Factors controlling virus-vector-host plant interactions: The model system *Frankliniella occidentalis* and *Tomato spotted wilt virus*

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ABSTRACT

Tospovirus-vector-host plant interaction is complex and very specific leading to great economic losses globally. Consequently, the interaction has garnered major attention within the scientific community in the recent past. Together with the worldwide distribution and the broad host-range, tospoviruses have fascinating biology and are also reported to manipulate their thrips vectors, thus challenging the management of both the viruses and the vectors. Additionally, under natural conditions, thrips populations exhibit high variability in the transmission efficiency of tospoviruses. This intraspecific variation has been related to the rapid co-evolution between thrips and tospoviruses. Several factors, either phenotypic or genetic, have been linked to this instability, thus, making it a very delicate and intricate area of research. These factors include: virus isolate, environmental conditions, host plant age, vector gender, individual vector genetics, among others.

A number of interaction studies have been carried out to try to broaden the understanding of this complex relationship, however, their main shortcoming is the missing information concerning the intrinsic potential of individual thrips to serve as efficient vector, both at phenotypic as well as genotypic level. Therefore, using the model system: *Frankliniella occidentalis*, *Tomato spotted wilt virus* (TSWV) (Tospovirus) and their shared host plant, *Capsicum annuum*, we evaluated different relationships in the triangle virus-vector-host plant, presuming that the multiplication and dispersal of TSWV in a crop stand strongly depends on mutualistic effects, individual vector genetics and vector gender. Controlled micro-cosmos conditions and leaf disk assay (one individual *F. occidentalis* per leaf disk) were used in all the experimental settings to first unravel this complex biological system, which can then be later up-scaled to larger systems. Our main findings were: (a) Analysis of genetic composition of individual vectors revealed that the trait vector competence is heritable in a haplodiploid pattern in *F. occidentalis* and it is linked to a recessive allele, with males only able to inherit the trait from their mothers. Evaluation of the expected allele frequencies showed that the recessive allele for the trait vector competence had the highest frequency in a population at any given time. (b) Transmission efficiency is male biased due to their shorter development time, higher survival, and shallower feeding behaviour compared to females, which leads to higher transmission efficiency. (c) A switch in diet status (i.e. +/-TSWV) during vector development, resulted in different influences of the virus on the vector, which we assumed to be the manipulation of *F. occidentalis* by TSWV to favour its multiplication and dispersal in a crop stand. (d) Using a mathematical model for the prediction of disease spread, we were able to

quantify the influences of the virus induced vector life processes on the virus propagation dynamics in the field. (e) Proteomics analysis of the differentially expressed proteins in *F. occidentalis* after exposure to TSWV showed that proteins associated with the innate immune system of the vector were abundantly increased in response to viral infection which corresponds to the reported improved fitness and performance of the vector after exposure to the virus.
From a basic viewpoint, these findings are fundamental for a deeper understanding of the evolutionary interaction of virus-vector-host plant, and an essential basis for further molecular genetic studies of the trait vector competence. From an application point of view, the development of vector–based models could further help in elucidating the role of tri-trophic interactions in such complex disease systems, and stimulate the development of efficient control strategies for both the virus and the vector.

Key words: *Frankliniella occidentalis*, *Tospovirus*, *Tomato spotted wilt virus*, vector competence, haplodiploidy, intraspecific variation, predictive models, vector manipulation hypothesis, innate immunity.

ZUSAMMENFASSUNG

Interaktionen zwischen Tospoviren, Vektoren und Wirtspflanzen sind komplex und sehr spezifisch und führen weltweit zu hohen wirtschaftlichen Verlusten. Infolgedessen haben diese Interaktionen in den letzten Jahren vermehrt Beachtung in der wissenschaftlichen Gemeinschaft auf sich gezogen. Tospoviren haben nicht nur eine weite Verbreitung und ein großes Wirtsspektrum, sondern auch eine faszinierende Biologie. Es gibt zunehmend Hinweise, dass sie ihre Vektoren (Thripse) in komplexer Art und Weise beeinflussen. Damit wird das Management von Virus und Vektor zu einer großen Herausforderung. Außerdem weisen Thripspopulationen eine hohe Variabilität bei der Transmissionseffizienz von Tospoviren auf. Diese intraspezifische Variabilität wird mit der schnellen Koevolution von Thripsen und Tospoviren in Verbindung gebracht. Auch werden verschiedene phänotypische oder genetische Faktoren für Schwankungen im Vektorpotential verantwortlich gemacht, so daß Kausalanalysen der zugrunde liegenden Meanismen sehr anspruchsvoll werden. Zu den wesentlichen Variabilitätsfaktoren gehören unter anderem Virusisolat, Wirtspflanzenalter, Umweltbedingungen, Geschlecht des Vektors und die individuelle Genetik des Vektors.

Es wurden bereits mehrere Studien zu diesen Interaktionen durchgeführt, um deren Verständnis zu vertiefen, jedoch war bislang eine Schwäche dieser Studien, dass Informationen zu dem intrinsischen Potential von individuellen Thripsen, sowohl auf phänotypischer als auch auf genetischer Ebene als effizienter Vektor zu fungieren, fehlten. Daher wurden in der vorliegenden Arbeit unter Verwendung des Modelsystems *Frankliniella occidentalis*, *Tomato spotted wilt virus* (TSWV) (Tospovirus) und der gemeinsamen Wirtspflanze *Capsicum annuum* verschiedene Beziehungen in dem Dreieck Virus-Vektor-Wirtspflanze unter sucht. Zugrunde lag die Annahme, dass die Vermehrung und Dispersion von TSWV in einem Pflanzenbestand von mutualistischen Effekten, individueller Vektorgenetik und Geschlecht des Vektors abhängt. Bei allen Versuchen wurden Mikrokosmen und Blattscheiben-Assays (ein Individuum von *F. occidentalis* pro Blattscheibe) unter kontrollierten Bedingungen verwendet, um zunächst Basisdaten in diesem komplexen biologische System zu gewinnen, die dann später auf größere Systeme hochskaliert werden können.

Die wichtigsten Ergebnisse waren: (a) Genetische Untersuchungen an einzelnen Vektorindividuen zeigte, dass das Merkmal Vektorkompetenz bei *F. occidentalis* in einem haplodiploiden System vererbt wird und an ein rezessives Allel gebunden ist, wobei Männchen dieses Merkmal nur von Weibchen erben können. Die Berechnung der erwarteten Allelfrequenzen für Populationen zeigte, dass das rezessive Allel für das Merkmal

Vektorkompetenz die höchste Frequenz in einer Population zu jeder beliebigen Zeit hatte. (b) Männliche Thripse haben im Vergleich zu Weibchen eine erhöhte Transmissionseffizienz, was auf deren kürzere Entwicklungszeit, die höhere Überlebensrate und die geschlechtspezifische Ausprägung der Nahrungsaufnahme mit kürzerern und weniger destruktiven Saugtätigkeiten im Vergleich zu Weibchen zu begründen ist. (c) Ein Wechsel der Nahrungssituation (d. h. +/- TSWV) während der Entwicklung des Vektors führte zu Veränderungen in wichtigen biologischen Parameteren des Vektors, die nur als gezielte Manipulation von *F. occidentalis* durch TSWV gedeutet werden können, um dessen Verbreitung und Dispersion im Pflanzenbestand zu begünstigen. (d) Anhand eines mathematischen Modells zur Vorhersage der Krankheitsausbreitung konnten wir die Einflüsse der Virus-induzierten Ausprägungen in der Vektorbiologie auf die Dynamik der Virusausbreitung im Freiland quantifizieren. (e) Eine Proteomanalyse der unterschiedlich exprimierten Proteine von *F. occidentalis* nach Kontakt mit TSWV zeigte, dass als Antwort auf eine Virusinfektion, Immunproteine des Vektors exprimiert wurden, was mit der erhöhten Fitness und Leistungsfähigkeit des Vektors nach Viruskontakt korrespondierte.

Aus grundlegender Sicht können diese Ergebnisse als Basis für ein tieferes Verständnis der evolutionären Interaktion von Virus-Vektor-Wirtspflanze dienen und sind eine essentielle Basis für weitere molekularbiologische Untersuchungen zur Vektorkompetenz. Von einem angewandten Standpunkt aus betrachtet kann die Entwicklung von Vektor-basierten Modellen weiter helfen, die Rolle von tritrophischen Interaktionen in komplexen Systemen aufzuklären und die Entwicklung von effizienten Bekämpfungsstrategien für Viren und deren Vektoren zu stimulieren.

Stichworte: *Frankliniella occidentalis*, *Tospovirus*, *Tomato spotted wilt virus*, Vektorkompetenz, Haplodiploidie, intraspezifische Variation, Vorhersagemodelle, vector manipulation hypothesis, angeborene Immunität

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ABBREVIATIONS

% RH	Relative Humidity
°C	Degree Celsius
Ø	Diameter
2D-IEF/SDS/PAGE	Two-dimensional isoelectric focusing/sodium dodecyl sulfate/polyacrylamide gel electrophoresis
AFLP	Amplified fragment length polymorphism
ATP	Adenosine triphosphate
AAP	Acquisition Access Period
AMP	Antimicrobial peptides
ANOVA	Analysis of Variance
c	Recessive allele for the trait vector competence
C	Dorminant allele for the trait vector competence
$CaSO_4$	Calcium Sulphate (gypsum)
cDNA	Complementary Deoxyribonucleic acid
cm	Centimeter
CMA	Chaperone-mediated autophagy
DAS-ELISA	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay
DI	Defective interference
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - German Collection of Microorganisms and Cell Cultures
dsRNA	Double Stranded RNA
Gn	Glycoprotein amino-terminal
Gc	Glycoprotein carboxyl-terminal
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EPG	Electrical Penetration Graph
F0	Parental Generation
F1	First Filial Generation
F2	Second Filial Generation

Fo2	*Frankliniella occidentalis* Netherlands strain 2
Hsc	Heat shock proteins
HWE	Hardy Weinberg Equilibrium
IAP	Inoculation Access Period
IgG	Immunoglobulin G antibody
IgG AP	Immunoglobulin G Antibody Alkaline phosphatase
IFN	Interferon
IkB	NF-kB Inhibitor
IKK	IkB Kinase
IPP	Institute of Plant diseases and Plant Protection
JA	Jasmonic Acid Pathway
kb	Kilo base
kDa	Kilo Dalton
L.	Linnaeus
L1 /LI	First Instar Larva
L2/LII	Second Instar Larva
L: D	Light: Dark photoperiod
L-RNA	Long Ribonucleic Acid Segment
$MgCl_2$	Magnesium Chloride
miRNAs	MicroRNAs
M-RNA	Medium Ribonucleic Acid Segment
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
N	Nucleocapsid Protein
N12	TSWV isolate, Netherlands 2012
NF-kB	Nuclear factor Kappa-light-chain-enhancer of activated B cells
NL	Non-linear
NS	Non Structural Proteins
nt	Nucleotide
OD	Optical Density
ORF	Open Reading Frame
PAMPs	Pathogen Associated Molecular Patterns
PBS-TPO	Phosphate Buffer Saline-Tween- Polyvinylpyrrolidone Egg albumine

PBS-T	Phosphate Buffer Saline-Tween
piRNAs	Piwi Interacting RNAs
pNPP	para-Nitrophenyl phosphate
PRR	Pattern Recognition Receptors
PVP	Polyvinylpyrrolidone
RAPD	Random Amplification of Polymorphic DNA
RNA	Ribonucleic Acid
RNPs	Ribonucleoproteins
rpm	Revolutions per Minute
RdRp	RNA dependent RNA polymerase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SA	Salicylic Acid Pathway
SAS	Statistical Analysis System
siRNAs	Small Interfering RNAs
SPSS	Statistical Package for Social Sciences
S-RNA	Small Ribonucleic Acid Segment
SSR	Simple sequence repeats
ssRNAs	Single stranded RNAs
TLRs	Toll-like Receptors
v	Viral sense
vc	Viral complementary sense
VCIP135	Valosin-containing interacting protein 135
VSV -G	*Vesicular stomatitis virus* glycoprotein
WFT	Western Flower Thrips
W/V	Weight per Volume

Viruses Acronyms

BYDV	*Barley yellow dwarf virus*
CaCV	*Capsicum chlorosis virus*
CHMV	*Human cytomegalovirus*
CNSV	*Chrysanthemum stem necrosis virus*
GRSV	*Groundnut ringspot virus*
HSV-1	*Herpes simplex virus* 1
INSV	*Impatiens necrotic spot virus*
M-MLV	*Moloney Murine Leukemia Virus*
MMTV	*Mouse mammary tumor virus*
MV	*Measles virus*
PLRV	*Potato leafroll virus*
TCSV	*Tomato chlorotic spot virus*
TSWV	*Tomato spotted wilt virus*
TYLCV	*Tomato yellow leaf curl virus*
VSV	*Vesicular stomatitis virus*

1 General introduction

1.1 *Tomato spotted wilt virus*

1.1.1 Taxonomy, distribution and economic importance

Family *Bunyaviridae* is the largest family consisting of viruses that are mainly amplified in vertebrate hosts and are vectored by arthropods or rodents. It is made up of 5 main genera; *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus* and *Tospovirus* (Nichol *et al.*, 2005a). Only *Tospovirus* genus is plant infecting, and it is named after *Tomato spotted wilt virus* (TSWV) as the type species (Elliott, 1997; van de Wetering *et al.*, 1999a; Whitfield *et al.*, 2005). Tospoviruses make only a very small proportion out of more than 300 species of primarily arthropod-borne viruses described in the family *Bunyaviridae*.

