Ceratothripoides claratris, Capsicum chlorosis virus and *Solanum lycopersicum*: A Case Study of Thrips - Tospovirus - Plant Interaction

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Summary

The protected cultivation of tomatoes in central Thailand is constrained by the oriental tomato thrips, *Ceratothripoides claratris*, and the tospovirus, Capsicum chlorosis virus (CaCV), transmitted by the thrips. The epidemiology of the tospovirus is characterized by the behaviour (e.g. distribution pattern), transmission efficiency of the vector and properties (e.g. nutrional quality, defence) of the common host plant. However, little was known about this triangle tospovirus-thrips-plant interaction. Therefore, in depth studies of the tospovirus-thrips interrelationships and the role of the host plant in this trilateral relationship were performed. All experiments were realized in laboratories or greenhouses at the Asian Institute of Technology (AIT), within the frame of the program of the DFG research group FOR 431 “Protected cultivation – an approach to sustainable vegetable production in the humid tropics”.

First a new leaflet assay was developed and proved to be superior to other conventionally used methods. Consequently, this leaflet assay was used to study the vector biology of *C. claratris*. The results showed that only first and early second instar larvae can acquire the tospovirus, CaCV (isolate AIT), resulting in 10-22% of the resultant adults being viruliferous. Though, 80% of viruliferous thrips started transmitting the CaCV at the first after emergence as adults, still 20% of the viruliferous thrips could transmit also during their late second larval stage. All viruliferous thrips retained their ability to transmit the virus during their whole life span. Adults of the thrips *C. claratris* were unable to transmit the CaCV-AIT when feeding on virus infected leaves first happened as adults.

The percentages of viruliferous thrips within the tested populations was unexpectedly low, moreover we observed a progressive and finally complete loss of transmission ability in a sub-population kept isolated for about 20 generations. Consequently we hypothesized significant intra population variability of the property “viruliferous” and heritability of this trait. In *C. claratris*, indeed, the results of the second study provided strong support of the proposed hypothesis as the trait ‘vector competent’ was vertically inherited from uninseminated mothers to their offspring. 81% of the offspring of viruliferous uninseminated females were viruliferous too. On the other

hand, none of the offspring of the non-viruliferous uninseminated females developed to viruliferous individuals. Further crosses between viruliferous and non-viruliferous individuals suggested that the competence of the thrips *C. claratris* as a vector for CaCV is a heritable trait controlled by a recessive allele, and that the genetic background of the thrips is a key factor determining vector competence.

In the third part possible effects of CaCV-infected leaflets on *C. claratris* fitness (in terms of size, fecundity, and feeding activity) were evaluated. Results showed a reduction in the size of male thrips feeding throughout their larval period on CaCV-infected tomato leaflets compared to cohorts feeding on uninfected leaflets. Anatomical features of females were not affected on infected leaflets, however the fecundity was lowered. Further evaluation with individual females showed that the virus CaCV direct negative effects were much less than indirect plant-mediated effects. Unexposed virus free control females fed more intensively than CaCV-exposed viruliferous females on uninfected leaflets, and the CaCV-exposed non-viruliferous females were in-between. However, all cohorts of tested females fed less on infected leaflets than on uninfected ones with no significant differences between the cohorts; Mean daily fecundity was reduced in the CaCV-exposed thrips, yet only significant with the viruliferous females, whereas the fecundity of the unexposed control females was not affected. This suggests that the pre-imaginal nurture period is crucial to the fitness of the resultant adults.

When assessing a possible role of the common host plant in the CaCV-*C. claratris*-Tomato system, results of the fourth part showed that ontogenetic stages of the tomato plant (i.e., cotyledon, seedling and juvenile) influenced the amount of settling and colonisation by *C. claratris*. Moreover, the plant/leaf age affected the feeding intensity of the thrips. In a greenhouse choice experiment with young tomato plants of five different age categories, the infestation of the plants by *C. claratris* and the feeding-damage, as well as tospovirus infection increased significantly with the age of the plants. In no-choice experiments when thrips were confined inside a microcosm with one plant of different ontogenetic stages only 28% and 61% of plants in the cotyledon and seedling stages, respectively, showed feeding-damage, while 100% of juvenile plants had visible feeding-damaged leaflets. The results also suggest that cotyledons may have negative effect on tospovirus infection.

Summary

In conclusion the results of this study clearly indicate that many factors determine vector competence of *C. claratris* for the tospovirus CaCV and therefore efficient plant infection and virus spread: First, the thrips must feed on an infected source plant during a short and defined larval stage. Second, the thrips will develop to a successful transmitter of the tospovirus only if the individual genetic constitution (recessive allele) is fitting. Third, the thrips sex is a crucial factor. Fourth, the host plant sensitivity is variable during its development with young plant/leaf age stages being more resistant in terms of thrips settling and feeding behaviour and subsequent inoculation of the virus. Finally, the interaction between all or some of these factors makes the vector competence a highly complex trait. Yet, the here presented results are contributing to the understanding of the tospovirus-thrips-plant system.

Keyword: CaCV, vector competence, inheritance.

Zusammenfassung

Die Produktion von Tomaten im geschützten Anbau in Thailand (warme und wechselfeuchte Tropen) wird durch den Befall mit einer tropischen Thripsart *Ceratothripoides claratris*, vor allem aber durch das Tospovirus, Capsicum chlorosis virus (CaCV), welches durch diesen Thrips übertragen wird, stark beeinträchtigt. Die Epidemiologie des Tospovirus wird durch das Verhalten (Mobilität, Verteilungsmuster) und die Übertragungseffizienz (Vektorkompetenz) des Thrips aber auch durch Eigenschaften der gemeinsamen Wirtspflanze (Nahrungsqualität für den Vektor, Abwehrpotential) geprägt. Über Interaktionen in diesem Beziehungsdreieck zwischen dem Tospovirus (CaCV), dem Thrips (*C. claratris*) und der Wirtspflanze (Tomate) war zu Beginn der Studie wenig bekannt. Deshalb wurden detaillierte Untersuchungen zum genannten Themenkomplex durchgeführt. Alle Untersuchungen fanden in Laboratorien und tropischen Gewächshäusern (Netzhäuser mit Foliendächern) am Standort des Asian Institutes of Technology (AIT) statt und waren Teil des Forschungsprogramms der DFG Forschergruppe FOR 431 “Protected cultivation – an approach to sustainable vegetable production in the humid tropics”.

Zunächst wurde ein neues Biotest-Verfahren („Leaflet–assay“), dass eine längere Haltung von *C. claratris* auf isolierten Blättern der Tomate und eine einfache und präzise Bestimmung von Virusübertragungsraten ermöglicht, entwickelt. Untersuchungen zur stadienabhängigen Virusübertragung zeigten, dass nur die Virusaufnahme während des ersten und zweiten Larvenstadiums *C. claratris* zu einem infektiösen („viruliferous“) und effektiven Vektor machen kann. 10 -22% der adulten Thripse, die sich aus Larven mit Virusaufnahme im ersten und zweiten Stadium entwickelten, waren erfolgreiche Überträger. 80% der potentiellen Vektoren konnten das CaCV Virus aber erst nach Abschluss der Entwicklung zum Adultstadium übertragen. 20% dieser Kohorte waren auch schon im späten zweiten Larvenstadium erfolgreiche Vektoren. Alle virusübertragenden Thripse behielten diese Fähigkeit bis zum Lebensende. Adulte waren allerdings nicht zur Virusübertragung in der Lage, wenn sie erstmals im Adultstadium an virusinfizierten Blättern saugten.

Zusammenfassung

Der Prozentsatz übertragender Thripse in der Testpopulation war unerwartet niedrig. Zudem konnte eine zunehmende Abnahme der Übertragungsrate bis zum völligen Verlust dieser Fähigkeit in einer über 20 Generationen isolierten und ingezüchteten Subpopulation beobachtet werden. Daraus konnte die Hypothese abgeleitet werden, dass die Fähigkeit (der Phänotyp) zur Übertragung eine vererbbare Komponente besitzt. Diese Vermutung konnte durch Kreuzungsversuche bestätigt werden: Nachkommen übertragender Weibchen, die aus unbefruchteten Eiern hervorgingen, waren zu 81% Überträger. Andererseits entwickelten sich aus unbefruchteten Eiern nicht übertragender Weibchen in keinem Fall Überträger. Weitere Kreuzungen zwischen übertragenden („viruliferous“) und nicht übertragenden („non-viruliferous“) Individuen bestätigten, dass die Übertragungsfähigkeit erblich ist und vermutlich durch ein rezessives Allel kontrolliert wird. Somit konnte der genetische Hintergrund individueller Thripse als wesentlicher Variabilitätsfaktor für die Vektorkompetenz identifiziert werden.

Der dritte Abschnitt dieser Studien befasste sich mit dem möglichen Einfluss des Virus auf wichtige Fitnessparameter (Größe, Fruchtbarkeit, Saugaktivität) von *C. claratris*. Es zeigte sich, dass Männchen, die während ihrer gesamten Larvalentwicklung an CaCV infizierten Tomatenblättern saugten, eine geringere Größe als Männchen aus Vergleichskohorten an virusfreien Blättern aufwiesen. Bei Weibchen ergaben sich keine Unterschiede in morphologischen Parametern. Bei letzteren war allerdings die Fruchtbarkeit bei der Entwicklung an virusinfizierten Blättern reduziert. Dabei überwogen die indirekten Effekte der „Nahrungsqualität“ aus der infizierten Pflanze die direkte Wirkungen der Viren auf die Weibchen bei weitem: Nicht exponierte virusfreie Weibchen (Kontrollen) saugten intensiver als CaCV-exponierte virustragende Weibchen an nicht infizierten Blättern. Alle Kohorten überprüfter Weibchen saugten aber an infizierten Blättern grundsätzlich weniger intensiv als an virusfreien Blättern, wobei es keine signifikanten Unterscheide zwischen den Kohorten gab. Die mittlere tägliche Fruchtbarkeit von CaCV exponierten Weibchen war geringer als die von nicht exponierten. Die Ergebnisse lassen vermuten, dass die pre-imaginale Reifungsperiode eine besondere Bedeutung für die Fitness der adulten Thripse besitzt.

Zusammenfassung

Im vierten Teil der Arbeit wurde die mögliche Rolle des Pflanzen– oder Blattalters für die Besiedlung der Pflanzen und die Entwicklung der Thripspopulation einschließlich der Empfindlichkeit für die Aufnahmen und Vermehrung der Viren hinterfragt. Es zeigte sich, dass die Intensität der Ansiedlung und Entwicklung vom ontogenetischen Stadium der Wirtspflanze (Keimblatt-, Sämlings- und Juvenilstadium im Vergleich) abhing. Zudem wurde die Saugintensität der Thripse durch das Pflanzen-/Blattalter beeinflusst. In Wahlexperimenten bevorzugte *C. claratris* ältere Entwicklungsstadien der Tomate, zudem nahmen Saugschäden und Virusbefall mit dem Alter der Pflanzen zu. In Mikrokosmosversuchen an einzelnen Pflanzen mit definierten Thripsdichten ("no-choice") zeigten lediglich 28% der Pflanzen im Keimblattstadium und 61% der Sämlinge Saugschäden im Gegensatz zu 100% der älteren Pflanzen. Die Ergebnisse lassen vermuten, dass insbesondere im Keimblattstadium die Pflanzen eine gewisse partielle Resistenz gegenüber Vektor und Virus aufweisen.

Zusammenfassend betrachtet, zeigen die Ergebnisse deutlich, dass die Fähigkeit zur Virusübertragung – die Vektorkompetenz - von *C. claratris* durch einen Faktorenkomplex determiniert wird: (1) Die Thripse müssen an der virusinfizierten Pflanze während eines ganz bestimmten kurzen Abschnittes der Larvalentwicklung saugen, (2) Thripse können sich nur zu erfolgreichen Überträgern entwickeln, wenn ihre genetische Konstitution entsprechend ist, (3) Das Geschlecht ist ein wesentlicher Faktor für die Variabilität in der Übertragungseffizienz, (4) Die Empfindlichkeit der Wirtspflanze (Tomate) für Thrips und Virus nimmt mit zunehmendem Alter zu.

Die hier zusammengestellten Ergebnisse können das Verständnis der Interaktionen im Dreieck Pflanze-Tospovirus-Thrips vertiefen helfen.

Stichworte: CaCV, Vectorkompetenz, Vererbung.

Contents

Abbreviations

AAP	Acquisition Access Period
AIT	Asian Institute of Technology
CaCV	Capsicum chlorosis virus
DAS-ELISA	Double-Antibody Sandwich Enzyme-Linked ImmunoSorbent Assay
EPG	Electrical Penetration Graph
F1	First Generation
GBNV	*Groundnut bud necrosis virus*
IAP	Inoculation Access Period
ICTV	International Committee on Taxonomy of Viruses
INSV	*Impatiens necrotic spot virus*
L1	First Instar Larva
L2	Second Instar Larva
L: D	Light: Dark period
MYSV	Melon yellow spot virus
ORF	Open Reading Frame
RH	Relative Humidity
TSWV	*Tomato spotted wilt virus*
WSMoV	*Watermelon silver mottle virus*
χ^2	Chi-square tests

General introduction

Thrips are insects of the order Thysanoptera, which encompasses about 5500 described species. The majority are herbivorous; however, hardly a hundred thysanopterans are recorded as serious pests (Lewis, 1997). Because of their minute size (1-2 mm in length), cryptic behaviour, and they deposit their eggs inside plant tissues a few of these thrips pests had been successful invaders who now occur worldwide (Morse & Hoddle, 2006). The occurrence of optimal environmental conditions, in particular, inside greenhouses allows high populations build up in a short time due to their high intrinsic rate of population increase and parthenogenesis. This facilitates the fast development of insecticide resistance thrips strains that made the chemical control especially difficult (Bielza et al., 2008). Thrips pest have mostly a polyphagous feeding behaviour thus affecting a wide range of crops by direct feeding. Coupled with the potential ability to vector tospoviruses, highly destructive pathogens, thrips had arguably become one of the most damaging insect pests in the world (Lewis, 1997).

The thrips

Ceratothripoides claratris

The oriental tomato thrips, as it is commonly named, is found in India and Southeast Asia (Jones, 2005). It is a polyphagous foliage-feeding thrips on many cucurbitaceous and solanaceous hosts (Steenken, 2007). In Thailand, in particular its central part, it is a serious pest of tomatoes, *Solanum lycopersicum* L. (Solanaceae) (Murai et al., 2000; Premachandra et al., 2004). The thrips feeds on leaves using the ‘punch and suck’ feeding method. The single mandible punches a hole in the plant surface through which the maxillary stylets are then inserted (Kirk, 1997). Thrips feed on the epidermal, palisade and spongy cells, leaving collapsed or emptied cells. Typical feeding symptoms are silvery localized scarring accompanied by black faecal droplets (Childers, 1997). Hence, direct infestation of leaves causes severe

damage to the crop, especially, when infestation is commenced at a very early growth stage. Normally under the conditions of Thailand immigration starts immediately after access, e.g. after moving the plants from thrips free nurseries to the greenhouse (transplanting) (Premachandra et al., 2004). If direct damage is combined with transmission of tospovirus, damage multiplies and is very detrimental to the crop.

Fig.1. A female of *Ceratothripoides claratris* (magnified 50×)

Ceratothripoides claratris females lay their eggs inside the plant tissues using their ovipositor. As with other thrips species the reproduction system is haplodiploid: Fertilized eggs produce females, while unfertilized eggs develop to males (arrhenotoky) (Moritz, 1997). Development rate is temperature dependent and the life cycle of *C. claratris* will be complete in 9-20 days, at 30 and 22° C, respectively (Premachandra et al., 2004). The optimal temperature of *C. claratris* was determined with 30° C, with highest pre-adult survivorship and net reproductive rate resulting in a high intrinsic rate of increase, and short mean generation and doubling times. At this optimal temperature (30° C), eggs will hatch in three days, and the following first larval instar (L1) will start moulting into the second larval instars (L2) after two days. L2 will last for only a day thereafter late L2 will search for a pupation site. The pupal stage is subdivided into two stages; prepupa (less than a day) and pupa (2 days). Thus, at 30° C the egg-to-adult time will complete in nine days. Sex ratio is female biased (71%), and adult longevity varies with males living longer than

females at the optimal temperature; longevity of males and females is 17 ± 1 and 12 ± 1, respectively. The pre-oviposition and the post-oviposition period takes 1-2 days, and the daily fecundity amounts to around 10 eggs per female (Premachandra et al., 2004).

Tospovirus-thrips interaction

The tospovirus-thrips interaction is exceptional in that adult thrips can only transmit the tospovirus if they had been feeding on infected plants as young larvae. Hence, the acquisition of the virus by young larvae is a determinant of adult vector competence (van de Wetering et al., 1996; Whitfield et al., 2005). Moreover, the ability to develop to a virus transmitter after acquiring the tospovirus declines during the larval development of the thrips from the first to the second larval instar (van de Wetering et al., 1996) and only the very early second larval instar is potentially able to transmit the virus after acquiring it (Moritz et al., 2004). Adults, on the other hand, cannot transmit the virus even though the virus can be successfully acquired by the midgut epithelial cells (Ohnishi et al., 2001). Transmission mode is persistent-propagative. Once successfully acquired by the thrips, the tospoviruses will start to replicate inside the thrips midgut cells until it finally reaches the salivary gland (Nagata et al., 1999, 2002). There the virus propagates and is accumulated until transmitted mainly by the adults (Whitfield et al., 2005). The second larvae can also transmit the virus, however in a lower ratio than adults (Nagata et al., 1999; Premachandra et al., 2005). Moritz et al. (2004) explained this relation by providing anatomical evidences of a temporary (L1 stage) association between the thrips midgut and the salivary gland, as a result of brain displacement into the prothoracic region. This temporary association occurs only during the first and early second larval developmental stages, thus facilitating the spread of the virus from the midgut to the salivary gland, which explains why only the first and early second larva can acquire the virus and then transmit it. As thrips develop this association will be lost and hence no virus particles are able to reach the salivary gland, which in turn explains why late second larvae and adults can not develop to transmitters even though the virus can infect their midgut.

Moreover, transmission efficiency by the thrips may be influenced by sex, species, and even population properties as well as the tospovirus isolates and the host plant.

Males of the western flower thrips *Frankliniella occidentalis* (Pergande) were reported as more efficient transmitters than females (Sakurai et al., 1998; Nagata et al., 2004), a phenomenon that was attributed to the differences in the feeding behaviour between males and females of the western flower thrips. Because males make more frequent punctures but ingest less of the plant cellular contents compared to females. Thus the punctured but not destroyed cells could support primary virus propagation after transmission. Females on the contrary puncture the leaf less frequent but remain longer at a distinct feeding site and ingest much more of the cell contents, leaving behind complete collapsed or empty cells (Van de Wetering et al., 1998, 1999b). Males and females of *C. claratris*, on the other hand, showed similar transmission efficiency (Premachandra et al. 2005). Not all thrips species vectoring tospovirus are able to transmit all tospovirus species. For instance, both *Frankliniella schultzei* (Trybom) and *Frankliniella intonsa* (Trybom) were able to transmit *Tomato spotted wilt virus* (TSWV) but not *Impatiens necrotic spot virus* (INSV) (Wijkamp et al., 1995), and *F. occidentalis* had failed to transmit CaCV in Australia (Persley et al., 2006). Hence, vector competence is a species specific interrelationship. Moreover, different populations of *F. occidentalis* and *Thrips tabaci* Lindeman originating from different countries possessed marked differences in their transmission efficiency (van de Wetering et al., 1999ab; Cabrera-La Rosa & Kennedy, 2007). Finally, transmission efficiency is influenced by the interaction between the tospovirus isolates and the infected host plant. For example, the transmission percentage by the thrips *Frankliniella fusca* (Hinds) that developed on TSWV-infected *Emilia sonchifolia* (L.) Moench. (Asteraceae) were significantly more than their cohorts that were developing on TSWV-infected *Datura stramonium* L. (Solanaceae) (Stumpf & Kennedy, 2005).

All findings discussed above, indicate that the expression of vector competence is determined by a complex of interrelated factors between the tospovirus, the thrips and the host plant. As young larvae feed on an infected host they will take up the virus particles. The primary entry site of virions is the midgut where also the initial replication takes place (Nagata et al., 1999; Assis Filho et al., 2002). The virions have then to pass the thrips internal tissues and their different membranes before they finally enter the salivary gland (Whitfield et al., 2005). Where the virions propagate

and maintain a high titre before they will be transmitted successfully into new plants through feeding of the thrips (Nagata et al., 1999).

The abovementioned work by Moritz et al., (2004) is very important in providing evidence of anatomical determinants of vector competence. However, it does not explain why different individuals in a thrips population and different populations of the same thrips species exhibit strong variability in their transmission efficiency. In this context the question of within population variability in terms of heritability becomes important. Recent work by Cabrera-La Rosa & Kennedy (2007) has indicated that the ability to transmit TSWV by *T. tabaci* is potentially inherited as a recessive trait. However, in their study genetic crosses were performed between different populations of *T. tabaci*. Studying thrips inheritance as an important determinant factor in vector competence and not only on a population but also on an individual level is meriting further and more detailed studies.

Tospoviruses

Molecular biology

The Tospovirus family Bunyaviridae includes, to date, 20 recognised tospovirus species (Campbell et al., 2008). A Tospovirus virion is an 80-120 nm particle formed from a host-derived membrane studded with many surface projections, which are composed of two viral glycoproteins, G_N and G_C. This membrane encloses the ribonucleoproteins (RNPs) and a few copies of the viral RNA-dependent RNA polymerase (RdRp or L protein). The three circular RNPs complex are comprised of the three ssRNAs (designated L (8.9 kb), M (4.8 kb), and S (2.9 kb)) and the nucleoproteins (N). The ambisense S RNA encodes for two proteins, a 52.4-kDa non-structural protein (NSs) in the viral sense and the 29-kDa N protein in the viral complementary sense. The NSs proteins are suppressing RNA silencing during plant infection. The ambisense M RNA encodes a 33.6-kDa another non-structural protein (NSm) in the viral sense and a 127.4-kDa protein in the viral complementary sense. The later is a precursor of the glycoproteins, G_N and G_C. The NSm plays a role in cell-to-cell movement in plants. In contrast, the L RNA has only one open reading

frame (ORF) that encodes a 331-kDa in the viral complementary sense, that is, the L protein or the RdRp (reviewed in Whitfield et al., 2005).

Tospoviruses in Thailand

Currently, three tospovirus species are recognized from Thailand; namely, *Watermelon silver mottle virus* (WSMoV), Melon yellow spot virus (MYSV), and Capsicum chlorosis virus (CaCV). These viruses are infecting many important Solanaceous and Cucurbitaceous crops, as well as peanuts (*Arachis hypogaea*) (Fabaceae) (Chiemsombat et al., 2008). Symptoms are typical to tospoviruses that include; chlorotic spots, necrotic spots, necrotic concentric rings, chlorotic concentric rings, systemic necrosis, stunting, leaf deformation (Chiemsombat et al., 2008; Knierim et al., 2006).

