accessions Pikante Reuzen, Mazurka RZ, CPRO-1 and CPRO-2 were equally susceptible to TSWV, as they had similar virus titers two weeks after mechanical inoculation (Table 2.2). Accessions Perla RZ, PI 152225 and PI 159236 on the other hand, were not systemically infected, as expected because these accessions contained the *Tsw* resistance gene (Boiteux & de Ávila, 1994). Similar results were obtained for the plants inoculated with a 100-fold diluted inoculum.

Based on the results summarised in Tables 2.1 and 2.2, accession CPRO-1 was identified as the most thrips-resistant line and accession Pikante Reuzen as the most thrips-susceptible line with both being equally susceptible to TSWV.

Table 2.2. Disease symptoms of TSWV and its titers determined by enzyme-linked immunosorbent assay (ELISA) in systemically infected leaves of seven pepper (*Capsicum*) accessions 2 weeks after mechanical inoculation.

Pepper accession	Symptoms	ELISA-reading[z]
Pikante Reuzen	Mosaic, chlorotic veins	1.23 ± 0.08
Mazurka RZ	Mosaic, chlorotic veins	1.36 ± 0.07
CPRO-2	Leaf curl, chlorotic veins	1.38 ± 0.12
CPRO-1	Leaf curl, chlorotic veins	1.19 ± 0.07
PI 159236	None	0.04 ± 0.01
Perla RZ	None	0.05 ± 0.01
PI 152225	None	0.00 ± 0.01

[z] mean ELISA reading (A_{450} values) of 10 plants ± the standard error of the mean (sem). The threshold value of the control leaf disks was 0.11± 0.01.

Effect of TSWV infection on the level of thrips resistance.

To address the value of thrips resistance for virus control, it is of prime importance to evaluate whether virus infection would alter this trait. This possibility was determined in a leaf disk assay. Thrips preferred to feed on leaf disks of the thrips-susceptible accession over disks from the thrips-resistant accession (Table 2.3) regardless of infection status. Although less thrips were found "off" the infected disks, the ratio of *F. occidentalis* preferring the susceptible over the resistant leaf disks was not significantly affected by virus infection (Table 2.3). The experiments also demonstrate that thrips resistance can be reliably monitored using leaf disk assays.

Table 2.3. Percent dispersal of *F. occidentalis* over leaf disks of the thrips-resistant accession CPRO-1 (TR) and thrips-susceptible accession Pikante Reuzen (TS), either healthy or TSWV infected in a petri dish.

	TR disk	TS disk	"off" disk[y]
Non-infected	22 ± 7 [a z]	55 ± 7 [b]	23 ± 5 [b]
Infected	24 ± 17 [a]	64 ± 15 [b]	12 ± 2 [c]

[y] Thrips found on the agar, wall and lid, were scored as "off" disk.

[z] Different superscript letters in a row indicate significant differences between values ($P<0.05$).

Effect of vector resistance on TSWV acquisition and inoculation.

To assess to what extent thrips resistance may affect TSWV acquisition and inoculation, and therefore virus spread in the field, the effect of this resistance on both properties was analysed in leaf disk assays. The acquisition efficiencies obtained on the disks from thrips-susceptible and resistant plants were plotted for each accession as function of the AAP (Fig. 2.4). For both accessions, approximately 70% of the thrips transmitted the virus after an AAP of 24 h, allowing reliable comparisons between the thrips-susceptible and resistant accessions. Virus acquisition on thrips-susceptible and resistant leaf disks did not differ significantly for all AAPs ($P>0.10$). No differences in mortality were found during the periods that the larvae had acquisition access to infected leaf disks of the thrips-susceptible or resistant accession. TaqMan PCR-assays confirmed the results of the virus acquisition studies. C_T -values found for thrips that fed on resistant and susceptible plants did not differ significantly over a wide range of acquisition access periods (Figure 2.5).

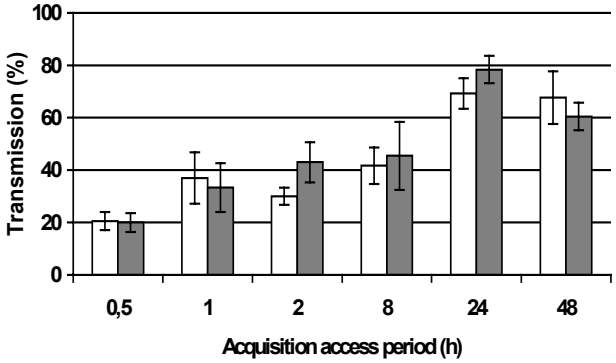

Figure 2.4. Mean *Tomato spotted wilt virus* acquisition efficiencies by *Frankliniella occidentalis* from leaf disks of a thrips-susceptible (Pikante Reuzen; white bars) and a thrips-resistant (CPRO-1; dark bars) accession as function of the acquisition access period. Error-bars indicate the standard error of the mean.

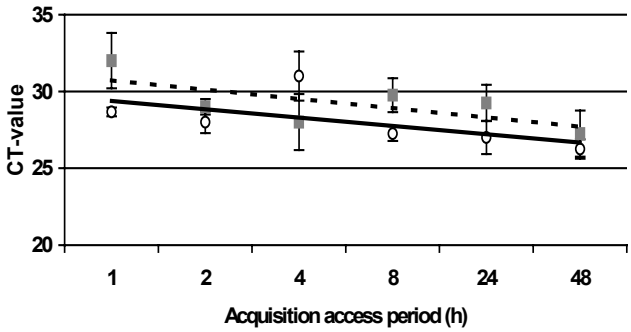

Figure 2.5. Relative amount of *Tomato spotted wilt virus* particles ingested by *Frankliniella occidentalis* larvae from infected leaf disks of a thrips-resistant (CPRO-1; open circles) and thrips-susceptible (Pikante Reuzen; closed squares) accession as function of the acquisition access periods. Lines indicate the regression curves for the TSWV-load of thrips feeding on infected thrips-resistant (solid line) or thrips-susceptible (dashed line) plants. Error bars indicate the standard error of the mean.

Inoculation efficiencies to disks of the thrips-susceptible accession and *Petunia* after exposure to individual viruliferous thrips showed that 74 and 72% of the adults, respectively, transmitted the virus, allowing us to make reliable comparisons between inoculation of thrips-susceptible and resistant leaf disks. Identical inoculation efficiencies were found for both accessions when short IAPs were given, but they differed significantly after IAPs of 24 and 48 h ($P<0.01$; Fig. 2.6), indicating that vector resistance reduced virus inoculation significantly only after long IAPs. No differences in mortality were recorded between thrips feeding on thrips-susceptible or resistant leaf disks.

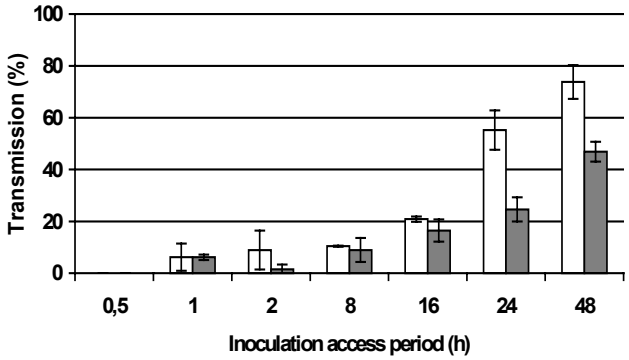

Figure 2.6. Mean *Tomato spotted wilt virus* inoculation efficiencies of *Frankliniella occidentalis* on leaf disks of a thrips-susceptible (Pikante Reuzen; white bars) and thrips-resistant accession (CPRO-1; dark bars) as function of the inoculation access period. Error bars indicate the standard error of the mean.

DISCUSSION

The comparative study presented in this chapter shows that the seven pepper accessions varied considerably with respect to thrips resistance levels. Differences in thrips resistance were defined by three parameters, i.e. quantity of direct feeding damage, host preference, and thrips population development on the accessions. The initial feeding damage produced on the resistant accessions CPRO-1 and PI 152225 after release of the adults did not increase. This might indicate that, besides a poor population development on the resistant accessions, dispersal of the thrips between the susceptible and resistant plants was low and/or that the thrips left the resistant plants without feeding. The almost complete absence of feeding scars on the resistant accession, showing that the plant is a less suitable host for thrips, does not result in a reduced acquisition of TSWV and gives only a reduction of the inoculation efficiency after long IAPs. Apparently, the (duration of) probing activity of the thrips is not affected by thrips resistance and is long enough to transmit the virus. The acquisition efficiencies found for both pepper accessions were not affected by thrips resistance and were in good agreement with those reported for *Impatiens* sp. (Wijkamp *et al.*, 1996b), known to be a permissive host. Thrips resistance had also no effect on the amount of virus ingested by *F. occidentalis* larvae, as the C_T –values for thrips which fed on resistant or susceptible plants were similar for each AAP tested. Virus load was positively related with the AAP, resulting in low C_T -values after long AAPs. The regression curves of C_T –values for thrips feeding on both accessions indicate that doubling the AAP does not lead to doubling of virus load, but a relatively lower load. This might indicate that soon after the first acquisition excretion occurs at almost the same rate as ingestion takes place or that viral RNA is rapidly degraded in the thrips' midgut. The slight increase of virus in the thrips with longer AAPs is also affected by the growth of the larvae, hence an increase of midgut volume, and the

onset of virus replication in thrips (Wijkamp *et al.*, 1993). The relative contribution of the different parameters on the virus load remains unknown. The variation between C_T –values for each treatment might be explained by a possible patchy distribution of TSWV in leaves of the pepper accessions used, as was found for burdock (*Arctium lappa*) (Ullman *et al.*, 1992a).

The inoculation efficiency was found to be similar when short IAPs were given. Long IAPs resulted in a significant lower number of disks from the thrips resistance accession infected with TSWV than of disks from the susceptible accession. This indicates that virus incidence will be lower when the thrips continuously feed on plants of thrips-resistant crops, but this remains to be studied under field conditions.

The minimum time, required for a successful inoculation on both the thrips-susceptible and resistant phenotype in this study was 1 h (Fig. 2.5), comparable to the values found for GBNV transmission by *Thrips palmi* to groundnut (Vijayalakshmi, 1994). However, this time span is considerably longer than the minimum IAPs reported for the transmission of TSWV to *Petunia* spp. and *Nicotiana rustica* by *F. occidentalis* and *Thrips tabaci* (Wijkamp *et al.*, 1996b; Razvyazkina, 1953). In our study, only 9% of the disks from thrips-resistant plants and 10% of the disks from susceptible plants became infected after an IAP of 8 h, whereas 80% and 65% of the *Petunia* and *D. stramonium* leaf disks, respectively, became infected after an IAP of 5 h (Wijkamp *et al.*, 1996b). These differences in inoculation efficiencies might be explained by different adaptation rates and feeding responses of the adults to these host plants. The thrips-resistant pepper accession used is likely to be a less suitable host for adult feeding than *Petunia* and *D. stramonium*, resulting in lower number of TSWV infections per unit of time.

After IAPs of 16 h or longer, the percentage of infected thrips-susceptible leaf disks increased rapidly whereas a slower increase was observed for thrips-resistant disks. The explanation for this phenomenon might be that the adults adapted more readily to the susceptible pepper accession than to the resistant accession. This quicker adaptation would then result in a different feeding behaviour on the first accession leading to more effective inoculations. It is not known which activity in the feeding behaviour, e.g. penetration, saliva production or food ingestion, is less or more frequently performed as a result of this adaptation. In case thrips make more penetrations on resistant plants in search for suitable feeding sites, it can be concluded that the virus is not inoculated during stylet penetration, but during a later activity by which cells remain sufficient viable to replicate the virus.

The lower inoculation efficiency found after long IAPs on the resistant pepper accession is in contrast with the higher inoculation rate found on a thrips-resistant *versus* a thrips-susceptible chrysanthemum (van de Wetering, 1999). This increase was explained by the assumption that a higher number of punctures were made by thrips on the resistant plants. These results are compatible with those obtained in

arena tests in which the transmission rate of a non-persistently transmitted virus to a non-host for its (aphid) vector was significantly higher than to hosts of the vector (Yuan & Ullman, 1996). It remains to be analysed why such, apparently host-dependent differences exist.

Vector resistance in plants has often been associated with a decrease of virus incidence. Decreases have been found for *Cowpea mottle virus*, *Wheat streak mosaic virus*, *Grapevine fanleaf virus*, *Potato leaf roll virus*, *Tomato yellow leaf curl virus* and *Rice ragged stunt virus* which are transmitted by beetles, mites, nematodes, aphids, whiteflies and planthoppers, respectively (Allen *et al.*, 1982; Berlinger *et al.*, 1986; Bouguet, 1981; Martin *et al.*, 1983; 1984; Parejarearn *et al.*, 1984; Rizvi & Raman, 1983; Shukla *et al.*, 1994). On the other hand, an increase of *Cowpea aphid-borne mosaic virus* (CABMV) incidence has been demonstrated in an aphid-resistant cowpea cultivar (Atiri *et al.*, 1984). Differences in incidences may be determined by the number of incoming vectors, their reproduction and mortality rates, dispersal in the crop and the number of punctures made by the vector on a plant. These parameters controlling virus incidences in vector-resistant crops are not fully understood.

The results presented in this study demonstrate that an operational level of thrips resistance does not necessarily lead to impeded TSWV acquisition or inoculation during the transmission of this virus. Although a thrips-resistant accession was selected based on a lower preference, decreased feeding damage as well as decreased reproduction, our study reveals that feeding damage cannot be used as a reliable marker for thrips resistance when the thrips spent only limited periods on the resistant plants. Due to the lower reproduction rate and lower preference of thrips for the resistant plants, as found in this study, beneficial effects of thrips resistance might still be expected under field conditions. As the pepper accessions were screened for their level of resistance to thrips feeding, to thrips population development and to host preference, these accessions represent suitable material for studying the underlying mechanism(s) of possible effects of vector resistance on virus spread under field conditions.

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Chapter 3

Restricted spread of *Tomato spotted wilt virus* in thrips-resistant pepper

Spread of *Tomato spotted wilt virus* (TSWV) and population development of its vector *Frankliniella occidentalis* were studied on the pepper accessions CPRO-1 and Pikante Reuzen, which are resistant and susceptible to thrips, respectively. Viruliferous thrips were released on plants of each accession (non-choice tests) or on plants in a 1:1 mixture of both accessions (choice tests) in small cages containing 8 or 16 plants. Significantly fewer CPRO-1 plants became infected in the primary infection phase in both tests. In the non-choice test, virus infection of the resistant plants did not increase after the initial infection, but all plants eventually became infected when mixtures of both cultivars were challenged in the secondary infection phase. Secondary spread of TSWV from an infected resistant or susceptible source plant was significantly slower to resistant plants than to susceptible plants, independent of source plant phenotype.

The restricted introduction and spread of TSWV in the thrips-resistant cultivar was confirmed in a large-scale greenhouse experiment. The restricted and delayed TSWV spread to plants of the resistant accession in both the cage and the greenhouse experiment was explained by impeded thrips population development. The results obtained indicate that thrips resistance may provide a significant protection to TSWV infection, even when the crop is fully susceptible to the virus.

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INTRODUCTION

Tomato spotted wilt virus (TSWV), the type species of the genus *Tospovirus* within the family Bunyaviridae, is propagatively transmitted by thrips (Thysanoptera: Thripidae) and has a broad host range of over 1.000 plant species (Chatzivassiliou *et al.*, 2001). Eight thrips species have been identified as vectors, of which *Frankliniella occidentalis* Pergande currently is considered to be the most important (Daughtrey *et al.*, 1997; Ullman *et al.*, 1997; Wijkamp *et al.*, 1995a). The broad host range of this vector and its increasing resistance to insecticides (Brødsgaard, 1994; Robb *et al.*, 1995; Zhao *et al.*, 1995) impede control of TSWV by insecticide application.

Current control strategies for TSWV include rouging infected plants, the use of clean stock material (Ochoa *et al.*, 1996), excluding thrips by greenhouse screens (Robb & Parella, 1989), air locks, or introducing natural enemies (Funderburk *et al.*, 2000; Loomans *et al.*, 1997). As these control strategies are only partially successful, additional measures are needed to limit virus spread.

Host plant resistance to TSWV is available for a number of crops, including chrysanthemum (Daughtrey *et al.*, 1997), lettuce (Cho *et al.*, 1987), pepper (Black *et al.*, 1991; Boiteux & de Ávila, 1994; Boiteux *et al.*, 1993) and tomato (Stevens *et al.*, 1992). In addition, significant levels of resistance to thrips have also been reported in cabbage (Stoner & Shelton, 1986), chrysanthemum (Broadbent *et al.*, 1990; de Kogel *et al.*, 1998), groundnut (Kinzer *et al.*, 1973; Rhoda *et al.*, 1991), pepper (Fery & Schalk, 1991; Maris *et al.*, 2003c; Chapter 2), and tomato (Kumar *et al.*, 1995). Thrips resistance might affect the spread of TSWV either in a negative or positive way. Reduced spread of *Groundnut bud necrosis virus* (GBNV) was found in the groundnut accession Robut 33-1, which was attributed to fewer thrips on the plants of this accession than on thrips-susceptible accessions (Amin, 1985). In contrast, increased virus spread on a thrips-resistant cultivar has been reported for chrysanthemum (van de Wetering, 1999). This outcome was explained by altered feeding behaviour of *F. occidentalis* on the resistant chrysanthemum plants. Disturbed feeding behaviour of *F. occidentalis* and *Thrips tabaci* also was reported on resistant cucumber and leek, respectively, resulting in a fewer penetrations and reduced feeding time (Harrewijn *et al.*, 1996).

Previous studies using pepper cultivars have revealed that resistance to thrips has little effect on the acquisition and inoculation of TSWV (Maris *et al.*, 2003c; Chapter 2). In those studies, however, the impact of the thrips population development on the spread of the virus was not analysed. Thrips reproduction was significantly lower on the thrips-resistant plants (Maris *et al.*, 2003c); therefore, it may reduce spread of TSWV in such cultivars.

In this study, the effects of thrips resistance on the spread of TSWV by *F. occidentalis* in pepper was analysed, taking effects of thrips population development into account. To this end, both small-scale infection experiments as well as a large-scale greenhouse experiment were set up to validate the results.

MATERIALS AND METHODS

Thrips population, virus isolate, and pepper material used.

The *F. occidentalis* population originated from a greenhouse infestation in lucerne (*Medicago sativa*). To produce viruliferous thrips, 0- to 4-h-old first instar larvae were confined to leaf disks (2.5 cm in diameter [i.d.]) from TSWV-infected *Datura stramonium* plants in a petri dish (3.4 cm i.d.) on 1.5% agar. After an acquisition access period (AAP) of 48 h, the larvae were transferred to virus-free leaf disks of *D. stramonium* until the adults emerged. The percentage of viruliferous adults was determined by testing 100 randomly sampled adults on Petunia leaf disks (Wijkamp & Peters, 1993).

The TSWV isolate BR-01 (de Ávila *et al.*, 1990) was maintained by thrips-mediated passages on *D. stramonium* plants. Extracts of these plants were mechanically inoculated on 2-week-old pepper seedlings, which served as virus source in transmission studies 2 weeks post inoculation.

In all studies, the *Capsicum annuum* accessions “Pikante Reuzen” and “CPRO-1”, susceptible and resistant, respectively, to *F. occidentalis* (Maris *et al*., 2003c; Chapter 2), were used. Both accessions, hereafter referred to as TS (thrips-susceptible) and TR (thrips-resistant), respectively, were verified to be equally susceptible to TSWV by mechanical inoculation (Maris *et al*., 2003c; Chapter 2). Resistance to thrips in the TR cultivar CPRO-1 was defined previously by lack of reproduction, low preference and minimal feeding damage (Maris *et al*., 2003c; Chapter 2).

Spread of TSWV to TR and TS plants in choice and non-choice tests.

Primary spread of TSWV (i.e. virus spread after introduction of viruliferous thrips) was studied by releasing 30 randomly selected adults that were allowed to acquire the virus as 0- to 4-h-old larvae, in the middle of thrips-proof cages (0.75x0.75x1 m) with 8 or 16 TR or TS plants (non-choice tests). The 4-week-old plants (sixth-leaf stage) were placed in a square at a distance of approximately 20 cm apart. In choice tests, 4 or 8 plants of each accession were placed together in cages, instead of using 8 or 16 plants of one accession. The choice and non-choice tests each were performed three times. Development of TSWV symptoms and the number of larvae and adult thrips were scored on each plant at weekly intervals. The thrips were counted visually while turning and inspecting the leaves carefully to prevent dispersal of the adults. In addition, infection of test plants was monitored weekly by enzyme-linked

immunosorbent essay (ELISA) using extracts of a leaf disk (1.3 cm i.d.) cut from a top leaf of each plant.

Spread of TSWV from an infected source plant to TS and TR plants.
Secondary spread of TSWV (i.e. virus spread from infected to healthy plants) was studied after the release of non-viruliferous thrips to a TSWV-infected TR or TS plant surrounded by either 8 or 16 healthy plants of either accession. This resulted in four treatments with 8 plants and four treatments with 16 plants. Each treatment was replicated twice. Thirty non-viruliferous *F. occidentalis* adults were released at the base of the infected source plant in the middle of each cage. The number of thrips and the spread of the virus from the infected plant to the non-infected plants were scored at weekly intervals.

Greenhouse validation of thrips resistance on the spread of TSWV.
Spread of TSWV and thrips population development in TR and TS pepper were analysed in a greenhouse experiment in which these cultivars were exposed to the same thrips population. In total 15 plots were arranged, with each containing 25 plants. Five plots contained TS plants, 5 plots contained TR plants (non-choice plots), and 5 plots contained a 1:1 mixture of TR and TS plants (choice plots). Plants were grown in 5-liter plastic pots at a 45-cm distance within a row and at a 50-cm distance between rows. The 15 plots were surrounded by 'edge-plots' to prevent environmental influences affecting the outer-plots. Treatments were randomly assigned to the plots. Forty randomly selected adults, that were allowed to acquire TSWV as 0- to 4-h-old larvae on infected leaf disks, were released at 5 points in each plot when the plants were 4 weeks old. The experiment was conducted from February to April in Wageningen (the Netherlands), during which the temperature ranged from 23 to 31ºC during the day and was maintained at 20ºC during the night. Plants were watered daily with 200 ml of water using a dripping system, and grown under a 16-h light and 8-h dark cycle.
Infected plants and the number of thrips were scored at weekly intervals. Infection of plants was confirmed by ELISA. At the end of the experiment, thrips were collected from each plot and tested for their ability to transmit TSWV in a Petunia leaf disk assay (Wijkamp & Peters, 1993) to determine the percentage of viruliferous thrips per plot.