Disease attributed to tomato spotted wilt was first described in 1915 in Australia. It was later found to be caused by a virus and transmitted by thrips (Pittman, 1927), thus it was named *Tomato spotted wilt virus* (Samuel *et al.*, 1930). The uniqueness of TSWV was first realized in 1990, prior to that, TSWV was the only plant virus assigned to genus *Tospovirus*. *Tospovirus* is now recognized as a group consisting of several different viruses rather than monospecific genus as earlier thought (German *et al.*, 1992). All viruses of this genus are distinguished on the basis of N protein RNA sequences, N protein serology and vector specificity (de Ávila *et al.*, 1990; de Ávila *et al.*, 1993; Goldbach and Kuo, 1996). Recognition of viruses as new *tospovirus* species is only possible in cases where the N protein sequence shows less than 90% homology to other established *tospovirus* species (Goldbach and Kuo, 1996). So far, 16 species have been identified based on this criteria (Fauquet *et al.*, 2005; King *et al.*, 2011). There is no serological relationship between tospoviruses and the other genera in the family *Bunyaviridae* on a higher taxonomic level. However, a minimum but significant level of amino acid sequence homology is reported for the glycoproteins precursor and for the RNA polymerase between TSWV and members of the genus *Orthobunyavirus* (Kormelink *et al.*, 1992; Cortês *et al.*, 2002; Haan *et al.*, 1991).

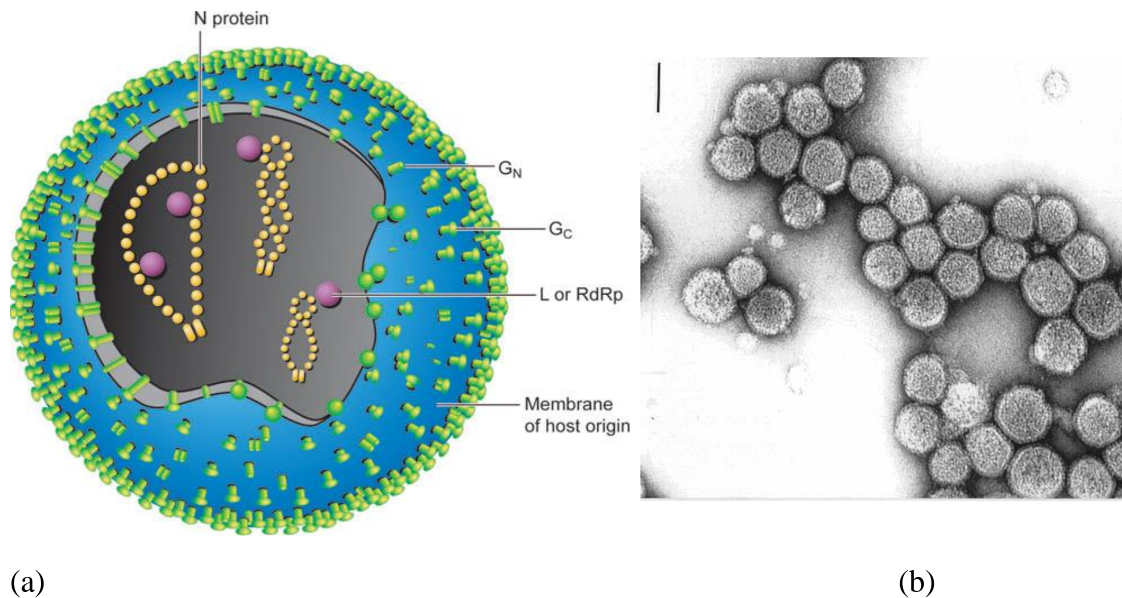
TSWV has a wide host range, infecting more than 1,000 plant species from over 85 families that include numerous crops and weeds (Stumpf and Kennedy, 2007; Chatzivassiliou *et al.*, 2002; Parrella *et al.*, 2003) mainly vectored by *F. occidentalis* (Wijkamp *et al.*, 1995; Whitfield *et al.*, 2005), leading to serious economic losses worldwide (Goldbach and Peters, 1996). Most of the plants are also host for the vectors, and thus serve as reservoirs of infection that contribute

to epidemics in crop plants (Stobbs *et al.*, 1992). By 1994, worldwide yield losses caused by this virus were roughly estimated to be in excess of US$1 billion annually (Pappu, 1997; Goldbach and Peters, 1994). Thus, TSWV remains one of the top 10 most economically destructive and scientifically challenging plant virus worldwide (Scholthof *et al.*, 2011).

TSWV is prevalent in warm climates, in regions with high population of convenient thrips vectors. In relation to increasing thrips occurrence as an agricultural pest in Asia, America, Europe and Africa, over the past 15 years, resurgence of TSWV have become more prevalent in these regions, hence, it's economic and scientific importance (Mumford *et al.*, 1996b; Sherwood *et al.*, 2000; Parrella *et al.*, 2003). Therefore, the increased prevalence is largely because of the successful distribution of the predominant thrips vector *Frankliniella occidentalis* (Pergande) (Kirk and Terry, 2003; Mound and Lewis, 1997), (See Fig. 1.4 for the distribution of *F. occidentalis* in Europe).

1.1.2 Molecular Structure

The morphology and organization of tospoviruses share several features typical of members of the family *Bunyaviridae*. Virions are spherical lipid-bound membrane particles, 80-120 nm in diameter, covered with surface projections of about 5-10 nm in length, consisting of two glycoproteins Gn and Gc (according to amino and carboxyl terminals respectively; location within the precursor protein) (Fig. 1.1a), which form the integral part of the membrane and can be observed in electron micrographs (Fig. 1.1b) (Mohamed, 1981; Tas *et al.*, 1977). The virion consists of four structural proteins which include, a putative 330.5 kDa viral polymerase (L), a 29 kDa nucleocapsid protein (N) and the already mentioned glycoproteins Gn and Gc; 78 kDa and 58 kDa respectively (Mohamed, 1981) (Fig. 1.1a). The N proteins are tightly bound to RNA within the particles and form pseudocircular nucleocapsid structures attributable to noncovalent bonding of the complementary RNA termini.



(a) (b)

Figure 1.1: Molecular structure of TSWV. (a) Schematic representation of TSWV virion. Graphic design by D.E. Ullman and E. Rendahl (Hogenhout *et al.*, 2008) (b) Electron micrograph of purified TSWV particles. Size bar indicates 100 nm. TS (Snippe *et al.*, 2007)

Furthermore, virus of the family *Bunyaviridae* are recognized by their tripartite genome consisting of single stranded linear RNA fragments, all held together in a membrane like envelope. The three RNA strands in the virus genome are called large (L), medium (M) and small (S), according to their sizes, i.e. 8897 nucleotides (nt), 4821 nt, and 2918 nt respectively (Fig. 1.2), and together contain 5 open reading frames (ORF) (Haan *et al.*, 1991; Kormelink *et al.*, 1992). The negative sense L-RNA strand contains one large ORF which encodes for the 331.5 KDa RNA-dependent RNA-polymerase (RdRp) (Haan *et al.*, 1990; Mohamed, 1981; Chapman *et al.*, 2003; van Poelwijk *et al.*, 1993) which is necessary for replication. The ambisense M-RNA encodes in the viral (v) sense, a nonstructural protein (NSm) of 33.6 KDa, which is implicated in cell-to-cell movement (Kormelink *et al.*, 1994; Storms *et al.*, 1995; Storms *et al.*, 1998), and in the viral complementary (vc) sense, a 127.4 KDa protein which is the precursor to the glycoproteins (Gn and Gc) which constitute the envelope. The ambisense S-RNA encodes a nonstructural (NSs) protein of 52.4 KDa in the v sense, which has been found to be a suppressor of RNA silencing (Bucher *et al.*, 2003; Ananthakrishnan and Annadurai, 2007; Takeda *et al.*, 2002), and a nucleoprotein (N) of 28.8 KDa in the vc sense (Haan *et al.*, 1990; Kormelink *et al.*, 1991), which form part of ribonucleoproteins (RNPs), serve as the structural protein, and may also have some regulatory role in modulating the transcription and replication. All members of *Bunyaviridae*, including tospoviruses, replicate in the cytoplasm of the host cell (Whitfield *et al.*, 2005; Moyer, 1999; Pappu *et al.*, 2009).

Figure 1.2: A schematic representation of genomic organization of the TSWV. This organization applies to all members of *Tospovirus* genus studied so far. L, M and S represent the three ssRNAs genomic segments of the virus. Graphic design by P. A. Ogada, 2016.

The membrane glycoproteins Gn and Gc, are essential for acquisition and transmission by the vector thrips. During acquisition, Gc binds to the gut of the vector. Consequently, TSWV acquisition by thrips vector is inhibited by the absence of Gc, suggesting that this protein may act as the viral ligand that mediates the attachment to receptors in the midgut epithelium of the vector (Whitfield *et al.*, 2004). Therefore, only enveloped virus particles are transmissible by thrips (Nagata *et al.*, 2000; Resende *et al.*, 1991; Wijkamp *et al.*, 1995).

Serial mechanical passages of TSWV on host plants at high inoculum concentration and low temperature regimes, have been reported to result in defective interference (DI) leading to RNA molecules of sub-genomic lengths, derived from the L RNA (Inoue-Nagata *et al.*, 1997; Resende *et al.*, 1991). These defective RNAs have been shown to interfere with the replication of the wild type genome (Resende *et al.*, 1991), causing expression of attenuated symptoms. The mode by which the internal deletions are generated is still unknown. The presence of the DI RNAs is also reported to affect negatively the transmission of TSWV by the thrips vector (Wijkamp *et al.*, 1995; Nagata *et al.*, 2000).

1.1.3 TSWV infection and detection

In nature, TSWV is transmitted by several thrips species (Sherwood *et al.*, 2000; Whitfield *et al.*, 2005) it is also mechanically transmissible, but not seed transmissible. Infections and symptoms of TSWV vary across plant species (German *et al.*, 1992; Goldbach and Peters, 1996; Mandal *et al.*, 2007; Roselló *et al.*, 1996), within the same host plant species under varying environmental conditions (Díaz-Péreza *et al.*, 2007; Llamas-llamas *et al.*, 1998) and specifically according to the plant age at the time of infection (Mandal *et al.*, 2007; Soler *et al.*, 1998). Also, biologically distinct isolates of TSWV exist in nature, and they differ in thrips transmissibility,

host range, symptomatology and symptom severity. Two types of host plants are known; those characterized by localized sites of infection in tissues inoculated by the vector and the other type is called a systemic host, in which the virus spreads systemically from the inoculation site to other parts of the plant (Persley *et al.*, 2006; 2007). General symptoms include chlorosis (yellowing) of leaves and subsequently necrotic local lesions on the leaf sites that thrips "injected" the virus, ring patterns (Fig. 1.3a), bronzing/silvering and curling of terminal shoots (Fig. 1.3b), stunting and near cessation of plant growth, and distortion of affected plant areas (Mandal *et al.*, 2007). In most of the crops affected, TSWV causes systemic infection (Fig. 1.3c). In addition, infected fruits are misshapen and ripen unevenly, often with a necrotic ring pattern, therefore, unmarketable with striking chlorotic/necrotic ringspots that often appear only when the fruits reach maturity (Chiemsombat and Adkins, 2006).

The main detection methods used for TSWV include; visual observation, serological methods e.g. ELISA, and nucleic-acid based methods e.g RT-PCR (Ogada, 2011).

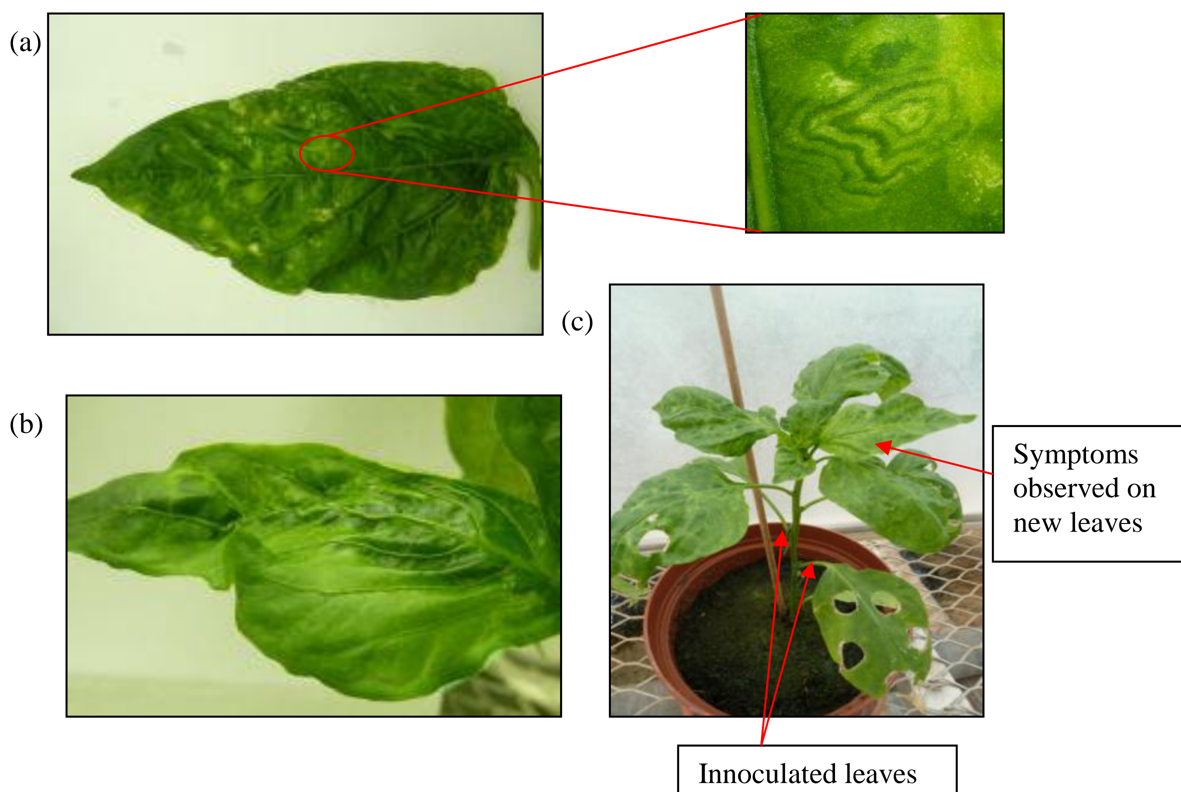


Figure 1.3: Observed symptoms of TSWV; (a) Chlorosis and mosaic patterns with necrotic ring spots on infected leaf, (b) Deformity or mottling of leaves, (c) Systemic infection of the TSWV on *C. annuum*.