In central Thailand and in Pathumthani province in particular, the CaCV is infecting tomato and pepper plants (Murai et al., 2000; Premachandra et al., 2005). A few isolates of CaCV were indentified that originated from this area (Chiemsombat et al., 2008; Knierim et al., 2006). One of these isolates is CaCV-AIT (AIT = Asian Institute of Technology, Pathumthani, the main location where all experiments herein had taken place), is now completely sequenced (Knierim et al., 2006). Historically, McMichael et al. (2002) identified CaCV in symptomatic pepper and tomato in Australia in 1999. However, later evidence had traced its occurrence in that country seven years before the first reported identification (Persley et al., 2006). At present besides its widespread occurrence in Australia and Thailand, CaCV is present in China (Chen et al., 2006). Therefore, the expected geographical presence of this virus is expected to be wider than actually reported.

CaCV, as a characteristic of tospoviruses, is exclusively transmitted in nature by thrips. In Australia, the melon thrips, *Thrips palmi* Karny and the cotton bud thrips, *Frankliniella schultzei* (Trybom) had successfully transmitted the CaCV in laboratory tests (Persley et al., 2006). In Thailand, the transmission ability of both *T. palmi* and the chilli thrips, *Scirtothrips dorsalis* Hood that were collected from cucurbitaceous fields was equivocally suggested (Chiemsombat et al., 2008). In contrast, the transmission efficiency of CaCV by the oriental tomato thrips *Ceratothripoides claratris* (Schumsher) is unequivocal (Premachandra et al., 2005).

Objectives of the study

In the experimental greenhouses at the Asian Institute of Technology (AIT), Central Thailand, the oriental tomato thrips, *C. claratris* is the dominant thrips on tomatoes. There, the thrips shows high affinity towards tomato plants resulting in early foliar infestation and, more importantly, later infection by the tospovirus CaCV. However, little is known about the interaction between *C. claratris* and CaCV (Premachandra et al., 2005). The main aim of this research, therefore, was to study this interaction in more detail.

More specifically, the system (i.e., CaCV-*C. claratris*-Tomato) was used as a case study for biological interaction between tospovirus-thrips-plant. By using this system we aimed to analyze the thrips specific age requirements for successful acquisition of the virus, as well as the time at which the thrips starts successful inoculation of the virus. Moreover, potential sexual differences in vector competence besides the ability of adults to successfully inoculate the virus throughout their life span were investigated. As not all individuals are transmitters, thus we aimed to study the variability and heritability in determining vector competence. In later experiments, we aimed to use the CaCV-*C. claratris*-Tomato system to evaluate a possible pathological role of the virus on its vector, and in addition, the potential role of the host plant factors (e.g. age, size, and infection status) on the attraction and colonisation and subsequent viral infection were scrutinized.

CHAPTER 1

Studies on the vector biology of *Ceratothripoides claratris* (Schumsher) (Thysanoptera: Thripidae), using a new leaflet assay

Abstract

A new leaflet assay was used to study tospovirus transmission by the thrips *Ceratothripoides claratris*. Only first and early second instar larvae transmitted equally the tospovirus, Capsicum chlorosis virus-isolate AIT (CaCV-AIT). 10 and 46% of the concomitant late second instar larvae and adults, respectively, transmitted the virus. Once viruliferous, adults retain their regular ability to transmit the virus for life. More viruliferous thrips in the rainy season than in the hot dry season were collected from the natural thrips populations surrounding the greenhouses in central Thailand. The significance of using the leaflet assay and the test plant (in the microcosm) in studies concerning virus-vector-plant relations is discussed.

Introduction

Oriental tomato thrips *Ceratothripoides claratris* (Schumsher) (Thysanoptera: Thripidae) is a polyphagous species from south and Southeast Asia. Okajima et al. (1992) reported it on melons in northern Thailand. Later, Murai et al. (2000) detected it in central Thailand and observed that it was the sole thrips species on tomatoes there. Further studies by Premachandra et al. (2004, 2005) at the Asian Institute of Technology (AIT), greater Bangkok area, Thailand, confirmed its serious pest potential of tomato grown under protected cultivation. *C. claratris* showed a high vector competence for Capsicum chlorosis virus-isolate AIT (CaCV-AIT) (genus *Tospovirus*, family Bunyaviridae) which is a devastating pathogen for tomato (Premachandra et al., 2005). CaCV is a proposed species of Tospoviruses and a member of the *Watermelon silver mottle virus* (WSMoV) group (Knierim et al., 2006; Persley et al., 2006). It was first found in pepper and tomato in Australia (McMichael et al., 2002). In only nine weeks post-planting inside a nethouse at AIT, CaCV-AIT infected 80% of the tomato plants (then 12 week-old), which consequently led to almost a complete loss of the crop (Premachandra et al., 2005).

Ceratothripoides claratris, as all other vector thrips, acquires the tospovirus during feeding of the first larval instar (L1) and early second larval instar (L2) stages (van de Wetering et al., 1996, 1999a; Moritz et al., 2004; Premachandra et al., 2005). Tospoviruses are described to be transmitted in a persistent-propagative manner (Ullman et al., 1993; Wijkamp & Peters, 1993; Whitfield et al., 2005) by only viruliferous L2 and adults (Wijkamp & Peters, 1993; Premachandra et al., 2005) however detailed studies with *C. claratris* and CaCV are missing thus far. Moreover this study aimed on testing a newly developed leaflet assay method and a principal objective of this study was to scrutinize the significance of the use of the leaflet assay and test plants (in microcosms) in virus-vector studies. The leaflet assay can efficiently measure tospovirus transmission and hence it was used to further study the vector biology of *C. claratris* in more details.

Materials and methods

Thrips source

Initially, specimens of *C. claratris* were identified by R. zur Strassen and voucher specimens were deposited at the Senckenberg Museum, Frankfurt, Germany (Premachandra et al., 2005). Starting 2006, from a natural infested greenhouse population at AIT, a virus-free *C. claratris* colony was reared on potted tomato plants in thrips-proof Plexiglas cages (50×50×60 cm, covered at the top with 64µm nylon net) at 29 ± 1°C, 50-60% relative humidity (RH) and 12:12h L: D. To produce newly hatched L1 (<1h), initially adults (around 50) were isolated from the greenhouse population with an aspirator and transferred to a few potted three-week-old tomato plants contained in a thrips proof Plexiglas cage for egg deposition. On the following day all adults were discarded by means of aspirator and a fine brush before transferring the plants into another cage. Two days later, newly hatched L1 were collected with a very fine wet painting brush under a stereomicroscope and transferred for feeding on tomato plants inside the colony cage, thus, ultimately serving as the primary source for the colony. This colony was further maintained completely isolated from any other thrips source and served as thrips source for all laboratory experiments.

Test plants and tospovirus isolate

Three-five week-old tomato plants (*Solanum lycopersicum* L. cv. FMTT260) (Solanaceae) (AVRDC; Shanhua, Taiwan) were used throughout the experiments for thrips rearing and as host (i.e., both as leaflets source and test plants). Seeds were sown in peat moss then kept in a completely closed nursery greenhouse to avoid any pest immigration. The greenhouse was equipped with an evaporative fan and pad cooling system. Mean temperature and RH were 28-30°C and 90-100%, respectively. A culture of CaCV-AIT was maintained by thrips inoculation of tomato plants in a spatially separated greenhouse (20×10 m). Roofs and lower sidewalls of the greenhouse were clad with an UV-absorbing polyethylene (PE) film (Wepelen^{TM}, FVG, Dernbach, Germany) while sides were covered with 52-mesh UV-absorbing insect proof net screens (Bionet^{TM}, Klayman Meteor Ltd, Petah Tikva, Israel) to avoid any thrips immigration from outside (Kumar & Poehling, 2006). Irrigation was

performed by a dripping system radiation controlled by means of computerized central climate control unit. Nutrition application of minerals was combined with irrigation system and consisted of Kristallon™ 6+12+36+3+Micro (% N, P, K, Mg) and Calcinit™ 15.5+0+0+19Ca (both Yara, Oslo, Norway) in a ratio of 70:30 (both Yara, Oslo, Norway) Mean temperature and RH were 28-30°C and 70-80%, respectively. Initially, newly hatched (<1 h) L1 of *C. claratris* were given an acquisition access period (AAP) until adulthood onto systemically infected tomato leaflets. After adults' emergence several males were encaged inside a PE cylinder (15×60 cm with 4 (Ø3cm) ventilation holes) containing one three-week-old tomato plant for few days inoculation access period (IAP). Afterwards the plants were kept further un-caged in the greenhouse. Only males were chosen for the virus inoculation because males will die out after ten days latest at the greenhouse's temperature, hence avoiding insecticides to control thrips population growth in case of females. After a successful CaCV inoculation, in two to three weeks the plants started to exhibit typical tospovirus symptoms. Thereafter, samples of potentially infected plants were tested with double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to confirm the presence of CaCV. The Compound direct ELISA for (WSMoV) and *Groundnut bud necrosis virus* (GBNV) (AGDIA® Inc., Elkhart, IN, USA. Cat. No. SRA 61500) was employed following the supplier's protocols for the detection of CaCV. To confirm the virus isolate (i.e., CaCV-AIT) systemically infected leaflets were tested with PCR (see Knierim et al., 2006) at the Institute of Plant Diseases and Plant Protection, Hanover University.

Virus acquisition by L1 and transmission by adults, a comparison between two methods

Newly hatched L1 from the virus free colony population were collected as described above. Then L1 were allowed an AAP until pupation on systemically infected leaflets incubated in a gypsum-petri dish. For a comparison study between two adopted methods (namely: leaflet assay and microcosm) living pupae were then transferred separately and randomly to either the leaflet assay or the microcosm experimental set-ups i.e., 1 pupa/ 1 leaflet (the leaflet assay, see appendix) or 1 pupa/ 1 plant (the microcosm). The leaflet assay was composed of the gypsum-petri dish,

i.e., a petri dish (9cm in diameter) that was lined with a layer of a mixture of plaster ($CaSO_4$) and charcoal (9:1 ratio). After adding a few millilitres of double distilled water a small piece of filter paper was placed on the gypsum layer and a healthy leaflet on top of it. Subsequently, the gypsum-petri dishes were closed by their modified lids and sealed with laboratory film (Parafilm M®, Pechiney, Plastic Packaging, Inc., USA). The lids were perforated with three equally distanced marginal holes (Ø12 mm) that were closed with thrips proof net (64 µm mesh nylon) for air exchange. For virus transmission, thereafter, adults were kept to feed on the leaflet for 5d IAP when they were removed and sexed. The leaflets were tested for the presence of the virus by DAS-ELISA to determine the viruliferous status (i.e., viruliferous or non-viruliferous) of all adults individually. Forty replicates were done with two control treatments of five replicates each. The first control was thrips-free leaflet assay and in the second control thrips reared on healthy leaflets were used. The experiment was repeated two times over the time.

The microcosm, on the other hand, was composed of a three-week-old potted tomato plant encaged inside a PE cylinder (15×60 cm with 4 (Ø3cm) ventilation holes) then sealed with laboratory film. Five days after the emergence of the adults the PE cylinder were removed and the sex of the thrips was determined and recorded. The plants were kept further un-caged in the greenhouse after being sprayed with 1% spinosad. Thereafter, all plants were kept there for a maximum of sex weeks or until the development of tospovirus symptoms and all plants regardless infected or not were tested with DAS-ELISA for further confirmation. Forty replicates were done with two control treatments of five replicates each. The first control was thrips-free microcosm and in the second control thrips reared on healthy leaflets were used. The experiment was repeated two times over the time.

Only leaflets/ plants with feeding damage were considered as valid replicate for DAS-ELISA and hence used in further analysis to determine the percentage of viruliferous thrips and to assess the methods used. Wilted plants were subtracted from the total plants count. All assays and microcosms were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

Virus acquisition by L2 and transmission by adults

In the gypsum-petri dish newly hatched L1 from the colony were reared on healthy leaflets for 30h, late L1 then were marked dorsally with a little spot of India ink and were kept on the healthy leaflets where they were monitored regularly for the initiation of moulting. After shedding the old skin the new L2 instars were collected and placed to feed on CaCV-infected leaflets in another gypsum-petri dish until pupation. All living pupae were transferred separately each into a leaflet assay and the virus transmission was subsequently determined as above. Thirty replicates were done with two control treatments of five replicates each. The first control was thrips-free leaflet assay and in the second control thrips reared on healthy leaflets were used. The experiment was repeated two times over the time. Only leaflets with feeding damage were considered as valid replicate for DAS-ELISA and hence used in further analysis. All assays were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

Virus acquisition by adults and transmission by adults

To test the potential of adults to acquire CaCV, initially, newly hatched L1 were collected and randomly divided between two separate groups with each placed in separate gypsum-petri dishes, i.e., either with healthy leaflets or infected ones until pupation. Pupae fed on the systemically infected leaflets were then transferred to new infected leaflets while those who were feeding onto healthy leaflets were again divided randomly between two new separate gypsum-petri dishes containing either healthy leaflets or systemically infected ones. Subsequently, all emerged adults were kept in their set-ups for 2d AAP post-emergence to feed and ingest the virus.

Thereafter, all adults were individually transferred to leaflet assays where they were kept for 5d IAP until the leaflets were tested by DAS-ELISA. Twenty adults were used per treatment and the experiment was repeated two times. All assays were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

Virus acquisition by L1 and transmission by L2

To test the potential of virus transmission by L2, at first newly hatched L1 were collected and placed to feed on infected leaflets for a day in a gypsum-petri dish as

described above. On day two all larvae, still L1, were placed individually in a leaflet assay to feed and thus inoculate the virus onto a new healthy leaflet. On day three all larvae, now L2, were transferred individually to continue feeding onto a new healthy leaflet in new leaflet assay. The previous leaflets were further incubated in the same-labelled leaflet assays for at least five days to be tested with DAS-ELISA. On day four late L2 preparing for pupation now were transferred yet to a new leaflet assay and the same procedure as the day before was repeated. During pupation period the transfer steps were stopped because pupae do not feed. Three days later adults were starting to emerge, thus they were kept in their leaflet assay for feeding and virus inoculation for five days; afterwards all leaflets were tested with DAS-ELISA. Twenty larvae starting form infested leaflets were tested for transfer potential whereas further ten being reared on healthy leaflets only served as control. The whole experiment was repeated two times. All assays were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

Adults' life span transmission

Viruliferous and non-viruliferous adults were produced as in the above mentioned method. One day post-emergence adults were sexed and then transferred to new leaflet assay for two times three-day periods. From day seven onward the thrips were transferred daily to a new-labelled leaflet assay until the death of the thrips. All leaflets from all previous assays were further incubated for at least five days to be tested for virus presence by DAS-ELISA. The life span was recorded for each insect as well as the ELISA result for each day. Twenty larvae were tested with 10 as control being reared on healthy leaflets, and the experiment was repeated two times. All assays were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

Viruliferous adult population density and virus incidence

To study the relation of density of viruliferous adults and virus incidence, the microcosm as described above was used. Viruliferous adults were produced by the abovementioned method, the resultant pupae were picked up by means of a wet fine brush and placed randomly in different densities, i.e., 0, 1, 2, 4, 8 and 16 thrips, in a

small glass petri dish (5 cm in diameter) on the soil next to the plant and sealed. Post-emergence of the adults, five days later, the plants were un-caged and sprayed with spinosad. Thereafter, they were kept in the greenhouse. Number of infected plants and appearance time of tospovirus symptoms were recorded for each infected plant for six weeks. Ten plants were used for each thrips density and the experiment was repeated twice. All microcosms were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room thereafter in the greenhouse at 28-30°C and 70-80% RH.

Viruliferous adults in natural populations

From March to October 2006 *C. claratris* adults were collected monthly from the greenhouses vicinity and tested for their ability of virus transmission. At the beginning of each month few three-week old potted tomato plants were placed together at three meters distance from outer Northeast corner of the greenhouses complex. One week later the *C. claratris* infested plants were cut and thrips were sexed and singled out randomly (in case of low thrips numbers all thrips were tested) by means of a wet fine brush and confined individually within a leaflet assay. Five days later all leaflets were tested with DAS-ELISA.

Statistical analysis

Percentages of viruliferous thrips were calculated as the percentage of leaflets tested positive by DAS-ELISA. Over time the percentages of viruliferous thrips was summed across all transmission tests. Fisher's exact probability test was adopted when any of the expected frequencies was less than 5. Otherwise, Chi square test of independence was performed for comparison of the percentages of viruliferous thrips PROC FREQ command in SAS (SAS institute, 2001). One-way ANOVA was used to compare the symptoms appearance time with respect to number of introduced thrips. Probit analysis was chosen to analyse the median number of thrips needed for successful inoculation of the virus with 95% fiducial limits using PROBIT option of SAS.

Results

The comparison between the two experimental methods in table 1 shows no significant differences between the total accumulative percentages of viruliferous thrips (χ^2 (1,100) = 1.62, p= 0.2030). Specifically, the accumulative percentage of viruliferous females and males did not differ significantly between the two methods. No differences between sexes were detected in the both methods. Yet, there was a significant reduction in the percentage of valid replicates when the microcosm was used (χ^2 (1,146) = 14.27, p= 0.0002). Twelve out of the invalid 32 microcosm replicates were due to wilting caused by fungal infection of roots because of the very high humidity (100% RH) inside the microcosm. In contrast, though the humidity was similarly high inside the leaflet assay there were no fungal infections at all. Even though, very rarely, leaflets were drying within the leaflet assay they were still valid and used in DAS-ELISA analysis. Accordingly, the leaflet assay was chosen as the main assay for further experiments. When early L2 started feeding on the CaCV-AIT infected leaflets the resulting or consequential adults (females and males) have acquired and could transmit the virus similar to adults that acquired the virus as early L1 (table 1). Here again no sexual differences in acquiring the virus could be observed. While L1 and early L2 could acquire and transmit CaCV as adults, the adults of the thrips failed to acquire. Only the adults feeding as L1 on CaCV infested leaves developed to viruliferous thrips (table 2).

When the ability of L2 to transmit the CaCV-AIT was tested 21% (i.e., 4/ 19) of the viruliferous concomitant adults started viral transmission during the 48-72h-age period (i.e., as L2) while the rest 15 viruliferous thrips started to transmit for the first time only as adults (fig. 3). Hence the majority of *C. claratris* transmit CaCV-AIT significantly more post-emergence rather than preimaginal (χ^2 (2, 41) = 32.19, p= 0.0001).

Table 1: Accumulative percentage of viruliferous *Ceratothripoides claratris* adults tested in two experimental set ups

Acquisition life-stage	Experimental set up	Accumulative percentage of viruliferous[1] (%)			Valid replicates[2] (%)
		males	females	total	
L1	Leaflet assay	10.26(39) Aa	16.00(25) Aa	12.50(64) a	82.10(78) a
L1	Microcosm	21.43(14) Aa	22.73(22) Aa	22.22(36) a	52.94(68) b
L2	Leaflet assay	16.67(24) Aa	17.86(28) Aa	17.31(52) a	-

[1] newly hatched first instar larvae (L1) or newly moulted second instar larvae (L2) were given an acquisition access period until pupation on CaCV-AIT systemically infected tomato leaflets.
[2] percentage of leaflets/ plants exhibiting leaf damage due to thrips feeding and considered for DAS-ELISA. Same lowercase letters in columns and uppercase letters in rows indicate no significant differences (χ^2 or Fisher's exact test, p= 0.05). Numbers in parentheses represent sample sizes.

Table 2: Accumulative percentage of viruliferous *Ceratothripoides claratris* that were given an acquisition access period for two days post-emergence then tested in the leaflet assay

Thrips adults	Accumulative percentages of viruliferous adults (%)
V -	0 (40)
V +	20 (40)
V	0 (46)

V- = adults feeding all larval and adulthood stages on healthy leaflets
V+ = adults feeding all larval and adulthood stages on CaCV-AIT infected leaflets
V = adults feeding all larval time on healthy leaflets but as adults on CaCV-AIT infected leaflets
Numbers in parentheses represent sample sizes.

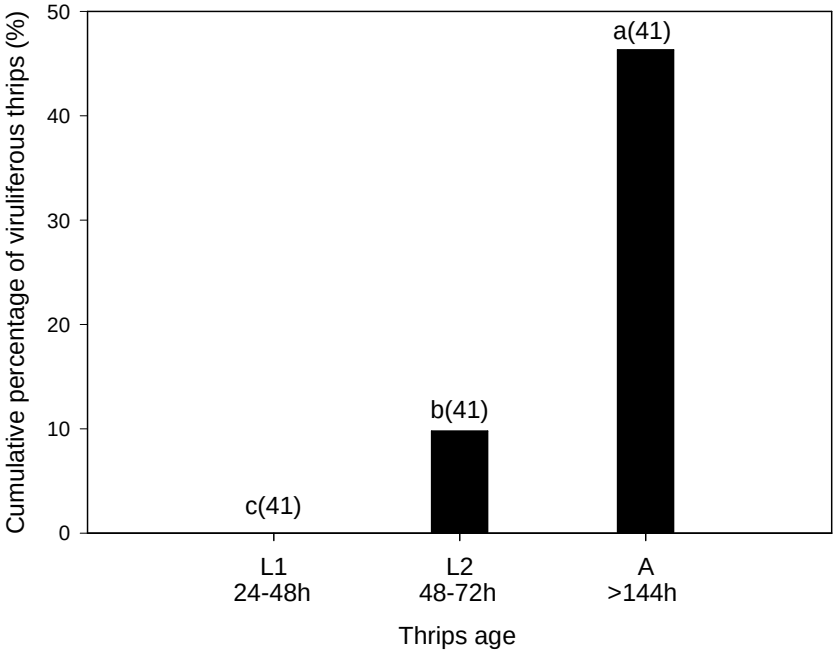

Fig.1. Cumulative percentage of viruliferous *Ceratothripoides claratris* tested for their ability to transmit CaCV-AIT during the larval stages and adulthood (i.e., L1, L2 and adults (A)). Same individuals were tested in every stage using the leaflet assay. Thrips previously had acquired the virus during the first day of L1 then they were transferred daily to a new healthy leaflet until pupation. At the end the emerged adults were placed on new leaflets, which were later tested with DAS-ELISA. (χ^2, p= 0.05); numbers in parentheses represent sample sizes.

Viruliferous thrips starts successful transmission on the first day post-emergence and retain the ability to inoculate the virus for life while non-viruliferous thrips were never able to successfully infect any leaflet throughout the whole testing period (fig. 2). Fig.2 as well illustrates the similar longevity range of viruliferous and non-viruliferous males (i.e., 7-12d and 8-11d for viruliferous and non-viruliferous, respectively) and females (i.e. 6-11d and 7-10d for viruliferous non-viruliferous, respectively).

From the first method comparison experiment (table 1) and plant infection with increasing densities of viruliferous thrips (fig. 3) it was obvious that a single viruliferous thrips was able to inoculate a host plant with CaCV. Moreover, fig. 3 shows the significant increase of infected plants in relation with the increase number of introduced thrips (χ^2 (5, 110) = 37.64, p< 0.0001).