Virus detection by ELISA.

TSWV infection was monitored weekly by analysis of 1.3-cm-i.d. leaf disks by double-antibody sandwich ELISA (Clark & Adams, 1977; Resende *et al*., 1991). Each disk was ground in 150 μl of phosphate-buffered saline, containing 0.05% Tween 20, pH 7.2. Polyclonal antibodies, raised in rabbits, against the TSWV nucleocapsid protein were used at a concentration of 1 μg/ml.

Statistical analysis.

The percentage of virus-infected plants obtained at each weekly interval in the different experiments was analysed as binomial distributed variables with Genstat (Payne *et al.*, 1993) and compared for each treatment. The number of thrips found on TS and TR plants was analysed by Kruskal-Wallis one-way analysis of variance.

RESULTS

Spread of TSWV to TR and TS plants in non-choice tests.

In the non-choice tests, plants of the TR and TS accessions were separately exposed to adult thrips, of which an average of 27% were viruliferous (data not shown). The first virus-infected plants were observed 2 weeks after release of thrips in these tests (Fig. 3.1). Plants of the TS accession became readily infected in both independent replications but the number of infected TR plants remained significantly ($P<0.05$) lower and did not increase after 3 weeks following thrips release (Fig. 3.1). On the TS plants, thrips numbers started to increase 3 weeks after release, whereas no thrips could be discerned on the TR plants 3 weeks following release. The difference in thrips numbers between the accessions became significant ($P<0.05$) from the third week onward (Fig. 3.1).

Spread of TSWV to TR and TS plants in choice tests.

In the choice tests, in which released thrips had access to equal numbers of TS and TR plants, the first infected plants were found 2 to 3 weeks after thrips release (Fig. 3.2). Although eventually all plants became infected in this test, virus spread to the TR plants was significantly delayed compared with the TS plants. Thrips numbers increased 3 weeks after release and were not significantly ($P>0.05$) lower on the resistant plants, indicating that thrips reproduction on TS plants resulted in similar vector pressure on both accessions.

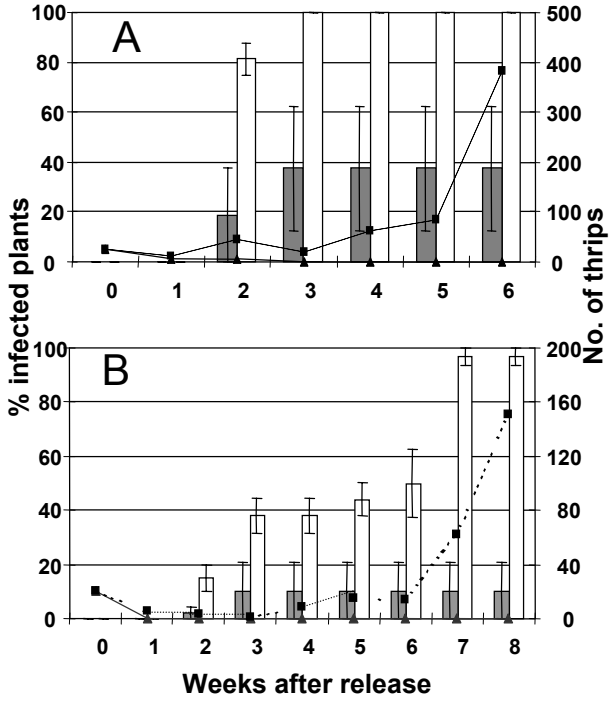

Figure 3.1. Development of *Tomato spotted wilt virus* infection in 8 (A) or 16 (B) thrips-susceptible (TS; white bars) or thrips-resistant (TR; dark bars) pepper plants in non-choice tests after release of viruliferous thrips. Error bars indicate the standard errors of the mean. Lines depict the average number of *Frankliniella occidentalis* on TS (dashed line with squares) or TR (solid line with triangles) plants.

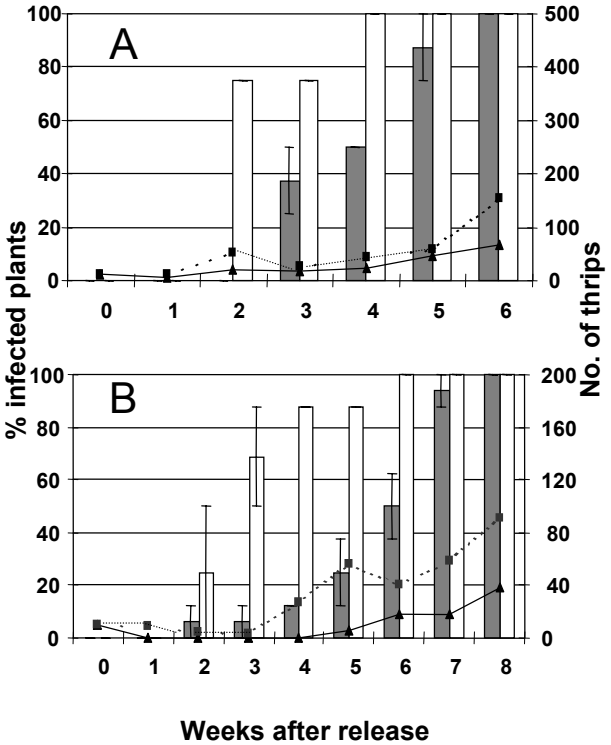

Figure 3.2. Development of *Tomato spotted wilt virus* infection in 8 (A) or 16 (B) pepper plants in choice tests after the release of viruliferous thrips. Half of the plants were thrips-susceptible (TS; white bars) and half were thrips-resistant (TR; dark bars). Error bars indicate the standard errors of the mean. Lines depict the average number of *Frankliniella occidentalis* on TS (dashed line) or TR (solid line) plants.

Spread of TSWV from an infected source plant to TS and TR plants.

Secondary spread of TSWV was assessed by releasing non-viruliferous adult thrips on an infected TS or TR source plant in cages together with non-infected plants of the TR or TS accession. In two independent experiments (using either 8 or 16 plants) the first infections were observed approximately 4 weeks after releasing the thrips (Figs. 3.3 and 3.4). After these initial infections, the number of TR plants becoming infected increased at a significantly lower rate ($P<0.05$) than the number of infected TS plants in the four different combinations of infected source and healthy test plants (Figs. 3.3 and 3.4). The rapid infection of TS plants occurred independently whether a TS or TR plant was used as virus source (Figs. 3.3 and 3.4). Compared with TS, the infection of TR plants was considerably delayed when 8 plants were exposed (Figs. 3.3A and 3.4A) and slightly lower when 16 plants were exposed to the virus sources used (Figs. 3.3B and 3.4B). This effect might be due to a lack of thrips population development on TR plants. Indeed, significantly ($P<0.05$) fewer thrips were consistently found on TR plants than on TS plants approximately 4 weeks after release. This trend was independent of virus source and number of plants per treatment (Figs. 3.3 and 3.4).

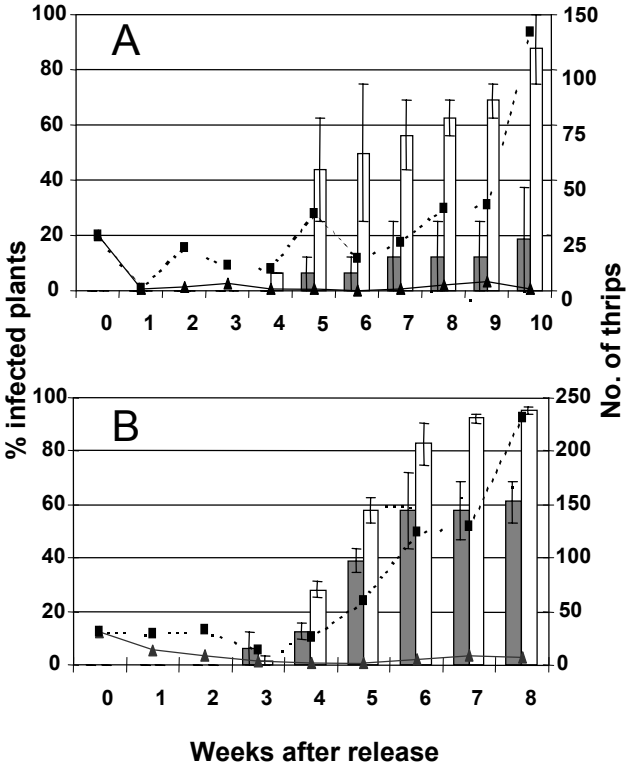

Figure 3.3. Spread of *Tomato spotted wilt virus* from an infected thrips-resistant (TR) plant after the release of non-viruliferous *Frankliniella occidentalis* to 8 (A) or 16 (B) thrips-susceptible (TS; white bars) or TR (dark bars) pepper plants. Error bars indicate the standard errors of the mean. Lines depict the average number of *Frankliniella occidentalis* on TS (dashed line with squares) or TR (solid line with triangles) plants.

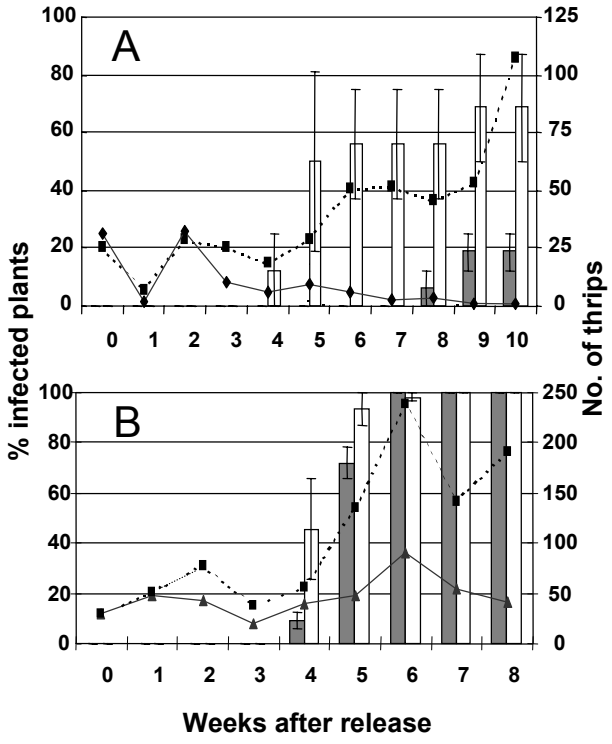

Figure 3.4. Spread of *Tomato spotted wilt virus* from an infected thrips-susceptible (TS) plant after the release of non-viruliferous *Frankliniella occidentalis* to 8 (A) or 16 (B) TS (white bars) or thrips-resistant (TR; dark bars) pepper plants. Error bars indicate the standard errors of the mean. Lines depict the average number of *Frankliniella occidentalis* on TS (dashed line with squares) or TR (solid line with triangles) plants.

Greenhouse validation of thrips resistance on the spread of TSWV.

The small-scale experiments described above indicate that a substantial level of thrips resistance may improve control of TSWV in pepper. To validate these results, performance of TR pepper was evaluated in a large-scale greenhouse experiment. Thrips were released at five points in each non-choice or choice plot. Of the released adults, 53% transmitted TSWV in a Petunia leaf disk assay (data not shown), hence approximately 21 of the 40 released adults in each plot were viruliferous. The first systemically infected TR and TS plants were observed 2 weeks after thrips release in both the non-choice and choice plots (Fig. 3.5). The number of initial infections in TR plots was considerably lower during the first 2 weeks and secondary spread was significantly delayed compared with TS plots ($P<0.05$). However, at 12 weeks after thrips release, 97% of the TR plants and all TS plants became infected. The delayed spread of the virus in the TR plots also was reflected in the percentage of viruliferous thrips sampled at the end of the experiment, being 14% (TR plots) compared to 25% (TS plots).

Thrips numbers were significantly lower on TR plants than on TS plants during the entire experiment in the choice and non-choice plots ($P<0.05$) (Fig. 3.5). Few thrips could be discerned on TR plants during the first 6 weeks after release, whereas high

numbers were found on the TS plants in this period. The increased thrips numbers on TR plants after 6 weeks might have been due the flowering of the plants and, hence, the availability of pollen, an essential food source for this thrips species.

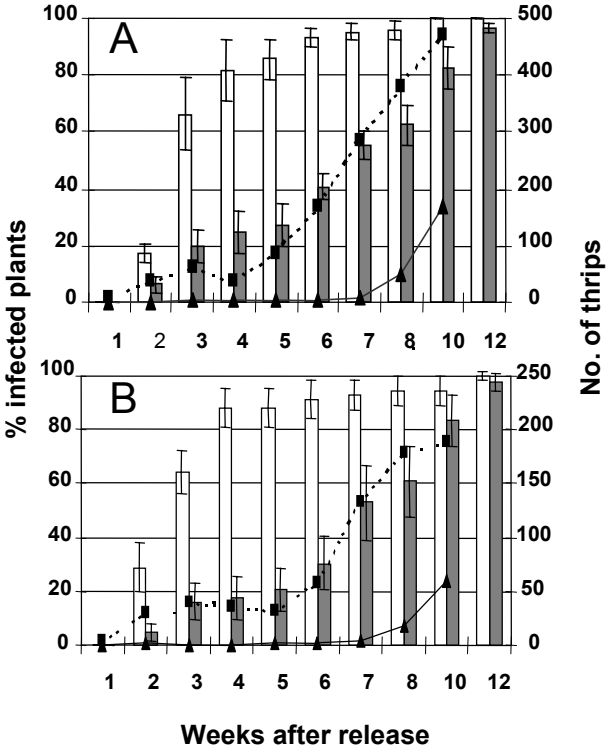

Figure 3.5. Development of *Tomato spotted wilt virus* infection in a thrips-resistant (TR; dark bars) or thrips-susceptible (TS; white bars) accession in greenhouse plots with plants of one accession (A) or in plots with a 1:1 mixture of TR and TS plants of both accessions (B) after the release of viruliferous *Frankliniella occidentalis*. Error bars indicate the standard errors of the mean. Lines depict the average number of *F. occidentalis* on TS (dashed line with squares) or TR (solid line with triangles) plants.

DISCUSSION

The effect of thrips resistance on spread of TSWV was assessed on TR and TS pepper accession in both choice and non-choice tests. In choice tests, released viruliferous thrips had access to plants of both accessions; whereas, in non-choice tests, access was limited to plants of one accession. Infections occurring during the first 2 weeks were considered primary infections, resulting from inoculations by introduced viruliferous thrips, whereas later, secondary infections were considered the result of inoculations by offspring (Figs. 3.1 and 3.2). In the small- scale studies, using 8 or 16 plants per accession, the primary infection rate was significantly lower for TR plants. Apparently, released thrips failed to reproduce on TR plants; therefore, no secondary spread occurred on these plants in the non-choice cage tests. In

contrast, the number of infected TS plants increased during the third and fourth week (Figs. 3.1A and 3.1B) due to successful vector reproduction.

In the choice tests, the number of primarily infected TR plants remained significantly lower than for TS plants (Fig. 3.2). The difference was larger than expected, because the inoculation rate was somewhat lower on TR than on TS leaf disks after inoculation access periods longer than 16 h (Maris *et al.*, 2003c; Chapter 2). This difference might be caused by factors playing a role in the transmission on plants, but not on leaf disks. These factors may be related either to a different (feeding) behaviour of thrips on TR plants, a more active dispersal of thrips to TS plants, or a higher preference for these plants. A different feeding behaviour of *F. occidentalis* and *Thrips tabaci* has been reported for thrips-resistant cucumber and leek, respectively (Harrewijn *et al.*, 1996), but the effect on TSWV transmission was not analysed in these studies.

In the choice tests, the rate of virus transmission to TR plants clearly differed from that in the non-choice tests. Spread in TR plants followed the same trend as for TS plants, but with a delay of 2 to 4 weeks (Figs. 3.2A and 3.2B). On TR plants thrips did not reproduce in the non-choice tests; hence, secondary spread to TR plants in the choice test was attributed to thrips that originated from the TS plants.

Secondary virus spread of TSWV was studied in choice tests by releasing non-viruliferous thrips in cages with an infected source plant and healthy plants. In two replicate experiments using either a cohort of 8 or 16 healthy plants, the rate of secondary spread of TSWV was significantly delayed among TR plants compared with TS plants.

Differences in virus spread to TR and TS plants were much smaller and reached higher levels when the virus was acquired from a TS source than from an infected TR source. Apparently, the efficient reproduction and development of the thrips on the TS source plant resulted in a more efficient virus spread. This indicates that infected TS plants support virus epidemics more efficiently than infected TR plants. Although TSWV is often spread in a greenhouse or field by primary infections (Camann *et al.*, 1995; Gitaitis *et al.*, 1998; Latham & Jones, 1997; Wilson, 1998), our results illustrate that the significance of secondary infections in TS crops should not be ignored.

The effect of thrips resistance on both virus spread and thrips population development, observed in small-scale experiments, was confirmed in a greenhouse experiment. Plots with plants of both pepper accessions were exposed to the same initial infection pressure. The rate at which TR plants became infected was significantly lower in both choice and non-choice plots, consistent with the results from small-scale experiments. On TR plants, fewer primary infections were produced by released viruliferous thrips and secondary spread was delayed. Thrips failed to reproduce on TR plants; therefore, the increase of infected TR plants is the result of secondary spread by thrips dispersing from TS plots. As the inoculation efficiency of

TSWV on TR and TS plants is similar (Maris *et al.*, 2003c; Chapter 2), the delayed secondary spread of TSWV to TR plants compared to TS plants may be due to slower dispersal of adults from TS to TR plants. An alternative explanation might be that adults prefer virus-infected TS plants over non-infected TR plants and, therefore, do not readily move from the infected plants in TS plots. The finding that a significantly higher percentage of viruliferous thrips were caught in the TS plots support limited dispersal between the plots.

The impeded virus spread due to thrips resistance of the host plant can be related to both restricted reproduction and low host preference, because fewer thrips are found on TR than on TS plants in the non-choice as well as choice plots (Fig. 3.5A and 3.5B). Restriction of virus spread on thrips-resistant accessions also has been found in other investigations using different virus-thrips-host systems. The lower incidence of *Groundnut bud necrosis virus* on thrips-resistant groundnut (cultivar Robut 33-1) was attributed to a lower thrips density compared to the more susceptible cultivar TMV 2 (Amin, 1985). Some *Lycopersicon esculentum* Mill. accessions were less susceptible to TSWV in thrips inoculation experiments than others, although all accessions were highly susceptible to mechanical inoculation of the virus (Kumar *et al.*, 1993). Vector resistance also reduced virus incidence in studies with *Grapevine fanleaf virus*, *Potato leafroll virus*, *Rice ragged stunt virus* and *Tomato yellow leaf curl virus,* which are transmitted by nematodes, aphids, planthoppers, or whiteflies, respectively (Berlinger *et al.*, 1986; Bouguet, 1981; Parejarearn *et al.*, 1984; Rizvi & Raman, 1983). The precise mechanism by which transmission is restricted remains unknown (Jones, 1987). Our previous work on the TR pepper accession revealed that thrips resistance does not necessarily lead to lower virus acquisition or inoculation frequency (Maris *et al.*, 2003c; Chapter 2). Apparently, the seemingly indirect effects of thrips resistance on vector pressure result in significant restriction of virus spread even in a host that is fully susceptible to the virus.

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Chapter 4

Spread of *Tomato spotted wilt virus* and population development of *Frankliniella occidentalis* in pepper resistant to thrips

The effect of thrips resistance on both the spread of *Tomato spotted wilt virus* (TSWV) and the population development of its major vector *Frankliniella occidentalis* was analysed on two resistant and susceptible pepper (*Capsicum* sp.) accessions. After release of viruliferous thrips, spread of TSWV was significantly lower in the primary and delayed in the secondary infection phase in plots with thrips-resistant accession CPRO-1 compared to plots with thrips-susceptible accession Pikante Reuzen. Similar results were obtained in plots with a 1:1 mixture of plants of both accessions.
Spread of TSWV to two virus-resistant accessions PI 152225 and PI 159236, resistant and susceptible to thrips, respectively, was delayed in the secondary phase to the former accession. The number of local lesions was considerably lower on the PI 152225 plants. Since the delay of the infection in the plots with thrips-resistant plants could only partly be explained by an impeded development of thrips populations, other factors, probably related to the behaviour of thrips, may also affect the transmission of TSWV to the resistant plants. The results obtained indicate that thrips resistance in pepper can be a useful tool in IPM strategies to control TSWV-infections.

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INTRODUCTION

Frankliniella occidentalis Pergande (Thysanoptera; Thripidae) is not only an important pest in many agricultural crops throughout the world, but is also one of the major vectors of *Tomato spotted wilt virus* (TSWV). This virus causes severe diseases in many crops like groundnut, sweet pepper, tobacco, and tomato in the subtropics and in many greenhouse-grown crops and ornamentals in temperate regions. The virus is transmitted in a propagative/circulative manner to a host range of at least 1100 plant species (Chatzivassiliou *et al.*, 2001; Peters, 2004). Control of TSWV is mainly based on the application of pesticides to reduce the vector population, and alternatively on measures as removal of infected plants and the use of virus-free stock material. Due to increased public awareness of potential hazards to the environment of pesticides, interest in breeding for pest resistance for controlling the spread of pathogens has increased ever since the 1960s (Kennedy, 1976).

Several studies have shown that vector resistance prevents virus spread (Amin, 1985; Berlinger *et al.*, 1986; Bouguet, 1981; Parejarearn *et al.*, 1984; Rizvi & Raman, 1983). However, resistance to insects in crops is not always effective in preventing virus-spread and even increases of virus incidence have been reported (Baerecke, 1958; van de Wetering, 1999). The effect of vector resistance on the incidence has to be explained by interactions between vector, virus, and plant, and mechanisms by which the virus is transmitted. However, a detailed analysis on virus-spread and vector population dynamics has not been made for most crops with vector resistance. The objective of this study was to analyse the spread of TSWV in thrips-resistant pepper under greenhouse conditions, in relation to thrips population development in both virus susceptible and virus-resistant accessions.

MATERIALS AND METHODS

Pepper accessions, thrips population and virus isolate.

The *Capsicum annuum* accessions CPRO-1 and Pikante Reuzen, and the *Capsicum chinense* accessions PI 152225 and PI 159236 were used in this study. The accessions CPRO-1, resistant to thrips, and Pikante Reuzen, susceptible to thrips, are equally susceptible to TSWV (Maris *et al.*, 2003c; Chapter 2). These accessions are hereafter indicated as TR (thrips-resistant) and TS (thrips-susceptible), respectively. The accessions PI 152225 and PI 159236 were known to be resistant to TSWV (Black *et al.*, 1991; Boiteux & de Ávila, 1994). However, these accessions are thrips-resistant and thrips-susceptible, respectively (Maris *et al.*, 2003c; Chapter 2).