1.2 Thrips as vectors of TSWV

1.2.1 Origin, distribution and economic importance

The order *Thysanoptera* (fringed-winged insects, their wings consisting of fine hairs rather than a membrane) and family *Thripidae* encompasses about 7,700 described species (CSIRO Australia, 2005), of which about 1% are recorded as serious pests (Lewis, 1997). A few genera and species are very successful invaders which now occur worldwide on a broad diversity of economically important crops (Morse and Hoddle, 2006). The genus *Frankliniella*, is the second largest in the family *Thripidae* with about 160 species, and is predominantly American (Kirk, 2002). Overtime, these species have expanded their geographical range to almost all the subtropical and temperate climate zones in all continents of the world (Kirk and Terry, 2003; Mound, 2005). *F. occidentalis* is one of the most damaging thrips pest in this order, having a very broad host range, infesting hundreds of plant species from more than 60 families (Jones, 2005), causing enormous annual economic losses both by direct damages primarily through actual or potential reduction in crop market values by its special kind of feeding. Additionally, actual yield reduction results from its ability to efficiently vector five tospoviruses; *Groundnut ringspot virus* (GRSV), *Impatiens necrotic spot virus* (INSV), *Tomato chlorotic spot virus* (TCSV), *Chrysanthemum stem necrosis virus* (CNSV), and in particular the *Tomato spotted wilt virus* (TSWV) (Jones, 2005; Persley *et al.*, 2006; Kirk, 2002). Moreover, potential infestation by both TSWV and *F. occidentalis* is often very important from economical stand point, as it leads to quarantine restrictions that shut out competitors from profitable markets (Mound, 2005).

F. occidentalis was first described in 1895 from specimens collected in California, USA where it was recorded as plentiful. In Europe it established first in greenhouses in the Netherlands in 1983. It was suspected to have come through immigration as a result of major imports and exports of infested plant materials between adjacent countries, and later spread over the whole western and northern Europe (Kirk and Terry, 2003) (Fig. 1.4). In northern Europe however, the *F. occidentalis* is restricted to glass houses and can only survive short periods outdoors because of its susceptibility to extremly cold temperatures (≤ 0 °C), whereas in southern Europe it has established in the field (Kirk and Terry, 2003; Brødsgaard, 1993; McDonald *et al.*, 1997). Furthermore, *F. occidentalis* is able to overwinter into protected micro-climates in soils and plant debris in their pupal stages, therefore this behavioral factor needs to be taken into account (Brødsgaard, 1993; McDonald *et al.*, 1997). The winter thrips populations are important for

maintaining TSWV in the resident weed plants populations. This sets the stage for TSWV epidemics in the following spring season (Riley *et al.*, 2009a).

Figure 1.4: Map indicating the years in which *F. occidentalis* was first recorded in the countries and larger islands of Europe and northern Africa, either in fields or greenhouses. (the prefix 19 has been omitted for the years due to space) (Kirk and Terry, 2003; Tommasini and Maini, 1995)

1.2.2 Biology of *F. occidentalis*

Immature *F. occidentalis* are cream to yellowish in color and adult females are either dark brown or yellow orange depending on the season. Males are generally paler and smaller (1 mm long) than the females (1.4 mm). Adults usually have narrow and fringed wings with long hairs (Parrella *et al.*, 2003; Mound and Lewis, 1997). Eggs are laid singly and usually inserted in plant tissue. The eggs hatch in 3-4 days, two larval stages last about 10-14 days, prepupa and pupa stages last around 5-7 days (in which the thrips are inactive, do not feed and are in the growing medium), and then they become adults (Fig. 1.5), at temperatures 25±2 °C, and 14:10 h L: D photoperiod.

F. occidentalis has arrhenotokous parthenogenesis reproduction system (Moritz, 1997) where females lay two kinds of eggs, fertilized eggs that have a diploid set of chromosomes which

develop to females, and unfertilized eggs with only one copy (i.e. haploid) of the mother's chromosomes producing only males (Crespi *et al.*, 1991; Hedrick and Parker, 1997). Thus, thrips can reproduce parthenogenetically, and if females remain unmated, all offspring produced are males. If thrips mate, they reproduce mostly female offspring but even inseminated females can deliver unfertilized eggs resulting into males (Moritz, 1997).

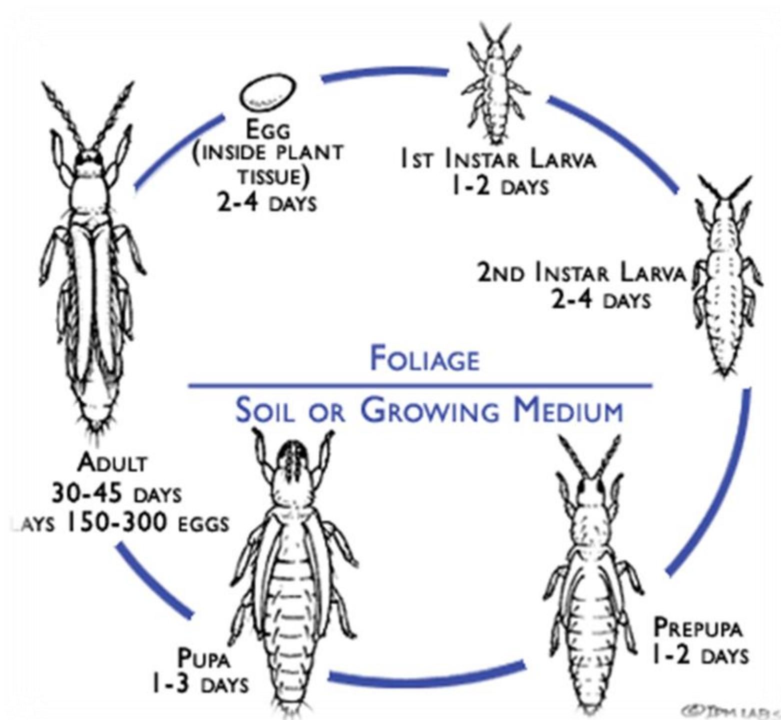


Figure 1.5: Life cycle of *F. occidentalis* (Zitter and Daughtrey, 1989)

1.2.3 Feeding

Thrips feed by piercing plant cells with their mouthparts and sucking out the contents (Harrewijn *et al.*, 1996), both adults and larvae feed this way, therefore contributing to plant damage. An initial opening is produced by penetrating the plant cuticle with their single mandible after rocking the head capsule downwards and upwards numerous times. With successful punching of an opening or food channel through the outer cell wall, a pair of maxillary stylets is then extended into the plant tissue. Saliva is injected into the plant and cellular contents are drawn up through a pumping action (Kindt, 2004). TSWV transmission occurs via saliva injection into the plant tissue during the adults feeding. Direct damage to the plant tissue can be caused by thrips feeding; however, the greatest loss to agriculture is caused by the species that vector (transmit) tospoviruses like TSWV. Since TSWV transmission occurs through thrips feeding, understanding factors that affect thrips feeding is critical for managing this pest-virus complex. Hence all possible factors affecting host preference and feeding

intensity such as host plant resistance, plant age, thrips gender, induced plant defense as well as application of chemicals (insecticides) can more or less influence virus spread (Ogada and Poehling, 2015; Kindt, 2004; Chaisuekul and Riley, 2005; Joost, 2003).

1.3 Plant - thrips – tospovirus interaction

The *Tospovirus*-thrips relationship is very specific and complex, like many insect vector/plant virus associations. Only seven among the many known thrips species are able to acquire and transmit viruses in this genus (German *et al.*, 1992). Transmission of TSWV by *F. occidentalis* is in a persistent and propagative manner (Sherwood *et al.*, 2000; Whitfield *et al.*, 2005), and only adult thrips that acquire the virus in their early larval stages are able to transmit. Hence, acquisition of the virus by the young larvae is one of the basic determinant for adult vector competence (de Assis Filho *et al.*, 2004; Ananthakrishnan and Annadurai, 2007; Ullman *et al.*, 1992; Wijkamp and Peters, 1993; van de Wetering *et al.*, 1996; Moritz *et al.*, 2004; Whitfield *et al.*, 2005; Hogenhout *et al.*, 2008). The aquisition by the young larvae favors internal virus transmission to the salivary glands (see below). Moreover, successful transmission of the virus by the adults requires heavy infection of the salivary glands during thrips development, thus the second important determinant of vector competence is the ability of a vector to multiply the virus internally (Nagata *et al.*, 2002; Nagata, Inoue-Nagata, *et al.*, 1999a) (Fig. 1.6). An increase in the viral protein in thrips vectors during their development is an indication of replication of TSWV in the thrips. TSWV initially infect the midgut, replicating first in the epithelium and then the muscle fibres surrounding the foregut and the midgut of the developing larvae. The virus then spreads via cell-to-cell movement to the salivary glands where it also replicates and from which it is transmitted to the plant during feeding (Ullman *et al.*, 1995; Ullman *et al.*, 1997; Nagata, Inoue-Nagata, *et al.*, 1999a; Nagata *et al.*, 2002; Moritz *et al.*, 2004). TSWV acquisition by the late larval stages and the adults support infections in the midgut tissues, but the virus does not spread to the salivary glands and thus, cannot be transmitted to plants (de Assis Filho *et al.*, 2002; de Assis Filho *et al.*, 2004). Therefore, vector competence, refers to the ability of a vector to acquire the virus, replicate it in the salivary gland to a substantial amount, and then successfully transmit it to a susceptible host plant. Additionally, TSWV has no trans-ovarial (vertical) transmission; therefore, each generation must re-acquire the virus for the disease epidemic to continue (Wijkamp *et al.*, 1995; van de Wetering *et al.*, 1996; Nagata, Carla, *et al.*, 1999). Once thrips acquire the virus, they remain viruliferous (infected with virus) throughout their life span (Riley *et al.*, 2009b).

The reason for the successful “loading” of the salivary glands with infectious virus particles only if young larvae acquire the virus has not yet been conclusively explained.

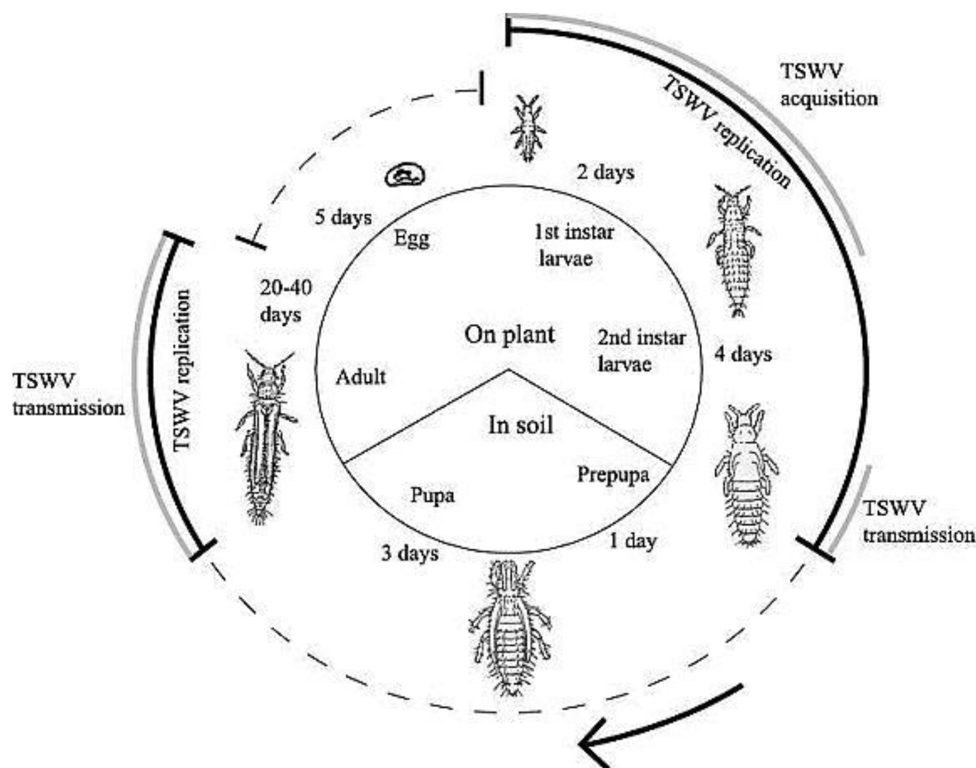


Figure 1.6: Life-cycle of *F. occidentalis* (inner circle) and the infection-cycle of *Tomato spotted wilt virus* (TSWV, outer circle) (Kindt, 2004)

Moritz *et al.*, (2004) related the pronounced changes in vector competence during the ontogenetic thrips development to the special morphological characteristics. They clearly showed a temporary association between the thrips midgut and the salivary gland, as a result of brain displacement into the prothoracic region, which occurs only during the first and early second larval stages, thus contingently facilitating the spread of the virus from the midgut to the salivary glands. Once having successfully passed this barrier and finally reached salivary gland cells, virus propagates further and is stored there until it is transmitted in a persistent-propagative manner partly by the second larval stage but mainly by the adults during feeding (Ullman *et al.*, 1993; Wijkamp, Vanlent, *et al.*, 1993; Whitfield *et al.*, 2005).