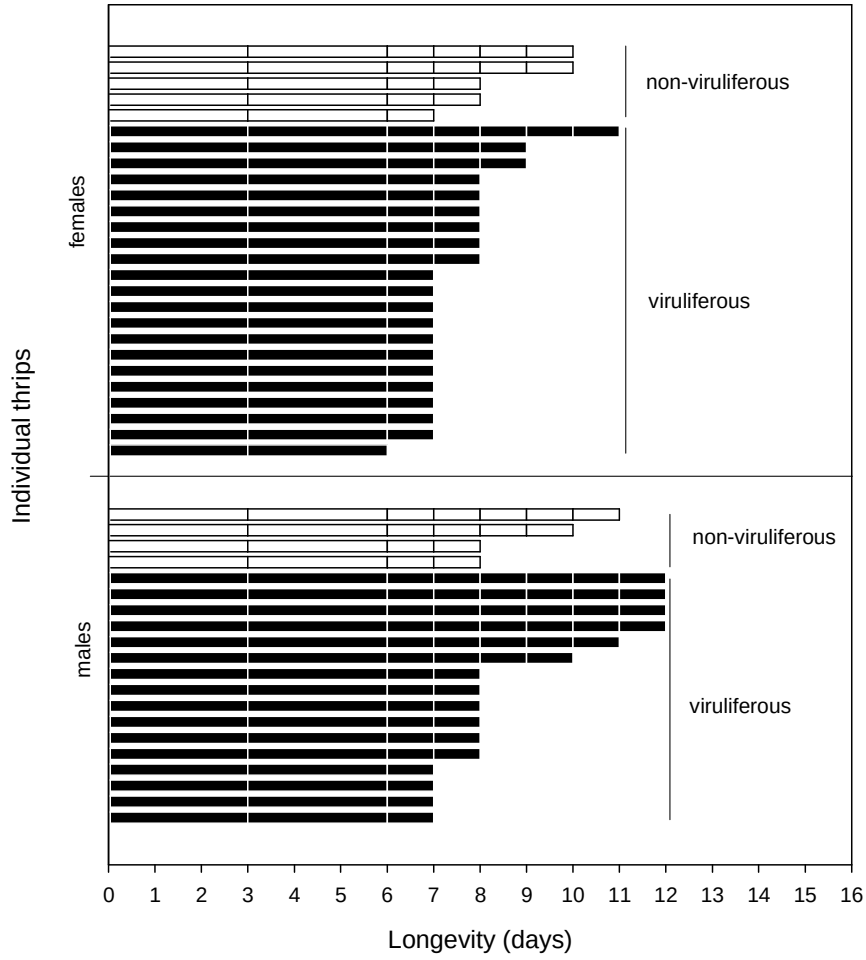

Fig.2. Longevity (in days) and transmission efficiency of CaCV-AIT by males and females *Ceratothripoides claratris*, both viruliferous and non-viruliferous thrips were reared until pupation on CaCV-AIT infected leaflets. Then after determining their viruliferous status on day zero, individual adults were transferred periodically (indicated here by the division line within each bar) to new leaflet assays. All leaflets on which thrips were feeding were later tested with DAS-ELISA and black or white column sections indicate positive or negative ELISA result per leaflet, respectively.

The number of thrips needed for successful inoculation of 50% of the plants as determined by the Probit analysis (y = -0.54 + 1.73x) was 2.0 thrips/ plant with 95% fiducial limits of 1.2-3.0 thrips/ plant and the number of thrips needed to cause 95% diseased plants was 18.4 thrips/ plant with 95% fiducial limits of 9.8-77.1 thrips/ plant. However, fig. 4 illustrates that the time until appearance of tospovirus symptoms was not influenced by the number of introduced viruliferous individuals ($F(4, 30) = 0.73$, $p = 0.5776$). Typical tospovirus symptoms first appeared after 10-15d inoculation.

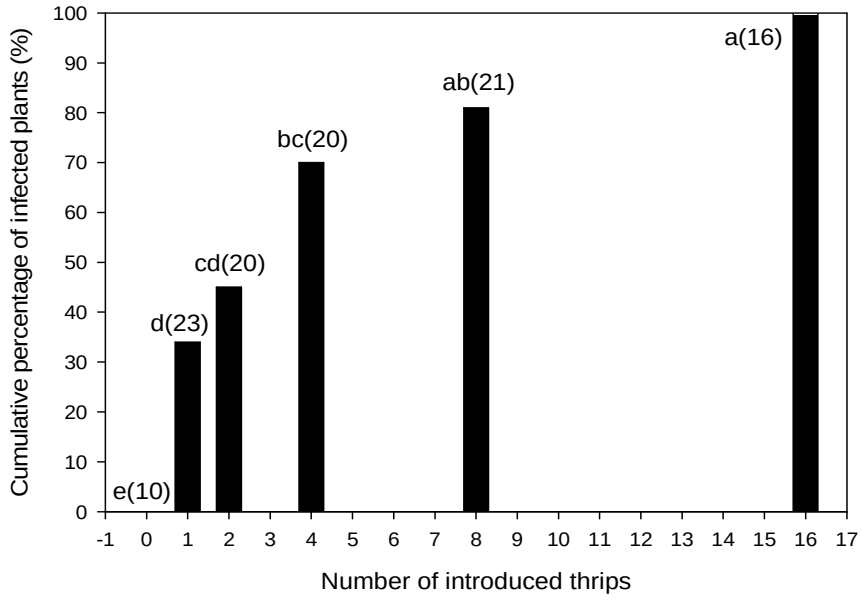

Fig.3. Relation between the cumulative number of infected plants and the number of thrips released per plant. One plant per microcosm was infested with different number of *Ceratothripoides claratris* (i.e., 0, 1, 2, 4, 8 and 16). All introduced adults were feeding during their larval stages on CaCV-AIT infected leaflets until pupation. (χ^2, p= 0.05) and numbers in parentheses represent sample sizes.

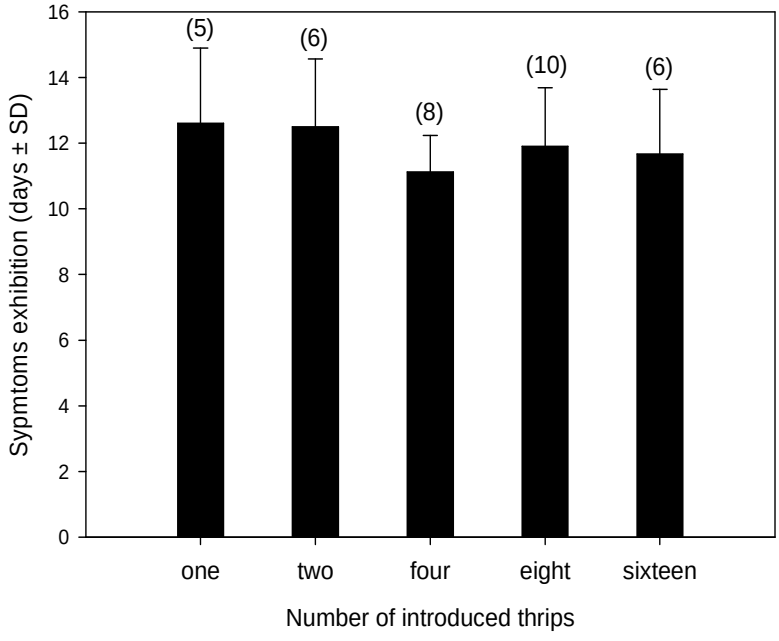

Fig.4. Relation between appearance of first CaCV symptoms (days) (mean ± SD) and the number of thrips released per plant. One plant per microcosm was infested with different number of *Ceratothripoides claratris* (i.e., 0, 1, 2, 4, 8 and 16). All introduced adults were feeding during their larval stages on CaCV-AIT infected leaflets until pupation. (One-way ANOVA, p= 0.05). Numbers in parentheses represent sample sizes.

Monthly collection and testing of natural populations surrounding the greenhouses shows in general a very strong increase in percentage of viruliferous individuals during the rainy season. In the hot dry season none of the tested males and only two females were viruliferous (i.e., 0/44 and 2/65, (viruliferous/total tested) males or females, respectively). In the rainy season, in contrast, the number of viruliferous males and females increased dramatically (i.e., 9/31 and 20/102, respectively) (table 3).

Table 3: Percentage of viruliferous *Ceratothripoides claratris* collected from vicinity of the greenhouses at AIT in 2006, then confined individually in the leaflet assay

Season	Month	Viruliferous adults (%)		
		Females	Males	Total
Hot dry season	March	6.2 (16)	0 (14)	3.3 (30)
	April	2.9 (34)	0 (21)	1.8 (55)
	May	0 (16)	0 (9)	0 (25)
Rainy season	June	44.4 (9)	25 (12)	33.3 (21)
	July	36.8 (19)	25 (8)	33.3 (27)
	August	5.9 (34)	0 (5)	5.1 (39)
	September	21.4 (28)	66.7 (6)	29.4 (34)

Numbers in parentheses represent sample sizes.

Discussion

In early work using the described leaf disk assay (Wijkamp and Peters, 1993; Premachandra et al., 2005) at the above-described experimental conditions (i.e., 30°C and 100% RH and direct contact with water film) many samples were lost as a result of fungi infections on the excised disks during the incubation period necessary for virus replication and propagation. For reliable ELISA measurements it was necessary to have 5-6d incubation period for the tomato leaf disk assay (Premachandra et al., 2005). This rendered the leaf disk assay inefficient for transmission studies in our system. Because of that a new assay was developed based on a single tomato leaflet placed on a solid gypsum layer (the leaflet assay). Using this assay the leaflets could

be incubated for up to two weeks without any fungal infection. Rarely leaflets were desiccated, yet they were still valid for the ELISA testing. This longer period of incubation is crucial when e.g., a slow replicating virus is being studied. Disks of tomato leaflets did not show any local lesions to indicate virus infection, which corroborates the results of Wijkamp et al. (1996a) when they used leaf disks of 14 different plant species (including tomato) and reported that lesions occurred only in petunia leaf disks.

The leaflet assay has a number of advantages over the use of a test plant in a microcosm. First, the handling is much easier and thrips specimens can be manipulated and observed readily. Larvae, pupae and adults are more easily recovered in the leaflet assay set ups than in the much bigger microcosm. Second, the percentage of valid replicates (i.e., identified leaflets/ plant exhibiting clear feeding damage and thus valid to be considered for further assessments following the ELISA test) was much higher in the leaflet assay due to the relatively difficult determination of feeding damage on plants, particularly with males, whereas it was much simpler using a stereomicroscope with the leaflets. Third, although similar and inevitable very high humidity (~100% RH) was characteristic for both assay environments, the number of fungal infections was negligible on the leaflets while fungal wilt reduced significantly the number of usable samples in the microcosm (i.e., 12 from the initial 80 microcosms used were lost due to fungal wilt). Finally, the thrips transmitted the virus as efficiently to the leaflet as to the plant, which responded with a systemic infection (table 1) in 10-15 days. Nonetheless, using the microcosm is still important for studies addressing factors e.g., plants age, number of thrips released and time of symptoms appearance (fig. 4).

The percentage of valid replicates in the leaflet assay was 82%, which should not be confused with pre-adult survivorship because few pupae and adults were found trapped between the parafilm layers used to seal the assay. Therefore, this percentage is acceptable and comparable to former experiments with *C. claratris* that was ranged between 72-95% at 27-34°C (Premachandra et al., 2004).

Results of this study demonstrate the ability of *C. claratris* to transmit CaCV-AIT, yet in low efficiency and inconsistently with time (i.e., in the first experiments the percentage of viruliferous was between 10-23% while in fig.1 it reached 46%). When

Premachandra et al. (2005) first report about *C. claratris* as a new vector of CaCV-AIT from the same location, they reported a very high transmission efficiency of 69-87% without any notes concerning the inconsistency. It is well documented that different populations of other thrips species like *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) or *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) also showed marked differences in their vector competence (van de Wetering *et al.*, 1999ab; Cabrera-La Rosa & Kennedy, 2007). Yet those populations were originated from different hosts and countries. Later studies show that the size of the primary selected population used to start the colony is influencing the transmission rates with time. A phenomenon attributed to founder effects and inheritance of traits affecting vector competency (Chapter 2).

The vector biology of *C. claratris* is similar to that of all vector thrips. *Ceratothripoides claratris* was able to acquire CaCV-AIT particles as first larval instar and early second larval instar (table 1) while failed to acquire the virus during adulthood (table 2). After a successful acquisition of the virus late second larval instar and adults were able to transmit the virus (fig. 1). Moritz et al. (2004) had explained this phenomenon and attributed a successful tospovirus acquisition by thrips vectors to a crucial time period when there is a temporary association between the mid-gut, visceral muscle and salivary glands during which the virions are able to reach eventually the salivary glands. This short period of time is included within the first and early second instar stages. As the second instar develops, this temporary association is lost and hence further movement of the virus to the salivary glands is prevented.

The optimum temperature for egg-adult development *C. claratris* was determined as 32-33°C and fecundity was highest at 30°C. Females' longevity was highest at 25°C and males at 30°C. The development of different life stages at 30°C was 3, 2, 1.2, 0.8 and 1.8 days for egg, L1, L2, prepupa and pupa respectively (Premachandra et al., 2004). Only 20% of individuals were able to start transmitting the virus as late L2 and the rest 80% of adults started to transmit the virus for the first time on the first day post-emergence and continued to do so regularly until death (fig. 2). Therefore, it was not possible with the data in fig. 1 to determine the latent period of CaCV-AIT within *C. claratris*. This latent period is included within the pupal stage in which no

feeding occurs, hence cannot be tested. With *F. occidentalis* 80-85% of the thrips transmitted the virus for the first time as L2. This and the longer life cycle of this thrips allowed the determination of the median latent period (LP_{50}) of *Impatiens necrotic spot virus* (INSV) and *Tomato spotted wilt virus* (TSWV) (Wijkamp and Peters, 1993).

In this study we report a similar efficiency in virus transmission between both larval instars (i.e., L1 and L2) (table 1). Premachandra et al. (2005), in contrast, reported that *C. claratris* second instars transmitted less than the first instars (48% and 69% for L2 and L1, respectively). Similarly, when cohorts of *F. occidentalis* commenced TSWV acquisition as first or second instars, 47% or 12%, respectively of the concomitant adults transmitted the virus (Nagata et al., 1999). This difference could be attributed to the low transmission rate of 10-23% achieved in this study, which made it difficult to statistically detect any difference. Moreover the different method used to identify the beginning of the second instar stage should be considered. Whereas the occurrence of casted skin (exuvia) was used to decide the initiation of the second instar stage (Premachandra et al., 2005), we used paint marking with India ink to decide the beginning of the L2 stage. Because the very short time during the second instars larval stage when there is still contact between the mid-gut and salivary gland. The accurate determination of the initiation of this stage directly after ecdysis is crucial for testing the potential of second instars to acquire the virus.

Ceratothripoides claratris adults did not transmit the virus when they commenced feeding on infected leaflets which corroborates results of other authors with different thrips species. None of the adults of *Thrips setosus* Moulton, *Frankliniella fusca* Hinds (both Thysanoptera: Thripidae) and *F. occidentalis* were able to transmit TSWV after 2, 16 and 16h AAP, respectively, as adults on infected leaves (Nagata et al., 1999 ; Ohnishi et al., 2001; Assis Filho et al., 2003).

Ceratothripoides claratris started regularly to transmit the CaCV-AIT on the first day post-emergence and continued to do on a daily bases (fig. 2). In pervious study the high majority of *C. claratris* transmitted CaCV-AIT repeatedly with the same rate for 8d in three subsequent IAPs of three days each (Premachandra et al., 2005). However, the first IAP covered the first three days together and was not testing or

considering day zero post-emergence. Unlike *C. claratris*, *F. occidentalis* transmission of TSWV decreased with age (van de Wetering et al., 1999b).

We achieved 100% infected plants with increasing thrips density of 16-thrips/plant. Though, as determined by Probit analysis 18.4 insects/plant is needed to cause almost a total infection of the tomato crop. But the time needed to develop symptoms was not correlated with the number of introduced thrips. Indicating that population size of invading thrips is contributing to viral incidence but not to symptoms exhibition. This result is valid only for the colony population used throughout the experiments and does not necessarily represent the naturally invading populations at different times into the greenhouses at AIT. The transmission efficiency was monthly and seasonally fluctuating from 0% in May 2006 to 33% in both June and July 2006 (table 3). There is an evident influence of the season on the thrips transmission rates. While only 2/109 adults were viruliferous during the hot dry season of 2006, in the rainy season 29/133 adults were viruliferous. In August the dramatic reduction in viruliferous adults is maybe due to the periodic removal of weeds at AIT. That eventually resulted in the removal of the assumed common host for both CaCV and *C. claratris*. Nonetheless, the short life cycle of *C. claratris* and its high reproductive capacity in tropical conditions will compensate the low seasonal transmission rates (Premachandra et al., 2004), hence may cause serious virus epidemics.

CHAPTER 2

Inheritance of vector competence by *Ceratothripoides claratris* (Schumsher) (Thysanoptera: Thripidae) *

Abstract

The thrips *Ceratothripoides claratris* is an efficient vector of the Capsicum chlorosis virus. Transmission studies with a natural population of *C. claratris* found in a greenhouse and a colony derived from this population by selection resulted in a lowering of the percentage of viruliferous individuals within the colony. After passing through about 20 generations, the colony finally lost the ability to transmit the Capsicum chlorosis virus. When either viruliferous or non-viruliferous virgin females parthenogenically reproduced, 81% of F1 arrhenotokous males inherited their viruliferous status from their mothers, whilst, no viruliferous offspring were found from the non-viruliferous virgin mothers. Crosses between viruliferous and non-viruliferous individuals suggest that the competence of the thrips *C. claratris* as a vector for this virus is a heritable trait controlled by a recessive allele.

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Introduction

Tospoviruses (genus *Tospovirus*, family Bunyaviridae) occur worldwide (Mumford et al., 1996; Persley et al., 2006) and infect hundreds of plants in 108 different families (Campbell et al., 2008). Capsicum chlorosis virus (CaCV) is one of the 16 species that has been recognized or proposed by the International Committee on Taxonomy of Viruses (ICTV) and is a member of the *Watermelon silver mottle virus* (WSMoV) group (Persley et al., 2006). CaCV was found first in pepper and tomato plants in Australia in 1999 (McMichael et al., 2002). In 2001, an isolate of CaCV (namely, CaCV-AIT) was found in tomato plants growing at the Asian Institute of Technology (AIT), greater Bangkok area, Thailand, where the complete nucleotide sequence was determined (Knierim et al., 2006). The tospoviruses are transmitted in a persistent-propagative manner (Ullman et al., 1993; Wijkamp et al., 1993; Whitfield et al., 2005). Whether the virus is picked up, or not, depends upon the development stage of the feeding thrips (Moritz et al., 2004). The ability to acquire tospoviruses is restricted to the first and the early-second instars of the larval stage (van de Wetering et al., 1996, 1999a). Although *Ceratothripoides claratris* (Schumsher) (Thysanoptera: Thripidae) is not the only vector of CaCV (Persley et al., 2006), it is the predominant thrips species in the greater Bangkok area and so has the potential to become a serious threat as a pest of tomato crops (Murai et al., 2000; Premachandra et al., 2004). In 2005, Premachandra et al. showed that adults of *C. claratris* could transmit CaCV-AIT with an efficacy of up to 70%. Using both plants and leaf discs, however, subsequent transmission studies with a selected population of *C. claratris*, originally from the same location, with the same virus isolate, indicated that the percentage of viruliferous adults rarely exceeded 30% and so was much lower than reported earlier (see chapter 1). Different populations of *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) or *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) originating from different hosts and countries also possessed marked differences in their vector competence (van de Wetering et al., 1999ab; Cabrera-La Rosa & Kennedy, 2007). However, in our study the observed reduction in vector competence occurred in a small subpopulation (colony) that had

been selected from a much larger greenhouse population of thrips. Therefore, we hypothesize that the reduction in vector competence of *C. claratris* was a result of the isolation of a few individuals (a kind of founder effect) and this indicates strongly that the effect is genetically based. Only a few authors have studied how herbivorous insects inherit the competence to transmit viruses (Storey, 1932; Cabrera-La Rosa & Kennedy, 2007). The main objective of this research was to determine how *C. claratris* inherited the competence to vector CaCV. Earlier studies confirmed the inheritance of vector competence by crossings made at the population level, whereas our study is based on the more refined method of crossing pre-tested (vector competent) individuals.

Materials and methods

Plants

Throughout the experiments, 3-5 week-old tomato plants (*Solanum lycopersicum* L. cv. FMTT260) (AVRDC; Shanhua, Taiwan) were used for rearing the thrips and as test plants. The tomato seeds were sown in peat compost and then, to prevent the immigration of pest insects, the plants were kept in a completely closed nursery greenhouse. The greenhouse was equipped with an evaporative fan and pad cooling system, and was maintained at 28-30°C and a relative humidity (RH) of 90-100%.

Thrips

The specimens of *C. claratris* were identified by R. zur Strassen and voucher specimens were deposited at the Senckenberg Museum, Frankfurt, Germany (Premachandra et al., 2005). Two different groups of the thrips were used in the experiments. The first was from a natural infestation found on tomato plants growing in a small (2×3×2.5 m) greenhouse covered with a 40-mesh screen (Econet M, Ludvig Svensson Inc., Kinna, Sweden) that was not sufficiently fine to keep out thrips (hereafter called the GH). The tomato plants were potted in 10-litre polyethylene (PE) pots filled with commercial compost (Dinwondeekankasat, Ayutthaya, Thailand). The nutrient solution consisted of a 70:30 mixture of Kristallon^{TM}: Calcinit^{TM} (both Yara, Oslo, Norway) and was applied to the plants via

a drip irrigation system. In this greenhouse, the irrigation was controlled through a computerized central unit and mean temperature and relative humidity (RH) were 28-30°C and 70-80%, respectively.

The second was a virus-free colony of *C. claratris* (hereafter called the colony). The colony was started in 2006 by using an aspirator to collect, at random, 50 thrips adults from the GH population These thrips were then put onto potted three-week old tomato plants that had been placed inside 50 × 50 × 60 cm Plexiglass cage, which top was made from thrips-proof, 64µm, nylon net The cage was kept under laboratory conditions at 29 ± 1°C, 50-60% RH and 12:12h L: D. Once in this cage, the thrips were allowed one day to lay eggs, after which they were removed using either an aspirator or a fine paint brush. The plants infested with thrips eggs were then placed into a clean cage. Two days later, about one-hundred newly-hatched larvae, which were less than 1h old and so were still white, were collected using a damp, extremely-fine paint brush. As thrips larvae are small, this work was done under a stereomicroscope. The newly-hatched larvae were then put onto tomato plants inside the cage used to house the colony and thus served ultimately as the source for the virus-free thrips. This virus-free colony was kept under laboratory conditions, so that it was isolated completely from any other source of thrips.