The *F. occidentalis* population used was isolated from a greenhouse infestation in lucerne (*Medicago sativa*). To produce viruliferous thrips, 0- to 4-h-old larvae were confined to leaf disks (2.5 cm in diameter, i.d.) from TSWV-infected *Datura*

stramonium leaves in a Petri-dish (3.4 cm i.d.) on 1.5% agar. After an acquisition access period of 48 h, the larvae were repeatedly transferred to virus-free leaf disks of *D. stramonium* until the adults emerged. The percentage of viruliferous adults was determined by testing 100 randomly sampled adults in the Petunia leaf disk assay (Wijkamp & Peters, 1993).
The TSWV isolate BR01 (de Ávila *et al.*, 1990) was used in this study.

Spread of TSWV and population development of its vector in thrips-resistant and susceptible pepper.
Spread of TSWV in thrips-resistant pepper was studied in a greenhouse (22x17x4 m) experiment. Twenty-five plots with 25 4-weeks-old pepper plants were laid out in a greenhouse. The analysis was done in 20 plots with plants of the pepper accession TR, TS, PI 152225 or PI 159236 (non-choice plots), and in 5 plots with a 1:1 mixture of TR and TS plants (choice plots). Treatments were assigned to the plots in a Latin-square design. Plants were grown in 5-l plastic pots and placed at a distance of 50 cm between and 45 cm within the rows. The plots were surrounded by 'edge-plots' to prevent environmental influences affecting the outer-plots as much as possible. Plants were watered daily with 200 ml water using a dripping-system, and grown in a daily 16-h light and 8-h dark regime. The experiment was conducted from February to April in Wageningen, the Netherlands. The temperature in the greenhouse ranged from 23-31ºC at daytime and was kept at 20ºC during the night.
Forty randomly selected adults that were allowed to acquire TSWV as 0-4 old larvae on infected leaf disks were released in each plot at 5 spots. Plants were monitored for infection by visual inspection and by ELISA. The number of thrips on the plants was counted by visual inspection weekly.
The percentages of virus-infected plants found after weekly intervals were analysed as binomial distributed variables with Genstat and compared for each treatment (Payne *et al.*, 1993). The number of thrips found on the plants was analysed by the Kruskal-Wallis one-way analysis of variance method.

Susceptibility of PI 152225 and PI 159236 accessions to TSWV by thrips-mediated inoculation.
The inoculation efficiency of viruliferous thrips on the virus-resistant PI 152225 and PI 159236 accessions was studied by exposing 4-weeks-old plants to 1, 2, 4, 8 or 16 viruliferous adult thrips. In non-choice tests, plants were individually caged with a transparent cylinder closed with thrips proof gauze at the top, whereas in choice tests a PI 159236 plant was placed in a thrips-proof cage (45 by 30 by 40 cm) with a PI 152225 plant. Thrips could feed on these plants for 7 days, after which they were removed from the plants with a suction tube. During this inoculation period the thrips were daily counted. The local lesions produced were counted on the plants seven

days after removal of the thrips. There were 10 replicates per pepper accession in the non-choice tests, and 15 replicates in the choice-tests for each treatment.

RESULTS

Lower spread of TSWV and development of thrips in thrips-resistant pepper.
The spread of TSWV was studied in thrips-resistant pepper by releasing viruliferous thrips in plots with plants of thrips-resistant (TR and PI 152225) and susceptible (TS and PI 159236) accessions. Using a *Petunia* leaf disk assay (Wijkamp & Peters, 1993), 53% of the released adults were capable to transmit TSWV. The release of 40 thrips in each plot at five spots resulted in an equal infection pressure in each plot and over the whole experiment.
The first systemically infected TR and TS plants in the non-choice plots were observed two weeks after thrips release (Fig. 4.1). After the appearance of the first infected plants, the virus incidence in TR plots progressed at a significant lower rate than in the TS plots ($P<0.05$). Nevertheless, 12 weeks after the release of the thrips, 97% of the TR plants and 100% of the TS plants were infected. The number of thrips observed was significantly lower on the former than on the latter plants during the whole experiment ($P<0.05$). Thrips could rarely be observed on the TR plants in the first 7 weeks after their release, but their number increased rapidly after the appearance of the first flowers. With the increase of thrips on the TR plants, the number of infected plants started to increase more rapidly (Fig. 4.1).

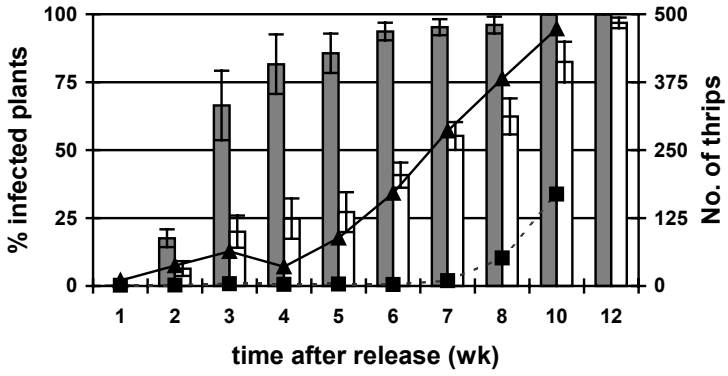

Figure 4.1. The development of a TSWV-infection in virus-susceptible TR plants (white bars) and TS plants (grey bars) after the release of viruliferous *F. occidentalis* thrips in plots with plants of one accession (non-choice plots). Lines depict the thrips population development on the TS (solid line with triangle) or TR (dashed line with squares) plants. Error bars indicate the standard errors of the mean.

The first infected TR and TS plants in the plots with a 1:1 mixture of plants were also found two weeks after the release of the thrips (Fig. 4.2). Virus incidence on TR and TS plants in these plots developed at a similar rate as in the single accession plots. Again the thrips numbers on the TR plants were significantly lower than on the TS plants ($P<0.05$).

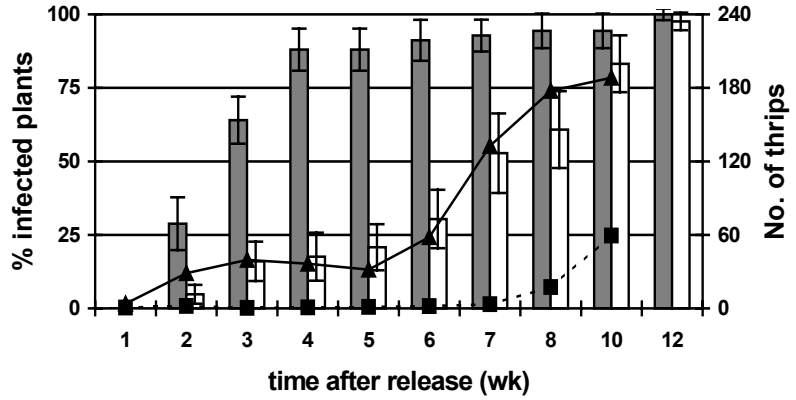

Figure 4.2. The development of a TSWV-infection in virus-susceptible TR plants (white bars) and TS plants (grey bars) accession after the release of viruliferous *F. occidentalis* thrips in plots with plants of both accessions (choice-plots). Lines depict the thrips population development on TS (solid line with triangle) or TR (dashed line with squares) plants. Error bars indicate the standard errors of the mean.

Reduced local lesion and thrips development in virus-resistant pepper.

In the previous paragraph the effect of vector resistance on the spread of TSWV in virus-susceptible pepper accessions was described. The use of virus-resistant plants will be the favoured way to control virus infections in crops. This trait is present in the thrips-resistant accession PI 152225 and the thrips-susceptible accession PI 159236, reacting with the formation of local lesions (cosmetic damage) on leaves and fruits after inoculation in consequence of a hypersensitive response. The effect of thrips resistance on the production of local lesions and the development of the thrips was assessed on these virus-resistant accessions.

The percentage of plants with local lesions developed at a significantly higher rate on the thrips-susceptible PI 159236 than of the thrips-resistant PI 152225 plants (Fig. 4.3). An average of 136 lesions per PI 159236 plot was significantly higher than the 4 lesions found in PI 152225 plot after 7 weeks (data not shown). Only few thrips could be observed on both accessions during the first 7 weeks after their release, but their number increased rapidly after the appearance of the first flowers (Fig. 4.3). The number of thrips observed on the PI 152225 plants was only significantly lower in week 6 after the onset of this experiment ($P<0.05$).

The rate at which the virus-resistant accessions became infected was considerably lower than that of both virus-susceptible accessions (Fig. 4.3 vs. Figs. 4.1 and 4.2).

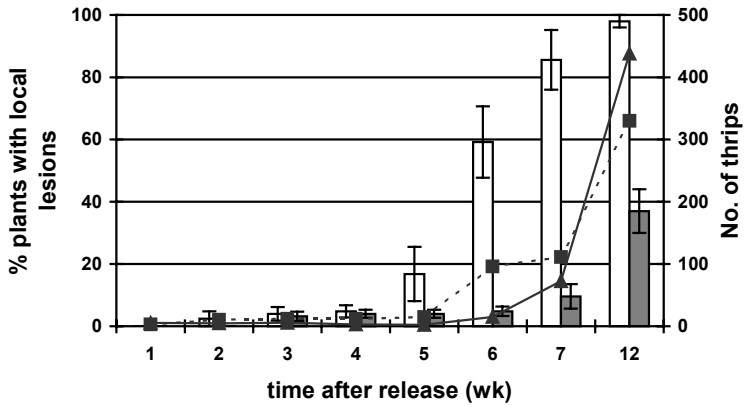

Figure 4.3. Development of local lesions on virus-resistant plants of a thrips-resistant (PI 152225; dark bars) and susceptible (PI 159236; white bars) accession after the release of viruliferous *F. occidentalis* thrips in plots with plants of only one accession (non-choice plots). Lines depict the thrips population development on the PI 152225 (solid line with triangles) or PI 159236 (dashed line with squares) plants. Error bars indicate the standard errors of the mean.

Development of local lesions on the virus-resistant accessions.

Plants of both virus-resistant accessions PI 152225 and PI 159236 were exposed to increasing numbers of viruliferous thrips to analyse the relation between the number of thrips and the number of local lesions produced. The number of local lesions on both accessions increased with increasing number of thrips placed on the plant and appeared to be almost similar when the plants were infested with the same number of viruliferous thrips in non-choice tests (Fig. 4.4).

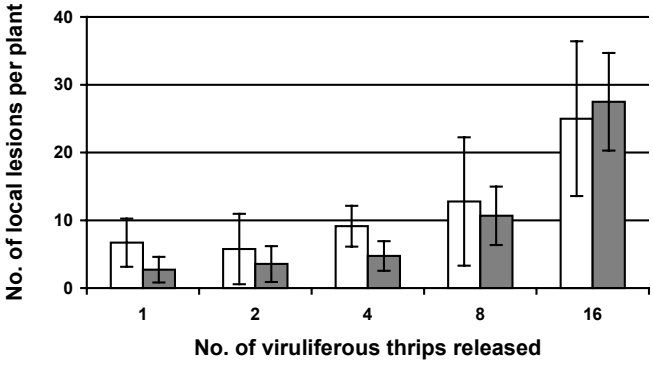

Figure 4.4. Development of local lesions on PI 159236 (white bars) and PI 152225 (dark bars) plants after confining viruliferous thrips to individual plants (non-choice tests).

Plotting the number of local lesions against the product of the number of thrips and the number of days they survived shows that the thrips produced similar numbers of local lesions on PI 159236 and PI 152225 plants and that they survived at an almost identical rate on plants of both accessions (Fig. 4.5).

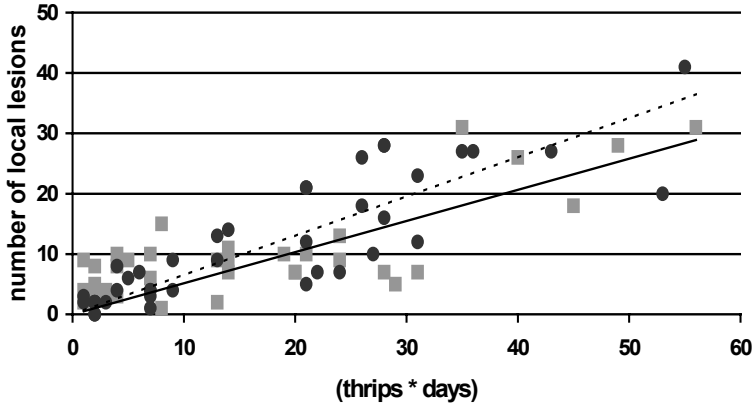

Figure 4.5. Infection of PI 152225 and PI 159236 plants by thrips confined on individual plants (non-choice tests). The thrips population is expressed as the product of thrips numbers released on the plants and the number of days they could visually be recovered (thrips * days). Number of local lesions on PI 152225 plants: black dots, on PI 159236 plants: grey squares. Lines depict regression curves of the infection for the PI 152225 (dashed line) and the PI 159236 (solid line) accession.

However, in choice tests in which thrips could infest both types of plants, significantly more local lesions were found on thrips-susceptible PI 159236 plants than on the thrips-resistant PI 152225 plants after infestation with 2, 4, or 8 viruliferous thrips, ($P<0.05$; Fig. 4.5). Remarkably, an almost identical number of local lesions was found on plants of both accessions when 16 viruliferous thrips were released.

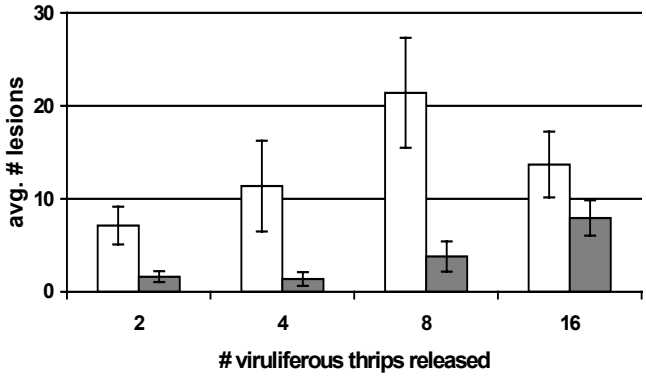

Figure 4.6. The average number of local lesions produced on PI 159236 (white bars) and PI 152225 (dark bars) plants after the release of viruliferous thrips in cages with one plant of both accessions (choice tests).

DISCUSSION

The influence of thrips resistance on both the spread of TSWV and the population development of *F. occidentalis* was analysed in pepper accessions differing in their resistance to either the vector or the virus. This study was made on a semi-large scale in a greenhouse with 25 plots of the four pepper accessions, and in which thrips could freely move between the accessions. A primary and secondary infection phase can be distinguished after the introduction of cohorts of viruliferous thrips. The primary infections are the result of the inoculations made by the released thrips at the start of the experiment and the secondary infections by the offspring of the released thrips.

At the onset of the primary phase, a uniform infection pressure may have existed following the release of equal numbers of thrips in each plot. On application of such an infection pressure it could be expected that a similar number of TR and TS plants would become primary infected, as the plants are equally susceptible to TSWV (Maris *et al*., 2003c; Chapter 2). However, the incidence of primary infected plants was significantly lower in the vector-resistant TR plots than in the vector-susceptible TS plots. This lower initial infection might indicate that either the thrips rapidly left the thrips-resistant plants before making a successful inoculation, or that when they do not move to the TS plants, other factors negatively affect the successful inoculation of the TR plants. In a previous study using leaf disks it has been demonstrated that thrips resistance hardly affects the inoculation efficiency (Maris *et al*., 2003c; Chapter 2). In that study the thrips were more or less forced to feed, and thus to inoculate the disks. In the present greenhouse experiment the thrips could freely move and were not forced to feed on the resistant plants, which might explain the difference between the results in these experiments. It remains unknown from the present results whether thrips differ in feeding behaviour on thrips-susceptible and resistant plants resulting in less virus inoculations on the latter. Evidence for an altered feeding behaviour of vectors on resistant plants was shown in other studies, with plants resistant for leafhoppers (Khan & Saxena, 1985), for aphids (Haniotakis & Lange, 1974) or for thrips (Harrewijn *et al.*, 1996). Leafhoppers made more probes on resistant cultivars (Khan and Saxena, 1985), while a greater restlessness of thrips was observed on resistant than on susceptible cucumber plants (Harrewijn *et al.*, 1996). This altered feeding behaviour may also result in more virus spread as shown in studies on chrysanthemum resistant to thrips (van de Wetering, 1999) and with aphids in resistant potato (Baerecke, 1958).

Since the first larval instars can acquire virus from systemically infected TR and TS plants approximately 8 days after the release of adults, symptoms of secondary spread of TSWV can be expected 3 weeks later. The incidence progressed in this

phase at a significant lower rate on the thrips-resistant TR than on the susceptible TS plants, although almost all plants became finally infected (Figs. 4.1 and 4.2). This difference in virus spread has to be explained by the poor reproduction or absence of any reproduction of thrips on the TR plants (Maris *et al.*, 2003c; Chapter 2). The increase, although slow, of the number of infected TR plants will be the result of a dispersal of thrips from the TS to the TR plots. This dispersal does not result in similar numbers of thrips in both plots (Fig. 4.1 and 4.2) by which the incidence of infected plants in the TR plots remains lower. The absence of an equilibrium in thrips numbers on the TR and TS plants demonstrates that the dispersal between the plots was rather restricted or that the thrips moved more frequently from the TR to the TS plants than vice versa.

To assess whether thrips resistance would result in a decreased local lesion induction and, therefore, less cosmetic damage in TSWV-resistant pepper, spread in plots with the two TSWV-resistant accessions PI 152225 and PI 159236, which are thrips-resistant and thrips–susceptible, respectively, was studied.
Both the number of thrips-resistant PI 152225 plants showing local lesions (Fig. 4.3), and the number of local lesions produced per plot on the leaves of the resistant plants were significantly lower than on PI 159236 plants. The difference in the number of infected PI plants and local lesions can not be explained by the slightly higher thrips numbers on the PI 159236 plants (Fig. 4.3). Moreover, a difference in susceptibility for TSWV can be excluded, as mechanical inoculation of both PI accessions using a dilution series of inocula produced similar numbers of local lesions (data not shown). Both accessions appeared also to be equally susceptible in non-choice tests in which thrips were confined to single plants of either accession (Fig. 4.4). This similar susceptibility of both accessions after thrips-mediated virus inoculation and the large difference between the number of infected plants in the greenhouse indicates that other factors than population density play a large role in the spread of TSWV when the thrips have free access to plants. This conclusion was confirmed in the choice-test cage experiments, in which viruliferous thrips had free access to thrips-resistant PI 152225 and susceptible PI 159236 plants. The higher number of local lesions on PI 159236 plants indicate that the thrips made more successful inoculations on this accessions than on PI 152225 plants as both accessions are equally susceptible to TSWV. No difference between the number of lesions on the thrips-resistant and susceptible plants was obtained when 16 viruliferous thrips were released on the plants. This was mainly due to lower numbers of local lesions found on thrips-susceptible PI 159236 plants. The thrips preferred to feed on the edge of the leaves where also most of the lesions were found. Therefore, feeding damage and local lesions produced could not be differentiated clearly.

In the present study, thrips resistance was found to have a significant positive effect on reducing virus spread in both virus-susceptible accessions, resulting in lower numbers of infected plants, and in virus-resistant accessions, resulting in less cosmetic damage (local lesions). One of the main factors responsible for the reduced virus spread in the thrips-resistant accessions was the low thrips reproduction on and low preference for plants of these accessions, but these factors can not completely explain the low primary infections and the delay of the infection of the thrips-resistant plants. Nevertheless, the results show that thrips resistance will be a useful tool in IPM programs to control TSWV spread, in combination with cultural measurements, e.g. using thrips and virus-free stock material, biological control of thrips and removing virus infected plants.

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Chapter 5

Decreased preference and reproduction, and increased mortality of *Frankliniella occidentalis* on thrips-resistant pepper plants

The effect of thrips resistance in pepper (*Capsicum annuum*), previously shown to result in impeded thrips population built-up (Maris *et al.*, 2003a,b; Chapters 3 and 4), on thrips' (*Frankliniella occidentalis* Pergande) reproduction, mortality, host preference and behaviour was investigated. With respect to reproduction, both oviposition and larval survival were negatively affected by the thrips-resistant (TR) phenotype, whereas the offspring's developmental rate from egg to adult was not affected. A significant preference for thrips-susceptible (TS) control plants over the TR plants was observed in different tests, although thrips' behaviour was hardly affected by thrips resistance.
When released on either a TR or a TS plant, thrips dispersed at higher rates from the TR plants, demonstrating that not only an impeded reproduction, but also a higher emigration adds to the reported lower thrips numbers on TR plants.

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INTRODUCTION

The western flower thrips, *Frankliniella occidentalis*, is a polyphagous insect species. With its 'piercing-sucking' stylets, *F. occidentalis* penetrates epidermal and sub-epidermal cells from which the contents are ingested, causing extensive damage on the plants (Hunter & Ullman, 1989). Damage consists of silvery scars on fruits and leaves, of growth reduction and deformation of plants. Cosmetic damage can often been found on fruits and flowers (van Dijken *et al.*, 1994; de Jager *et al.*, 1995), making them less valuable or even unmarketable (Parrella & Jones, 1987). In addition to this direct feeding damage, the western flower thrips causes indirect damage by transmitting tospoviruses (family *Bunyaviridae*) (German *et al*., 1992; Ullman *et al.*, 1997; Wijkamp *et al.*, 1995a). *Tomato spotted wilt virus* (TSWV), the type species of the *Tospovirus* genus, is worldwide considered as one of the economically most important viruses (German *et al.*, 1992; Goldbach & Peters, 1994). TSWV can infect over 1100 plant species including a considerable number of agricultural and horticultural crops, as well as many weed species (Chatzivassiliou *et al.*, 2001; Peters, 2004).

Significant levels of thrips resistance have been reported in commercial accessions of cabbage (Stoner & Shelton, 1986), chrysanthemum (de Kogel *et al.*, 1998), groundnut (Kinzer *et al.*, 1973; Robb & Parella, 1989), pepper (Fery & Schalk, 1991; Maris *et al.*, 2003c; Chapter 2) and tomato (Kumar *et al.*, 1995). Although thrips resistance negatively affects the vector's population development, virus spread may be positively affected. As tospoviruses can be transmitted in short inoculation access periods and thrips may move more frequently between plants of resistant accessions in search for a better host, the use of thrips-resistant cultivars may thus potentially lead to enhanced virus spread. Evidence for such adverse effect of thrips resistance comes for instance from comparative studies on chrysanthemum cultivars (van de Wetering, 1999). However, for pepper it was observed that primary TSWV-infection rates were considerably lower in a thrips-resistant (TR) than in a thrips-susceptible (TS) pepper accession when exposed to an equal vector pressure (Maris *et al.*, 2003a,b; Chapters 3 and 4). This lower rate of primary virus-infections was not caused by differences in virus acquisition and inoculation rates (Maris *et al.*, 2003c; Chapter 2) and should therefore be due to other factors such as initial host acceptance or altered thrips' behaviour on the TR host. Thrips resistance in cucumber and leek has been shown to lead to a disturbed feeding behaviour of *F. occidentalis* and *Thrips tabaci*, respectively, resulting in a lower number of penetrations and a reduction of feeding time (Harrewijn *et al.*, 1996). Thrips also showed an increased restlessness and searched more often for new feeding sites on thrips-resistant plants (Harrewijn *et al.*, 1996). Hence, altered (feeding) behaviour has

indeed been reported for thrips-resistant host plants and this might potentially lead to undesired enhanced spread of TSWV.