However, this pictures the virus transmission cycle in general and the stage specific acquisition but cannot explain the high individual variability in vector competence when same aged L1 thrips are subjected to the same virus source (infected plant), nor the dynamic change of relative amounts of vector competent individuals in populations of different sizes or isolation (inbreeding) (Halaweh and Poehling, 2009). Recent studies showed that the efficiencies of TSWV transmission were different among populations which can be separated by their ribosomal RNA (Gillings *et al.*, 1995; van de Wetering *et al.*, 1999a) and between sexes (Ogada

and Poehling, 2015; Sakurai *et al.*, 1998; van de Wetering *et al.*, 1999b). The variation in transmission efficiency may also result from different abilities of individuals to acquire and transmit the virus (van de Wetering *et al.*, 1999a) which could be influenced by factors like, virus induced changes in vector's life processes such as preferential behavior and survival, gender of the vector, individual vector genetics and virus isolate, among others. Additionally, first genetic studies of some virus-thrips-host plant systems have shown that the trait vector competence can been linked to a recessive gene and it is inherited in a haplodiploid manner (Halaweh and Poehling, 2009; Cabrera-La Rosa and Kennedy, 2007), with its allele frequency varying between males and females, and across generations (Hedrick and Parker, 1997; Crespi *et al.*, 1991). Immunochemical studies with non-competent thrips individuals showed that virus particles are always infecting the midgut and later surrounding muscle cells and propagate there but failed to move along the ligaments to the salivary glands (Nagata *et al.*, 2002). Information about such intraspecific variations is very important because they influence virus spread based on the composition of the vector population (Inoue and Sakurai, 2006).

TSWV replication in the midgut tissue of its thrips host suggests the likelihood for pathological effects that could affect the fitness and performance of TSWV vectors. However, consistent demonstration of such effects have proven difficult due to the complex virus-vector-host plant interaction (Stumpf and Kennedy, 2007). Earlier studies have reported improvement in fitness and performance of *F. occidentalis* after exposure to TSWV (Ogada *et al.*, 2013), which correlates to a presumed triggered immune system in the infected thrips (de Medeiros *et al.*, 2004) as the insect lack an adaptive immune system (Irving *et al.*, 2001), consequently leading to longer survival of the infected vector and thus promoting the virus spread. Moreover, several examples of arthropod behavior changing with pathogens infections have been reported (Thomas *et al.*, 2005). For example, varying reports are emerging on the effect of TSWV on the development rate, survival, and reproduction rate of its thrips vectors (Sakimura, 1963; Robb, 1989; Wijkamp *et al.*, 1996; Maris *et al.*, 2004; Ogada *et al.*, 2013; Shalileh *et al.*, 2016). These variations could be due to different TSWV isolates, host plants, thrips populations, as well as different experimental conditions and protocols involved in these studies (Stumpf and Kennedy, 2007). Interestingly, many of these studies have indicated a mutualistic relation between the vector and the virus, for example, TSWV benefits by modifying the preferential behavior and fitness of its vector, as well as improving the host plant quality for its vector (Shalileh *et al.*, 2016). This promotes TSWV multiplication and spread as the virus circulates and replicates in thrips larvae (Nagata, Inoue-Nagata, *et al.*, 1999b; Kritzman *et al.*, 2002), and once infected the adults become restless and move from one plant to the other, with a switched

preferential behaviour towards the host plants, spreading the virus (Ogada, Moualeu, *et al.*, 2016; Shalileh *et al.*, 2016; Sherwood *et al.*, 2000).

1.3.1 Management of TSWV and *F. occidentalis*

The management of TSWV in field crops is mainly based on vector control using insecticides which are often undesirable to the environment, and at the same time the thrips develop resistance overtime (Brødsgaard, 1994). An infected adult thrips can migrate long distances to new host plants and quickly transmit the virus, often before thrips can be controlled (Wijkamp *et al.*, 1995; van de Wetering *et al.*, 1996; Nagata, Inoue-Nagata, *et al.*, 1999b). Desired integrated control measures either using selective synthetic pesticides or biological control options such as natural enemies or biorational pesticides (Otieno *et al.*, 2016), require not only precise thrips monitoring but in particular modeling of vector and virus dynamics within or even between crop stands in time and space.

1.4 Objectives

In the complexity and specificity of virus-vector-host plant relationship, many factors and their interactions affect the stability of transmission efficiency of the virus by the vector, hence, greatly challenging predictive systems for the management of both the virus and its vector. In an attempt to contribute to deeper understanding of this research gap, we set out different studies dealing with basic factors influencing vector competence at individual vector level, using the model system of *F. occidentalis*, TSWV and their shared host plant *C. annuum*.

Motivated by reported studies from other researchers, as well as our earlier studies (Halaweh and Poehling, 2009; Ogada *et al.*, 2013), this project contributed to five broad areas affecting the stability of TSWV transmission by *F. occidentalis*, forming the main objectives of our study, which were; (a) To determine the contribution of individual vector genetics to the interaction, (b) To determine the contribution of vector gender to the interaction, (c) To evaluate the mutualistic relationship between the TSWV and *F. occidentalis* by studying the virus induced vector's life processes, (d) To develop predictive models for the TSWV spread dynamics incorporating the observed virus induced influences on the vector life's processes and finally (e) To study the vector's immune response to viral infection. These formed the research chapters that follow:

Chapter 2: Inheritance genetics of the trait vector competence in *Frankliniella occidentalis* (Western Flower Thrips) in the transmission of *Tomato spotted wilt virus*.

Chapter 3: Sex-specific influences of *Frankliniella occidentalis* (Western Flower Thrips) in the transmission of *Tomato spotted wilt virus* (Tospovirus).

Chapter 4: Manipulation of *Frankliniella occidentalis* by *Tomato spotted wilt virus* (Tospovirus) via the host plant nutrients to enhance its transmission and spread.

Chapter 5: Predictive models for *Tomato spotted wilt virus* spread dynamics, considering *Frankliniella occidentalis* specific life processes as influenced by the virus.

Chapter 6: Differential proteomics study of *Frankliniella occidentalis* immune response proteins, after infection with *Tomato spotted wilt virus* (Tospovirus).

Each of the research chapters has been prepared as an independent manuscript, therefore, there are some repetitions between chapters. However, here, thesis formatting has been adopted in terms of citations, font, figures captions and general arrangement.

2 Inheritance genetics of the trait vector competence in *Frankliniella occidentalis* (Western flower thrips) in the transmission of *Tomato spotted wilt virus*

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2.1 Abstract

The complexity of *tospovirus*-vector-host plant interaction is linked to a range of factors influencing vector's efficacy in virus transmission, leading to high variability in the transmission efficiency within vector populations. Main shortcomings of most studies is the missing information on the intrinsic potential of individual insects to serve as efficient vectors, both at phenotypic and genotypic levels. Moreover, detailed analysis of vector competence heredity and monitoring the splitting of both genotypes and phenotypes in filial generations have not been reported. In this study, using the model system *Frankliniella occidentalis* and *Tomato spotted wilt virus* (TSWV), we evaluated the inheritance and stability of the trait vector competence in a population through basic crossings of individually characterised partners, as well as virgin reproduction. We hypothesized that the trait is heritable in *F. occidentalis* and is controlled by a recessive allele. From the results, 83% and 94% of competent and non-competent males respectively, inherited their status from their mothers. The trait was only expressed when females were homozygous for the corresponding allele. Furthermore, the allele frequencies were different between males and females, and the competent allele had the highest frequency in the population. These suggest that the trait vector competence is inherited in single recessive gene in *F. occidentalis*, for which the phenotype is determined by the haplodiploid mechanism. These findings are fundamental for our understanding of the temporal and spatial variability within vector populations with respect to the trait vector competence, and at the same time offer an essential basis for further molecular studies.

Key words: Vector competence, Haplodiploidy, Intraspecific variation, Inheritance, *Frankliniella occidentalis*, *Tospovirus*, *Tomato spotted wilt virus*,

2.2 Introduction

The order *Thysanoptera* encompasses about 7700 described species (CSIRO Australia, 2005), out of which only about 14 species have been identified as vectors of tospoviruses (Riley *et al.*, 2011), indicative of the specificity of these virus-vector interactions. All the reported vector species belong to the subfamily *Thripinae* of the family *Thripidae* (Mound, 2002). *Frankliniella occidentalis* (Pergande) (Western Flower Thrips), is considered the most economically important pest among thysanopterans causing enormous annual economic losses worldwide (Kirk, 2002) both by direct damage as well as competence to transmit five of the 14 recognized *tospovirus* species (Whitfield *et al.*, 2005). It is reported to be the most efficient vector of *Tomato spotted wilt virus* (TSWV) (Whitfield *et al.*, 2005), which currently ranks among the top ten most economically important plant viruses worldwide (Sherwood *et al.*, 2000; Parrella *et al.*, 2003; Scholthof *et al.*, 2011) causing serious losses in a wide range of crops and flowers all over the world (Goldbach and Peters, 1994). However, there is inter as well intraspecific variation in the capability to vector specific tospoviruses which has been associated to rapid co-evolution between thrips and tospoviruses (Nagata *et al.*, 2004). For instance, transmission of TSWV differs considerably between and within populations of *F. occidentalis* (van de Wetering *et al.*, 1999a) as well as in *Thrips tabaci* (Lindeman) (Jacobson *et al.*, 2013; Westmore *et al.*, 2013; Cabrera-La Rosa and Kennedy, 2007).

The transmission of TSWV to healthy plants by *F. occidentalis* follows a persistent and propagative manner, mainly by the adults during feeding (Sherwood *et al.*, 2000; Whitfield *et al.*, 2005), but adults are only efficient vectors if the acquisition of the virus occurs at the early larval stages (Moritz *et al.*, 2004; Whitfield *et al.*, 2005), followed by replication inside the host midgut, passing the midgut barrier and uptake and multiplication in the salivary glands during thrips development (Nagata, Inoue-Nagata, *et al.*, 1999a). The pronounced change in virus acquisition during thrips ontogeny has been related to a temporary displacement of the thrips' brain into the prothoracic region in the early developmental stages, which leads to casual association of the midgut and the salivary gland, enabling the flow of the virus between these two chambers (Moritz *et al.*, 2004). However, this association is broken as the thrips develops, which explains the age specific acquisition/transmission characteristics of this vector. Furthermore, transovarial transmission of TSWV is not possible in *F. occidentalis*, so each generation must re-acquire the virus for the disease epidemic to continue (Nagata, Inoue-Nagata, *et al.*, 1999a). However, this specificity in the virus transmission cycle can neither explain the always high individual variability in vector competence when same aged L1 thrips are subjected to the same virus sources (*tospovirus* infected host-plants), nor the dynamic

change of relative amounts of vector competent individuals in populations of different sizes, or in isolation (inbreeding).

Several studies have postulated that the variability in transmission efficacy observed in thrips populations is due to differences in sexes (van de Wetering *et al.*, 1999a), or in genotypes, which can be separated for instance by random amplification of polymorphic DNA (RAPD) analysis (Gillings *et al.*, 1995), as well as using mitochondrial cytochrome c oxidase 1 gene (COI) sequence analysis (Jacobson *et al.*, 2013; Westmore *et al.*, 2013), indicating that the trait vector competence is manifested in the genome and it is variable. Cabrera-La Rosa and Kennedy, (2007) hypothesized that vector competence trait is recessively inheritable, in the case of TSWV and *T. tabaci* Lindeman. Furthermore, Halaweh and Poehling, (2009) reported in their preliminary crossing experiments with *Ceratothriopoides claratris* (Shumsher) (Thysanoptera: Thripidae) vectoring *Capsicum chlorosis virus* (CaCV), that males inherit the trait vector competence only from their mothers. Additionally, under inbreeding conditions in an isolated colony the ratio of competent versus non-competent individuals strongly declined with increasing homozygosity, suggesting also that vector competence in *C. claratris* is controlled by a recessive allele (Halaweh and Poehling, 2009). It is however not obvious yet, whether this mechanism of inheritance found in *C. clarathris* and *T. tabaci* is common in all thrips–*tospovirus* relationships.

Based on the hypothesis that vector competence of *F. occidentalis* is a heritable trait linked to a recessive allele, we performed inheritance experiments with individually characterized *F. occidentalis* (competent (transmitters) or non-competent (non-transmitters)), and evaluated the competence status of offspring produced both parthenogenetically by individual virgin females, and from controlled crossings of individual partners with determined competence status. The focus on individual crossings is based on our hypotheses that the available inheritance studies on the trait vector competence in *C. clarathris* and *T. tabaci* (Cabrera-La Rosa and Kennedy, 2007; Halaweh and Poehling, 2009), crossing experiments were performed only at population levels, which might have obscured the contribution of individual’s genetic constitution.

2.3 Materials and Methods

2.3.1 Host plant

For the study, *Capsicum annuum* L. (Solanaceae) (4-5 leaf stage) was selected as a host plant for *F. occidentalis* and TSWV maintenance, and was used in all the experiments. To facilitate propagation of the virus for stock inoculum, *Nicotiana benthamiana* L. (Solanaceae) (3-4 leaf stage) served as a reservoir host plant alongside *C. annuum*, because of its susceptibility to the

virus and ease in handling during mechanical inoculation. *Phaseolus vulgaris* L. (Fabaceae) was used for the maintenance of *F. occidentalis* (see below) stock culture. All clean host plants were maintained in a thrips proof nursery chamber at green house conditions (28-30 °C and 70-80% r.h.).

2.3.2 Thrips culture and maintenance

F. occidentalis strain (*Fo*2) was obtained from Wageningen University Laboratory of Virology in the Netherlands, and maintained as stock culture on bean plants (*P. vulgaris*) at 2–3 leaf stage, in cages covered with thrips tight gauze in climate cabins at constant conditions (25± 2 °C; 60–70% r.h.; L16: D8). A synchronised rearing which served as *F. occidentalis* source for all the experiments, was established from the stock culture on young fresh green bean pods (*P. vulgaris*), supplemented with commercial honey-bee pollen mixture (Naturprodukte-mv.de; Naturprodukte Lembcke, Faulenrost, Germany) in Plexiglas cages closed on top with thrips-proof 64 µm nylon gauze. Synchrony was achieved by transferring the old bean pods with a cohort of freshly deposited eggs, into new cages for L1 hatching and replacing them with new bean pods for further egg laying, this was done at one day intervals. Isolated rearing of such same aged cohorts ensured the availability of all life stages of *F. occidentalis* at any one time.