Tospovirus isolate

The isolate (AIT) of the CaCV tospovirus was maintained by allowing infected thrips to feed on tomato plants kept in a different 20×10 m greenhouse. To prevent thrips from outside sources migrating into this greenhouse (Kumar & Poehling, 2006), the roof and lower sidewalls of the greenhouse were made from a UV-absorbing polythene film (Wepelen^{TM}, FVG, Dernbach, Germany) and the sides from a 52-mesh UV-absorbing insect-proof net screen (Bionet^{TM}, Klayman Meteor Ltd, Petah Tikva, Israel). The plants were watered and fed using the system described earlier.

To produce viruliferous thrips, newly-hatched (L1) thrips from the *C. claratris* colony were allowed to feed until adulthood on CaCV-infected tomato leaflets collected from the GH. After the thrips adults emerged, several males were put onto a three-week-old tomato plant placed inside a 15×60 cm cylindrical polythene cage that had four 3cm in diameter holes for ventilation. The male thrips were allowed to

feed for a few days, after which the plants were taken out of the cages and kept in the greenhouse. Only thrips males were used for the virus transmission work, as at greenhouse temperatures, males die within seven days (Premachandra et al., 2004). Using only males prevents the establishment of new infestations of thrips which would then have to be controlled with insecticide. After 2-3 weeks, some of the test plants started to show typical symptoms of the CaCV tospovirus (see chapter 1 & 4). Once this occurred, samples of the infected plants were tested, for the presence of CaCV, using a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The Compound direct ELISA for *Watermelon silver mottle virus* (WSMoV) and *Groundnut bud necrosis virus* (GBNV) (AGDIA® Inc., Elkhart, IN, USA. Cat. No. SRA 61500) was used, following the manufacturers instructions, for detecting CaCV in the leaflets of plants.

To ensure the virus isolate (i.e., CaCV-AIT) remained unchanged during the course of the experiments, samples of the plant leaflets, that were infected with CaCV, were tested at regular intervals with PCR at the Institute of Plant Diseases and Plant Protection, Hanover University (Knierim et al., 2006).

Vector competence test

For testing vector competence, newly hatched L1 were produced then reared on CaCV-infected leaflets in gypsum-petri dish until pupation. Newly-hatched thrips larvae were produced using the method described earlier. Basically, this entailed allowing selected thrips adults to lay eggs for one day and then collecting the newly-emerged larvae 2 days later.

The tests for vector competence within the colony were done in Petri dishes whose bases had been lined with a 9:1 mixture of gypsum ($CaSO_4$) and charcoal (9:1 ratio). After adding a few millilitres of double distilled water, a small piece of filter paper was placed onto the gypsum layer to absorb any free water. A few tomato CaCV-infected leaflets (with high virus titre as pre-tested with DAS-ELISA (see appendix)) were then placed onto the filter paper. Subsequently, the newly-hatched thrips were then placed onto the infected tomato leaflets and then the lids were put on the Petri dish. For ventilation purposes, the lid of each Petri dish had three equally-spaced marginal 12mm diameter holes that were covered with thrips-proof (64 µm) nylon

mesh. Once closed, all of the Petri dishes were sealed with Parafilm M® (Pechiney, Plastic Packaging, Inc., USA) which retained sufficient moisture to keep the excised leaflet turgid for up to 12 days.

Once pupae were formed, they were transferred individually onto a single healthy tomato leaflet in a clean gypsum-lined Petri dish. This process will be referred to in future as the (leaflet assay). The thrips adults that emerged were allowed to feed on the leaflet for at least five days before being collected from the leaflets and sexed. All leaflets were subsequently detected for the virus using the DAS-ELISA test. A thrips was considered to be viruliferous (vir) if the leaflet on which it had been feeding gave a positive result in the DAS-ELISA test (Storey, 1932; Boonham, et al., 2002), and a negative result was considered to be non-viruliferous (non-vir). Thrips that were allowed to feed on virus-free (healthy) leaflets were used as the control treatment. All leaflet assays were done in a laboratory maintained at 29 ± 1°C, 50-60% RH and 12:12h L: D.

The exact method described above was also used to assess the vector competence of the GH population of thrips. The only difference was that the newly-hatched larvae were collected directly from leaflets cut from infested plants in the GH. As previously, newly-hatched larvae provided with virus-free leaflets were used as the control treatment.

Vector competence by offspring of virgin females

Several of the resultant females (all virgins) with previously determined viruliferous status by the mentioned vector competence test were confined separately in gypsum-petri dishes to reproduce parthenogenically on healthy leaflets. Three days later all newly hatched L1 (offspring) were picked up and placed to feed on infected leaflet until pupation. Then the offspring were sexed and the aforesaid method to test the vector competence was used for determining the viruliferous status of the offspring.

Crossing experiments

As the original colony eventually contained only non-viruliferous individuals, thrips from the colony were not suitable for the crossing experiments. It is important to stress that only thrips from the GH infestation that was fortified from time to time by

the immigration of new thrips from outside the greenhouse were used for the crossing experiments. As described earlier, newly-hatched larvae were collected directly from the GH plants and were reared until pupation on CaCV-infected tomato leaflets. Males and females that were found to be viruliferous or non-viruliferous, from the results of the leaflet assay, were used as the parents for the subsequent crosses. All four possible parental crosses were repeated on two or three different occasions. The crosses tested were: (i) vir ♀× vir ♂ (3×2, 3×2 and 3×3 individuals used in the first, second and third crosses, respectively); (ii) non-vir ♀ × non-vir ♂ (3×2, 2×3 and 3×3 individuals used in the first, second and third crosses, respectively); (iii) vir ♀× non-vir ♂ (4×2, 8×4 and 13×4 individuals used in the first, second and third crosses, respectively); (iv) non-vir ♀× vir ♂ (10×2 and 9×3 in the first and second crosses, respectively). Parents for each cross at each occasion were confined together in a gypsum Petri dish to allow them to mate and then lay eggs on a few healthy tomato leaves. All offspring were collected as newly-hatched larvae and reared, until pupation, on infected leaflets. The viruliferous status of the adults (F1) that emerged was determined using the leaflet assay. Throughout the experiment, the infected leaflets used for both feeding the thrips and for the virus acquisition work were divided equally between the various crosses. In addition, the leaflets were monitored for the presence of CaCV by subjecting them to the DAS-ELISA test either before or after the thrips had fed on them. The tests were considered valid only when a high virus titre was found in the tomato leaflets used as the food source (see appendix for an example of such an infected leaflet with homogenous high virus titre).

Statistical analysis

The percentage of viruliferous thrips was considered to be the same as the percentage of leaflets that gave positive readings in the DAS-ELISA test. As there were considerable difficulties both in making the crosses and in rearing the subsequent offspring, each cross was repeated on at least three different occasions and the numbers of viruliferous and non-viruliferous offspring within each cross was summed over all tests. Fisher's exact probability test was used when any expected frequency was less than 5. Otherwise, Chi square tests were done to compare how

thrips sex, different populations of thrips, different times of testing, and different batches of F1 offspring, affected the frequency of the number of leaves that tested positive for virus. The Chi-square tests were done using PROC FREQ command in SAS (SAS institute, 2001).

Results

Table 1 and 2 shows the percentage of viruliferous adults during a five months period for the selected colony and during a three months period for the GH population respectively. The percentage of viruliferous males in the colony (table 1) was more or less constant (11.9 – 12.5%) whereas the percentage of females ranged from 18 – 39%. The percentage of viruliferous adults in the colony fluctuated considerably with time (total χ^2 (2, 203) = 19.22, $p < 0.0001$). Within the colony, no viruliferous thrips were found during the tests done in August, by which time the colony had passed through about 20 generations. The initial tests done in August involved 12 uninseminated females. These produced 54 offspring, which were also tested in August. All of these were non-viruliferous males, which explain the high number of males (63) in the August results (table 1). Compared to the selected colony, the GH population (table 2) was much less variable and the percentage of viruliferous thrips ranged from 31-49% for the males and 62-68% for the females. Hence, this population had a relatively stable percentage of viruliferous adults.

When comparisons were made between the data pooled over time for the two populations, there was a clear difference (total χ^2 (1, 260) = 39.94, $p < 0.0001$, males χ^2 (1, 112) = 14.21, $p < 0.0002$ and females χ^2 (1, 148) = 21.00, $p < 0.0001$) in the efficiency of virus transmission between the GH population and the colony derived from it (fig. 1).

Significant differences between males and females from the same batch could be detected only in June (colony) and February (GH) (χ^2 (1, 47) = 4.38, $p = 0.0363$ and χ^2 (1, 68) = 4.11, $p = 0.0426$ respectively) with lower proportions of viruliferous males, however this strong difference resulted for both populations in significant

differences between the sexes regarding the pooled data (colony (χ^2 (1, 128) = 3.93, p < 0.0474); GH (χ^2 (1, 132) = 5.12, p < 0.0236)) (tables 1 and 2). Excluding August (colony), in addition, no significant differences were detected overtime for both males and females as well as for the total proportion of viruliferous thrips in both the colony and the GH populations (tables 1 and 2)(the colony: males, Fisher's exact test, p = 1.0000, females χ^2 (1, 62) = 3.39, p = 0.0656 and total χ^2 (1, 128) = 2.24, p = 0.1343; The GH: males, χ^2 (1, 46) = 1.19, p = 0.2751, females χ^2 (1, 86) = 0.30, p = 0.5828 and total (χ^2 (1, 132) = 0.05, p = 0.8230).

Table 1: The percentages of viruliferous adults *Ceratothripoides claratris* from the colony as determined by DAS-ELISA following the leaflet assay

Testing period	% viruliferous adults[1]		
	Females	Males	Total
April	17.9 (39)aA[2]	11.9 (42)aA	14.8 (81)a
June	39.1 (23)aA	12.5 (24)aB	25.5 (47)a
August	0 (12)b	0 (63)b	0 (75)b
Pooled data	25.8 (62)A	12.1 (66)B	18.8 (128)

[1] Adults as newly hatched L1 were collected and provided with acquisition access period on CaCV systemically infected tomato leaflets until pupation. Numbers in parentheses represents sample size.

[2] % followed by the same lowercase letters in columns and uppercase letters in rows indicate no significant differences (χ^2 or Fisher's exact test, p = 0.05).

Table 2: The percentages of viruliferous adults *Ceratothripoides claratris* from the GH as determined by DAS-ELISA following the leaflet assay

Testing period	% viruliferous adults[1]		
	Females	Males	Total
December	67.7 (31)aA[2]	48.5 (33)aA	57.8 (64)a
February	61.8 (55)aA	30.8 (13)aB	55.9 (68)a
Pooled data	63.9 (86)A	43.5 (46)B	56.8 (132)

[1] Adults as newly hatched L1 were provided with acquisition access period on CaCV systemically infected tomato leaflets until pupation. Numbers in parentheses represents sample size.
[2] % followed by the same lowercase letters in columns and uppercase letters in rows indicate no significant differences (χ^2, $p = 0.05$).

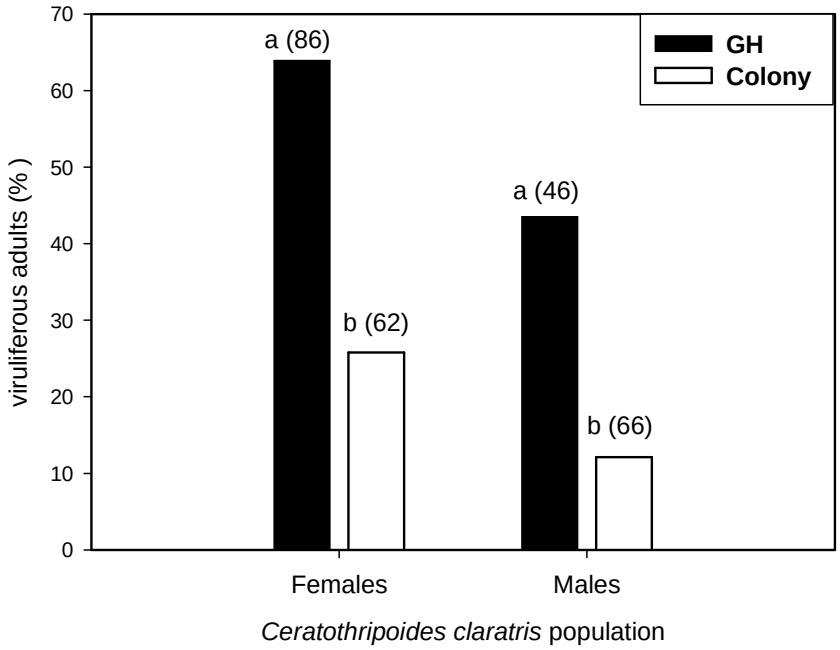

Fig.1. Comparison between sexes originated either from the colony or the Greenhouse (GH). Adults as newly hatched L1 were provided with acquisition access period on CaCV systemically infected tomato leaflets until pupation. Then the percentages of adult viruliferous *Ceratothripoides claratris* were determined by DAS-ELISA following the leaflet assay percentages followed by the same lowercase letters within sex categories indicate no significant differences (χ^2, $p = 0.05$). Numbers in parentheses represents sample size.

All offspring of the virgin females were males. 81 % of the offspring of viruliferous virgin females were as well as viruliferous. In contrast, none of the offspring of the non-viruliferous females were viruliferous (χ^2 (1, 81) = 60.41, $p < 0.0001$) (fig. 2).

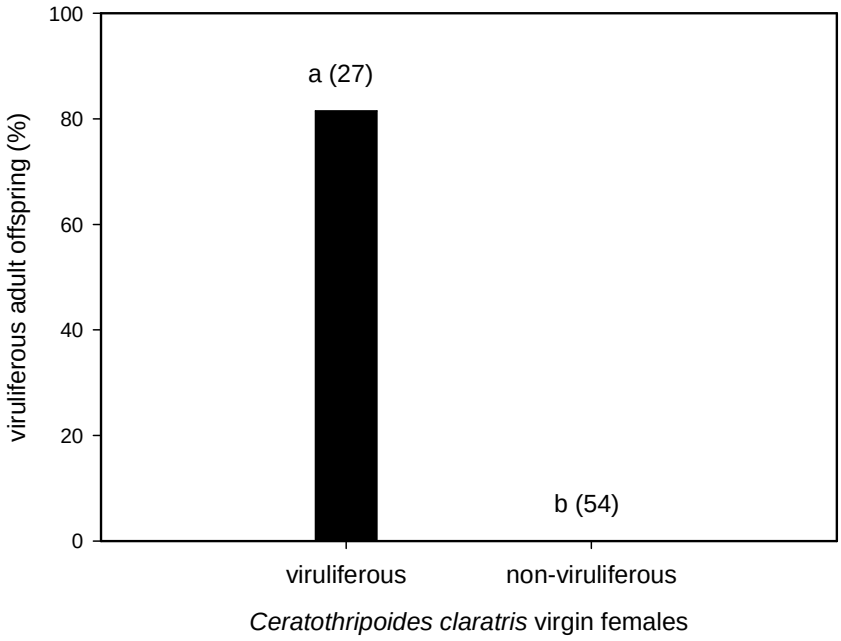

Fig.2. Percentages of adult viruliferous offspring *Ceratothripoides claratris* produced from either viruliferous or non-viruliferous virgin mothers. Adult offspring as newly hatched L1 were provided with acquisition access period on CaCV systemically infected tomato leaflets until pupation. Then the percentages were determined by DAS-ELISA following the leaflet assay. The percentages followed by the same lowercase letters indicate no significant differences (χ^2, $p = 0.05$) Numbers in parentheses represent sample size.

The crossing experiments, repeated on at least three occasions, gave consistent results. The data shown in table 3 are the cumulative number of all thrips tested. The results of the crossing experiments show clearly how the choice of parents determines the viruliferous status of their offspring. The percentage of viruliferous offspring found in F1, when both parents were viruliferous, was significantly higher than any other parents combination (i.e., 80% and 100% for males and females respectively) (total, χ^2 (3, 197) = 32.23, $p < 0.0001$, males, χ^2 (3, 100) = 20.29, $p = 0.0001$ and females, χ^2 (3, 97) = 16.52, $p = 0.0009$) (table 3). When non-viruliferous females were crossed with either non-viruliferous males or viruliferous males, the percentages of the male offspring that were viruliferous were 17% and 35%,

respectively. These two values did not differ (χ^2 (1, 43) = 1.74, p = 0.1868). In contrast, when the parental female was viruliferous, 80% of the male offspring were viruliferous when the parental male was also viruliferous and 50% when it was not (χ^2 (1, 57) = 5.43, p = 0.0198). The percentages of viruliferous individuals in the female offspring followed a different pattern, as only the female offspring produced when both parents were viruliferous differed (χ^2 (3, 97) = 16.52, p = 0.0009) from the other combinations. The production of viruliferous female offspring in the other three crosses were similar and ranged from 41-50% (χ^2 (2, 81) = 0.61, p = 0.7367). Again no difference within sexes of the same batch could be detected in any combination (for vir × vir (Fisher's exact test, p = 0.1374), non-vir × non-vir (χ^2 (1, 60) = 1.21, p = 0.2709), vir × non-vir (χ^2 (1, 59) = 0.50, p = 0.4769) and non-vir × vir (χ^2 (1, 37) = 2.86, p = 0.0907)).

Table 3: Crossing experiments results presented as the percentages of viruliferous offspring adults *Ceratothripoides claratris* as determined by DAS-ELISA following the leaflet assay

Parents	% viruliferous offspring[1]		
Females × Males	Females	Males	Total
Vir (9) × Vir (7)	100 (16)aA[2]	80 (25)aA	87.8 (41)a
Non-vir (8) × Non-vir (8)	50 (40)bA	35 (20)bcA	45 (60)b
Vir (25) × Non-vir (10)	40.7 (27)bA	50 (32)bA	45.8 (59)b
Non-vir (19) × vir (5)	42.9 (14)bA	17.4 (23)cA	27 (37)b

[1] Adults offspring as newly hatched L1 were collected and provided with acquisition access period on CaCV systemically infected tomato leaflets until pupation. Numbers in parentheses represents sample size.
[2] % followed by the same lowercase letters in columns and uppercase letters in rows indicate no significant differences (χ^2 or Fisher's exact test, p = 0.05).

Discussion

The competence of an insect to act as a vector for plant viruses depends upon a range of interactions between the plant virus, the insect vector and the host-plant. Of the 13

insect species that have been identified to date as vectors, all are members of the subfamily Thripinae of the family Thripidae (2005 species) (Mound, 2002). These 13 species differ in their vector competence both between species (Wijkamp et al., 1995; Nagata et al., 2004; Sakurai et al., 2004) and within the populations of a given species. For example, the ability to transmit *Tomato spotted wilt virus* (TSWV) differs considerably between populations of *F. occidentalis* (van de Wetering et al., 1999ab; Sakurai et al., 2002) and *T. tabaci* (Chatzivassiliou et al., 2002; Cabrera-La Rosa & Kennedy, 2007). In addition, both the tospovirus species themselves and their various isolates also play an important role in affecting the competence of the vector (Wijkamp et al., 1995; Roca et al., 1997; Nagata et al., 2004). Finally, how the host plant responds to viral infection will obviously affect how the virus is transmitted subsequently by any population of thrips (Wijkamp et al., 1995; Roca et al., 1997). To avoid such problems, all of these variables were kept constant during the present study. This was done by, using just one original stock population of thrips, and a subpopulation derived from it, one CaCV isolate, and one cultivar of tomato.

The difference between individuals from the GH population and its colony to transmit the virus may be attributed to a kind of founder effect during establishment of the colony. The colony population was built-up initially from a small number of individuals selected from the GH population. After about 20 generations of inbreeding, the thrips within the colony had lost completely the ability to transmit the virus. This phenomenon was observed in two different colonies of *C. claratris* (Halaweh, personal observations). This progressive loss of the ability of the thrips to transmit virus indicated that a recessive gene appears to be linked to the "viruliferous" trait (Cabrera-La Rosa & Kennedy, 2007). A loss of the ability to transmit another virus, TSWV, had been recorded earlier for some populations of *Frankliniella schultzei* Trybom (Thysanoptera: Thripidae) (Nagata et al., 2004), but no attempts were made to explain why this loss occurred. Similar effects have been described in the transmission of viruses by mosquitoes. For example, Grimstad et al., (1977) reared various strains of *Aedes triseriatus* (Say) (Diptera: Culicidae) in the laboratory for many generations and tested, as a routine procedure, their efficacy for transmitting virus. Their data showed that selected small colonies had much lower

rates of transmission (24%) of the *La Crosse virus* (Bunyaviridae) than large colonies (71%). Hence, the numbers of individuals used to maintain a robust subpopulation need to be considered carefully.

Sex in Thysanopteran, with few exceptions, is determined by the haplodiploid sex-determination system (Moritz, 1997). In this system females lay two kinds of eggs; fertilized eggs that have a diploid set of chromosomes develop to females, whereas unfertilised eggs with only one copy (i.e. haploid) of the mother's chromosomes produce only males. The results presented herein clearly demonstrate the arrhenotokous parthenogenesis reproduction in *C. claratris* as all F1 from virgin females comprised only males (fig. 2). Moreover, since more then 80% of virgin viruliferous females produced also viruliferous male offspring (see fig. 2) it is evident that the phenotypic trait "viruliferous" is inherited from mothers to their offspring. On the other hand, tests with non-viruliferous virgin females resulted in none of the offspring to be viruliferous.

Crosses were done to study the inheritance of the competence of the thrips *C. claratris* to vector CaCV. Unfortunately, it was not possible to make F1 test crosses as, at the temperatures used throughout the experiments (around 30°C), the females lived for a maximum of 7 days. However, as it took 8 days for the thrips to develop from the egg to the adult stage (Premachandra et al., 2004), there was no possibility of doing test crosses. The results from the crossing experiments confirm the existence of a vector competent phenotype, as most offspring were viruliferous in the crosses in which both parents were also viruliferous (i.e. vir × vir). When compared to the other combinations this indicates that a recessive gene hypothesis is supported.

From the crosses in which both the female (genotype = vv) and male (genotype = v) parents were viruliferous, 80% of the male offspring were viruliferous, which supports a simple vertical inheritance as seen with the virgin viruliferous females in fig. 1. However, the vertical inheritance is not supported, in particular, by the cross in which viruliferous (vv) females were crossed with non-viruliferous (V) males. In this situation, based on the arrenotokous sex determination that occurs in thrips, the male offspring should inherit only the genes from the mother. Therefore, the percentage of male viruliferous offspring from this cross (50%) was much lower than the 80-100% needed to support the vertical inheritance.

From all crosses, the male offspring were less effective in transmitting CaCV than the female offspring and at least 20% of the male offspring were not viruliferous even though the genetics indicate that they should have been. At present, we cannot explain this result. However, it could indicate that while vector competence is under simple genetic control, the expression of this competence can be affected by interactions between the tospovirus and the thrips and that this results, especially in the males, in a percentage of the genetically viruliferous individuals being unable to transmit the virus.