Secondary TSWV-spread was found to be delayed in thrips-resistant plants, even in mixed stands with thrips-susceptible plants (Maris *et al*, 2003a,b; Chapter 3 & 4). This delayed infection rate can at least for a major part be explained by a strongly impeded reproduction of thrips on this cultivar (Maris *et al*., 2003c; Chapter 2). It has remained unknown, though, whether decreased oviposition, poor egg hatching, hampered larval development, or increased mortality were responsible for the limited thrips reproduction on TR plants. Besides an impeded population built up, a net migration balance to the susceptible plants in mixed stands may have contributed to the lower infection rate of the thrips-resistant plants.

To investigate the population on TR plants further, the oviposition rate, egg hatching rate and development from egg to adult were compared on thrips-resistant and thrips-susceptible pepper plants. Besides, migration of *F. occidentalis* between and mobility on resistant and susceptible plants were determined in the present study.

MATERIALS AND METHODS

Thrips population and pepper material used.

A population of *F. occidentalis* 'IS2' (van de Wetering *et al.*, 1999), isolated from mango, was maintained on greenhouse chrysanthemum. The *Capsicum annuum* accessions "CPRO-1" and "Pikante Reuzen", resistant and susceptible to *F. occidentalis*, respectively, were used (Maris *et al.*, 2003c; Chapter 2). These accessions will further be referred to as TR (thrips-resistant) and TS (thrips-susceptible), respectively.

Oviposition, egg hatching rate and life history parameters of thrips on TR and TS plants.

To study the effect of thrips resistance on oviposition of *F. occidentalis*, two females were allowed to lay eggs on caged TR or TS plants for 12 hours after which they were removed from the plants using an aspirator. Hatched larvae were counted daily and then removed. After 7 days, when all vital larvae should have hatched, leaves were stained with methyl-red to detect unhatched eggs in the leaf tissue. This experiment was performed with 12 TR and 12 TS plants.

Duration of the development of the larvae and pupae was studied by transferring 0- to 4-h-old larvae that hatched on bean pods to caged TR or TS seedlings. After 4 days, before pupation, 34 second instars were individually transferred for pupation to leaf disks from plants of the same accession. Plants and leaf disks were incubated in a climate chamber at 24°C under a light and dark regime of 16 and 8 h, and the developmental stages of the thrips were scored at intervals of 12 h. Possible effects

of thrips resistance on the developmental rate of thrips were analysed by simple linear regression models. Computations to indicate statistical significance were based on 95% confidence intervals.

Migration of thrips between TR and TS plants.

Thrips migration among thrips-resistant or -susceptible pepper plants was studied in two experimental set-ups. In one set-up, dispersal of thrips towards 4-week-old TR or TS plants was determined after releasing 20 adult thrips from a spot between two plants placed at a distance of 30 cm from each other in transparent plastic cages (45 by 30 by 40 cm). In the second set-up, dispersal from either a TR or a TS plant was monitored after releasing 20 adults on one of the two plants. In both set-ups, the thrips could move between a TR and a TS plant (choice test) or between two TR or TS plants only (non-choice tests). The dispersal was determined by counting the thrips on each plant at every hour in the first 8 hours and then at 24, 48, 72 and 96 h after their release. The choice and non-choice tests were replicated 18 times. Cages were placed in climate chambers at 24°C under a light and dark regime of 16 and 8 h. Dispersal rates of thrips from TR and TS plants were compared by analysing the log-transformed numbers of thrips recovered on the plants as binomial distributed variables by analysis of variance using general linear models and Genstat (Payne *et al.*, 1993).

Host finding of thrips.

To study whether the dispersal of an adult thrips is directed towards a plant or is made at random, and whether this is affected by the thrips-resistant phenotype, thrips were released in cages of different sizes. In one set-up, a 5-week-old TR or TS plant was transplanted in soil and covered with the cage. Cages measured 15 by 15 by 15 cm, 30 by 20 by 30 cm and 30 by 30 by 42 cm, respectively. Twenty thrips were released from a small vial placed on the soil at 6 cm distance from the base of the plant. The number of thrips present on the plant was counted every 24 h after their release for 1 week.

In a second set-up, two Xerox transparencies were covered with Tanglefoot insect glue and vertically placed between two sticks at 12 cm from the base and the vial. The transparencies placed parallel in the small, medium and large cages were 10 by 14 cm, 15 by 29 cm and 25 by 29 cm, respectively. The thrips present on the plants and caught on the transparencies were counted every 24 h after their release for 1 week. Both type of experimentals were twice replicated with 4 cages with plants of either the TR or TS accession. The experiment was performed at 24°C under a light and dark regime of 16 and 8 h. The number of thrips present on the TR and TS plants were compared per cage size for each time interval by analysis of variance method.

Behaviour of thrips on TR and TS plants.

To analyse the behaviour of *F. occidentalis* adults on TR and TS plants, twenty thrips were released on one of the top leaves of either plant. The duration of periods that a randomly selected individual thrips was "walking" or "resting" in a period of 10 min on one of the leaves was visually monitored. This was done at 5 min and at 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72 and 96 h after thrips release and in six replications for each time point on both TR and TS plants. The experiment was performed at room temperature under a light and dark regime of 16 and 8 h. Total fraction of time and number of periods that thrips were "resting " on plants of both accessions in the behaviour studies were analysed as binomially and Poisson distributed variables, respectively, by regression analysis.

RESULTS

Oviposition, egg hatching rate and life history parameters of thrips on TR and TS plants.

As previously shown, the built up of a *Frankliniella occidentalis* population on the thrips-resistant pepper accession CPRO-1 is drastically impeded (Maris *et al.*, 2003a,b,c; Chapter 2 & 3 & 4). To investigate which life-stage(s) in the thrips reproduction cycle is negatively affected by the thrips-resistant (TR) phenotype, oviposition rate, egg-hatching rate, larval development and mortality rates on this TR accession were compared with those on thrips-susceptible (TS) plants. Significantly ($P<0.05$) less offspring were produced per adult on the TR plants (0.2 ± 0.1) compared to the TS plants (1.2 ± 0.3). Unhatched eggs were not discerned in TR leaves after staining with methyl-red, while only 0.3 eggs per adult did not hatch on the TS leaves (data not shown). The larval mortality rate, though, was significantly higher on the TR (28/34 *versus* 7/34 for TS), whereas none of the pupae on either phenotype died (data not shown). Duration of larval development and of the pupal stage was similar on both pepper accessions (Table 5.1).

These results indicate that decreased oviposition at one hand, and increased larval mortality at the other, contribute to the previously reported lower thrips densities on TR pepper plants (Maris *et al.*, 2003c; Chapter 2).

Table 5.1. Duration (days) of different life-stages of *Frankliniella occidentalis* on the thrips-resistant (TR) and susceptible (TS) *Capsicum annuum* plants (± sem). Numbers between brackets indicate the number of thrips that completed a certain life-stage.

Host	Larval	Prepupal	Pupal	Total
TR	7.8 ± 1.1[a*] (6)	2.1 ± 1.6[a] (5)	2.3 ± 0.8[a] (5)	12.7 ± 1.3[a] (5)
TS	8.1 ± 0.5[a] (27)	1.5 ± 0.5[a] (25)	1.6 ± 0.7[a] (25)	11.2 ± 0.7[a] (25)

[*]The same superscript letters in a column for each life-stage indicate no differences within the treatments TR and TS (P>0.05).

Increased dispersal of thrips from TR plants.

A second important feature of the TR phenotype keeping thrips numbers low, might be an altered balance between immigration (preference) and emigration (dispersal). To study this possible effect, cohorts of 20 females were allowed to choose between one TR and one TS plant (choice tests), and their preference for either host plant was monitored. Fewer thrips were consistently found on TR than on TS plants (Fig. 5.1A). The number of thrips recovered on TR and TS plants differed significantly at 72 and 96 h after their release. The cumulative numbers of thrips found at each time point on the TR or TS plants, 7.9 ± 3.2 and 18.6 ± 4.1 respectively, differed also significantly (P<0.05).

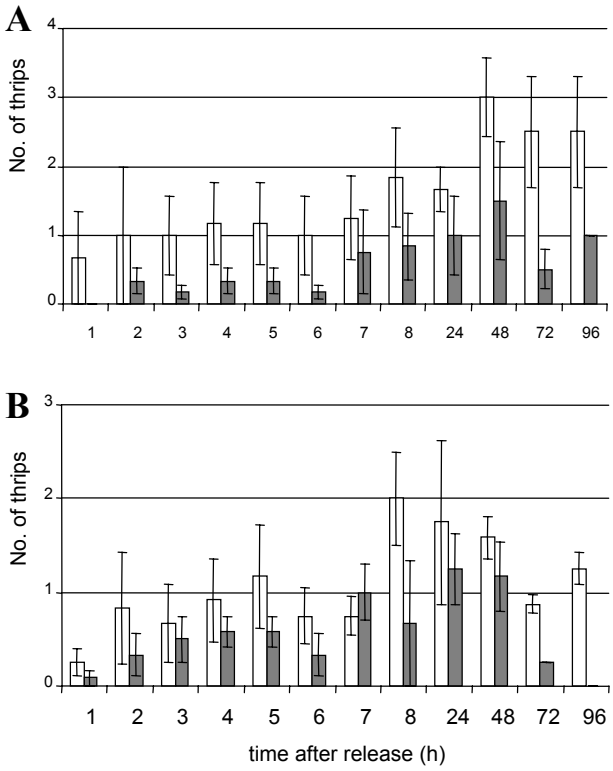

Figure 5.1. Number of *Frankliniella occidentalis* females recovered on individual plants of the thrips-resistant (TR; closed bars) and of the thrips-susceptible accession (TS; open bars) in choice (A) and non-choice tests (B). Twenty adults were released at a spot between the two plants. Error bars indicate the standard error of the mean.

A similar trend was found in the non-choice tests, in which the thrips were released between two plants of the same accession. After 72 h and 96 h, thrips numbers were significantly lower on the TR plants than on the TS plants. Also, the cumulative number of thrips recovered on the TR plants was significantly ($P<0.05$) lower than on the TS plants, 6.8 ± 0.9 and 12.8 ± 0.7, respectively (Fig. 5.1B). The majority of the 20 released thrips could not be recovered on the plants in both the choice (Fig. 5.1A) and non-choice (Fig. 5.1B) tests.

In a slightly different set-up, females were released on one of the two plants to be compared in stead of on a spot between the plants. Thrips could move between two TR or between two TS plants (non-choice tests; Fig. 5.2), or between a TR and a TS plant (choice tests; Fig. 5.3). Of the 20 females released in each treatment, approximately 12 were recovered after 1 h on the TR or TS plant on which they were released (Figs. 5.2 and 5.3). Beyond this first hour, the thrips dispersed slowly from the source plant and colonised the companion plant at low rate, whereas most of the thrips went astray in the cage. When two plants of the same pepper accession were exposed, similar numbers of thrips were recovered on both plants after approximately 48 h (Fig. 5.2), but numbers were significantly lower on the TR plants (Fig. 5.2B) than on the TS plants (Fig. 5.2A) after 48 h.
In tests in which the thrips could choose between a TS and a TR plant, significant more thrips were recovered on the TS plant on which they were released during the whole test period (Fig. 5.3A). When released on a TR plant, only the first 24 h most thrips were recovered on this TR plant (Fig. 5.3B), after which the majority had migrated to the TS plants. Thrips dispersal was signifiancly higher from TR (Figs. 5.2B and 5.3B) plants than from TS (Figs. 5.2A and 5.3A) plants independent of whether the neighbouring plant was resistant or not ($P<0.05$). Log-transformation of thrips numbers on the plants and the intervals confirmed this conclusion (Table 5.2). Average slopes of the regression lines show that the thrips left the TR plants (slope: -0.75) at higher rates than TS plants (slope: -0,26).

Table 5.2. Regression functions after log-transformation of the number of *Frankliniella occidentalis* (Y) recovered on thrips-resistant (TR) and susceptible (TS) *Capsicum annuum* plants. Twenty thrips were released on a source plant that was placed with another plant (neighbouring plant) in a thrips-proof cage. "R^2"-column indicates the correlation coefficient (R^2) of the regression function. Last column gives the corresponding figure in which the non-transformed thrips numbers are given.

Source plant	Neighbouring Plant	Regression function	R^2	Corresp. Figure
TS	TS	Y = -0.28x +1.12 [a*]	0.94	5.2A
TR	TS	Y = -0.75x +1.38 [b]	0.85	5.3B
TS	TR	Y = -0.23x +1.09 [a]	0.94	5.3A
TR	TR	Y = -0.75x +1.34 [b]	0.86	5.2B

* different letters in two subsequent rows indicate significant differences between dispersal rate of thrips from a TR source plant and a TS source plant to a neighboring plant.

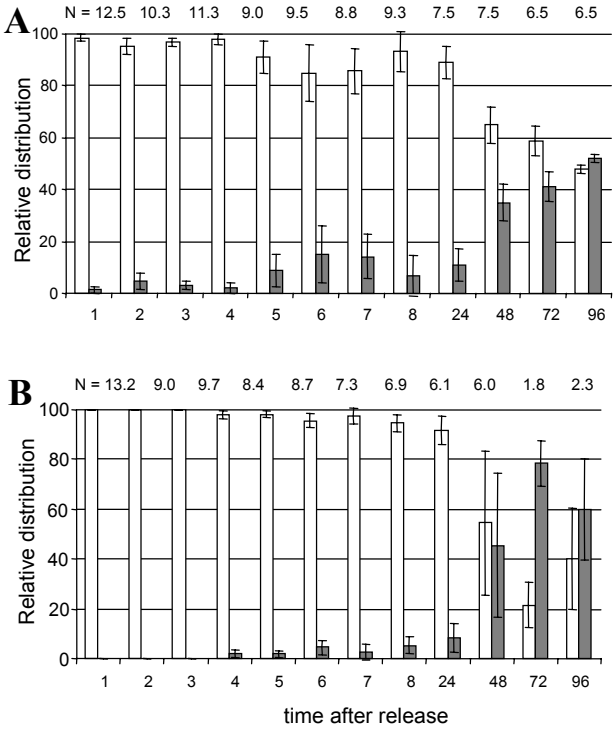

Figure 5.2. Average relative distribution of *Frankliniella occidentalis* over two thrips-susceptible (TS) (A) or thrips-resistant (TR) (B) plants placed in a cage (non-choice tests). Twenty adults were released on one plant (white bars) and could disperse to a neighbouring plant (dark bars). Thrips were counted every hour in the first 8 h after release and then every 24 hours. Error bars indicate the standard error of the mean. Numbers presented above the figures indicate the average total number of thrips recovered for each time point.

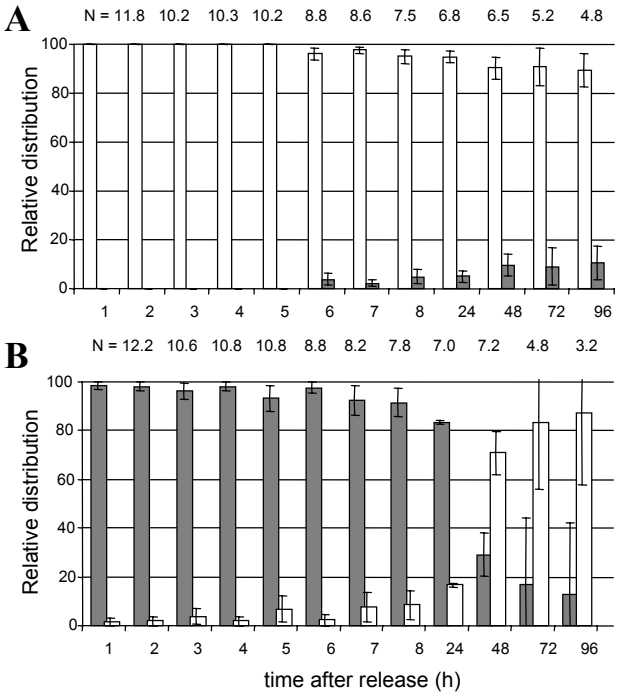

Figure 5.3. Average relative distribution of *Frankliniella occidentalis* over a thrips-susceptible (TS; white bars) or a thrips-resistant (TR; dark bars) plant placed in a cage (choice tests). Twenty adults were released on the TS plant (A) or on the TR plant (B). For further details: see legend Fig. 5.2.

Host finding of thrips.

Since most of the thrips went astray in the cages in the experiments described above, the question arose whether thrips would disperse randomly rather than being predetermined to host plants. To answer this question, a study was made in cages of different size to determine whether the recovery of thrips on the plants would depend on cage size. Thrips numbers recovered on the TR plants were consistently lower than on the TS plants (Fig. 5.4). The average number of thrips on the TS plant in the smallest cage was only 24 h after release significantly ($P<0.05$ higher) than on the TS plant in the larger cages (Fig. 5.4). These results demonstrate that the cage size did not affect the number of thrips recovered on both the TR and TS accession at all other intervals sampled (Fig. 5.4).

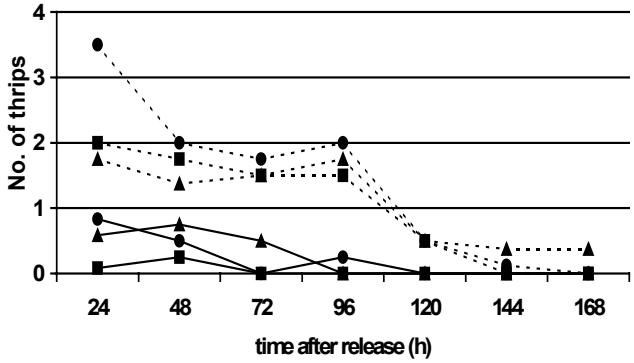

Fig. 5.4. Average number of *Frankliniella occidentalis* on individual TR (solid lines) or TS (dashed lines) plants placed in small (lines with dots), medium (lines with squares) or large (lines with triangles) cages. Twenty adults were released from a pipette tip at 6 cm distance from the stem base.

When sticky traps were placed at two sides of the plant in the cages, considerable numbers were caught on these traps within 24 h after their release, whereas hardly any thrips could be recovered on the TS (Fig. 5.5) or TR (Fig. 5.6) plants. The large number of thrips recovered on the traps suggests that thrips disperse randomly rather than in a predetermined way towards the plants.

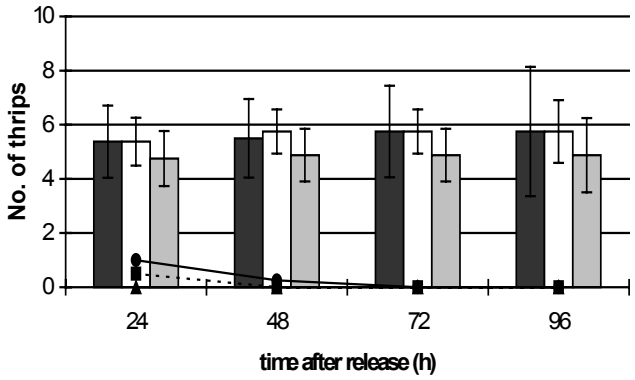

Fig. 5.5. Average number of *Frankliniella occidentalis* recovered on sticky traps in small (black bars), medium (white bars) or large (grey bars) cages with a thrips-susceptible (TS) plant. Lines depict the average number of thrips on the TS plant in the small (solid line with dots), medium (dashed line with squares) or large (solid line with triangles) cages and are (partly) not separately visible as the data coincide with each other. Traps were placed on two sides of the plants and 20 adults were released from a pipette tip at 6 cm distance from the stem base. Error bars indicate the standard deviation.

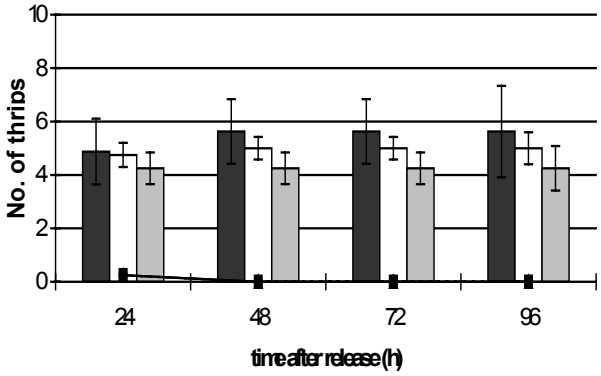

Fig. 5.6. Average number of *Frankliniella occidentalis* recovered on sticky traps in small (black bars), medium (white bars) or large (grey bars) cages with a thrips-resistant (TR) plant. Lines depict the average number of thrips on the TR plant in the small (solid line with dots), medium (dashed line with squares) or large (solid line with triangles) cages, and are (partly) not separately visible as the data coincide with each other. Traps were placed on two sides of the plants and 20 adults were released from a pipette tip at 6 cm distance from the stem base. Error bars indicate the standard deviation.

Behaviour of thrips on TR and TS plants.

The behaviour of thrips on TR and TS plants was analysed by monitoring the duration and frequency of “walking” and “resting” on the leaves of TR and TS plants as described in Materials and Methods. Although it remained unknown with the experimental set-up used whether “resting” indicated “feeding”, the average fraction of time that thrips were “resting” or “walking” did not differ between TR and TS plants (Table 5.3). The most passive thrips did not walk at all while the most active individuals were mobile during 4.5 min within a 10 min interval. Also the number of “resting” periods were similar on both accessions (Table 5.3). The average duration of one “resting” period ranged from 74 sec up to over 5 min. Only 1 thrips laid an egg (on a TS plant) when its behaviour was observed 5 h after its release.

Table 5.3. Average fraction of time (± standard error of the mean (sem)) that a thrips "rested" in a period of 10 min observation at 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72 and 96 h after its release on a thrips-resistant (TR) or a thrips-susceptible (TS) *C. annuum* plant, and the average number of periods within these 10 min (± sem) that thrips "rested".