2.3.3 TSWV isolate and maintenance

The TSWV isolate (TSWV-12) was also obtained from Wageningen University Laboratory of Virology in the Netherlands, and confirmed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The isolate was maintained at green house conditions (28-30 °C and 70-80% r.h.) by series of mechanical inoculations done after every 2 to 3 weeks on *C. annuum* (4-5 leaflet stage) and *N. benthamiana* (4-5 leaflet stage). To reduce the chances for defective interference of the virus, feasibly caused after several serial passages by mechanical inoculation, and to ensure viability of the virus for transmission by *F. occidentalis*, fresh inoculations with the original source inoculum were made after every 5th serial passage. Moreover, as a backup, a parallel thrips-mediated inoculation was maintained on *C. annuum* (4-5 leaflet stage) in an isolated climate chamber (25±2 °C, 50-60% RH and L16: D8) in thrips proof cages.

Protocol developed by Mandal *et al.* (2008) was used for the mechanical inoculation. In summary: the inoculum contained TSWV infected leaf sap prepared in 0.1 M phosphate buffer, 0.2% sodium sulphite, 0.01M 2-mercaptoethanol and 1% each of celite 545 and carborundum 320 grit. A soft finger rubbing technique was used in delivering the inoculum onto the test plants leaves. The inoculated plants were kept under greenhouse conditions. The first symptoms

appeared 10-14 days after inoculation, and successful transmission of the virus in systemically infected leaflets was confirmed using DAS-ELISA, targeting the viral nucleocapsid protein. The infected leaves were then used as the inoculum source for further series of mechanical inoculation as well as for virus acquisition by the newly-hatched first instar larvae (L1) in the experiments.

2.3.4 Detection of TSWV in host plants by double antibody sandwich Enzyme-linked immunosorbent assay (DAS ELISA)

DAS ELISA was used to confirm the observed symptoms on the host plants. Two TSWV specific antibodies (polyclonal mixture ex rabbit) (Loewe) were used. A polystyrol-96-well micro titre plate (medium binding) was first coated with the antigen-specific coating-antibody (IgG), followed by addition of sap extracted from leaf disks to the coated wells: samples were extracted in phosphate buffered saline with Tween plus Polyvinylpyrrolidone (PVP) (Loewe) and egg albumin (Loewe) (PBS-TPO). The third step involved the addition of the enzyme labelled antibody-AP-conjugate, forming the double antibody sandwich. *p*-Nitrophenyl phosphate (Loewe) dissolved in a substrate buffer was then added to initiate enzymatic reaction, resulting in yellow coloured 4-nitrophenol as product. All samples and known TSWV-positive and negative controls were tested in duplicate. Colour development was measured in a spectrophotometer at 405 nm using Multiskan FC microplate photometer, from Thermo Scientific. The average value of the non-infected controls from the plants, plus three times their standard deviation made the minimum threshold for positive ELISA values. Therefore, samples were considered TSWV positive if the mean optical density reading exceeded the minimum threshold.

2.3.5 Characterization of individual *F. occidentalis* competence status (biotest-phenotyping)

Newly hatched L1 larvae (<12h old) were collected from the synchronized rearing by softly blowing them off the bean pod onto a TSWV infected C. annuum leaf (high virus titer confirmed by DAS-ELISA) in a gypsum ($CaSO_4$) petri dish (9 cm diameter) for an Acquisition Access Period (AAP) throughout their larval stages until pupation. The gypsum petri dishes were made up of a thin layer of gypsum and charcoal mixture (ratio 9:1) covering the bottom, moistened with a few millilitres of distilled water, and then overlaid by a piece of filter paper to absorb excess water. The lid of each petri dish had three equally spaced holes (12 mm diameter) covered with thrips-proof (64 µm) nylon mesh for ventilation. Once closed, all of the petri dishes were sealed with Parafilm M® (Pechiney Plastic Packaging, Inc., USA) to avoid thrips

escape. After 4-6 days, the resulting pupae were individually transferred onto a virus free *C. annuum* leaf disk (17 mm diameter) placed in a new petri dish (6 cm diameter) lined with moist filter papers for adult emergence. The emerging adults were allowed to feed on the virus free leaf disk for 36 hours Inoculation Access Period (IAP) before being transferred onto a new leaf disk for further experiments (virgin reproduction or crossing, see below). The old leaf disks were incubated for three days before being subsequently assayed for successful virus transmission using Amplified DAS-ELISA (an improved form of DAS-ELISA in terms of sensitivity, at least 10-fold higher), which amplifies the ELISA signals by using amplification kit from Invitrogen Life Technologies GmbH, Cat. No. 19589-019, following the manufacturer protocol. Based on the results, individual thrips were characterized as either competent or non-competent virus transmitters. Further confirmation of the individual competence status was done using the status of their offspring. In both virgin reproduction and basic crossing experiments, the percentage of competent thrips at population level was considered to be the same as the percentage of leaf disks that gave positive readings in the Amplified DAS-ELISA tests. We presumed that the allele for the trait vector competence is recessive (c) while non-competence is dominant (C). All the experiments were performed in the climate chambers at 25±2 °C, 50-60% RH and 16:8h L: D.

2.3.6 Virgin reproduction and inheritance evaluation

Ten non-inseminated female *F. occidentalis* were randomly selected after IAP prior to the characterization step (described above), and individually used as parents in the virgin reproduction (parthenogenetic) experiment. Each virgin female was placed on a healthy leaf disk for egg laying, and the leaf disk was replaced at one-day interval with a new one for further oviposition. The old leaf disks with cohorts of eggs were individually kept for collecting the newly-hatched F1 larvae, which were allowed an AAP on infected *C. annuum* leaves until pupation. Several batches of F1 larvae were collected until the death of the mother thrips. After the AAP, the resulting pupae were individually transferred onto a new virus free leaf disks, and the hatching adults (F1, all males) were tested for successful transmission of the acquired virus. This way, the vector competence of each individual offspring was determined, and the percentage of competent and non-competent individuals calculated per virgin parent. Additionally, the resulting F1 status was used to distinguish between the homozygous and heterozygous non-competent parents based on the hypothesised F1 phenotypes per parental status (Table 2.1). A threshold of 30% was used: that is, if 30 % of the resulting offspring from a non-competent female parent were competent, then the female parent was considered heterozygous for this trait. This experiment was repeated 5 times.

Table 2.1: The possible parental genotypes virgin females and the resulting F1 outcome, with the expected phenotypes and the possible genotypes. CC & C-: homozygous non-competent female & non-competent male status respectively; Cc: Heterozygous non-competent female; cc & c-: Homozygous competent female & competent male status respectively.

Parental phenotypes (virgin females) (Possible genotypes)	**F1 offspring phenotypes (all males)** (Possible genotypes)
♀ com (cc)	♂ com (c-)
♀ n-com (CC)	♂ n-com (C-)
♀ n-com (Cc)	♂ n-com:com (½C-:½ c-)

2.3.7 Basic crossing experiments and segregation analysis

Males and non-inseminated females selected randomly after the IAP prior to characterization (see above), were used as parents in subsequent crosses. The presumed cross combinations for the experimental series were based on the hypothesis that the trait vector competence is recessive (c). Table 2.2 summarises the hypothesised crosses and the expected offspring status. Each cross was made up of a couple consisting of 1 male and 1 female *F. occidentalis*, placed together on a single virus free leaf disk to mate and reproduce. The leaf disks were replaced at one-day interval with new ones for further oviposition until the death of the mother thrips, and the old leaf disks were individually kept for collecting the cohorts of daily hatched F1-larvae, which were transferred onto TSWV infected *C. annuum* leaves for an AAP until pupation. The resulting pupae were individually transferred onto healthy leaf disks and the emerging adults were tested for the trait vector competence by leaf disk assay (described above). The percentage of competent male and female offspring from each parental cross was calculated, and further used to confirm the characterised competence status of their parents. Non-competent F1 females from all the possible cross combinations (Table 2.2) were allowed to reproduce parthenogenetically in a separate leaf disk assay. The competence status of the resulting offspring (F2 generation, all males) was tested as described to determine the homo/heterozygous specificity of the non-competent F1generation females. 8 repeats each consisting of 10 random crosses of the possible 6 combinations (Table 2.2) were done, and ideally all offspring of individual pairs were tested for vector competence in consideration of the expected stochastic component given e.g. by variability in virus acquisition and transmission independent from the trait vector competence.

Table 2.2: Intended parental cross combinations and the expected F1 offspring outcome with the expected phenotypes and the possible genotypes. CC & C-: homozygous non-competent female & non-competent male status respectively; Cc: Heterozygous non-competent female; cc & c-: Homozygous competent female & competent male status respectively.

Cross Combinations	Possible parental crosses genotypes			Expected F1 offspring phenotypes (genotypes)	
#	Female (♀)	x	Male (♂)	♀	♂
1	comp (cc)	x	n-comp (C-)	n-com (Cc)	com (c-)
2	n-comp (CC)	x	comp (c-)	n-com (Cc)	n-com (C-)
3	n-comp (Cc)	x	comp (c-)	n-com:com (½Cc:½ cc)	n-com:com (½C-:½ c-)
4	comp (cc)	x	comp (c-)	com (cc)	com (c-)
5	n-comp (CC)	x	n-comp (C-)	n-com (CC)	n-com (C-)
6	n-com (Cc)	x	n-comp (C-)	n-com (½CC:½ Cc)	n-com:com (½C-:½ c-)

2.3.8 Expected allele and genotype frequencies

Hardy Weinberg Equilibrium (HWE) was taken as the basis for analysis of both allele and genotype frequencies. It assumes stable allele and genotype frequencies in a population if there are no evolutionary influences. When assuming random mating in diploid thrips (females), and vector competence as recessive (c) while non-competence as dominant (C) allele, HWE is given as;

$$p^2 + 2pq + q^2 = 1 \text{ (For genotype frequency)} \quad 1.0$$

$$p + q = 1 \text{ (For allele frequency)} \quad 1.1$$

Where, p is the frequency of the dominant allele C; q is the frequency of the recessive allele c; p^2 = homozygous dominant; q^2 = homozygous recessive; and $2pq$ = heterozygous. Equation 1.0 was used for the genotype frequencies and 1.1 for allele frequencies, for the diploid population (females). While for the males, we assumed that the allele frequencies are similar to their mothers, and since they are haploids, equation 1.1 was used for both the allele and genotype frequencies. Discrepancy between the observed and the expected frequencies in the test population were evaluated for the fit to the Hardy-Weinberg Equilibrium.

2.3.9 Statistical analysis

The classical Mendelian values (assuming single gene inheritance) were used to calculate the expected ratios, which were further adjusted using the misclassification probabilities determined from the parental status. Chi-square analysis was used to test the observed ratios against the expected ratios. Male and female offspring data per cross combination were analysed separately. Parents-offspring relatedness was analysed using chi-square test of

independence (Fisher's exact test only for small sample numbers), to test if the observed phenotype frequencies in the offspring were independent from the parental status for the trait vector competence. The expected genotype and allele frequencies of the observed phenotype (biotest) were analysed and compared using the equations 1.0 and 1.1, and the discrepancy between them evaluated using chi-square, to determine the HWE status of the population. The chi-square test was done using PROC FREQ/ Chi-square command in Statistical Analysis System 9.0 for Windows (SAS/STAT Software, 2002), and a significance level of 0.05 was used.

2.4 Results

2.4.1 Parental competence status represented in the experimental population

The representation of the three possible genotypes in the entire test population of virgins was uneven according to the random samples taken. 64% of the population were determined to be homozygous competent (cc), 22% were heterozygous non-competent (Cc) and 14 % were homozygous non-competent (CC) (Fig. 2.1 A). Determinations were based on both the Amplified DAS-ELISA results as well as the offspring status. On the other hand, from the crossing experiments out of the 6 hypothesised cross combinations, only 5 were achieved, with the highest representation being the combination where the couple were competent (i.e., cc x c-) at 54%, while a combination of heterozygous non-competent female and non-competent male (Cc x C-) could not be ascertained (Fig. 2.1 B)

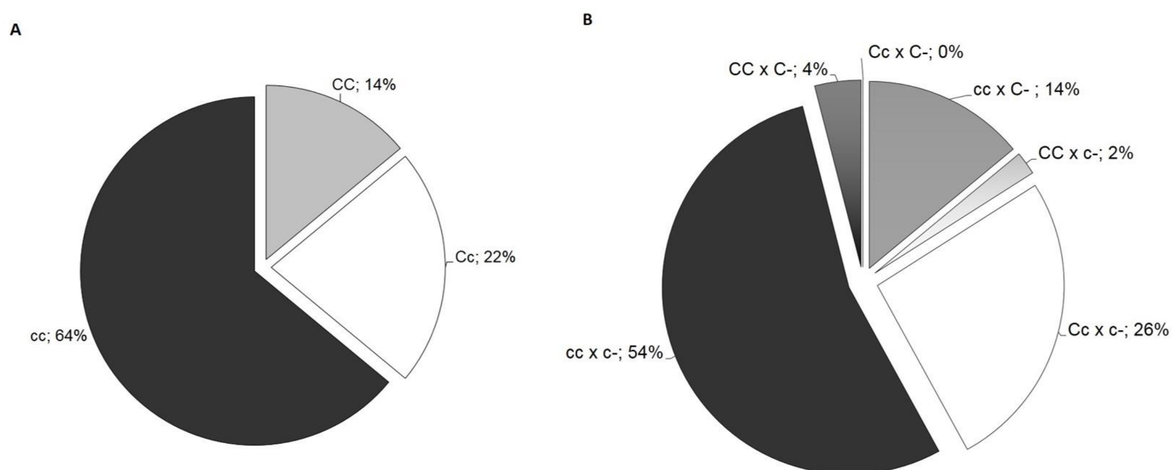

Figure 2.1: Representation of competence status (genotypes) in the test populations of *F. occidentalis*; A. Representation of different genotypes in virgin females' population, B. Cross combinations representation of different genotypes. CC & C-: homozygous non-competent female & non-competent male status respectively; Cc: Heterozygous non-competent female; cc & c-: Homozygous competent female & competent male status respectively.