Unlike males, females inherit one allele from each parent. Therefore, the percentage of viruliferous individuals in the female offspring provides an indication of whether dominant or recessive allele/s control vector competence. Assuming vector competence is under the control of a recessive allele, females from the non-viruliferous phenotype could be either heterozygous (Vv) or homozygous (VV) for the dominant allele. The male offspring results provide an estimation of the ratio of heterozygous mothers. In the cross in which non-viruliferous females were mated with viruliferous male, 17%, of the male offspring were viruliferous. This indicates that about half of the parental females were heterozygous (i.e. ½Vv & ½VV) and, as a result, 25% of the male offspring should have been viruliferous. The value that was actually obtained, 17%, was not unexpected, as it was shown earlier that at least 20% of males do not express the viruliferous trait, even though they have the viruliferous genotype. May be that even if males are carrying the genetic scheme for being viruliferous other factors like intensity of taking up the virus as L1 or progressing the virus inside the vector before establishing in the salivary glands may reduce the potentially possible transmission efficacy.

Likewise, in the cross in which both parents were non-viruliferous, 35% of the male offspring were viruliferous (i.e. 65% = V; 35% = v). If we assume again that not all of the genetically viruliferous males manage to express the viruliferous trait, the result implies that most, if not all, of the parental females were heterozygous. However, in this particular cross, the percentage (50%) of the female offspring that were viruliferous was much higher than expected theoretically. As the parental females were non-viruliferous, we have to assume that they were heterozygous (Vv). Therefore, when these females mated with non-viruliferous males (V) we expected

all of the female offspring in the F1 to be non-viruliferous (i.e. VV & Vv). Obtaining a value of 50% instead of 0% does not support the recessive gene hypothesis. However, the difference recorded could be accounted for if the male thrips selected as the non-virulent phenotype, were in fact a virulent genotype that was unable to express the virulent trait. In this test, the numbers of parents were low and of the 40 female offspring produced, 27 were from one cross involving only 3 males and 3 females. The chance that one of these three "non-viruliferous" males was of the virulent genotype cannot be discounted nor can the fact that it might have been the only male to mate with the females. A major difficulty in experiments of this type is the production of high numbers of adult offspring from a single pair of thrips, when only about 20% of the offspring survive to the adult stage. As some of the male offspring from the viruliferous genotype were not capable of transmitting the virus, the expected genotype frequency was not reflected accurately in the percentage frequency of the phenotype. In addition, as described earlier, the proportions of the heterozygous and the homozygous females varied considerably within a given population. Consequently, the differences between the results expected (theoretical) from the recessive gene hypothesis, and the actual results can only be explained partially. In one crossing trial (data not shown) however, a non-viruliferous male (♂V) was confined to mate with two viruliferous females (♀vv). The viruliferous status of their F1 was 0% viruliferous females (0/6, viruliferous females/ total females tested) and 55% viruliferous males (5/9, viruliferous males/ total males tested), further supporting that a responsible recessive gene is involved since all heterozygous females were not viruliferous (♀Vv).

Generally, if we look at our pooled data over time males were less efficient than females, but regarding selected time periods differences between males and females from the same batch was not a consistent phenomenon.

Sexual influence on vector competence was intensively studied before with other thrips species resulting in contradictory conclusions. No significant differences between sexes were found in *F. schultzei* (Sakurai et al., 2004) and two Japanese populations of *F. occidentalis* transmitting *impatiens necrotic spot virus* (INSV) (Sakurai et al., 2004) and another two populations of *F. occidentalis* in transmitting TSWV (Sakurai et al., 1998). Later tests revealed, however, that one of the two latter

F. occidentalis populations comprised males significantly more efficient than females in transmitting TSWV. Moreover in the same study five out of nine Japanese *F. occidentalis* populations additionally tested, contained males significantly more efficient than the females (Sakurai et al., 2002). Similarly, in a study of van de Wetering et al., (1999b), seven out of 14 different populations of *F. occidentalis* collected from different hosts and distinct geographic regions showed significant differences between sexes in transmitting TSWV. Nevertheless, in all the populations males had higher transmission efficiency than females (Sakurai et al., 1998; van de Wetering et al., 1999; Sakurai et al., 2002; Sakurai, 2004; Sakurai et al., 2004). In conclusion, sexual influence on vector competence seems to be not a stable phenomenon across thrips species, populations, time and host plant.

Ceratothripoides claratris females produced 20-times feeding area damage than males (chapter 3). Males' lower feeding intensity and hence the lower inoculation of virus particles than females might be the reason behind the lower efficiency for the males in comparison with the females. Although *F. occidentalis* males transmitted TSWV more efficiently than females, feeding area damage by males and females was not significantly different in *F. occidentalis* (van de Wetering et al., 1998). Therefore, the behavioural difference between the two species in general and feeding behaviour in particular may explain this difference between them in viral transmission.

In our study, although the thrips fed throughout their larval stage on CaCV-infected tomato leaflets, about half of them, maximum, developed into viruliferous adults (55% females, 47% males). We confirmed that the CaCV was distributed evenly throughout the leaflet tissue, by dividing the CaCV infected leaflets into $1cm^2$ portions and checking the titre of virus using DAS-ELISA (see appendix). This phenomenon of only a percentage of the thrips becoming viruliferous, after feeding on plants infected with virus, has been recorded also with *T. tabaci* (Chatzivassiliou et al., 2002). These authors found that for the seven populations of *T. tabaci* they collected from seven different tobacco fields, in which 100% of the plants were infected with Tomato spotted wilt virus, only 0-50% of the males and 10-50% of the females were able subsequently to transmit the virus.

A combination of transmission studies, histological studies and ELISA of individual viruliferous and non-viruliferous thrips (Nagata et al., 1999) indicated that even within a single population of thrips there can be three different types of thrips (Wijkamp et al., 1993, 1995; van de Wetering et al., 1996; Nagata et al., 2004). The first type consists of individuals that can successfully transmit the virus and that also have a high titre of virus in the salivary glands. In the second and third types, although ELISA showed that these thrips had taken up the virus, these thrips had only low levels (type 2) or no detectable virus (type 3) in their salivary glands. Both type 2 and type 3 thrips were unable to transmit the virus. Hence, the percentage of thrips that show a positive reaction in the ELISA test could be much higher than the percentage of thrips that are actually viruliferous, particularly as the salivary glands must contain a high titre of virus for the virus to be transmitted successfully (Nagata et al., 1999).

Once virus particles are taken up by the feeding thrips, the particles have to pass six different membranes before they enter the salivary gland (Whitfield et al., 2005). Unfortunately, the virus receptors present in membranes of the salivary gland and intestines of thrips are unknown (see Whitfield et al., 2005). It is agreed generally, that the primary site of virions entry and initial replication is in the midgut (Nagata et al., 1999; Assis Filho et al., 2002). However, the inability of the thrips to transmit is not likely to be related to what happens to the tospoviruses in the mid-gut, as the virus can be found in the midgut of *T. tabaci* populations that are unable to transmit the virus (Nagata et al., 2002). Following ingestion, TSWV could successfully infect and replicate in the mid-gut of adults of *Thrips setosus* Moulton (Thysanoptera: Thripidae). However, this replication was restricted to the epithelial cells of the mid-gut (Ohnishi et al., 2001). Therefore, the differences observed in vector competence cannot be explained qualitatively by the amounts of receptor-mediated virus found in the epithelial cells of the mid-gut. Therefore, the site of supposed receptors that could differentiate between the two phenotypes, viruliferous and non-viruliferous, and be regulated by a recessive gene, seems unlikely to be located in the midgut. Whether such receptors are present in the salivary glands, or elsewhere, remain to be seen.

Sin et al. (2005) showed that the genetics reassortments of a particular tospovirus determine largely whether the virus will be transmitted by insects. In addition, the

genetics of the host plant also has a crucial role in resisting the establishment of tospoviruses (Roselló et al., 1998). The present study showed that the genetics of the thrips *C. claratris* also has a considerable impact in the expression of vector competence. In future studies, the role of the inheritance of vector competence in thrips will be determined for combinations involving different species of thrips and different tospoviruses. This should enable us to unravel at least a part of the complicated interrelationships between thrips, tospoviruses and plants.

CHAPTER 3

Effects of Capsicum chlorosis virus on, size, feeding, fecundity and survival of *Ceratothripoides claratris* (Schumsher) (Thysanoptera: Thripidae)

Abstract

The effect of CaCV-infected leaflets on *Ceratothripoides claratris* fitness was studied in the laboratory. Thrips were reared until adult eclosion on either infected or uninfected tomato leaflets. Anatomical features measurements reveal a reduction in sizes of exposed males but not females, yet the fecundity of cohort females was lowered. Further evaluation with individual females showed that CaCV has small negative direct effect, but the negative indirect plant-mediated effects were more pronounced. The unexposed females have fed more than the exposed viruliferous females on the uninfected leaflets, and the non-viruliferous females were in-between. However, all categories of females had similarly fed on the infected leaflets. Mean daily fecundity was reduced in the exposed thrips, yet only significant with the viruliferous females. The unexposed fecundity of the females was not affected. This suggests that nurture during the pre-imaginal period is determinant to the fitness of the adults. The role of virus on plants as thrips food quality as well as the role of trichomes is discussed.

Introduction

Tospoviruses (genus *Tospovirus*, family Bunyaviridae) are economically important plant pathogens that are exclusively transmitted by a few thrips vectors (Jones, 2005; Whitfield et al., 2005; Persley et al., 2006). Capsicum chlorosis virus (CaCV) is a tospovirus species and a member of the *Watermelon silver mottle virus* (WSMoV) group (Persley et al., 2006). It was found in pepper and tomato in Australia (McMichael et al., 2002). Currently, it is a major threat for the nethouse tomato production in Central Thailand, where it is vectored by the Oriental tomato thrips *Ceratothripoides claratris* (Schumsher) (Thysanoptera: Thripidae) (Premachandra et al., 2005). *Ceratothripoides claratris* is a polyphagous pest species in south and Southeast Asia and the predominant thrips species on tomato plants (Murai et al., 2000; Premachandra et al., 2004, 2005).

Before a thrips becomes viruliferous it must commence feeding as young larvae on a tospovirus infected host followed by successful virus replication in a persistent-propagative manner within the thrips organs especially the salivary glands (Moritz et al., 2004; Whitfield et al., 2005), provided that the individual thrips has the necessary genetic background to realize successful virus processing from acquisition to transmission. Only a certain contingent of actively feeding and virus ingesting thrips will be really viruliferous, means actively transmitting the picked up virus (Chapter 2). Hence normally three thrips categories (our terminology below) can be found in vector populations: individuals developed on virus free plants (control), individuals from infested plants having acquired the virus will develop to either and are viruliferous (transmitters) or non-viruliferous (non-transmitters). The epidemiology of the tospoviruses is strongly linked to the dispersion dynamics of the adult thrips as main vectors. The interaction pathways can be complicated: The tospovirus infection of the host plant can alter plant physiology and thereby nutritional quality for the thrips, nutritional quality is a key parameter of indirect effects on thrips dispersion dynamics and finally the virus is infecting and propagating within the thrips, hence closely related to the vector physiology. Consequently thrips fitness may be affected directly and indirectly. The direct and indirect (plant-mediated)

effects of tospoviruses on their thrips vectors have been studied mainly with *Frankliniella* spp. and *Thrips* spp. (both Thysanoptera: Thripidae) and the tospoviruses *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV).

Conflicting results were reported ranging from negative through neutral to positive effects. TSWV was found to have no effects on *Frankliniella occidentalis* (Pergande) survival (Wijkamp et al., 1996b; Roca et al., 1997). Moreover, Wijkamp et al. (1996b) found no significant differences in development time and reproduction rate among viruliferous, non-viruliferous and control *F. occidentalis* that had been feeding on TSWV infected Datura. Inoue & Sakurai (2006) reported that the development time and mortality was not significantly different between TSWV exposed and unexposed thrips in two populations of *Thrips tabaci* Lindeman. Whereas, mean adults longevity of TSWV exposed *T. tabaci* was shortened in comparison with the unexposed control. Additional negative effects were reported about INSV exposed *F. occidentalis* with lower survival time and reproduction rate and slower development rate than unexposed thrips (DeAngelis et al., 1993). In addition, Medeiros et al. (2004) provided first evidence of activated immune response of *F. occidentalis* due to direct TSWV infection. Stumpf & Kennedy (2005) and (2007) reported evidence for combined direct and indirect plant-mediated effects of TSWV on *Frankliniella* spp. They reported a smaller direct effect on the thrips that lowered the development time and reduced the size of *Frankliniella fusca* (Hinds). In addition, the improved survival of *F. occidentalis* larvae to adulthood on infected plants compared to uninfected plants is considered as an indirect positive effect of tospovirus. Corroborating Belliure et al. (2005) demonstrated positive plant-mediated effects; the survival and development rate of *F. occidentalis* was lower on virus-free thrips damaged pepper plants compared to thrips/mechanical-inoculated plants and uninfected control plants. They suggested that TSWV infection of the plant improved its suitability for *F. occidentalis* due to a negative cross-talk between herbivore and pathogen (virus) induced defence pathways in pepper. Maris et al. (2004) found a mutualistic relationship between *F. occidentalis* and TSWV as infected pepper plants attracted more thrips than healthy plants. Besides, thrips development time was faster on infected than on healthy plants due to both earlier

hatching and faster larval development. In addition, Chaisuekul & Riley (2005) found that both *F. occidentalis* and *F. fusca* oviposited significantly but slightly more on TSWV-infected tomato leaf disks than on healthy leaf disks.

In conclusion the described examples of interactions studied demonstrate that the direction of effects can be significant but quite variable according to the specific system (plant, thrips, virus, environmental conditions) (Stumpf & Kennedy, 2005, 2007). Hence the aim of this work was to study whether CaCV mediated plant quality of tomato, viral infection of *C. claratris* or both are affecting the fitness of *C. claratris* in terms of size, feeding damage area and daily oviposition.

Materials and methods

Thrips, test plants and tospovirus isolate

Initially, specimens of *C. claratris* from a naturally established greenhouse population at the Asian Institute of Technology (AIT), central Thailand were identified by R. zur Strassen and voucher specimen were deposited at the Senckenberg Museum, Frankfurt, Germany (Premachandra et al., 2005). A virus-free *C. claratris* colony was established in 2006 from the greenhouse population. The colony had been reared on potted tomato plants in thrips-proof Plexiglas cages (50×50×60 cm, covered at the top with 64µm nylon net) at 29 ± 1°C, 50-60% relative humidity (RH) and 12:12h L: D.

To produce newly hatched L1 (<1h), initially around 50 adults were transferred to oviposit onto few potted three-week-old tomato plants, which were enclosed by a thrips proof Plexiglas cage. On the following day all adults were discarded by means of an aspirator and a fine brush before transferring the plants into another cage. Two days later, newly hatched L1 were collected with a very fine wet painting brush under a stereomicroscope and transferred for feeding on tomato leaflets.

Three-five week-old tomato plants (*Solanum lycopersicum* L. cv. FMTT260) (AVRDC; Shanhua, Taiwan) were used throughout the experiments for thrips rearing and as host plants. Seeds were sown in peat moss then kept in a completely closed nursery greenhouse to avoid any pest immigration. The greenhouse was equipped

with an evaporative fan and pad cooling system. Mean temperature and RH were 28-30°C and 90-100%, respectively.

CaCV-AIT was maintained by thrips inoculation of tomato plants in a greenhouse (20×10 m). Roofs and lower sidewalls of the greenhouse were clad with an UV-absorbing polyethylene (PE) film (Wepelen™, FVG, Dernbach, Germany) while sides were covered with 52-mesh UV-absorbing insect proof net screens (Bionet™, Klayman Meteor Ltd, Petah Tikva, Israel) to avoid any thrips immigration from outside (Kumar & Poehling, 2006). Irrigation was combined with fertigation, consisting of Kristallon™ 6+12+36+3+Micro (% N, P, K, Mg) and Calcinit™ 15.5+0+0+19Ca (both Yara, Oslo, Norway) in a ratio of 70:30 and delivered to the soil near the stem of potted plants by drip irrigation. Intensity (frequency and time) was radiation controlled by means of computerized central irrigation and climate control unit. Mean temperature and RH were 28-30°C and 70-80%, respectively.

To produce viruliferous male thrips for plant inoculation, initially, newly hatched (<1 h) L1 of *C. claratris* were given an acquisition access period (AAP) until adulthood onto infected tomato leaflets. After adults' emergence several males were encaged inside a polyethylene (PE) cylinder (15×60 cm with 4 (Ø3cm) ventilation holes) containing one three-week-old tomato plant for a few days inoculation access period (IAP). Afterwards the plants were kept further un-caged in the greenhouse. Only males were chosen for the virus inoculation because males will die out in ten days latest at the greenhouse's temperature, hence avoiding the use of insecticides to control thrips population growth necessary if females were the primary vectors. After successful CaCV inoculation, the plants started to exhibit typical tospovirus symptoms in two to three weeks (chapters 1 & 4). Thereafter, samples of the infected plants were tested with double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to confirm the presence of CaCV. The Compound direct ELISA for *Watermelon silver mottle virus* (WSMoV) and *Groundnut bud necrosis virus* (GBNV) (AGDIA® Inc., Elkhart, IN, USA. Cat. No. SRA 61500) was employed for the detection of CaCV (Premachandra et al., 2005). To confirm the virus isolate (i.e., CaCV-AIT) systemically infected leaflets were tested with PCR at the Institute of Plant Diseases and Plant Protection, Hanover University as described by Knierim et al. (2006).

Influence of CaCV-AIT exposure on C. claratris *reproduction and size*

We employ the term exposed for adults who were feeding throughout their larval stages on infected plants. Alternatively, unexposed thrips are those who were feeding on uninfected plants. Exposed thrips are further classified to viruliferous and non-viruliferous depending on their ability for virus transmission while the unexposed thrips are always the control.

Newly hatched L1 from the colony were collected as described above and given an AAP until pupation on either CaCV infected leaflets or uninfected leaflets, hence producing adults that were either exposed or unexposed to the virus, respectively. Five females and five males were then released in a microcosm. The microcosm was composed of a single three-week-old potted tomato plant encaged inside a PE cylinder (15×60 cm with 4 (Ø3cm) ventilation holes) then sealed with laboratory film (Parafilm M®, Pechiney, Plastic Packaging, Inc., USA). Five days later the plants were cut and all eggs and larvae were counted under a stereomicroscope. The experimental design was completely randomised design with five replicates per treatment and two repetitions over time. All microcosms were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

To assess the affect of virus exposure on thrips size, adult thrips (exposed or unexposed) were produced as above and collected in 95% ethanol and then measured directly using a stereomicroscope equipped with an ocular micrometer. The measurements were done three times over the time with 38, 30, 35 and 25 total insects (i.e. males exposed, males unexposed, females exposed and females unexposed, respectively).

Female host-preference between infected or uninfected leaflets

In a choice experiment, the host-preference of females for infected or uninfected leaflets was determined in the laboratory. Thrips females were initially reared as L1 either on infected (exposed) or uninfected leaflets (unexposed). All resulting females were individually confined in a leaflet assay (Chapter 1) containing both an infected and an uninfected leaflet of an equal size. The uninfected leaflets were mostly cut to

reduce their size. On the next day the females' choice was determined by recording the feeding damage (scars) on the leaflets.

The leaflet assay, briefly, was composed of a petri dish that was lined with a layer of a mixture of plaster and charcoal (9:1 ratio). After adding few millilitres of double distilled water a small piece of filter paper was placed on the gypsum layer and a the leaflet on top of it. Consequently, the gypsum-petri dishes were closed by their modified lids and sealed with laboratory film. The lids were perforated with three equally distanced marginal holes (Ø12 mm) that were closed with thrips proof net (64 µm mesh nylon) for air exchange.

The experimental design was a completely randomised design with a minimum of thirty replicates per treatment and two repetitions over time. All leaflet assays were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

Influence of CaCV-AIT exposure on fecundity and feeding

Thrips that had been exposed to the tospovirus developed to either viruliferous or non-viruliferous. Therefore, it was possible to evaluate the direct effect of the virus on the thrips by comparing the performance of these two categories in comparison to unexposed thrips on same quality plants. To evaluate the plant-mediated indirect effects it was adequate to compare the exposed and unexposed (control) thrips on infected and uninfected plants.

Newly hatched L1 were allowed to feed until adulthood on either infected or uninfected leaflets (the unexposed control). Then only females were selected and confined individually in a leaflet assay containing an uninfected leaflet for five days. Then the leaflets were changed and the old ones were tested with DAS-ELISA. After determining their individual viruliferous status (i.e., viruliferous or non-viruliferous) all females were randomly and separately allocated into leaflet assays either with infected leaflets or uninfected leaflets. The same was done with same aged unexposed females either on infected or uninfected leaflets. Subsequently, 24h later, the six combinations (i.e., control, viruliferous and non-viruliferous on either uninfected or infected leaflets) were evaluated for the number of eggs and area of feeding damage. The number of eggs was counted as above. The area of feeding damage was measured as mm^2. Initially, all leaflets were digitally photographed

(both the adaxial and the abaxial surfaces). Subsequently, the area of feeding damage was marked and calculated as pixels^2 by means of the software AxioVisionLE 4.2 (Carl Zeiss Vision, Germany). Then the area was recalculated as mm^2. A reference square of 1 mm^2 was included in each photo. The experimental design was a completely randomised design with ten replicates per treatment and two repetitions over time. All leaflet assays were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

Influence of trichomes on L1 survival

Infected tomato plants develop significantly smaller leaflet areas than the uninfected plants. In consequence, those infected leaflets have higher density of trichomes / area on their surfaces compared to healthy leaflets. Tomato leaves are compound leaves that are composed of compound leaflets, which in turn are composed of big and small leaflets. Hence, four groups of leaflets were categorised and used to study the influence of trichomes on L1 survival. Namely, (i) leaves with clear virus symptoms and, (ii) symptom less leaflets from the infected plants, as well as (iii) small and (iv) big leaflets from the healthy plants. Thirty newly hatched L1 were introduced onto each leaflet in a leaflet assay and the numbers of surviving pupae were counted 5d later. Pupae were chosen simply because they are relatively easier to recover than adults. Four replicates per treatment were performed. All leaflet assays were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

Statistical analysis

Data were subjected to Sapiro-Wilk's test for normality using PROC UNIVARIATE and data of experiments repeated over time were checked for homogeneity of variance using the HOVTEST=LEVENE option of SAS (SAS, 2001) and pooled only when variance homogeneity could be assumed. To evaluate the effect of virus exposure on reproduction and size, the independent-samples t test was used. For the choice experiments Chi square test of independence was performed by PROC FREQ command in SAS. The rest of the analysis was used using the PROC GLM procedure in SAS. When significant factor effects were detected in the ANOVAs, means (using the SAS command MEANS or LSMEANS (least-squares means for unbalanced

design)) were compared using Tukey's multiple means comparison procedure. A significance level of p =0.05 was used in all analyses.