	Fraction of 10 min that a thrips "rested"		Number of periods that a thrips "rested"	
Time (h)	TR	TS	TR	TS
0.1	0.78 ± 0.09[a]*	0.87 ± 0.05[a]	6.3 ± 1.6[a]	5.3 ± 0.8[a]
1	0.77 ± 0.11[a]	0.89 ± 0.04[a]	4.7 ± 1.0[a]	3.7 ± 0.9[a]
2	0.78 ± 0.11[a]	0.83 ± 0.06[a]	6.2 ± 1.3[a]	5.5 ± 1.3[a]
3	0.84 ± 0.10[a]	0.90 ± 0.04[a]	3.5 ± 1.3[a]	5.0 ± 1.5[a]
4	0.98 ± 0.02[a]	0.97 ± 0.01[a]	1.8 ± 0.5[a]	2.8 ± 0.6[a]
5	0.97 ± 0.03[a]	0.91 ± 0.05[a]	2.2 ± 0.8[a]	2.5 ± 0.7[a]
6	0.91 ± 0.04[a]	0.91 ± 0.05[a]	3.2 ± 0.6[a]	3.7 ± 0.3[a]
7	0.90 ± 0.04[a]	0.92 ± 0.05[a]	3.3 ± 0.5[a]	2.5 ± 0.3[a]
8	0.87 ± 0.07[a]	0.92 ± 0.04[a]	3.5 ± 0.8[a]	3.2 ± 0.8[a]
24	0.95 ± 0.02[a]	0.89 ± 0.07[a]	3.2 ± 0.8[a]	2.8 ± 0.7[a]
48	0.93 ± 0.06[a]	0.96 ± 0.03[a]	2.8 ± 0.8[a]	1.8 ± 0.4[a]
72	0.84 ± 0.01[a]	0.90 ± 0.03[a]	5.3 ± 1.0[a]	4.3 ± 0.6[a]
96	0.83 ± 0.08[a]	0.95 ± 0.03[a]	4.0 ± 1.3[a]	4.2 ± 1.2[a]

* The same superscript letters in a row for each parameter indicate no differences within the treatments TR and TS (P>0.05).

DISCUSSION

In previous studies it was demonstrated that both thrips population built-up and spread of TSWV are significantly restricted in a thrips-resistant (TR) pepper accession compared to thrips-susceptible (TS) control plants (Maris *et al.*, 2003a,b; Chapters 3 and 4). To gain further information on the critical steps in the vector's population dynamics that are affected by the host's TR phenotype, thrips development as well as thrips migration was studied in the present paper. The results show evidently that a decreased oviposition rate and an increased larval mortality rate are main factors resulting in the impeded population built-up on TR plants. Besides, it was found that thrips show an increased emigration/immigration ratio on TR plants, which also contributes to the observed lower thrips numbers on the TR phenotype (Maris *et al.*, 2003a,b,c; Chapters 2, 3 and 4).

The rate of primary spread of TSWV in a crop depends on several parameters, such as the number of incoming viruliferous thrips landing on the plants, their (feeding) behaviour on the plant and the virus inoculation efficiency. It is clear that the increased emigration/immigration balance on thrips-resistant host plants, as documented in the current paper, will have a major limiting effect on the primary TSWV infection rate, thus explaining the outcomes of previous studies (Maris *et al.*,

2003a,b; Chapters 3 and 4). Secondary spread of TSWV is dependent on the reproduction of a colonising population of thrips on a crop, the virus acquisition efficiency by young larvae and the subsequent dispersal of viruliferous progeny to healthy plants. The significantly reduced reproduction on TR plants as caused by a decreased oviposition rate at one hand and an increased larval mortality at the other as found in this study explains evidently the delayed secondary spread.

Experiments with individual caged plants made clear that the size of the cages did not affect the number of thrips recovered on the plant. The low thrips numbers recovered on the plants and the high numbers found on the sticky traps suggest that the thrips dispersed more at random through the cages rather than being predetermined to find the plant. This was found for both the TR and TS plants. Nevertheless, the slightly higher number of thrips on the TS plants (Fig. 5.2) suggest that thrips might perceive stimuli resulting in more thrips on these plants than on TR plants. Visual stimuli could have been involved as the TS plants have light green coloured leaves to which thrips might be more attracted than to the dark green coloured leaves of the TR plants. Similar observations were made in previous studies in which more thrips were found on groundnut accession “TMV 2”, which had yellowish green foliage, than on the dark green “Robut 33-1” accession (Amin 1985). Later dispersal of thrips from TS plants than from TR plants, as shown in current study, will also contribute to the higher number of thrips found on former plants.

The average time span and number of periods that thrips were “resting” were similar for both accessions, but it remains unknown from the results whether the feeding behaviour (e.g. number of feeding probes, feeding time) was also different, as found for thrips feeding on resistant leek and cucumber (Harrewijn *et al.*, 1996). Differences in feeding behaviour of thrips on TR plants will undoubtedly affect the spread of TSWV. Irrespective of this undetermined effect, the experiments described in this paper evidently demonstrate that a TR host phenotype may lead to decreased numbers of both colonising thrips (due to decreased oviposition and increased larval mortality) and invading thrips (due to an altered emigration/immigration balance). In turn, this not only leads to decreased levels of direct thrips damage, but also to restricted spread of vectored TSWV, as indeed previously reported in greenhouse experiments (Maris *et al.*, 2003 a,b; Chapters 3 and 4).

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Chapter 6

Tomato spotted wilt virus infection improves host suitability for its vector *Frankliniella occidentalis*

The effect of *Tomato spotted wilt virus* (TSWV) infection on plant attractiveness for the western flower thrips (*Frankliniella occidentalis*) was studied. Significantly more thrips were recovered on infected than on non-infected pepper (*Capsicum annuum*) plants in different preference tests. In addition, more offspring was produced on the virus infected pepper plants, and this effect was also found on *Datura stramonium* upon infection. Thrips behaviour was hardly influenced by TSWV-infection of host plants, with only a slight preference to feed on infected plants. Offspring development, though, was positively affected as larvae hatched earlier from eggs and subsequently pupated significantly faster on TSWV-infected plants. The implications of these findings for TSWV-epidemics are discussed.

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INTRODUCTION

The western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), often forms a serious pest in many vegetable and ornamental crops world-wide. Among the direct damage caused by this thrips species are growth reduction and cosmetic damage on fruits and flowers. *F. occidentalis* is also the main vector for some tospoviruses (family *Bunyaviridae*) as it is able to vector at least 4 species (Wijkamp *et al.*, 1995a) of which *Tomato spotted wilt virus* (TSWV) is the most detrimental (German *et al.*, 1992; Goldbach & Peters, 1994). For TSWV transmission, young larvae (L1 stage and early L2 stage) have to acquire the virus from infected plants (van de Wetering *et al.*, 1996), usually the plants on which they emerge. The virus is propagatively transmitted, i.e. replicates in the vector (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993), and viruliferous adults are able to transmit the virus during their whole life (Wijkamp *et al*., 1993).

TSWV-epidemics would benefit from a high suitability of infected host plants to support the reproduction of thrips and the development of viruliferous offspring. As larvae develop on the plants on which they hatch, it is obvious that spread of TSWV is dependent on female thrips to oviposit on virus-infected plants. Studies with *Lactuca sativa*, *Arctium lappa* and *D. stramonium* have demonstrated that *F. occidentalis* produced more offspring on TSWV-infected plants than on non-infected plants (Bautista *et al.*, 1995). It was not established whether this enhanced offspring production was due to higher numbers of females being attracted to virus-infected plants, or whether this was caused by a higher oviposition rate, a higher egg hatching rate, or a lower mortality of developing individuals on these plants. TSWV-infection of thrips has no deleterious effects, as developmental time, reproduction rate and survival were similar for viruliferous thrips and non-viruliferous thrips, when they had fed for short periods on tospovirus-infected *Nicotiana rustica* plants (Wijkamp *et al.*, 1995b; 1996a). However, exposing thrips for long periods to infected plants might affect the longevity of thrips. TSWV-infection of chrysanthemum plants had no effect on the mortality of *F. occidentalis* (Robb, 1989), but thrips development on *Impatiens necrotic spot virus* infected *Lobelia* plants was significantly slower than on non-infected plants (DeAngelis *et al.*, 1993).

In the current study, the possible preference of *F. occidentalis* for TSWV-infected *versus* non-infected plants was analysed, as well as possible differences in oviposition rates on these hosts. Two different host plant species were used in these studies. *Capsicum annuum* was used as example of an important field crop grown both in the open field and in greenhouses and suffering from thrips-infestation and TSWV-infection, and *D. stramonium* was used as this species is often used in thrips and tospovirus research. In addition, the effect of TSWV-infection of the host on female thrips' behaviour and on thrips development was determined.

MATERIALS AND METHODS

Thrips population, plants, virus isolates and inoculations.

A population of *F. occidentalis* 'IS2' (van de Wetering *et al.*, 1999), which originated from an infestation on mango in Israel, was used. This virus-free population was reared on chrysanthemum plants at 23 ± 2.0°C under a 16-h light and 8-h dark cycle in the greenhouse.

Three *Capsicum annuum* accessions were used in the studies, i.e. "Pikante Reuzen", highly susceptible to *F. occidentalis* (further referred to as TS: "thrips susceptible"), "CPRO-1", resistant to *F. occidentalis* (referred to as TR: thrips resistant") (Maris *et al.*, 2003c; Chapter 2), and Mazurka RZ. Besides, *D. stramonium* was used in the comparative studies.

The TSWV-isolate BR01 (de Ávila *et al.*, 1990) was used in this study. To preserve its virulence, this isolate was maintained by thrips-mediated passages on *D. stramonium* L. plants. To obtain TSWV-infected plants, leaf tissue from systemically infected *D. stramonium* plants was ground in 0.05 M of sodium phosphate buffer, pH 7.0. Inocula were rubbed on carborundum-dusted leaves of 3- to 4-week-old seedlings. Control plants were inoculated with buffer only. Plants with clear symptoms were used in the experiments about 2-3 weeks after inoculation.

Preference of thrips for TSWV-infected versus non-infected pepper plants.

The effect of TSWV-infection in plants of both the TR and TS accession on the preference of thrips was determined in two different experimental designs. In the first design, the thrips could disperse towards 4- to 5-week-old TSWV-infected or non-infected plants after releasing 20 adults at a spot between two plants placed at a distance of 30 cm from each other in transparent plastic cages (45x30x40 cm). In the second design, the dispersal from an infected or non-infected plant to a neighboring plant was studied by releasing 20 adults on one of the two plants. The thrips could move between an infected and non-infected plant (choice test) or between two infected or two non-infected plants (non-choice tests). The dispersal of the thrips was measured by scoring the number of thrips present on each plant every hour on the first 8 hours, and then at 24, 48, 72, 96 and 168 h after their release. Leaves were visually inspected by turning carefully so that the adults did not disperse. Each experiment contained six sets of plants and was replicated twice. Experiments were performed at 23 ± 2.0°C under a 16-h light and 8-h dark cycle in a climate chamber. Dispersal rates of thrips from TSWV-infected and non-infected plants were compared by analysing the log-transformed numbers of thrips recovered on the plants as binomial distributed variables by analysis of variance using general linear models and Genstat (Payne *et al.*, 1993).

Preferential oviposition on TSWV-infected plants.

Preference for and reproduction of *F. occidentalis* females on TSWV-infected or non-infected plants were compared. Twenty virus-free females were released in plastic cages (45x30x40 cm) with a transparent lid. Four virus-infected or non-infected seedlings of TS, TR, Mazurka RZ or *D. stramonium* were placed in the cages for non-choice tests, whereas two infected and two non-infected plants of an accession were arranged side-by-side for choice-tests. Each non-choice and choice test contained six sets of plants and was repeated twice. Cages were placed in the greenhouse at 23 ± 2.0°C under a 16-h light and 8-h dark cycle. Females were allowed to oviposite on the plants for 3 days after which they were counted and subsequently removed from the plants using an aspirator. Offspring were collected and counted every 24 h for 7 days after removing the adults. Then, leaves of each plant were stained with 1.5% methyl-red to detect unhatched eggs which were counted under a binocular microscope.

The number of females and offspring per female recovered from virus-infected and non-infected plants was analyzed and compared for each cultivar by Kruskal-Wallis one-way analysis of variance using Genstat (Payne *et al.*, 1993).

Behaviour of female thrips on TSWV-infected and non-infected plants.

The behaviour of *F. occidentalis* females on TSWV-infected and non-infected plants was visually assessed by releasing 20 thrips on one of the top leaves of either plant. The duration of periods that a randomly selected thrips was “walking” or not-walking (“resting”) on the leaves and the number of these periods were visually monitored. These parameters were scored for 10 min at 5 min and 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72 and 96 h after thrips release. For each time point, the behaviour of six individual thrips was scored on infected and non-infected host plants. Total fraction of time and number of periods that thrips were “resting“ on plants of both accessions were analyzed as binomial and Poisson distributed variables, respectively, using regression analysis.

Thrips development on TSWV-infected plants.

To study whether TSWV-infection in plants affects thrips development, three females were confined to leaf disks (2.5 cm in diameter (i.d.)) of TSWV-infected or non-infected plants. Disks were placed on 1.5% agar in a petri-dish (3.5 cm i.d.) and covered with cling film, and incubated at 24°C. Adults had access to the leaf disks for an oviposition period of 12 h, after which they were removed. The larvae hatching from eggs were individually transferred to new leaf disks of either infected or non-infected plants depending on their original host. Their development was monitored every 12 h till adult emergence. The duration of each developmental stage of thrips feeding on TSWV-infected disks and on non-infected disks was compared. This

experiment was performed in duplo with pepper (TS and Mazurka RZ) and *D. stramonium* plants, and development of 20-30 individual thrips was scored for each treatment. Possible effects of thrips-resistance on the developmental rate were analysed by simple linear regression models. Computations to indicate statistical significance were based on 95% confidence intervals.

RESULTS

Preference of thrips for TSWV-infected versus non-infected pepper (*C. annuum*) plants.

To obtain a first indication for possible beneficial effects of TSWV-infection on host suitability for thrips, cohorts of 20 females were released in a cage containing a single virus-infected and a non-infected pepper (*C. annuum*) plant, and their preference for either host plant was followed during 168 h.

Consistently higher numbers of thrips were found on infected plants than on non-infected plants when the thrips were released at a spot in between an infected and a non-infected plant. This was found for both the relatively highly thrips susceptible (TS) and the thrips resistant (TR) accessions (Figs. 6.1A and 6.2A). While the number of thrips did not significantly differ ($P>0.05$) at each time interval, the cumulative numbers of thrips recovered on TSWV-infected TS (38.3 $\pm$ 3.9) and TR (15.1$\pm$ 5.3) plants were significantly ($P<0.05$) higher than on non-infected TS (20.3 $\pm$ 1.1) and TR (6.4 $\pm$ 1.8) plants, respectively.

A similar trend was found in preference tests in which the thrips were allowed to choose between two infected or two non-infected TS (Fig. 6.1B) or TR (Fig. 6.2B) plants. The cumulative number of thrips recovered was again significantly ($P<0.05$) higher on infected TS (26.8 $\pm$ 6.9) and TR (17.2 $\pm$ 4.7) plants than on non-infected TS (13.5 $\pm$ 3.2) and TR (12.1 $\pm$ 0.9) plants.

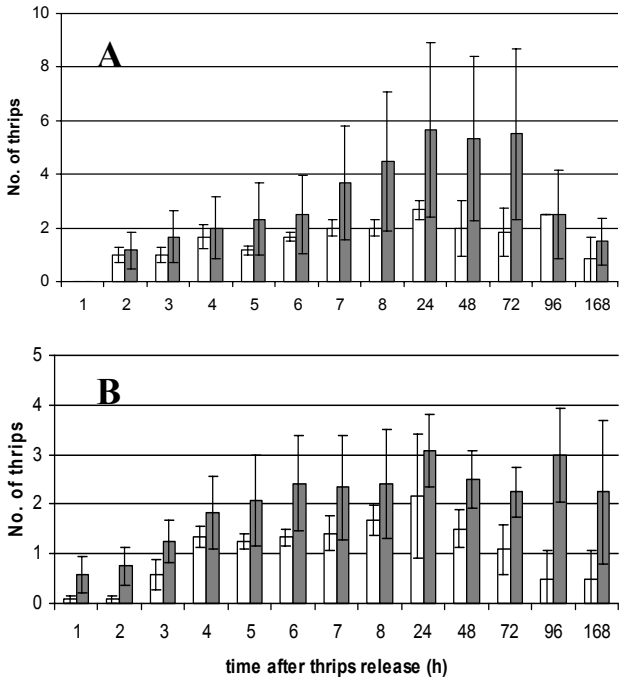

Figure 6.1. Number of thrips recovered on *Tomato spotted wilt virus*-infected (dark bars) or non-infected (white bars) pepper (*Capsicum annuum*) TS plants in choice (A) and non-choice tests (B). Twenty adults were at the beginning of the experiment released at a spot between the two plants. Error bars indicate the standard error of the mean.

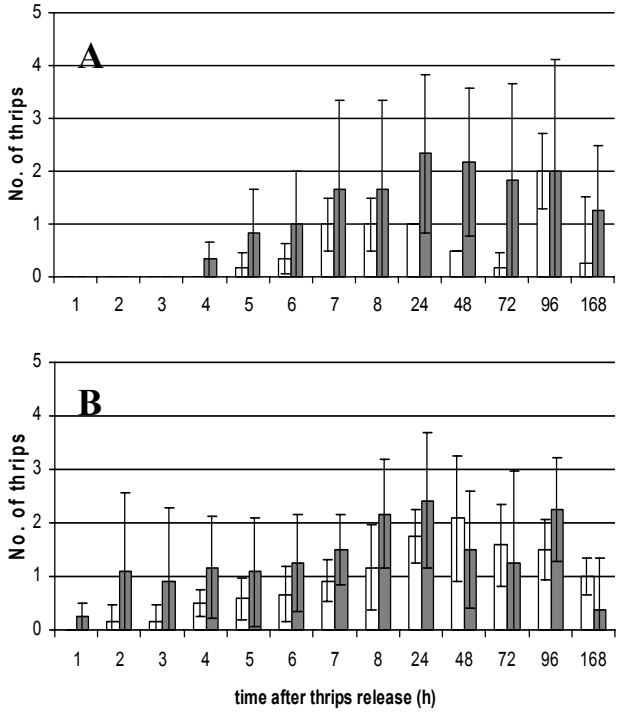

Figure 6.2. Number of thrips recovered on *Tomato spotted wilt virus*-infected (dark bars) or non-infected (white bars) pepper (*Capsicum annuum*) TR plants in choice (A) and non-choice tests (B). Twenty adults were at the beginning of the experiment released at a spot between the two plants. Error bars indicate the standard error of the mean.

In a slightly different set-up, females were released on one of the two plants to be compared instead of in between these plants. The thrips were again allowed to choose between two infected or two non-infected plants in a cage (non-choice tests) of the TS (Fig. 6.3) or TR (Fig. 6.4) accession, or between one infected and one non-infected TS (Fig. 6.5) or TR (Fig. 6.6) plant (choice tests). Of the 20 females released in each treatment, 10-15 were recovered after 1 h on the plant on which they were released (Figs. 6.3 - 6.6). Beyond this first hour, the thrips dispersed slowly from the source plant and colonised the companion plant at low rate, whereas most of the thrips went astray in the cage. Thrips dispersal was lower from infected TS (Figs. 6.3A and 6.5A) or TR (Figs. 6.4A and 6.6A) plants than from non-infected TS (Figs. 6.3B and 6.5B) or TR (Figs. 6.4B and 6.6B) plants independent of whether the neighbouring plant was infected or not.

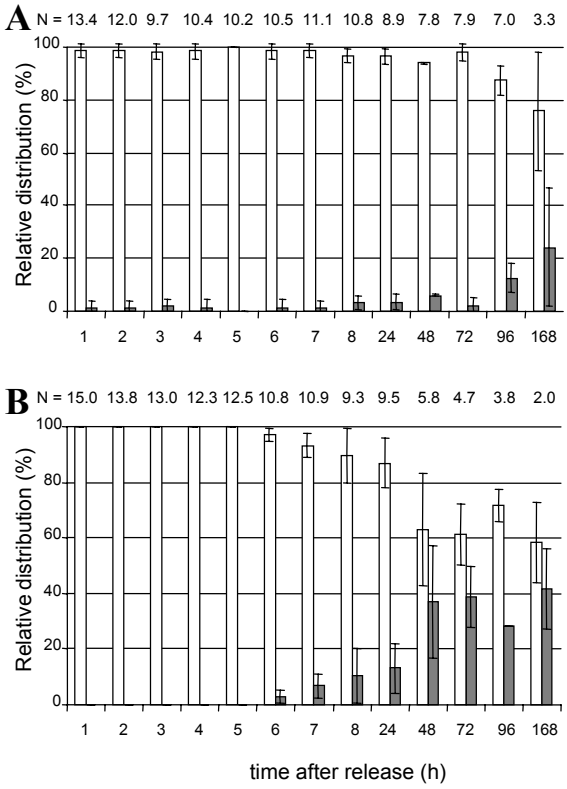

Figure 6.3. Average distribution of *Frankliniella occidentalis* on two *Tomato spotted wilt virus*-infected (A) or two non-infected (B) pepper (*Capsicum annuum*) TS plants placed in a cage (non-choice tests). Twenty adults were released on one plant and counted every hour in the first 8 h after release and then every 24 h. White bars: average thrips numbers on the plants on which they were released; dark bars: average thrips numbers on the plant to which they could migrate. Error bars indicate the standard error of the mean. Numbers presented above the figures indicate the average total number of thrips recovered for each time point.

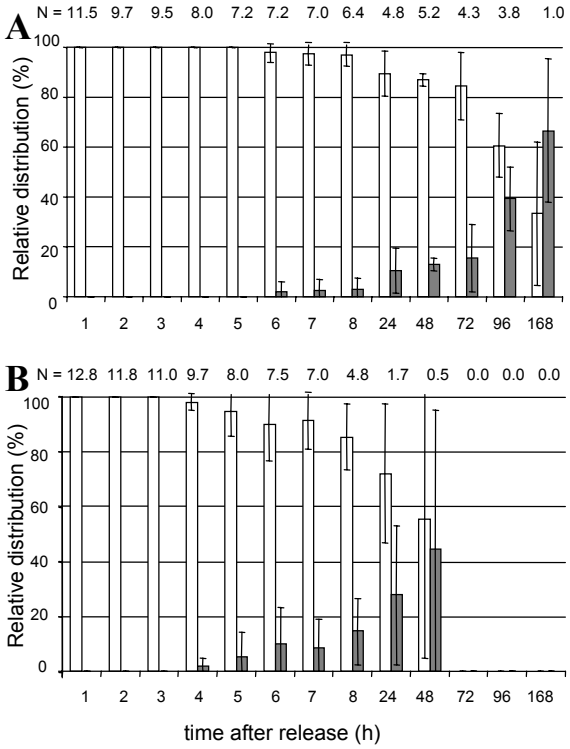

Figure 6.4. Average distribution of *Frankliniella occidentalis* over two *Tomato spotted wilt virus*-infected (A) or two non-infected (B) pepper (*Capsicum annuum*) TR plants placed in a cage (non-choice tests). For further details: see legend Fig. 6.3.