2.4.2 Evaluation of inheritance of the trait vector competence in virgins' reproduction

Parthenogenetic reproduction by the virgin females resulted in 100 % male F1 offspring. Of the progeny from the homozygous non-competent virgin females, 94% of the F1 were non-competent, while from the heterozygous non-competent virgin parents 56% of the F1 were non-competent and 44% were competent. From the competent parents, 83% of the resulting F1 were competent and 17% were non-competent (Fig. 2.2). The competence status of the resulting offspring significantly related to the status of their mothers, χ^2 (df=2, N =397) = 123.34, $P <$ 0.0001.

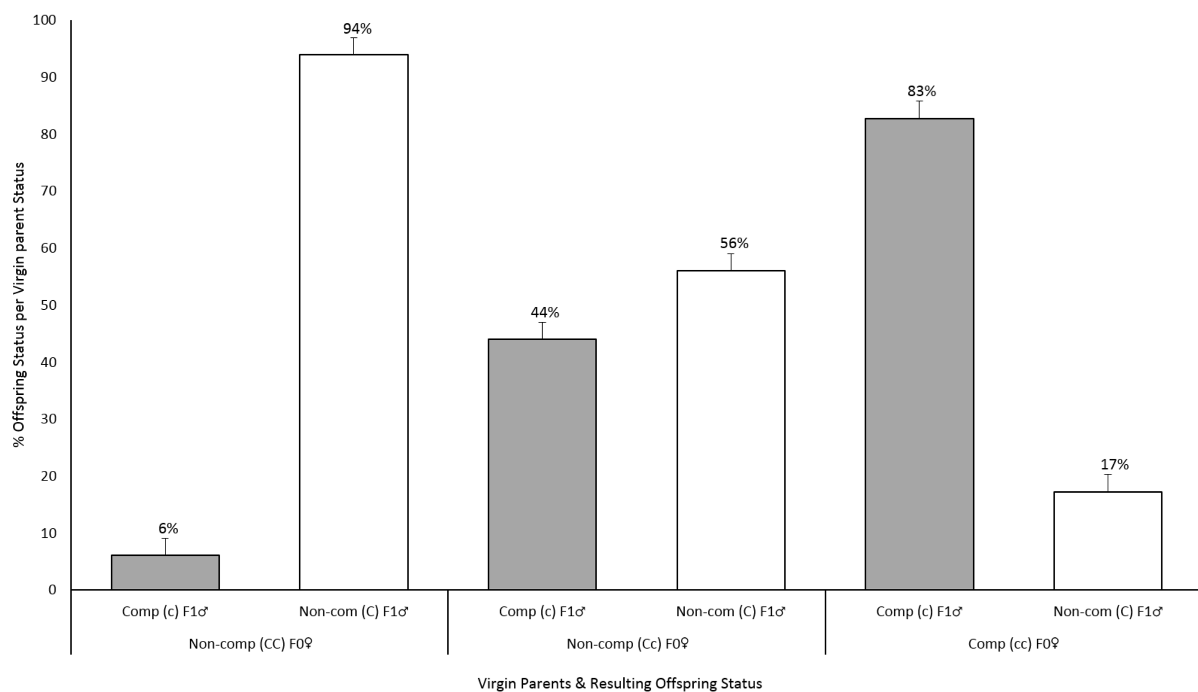

Figure 2.2: Evaluation of inheritance of the trait vector competence by *F. occidentalis*: Status of progeny from virgin females. F0♀ represent the virgin female parents and F1♂ represent the male offspring. Comp = competent status, Non-comp = non-competent. CC & C-: homozygous non-competent female & non-competent male status respectively; Cc: Heterozygous non-competent female; cc & c-: Homozygous competent female & competent male status respectively.

2.4.3 Basic crossings - Analysis of inheritance of the trait vector competence in F1

We considered the total number of F1 per cross combination in all the repeats for our analysis, as the offspring status of similar crosses were comparable. Cross combinations involving a homozygous non-competent female parent (CC) resulted in 100% non-competent male and female offspring (Table 2.3: combinations number 2 ($\chi^2_{\text{(df=1, N=27)}} = 0$, P =1) and 5 ($\chi^2_{\text{(df=1, N=17)}}$ = 0, P =1)), regardless whether the male parent was competent (c-) or not (C-). Crossing of competent females (cc) with non-competent males (C-) resulted in 100% non-competent female offspring and 89% competent male offspring (Table 2.3: combination number 1($\chi^2_{\text{(df=1, N=52)}}$ =

36.98, P <0.0001)). A cross of competent parents (cc x c-) resulted in 87% female offspring being competent (cc), and 84 % of male offspring being competent (c-) (Table 2.3: combination number 4 ($\chi^2_{(df=1, N=289)}$ = 0.51, P =0.48)). In the cross combination involving a heterozygous non-competent female (Cc) and a competent male (c-); the status of the female parent was determined as being heterozygous non-competent (Cc) indirectly based on the resulting percentages of the offspring status (see above), approximately 57% and 44% of F1 females and males respectively, were competent (Table 2.3: combination number 3 ($\chi^2_{(df=1, N=119)}$ = 1.83, P =0.176)). However, it was not possible to find cross combination number 6 (Cc x C-) (Table 2.3) in our test population. When further evaluating the F2 of the non-competent virgin F1 females from the crosses, 100% of the resulting F2 males from the cross combinations 4 and 5 (see Table 2.3) were competent (c-) and non-competent (C-) respectively, indicating homozygosity of the F1 virgin parents. For the cross combinations 1, 2 and 3, the resulting competence status of the F2 were approximately 50% of both competent (c-) and non-competent (C-) individuals, indicating heterozygosity of the virgin F1 females in these combinations.

Table 2.3: The relative numbers of the resulting offspring (F1) competent and non-competent per parental cross combination

The relative number of competent and non-competent F1 per parents cross combination									
	Possible parental crosses phenotypes			**Competent F1**			**Non-competent F1**		
#	**Female (♀)**	**x**	**Male (♂)**	♀	♂	**Total**	♀	♂	**Total**
1	comp (cc)	x	n-comp (C-)	0	32	32	16	14	20
2	n-comp (CC)	x	comp (c-)	0	0	0	11	16	27
3	n-comp (Cc)	x	comp (c-)	24	34	58	18	43	61
4	comp (cc)	x	comp (c-)	100	146	246	15	28	43
5	n-comp (CC)	x	n-comp (C-)	0	0	0	8	9	17
6	n-com (Cc)	x	n-comp (C-)	0	0	0	0	0	0
	Total			124	212	336	68	100	146

2.4.4 Analysis of relatedness based on the trait vector competence

Parents-offspring relatedness was evaluated using chi-square analysis. We found high significance between mother-sons, mother-daughters and father-daughters' relations. However, there was no relation whatsoever between father-sons ($\chi 2$ (df=1, N=301) =0.0117, P=0.9138, Table 2.4). This calculation was based on the inheritance probability of an allele by the offspring from the parents.

Further evaluation of the F2 males from the F1 virgins produced in the crossing experiment showed a relationship between grandmother-grandson, as well as between grandfather-grandson (results not shown).

Table 2.4: Chi-square analysis of the parental-offspring relatedness

Relations	$\chi 2$	df	*P*	N
Mother <=> Sons	79.58	2	<0.0001	301
Mother <=> Daughters	23.64	2	<0.0001	181
Father <=> Sons	0.0117	1	0.9138	301
Father <=> Daughters	60.19	1	<0.0001	181

2.4.5 Expected genotype and allele frequencies

The allele and genotype frequencies of the virgin female parents (diploids) were determined based on the HWE formulas 1.0 and 1.1. We assumed that the males' allele frequencies were similar to their mothers. The expected frequency of the competent alleles (c) in both males and females was higher (0.8) compared to the non-competent alleles (C) (0.2). From the determined allele frequencies, the expected proportions of genotypes within our test population were evaluated for both males and females based on the described formulas. The expected genotype frequencies in males were similar to their allele frequencies since they are haploids. In females, the homozygous competent genotype had the highest frequency (0.64), followed by the heterozygous non-competent (0.32), while the homozygous non-competent genotype had the lowest frequency (0.04) (Table 2.5).

Table 2.5: Expected proportions of genotypes for the trait vector competence in both males and females in the test population.

Males		**Females**	
(Genotypes (♂)	Expected genotype Frequencies	Genotypes (♀)	Expected genotype Frequencies
C-	$p = 0.2$	CC	$p^2 = 0.04$
c-	$q = 0.8$	Cc	$2pq = 0.32$
		cc	$q^2 = 0.64$

In the analysis of deviation from the HWE, the gene frequencies in the samples were first calculated from the observed numbers using the equation 1.1; the observed numbers in the sample population are presented in Table 2.6. χ^2 test comparison between the observed and the expected numbers showed low amount of heterozygotes but an excess of competent (recessive) homozygotes in the diploid females. The differences between the observed and the expected numbers were of low significance (P=0.0382) in females, while in males, the difference was highly significant (P< 0.0001). Evaluation of the difference in the population as a whole (both

males and females) was also highly significant (P<0.0001) (Table 2.6), which is an indication of deviation from the HWE. Since the allele frequencies were estimated from the same data, χ^2 has only one degree of freedom, to ensure that the observed and the expected numbers agree both in their allele frequencies and in their totals.

Table 2.6: Chi-square analysis for the agreement with the Hard-Weinberg Equilibrium using the observed and the expected numbers of genotypes in the test population

	Females genotypes				Males genotypes		
	CC	Cc	cc	**Total**	C-	c-	**Total**
Numbers observed	8	45	139	**192**	100	212	**312**
Numbers expected	7.68	61.44	122.88	**192**	62.4	249.6	**312**

Females	$\chi^2_{(1, 192)}=6.53$	P=0.0382
Males	$\chi^2_{(1,312)}=88.06$	P<0.0001
Total	$\chi^2_{(1,504)}=94.58$	P<0.0001

From the observed genotype numbers in the test population, we calculated the allele frequencies using the HWE formulas. Females had higher frequencies of the competent allele (c) (0.841) compared to males (0.680) (Table 2.7).

Table 2.7: Allele frequencies calculated from the observed numbers in the test population.

	Number of alleles in the population			Frequency of the competent allele (c)
	C	c	Total	q
In females	61	323	384	0.841
In males	100	212	312	0.680

With the observed high allele and genotype frequencies for the competent trait, the same data was used to evaluate the frequencies for the listed possible cross combinations (Table 2.8) within a hypothesised one million pairs of parents. The results show that a cross combination with both parents having the competent trait (cc x c) will have the highest frequency (0.512) within the population. On the other hand, the cross combination that will have the lowest frequency of occurrence in the population (0.008) is the one with both parents being non-competent (CC x -C) (Table 2.8).

Table 2.8: Estimation of the expected allele frequencies for the hypothesised cross combination per million pairs of parents

Mating types	Expected frequencies				
	Proportions			%	Per million pairs of parents
CC x -c	p^2q	=	0.032	3.20	32,000
cc x -C	pq^2	=	0.128	12.80	128,000
Cc x -C	$2p^2q$	=	0.064	6.40	64,000
Cc x -c	$2pq^2$	=	0.256	25.60	256,000
cc x -c	q^3	=	0.512	51.20	512,000
CC x -C	p^3	=	0.008	0.80	8,000
Totals			1	100	1,000,000

2.5 Discussion

Sex determination in *F. occidentalis* is by haplodiploid mechanism, with females laying two kinds of eggs; fertilized eggs that have diploid sets of chromosomes from both parents developing to females, whereas unfertilised eggs with only one copy (haploid) of the mother's chromosomes produce males (Crespi *et al.*, 1991; Hedrick and Parker, 1997). Therefore, females have twice as many copies of alleles compared to males, which is an important factor in the determination of the overall allelic frequencies in a population (Hedrick and Parker, 1997). Haplodiploid insects are reported to have lower levels of heterozygosity compared to the diploid insects, and hence lower levels of genetic variability (Crespi *et al.*, 1991). This could partly explain the high percentage of individuals bearing recessive allele for the trait vector competence (c) observed in our test population, and also the low and even complete lack of certain cross combinations. Reduced heterozygosity is also predicted in X-linked genetic systems (Hedrick and Parker, 1997), which are very similar to the haplodiploid systems (Hedrick and Parker, 1997). Furthermore, the observed reduction in heterozygosity could be due to evolutionary selection by fitness processes in our test population (Li, 1976), since the samples (cohorts) were obtained from a stock culture which had been maintained for a long time in isolated rearing.

From this study we can strongly infer that the trait vector competence in *F. occidentalis* is inherited in a haplodiploid recessive single-gene (Mendelian) pattern, which is similar to the X-linked recessive inheritance pattern (Hedrick and Parker, 1997; Falconer and Mackay, 1996). This inheritance pattern was obvious from the virgin reproduction results, where all offspring were males (as expected) and inherited their competence status from their mothers. This result confirms earlier findings by Halaweh and Poehling, (2009), who reported a vertical inheritance of the trait vector competence from the mothers to their male offspring in *C. claratis* when

studying the transmission of CaCV. Moreover, by analysing relatedness between parents and offspring in the crossing experiment, we found no relation of male offspring to their fathers with regards to inheritance of the trait vector competence, which confirms the above characterised pattern of inheritance. Additionally, we were able to examine the mode of inheritance by analysing F2 males from the virgin F1 females in the crossing experiment, eliminating the need for backcrossing or crossing F1 males and females as commonly done with diploid species (Crowder *et al.*, 2009). The results confirmed the recessive nature of the trait vector competence since it was only manifested in females (diploids) that were homozygous for this allele, which was obvious from the ~100% of their male offspring being competent. However, the 30% competent F1 used as the cut-off in the determination of the hetero/homozygous non-competence status of the female parents, could have led to the observed 6% competent offspring in the homozygous non-competent parents, where 100% non-competent offspring was expected.