Results

Table 1: Mean dimensions (mm ± SD) of some anatomical features of *Ceratothripoides claratris* adults which developed * on CaCV infected (exposed) and uninfected (unexposed) tomato plants

Sex	Anatomical features	Unexposed	Exposed
Male	Total body length	0.839 ± 0.054 (30)A	0.821 ± 0.086 (37)A
	Abdomen length	0.477 ± 0.036 (30)A	0.471 ± 0.080 (37)A
	Left forewing length	0.506 ± 0.028 (30)A	0.483 ± 0.030 (38)B
	Head capsule width	0.103 ± 0.005 (30)A	0.098 ± 0.005 (38)B
	Pronotum width	0.127 ± 0.008 (30)A	0.119 ± 0.007 (36)B
	Tergite V width	0.149 ± 0.003 (30)A	0.144 ± 0.006 (36)B
Female	Total body length	0.989 ± 0.088 (25)A	0.992 ± 0.092 (34)A
	Abdomen length	0.572 ± 0.070 (25)A	0.601 ± 0.083 (35)A
	Left forewing length	0.603 ± 0.020 (23)A	0.578 ± 0.040 (35)B
	Head capsule width	0.115 ± 0.007 (25)A	0.113 ± 0.008 (35)A
	Pronotum width	0.144 ± 0.006 (25)A	0.144 ± 0.006 (35)A
	Tergite V width	0.209 ± 0.010 (24)A	0.204 ± 0.016 (34)A

* Newly hatched first instar larvae (L1) were given an acquisition access period until adulthood on either CaCV-AIT infected or uninfected tomato leaflets.
Numbers followed by the same letters in rows indicate no significant differences (t test, p= 0.05). Numbers in parentheses represent sample sizes.

Ceratothripoides claratris males exposed to CaCV-AIT infected leaflets during their preimaginal period showed slight but significant reductions in the width of the head capsule by 5% ($t(66) = 4.17$, $p < 0.0001$), the pronotum by 6% ($t(64) = 3.87$, $p = 0.0003$) and the fifth tergite by 3% ($t(51.8) = 4.22$, $p < 0.0001$) and had 4,5 % shorter left forewings ($t(66) = 3.18$, $p = 0.0022$) in comparison to the unexposed males. In contrast, virus-exposed females had only slightly 4% shorter wings ($t(52.5) = 3.24$, $p = 0.0021$) (table 1).

Exposed females oviposited less eggs (though not significant) than unexposed females (t (12) = 1.93, p = 0.078). Accordingly, the number of hatched larvae was lower but not significantly different between both treatments (t (14) = 0.87, p = 0.3994) (fig. 1).

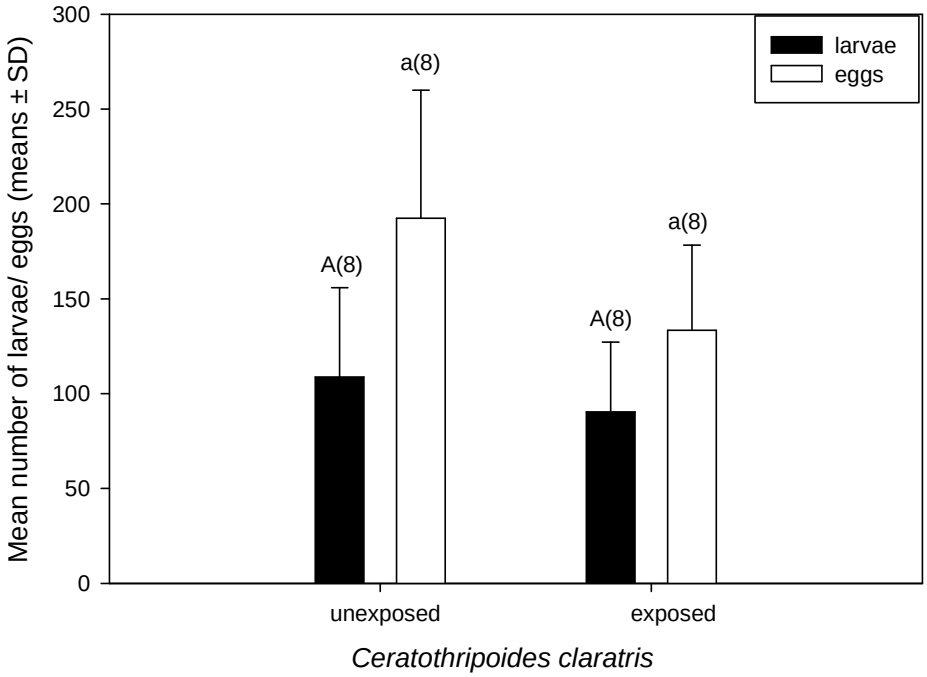

Fig. 1. Reproduction of CaCV-AIT exposed and unexposed *Ceratothripoides claratris*. Five pairs of each thrips category (exposed/unexposed) were discharged to a single three-week-old tomato plant inside a microcosm. Five days later, the number of eggs and larvae were counted. Bars marked by the same letters were not significantly different (t test, p= 0.05), numbers in parentheses represent sample sizes.

In the choice experiment exposed females showed no preference between infected or healthy leaflets (χ^2 (1, 69) = 0.70, p = 0.4039). Similarly, unexposed females did not prefer infected or uninfected leaflets (χ^2 (1, 68) = 0.14, p = 0.7081). Worth mentioning is that only two females out of the tested 139 had selected to feed on both leaflets, a result that demonstrate the sluggish behaviour of this thrips species after selecting a feeding site (fig. 2).

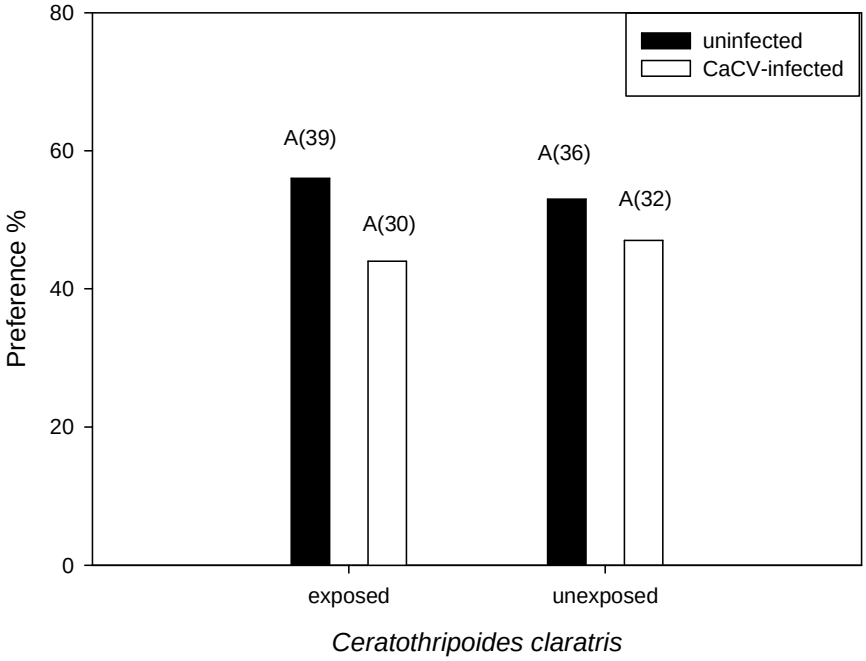

Fig. 2. Preference of CaCV-AIT exposed and unexposed *Ceratothripoides claratris* to infected or uninfected leaflets, females were individually introduced per leaflet assay that contained both infected and uninfected leaflets of same area for one day. Then the presence of feeding damage was recorded. Bars followed by the same letters indicate no significant differences (χ^2, p= 0.05) and numbers in parentheses represent sample sizes.

Although exposed to virus-infected plants in the sensitive acquisition stage (L1) developed adults can be viruliferous or non-viruliferous and it is unknown whether this feature modifies thrips performance on infected and uninfected plants. To test this in detail individual virus exposed females were pre-tested for their viruliferous status before being separately confined in a leaflet assay for oviposition on either infected or uninfected leaflets. Females' daily feeding damage was significantly 43-52% less on infected leaflets compared to uninfected leaflets, regardless whether the females were unexposed or exposed (F (5, 63) = 12, $p < 0.0001$) (unexposed: Tukey, $p = 0.0021$; and within the exposed group viruliferous or non-viruliferous: non-viruliferous: Tukey, $p = 0.0072$; viruliferous: Tukey, $p = 0.0318$) (fig. 3). Feeding damage area was not significantly different on infected leaflets when caused by control (unexposed), viruliferous or non-viruliferous females. In contrast, the damaged area was significantly different on the uninfected leaflets between the unexposed control and the viruliferous females (Tukey, $p = 0.0274$) but not with the

non-viruliferous females (Tukey, p = 0.1674). The viruliferous and non-viruliferous females showed same feeding intensity. Nonetheless, the sum of damaged area as causes by the unexposed females on both the infected and uninfected leaflets was significantly more than that of exposed females (F (2, 66) = 6.37, p = 0.0030) (fig. 3).

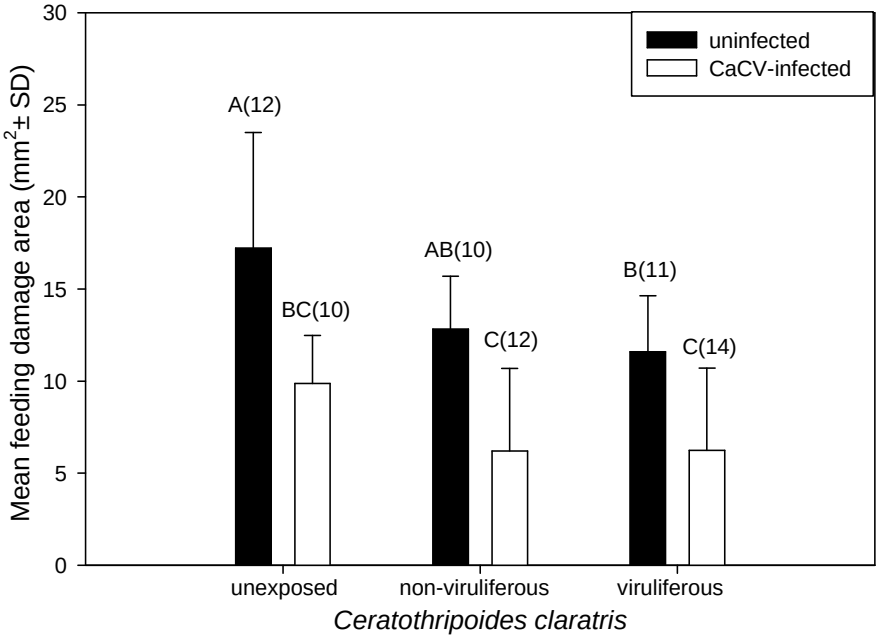

Fig. 3. Mean feeding damage area (mm^2 ± SD) by CaCV-AIT exposed (i.e., viruliferous and nonviruliferous) and unexposed *Ceratothripoides claratris*. Females were confined individually in a leaflet assay for one day on either infected or uninfected leaflets. Bars marked by the same letters indicate no significant differences (ANOVA, least-square means, p= 0.05) and numbers in parentheses represent sample sizes.

The fecundity differed significantly between the treatments (F (5, 66) = 5.25, p = 0.0004). There was a reduction in the mean daily oviposition per female on healthy leaflets compared to infected leaflets in exposed females only, regardless of they were viruliferous or non-viruliferous (43% and 36% less, respectively) (fig. 4). The reduction was only significant in case of viruliferous females (Tukey, p = 0.0018). The unexposed control females, alternatively, deposited eggs equally on both infected and healthy leaflets (Tukey, p = 1). The mean fecundity of unexposed females (12.6 ± 1.8) and exposed females (10.8 ± 5.7 and 12.1 ± 5.7 for non-

viruliferous and viruliferous, respectively) were not significantly different (F (2, 69) = 0.79, p = 0.4563).

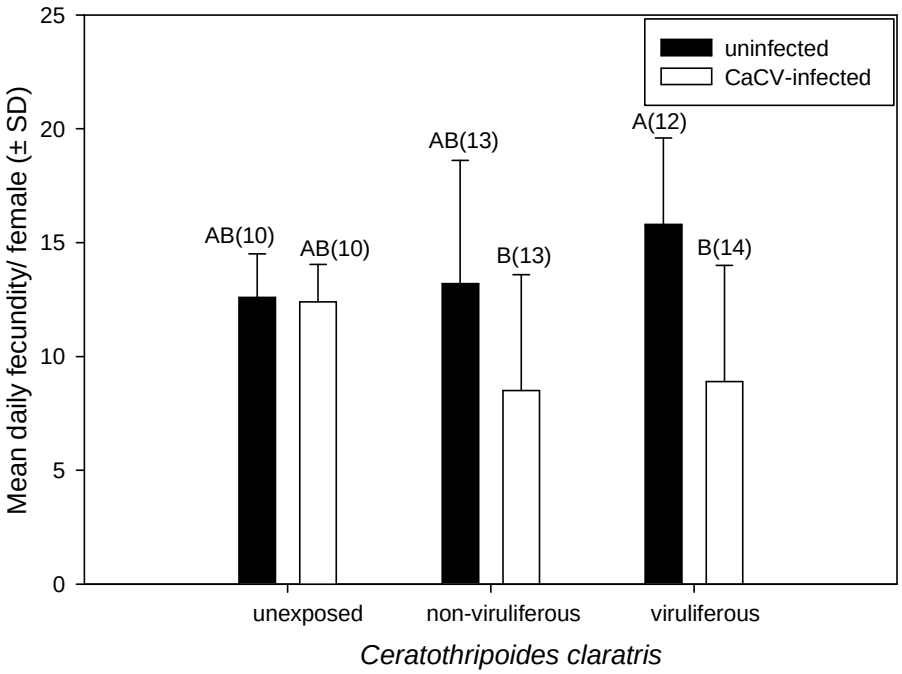

Fig. 4. Mean daily fecundity of CaCV-AIT exposed (i.e., viruliferous and nonviruliferous) and unexposed *Ceratothripoides claratris*. Females were confined individually in a leaflet assay for one day on either infected or uninfected leaflets. Bars marked with the same letters are not significantly different (ANOVA, least-square means, p= 0.05), numbers in parentheses represent sample sizes.

In the normal uninfected control 89.17 ± 4.19 % of L1 survived to the pupal stage (fig. 5). Survival to pupa did not show any significant difference either L1 larvae were established on trichome-dense infected or trichome-dense uninfected leaflets (fig. 5). However, the mere tangible presence of trichomes significantly reduced L1 survivorship from 20-31% compared to the normal healthy control (F (3, 12) = 8.44, p = 0.0028).

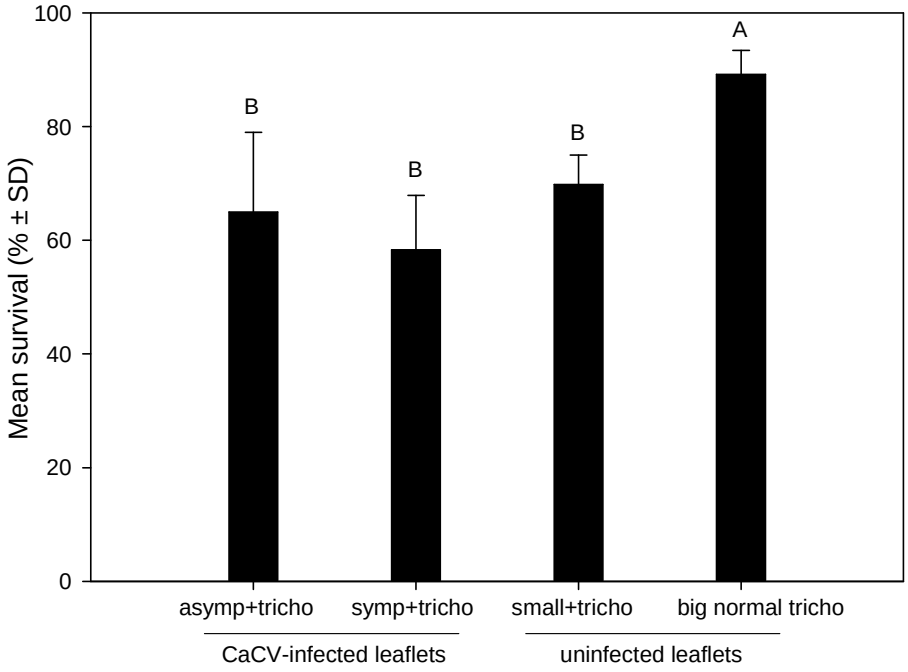

Fig. 5. Trichomes effects on *Ceratothripoides claratris* L1 survival, thirty L1 were introduced per leaflet assay that contained different leaflets type (i.e., (i) Symptomatic, (ii) asymptomatic leaflets from the CaCV-infected plants, (iii) small and (vi) big leaflets from the healthy plants; +tricho = high density trichomes, normal tricho = normal density trichomes). Five days later living pupae were counted. Bars followed by the same letters indicate no significant differences (ANOVA, Tukey, p= 0.05) and numbers in parentheses represent sample sizes.

Discussion

The herein presented results clearly demonstrate a negative influence of CaCV infected leaflets as feeding source on pre-imaginal stages of *C. claratris*. In terms of morphological parameters this effect was more pronounced on males that developed on virus infected plants rather than females. All anatomical features measured, but the total body and abdomen length, were slightly but significantly reduced. Ethanol used during measurements stretched the body and abdomen, hence affected stretchable body parts such as body length while it did not affect the width of all the measured exoskeletal plates (table 1). In contrast, regarding females that were feeding on CaCV-infected leaflets only showed shorter wings. However, a tested cohort of females used for morphometric measurements had reduced fecundity, although not significant (fig. 1), which indicates a possible moderate negative effect

of infected leaflets on females too. Stumpf & Kennedy (2005) reported that TSWV-infected *F. fusca* reared on TSWV-infected leaves had smaller head capsule width than their noninfected counterparts. But unlike *C. claratris* the effect was limited to females. The head capsule of *F. occidentalis*, on the contrary, did not differ between the viruliferous and non-viruliferous thrips reared on TSWV-infected leaves (Stumpf & Kennedy, 2007). Although, the evident negative effect of infected plants, female *Ceratothripoides claratris* have shown no preference when having the choice between the CaCV-infected leaflets and the noninfected leaflets in the leaflet assays (fig. 2).

From the first experiments described it is not possible to differentiate between direct and plant-mediated indirect effects of the tospovirus that both could have been responsible for the observed results. The effects could have been directly resulted from the CaCV-infection of the thrips and caused by physiological reactions or plant suitability as host for *C. claratris* may have been altered by CaCV infection or the reaction is simply a combined effect of plant and thrips infection by CaCV.

Further experiments with individual viruliferous and non-viruliferous females showed that the virus both directly and indirectly has negatively affected the fitness of thrips. Cohort female thrips always fed less on the infected tomato leaflets than on the noninfected leaflets regardless of their pre-imaginal exposure history, which indicated a host quality effect on female feeding intensity. However, the total feeding damage of unexposed females was found to be higher than that of the exposed females (fig. 3). This suggests that the nurture period of pre-imaginal larvae has to some kind determined the fitness of the resultant females, which is supported also by reduced oviposition of exposed females only on the infected leaflets while the unexposed females oviposited similarly on both healthy and infected leaflets. On the other hand the fecundity of exposed females (both viruliferous and non-viruliferous) was similar to unexposed on the uninfected leaflets. It may be concluded that the higher food quality of the uninfected plants could compensate the adverse indirect and direct negative effects of virus infection on the thrips.

In our experiments unexposed *C. claratris* laid the same daily number of eggs on both infected and uninfected leaflets. In contrast, both *F. occidentalis* and *F. fusca* had oviposited weekly a little more on TSWV-infected leaf disks than on uninfected

tomato leaf disks (Chaisuekul & Riley, 2005). The main differences were in the duration of the testing period (one day in comparison to one week), in addition to the host plant and thrips species tested which together may account for the different results achieved in this study and the study of Chaisuekul & Riley (2005) (Stumpf & Kennedy, 2005, 2007).

On the contrary, the number of daily-oviposited eggs was reduced only when exposed females had been ovipositing on CaCV-infected leaflets in comparison with their cohort on uninfected leaflets. Yet, the reduction was slightly significant in viruliferous females only, which may be considered as a direct rather weak negative effect of the CaCV circulation and replication within the females (i.e., the only major difference between the viruliferous and non-viruliferous females). That is corroborating previously observed direct effects of tospovirus that lowered thrips development time and reduced size of *F. fusca* (Stumpf & Kennedy, 2005, 2007).

Plant host quality may be altered as a result of tospovirus infection, through reduction of nutritional compounds and changes in plant architecture and foliage surface (e.g. higher trichomes density). The achieved lower fecundity assumes deterioration in nutritional quality of the infected leaflets (Awmack & Leather, 2002). We did not compare the nutritional quality (e.g. total proteins and amino acids) of CaCV-infected leaflets with uninfected leaflets. And we are not aware of such work concerning thrips and tospoviruses. However, it is reported in the literature that virus infections can reduce significantly the concentration of total amino acids in the infected plants, as e.g. shown with *Barley yellow dwarf virus* (BYDV) in cereals and the effects on cereal aphids (Fiebig et al., 2003). The concentration of amino acids, particularly the aromatic amino acids influence the extent of feeding by thrips. The feeding damage by *F. occidentalis* was significantly higher on tomato genotypes with high concentrations of aromatic amino acids (as a proportion of total proteins) than on genotypes with low concentrations of aromatic amino acids (Mollema & Cole, 1996). Therefore we hypothesise that the reduced fitness of *C. claratris* is attributed to the nutritional reduction of infected leaflets. More studies on the influence of tospovirus on the nutritional ecology of thrips are needed to advance our knowledge about the tospovirus-thrips-plant biological interactions.

Trichomes, both glandular and nonglandular, are prominent features of the tomato foliage and are essential in arthropod resistance (Kennedy, 2003). Results of the presented trichome experiment clearly demonstrated the negative effect of trichomes on the survival of first instar larvae, hence the suspected effect on *C. claratris* population build up. Boughton et al. (2005) reported that increased trichome densities on tomatoes, entrapped higher number of immature *F. occidentalis* than the control plants. Hence, the reduction in feeding on the infected plants could be due to the increased trichome density and reduced nutritional quality in the infected leaflets.

As viruliferous females settle on an uninfected plant they will infect it. Consequently, their offspring will develop on a thrips-inoculated plant with activated herbivore and pathogen-induced pathway defences, before some will eventually develop to viruliferous insects. At this point, and before the appearance of virus symptoms and the deterioration of the plant quality, the positive plant-mediated indirect effects could be expected (Maris et al., 2004; Belliure et al., 2005). Finally, as the virus replicates and spread within the plant, virus symptoms will develop and the plant quality will deteriorate, hence emigration from the crop is expected. Such hypotheses merit further research.

CHAPTER 4

Influence of tomato ontogeny on invasion and colonisation of the thrips *Ceratothripoides claratris* (Schumsher) (Thysanoptera: Thripidae) and subsequent tospovirus incidence

Abstract

Plants develop through conspicuous ontogenetic stages. Therefore, the potential influence of three main stages (i.e., cotyledon, seedling and juvenile) on the invasion and colonisation of tomato plants by the thrips *Ceratothripoides claratris* was studied. In a choice experiment with tomato juveniles of five different age categories, the thrips natural invasion, feeding-damage, and eventual tospovirus infection increased significantly with the age (size) of the plant. In the no-choice experiment, three thrips were confined inside a microcosm with one plant of different ontogenetic stages. Only 28% and 61% of plants in the cotyledon and seedling stages, respectively, showed feeding-damage, while 100% of the plants in the juvenile stages had feeding-damaged leaflets. Even though, the percentage of infected plants increased with plants ontogenetic stage, the results can only suggest that cotyledons may have negative effect on tospovirus infection. However, cotyledons have differently affected the males and females preference and feeding behaviour.