Log-transformation of thrips numbers on the plants and the intervals confirmed this conclusion (Table 6.1). Average slopes of the regression lines confirmed that the thrips left non-infected TS plants (slope: -0.51) and TR plants (slope: -0.70) at higher rates than infected TS (slope: -0.22) and TR plants (slope: -0.39). Thrips were no longer detectable on the two non-infected TR plants after 72 h, while some thrips could still be found on infected TR plants (Fig. 6.4).

Table 6.1. Regression functions after log-transformation of the number of thrips (Y) recovered on *Tomato spotted wilt virus*-infected and non-infected *Capsicum annuum* (cv. TS or TR) plants. Twenty thrips were released on a source plant that was placed next to another plant (neighbouring plant) in a thrips-proof cage. "R^2"-column indicates the correlation coefficient (R^2) of the regression function. Last column gives the corresponding figure in which the non-transformed thrips numbers are given.

Capsicum accession	Source Plant	Neighbouring Plant	Regression function	R^2	Corresp. figure
TS	Infected	Infected	Y = -0.22x +1.17[a*]	0.68	6.3A
	Non-infected	Infected	Y = -0.54x +1.29[b]	0.94	6.5B
	Infected	Non-infected	Y = -0.21x +1.15[a]	0.76	6.5A
	Non-infected	Non-infected	Y = -0.47x +1.35[b]	0.90	6.3B
TR	Infected	Infected	Y = -0.48x +1.20[a]	0.71	6.4A
	Non-infected	Infected	Y = -0.72x +1.27[b]	0.88	6.6B
	Infected	Non-infected	Y = -0.29x +1.10[a]	0.92	6.6A
	Non-infected	Non-infected	Y = -0.68x +1.30[b]	0.94	6.4B

* different letters in two subsequent rows indicate significant differences between dispersal rate of thrips from an infected source plant and a non-infected source plant to a neighboring plant with the same infection status.

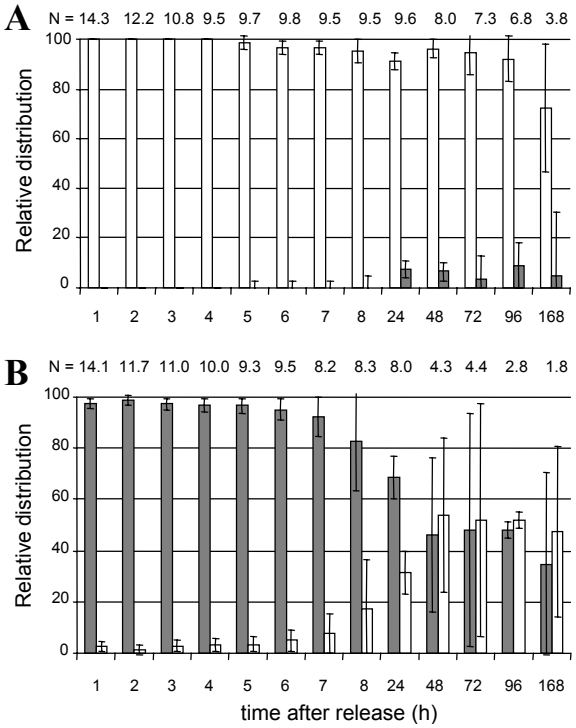

Figure 6.5. Average distribution of thrips over a *Tomato spotted wilt virus*-infected (white bars) and non-infected (dark bars) pepper (*Capsicum annuum*) TS plant placed in a cage (choice tests). Twenty adults were released on the infected plant (A) or on the non-infected plant (B) and counted every hour in the first 8 h after release and then every 24 h. Error bars indicate the standard error of the mean. Numbers presented above the figures indicate the average total number of thrips recovered for each time point.

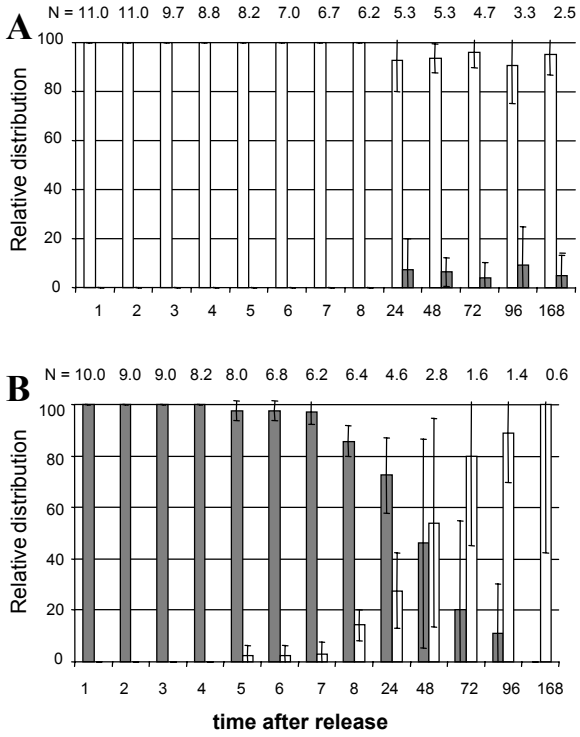

Figure 6.6. Average distribution of thrips over a *Tomato spotted wilt virus*-infected (white bars) and non-infected (dark bars) pepper (*Capsicum annuum*) TR plant placed in a transparent cage (choice tests). For further details: see legend figure 6.5.

A lot of thrips could not be recovered on the plants in these experiments. This was mainly due to dispersal of the thrips through the cage and not by forced escapes when they were counted. In a control experiment the thrips were counted for the first time eight hours after their release in stead of after one hour. The number of thrips recovered in both situations were similar (data not presented).

Preferential oviposition on TSWV-infected plants.

The effect of TSWV-infection on oviposition rate and offspring production was studied, using two different hosts, i.e. *C. annuum* and *D. stramonium*. Significantly more females were recovered and more offspring were produced on TSWV-infected plants than on non-infected plants in both choice and non-choice tests for all four hosts (three pepper accessions and *D. stramonium*) tested (Tables 6.2 and 6.3). The number of females recovered and the offspring produced on the TSWV-infected plants were positively related. Staining leaves with methyl-red indicated that the number of unhatched eggs in the TSWV-infected leaves from TR, TS and *D. stramonium* did not differ from that in non-infected leaves (data not shown).

Table 6.2. Average numbers of *Frankliniella occidentalis* females and of offspring per female recovered on *Tomato spotted wilt virus*-infected and non-infected *Capsicum annuum* and *Datura stramonium* plants. Twenty adults were released in cages each with two infected and non-infected plants (choice tests), or in cages with four virus-infected or non-infected plants (non-choice test). Data are presented with the standard error of the mean.

	Average number of females recovered			
	Choice test		Non-choice test	
Test plant	Infected	Non-infected	Infected	Non-infected
C. annuum TR	$4.0 \pm 1.2^{a*}$	0.2 ± 0.2^{b}	$5.0 \pm 0.5^{a*}$	0.8 ± 0.3^{b}
C. annuum TS	5.2 ± 0.9^{a}	0.5 ± 0.1^{b}	6.8 ± 0.7^{a}	3.1 ± 0.9^{b}
C. annuum Mazurka RZ	4.5 ± 0.8^{a}	0.3 ± 0.2^{b}	7.2 ± 0.3^{a}	2.3 ± 1.0^{b}
D. stramonium	6.5 ± 0.5^{a}	4.3 ± 0.4^{b}	7.0 ± 0.6^{a}	3.5 ± 0.9^{b}

* Different letters in a row for either choice or non-choice tests indicate significant differences ($P<0.05$) between values for infected and non-infected plants.

Table 6.3. Average numbers of offspring per female thrips recovered on *Tomato spotted wilt virus*-infected and non-infected *Capsicum annuum* and *Datura stramonium* plants. Twenty adults were released in cages each with two infected and non-infected plants (choice tests), or in cages with four virus-infected or non-infected plants (non-choice test). Data are presented with the standard error of the mean.

	Average number of offspring recovered			
	Choice test		Non-choice test	
Test plant	Infected	Non-infected	Infected	Non-infected
C. annuum TR	$1.4 \pm 0.3^{a*}$	0.2 ± 0.1^{b}	$2.1 \pm 0.4^{a*}$	0.3 ± 0.1^{b}
C. annuum TS	2.3 ± 0.5^{a}	0.1 ± 0.1^{b}	7.1 ± 0.7^{a}	0.7 ± 0.2^{b}
C. annuum Mazurka RZ	4.9 ± 1.0^{a}	0.2 ± 0.1^{b}	6.1 ± 0.7^{a}	1.0 ± 0.1^{b}
D. stramonium	2.6 ± 0.3^{a}	0.8 ± 0.2^{b}	3.6 ± 0.5^{a}	1.1 ± 0.2^{b}

* Different letters in a row for either choice or non-choice tests indicate significant differences ($P<0.05$) between values for infected and non-infected plants.

Behaviour of female thrips on TSWV-infected and non-infected plants.

The behaviour of *F. occidentalis* females on TS and TR plants was visually assessed by scoring the length and number of intervals that they “walked” and “rested” on TSWV-infected and non-infected plants during a period of 10 min. The average time that an individually observed thrips “rested” and the average number of periods that they “rested” on infected and non-infected plants did not differ for both the TS and the TR accession at all time points scored (Table 6.4). The thrips “rested” significantly longer than they “walked”. The maximal period that a thrips walked was longer on infected plants than on non-infected plants, whereas the maximal resting period was smaller. No eggs were deposited during the 52 hours that these observations were made.

Table 6.4. Average total time (± sem) that a thrips "rested" in a period of 10 min observation at 5 min and 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72 and 96 h after its release on a non-infected or a *Tomato spotted wilt virus*-infected *C. annuum* TS or TR plant, and the average number of periods that the thrips "rested".

	Total time (min) that a thrips "rested" in 10 min				Number of periods that a thrips "rested"			
	TS		TR		TS		TR	
Time (h)	Non-Infected	Infected	Non-Infected	Infected	Non-infected	Infected	Non-infected	Infected
0.1	8.7 ± 0.5[a]*	8.7 ± 0.5[a]	9.5 ± 0.2[a]	7.8 ± 0.9[a]	5.3 ± 0.8[a]	6.8 ± 1.9[a]	6.3 ± 1.6[a]	4.6 ± 0.2[a]
1	8.9 ± 0.4[a]	9.2 ± 0.6[a]	8.8 ± 0.5[a]	7.7 ± 1.1[a]	3.7 ± 0.9[a]	2.6 ± 0.5[a]	4.7 ± 1.0[a]	3.4 ± 0.7[a]
2	8.4 ± 0.6[a]	8.6 ± 0.8[a]	8.7 ± 0.7[a]	7.8 ± 1.1[a]	5.5 ± 1.3[a]	4.0 ± 0.9[a]	6.2 ± 1.2[a]	4.8 ± 1.1[a]
3	9.0 ± 0.4[a]	9.2 ± 0.7[a]	9.3 ± 0.3[a]	8.4 ± 1.0[a]	5.0 ± 1.5[a]	3.0 ± 1.1[a]	3.5 ± 1.3[a]	4.4 ± 1.1[a]
4	9.7 ± 0.1[a]	9.3 ± 0.4[a]	8.8 ± 0.7[a]	9.8 ± 0.3[a]	2.8 ± 0.6[a]	2.4 ± 0.9[a]	1.8 ± 0.5[a]	2.4 ± 0.9[a]
5	9.1 ± 0.5[a]	8.3 ± 0.8[a]	9.5 ± 0.3[a]	9.7 ± 0.3[a]	2.5 ± 0.7[a]	2.4 ± 0.2[a]	2.2 ± 0.8[a]	3.2 ± 1.0[a]
6	9.1 ± 0.5[a]	8.9 ± 0.6[a]	8.7 ± 0.7[a]	9.1 ± 0.4[a]	3.7 ± 0.3[a]	2.8 ± 0.5[a]	3.2 ± 0.6[a]	2.6 ± 0.8[a]
7	9.2 ± 0.5[a]	9.8 ± 0.1[a]	9.3 ± 0.4[a]	9.0 ± 0.4[a]	2.5 ± 0.3[a]	3.2 ± 0.5[a]	3.3 ± 0.5[a]	3.2 ± 0.6[a]
8	9.2 ± 0.4	8.2 ± 1.1[a]	9.1 ± 0.3[a]	8.7 ± 0.7[a]	3.2 ± 0.8[a]	4.2 ± 1.0[a]	3.5 ± 0.9[a]	3.2 ± 0.5[a]
24	8.9 ± 0.7[a]	9.1 ± 0.4[a]	8.7 ± 0.6[a]	9.5 ± 0.2[a]	2.8 ± 0.8[a]	2.8 ± 0.8[a]	3.2 ± 0.8[a]	4.7 ± 1.0[a]
48	9.6 ± 0.3[a]	7.2 ± 0.4[a]	6.8 ± 2.1[a]	9.3 ± 0.6[a]	1.8 ± 0.5[a]	3.6 ± 1.0[a]	2.8 ± 0.8[a]	2.8 ± 0.9[a]
72	9.0 ± 0.3[a]	8.6 ± 0.6[a]	9.1 ± 0.4[a]	8.4 ± 0.1[a]	4.3 ± 0.6[a]	3.3 ± 0.2[a]	5.3 ± 1.0[a]	3.8 ± 1.2[a]
96	9.5 ± 0.3[a]	9.6 ± 0.2[a]	8.2 ± 0.7[a]	8.3 ± 0.8[a]	4.2 ± 1.3[a]	3.4 ± 0.8[a]	4.0 ± 1.3[a]	5.8 ± 0.4[a]

* The same superscript letters in a row for two infection statuses per host indicate no differences within the treatments infected and non-infected ($P<0.05$).

Thrips development on TSWV-infected plants.

Development of thrips from egg to adult on TSWV-infected plants was studied to detect possible positive or negative effects of virus infection in the host on its vector. Larvae hatched significantly ($P<0.05$) earlier from eggs on TSWV-infected leaf disks than from eggs on non-infected disks (Table 6.5). Larvae developed significantly ($P<0.05$) faster on TSWV-infected pepper (Mazurka RZ) and *D. stramonium* leaf disks (4,4 and 4.6 days, respectively) than on non-infected disks (5.9 and 6.1 days, respectively). Both pupal stages were not affected by TSWV-infection. Overall, the complete development from egg to adult was 1 to 2 days shorter on TSWV-infected leaf disks (Table 6.5). No difference in mortality of thrips was observed between the disks of non-infected and infected plants for the eggs, larvae and pupa (data not shown).

Table 6.5. Average duration (days) of the developmental stages of *Frankliniella occidentalis* on leaf disks from *Tomato spotted wilt virus*-infected and non-infected *Capsicum annuum* (TS, Mazurka RZ) and *Datura stramonium* plants at 24°C. Data are given with their standard deviation.

Plant	Health status	Length of a developmental stage (days)				# days from
		Egg	Larval	Prepupal	Pupal	egg to adult
C. annuum TS	Infected	$3.0 \pm 0.2^{a*}$	4.7 ± 0.4^{a}	1.0 ± 0.2^{a}	2.2 ± 0.3^{a}	10.9 ± 0.8^{a}
	Non-infected	3.4 ± 0.5^{b}	5.2 ± 0.3^{a}	1.0 ± 0.1^{a}	2.5 ± 0.4^{a}	12.1 ± 0.2^{b}
C. annuum	Infected	3.3 ± 0.4^{a}	4.4 ± 0.7^{a}	0.9 ± 0.3^{a}	2.0 ± 0.4^{a}	10.6 ± 0.5^{a}
Mazurka	Non-infected	3.6 ± 0.4^{b}	5.9 ± 0.4^{b}	1.2 ± 0.3^{a}	2.2 ± 0.3^{a}	12.9 ± 0.3^{b}
D. stramonium	Infected	3.1 ± 0.1^{a}	4.6 ± 0.3^{a}	1.1 ± 0.3^{a}	2.3 ± 0.3^{a}	11.1 ± 0.4^{a}
	Non-infected	3.4 ± 0.2^{b}	6.1 ± 0.9^{b}	1.1 ± 0.1^{a}	2.1 ± 0.3^{a}	12.7 ± 1.0^{b}

* Different superscript letters in a column for each plant species indicate significant differences between development of thrips on virus infected and non-infected leaf disks ($P<0.05$).

DISCUSSION

The present study demonstrates that TSWV infection improves host suitability for its vector *F. occidentalis*. TSWV-infected plants gained in attractiveness for female thrips, and also were preferred for feeding and, most importantly in TSWV epidemics, for oviposition. These parameters are of significant importance for the abundance of viruliferous vectors in the field. All larvae hatching from eggs laid on an infected plant may acquire the virus, as the wingless larvae are not able to disperse and will therefore feed (almost) exclusively on the plant on which they hatch. Besides the preference of thrips for infected plants and in consequence of that more ovipositions on these plants, the observed faster development of progeny on infected plants as demonstrated in this study also will contribute to larger populations of infected thrips and, hence, a larger infection pressure for the plant host.

Thrips had a preference for feeding on TSWV-infected over non-infected pepper plants. This effect was shown in experiments in which females were released on a spot between or on TSWV-infected or non-infected plants (Figs. 6.1 - 6.6). Most strikingly, a large number of thrips could not be recovered on the plants after their release. In those experiments in which thrips were released between plants, the number of thrips recovered increased slightly in the first days after their introduction in the cage (Figs. 6.1 and 6.2). This increase, although small, indicates that the dispersed thrips did not die, but went astray in the cage. The higher number of thrips recovered on the TSWV-infected plants than on non-infected plants show that TSWV-infected plants exerted some attraction to thrips. Thrips might be attracted by the yellow color of virus-infected host plants as reported for TSWV-infected lettuce plants (Yudin *et al.*, 1987). Alternatively, it can not yet be excluded that TSWV-infected

plants release specific volatiles that attract thrips, as was found for PLRV-infected potato plants attracting its aphid vector *Myzus persicae* (Eigenbrode *et al.*, 2002). Another explanation for the higher numbers of thrips on infected plants is that the thrips disperse at a lower rate from infected plants than from non-infected plants in consequence of the higher preference to feed on former plants. This explanation is supported by the results of experiments in which thrips were released on one of the two caged plants (Figs. 6.3 – 6.6).

Considerably more eggs were produced on infected than on non-infected plants (Table 6.1). The number of larvae that hatched on either plant was closely related to the number of females recovered. The preference of thrips for feeding and oviposition on TSWV-infected plants is supported by the findings of Bautista *et al.* (1995), although the underlying mechanism remained unknown in that study. The current study indicates that more ovipositions are mainly the result of the thrips' preference for infected plants as food source, rather than a higher oviposition rate per individual, a different behaviour, or a lower mortality rate of eggs on infected plants. Latter two parameters were similar on TSWV-infected and on non-infected plants.
A higher reproduction of *F. occidentalis* on TSWV-infected plants was found for both plant species (*C. annuum* and *D. stramonium*) used. Even a thrips-resistant (TR) pepper accession became a more attractive host for thrips after TSWV-infection, resulting in more feeding, lower dispersal rate and more ovipositions. As also the thrips-susceptible (TS) pepper accession gained in attractiveness by TSWV infection, the difference between the TR and the TS accessions compared sustained on the same level though.

Our findings show a mutual relationship between *F. occidentalis* and TSWV. The attraction of thrips to TSWV-infected plants results in a greater population of viruliferous thrips, increasing the probability of TSWV transmission. Thrips also benefit as TSWV-infected plants are more suitable hosts for thrips feeding, reproduction and development.

ACKNOWLEDGEMENTS

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Chapter 7

Attraction of *Frankliniella occidentalis* by volatiles released from *Tomato spotted wilt virus*-infected plants

Possibly enhanced attraction of the western flower thrips (*Frankliniella occidentalis*) to *Tomato spotted wilt virus* (TSWV)-infected pepper (*Capsicum annuum*) plants was investigated by Y-tube olfactometer analysis. It is shown that two different pepper cultivars, with distinct levels of thrips resistance, release volatiles attractive for thrips upon TSWV-infection. GC-MS analysis revealed that the identity of the thrips-attracting volatiles differed between the two cultivars compared, the thrips-susceptible cultivar producing 1,2-dimethoxybenzene upon virus-infection, while the thrips-resistant cultivar released five virus-induced compounds of which at least (3*E*)-4,8-dimethyl-1,3,7-nonatriene had a thrips-attracting activity. The importance of the release of thrips-attracting volatiles by TSWV-infected host plants for TSWV-epidemics will be discussed.

INTRODUCTION

Many plant viruses are transmitted between and within plant populations by invertebrate vectors (Hull, 2002). The efficiency by which viruses are transmitted by these vectors is the result of specific interactions between virus, vector and plant which, especially for the virus-vector interactions, ideally are of mutual benefit. Virus-infection in plants causes different responses, e.g. discolouring of the leaves or changes in nutritional values for insects. These responses in turn may affect the host's attractiveness for the vectoring insect, and therefore also epidemics of the virus. Changes in foliage colour have been reported to stimulate insects to land on infected plants, as reported for the aphid species *Sitobion avenae* (Fiebig & Poehling, 1998), *Metopolophium dirhodum* (Ajayi & Dewar, 1983) and *Myzus persicae* (Castle *et al.*, 1998; Fereres *et al.*, 1999). After landing, the aphid population development may also be positively affected by virus-infection. Thus it has been shown that the aphid species *M. persicae*, *M. ascalonicus*, *Aphis fabae*, and *Aulacorthum solani* produce more nymphs and have longer life spans on virus-infected sugar beet plants than on non-infected plants (Baker, 1960). Likewise, the growth rate of *M. persicae* on *Potato leafroll virus* (PLRV)-infected potato plants is significantly faster than on non-infected plants (Castle & Berger, 1993).