Recessive traits are often associated with deleterious effects (characterised by selective elimination of homozygotes) in most X-linked and haplodiploid inheritance patterns (Borgia, 1980). However, our study revealed the contrary. In this system of haplodiploids with the specifics of TSWV-*F. occidentalis* relationship, we found strong indications for an increase in the frequency of this trait over time, leading to homozygosity biased towards the recessive allele in the population. We could therefore presume that the trait vector competence is favourable, and that the increase in frequency of the recessive allele (competent) over time is due to the relatively small selective effects and dosage compensation by equalization of gene expression in the haplodiploids. This contradicts earlier reports by Halaweh and Poehling, (2009), and Cabrera-La Rosa and Kennedy, (2007), who observed an increase in homozygosity of the dominant allele when investigating inheritance of vector competence in *C. claratris* and *T. tabaci*, in the transmission of CaCV and TSWV respectively, linking it to inbreeding effects. They both used populations in their crossing experiments, and therefore, could have overlooked the important contribution of individual insect genetics. Furthermore, Hedrick and Parker, (1997) also reported a lower inbreeding coefficient in haplodiploid or X-linked genes compared to autosomal genes, since males are haploid (zero inbreeding coefficient) and therefore cannot be inbred.

Apart from individual genetics, the observed increase in frequency of the competent allele could also be associated with the hypothesis that exposure of *F. occidentalis* to TSWV triggers the vectors' immune response resulting in improved fitness in terms of survival and longevity (de

Medeiros *et al.*, 2004; Ogada *et al.*, 2013). On the other hand, prolonged feeding of thrips on virus infected plants has been reported to have deleterious (pathological) effects to the vectors, presumably due to high virus load and virus propagation in the vector´s body which could overpower the initially induced immune system (Shalileh *et al.*, 2016). However, reports are also emerging indicating vector manipulation by the virus in terms of preferential behaviour, which favours the multiplication and the spread of the virus and at the same time promoting longer survival of the vector (fitness) (Shalileh *et al.*, 2016), presumably blinding off the expected deleterious effects on the vector. Improved fitness is also reported in bees (Hymenoptera), as a result of the haplodiploid sex-determination system due to indirect selection (Foster and Ratnieks, 2001).

The evaluation of the expected allele frequencies for the haplodiploid females and males follows the estimation of alleles in diploid and haploid individuals respectively (Li, 1976). Studies have shown that if there is a difference in the initial frequencies between the two sexes with no overlapping of the generations, oscillation in the allele frequencies will occur between the two sexes, above and below the average frequency, but the difference is halved in successive generations and the population rapidly approaches an equilibrium with equal frequencies in both sexes (Hedrick and Parker, 1997; Li, 1976). Such differences are already obvious in our analysis of the observed allele frequency between the two sexes. Since males receive their entire chromosomes from their mothers, we assumed therefore, that their allele frequencies are identical to their mothers (Hedrick and Parker, 1997; Moritz, 1997; Cabrera-La Rosa and Kennedy, 2007; Halaweh and Poehling, 2009), and employed HWE for the analysis of genotype and allele frequency. Furthermore, by variation of specific allele frequencies due to the haplodiploid inheritance system (Moritz, 1997), phenotypic variation (vector competence) in subsequent generation can be expected (Gillings *et al.*, 1995; van de Wetering *et al.*, 1999a). Therefore, Mendel's law of segregation and the Hardy-Weinberg equilibrium, cannot be applied in the same way for both genders, and additionally, the ideal conditions required for the HWE (Crow, 1999) are not adhered to in our experiments. Other factors like the small population size of the parental genotypes used, could have brought along high sampling variation in gene frequencies in the successive generation. Moreover, different genotypes in the parental generation may have had different fertilities, which we did not control, and hence may have influenced the allele frequency over time (selection). Additionally, controlled mating in this experiment might have also contributed to the observed allele frequencies.

When discussing the mechanism of vector competence, steps under genetic control and targets for genetic variability are crucial factors. The virus recognition and infection process which involves key proteins (gene products) should be considered. Evidence exists of involvement of viral glycoprotein as determinants in the recognition process in the vector's midgut (Whitfield *et al.*, 2008), and of receptor based endocytosis of the virus during its entry via the midgut epithelial cell (Bandla *et al.*, 1998; Kikkert *et al.*, 1998). For interaction with the vector, the involvement of TSWV non-structural protein (NSs), the gene silencing suppressor in plants, is reported (Bucher *et al.*, 2003; Ding *et al.*, 2004). Moreover, identification of molecules that might influence the pathogen development within the insect tissues, and the isolation of genes that encode for these molecules, is another approach to clarify the mechanisms controlling *F. occidentalis* vector competence (Beerntsen *et al.*, 2000). Primary immune components are found within the hemolymph, and are involved in virus recognition and initiation of defence response (Paskewitz and Christensen, 1996). This mechanism of vector defence and the strategies used by the virus to escape recognition and destruction by the vector´s immune system, could be the other important determinant of vector competence, since in the non-competent vectors, despite the virus particles successfully passing the midgut barrier, they fail to further propagate or are destroyed by the vector's defence response (Beerntsen *et al.*, 2000). Additionally, the genetic make-up of the virus and the host plant can also play a role in the adaptability success of the pathogen within the vector, and thus influencing vector competence (de Oliveira Resende *et al.*, 1992; Jacobson *et al.*, 2013; Montero-Astúa *et al.*, 2014).

Another important aspect in allele frequencies consideration of the trait vector competence in *F. occidentalis,* is the difference between males and females in terms of feeding behaviour, survival time and transmission efficiency of the virus (Ogada and Poehling, 2015), which have also been reported to contribute to the transmission variability, as well as influencing virus spread. Therefore, the ratio of males and females in terms of the vector population composition is quite important in this regard (Inoue and Sakurai, 2006).

We can therefore conclude that the genetic makeup of individuals within a vector species population, to a larger extent is the main determinant of the success of the specific virus-vector interaction with regards to vector competence, considering that this trait is not present in all individuals (Cabrera-La Rosa and Kennedy, 2007; (Halaweh and Poehling, 2009). Therefore, studying the inheritance of the trait and the behaviour of the alleles in the filial generation is an important contribution to a more general understanding of this phenomenon. From an application point of view, the projected increase in allele frequency of the trait vector

competence in consecutive generation may partly explain the often aggressive spread of TSWV in crop stands, and should be considered when developing control strategies, for instance, predictive models.

This work lays a fundamental foundation in the development of molecular markers for the trait vector competence which would enable a more precise analysis of the trait's behaviour overtime, using molecular tools like microsatellites or AFLP. Additionally, development of microsatellite genetic linkage map would enable the assessment of the genetic background controlling vector competence of *F. occidentalis* in the transmission of TSWV. The identification of the molecular marker loci linked to genes that influence vector competence would provide the necessary starting point for map-based cloning, and furthermore the realization of linking molecular and bioassay data.

3 Sex-specific influences of *Frankliniella occidentalis* (Western flower thrips) in the transmission of *Tomato spotted wilt virus* (Tospovirus)

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3.1 Abstract

The tospovirus-plant-vector interaction is a specific and complex relationship. It is established that several factors influence this interaction leading to high variability of transmission efficiency of the virus within a vector population. We studied the contribution of vector gender to this interaction using *Frankliniella occidentalis* (western flower thrips) reared on either *Tomato spotted wilt virus* (TSWV) infected or uninfected *Capsicum annuum* leaflets throughout their larval stages. Later pupae were individually transferred onto healthy leaf disks to examine the differences in: survival, development rate, transmission efficiency and feeding behavior between males and females in all the treatments. The study was based on the hypothesis that sex of the vector influences transmission efficiency within the vector population. Our results showed that males had significantly higher longevity, lower mortality and shorter development time compared to females, while in the comparison of feeding behavior, females were found to feed more intensively than males, regardless of whether they were exposed or unexposed to TSWV. Conversely, transmission efficiency; which referred to successful inoculation and establishment of the virus in the host plant after a given period of inoculative feeding, was found to be significantly higher in males compared to females, despite females having the highest percentage of transmitting individuals. These findings account in part for the high variability in vector competence of *F. occidentalis* within a population, which further give insight into the biological factors that may influence the transmission of TSWV by *F. occidentalis*.

Key words: *Frankliniella occidentalis*, *Tomato spotted wilt virus*, Vector Competence, Intraspecific variation, Tospovirus, Vector Gender

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3.2 Introduction

Frankliniella occidentalis (Pergande) (Western Flower Thrips) is the most economically important insect pest among thysanopterans due to its extremely wide host range, worldwide distribution and competence to transmit 5 of the 16 recognized Tospovirus species (Whitfield *et al.*, 2005). It is reported to be the most efficient vector of *Tomato spotted wilt virus* (TSWV)(Whitfield *et al.*, 2005; Wijkamp *et al.*, 1995), which currently ranks among the top ten most economically important plant viruses worldwide (Mumford *et al.*, 1996b; Sherwood *et al.*, 2000; Parrella *et al.*, 2003; Scholthof *et al.*, 2011), causing serious losses in a wide range of crops and flowers all over the world (Goldbach and Peters, 1994). Out of the 7700 described species of Thysanoptera (http://www.ento.csiro.au/thysanoptera/worldthrips.php); only 14 are known vectors of tospoviruses, indicative of the specificity of these virus-vector interactions (Riley *et al.*, 2011).

TSWV is transmitted by *F. occidentalis* in a persistent and propagative manner partly by the second instar larvae but mainly by the adults (Ullman *et al.*, 1993; Wijkamp, Vanlent, *et al.*, 1993; Sherwood *et al.*, 2000; Whitfield *et al.*, 2005), only when the virus is acquired at the early larval stages of the thrips life cycle (Ullman *et al.*, 1992; Wijkamp and Peters, 1993; van de Wetering *et al.*, 1996; Moritz *et al.*, 2004; Whitfield *et al.*, 2005), and after the virus has replicated and circulated inside the host midgut and finally reached the salivary glands (Wijkamp, Vanlent, *et al.*, 1993; Ullman *et al.*, 1993). For successful transmission of the virus, infection of the salivary glands and replication thereafter, is mandatory to achieve a high virus titer in the saliva which is injected into the plant during feeding (Wijkamp *et al.*, 1995; van de Wetering *et al.*, 1996; Nagata, Inoue-Nagata, *et al.*, 1999b; Nagata *et al.*, 2002). Transmission efficiency has been reported to decrease as development proceeds at the time of acquisition (Nagata, Inoue-Nagata, *et al.*, 1999a; Naidu *et al.*, 2008; Moritz *et al.*, 2004). Moritz *et al.* (2004) associated the distinct changes in vector competence during the ontogenetic thrips development to special morphological characteristics of thrips. They clearly showed a temporary association between the thrips midgut and the salivary gland, as a result of brain displacement into the prothoracic region. However, this temporary association occurs only during the first and the early second larval developmental stages, thus contingently facilitating the spread of the virus from the midgut to the salivary glands (Nagata *et al.*, 2002; de Assis Filho *et al.*, 2004, 2005; Moritz *et al.*, 2004). Therefore, this may explain why only in the first and early second larvae the virus particles successfully infect the salivary gland cell and propagate there and are not flushed out via hemolymph and the malphigian tubules after passing the midgut muscle barrier. After having successfully passed this barrier and finally reached

salivary gland cells, virus propagate further and is stored there until it is transmitted via the saliva in a persistent and propagative manner, mainly by the adults (Ullman *et al.*, 1993; Wijkamp, van Lent, *et al.*, 1993; Whitfield *et al.*, 2005). TSWV acquisition by the adults is also possible and the virus infects and replicates in the midgut tissues, but it does not spread to the salivary glands and therefore cannot be transmitted to plants when acquired by this developmental stage (de Assis Filho *et al.*, 2004).

TSWV replication in the vector tissue may change vector physiology suggesting the likelihood for pathological effects influencing the fitness and performance of the vector (Stumpf and Kennedy, 2007; Nault, 1994). However, consistent demonstration of such effects has proven difficult due to the complex virus-host plant-vector interaction (Thomas *et al.*, 2005). Earlier studies have indicated that TSWV benefits by modifying the host plant quality and fitness of its vector, as well as the host preference behavior (Stafford *et al.*, 2011; Shrestha *et al.*, 2012; Ogada *et al.*, 2013). For instance, improved host plant quality can promote TSWV uptake, multiplication and circulation in thrips larvae (Nagata, Inoue-Nagata, *et al.*, 1999b), and once infected, adults become more restless and prefer the healthy plants, hence switching feeding sites and spreading the virus (Ingwell *et al.*, 2012). Earlier studies have also reported triggered immunity in the infected thrips (de Medeiros *et al.*, 2004) as the insect lack an adaptive immune system (Irving *et al.*, 2001), thus ensuring longer survival of the infected vector (Ogada *et al.*, 2013) and further promoting the spread of the virus.

Studies have also shown that the efficiency of transmission of TSWV is not always stable in a population of *F. occidentalis* (Gillings *et al.*, 1995; van de Wetering *et al.*, 1999a), as well as between the sexes, which may be related to variation in mobility, feeding behaviour and scar production between males and females (Sakurai *et al.*, 1998; van de Wetering *et al.*, 1999b; Rotenberg *et al.*, 2009). *F. occidentalis* feeds by piercing and sucking, and the virus could be transmitted to a permissive host plant by both males and females during non-destructive brief probes, however, the viral load per individual, the intensity of feeding and sequences of foraging including intervals of probing and non-probing are different between the sexes (Rotenberg *et al.*, 2009; Stafford *et al.*, 2011; Kindt *et al.*, 2006).