Introduction

The Oriental tomato thrips *Ceratothripoides claratris* (Schumsher) (Thysanoptera: Thripidae) is mainly a foliage feeding thrips that become a pest of tomato crops in central Thailand (Murai et al., 2000; Premachandra et al., 2004). *Ceratothripoides claratris* is an especially serious pest of crops grown under protected cultivation where optimal environmental conditions for the thrips development are prevailing (Premachandra et al., 2005). After infestation the thrips population build up is fast and will damage the crop directly through feeding and indirectly through inoculating a devastating pathogen for the tomato, the Capsicum chlorosis virus (CaCV) (genus *Tospovirus*, family Bunyaviridae) (Premachandra et al., 2005).

Fig.1. A few examples of tomato (*Solanum lycopersicum* L. cv. FMTT260) ontogenetic stages and their relative sizes that was used in this study; 1 = cotyledon (3d-old), 2 = seedling (10d-old), 3 = juvenile (17d-old), and 4 = juvenile (24d-old).

Plants develop through a sequence of stages during their ontogeny: seed, cotyledon, seedling, juvenile, reproductive, and senescent phase (Boege & Marquis, 2005) (fig. 1). Throughout their development, plants may encounter pests and pathogens. The subsequent foliage damage by herbivores causes the induction of proteinase inhibitors and oxidative enzymes, which negatively affect the attacker (Stout & Duffey, 1996). Resistance to herbivory in plants, however, is likely to change during their ontogeny, owing partly to resource allocation (Boege & Marquis, 2005) and alteration in defensive compounds (Stout et al., 1996). For instance, protein

concentration inside a tomato seedling is decreasing in the cotyledons while it is increasing in the new leaves (Hall & Cocking, 1966). Larvae weight and percent survival of *Spodoptera eridania* (Lepidoptera: Noctuidae) increased with the age of *Brassica juncea* cotyledon, which was considered as a direct result of the reduction in glucosinolate activity and myrosinase concentration in the aging cotyledons (Wallace & Eigenbrode, 2002). In the tomato leaves, the induction of protein inhibitors and the oxidative enzymes polyphenol oxidase, peroxidase, and lipoxygenase (all are involved in foliar resistance) are dramatically reduced in the ageing plants/leaves (Stout et al., 1996).

The effect of leaf age on the feeding behaviour of the western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) was studied with many plants. On cucumber, *F. occidentalis* females preferred young leaves to older leaves for oviposition (de Kogel et al., 1997). The western flower thrips similarly favoured young leaves of Chrysanthemum compared to old leaves (Fung et al., 2001). The effects of tomato plant and leaf age on the feeding and settling behaviour of the foliage feeding thrips *Frankliniella fusca* (Hinds) and the flower thrips *F. occidentalis* have been recently addressed (Joost & Riley, 2008). Using the electrical penetration graph technique (EPG), they observed no effect of plant age on *F. occidentalis* feeding behaviour, simply because tomato leaves are not the preferred host of *F. occidentalis*. In contrast, the foliage feeding thrips *F. fusca* settled, probed and ingested more frequently on young plants (3-week-old and 4-week-old (still juveniles) than on older plants (6-week-old and 8-week-old, in the flowering stage). Important to note, that the authors used 1-week-old leaves from each plant age, thus the leaf age was fixed. In contrast, the leaf age (1, 2, and 4-week-old leaves taken from 6 week-old plants) had opposite effect and *F. fusca* preferred to settle and probe on the older leaves compared to younger ones. These results suggest that plant age affected *F. fusca* behaviour in a different way than leaf age.

Controlling of *C. claratris*, as a foliage feeding thrips, on pre-flowering tomatoes is important, since early infestation of the crop will result in fast population built-up and spread of the thrips reaching rates of up to 80% infected plants (Premachandra et al., 2005). Thus slowing down the thrips development on young plants can strongly reduce resulting yield losses. Therefore, we aim by this work to assess the role of

plant ontogenic stage on thrips performance with special consideration to a subsequent potential of tospovirus infection.

Materials and methods

Test plants, tospovirus isolate and thrips

Tomato plants (*Solanum lycopersicum* L. cv. FMTT260) (AVRDC; Shanhua, Taiwan) were used throughout the experiments and thrips rearing. Seeds were sown in peat moss then kept in a completely closed nursery greenhouse to avoid any pest immigration. The greenhouse, located within a greenhouse complex on the campus area of the Asian Institute of Technology (AIT), Bangkok, Central Thailand, was equipped with an evaporative fan and pad cooling system. Mean temperature and relative humidity (RH) were 28-30°C and 90-100%, respectively.

A virus-free colony of *C. claratris* (henceforth the colony) was established in 2006 from a naturally established greenhouse population at AIT. Initially, specimens of *C. claratris* from the greenhouse population were identified by R. zur Strassen and a voucher specimen was deposited at the Senckenberg Museum, Frankfurt, Germany (Premachandra et al., 2005). The colony had been reared on potted tomato plants in thrips-proof Plexiglas cages (50×50×60 cm, covered at the top with 64µm nylon net) at 29 ± 1°C, 50-60% RH and 12:12h L: D.

To produce newly hatched L1 (<1h), around 50 adults from the colony were transferred onto three-week-old potted tomato plants to oviposit. The plants were put in a thrips proof Plexiglas cage. On the following day, all adults were discarded by means of an aspirator and a fine brush. Then the plants were transferred into another cage. Two days later, newly hatched L1 were collected with a very fine wet painting brush under a stereomicroscope and transferred to tomato leaflets.

A culture of CaCV-AIT was maintained by thrips inoculation of tomato plants in a spatially separated greenhouse (20×10 m). Roofs and lower sidewalls of the greenhouse were cladded with an UV-absorbing polyethylene (PE) film (Wepelen^{TM}, FVG, Dernbach, Germany) while sides were covered with 52-mesh UV-absorbing insect proof net screens (Bionet^{TM}, Klayman Meteor Ltd, Petah Tikva, Israel) to

avoid any thrips immigration from outside (Kumar & Poehling, 2006) (henceforth 52-UV_{ab}GH). Fertigation was combined with the irrigation system and consisted of Kristallon™ 6+12+36+3+Micro (% N, P, K, Mg) and Calcinit™ 15.5+0+0+19Ca (both Yara, Oslo, Norway) in a ratio of 70:30 and delivered to the plants by single dripper irrigation that was radiation controlled by means of computerized central irrigation and climate control unit. Mean temperature and RH were 28-30°C and 70-80%, respectively.

To produce viruliferous male thrips for plants inoculation, newly hatched (<1 h) L1 of *C. claratris* were allowed an acquisition access period (AAP) until adulthood onto systemically infected tomato leaflets. Following emergence, several males were confined inside a polyethylene (PE) cylinder (15×60 cm with 4 (Ø3cm) ventilation holes) with one three-week-old tomato plant for five days inoculation access period (IAP). Afterwards the plants were kept further un-caged in the 52-UV_{ab}GH. Using only males prevents the establishment of new infestations of thrips which would then have to be controlled with insecticides. After a successful CaCV inoculation, infected plants started to exhibit typical tospovirus symptoms in two to three weeks (chapters 1 & 4). To confirm the presence of CaCV, thereafter, samples of the infected plants were tested with double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The Compound direct ELISA for *Watermelon silver mottle virus* (WSMoV) and *Groundnut bud necrosis virus* (GBNV) (AGDIA® Inc., Elkhart, IN, USA. Cat. No. SRA 61500) was employed following the supplier's protocols for the detection of CaCV. To confirm the virus isolate (i.e., CaCV-AIT) systemically infected leaflets were tested with PCR at the Institute of Plant Diseases and Plant Protection, Hanover University as described by Knierim et al. (2006).

Influence of plant ontogenetic stage on C. claratris *invasion and tospovirus incidence: a choice experiment*

This choice experiment was performed at AIT in a greenhouse (6 × 6 m) that was covered with 40-mesh (non thrips-proof) screens (Econet M, Ludvig Svensson Inc., Kinna, Sweden) (henceforth 40-mesh GH). Hence thrips from outside could pass the barrier and infest the plants. Three plant ontogenetic stages and five ages (expressed in d = days after seedling emergence) were chosen: cotyledon (3d), seedling (10d),

and juveniles (17d), (24d) and (31d). For a five-week period, seeds were sown weekly and seedlings were kept in the nursery greenhouse described above until transplanting. Plants were transplanted simultaneously on May 15, 2006 into a 30 cm Ø pot containing a local potting mix substrate (Dinwondeekankasat, Ayutthaya, Thailand; textural classes: 30% sand, 39% silt and 31% clay; organic matter: 28%; pH 5.3). Two weeks later, when all plants showed feeding damage, data were collected and then plants were sprayed with spinosad. To prevent further infestation, henceforth, spinosad was sprayed weekly until the end of the experiment on June 23, 2006. Throughout the experiment the incidence of infected plants were recorded weekly. Twenty replicates per age category were used and the experiment was performed in a randomised block design. The 40-mesh GH mean temperature and RH were 28-30°C and 70-80%, respectively. Irrigation and fertigation was conducted as described above.

Influence of plant ontogenetic stage on C. claratris *colonization and tospovirus incidence: a no-choice experiment*

For this no-choice experiment, the same five plant ages as above were chosen. Each plant was individually transplanted to a 15cm Ø pot in the potting substrate. Plants were individually caged by a PE cylinder (15×60 cm with 4 (Ø3cm) ventilation holes) (henceforth a microcosm). Three potentially viruliferous pupae were released per microcosm. The number of released pupae was selected according to previous results, which had shown that three potentially viruliferous pupae are needed to cause 50% incidence of infected plants (Chapter 1). Pupae were preferred over adults because they are easier to handle. In order to produce potentially viruliferous pupae, newly hatched L1 from the colony were collected as described above and allowed an AAP until pupation on symptomatic CaCV-infected leaflets. On the second day after the pupal introduction feeding damage started to be obvious, hence the microcosms were kept for further three days before they were finally opened and the presence of feeding damage was recorded. In total eighteen replicates per age were used and the experiment was performed in a randomised block design and the experiment was done on two occasions. All microcosms were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room. Irrigation and fertigation with the same

above mentioned concentrations was conducted manually inside the room. After spinosad treatment the plants were transferred to the 52-UV_{ab}GH until symptoms exhibition and the presence of the tospovirus was confirmed with DAS-ELISA. At the end of the experiment, two months later, all asymptomatic plants were tested with DAS-ELISA to confirm the absence of the virus.

Cotyledon versus true leaf: a preference study under choice and no-choice conditions

In a choice experiment, adult thrips (males and females) were individually allowed to chose between a cotyledon and a true leaflet of a similar area in a leaflet assay. The leaflet assay, briefly, was composed of a petri dish (10 cm Ø) that was lined with a layer of a mixture of plaster and charcoal (9:1 ratio). After adding a few millilitres of double distilled water, a small piece of filter paper was placed on the gypsum layer and the leaflet on top of it. Consequently, the gypsum-petri dishes were closed by their modified lids and sealed with laboratory film (Parafilm M®, Pechiney, Plastic Packaging, Inc., USA). The lids were perforated with three equally distanced marginal holes (Ø12 mm) that were closed with thrips proof net (64 µm mesh nylon) for air exchange. The experimental design was completely randomised with 20 replicates per sex and the experiment was done on two occasions.

In a no-choice experiment, male and female adults were divided between leaflet assays containing either a cotyledon or a true leaf. Then the area of feeding damage was measured as mm^2 on the following day. Initially, all leaflets were digitally photographed (both the adaxial and the abaxial surfaces). Consequently, the area of feeding damage was marked and calculated as $pixels^2$ by means of a software AxioVisionLE 4.2 (Carl Zeiss Vision, Germany). Then the area was recalculated as mm^2. A reference square of 1 mm^2 was included in each photo. The experimental design was completely randomised with a minimum of ten replicates per sex and the experiment was done on two occasions. All leaflet assays were kept at 29 ± 1°C at 50-60% RH and 12:12h L: D in an air-conditioned room.

Statistical analysis

Data were subjected to Sapiro-Wilk's test for normality using PROC UNIVARIATE and data of experiments repeated over time were checked for homogeneity of variance using the HOVTEST=LEVENE option of SAS (SAS, 2001) and pooled only when variance homogeneity could be assumed. One-way ANOVA was used to compare the leaflets and thrips numbers, and symptoms exhibition time across the different ontogenetic stages. When significant factor effects were detected in the ANOVAs, means were compared using Tukey's multiple means comparison procedure. To compare feeding-damage areas, the independent-samples t test was used. For the percentages of infected plants and the choice data, the Fisher's exact probability test was adopted when any of the expected frequencies was less than 5. Otherwise, Chi square test of independence was performed using PROC FREQ command in SAS (SAS institute, 2001). A significance level of $p = 0.05$ was used in all analyses.

Results

Even though thrips infestation to the older (bigger) plant has started on the first day post transplanting, the numbers of thrips were still very low during the first week. That required extending the experiment one more week in order to achieve higher level of infestation. At time of evaluation as shown in table 1, results as expected, clearly demonstrate that the number of leaflets increased with plant age (size) ($F(4, 94) = 161.53$, $p < 0.0001$). Even though, all juvenile plants but one (a 17d-old juvenile) exhibited feeding damage and the percent of the total damaged leaflets per plant from the total leaflets per plant at each plant age was more or less similar. However, the average number of the damaged leaflets increased significantly with the age of the plant ($F(4, 94) = 19$, $p < 0.0001$). It was obvious throughout the experiment that the number of thrips per plant was very low. Nevertheless, there is a trend of increase in the attacking thrips numbers with the plant age ($F(4, 95) = 2.9$, $p = 0.0225$) (table 1). As a direct result of attracting more thrips and having more

damaged leaves, the incidence of tospovirus infection significantly increased in the bigger plants (Fisher, $p = 0.0252$) (table 1).

Because of the inevitable two weeks needed for natural thrips infestation in the abovementioned 40-mesh GH experiment, unfortunately, important ontogenetic stages were lost (i.e., cotyledon and seedling stages). Therefore, we conducted the no-choice experiment under controlled conditions. In this experiment the plant growth was slow and plant growth stage did not change considerably during thrips feeding period. In this experiment, it was sufficient to consider the presence or absence of feeding damage visually because a specific number of thrips were released per microcosm. As shown in table 2, the incidence of feeding-damaged plants increased significantly with the ontogenetic stage (Fisher, $p < 0.0001$). Less than one third and two-third of the plants in the cotyledon and the seedling stages, respectively, exhibited visible feeding damage, even though the thrips were released to the microcosm containing the plants for a week. In contrast, thrips feeding symptoms were visible on all of the other stages. Consequently the incidence of virus infected plants increased as well with the stage of the plant (χ^2 (4, 90) = 15.1, $p = 0.0044$). No infection was observed in cotyledons, however, 17 to 50% of the plants (total used) in the seedling or juvenile stages, respectively developed virus symptoms. None of the plants that had not had feeding damage tested positive with DAS-ELISA. On the other hand, when considering the percentage of infected plants in relation only to the total number of plants damaged by feeding, no significant differences in the incidence of infected plants across the tested stages (χ^2 (4, 70) = 5, $p = 0.2886$) could be calculated. Though the three infected juvenile plants developed virus symptoms slightly faster than other stages, the time was not statistically significant (F (3, 23) = 1, $p = 0.3958$).

The special attractivity of the cotyledons for the thrips is illustrated by the choice experiment in fig. 2, the data clearly show that males preferred the leaf to the cotyledon (χ^2 (2, 34) = 37.7, $p < 0.0001$) and fed less on the cotyledon compared to leaves (t (9) = 2.6, $p = 0.0297$) (fig. 3). Unlike males, females showed no preference (χ^2 (2, 37) = 1.5, $p = 0.4629$) (fig. 2), however, they fed significantly more often on the cotyledons than the leaves (t (10) = 3.4, $p = 0.0063$) (fig. 3). Moreover, males significantly preferred feeding on the leaves than females (χ^2 (1, 71) =7.8, $p = 0.005$).

Table 1: Effect of tomato juvenile age (size) on the thrips *Ceratothripoides claratris* natural infestation and subsequent tospovirus incidence in 40-mesh GH.

Age of juvenile[1] (d)	N	Leaflets no. /plant (mean ± SD)	Feeding-damaged leaflets no. /plant		Thrips no. /plant (mean ± SD)	Infected plants (%)(N_{inf}/N)[2]
			(mean ± SD)	(% damage)		
17	20	11.45 ± 2.66 d	2.15 ± 1.18 d	19 a	1.80 ± 2.17 b	5 (1/20) ab
24	20	28.65 ± 4.61 c	5.00 ± 2.75 cd	17 a	4.15 ± 3.25 a	0 (0/20) b
31	20	55.31 ± 12.82 b	6.78 ± 3.35 bc	12 a	3.00 ± 2.51 ab	25 (5/20) a
38	20	67.35 ± 8.37 a	8.45 ± 3.87 ab	12 a	4.10 ± 2.59 a	20 (4/20) a
45	20	60.75 ± 9.43 ab	11.10 ± 5.02 a	18 a	3.85 ± 2.41 ab	30 (6/20) a

[1] Plants age on evaluation day.
[2] (Number of infected plants / total number of plants).
Means or percentages followed by a different letter in columns indicate significant differences (Tukey or Fisher, respectively, $p = 0.05$).
Numbers in parentheses = sample size.

Table 2: Effect of tomato ontogenetic stage on the thrips *Ceratothripoides claratris* colonization and subsequent tospovirus infection in a no-choice experiment.

stage (d) [1]	N	Feeding-damaged plants (%)(N_d/N)	Infected plants (%)		Symptoms exhibition (d) (mean ± SD)
			(N_{inf}/N_d)	(N_{inf}/N)	
Cotyledon (3)	18	28 (5/18) c	0 (0/5) a	0 (0/18) c	-
Seedling (10)	18	61 (11/18) b	27 (3/11) a	17 (3/18) bc	10.3 ± 0.6 a
Juvenile (17)	18	100 (18/18) a	50 (9/18) a	50 (9/18) a	16.0 ± 4.2 a
Juvenile (24)	18	100 (18/18) a	39 (7/18) a	39 (7/18) ab	15.6 ± 7.4 a
Juvenile (31)	18	100 (18/18) a	44 (8/18) a	44 (8/18) ab	16.1 ± 4.7 a

[1] the stage of the plant at time of thrips release.
N_{inf} = Number of infected plants, N_d = number of plants that were showing only visible feeding-damage symptoms (plants without feeding symptoms were excluded from the calculations).
Percentages or means followed by a different letter in columns indicate significant differences (χ^2 or Fisher, ANOVA, respectively, p = 0.05). Numbers in parentheses = sample size.

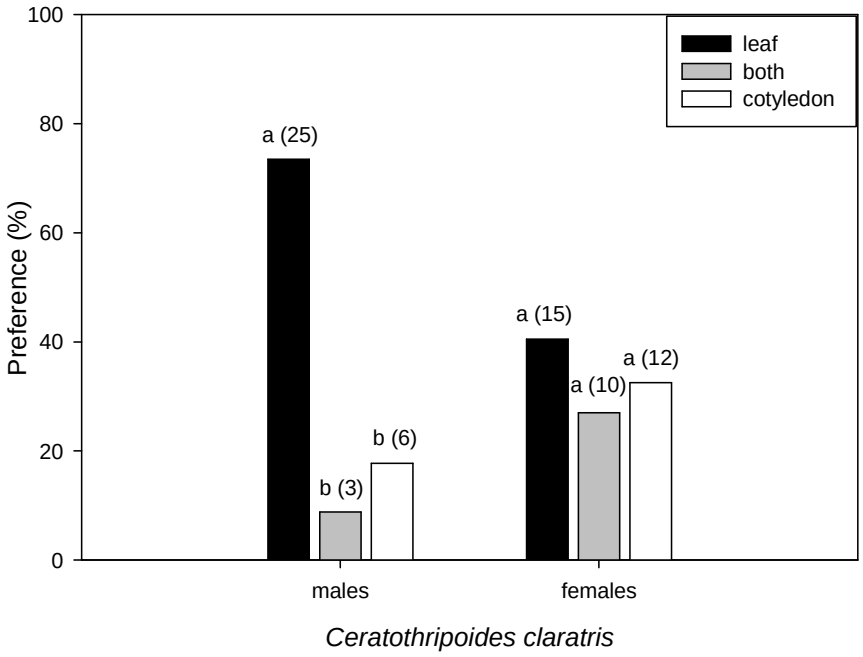

Fig. 2. The thrips *Ceratothripoides claratris* Preference between leaf and cotyledon (leaflet assays for one day). Within each sex, percentages followed by a different lower case letter indicate significant differences (χ^2, p = 0.05). Numbers in parentheses = sample size.

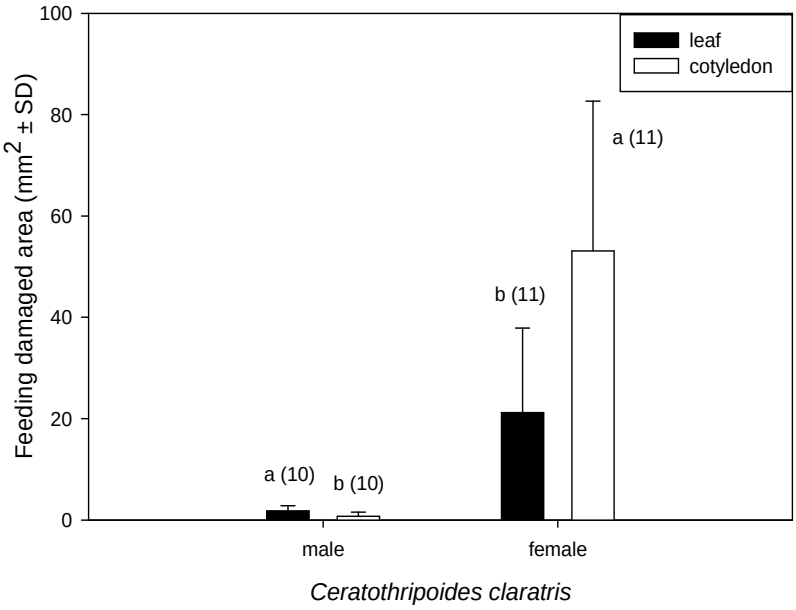

Fig. 3. Mean feeding-damaged area in mm^2 (± SD) as caused by the thrips *Ceratothripoides claratris* adults on either leaves or cotyledons (leaflet assay for one day). Within each sex, percentages followed by a different lower case letter indicate significant differences (t test, p = 0.05). Numbers in parentheses = sample size.