Specific virus-host-insect relations have also been reported for tospoviruses (*Bunyaviridae*) during their transmission by thrips vectors (Bautista *et al.*, 1995; Lewis, 1973; Maris *et al*., 2004; Chapter 6; Selman *et al.*, 1961; Yudin *et al.*, 1988). *Tomato spotted wilt virus* (TSWV), the type species of the tospoviruses, is transmitted by a limited number of thrips species (*Thysanoptera: Thripidae*), and has a wide host range (Peters, 2004). The virus can only be acquired by young larvae (L1 stage) whereas both old larvae (late L2 stage) and adults are capable to transmit the virus again (van de Wetering *et al.*, 1996; Wijkamp *et al.*, 1993). Previous studies indicated that thrips prefer to feed and oviposit on TSWV-infected plants (Bautista *et al.*, 1995; Maris *et al.*, 2004; Chapter 6; Yudin *et al.*, 1988) and that thrips development proceeds significantly faster on infected plants than on healthy plants (Maris *et al.*, 2004; Chapter 6). Nevertheless, the causal mechanism for the enhanced attraction of thrips to virus-infected plants has remained unclear. Changes in the physiology of virus-infected plants such as increased concentration of nitrogenous compounds (Lewis, 1973; Selman *et al.*, 1961) or the yellow hue of chlorotic leaves (Yudin *et al.*, 1988) might be responsible for this attraction. Alternatively, thrips might also be attracted by volatiles specifically released from virus-infected plants, as found in the interaction between *Potato leaf roll virus* (PLRV)-infected plants and the aphid vector *M. persicae* (Eigenbrode *et al*., 2002).

The objective of the present study was to analyse whether pepper (*Capsicum annuum*), a crop often suffering from TSWV-infections, emits volatiles after virus-infection which attract thrips (*F. occidentalis*). The recent finding that upon TSWV-infection pepper plants become more suitable hosts for thrips, in terms of higher preference, higher oviposition rate, faster development and lower mortality compared to non-infected plants (Maris *et al.*, 2004; Chapter 6) may certainly suggest emission of attractive volatiles. This has now been investigated by analysing the volatiles from TSWV-infected pepper plants using an Y-tube olfactometer (Koschier *et al.*, 2000). In these studies, two different pepper accessions were compared which considerably differ in their level of thrips resistance, i.e. cv. Pikante Reuzen (being thrips-susceptible) and cv. CPRO-1 (being thrips-resistant) (Maris *et al.*, 2003c; Chapter 2). Separate volatile compounds of the odour blend from TSWV-infected and non-infected plants of these accessions are compared by gas chromatography combined with mass spectrometry (GC-MS) and different compounds released from TSWV-infected plants affecting thrips response identified are tested for their attractiveness for *F. occidentalis*.

MATERIALS AND METHODS

Thrips population, virus isolates and plants.

A population of *F. occidentalis* 'IS2' (van de Wetering *et al.*, 1999), which originated from an infestation on mango in Israel, was used. This virus-free population was reared on greenhouse chrysanthemum plants at 23 ± 2.0°C under a 16-h light and 8-h dark cycle. Female thrips were starved overnight supplied with water for 16 h before they were used in the experiments.

The *C. annuum* cv. Pikante Reuzen, susceptible to thrips, and cv. CPRO-1, resistant to thrips, were used in the different experiments (Maris *et al.*, 2003c; Chapter 2). Both accessions are hereafter referred to as TS (thrips-susceptible) and TR (thrips-resistant), respectively. Resistance to thrips in the TR accession was defined previously by lack of reproduction, low preference and minimal feeding damage (Maris *et al*., 2003c; Chapter 2).

The TSWV-isolate BR01 used (de Ávila *et al.*, 1990) was maintained by thrips-mediated passages on *Datura stramonium* L. plants to preserve the virulence of the isolate. *Pepper severe mosaic virus* (PepSMV), an aphid-transmitted virus, was maintained by mechanical inoculation on TS plants. To obtain TSWV- or PepSMV-infected plants, leaves from systemically infected *D. stramonium* or pepper plants, respectively, were ground in 0.05 M of sodium phosphate buffer, pH 7.0, and inoculated on carborundum-dusted leaves of 3- to 4-week-old seedlings. Control plants were inoculated with buffer only.

Attraction of *F. occidentalis* by volatiles from TSWV-infected pepper plants.

The response of female thrips to volatiles from virus-infected plants was tested using a Y-shaped glass tube olfactometer (Koschier *et al.*, 2000). The inner diameter of the Y-tube was 5 mm, and air flew through each arm of the tube at 5 cm/sec. Individual thrips were released at the base of the Y-tube and allowed to walk wind upwards to the Y-junction of the tube where they had to chose between volatiles released from a leaf (100-200 mg) of a virus-infected plant in one arm of the tube or volatiles released from a leaf of a non-infected plant in the other arm. Thrips that did not reach the end of one of the arms within 3 min were discarded from the test.

The test was performed with leaves infected with TSWV or PepSMV. Each treatment was replicated 3 times with at least 30 thrips making a choice in each replicate. The different treatments were not performed at the same day to prevent any effect of the preceding experiment. The Y-tube was rotated 180° after testing 5 thrips to avoid positional effects.

To test whether the experimental set-up was suitable to make volatile analyses, *p*-anisaldehyde (1%), a volatile compound known to be attractive to *F. occidentalis* in Y-tube olfactometer experiments (Frey *et al.*, 1994; Hollister *et al.*, 1995; Koschier *et al.*, 2000) was used as positive control. A second control treatment consisted of volatiles from non-infected TS led through both arms of the Y-tube. Controls were made after every 7th test.

Analysis of volatile compounds from TSWV-infected plants.

To analyse the compounds released by TSWV-infected and non-infected *Capsicum* plants, volatile blends were collected of 5 to 6 weeks old plants. The plants were severed with a sharp knife just above the cotyledons and placed on wet cotton wool folded in a piece of aluminium foil and subsequently placed in a 5 l glass jar. The jar was closed with a glass lid with an air-inlet and –outlet. Between the lid and jar a vitron O-ring was placed, and a metal clamp tightly closed both. Pressurised air was filtered over silica gel, a molecular sieve, and activated charcoal, and the resulting clean air led through the jar for 3 h at a flow rate of approximately 30 ml/min. To collect the volatile compounds of plants, the airstream from the outlet of the jar was led through a 90 mg Tenax-TA glass tube. After 2 h, the Tenax tube was removed from the jar and closed with ¼'' Swagelok caps for analysis by GC-MS.

Volatile blend collections of non-infected plants were carried out simultaneously with the collection of volatiles from virus-infected plants by using a glass connection to divide the clean air stream equally over two 5 l jars. Collections were made in duplo for each treatment.

The collected volatiles were released from the Tenax tube using a Thermodesorption Cold Trap set-up (Chrompack, Middelburg, the Netherlands) by heating the tube at 250ºC for 10 minutes with a helium flow of 12 ml/min. The desorbed compounds were collected in a cold trap at -90ºC. The volatiles were injected in splitless mode into the capillary linear column (60 m DB%, 0.25 mm i.d., 0.25µm film thickness) of a gas chromatograph by ballistical heating the cold trap to 220ºC. The column head pressure was 18 psi, and the initial linear velocity of the helium carrier gas was 22 cm/s. After an initial linear column temperature of 40ºC for 4 min, temperature was increased to 280ºC at a rate of 4ºC/min. The column was directly coupled to the ion source of a Finnigan MAT 95 mass spectrometer, which was operating in the 70 eV EI ionisation mode and scanning from mass 24 to 300 at 0.5 s/d. Compounds were identified by comparison of the mass spectra with those in the NIST98 library and in the Wageningen Mass Spectral Database of Natural Products, and by checking the retention index.
A compound was considered to be emitted in significant larger amounts if its relative contribution to the total volatile blend was higher in both replicates of infected plants, than of both replicates of non-infected plants. Additionally, the smallest relative contribution of a compound to the blend of TSWV-infected plants had to be bigger than the largest relative contribution of that compound to the blend of non-infected plants.

Attraction of *F. occidentalis* by individual volatile compounds.
The response of thrips to two individual volatile compounds that were released from TSWV-infected TS or TR plants but not or in a low amounts from non-infected plants was tested in a Y-tube olfactometer. The experimental set-up and procedures used were similar as described above. 1,2-Dimethoxybenzene (DMB; 99+%; Acros Organics, Geel, Belgium) and (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT; supplied by Miss. J. de Boer, Laboratory of Entomology, Wageningen University, the Netherlands) were diluted in paraffin oil (Merck, Darmstadt, Germany) to different concentrations. One µl of each solution was applied on a filter paper disk (5.5 mm in diameter), which was then placed in the vial of the olfactometer set-up and tested against 1µl of paraffin oil.

RESULTS

Attraction of *F. occidentalis* by volatiles from TSWV-infected pepper plants.
The suitability of the Y-tube olfactometer set-up for identifying thrips-attracting volatiles was validated prior to testing thrips responses to virus-infected and non-infected plants. To this end, *p*-anisaldehyde, a compound known to attract thrips (Frey *et al.*, 1994; Hollister *et al.*, 1995; Koschier *et al.*, 2000), was led through one arm of the Y-tube and clean air through the other arm. The percentage of thrips choosing the arm with airflow over 1% *p*-anisaldehyde was 68%. An additional control comprised the use of volatiles from non-infected pepper leaves (accession TS) in both arms, resulting in 51/49% proportions over both arms, indicating that the Y-tube olfactometer could reliably be used to test for thrips-attracting volatile blends.

Volatiles from two different infected pepper cultivars, i.e. a thrips-susceptible (TS) accession and a thrips-resistant (TR) accession, were tested for their potentials to attract thrips to verify whether within a single crop species, independent of their level of thrips resistance, thrips-attracting volatiles are present and conserved.
Significantly more thrips were attracted to volatiles released from TSWV-infected TS and TR plants than to volatiles from non-infected leaves of these plants ($P<0.05$; Table 7.1). As a control also the volatiles from PepSMV-infected leaves of these plants were tested against non-infected leaves, but these did not significantly affect thrips' responses (Table 7.1).

Table 7.1. Response of *F. occidentalis* to volatiles released from *Tomato spotted wilt virus* or *Pepper severe mosaic virus*-infected and non-infected leaves of two *Capsicum annuum* accessions (Pikante Reuzen and CPRO-1) in an Y-tube olfactometer.

	TSWV			PepSMV		
	Preference[&] (%)		No	Preference[&] (%)		no
Pepper accession	Infected	Healthy	Choice[&]	Infected	healthy	choice[&]
Pikante Reuzen ("TS")	65[a]*	35[b]	0[c]	50[a]*	41[a]	9[b]
CPRO-1 ("TR")	52[a]	35[b]	13[c]	40[a]	51[a]	9[b]

* Different superscript letters in a row for each virus indicate significant differences within the choice test ($P<0.05$).
[&] 3 min observation time

Analysis of volatile compounds from TSWV-infected and non-infected plants.
Volatile blends from TSWV-infected and non-infected leaves of the TS and TR pepper plants were analysed by GC-MS. In total 46 compounds were identified in these blends, comprising terpenoids, green leaf volatiles, alcohols, aldehydes, and ketones (Figs. 7.1 and 7.2). TSWV-infected TS plants emitted slightly more volatile compounds (34) than non-infected plants (32) (Fig. 7.1). Strikingly, 31% of the blend from TSWV-infected TS plants comprised 1,2-dimethoxybenzene (DMB; compound

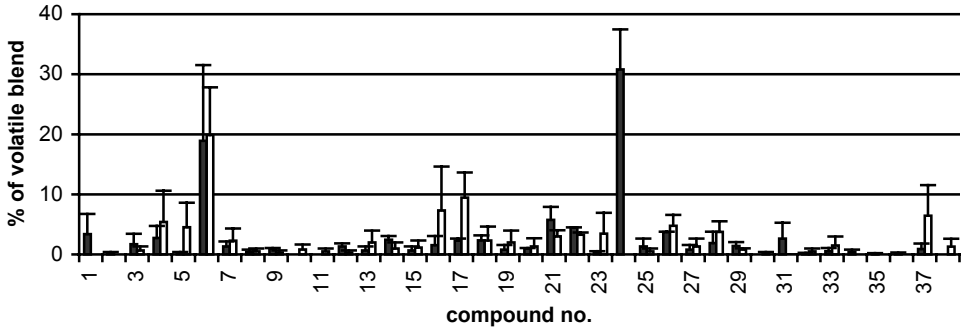

Figure 7.1. Volatile compounds released from TSWV-infected (grey bars) and non-infected plants (white bars) TS plants. Average peak areas of 2 replicates are shown. Error bars indicate the standard error of the mean.
Compound numbers are:
1. acetic acid **2.** (*E*)-3-hexen-1-ol **3.** hexanal **4.** (*E*)-2-hexenal **5.** (*E*)-2-hexen-1-ol **6.** (*Z*)-3-hexen-1-ol **7.** 1-hexanol **8.** cyclohexanone **9.** heptanal **10.** α-pinene **11.** β-pinene **12.** myrcene **13.** β-carene **14.** octanal **15.** 3-carene **16.** (*Z*)-3-hexen-1-ol, acetate **17.** 2-ethyl-1-hexanol **18.** limonene **19.** β-phellandrene **20.** (*E*)-β-ocimene **21.** linalool **22.** nonanal **23.** (3*E*)-4,8-dimethyl-1,3,7-nonatriene **24.** 1,2-dimethoxyben-zene **25.** methyl salicylate **26.** decanal **27.** benzothiazole **28.** 2-phenoxyethanol **29.** indole **30.** undecanal **31.** longifolene **32.** β-caryophyllene **33.** 1-dodecanol **34.** methyl-gamma-ionone **35.** β-selinene **36.** α-selinene **37.** (3E,7E)-4,8,12-trimethyl-trideca-1,3,7,11-tetraene **38.** 1-octadecanol.

nr. 24; Fig. 7.1), whereas non-infected plants did not release this compound. Absolute amounts of the volatile compounds could be estimated from results based on earlier GC-MS analyses on five different compounds (data not shown). The total amounts of volatile compounds released from TSWV-infected (110 ± 32 ng) and non-infected TS plants (133 ± 39 ng) were similar.

The composition of the volatile blend of TR plants differed noteworthy from that of TS plants. Only 17 compounds were detected in the volatile blend from non-infected TR plants, while TSWV-infected TR plants released 23 compounds (Fig. 7.2). TSWV-infection of TR plants did not result in increased release of DMB as found for the TS accession, but in an virus-induced release of 2-phenoxyethanol (no. 20) and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (no. 27), and in the enhanced release of (*Z*)-3-hexen-1-ol (no.5), linalool (no. 14) and (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT; no. 16), (Fig. 7.2). These five compounds were also detected in the blend of TS plants, but only (*Z*)-3-hexen-1-ol occurred in relatively high amounts. For the TR cultivar the absolute amount of volatiles released from TSWV-infected (88 ± 26 ng) was considerably higher than that from non-infected plants (25 ± 7 ng).

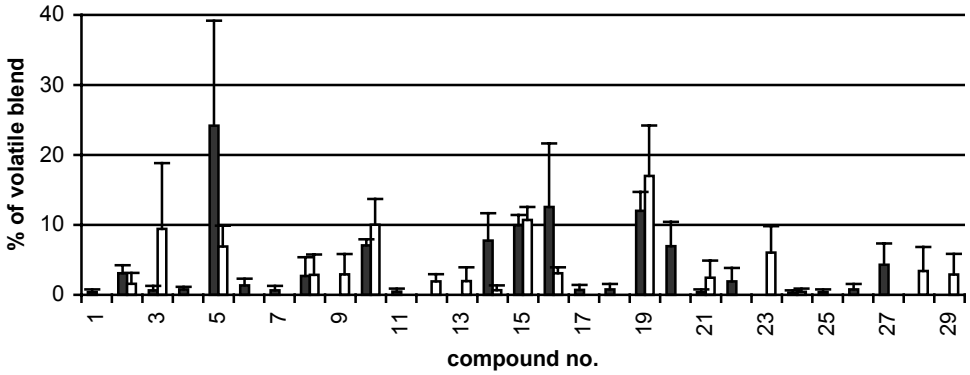

Figure 7.2. Volatile compounds released from TSWV-infected (grey bars) and non-infected plants (white bars) TR plants.
Compound numbers are:
1. (*E*)-3-hexen-1-ol **2.** hexanal **3.** butyl acetate **4.** (*E*)-2-hexenal **5.** (*Z*)-3-hexen-1-ol **6.** 1-hexanol **7.** heptanal **8.** octanal **9.** (*Z*)-3-hexen-1-ol, acetate **10.** 2-ethyl-1-hexanol **11.** phenylacetaldehyde **12.** limonene **13.** 2-phenyl-2-propanol **14.** linalool **15.** nonanal **16.** (3*E*)-4,8-dimethyl-1,3,7-nonatriene **17.** β-phenylethanol **18.** 1,2-di-methoxybenzene **19.** decanal **20.** 2-phenoxyethanol **21.** indole **22.** β-elemene, cis- **23.** undecanal **24.** dodecanal **25.** β-selinene **26.** α-selinene **27.** (3E,7E)-4,8,12-tri-methyltrideca-1,3,7,11-tetraene **28.** benzophenone **29.** 2-phenylacetophenone.

Attraction of *F. occidentalis* by individual volatile compounds.

The results from the GC-MS analysis showed that some volatile compounds were released only from TSWV-infected plants and not from non-infected plants, or at higher rates from former plants upon virus-infection. For the TS cultivar DMB was exclusively released from infected plants, while for the TR cultivar 2 compounds were exclusively released from infected plants and 3 at enhanced levels. Two of these compounds, i.e. DMB (from infected TS plants) and DMNT (from infected TR plants), were tested in a Y-tube olfactometer to determine their potential for attracting thrips. Thrips were significantly attracted when using 1 and 10 μg DMB diluted in paraffin oil, but not to higher or lower amounts (Table 7.2). Similarly, 1 μg DMNT attracted thrips significantly, but 0.1, 10 and 100 μg did not (Table 7.2). These results indicate that DMB and DMNT are volatile compounds released from TS and TR plants, respectively, at least in part responsible for the observed higher attractiveness of infected plants of these cultivars.

Table 7.2. Responses of *F. occidentalis* to 1,2-dimethoxybenzene (DMB) or (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) in different concentrations in Y-tube olfactometer tests. DMB and DMNT were diluted in paraffin oil.

	DMB			DMNT		
	Preference (%)			Preference (%)		
Amount (µg)	DMB	Parafin oil	No choice[&]	DMNT	parafin oil	no choice[&]
0.1	56[a]*	44[a]	0[b]	52[a]	48[a]	0[b]
1	59[a]	40[b]	1[c]	68[a]	31[b]	1[c]
10	71[a]	20[b]	9[c]	46[a]	47[a]	7[b]
100	58[a]	42[a]	0[b]	54[a]	43[a]	3[b]

* Different superscript letters in a row for each volatile compound indicate significant differences within the choice test ($P<0.05$).

DISCUSSION

This is the first report demonstrating that TSWV-infected host plants produce volatiles that enhance the attraction of the virus-vector *F. occidentalis*. By finding such volatiles in two different pepper accessions, one susceptible (TS) and the other resistant to thrips (TR), it can be concluded that the level of thrips resistance, previously based on lack of reproduction, low preference and minimal feeding damage (Maris *et al.*, 2003c; Chapter 2), does not determine whether the host plant does produce thrips-attractive volatiles after TSWV-infection or not. However, the nature of the TSWV-induced volatile compounds differ among both accessions, the infected TS accession releasing DMB and infected TR accession releasing at least one other thrips-attracting compound i.e. DMNT. It should be emphasised here that the GC-MS analysis as well as the testing of the purified compounds are not yet complete and rather preliminary. Sofar only 1 out of the 5 virus-induced compounds of the TR accession has been tested, while also the difference between DMB and DMNT in thrips attraction rate still needs to be determined. Nonetheless, at this point an important conclusion may already be drawn: within a single crop species a possible thrips-attracting volatile(s) is not conserved.

To date DMB and DMNT have not been associated with neither vector attraction nor with virus-infection of host plants. Both compounds, though, have been implicated in the behaviour of other insect species. DMB has been reported as a major behaviourally active compound in the oviposition aggregation pheromone of the desert locust, *Schistocerca gregaria* (Rai *et al.*, 1997). This compound also elicited a high electro-antennogram (EAG) response of the vine weevil *Otiorhynchus sulcatus* (van Tol & Visser, 2002). DMNT has been reported to be released from lima bean (*Phaseolus lunatus*) plants after infestation with the spider mite *Tetranychus urticae* and is able to attract females of the predatory mite species *Phytoseiulus persimilis*

and *Amblyseius potentillae* to the plants under attack (Dicke *et al.*, 1990; van den Boom, 2003). DMNT is also produced in cowpea (*Vigna unguiculata*), sweet pepper (*Capsicum annuum* cv. Lambada), *D. stramonium*, and grapevine (*Vitis vinifera*) plants after infestation with *T. urticae* (van den Boom, 2003).

Plants infected with the aphid-transmitted virus PepSMV did not attract thrips, indicating that the induction of thrips-attracting volatiles is virus specific. A similar specific relation has been found for *Myzus persicae* and *Potato leafroll virus* (Eigenbrode *et al.*, 2002).

Interpretation of our GC-MS-data in relation to biological activity at plant level is difficult. The average amount of DMB released from TS plants and trapped in the Tenax-tube was 1.4 ± 0.4 ng per gram of infected plant (data not shown). This was released in 2 h in which 3.6 l air was led through the Tenax tube. The 1.4 ng DMB released from these plants might be considerably lower than the amount of pure DMB to which the thrips were exposed in the Y-tube tests (1 μg and 10 μg). The rate of DMB release from the paraffin in these tests is unclear, but it has to be stated that these amounts are not released from the paraffin at the same time but slowly in time. Also the exposure time in the Y-tube only 3 min, which makes an estimation of real amount to which thrips are exposed difficult. As thrips were able to distinguish between volatiles of TSWV-infected and non-infected leaves at only a 100-200 mg level in Y-tube tests, it seems therefore most likely that also the composition of the total volatile blend including possible synergistic effects of different compounds determines the thrips' preference and not a single compound. The same might hold for DMNT released from infected TR plants. On average, 0.20 ± 0.1 ng DMNT was released per gram TSWV-infected plants, whereas 1 μg DMNT (slowly released from the paraffin) attracted thrips significantly in the Y-tube tests. It remains to be tested whether the other four compounds induced in TR plants after TSWV-infection also contribute in thrips attraction, either individually or synergistically.