Discussion

The oriental tomato thrips, *C. claratris* is a foliage feeder with a sluggish behaviour (when not disturbed) (see Chapter 3). Soon after transplanting in the 40-mesh GH, this thrips commenced invading the crop. The incidences of attacks were positively related to the size of the juvenile plants (as determined by age in this case). As the plants grow up they possess more leaves, this was evidently an important factor in driving more thrips to attack the bigger plants. However, this was only obvious from the number of leaflets with feeding scars (table 1) and not from the number of thrips observed (counted) on the plants. The low number of thrips as was calculated by the visual observation may not represent the real thrips density on each plant, owing to the difficulty in counting adult thrips on intact plants. Due to the sluggish nature of this thrips, we assume that the number of feeding-damaged leaflets may be considered as a direct evidence of the presence of *C. claratris* and hence valid to represent the number of thrips on each plant. In addition, as the number of damaged leaflets increased the incidence of infected plants increased as well. Hence, we conclude that the size of the plant is evidently an important factor that plays a crucial role in the orientation of the thrips (or the attraction of thrips to the plant). The host shape and size do affect the thrips host selection, as shown with broad bean plants of lush growth which attract more thrips than smaller plants (Terry, 1997). Maybe the increase in leaf area with plant age (size) also increases the green colour area and the UV reflectance from them attracts more thrips.

Since the level of natural infestation was low in May 2006 when the 40-mesh GH trial was started (chapter 1), we lost in the first experiment an important ontogenetic stage (the cotyledon) because of the inevitable two weeks waiting time, which was necessary to achieve that nearly all plants would have feeding-damaged leaves, hence will be considered as valid when the ratios of infected plants are needed. Therefore, the more controlled conditions adopted in the no-choice experiment made it possible to assess the role of this stage on thrips colonisation. Plant ontogenetic stage affected thrips colonisation not only through the size but also due to the physiological changes of the tissue during growth from cotyledon to leaf stages. In the no-choice experiment, the thrips clearly fed less on the cotyledons than leaves.

Moreover, the thrips fed less on the seedling leaves than the juvenile leaves (table 2). The plant size may still have influenced the choice of the thrips, which may partly explain the difference between the seedlings and the juveniles. Nevertheless, in the tomato leaves, the induction of protein inhibitors and the oxidative enzymes, which are involved in foliar resistance, are reduced with plant/leaf age (Stout et al., 1996). These physiological changes in the tomato plants as they age may explain the preference of the thrips to the older plants compared to the seedlings. In our experiments, as in fig. 1, the seedlings had two young leaves while the juveniles had both young and old leaves. Inside the microcosms, we observed the feeding scars on the old leaves of the juvenile plants. Considering the leaf age effect, our results corroborates the finding of Joost & Riley (2008) who observed that *F. fusca*, another foliage feeding thrips of tomato, showed a settling and feeding preference to 4-week-old leaves over 1-week-old leaves. Thus, although, the younger leaves are more nutritious (Mollema & Cole, 1996; Fung et al., 2001; Awmack & Leather, 2002), the foliage feeding thrips tend to prefer the older less defended leaves (Stout et al., 1996).

Tomato plant age as well affects the feeding behaviour of *F. fusca*, where they prefer to feed on 3 to 4-week-old (juveniles at leaf stage) compared to 6 to 8-week-old plants (budding and flowering stage) (Joost & Riley, 2008). Our results show that *C. claratris* also preferred to feed and colonise tomato plants of similar growth stages. However, we did not test the flowering stage, as we were interested in the pre-flowering tomato stages. On the other hand, Joos and Riley (2008) did not test the seedling and cotyledon stages.

The percentage of infected plants increased with the ontogenetic stage. The percentage of infected plants reached the expected 50% in the juvenile stages (this expected ratio was predetermined, see chapter 1), while it was less than expected in the two younger stages. Our results can only suggest that the cotyledon stage may be resistant to tospovirus infection. Successful virus transmission of potentially transmitting thrips (the thrips genotype having the vector determinant recessive allele) (Chapter 2), which had taken up the virus during feeding as L1 on virus infected plants depends on the thrips sex (see Chapter 2) and on the feeding behaviour of the thrips (van de Wetering et al., 1998). The plant tissue on which the

thrips is feeding (fig. 2 & 3) in turn influences the later; hence, the ratio of successful transmission is influenced by multiple factors. A prerequisite for an accurate calculation of the number of infected plants in relation to thrips infestation is the identification of those plants upon which the thrips had fed. Yet, this is not easy especially since some of the thrips (mainly the males) probe only for short time on leaves leaving no visible scars behind but can transmit the virus even by the short feeding period. Only prolonged feeding at a distinct feeding site will produce larger and visible damaged areas. Hence, excluding those plants on which thrips had only probe-fed leaving no obvious damage will certainly cause an overestimation in calculating the ratio of the infected plants. On the other hand, considering all plants even those on which the thrips did never fed will certainly underestimate that ratio. Nevertheless, the younger ontogenetic stage regardless through physiological characteristics of the tissue causing reduced feeding activity or indirectly by attracting less thrips faces a lower risk to become infected with the tospovirus compared to older plant tissues.

Further assessments of the effect of cotyledons and leaves of seedlings on thrips behaviour have revealed sexual differences in preference behaviour (fig. 2 & 3). Whereas males favoured leaves over cotyledons on which they even fed less, females showed no preference but fed more on the cotyledons. The cotyledons and true leaves used here originated from seedlings. This sex-biased preference may be partially explained by nutritional demands of the thrips and resource allocation or a kind of trade-off between food quality and plant defence compounds (see above). As the concentration of proteins sink with time in tomato seedling cotyledons as they are allocated to the seedling leaves (Hall & Cocking, 1966). Males can satisfy their relatively low nutritional demand by short feeding periods on nutrient–rich tissue encountering only low risk from defence compounds enriched in the young leaf tissue. The females, on the other hand, compensate the lower concentration of nutrients by more intensive feeding but then prefer tissue with low defence potential (Awmack & Leather, 2002). We cannot find any paper supporting that hypothesis with thrips but remoter from other pests. Barrett & Agrawal (2004) compared body mass of neonates of the beet armyworm *Spodoptera exigua* (Lepidoptera: Noctuidae) that were feeding on cucumber cotyledons with those feeding on leaves. Neonates on

cotyledons developed two-third smaller body mass than those feeding on leaves, and the authors related this to the comparatively low nitrogen concentration in cotyledons.

In conclusion, our results only suggest that thrips faced with cotyledon and/or young seedling stages of tomato come across with a relatively unsuitable diet limiting feeding activity and consequently virus infection. However, with plant growth and aging of the infested tissue suitability for *C. claratris* will improve. The reason could be a trade-off between nutritional quality and harmful defence compounds, but that hypothesis should be tested with advanced studies

General discussion

The oriental tomato thrips, *Ceratothripoides claratris* is one of the major pests of tomatoes in central Thailand. Particularly, in protected cultivation environmental conditions are optimal for rapid population built up as shown in multiple studies in tropical greenhouses based at the Asian Institute of Technology (AIT) (Premachandra et al., 2004). Eventually, the mere pressure of the high population on the crop is economically detrimental. Furthermore, *C. claratris* is a known vector of a serious pathogen; namely the Tospovirus, Capsicum chlorosis virus (CaCV) (Premachandra et al., 2005). Hence, both thrips feeding damage and tospovirus transmission and spread cause very serious threats that potentially can completely destroy the entire tomato crop. With this background, we aimed to study in depth the tospovirus-thrips interrelationships as well as the role of the host plant in this trilateral relationship, by using the specific biological system; CaCV-*C. claratris*-Tomato.

Our results clearly show that *C. claratris* is present all the year round in the vicinity of the greenhouses at AIT, though their natural population density is fluctuating seasonally , with higher abundance in the rainy than in the dry season (chapter 1). Consequently, the absolute numbers of individual thrips that are viruliferous (= transmitters) is also seasonally influenced (chapter 1). However, there are always individuals within the existing natural population that are viruliferous even though their proportion can be occasionally very low, in particular during extreme hot and dry periods as in early May (chapter 1). The results of our studies show that a single viruliferous thrips can be sufficient to infect a tomato plant with the CaCV (chapter 1). Once infected, the virus spreads systemically in the tomato plant and typical symptoms are displayed within 10-21 days (chapters 1 & 4). However, at thrips feeding sites the virus can be locally detected, with DAS-ELISA, in less than five days post inoculation (i.e. before any appearance of the systematic symptoms) (chapters 1 & 2). Therefore, when a viruliferous female invades a two to three week-old uninfected juvenile tomato plant, the typical situation after transplanting the plants from the closed nursery to the greenhouses, it will start with feeding and subsequently virus transmission, but at the same time deposit its eggs within the leaf

tissue. After three days, the first white coloured larvae will hatch (the first generation) and start feeding within a few minutes (we observed a lag period of about 20 min only) on the same assumedly just infected leaf previously attacked by their own viruliferous mother. This is a crucial time for the virus epidemiology (chapter 1), because our results show that viruliferous adults of the thrips *C. claratris* can successfully inoculate the CaCV to the plants only when they had been feeding as first and early second instar larvae on infected plants (chapter 1). Late second instar larvae and adults did not develop to viruliferous thrips after commencing feeding on infected leaves at that older age (chapter 1). Though, the majority of viruliferous thrips will start to further inoculate and spread the CaCV not before emergence to adults, only about 20% of the thrips are able to start virus inoculation as late second instar larvae and continue to vector the virus after emergence as new adults (chapter 1). Adults are most important for the epidemiology of the disease (chapter 4). Once viruliferous an adult is a potential transmitter throughout its lifespan (at 30°C, 7 and 10 days for females and males, respectively) (chapter 1). Our findings concerning the vector biology of the CaCV and the thrips *C. claratris* is corroborating all other described relationships between any other tospovirus and its specific thrips vector (see the introduction part). The pioneer work of Moritz et al. (2005) provided anatomical evidences that the temporary association between the thrips midgut and the salivary gland, as a result of brain displacement into the prothoracic region during the early developmental stages of the thrips is facilitating the spread of the virus from the midgut to the salivary gland. This explains why only the first and early second larva can acquire then transmit the virus. As thrips develop this association will be lost and hence no virus particles are able to reach the salivary gland furthermore, and this explains why late second larvae and adults can not transmit the virus after acquiring it.

These anatomical peculiarities during development can explain why young larvae have to feed on infected plants in order to develop into successful vectors as adults. But why not all thrips individuals having fed during their sensitive acquisition period on virus infected plants were able to develop into transmitters? (chapters 1 & 2). Result of chapter 2 provided evidence that vector competence of *C. claratris* is not only related to the anatomical prerequisites during development but other factor

(factors) that show variability within the population and are genetically determined as a recessive trait (traits). The genetically based determination of vector competence of the thrips should be considered as a key factor in the tospovirus-thrips interaction studies. For instance effects of genetic drift within thrips metapopulations related to spatial distribution of pioneers responsible for infestation of new crops could result in very different risk potentials of spreading subpopulations. Hence, more research is needed to further elucidate the role of the thrips genetics in determining the vector competence in other economically more important vector thrips (e.g. the western flower thrips, *Frankliniella occidentalis,* for which species to date nothing has been reported about the genetic background of vector competence).

Transmission efficiency of thrips, as it is conventionally measured using leaf disc (Wijkamp and Peters, 1993; Premachandra et al., 2005) or leaf assays (chapters 1 & 2), depends on many factors. First, as mentioned above the acquisition of the virus from an infected leaf requires thrips in a specific developmental stage and second, the fitting genetic constitution of each individual larva (chapter 2). Third, the feeding behaviour of the thrips is assumed to be crucial causing a sex biased transmission efficiency. Males of the western flower thrips, *F. occidentalis* were assessed more efficient in transmitting the *Tomato spotted wilt virus* (TSWV) than females, because males make more frequent punctures but ingest less of the plant cellular contents compared to females. Thus shortly punctured cells could recover to almost intact cells giving convenient conditions to support virus survival and propagation after transmission. Females on the contrary puncture the leaf less frequent but remain longer at a distinct feeding site and ingest much more of the cell contents, leaving behind complete collapsed or empty cells (Van de Wetering et al., 1998). Corroborating these findings males of *C. claratris* feed considerably less than females and consequently they destroy less leaf area (chapter 3). However, in contrast (or unawares), our crossing experiments with individuals of *C. claratris* showed that males are less efficient transmitters than females (chapter 2) even those males which were genotypically expected to transmit the CaCV. This indicates that although vector competence is may be under simple genetic control, the expression of this trait is far more complex and is determined by a more complex interaction between the tospovirus and the thrips as well as the host plant. Unfortunately we do

not know any details about the way of virus particles after ingestion in the alimentary tract especially with non-viruliferous genotypes. Based on the above discussed findings we suggest further studies that aim to follow the route and tissue preference of the virus within the different tissues of the thrips, both potentially transmitter and non-transmitter genotypes. This may allow us to identify more of the determinant factors of vector competence.

If we assume that the trait vector competence is inherited in a recessive manner, what may be the sense behind it in unawares evolutionary terms? A possible hypothesis can be that, a recessive trait is a way to exclude the virus from the thrips population. But this would consider the virus as a kind of parasite for the thrips. Our results, though, do not strongly support such a theory but cannot principally exclude it (chapter 3). The problem is the difficulty to separate experimentally between direct negative effects of the CaCV on the thrips from those resulting from the infection of the host plant thus a food chain effect (chapter 3). Further work is certainly needed to clarify the symbiotic nature between the tospovirus and their thrips vectors. We suggest to establish subpopulations of specific thrips genotypes (i.e. transmitters vs. non-transmitters), which will facilitate further studies aiming to elucidate the effects of the virus on its vector as well as the effect of the host plant infection status on the thrips.

Regarding the population dynamics of *C. claratris*, in the new established tomato crop population build up in a tropical greenhouse will start immediately after initial colonization (immigration or artificial introduction) of a few founder thrips (chapter 4). Viruliferous individuals will then infect the plants upon which they are feeding with the CaCV and establish first hot spots of virus infection in the host plant population. With time and increasing thrips population the quality of the infected plant will continuously deteriorate, which is clearly demonstrated by stunting, smaller leaves and typical tospovirus symptoms. Decreasing food quality of the host plant will trigger spread of the thrips and subsequently the CaCV. Thinking in terms of trade-off (selection pressure) it could be assumed that selection of a virus free host plant is a win-win situation for both the virus regarding spread and thrips considering nutritional quality at least for short time (fast cycle) development (chapter 3): For the virus selection of already infected plants would focus the infection to a limited

number of plants and limit virus spread. From thrips point of view a viruliferous female of *C. claratris* choosing an infected leaf, will have to endure inferior food quality, manifested by lower fecundity (chapter 3), moreover its offspring will encounter inferior food quality too, and higher trichome density. The later is potentially able to reduce the surviving number of larvae by 20% (chapter 3). In addition, the food for the larva is important because it can affect the fecundity of the later resultant females (chapter 3). Females who were feeding as larvae on uninfected plants were fitter (as measured by daily fecundity and feeding activity) than those who were feeding as larvae on infected leaves (chapter 3). However, it is important to stress that the negative effects on the females that were feeding as larvae on infected leaves (exposed) are only apparent when females continue feeding on the infected leaves. Whereas, these effects can be reversed if the exposed females are leaving infected leaves and colonizing uninfected ones (chapter 3). This indicates that the food ecology of the thrips is important for the epidemiology of the tospoviral diseases, which merits further research.

From the herein presented results, there is no doubt that the tomato plant is a perfect double host for both the CaCV and the thrips *C. claratris*. However, the plant can clearly contribute to other important factors that affect the virus epidemiology as well as the vector competence. As seen in chapter 4, the development stages of the plant can affect the orientation or attraction of the thrips, as more thrips will fly towards the bigger plant, and as the thrips feed less on the younger leaves than the older leaves. The later is especially interesting as it demonstrates the potential of the host plant to defend its younger developing parts as well as its sensitive young developing stages, and this worth further studies. Eventually, the ratio of infected plants will be affected. The plant as a suitable host for both the virus and the thrips is important for the epidemiology of the pathogen (the virus) because an unsuitable virus host will certainly lead to a dead end of the tospovirus by ending its infection cycle. For instance, the eggplant, *Solanum melongena*, is a suitable host for the thrips *C. claratris* that attracts and then supports very high population build up similar to that on the tomato plants. Yet, we could not detect the CaCV using the DAS-ELISA within its leaves (Steenken, 2007). Therefore we recommend the compatibility of the

virus-thrips-plant system in experiments that are intended to study the interrelationships between those three organisms.

In conclusion, the tospovirus-thrips-plant system is indeed a complex biological system in which many factors are interacting. One major finding of the study presented here is the heritability of the trait vector competence by the thrips *C. claratris* which first of all strongly recommends to consider thrips genotype more in future multitrophic interaction research. However the mere assessment of vector competence heritability as a recessive trait is far from explaining the mechanism leading finally to the vector competent phenotype. Important factors like thrips sex, feeding behaviour, plant/leaf age and host suitability as well as the many other factors that are yet to be identified should be compiled for a complete picture. We think that only considering these multiple factor system and exploiting the vector competent genotype will help to further improve our understandings of the tospovirus-thrips-plant interaction.

Appendix

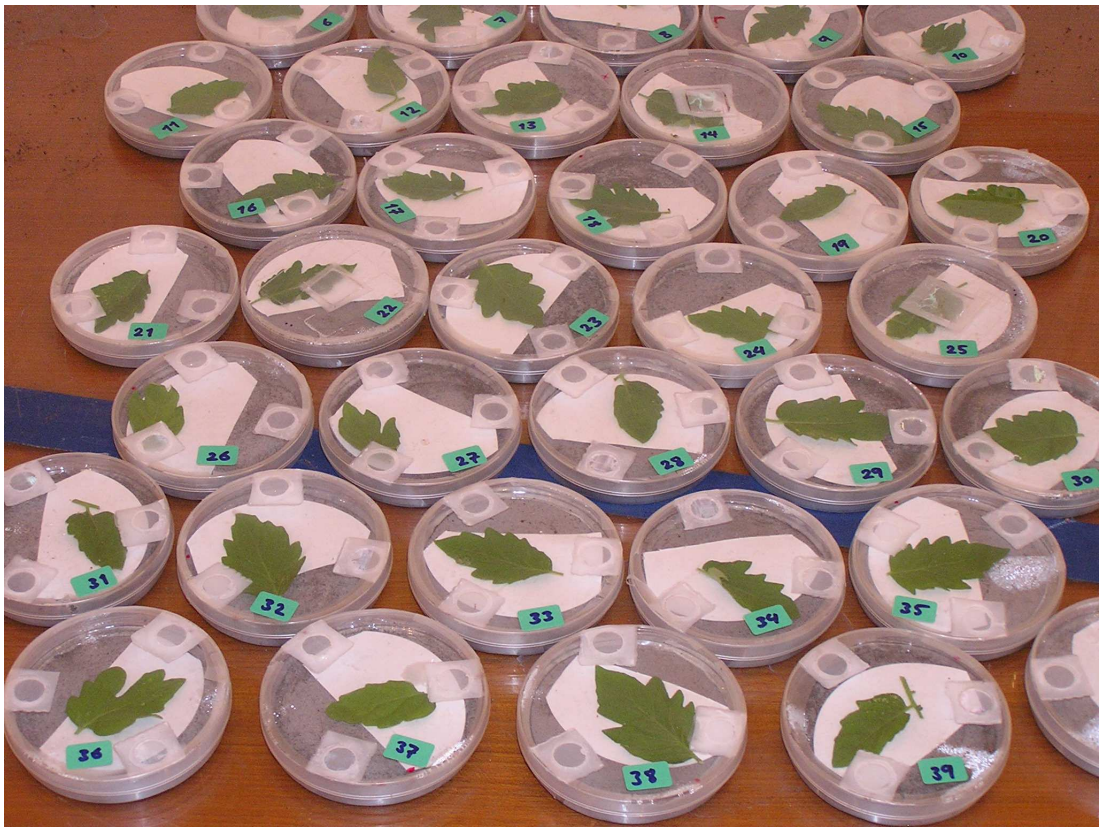

Fig.1. the leaflet assay used to test vector competence of individual thrips.

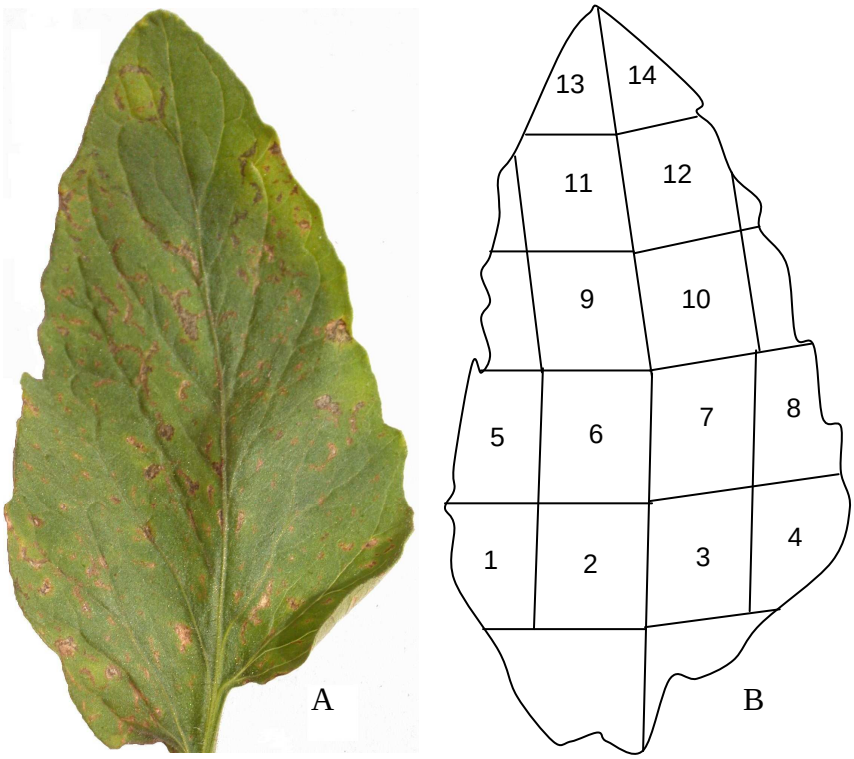

Fig.2. CaCV-infected tomato leaflet showing clear tospovirus symptoms of chlorosis, necrosis and necrotic rings (A). A schematic image of the same leaf is showing how it was sectioned into smaller parts of around 1 cm^2 (B). Each of the numbered sections was used to measure the CaCV titre by DAS-ELISA.

Table 1: Average readings of DAS-ELISA after 60min reaction

section	Readings
Blank	0.10
Uninfected leaf (negative control)	0.11
Infected leaf (positive control)	> 3.0
1	> 3.0
2	> 3.0
3	> 3.0
4	> 3.0
5	> 3.0
6	> 3.0
7	> 3.0
8	> 3.0
9	> 3.0
10	> 3.0
11	2.66
12	> 3.0
13	2.89
14	2.65

The CaCV infected tomato leaflet was divided to small sections of around 1 cm^2, as shown above, before each section was tested separately with the DAS-ELISA.

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Declaration by candidate

I, Nasser Halaweh, declare that this thesis, entitled ***"Ceratothripoides claratris*, Capsicum chlorosis virus and *Solanum lycopersicum*: a Case study of Thrips-Tospovirus-Plant Interaction"** is an original piece of work conducted by myself and has not been submitted for a degree in any other university.

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