The experimental Y-tube set-up used in the present studies precluded visual orientation of thrips to leaves. Also tactile or gustatory cues did not interfere with the interpretations, as thrips could not contact the leaves. To date, these cues have been hold responsible for attraction of thrips towards TSWV-infected plants (Bautista *et al.*, 1995; Lewis, 1973; Selman *et al.*, 1961; Yudin *et al.*, 1988). Previous studies demonstrated that thrips preferred to feed and oviposit on TSWV-infected *Lactuca sativa* (Yudin *et al.*, 1988), *Arctium lappa*, *D. stramonium* plants (Bautista *et al.*, 1995), and *C. annuum* plants (Maris *et al*., 2004; Chapter 6). The present study demonstrates evidently that besides beneficial changes in the physiology of virus-infected plants (Lewis, 1973; Selman *et al.*, 1961), yellow colouring of chlorotic leaves

(Yudin *et al.*, 1988), and curling of leaves offering satisfactory shelter for thrips (Carter, 1939), also volatile cues are involved in thrips attraction. The relative importance of these different cues induced in TSWV-infected plants still remains to be determined. It is clear, though, that attraction of thrips to TSWV-infected plants, by either cue(s), will be beneficial for both the virus and the thrips, the virus being acquired by increased numbers of hatched thrips larvae, and the thrips, in turn, being navigated to improved host plants.

ACKNOWLEDGEMENTS

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Chapter 8

Summary and concluding remarks

Introduction and aim of the study.

Tomato spotted wilt virus (TSWV) is an enveloped plant virus which is propagatively transmitted by a limited number of thrips species (Thysanoptera: Thripidae) all belonging to the genera *Thrips* and *Frankliniella*. *Frankliniella occidentalis* Pergande (generally known as the Western flower thrips) is currently considered to be the most important vector in both the US and Europe (Daughtrey *et al.*, 1997; Ullman *et al.,* 1997; Wijkamp *et al.*, 1995a). TSWV can only be acquired by young larvae (L1 stage) whereas both old larvae (late L2 stage) and adults are capable to transmit the virus (van de Wetering *et al.*, 1996; Wijkamp *et al.*, 1993). TSWV causes significant crop losses in many ornamental and agricultural crops. Control of TSWV has proven to be difficult due to its wide host range, the ability of its thrips vectors to colonise on many weeds and on cultivated plant species, the worldwide trading of thrips-infested plant material, and increased tolerance of thrips to insecticides. Breeding for resistance has remained cumbersome, as for only a few crops, resistance to TSWV is available, including chrysanthemum (Daughtrey *et al.*, 1997), lettuce (Cho *et al.*, 1997), pepper (Black *et al.*, 1991; Boiteux & de Ávila, 1994; Boiteux *et al.*, 1993) and tomato (Stevens *et al.*, 1992). Another potential control measure could be to introduce thrips resistance into threatened crops. Promising results on limiting virus spread in insect-resistant crops have indeed been reported for some plant virus/vector combinations (Berlinger *et al.*, 1986; Bouguet, 1981; Parejarearn *et al.*, 1984; Rizvi & Raman, 1983). Significant levels of thrips resistance have been found in cabbage (Stoner & Shelton, 1986), chrysanthemum (Broadbent *et al.*, 1990; de Kogel *et al.*, 1998), groundnut (Kinzer *et al.*, 1973; Rhoda *et al.*, 1991), pepper (Fery & Schalk, 1991; Maris *et al.*, 2003c; Chapter 2), and tomato (Kumar *et al.*, 1995).

However, at the onset of the study reported in this thesis, the potential of thrips resistance to control TSWV infections had hardly been investigated. It could be argued that thrips resistance could be beneficial in the control of TSWV, but it could equally be argued, even supported by some experimental data (van de Wetering, 1999), that thrips resistance would have an adverse effect on the spread of this virus.

In search for suitable hosts, thrips might exhibit increased activities (both in spread and in making feeding probes) within thrips-resistant crops, thus promoting virus inoculation and virus spread.
The objective of this study was therefore to analyse the effect of thrips (*F. occidentalis*) resistance in pepper (*Capsicum*) on the spread of TSWV. *Capsicum* is an economically important crop grown in the field in subtropical regions and in greenhouses in temperate climate zones. These environmental conditions also support the development of thrips populations and, hence, the spread of TSWV.

Selecting suitable pepper material for the study.
To assess the effects of thrips resistance on TSWV spread, it was of prime importance to select suitable accessions with different levels of thrips and virus resistance. A series of *Capsicum* accessions was therefore investigated and compared in the first phase of the project (Chapter 2). Based on three criteria, i.e. low preference (antixenosis), lack of reproduction, and minimal feeding damage (antibiosis), two thrips-resistant susceptible (CPRO-1 and PI 152225) and two fully susceptible accessions (Pikante Reuzen and PI 159236) were identified. The accessions CPRO-1 and Pikante Reuzen were equally susceptible to TSWV, whereas PI 152225 and PI 159236 were resistant to this virus. Accessions CPRO-1 and Pikante Reuzen were selected for further studies, as both were equally susceptible to TSWV and, hence, very suitable to assess the value of thrips resistance by comparative analysis. These accessions will be further referred to as TR (thrips resistant) and TS (thrips susceptible), respectively.
With respect to the mechanism of virus spread within a crop, a distinction was made between primary infection (introduction of TSWV by incoming viruliferous thrips) and secondary spread (radiation of virus from primary infected plants to healthy plants by colonising thrips).

Effect of thrips resistance on primary infections.
The rate of primary infections by thrips in a crop depends on different parameters, such as host preference, rate of thrips infestation, feeding behaviour, and virus inoculation efficiency. The work presented in Chapters 3 and 4 demonstrates that the rate of primary TSWV-infection is effectively limited in the TR pepper crop. Further studies indicated that this effect is based on a decreased preference of thrips for TR plants (Chapter 5). Decreased preference was evaluated by monitoring dispersal and remaining thrips on TR plants and TS control plants for a given period after releasing equal numbers of thrips on these phenotypes. Thrips exhibited a higher emigration/immigration ratio on TR plants than on TS plants when the insects could freely move between the plants (Chapter 5). Apparently, thrips can perceive some cues by which they are discouraged to fly to or to stay on TR plants. Nevertheless,

thrips which decided to stay on either phenotype on which they were released showed a similar behaviour, expressed as frequency and duration of “walking” or “resting” periods on both plant types. Inoculation efficiency was hardly affected by thrips resistance, as the efficiency was only lower on TR plants during longer inoculation access periods (Chapter 2). The almost unaffected inoculation efficiency on TR plants can be explained by the relatively short minimal inoculation access period (Wijkamp *et al.*, 1996b).
The increased emigration/immigration ratio of thrips on TR plants compared to TS plants would result in less primary TSWV-infections on the former plants. This prediction was confirmed in small-cage experiments with 8 or 16 plants, and in a large-scale greenhouse experiment (Chapter 3 & 4). Nine to 50% of the TR plants became primarily infected, whereas 39 to 100% of the TS plants became infected in these different experiments.

Effect of thrips resistance on secondary spread.
Secondary TSWV-infections in a crop depend on the built-up of a thrips population and on the dispersal of thrips from primarily infected source plants on which they developed (as to acquire the virus during their larval stage). The work presented in Chapters 3 and 4 demonstrates that secondary infections are effectively restricted in the TR pepper crop and it appears that this effect is based on a greatly reduced thrips population built-up (Chapter 5). Population built-up on TR plants was evaluated by monitoring the number of offspring after release of female thrips. Significantly fewer eggs were produced on the TR plants compared to the TS plants. Besides, the larval mortality rate was significantly higher on the TR plants.
The observed restricted population built-up was expected to reduce the rate of secondary infections in the TR plants. Both small-scale cage experiments and greenhouse experiments showed this was indeed the case. No secondary spread at all occurred in mono-cultural set-ups due to the extinction of the thrips population on these plants, whereas the spread in mixed plots of TR and TS plants was significantly delayed on the former plants (Chapters 3 and 4).

Synergistic effects of thrips- and TSWV resistance in pepper.
The studies described in this thesis and discussed in the previous paragraphs demonstrate that thrips resistance in pepper delayed the spread of TSWV significantly, even when the crop is fully susceptible to the virus. Nevertheless, since thrips resistance does not protect the crop sufficiently against virus infection when thrips susceptible plants are present around or in the field or greenhouse, additional measures are required to minimise or prevent economic losses. The use of virus-resistant plants will be the favoured way to control virus infections in crops. This trait is present in the selected thrips-resistant accession PI 152225 and the thrips-

susceptible accession PI 159236, responding with a hypersensitive reaction after infection with TSWV, resulting in necrotic local lesions on leaves and fruits. Under the same vector pressure, fewer local lesions were found in the thrips-resistant accession PI 152225 than in the thrips-susceptible PI 159236 accession indicating a synergistic effect of thrips- and virus resistance. Additional beneficial effects of virus-resistant accessions might be expected as the reduced initial population development of thrips on these plants lowers thrips infection pressure, and therefore also the virus infection pressure. This, in turn, reduces the chance that the TSWV resistance trait might become broken, a risk previously reported for tomato and pepper (Roggero *et al.*, 2002; Thomas-Caroll & Jones, 2003). Additionally, as the virus does not spread systemically in virus-resistant plants, thrips can not acquire TSWV from these plants, which will prevent secondary spread of the virus in the field. The synergistic effects of TSWV- and thrips resistance in *Capsicum* will therefore be a good tool in integrated pest management programs aimed to control TSWV-epidemics.

Comparing results with spread of other viruses in insect-resistant crops.

Restricted spread of TSWV in thrips resistant crops has also been demonstrated in studies with thrips resistant tomato (Kumar *et al.*, 1993; 1995). Significantly fewer plants of four *Lycopersicon* cultivars became infected with TSWV by thrips inoculations in the field than by mechanical inoculation of these accessions in the laboratory (Kumar *et al.*, 1993), indicating that (unknown) vector-mediated components were involved. It was suggested that the four accessions were thrips resistant and that the lower virus incidence was due to changed feeding behaviour of thrips on these accessions. A restricted virus spread was also found in laboratory and field studies on the non-ciculatively transmitted viruses *Beet yellow virus*, *Cucumber mosaic virus*, *Pea stunt virus* and *Watermelon mosaic virus* in aphid-resistant crops (Haniotakis & Lange, 1974; Lecoq *et al.*, 1979; Lecoq *et al.*, 1980; Martin *et al.*, 1998; Pitrat & Lecoq, 1982; Wilcoxon & Peterson, 1960). The resemblance between the restricted spread of these non-persistently transmitted viruses and persistently transmitted TSWV might indicate that the dispersal between plants, (feeding) behaviour on plants, and the reproduction of the vector seem to be of major importance on virus spread in resistant accessions rather than the mechanism of virus transmission. This was also demonstrated in studies on the transmission of *Cowpea aphid-borne mosaic virus* (CABMV), a non-persistently transmitted virus by *Aphis craccivora*. The increased CABMV-spread was explained by the higher number of probes made by the aphids on resistant plants compared with susceptible plants (Atiri *et al.*, 1984).

The study described in this thesis show evidently that thrips resistance is a proper trait to control the spread of TSWV, as both primary and secondary infections become restricted due to a higher emigration/immigration ratio and a significantly

decreased population development, respectively. At the onset of this research, though, it was reckoned that the outcome of our investigations could have been different, i.e.that virus spread might have been promoted by thrips resistance due to increased mobility and altered feeding behaviour, as previously suggested (van de Wetering, 1999). Our study demonstrates that thrips resistance in pepper (that may be used to reduce feeding damage in a crop) does not result in an increased TSWV-spread. Even in a sort of worst-case scenario, i.e. using mixed plots of TR and TS plants and high vector pressure, virus spread was significantly delayed in the trips resistant accession (Chapters 3 and 4).

Mutual benefits for TSWV and its vector in their virus-vector-plant relations.

The relationship between TSWV and thrips is very specific, the virus being fully dependent on its vector for its survival and spread. Only young larvae acquire the virus and only after subsequent multiplication (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993) and timely passage of a midgut barrier (Nagata, 1999) during larval development, TSWV can be transmitted by second instar larve (L2s) and adults (Wijkamp & Peters, 1993). Once the vector becomes viruliferous, it remains so throughout its lifespan. The ecology of TSWV would thus clearly benefit from an enhanced suitability of infected host plants with respect to thrips oviposition and larval development. The studies presented in Chapter 6 show that *F. occidentalis* is positively attracted to TSWV-infected plants (of both the TR and TS accession) resulting in more offspring on these plants and thereby enhancing the chance for virus transmission. Thrips attraction was already known to be based on visual cues (Bautista *et al.*, 1995; Lewis, 1973; Selman *et al.*, 1961; Yudin *et al.*, 1988) but Chapter 7 demonstrates that also volatiles released from TSWV-infected plants may be involved. Besides, thrips developed significantly faster on TSWV-infected plants than on non-infected plants (Chapter 6). This was due to shorter egg and larval stages, whereas the development of pupae was not affected by TSWV-infection.

These findings demonstrate that *F. occidentalis* and TSWV have mutual benefits from the attraction to virus-infected plants. The attraction results in a larger proportion of viruliferous individuals in a thrips population thereby enhancing virus spread. Thrips, in turn, will profit as TSWV-infected plants are more suitable hosts for both feeding and reproduction.

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Samenvatting

Het tomatenbronsvlekkenvirus (in het Engels “tomato spotted wilt virus”, afgekort TSWV) staat wereldwijd te boek als een van de meest schadelijke plantenvirussen. Het virus tast vele belangrijke land- en tuinbouwgewassen aan ongeacht of deze geteeld worden in het veld of in de kas. Belangrijke gewassen die te lijden hebben van TSWV-infecties zijn bijvoorbeeld aardappel, paprika, sla, tabak en tomaat. Daarnaast kan het virus ook diverse siergewassen en onkruiden infecteren. Het virus veroorzaakt een scala aan symptomen variërend van necrose (afsterving van geïnfecteerde plantendelen of van de gehele plant), verwelking, vruchtval, dwerggroei, misvorming van de plant en/of vrucht, verkleuring van bladeren, bladvlekken, en ringpatronen op de bladeren. Symptoomvorming is afhankelijk van diverse factoren zoals soort en genotype van de waardplant, seizoen, klimatologische omstandigheden en bemestingsfactoren.

TSWV behoort tot het genus *Tospovirus* binnen de grote virusfamilie *Bunyaviridae* en wordt uitsluitend overgedragen door tripsen (*Thripidae*). Zowel in Noord-Amerika als in Europa geldt de Californische trips (*Frankliniella occidentalis*) op dit moment als de meest belangrijke overbrenger (vector) van TSWV. Oorspronkelijk kwam deze trips alleen in Noord-Amerika voor, maar sinds de jaren tachtig van de vorige eeuw vertoont deze soort een sterke expansie en wist toen o.a. ook Europa te bereiken. Deze expansie hangt samen met de toegenomen wereldwijde handel van plantmateriaal.

Preventie of beheersing van TSWV-infecties is in de praktijk moeilijk vanwege het grote waardplantbereik van zowel het virus als de vector, de verborgen leefwijze van de vector, en, last-but-not-least, de toegenomen tolerantie van tripsen voor insecticiden. Het veredelen op virusresistentie heeft tot op heden beperkt resultaat opgeleverd omdat er slechts weinig natuurlijke resistentiebronnen voorhanden zijn. Daarnaast zou veredeld kunnen worden op resistentie tegen tripsen. Werkbare niveaus van tripsresistentie zijn vandaag de dag in een aantal gewassen, waaronder chrysant, aardnoot, paprika en tomaat, gerapporteerd. Echter, bij aanvang van het hier beschreven promotieonderzoek was slechts het directe effect van tripsresistentie op het beperken van tripsschade onderzocht, terwijl de mogelijkheid om hiermee ook TSWV-infecties te beheersen niet of nauwelijks onderzocht was. Enerzijds zou verondersteld kunnen worden dat tripsresistentie een goede bijdrage zou kunnen leveren aan beperking van virusverspreiding, anderzijds zou echter net zo goed kunnen worden verondersteld dat tripsresistentie juist verdere virusverspreiding in de

hand zou werken. Voortdurend zoekgedrag van tripsen op een minder geschikte voedingsplant zou immers kunnen leiden tot meer proefboringen en aldus zou dan tripsresistentie een negatief effect kunnen hebben op de TSWV verspreiding. Doel van het in dit proefschrift beschreven onderzoek was dan ook het effect van resistentie tegen de Californische trips in een belangrijk gewas, te weten paprika (*Capsicum annuum*), op de verspreiding van TSWV vast te stellen. Paprika is een economisch belangrijk gewas dat zowel in kassen (Noord-Amerika en grote delen van Europa) als in het open veld (mediterrane en subtropische gebieden) geteeld wordt. De omstandigheden in de kas en in de warmere klimaatzones bevorderen de ontwikkeling en verspreiding van zowel tripsen als TSWV, en aldus is paprika een interessant modelgewas.

In de aanvangsfase van het onderzoek werd allereerst de gevoeligheid van een aantal paprikarassen voor TSWV en tripsen onderzocht (Hoofdstuk 2). Om het effect van tripsresistentie op virusverspreiding te kunnen bepalen was het essentieel om uit te kunnen gaan van het juiste uitgangsmateriaal. De mate van tripsresistentie in de diverse paprikarassen werd onderzocht op basis van drie criteria, te weten preferentie, reproductie, en vraatschade. Op basis van deze drie criteria werden twee tripsresistente rassen (CPRO-1 en PI 152225) en twee tripsvatbare rassen (Pikante Reuzen en PI 159236) geselecteerd. De rassen CPRO-1 en Pikante Reuzen bleken even vatbaar voor TSWV-infectie te zijn, terwijl de rassen PI 159236 en PI 152225 TSWV-resistent bleken. Op basis van deze analyses werden de rassen CPRO-1 en Pikante Reuzen geselecteerd voor verder onderzoek, en worden hieronder kortheidshalve aangeduid als TR (tripsresistent) en TS (tripsgevoelig), respectievelijk.

In diverse proefopstellingen bleek de mate waarin TR planten primair geïnfecteerd worden (d.w.z. door vanuit de omgeving afkomstige virusdragende tripsen) significant lager te zijn dan voor TS planten (Hoofdstukken 3 en 4). D.m.v. verschillende experimenten kon worden vastgesteld dat de TR planten tot maximaal 50% geïnfecteerd werden terwijl TS controle planten allen besmet raakten (Hoofdstuk 5). Dit was toe te schrijven aan een verminderde voorkeur van tripsen voor de TR planten. Secundaire verspreiding van het virus (d.w.z. de verspreiding vanaf een geïnfecteerde plant in het gewas naar de omringende gezonde planten) was eveneens significant lager in TR dan in TS planten (Hoofdstukken 3 en 4). Deze geringere verspreiding kon vervolgens toegeschreven worden aan een verminderde populatieopbouw van tripsen op de TR planten (Hoofdstuk 5).
Vervolgens werd het effect van tripsresistentie op de verspreiding van TSWV in een virusresistent ras bestudeerd. De beschikbare virusresistente paprikarassen reageren met een “overgevoeligheidsreactie” op virusinfectie en hierbij ontstaan necrotische lokale lesies rond de primaire infecties in blad en vrucht. Op

tripsresistente PI 152225 planten ontstonden onder gelijke vectordruk minder necrotische lesies dan op de tripsvatbare PI 159236 planten, waardoor enerzijds opnieuw aangetoond werd dat tripsresistentie leidt tot minder virusinfecties, en anderzijds dat het zinvol is om tripsresistentie en virusresistentie in de plant te stapelen om aldus mogelijke cosmetische schade te beperken (Hoofdstuk 4). Al met al laten de resultaten duidelijk zien dat tripsresistentie een goede bijdrage kan leveren om de verspreiding van TSWV te beperken en dat deze eigenschap dus niet resulteert in een ongewenst grotere verspreiding van het virus via een veranderd voedingsgedrag van de vector.

In de loop van het promotieonderzoek werd vastgesteld dat *F. occidentalis* adulten een voorkeur hebben voor het voeden op TSWV-geïnfecteerde planten en als gevolg hiervan ook meer eieren op deze planten leggen. Deze resultaten, die beschreven staan in hoofdstuk 6, leiden tot een beter inzicht in de specifieke interacties tussen virus en vector. Uitsluitend in het jonge, larvale stadium kunnen tripsen TSWV opnemen, waarna oudere larven en (vooral) volwassen tripsen het virus kunnen overbrengen. Naarmate er meer volwassen tripsen eieren leggen op virusgeïnfecteerde planten en de uitgekomen jonge larven het virus daar opnemen, zal dit de kans op succesvolle verspreiding van TSWV ten goede komen. Omdat tevens werd aangetoond dat tripsen zich op TSWV-geïnfecteerde planten sneller ontwikkelen tot adult kan geconcludeerd worden dat er in de interactie tussen virus en vector voor beiden winst te halen valt (Hoofdstuk 6).
In Hoofdstuk 7 werd onderzocht of TSWV-geïnfecteerde planten specifieke geurstoffen produceren waarmee tripsen aangetrokken kunnen worden. Gebruikmakend van een olfactometer bleek dit inderdaad het geval. Nadere analyses toonden aan dat TSWV-geïnfecteerde TS planten 1,2-dimethoxybenzene (DMB) afscheiden terwijl gezonde TS planten dit niet doen. TSWV-infectie in TR planten resulteerde daarentegen niet in de inductie van DMB maar van vijf andere stoffen die in aanmerkelijk hogere concentraties voorkwamen dan in het geurmengsel van niet-geïnfecteerde TR planten. Een van deze stoffen, (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), en DMB bleken als zuivere component tripsen inderdaad aan te kunnen trekken. Aldus tonen de in hoofdstukken 6 en 7 verkregen resultaten aan dat bij de interacties tussen *F. occidentalis* en TSWV zowel het virus als de vector voordeel hebben bij de aantrekking van tripsen naar geïnfecteerde planten.

Nawoord

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Curriculum vitae

Op 1 juni 1976 werd ik, Paulus Cornelis Maris, geboren in het Hoekse Waardse Zuid-Beijerland. Tijdens de eerste 18 jaar van mijn leven groeide daar mijn interesse in het landbouwkundig onderzoek. Na het behalen van mijn VWO-diploma aan de Rijks Scholen Gemeenschap in Oud-Beijerland in 1994, ben ik “Plantenveredeling en Gewasbescherming” gaan studeren aan de toenmalige Landbouw Universiteit Wageningen. Tijdens de doctoraalfase heb ik me met name verdiept in de Nematologie. Achtereenvolgens heb ik afstudeeropdrachten gedaan bij het toenmalige Praktijkonderzoek voor Akkerbouw en Vollegrondsgroenten (PAV) in Lelystad, bij de University of California Riverside, USA, en tot slot bij het toenmalige Instituut voor Plantenziekenkundig Onderzoek (IPO-DLO) in Wageningen. In 1999 heb ik mijn doctoraaldiploma behaald en ben ik aansluitend begonnen met mijn promotie-onderzoek aan het Laboratorium voor Virologie van de Wageningen Universiteit. De resultaten van dit promotie-onderzoek staan beschreven in dit proefschrift. Sinds november 2003 ben ik werkzaam als Onderzoeker Fytopathologie bij De Ruiter Seeds in Bergschenhoek.

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DE RUITER SEEDS
HYBRID SEEDS

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