We therefore, hypothesized that *F. occidentalis* gender has a significant contribution to the reported intraspecific variations in vector competence within a population. Hence, by means of a model system; with *F. occidentalis* as the vector, TSWV, and *Capsicum annuum* as the shared host plant, we hereby report the possible clarification on the contribution of vector gender in the TSWV transmission efficacy by evaluating the differences between males and females in terms of developmental and behavioural aspects such as; survival time (longevity and

mortality), development time, feeding behaviour and transmission efficiency, as potential explanation for the variability in vector competence within a population. Also influence of TSWV on the considered parameters was evaluated.

3.3 Materials and methods

3.3.1 Host plants

Capsicum annuum (4-5 leaf stage) was selected for this study based on successful feeding and propagation of *F. occidentalis* as well as TSWV maintenance by mechanical inoculation. *Nicotiana benthamiana* (3-4 leaf stage) was also used (alongside *C. annuum*) for the maintenance of TSWV, due to its ease in handling and susceptibility to mechanical inoculation facilitating propagation of the virus for stock inoculum. However, it was only used as a reservoir for the virus as it was not able to support feeding and propagation of *F. occidentalis*, this was alleged to be due to the insecticidal effect of the nicotine content. Bean plant (*Phaseolus vulgaris*) was used for the rearing and maintenance of thrips culture. The planting and the maintenance of all the clean host plants were done in the nursery at green house conditions (28-30 °C and 70-80% r.h.).

3.3.2 Thrips culture

A stock culture of *F. occidentalis* was established from a strain obtained from Wageningen University, Laboratory of Virology in The Netherlands, and maintained on bean plants at 2–3 leaf stage, reared in cages covered with thrips tight gauze in climate cabins at constant conditions (25± 2 °C; 60 – 70% r.h.; L16: D8). From the stock culture, synchronised rearing was developed on young fresh green bean pods supplemented with some mm^3 of commercial honey-bee pollen mixture (Naturprodukte-mv.de; Naturprodukte Lembcke, Faulenrost, Germany) in Plexiglas cages closed on top with thrips-proof 64 µm nylon gauze. This served as *F. occidentalis* source for all the experiments.

3.3.3 *Tomato spotted wilt virus* isolates and mechanical inoculation

Three TSWV isolates, (T1) PV0182 -TSWV-L; (T2) PV0204 –TSWV and (T3) PV0393 – TSWV-AK 8, were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ- German Collection of Microorganisms and Cell Cultures Company Limited, Braunschweig, Germany) on *Nicotiana rustica* and confirmed for TSWV by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Appendix 1) and reverse transcription polymerase chain reaction (RT-PCR). The 3 isolates were used for all the experiments except for the transmission experiment where an isolate TSWV N12 obtained from

the Virology Department, Wageningen University (The Netherlands) was used. This was because individual transmission of TSWV by *F. occidentalis* was not successful with the 3 DSMZ-isolates (T1, T2, and T3) despite their successful acquisition (confirmed by testing 5 pupae from each treatment using RT-PCR); therefore, the TSWV N12 was selected for the transmission experiment as it was successfully transmitted. All isolates were maintained at green house conditions (28-30 °C and 70-80% r.h.) by series of mechanical inoculations done after every 2 to 3 weeks, on *C. annuum* and *N. benthamiana*.

The mechanical inoculation of the TSWV into the host plant was done following the rapid and efficient method developed by Mandal *et al.* (2008). In summary: the inoculum contained infected leaf sap prepared in 0.1 M phosphate buffer, 0.2% sodium sulphite and 0.01M 2-mercaptoethanol and 1% each of Celite 545 and Carborundum 320 grit. The inoculum application was done by soft finger rubbing technique (Appendix 3) onto the test plants leaves, and the inoculated plants kept under greenhouse conditions.

Initial symptoms appeared 10-14 days after inoculation. The infected plants displaying visual symptoms were tested using DAS-ELISA (Appendix 1) and RT-PCR (targeting the N-protein and the N-gene respectively) to confirm successful transmission of the TSWV virus isolates in systemically infested leaflets. After confirmation, infected leaves showing symptoms were used as the inoculum source for further series of mechanical inoculation as well as for virus acquisition by the newly-hatched first instar larvae (L1).

3.3.4 Acquisition of TSWV by *F. occidentalis*

F. occidentalis females from the synchronized rearing were allowed to lay eggs for a period of 1 day on young fresh green bean pods. Newly hatched L1 larvae (<12h old) were collected 2 days later by softly blowing them off the bean pods onto a TSWV infected *C. annuum* leaf in a gypsum ($CaSO_4$) petri dish (Ø 9 cm) for an Acquisition Access Period (AAP) throughout their larval stages until pupation. The bases of the petri dishes were covered with a mixture of gypsum ($CaSO_4$) and charcoal (9:1 ratio). A few millilitres of distilled water were added, and a small piece of filter paper was placed onto the gypsum layer to absorb any excess water before placing the virus-infected leaves (T1-T3 and TSWV N12; of high virus titer individually pre-tested using DAS-ELISA-Optical Density Value ≥1.0 measured at 405nm) or virus free control leaves (T4 – see below) on top of the filter paper, followed by infestation with the newly hatched L1 larvae for virus acquisition. The lid of each petri dish had three equally spaced holes (Ø 12 mm) that were covered with thrips-proof (64 µm) nylon mesh for ventilation purposes. Once

closed, all of the petri dishes were sealed with Parafilm M® (Pechiney Plastic Packaging, Inc., USA) to avoid escape. After 4-6 days, the resulting pupae were transferred individually onto a virus free *C. annuum* leaf disk (Ø 17 mm) in another clean gypsum petri dish (Ø 6 cm). Consequently, 5 pupae from each treatment were used to confirm acquisition using RT-PCR. The resulting adults were given 36 hours Inoculation Access Period (IAP) on the individual healthy leaf disks, to inoculate the acquired virus. Despite successful acquisition of the 3 TSWV isolates (T1 – T3) by *F. occidentalis*, the transmission results were negative. Therefore, resulting adults from these treatments (T1-T3 and the control T4) were used for evaluation of the behavioural and developmental parameters throughout their life time. While from the TSWV N12 which was successfully transmitted, the resulting adults were used to evaluate transmission efficacy between males and females (described below).

3.3.5 Experimental procedure and treatments

Exposed treatments refer to adults that fed on TSWV infected *C. annuum* leaflets throughout their larval stages, while unexposed fed on virus free leaflets (representing the control) (described above). Males and females *F. occidentalis* exposed to the three TSWV isolates, T1, T2 and T3 together with the unexposed individuals T4, were tested for survival time, development time as well as feeding behaviour. Mean and median comparison were made between males and females of *F. occidentalis* within each individual treatment (T1, T2, T3 and T4) as well as between the exposed (T1, T2 and T3) and the unexposed (T4) treatments, this was carried out simultaneously. However, for the transmission efficiency experiment, the comparison between males and females was done independently from other experiments using only *F. occidentalis* exposed to the TSWV N12 isolate by comparing the virus content per leaf disk inoculated by individuals after a 36 hours IAP, and a 3 days incubation period. A completely randomized design was used in all the experiments, where by the individuals were randomly assigned to the different treatments; either exposed or unexposed to TSWV, and comparison made between males and females. All the experiments were repeated three times. The experiments were carried out in a climate chamber at constant conditions of 25±2 °C, 60-70% r.h. and L16:D8.

3.3.6 Survival time

For comparison of survival time between males and females, 20 individuals (10 males and 10 females) were randomly selected on the last days of pupal development, from each of the four treatments (exposed (T1, T2 and T3) and unexposed (T4)), and individually transferred onto virus free leaf disks, which were replaced at an interval of one day. After adult emergence, daily

observation was done recording the individuals' survival time in each treatment throughout their lifetime, followed by statistical analysis. For the cumulative mortality evaluation, only 15 days survival time was used, as beyond that, mortality could have resulted from other factors apart from the treatments. The differences between sexes were compared within each treatment, as well as between the exposed and the unexposed. The experiment was repeated three times.

3.3.7 Development time

For comparison of the development time between males and females of *F. occidentalis*, 10 individual pupae (5 males and 5 females) were randomly selected from each of the four treatments and singly transferred onto virus free leaf disks until adult emergence, and the time taken from L1 larvae (from the time of exposure to the different treatments) to adult emergence was recorded for each individual. A comparison was made between sexes within each treatment and also between the exposed and the unexposed treatments. The experiment was repeated three times.

3.3.8 Feeding behaviour-manual scoring of scars made by individual thrips

L1 *F. occidentalis* of <12 hours old were first reared as described above to obtain exposed and unexposed individuals. This resulted in four groups of males and females from the four treatments. The resulting adults were individually reared on virus free leaf disks (Ø 17 mm) placed in gypsum petri dishes. 5 individuals were tested for each group; visual observations of the feeding scars (feeding intensity) per individual were recorded 2 and 5 days after placement (Fig. 3.1). Manual scoring of the feeding scars was done based on our predetermined scoreboard (Table 3.1) and comparison between males and females was made within each treatment and between the exposed and the unexposed treatments. The experiment was repeated three times.

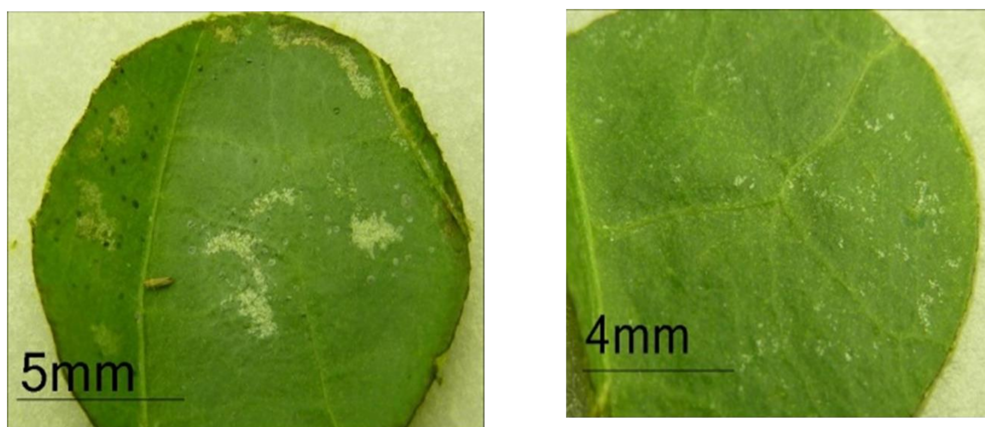

Figure 3.1: Feeding scars on leaf disks (size Ø 17 mm each); figure A; shows feeding scars caused by individual female after 2 days feeding period, and B; shows feeding scars caused by individual male after 5 days feeding period.

Table 3.1: Manual score board for the visual observation of feeding scars caused by different sexes.

Score	Estimated % leaf area covered by Scar
0	<1
1	1-15
2	16-30
3	31-50
4	51-70
5	>71%

3.3.9 Comparison of transmission efficiency between males and females

After the acquisition step (as described above but only for TSWV N12), 30 individual pupae were randomly picked and transferred individually onto a healthy leaf disk placed on a gypsum petri dish where they all emerged to adults. The adults were then given an Inoculation Access Period (IAP) of 36 hours on the leaf disk before being transferred onto another healthy leaf disk. The inoculated leaf disks were individually harvested into Eppendorf tubes and then incubated at climate chamber conditions for 3 days. After the incubation period, the virus content per leaf disk was evaluated using an Amplified DAS-ELISA (an improved form of DAS-ELISA in terms of sensitivity (at least 10-fold more) using ELISA Amplification kit from Invitrogen Life Technologies GmbH, Cat. No. 19589-019) which amplifies the ELISA signals (Appendix 2.1), and the individual's transmission efficiency for the exposed males and females determined using optical density readings at 450nm. From the results, at least 70% of the individuals transmitted the virus successfully. Percentage transmitters and non-transmitters were determined for both sexes, and the virus content per leaf disk transmitted by an individual was evaluated. From the results, the median values were then used in the comparison of the transmission efficiency between males and females. The experiment was repeated three times.

3.3.10 Statistical analysis

Survival time, which included longevity and mortality data was analyzed using the Kaplan–Meier survival curve analysis in SPSS version 15.0 Inc. (Chicago, IL). To compare the median and means for gender differences per treatment, Log-rank (Mantel-Cox) test was used. For the development time, feeding intensity, general comparison of 15 days cumulative mortality between males and females and the transmission efficiency experiments, as well as the comparison between the exposed and the unexposed treatments, data were analyzed statistically using PROC GLM procedures in Statistical Analysis System 9.0 for Windows (SAS/STAT Software, 2002). The significant factor effects were detected using ANOVA and the means

separated using Tukey's multiple means comparison procedure. A significance level of P = 0.05 was used in all analysis.

3.4 Results

3.4.1 Survival Time

Comparison of the survival between males and females of *F. occidentalis* showed significant differences regardless of whether they were exposed or unexposed to TSWV, with males having longer survival time than the females (Log rank; T1 $\chi^2_{(d.f.=1)} = 12.6$ $P < 0.0001$, T2 $\chi^2_{(d.f.=1)} = 10.45$ $P = 0.001$, T3 $\chi^2_{(d.f.=1)} = 14.24$ $P < 0.0001$, and T4 $\chi^2_{(d.f.=1)} = 8.66$ $P = 0.003$) (Fig. 3.2). Moreover, the cumulative mortality for a period of 15 days across all the treatments was significantly different between males and females ($F_{1,47} = 76.01$, $P < 0.001$), with females showing significantly higher mortality than males (Fig. 3.2). Both males and females in the exposed treatments (T1, T2 and T3) lived longer than the ones in the unexposed treatment (T4) ($\chi^2_{d.f.=3} = 23.79$ $P < 0.001$) despite the different TSWV isolates in the exposed treatments.

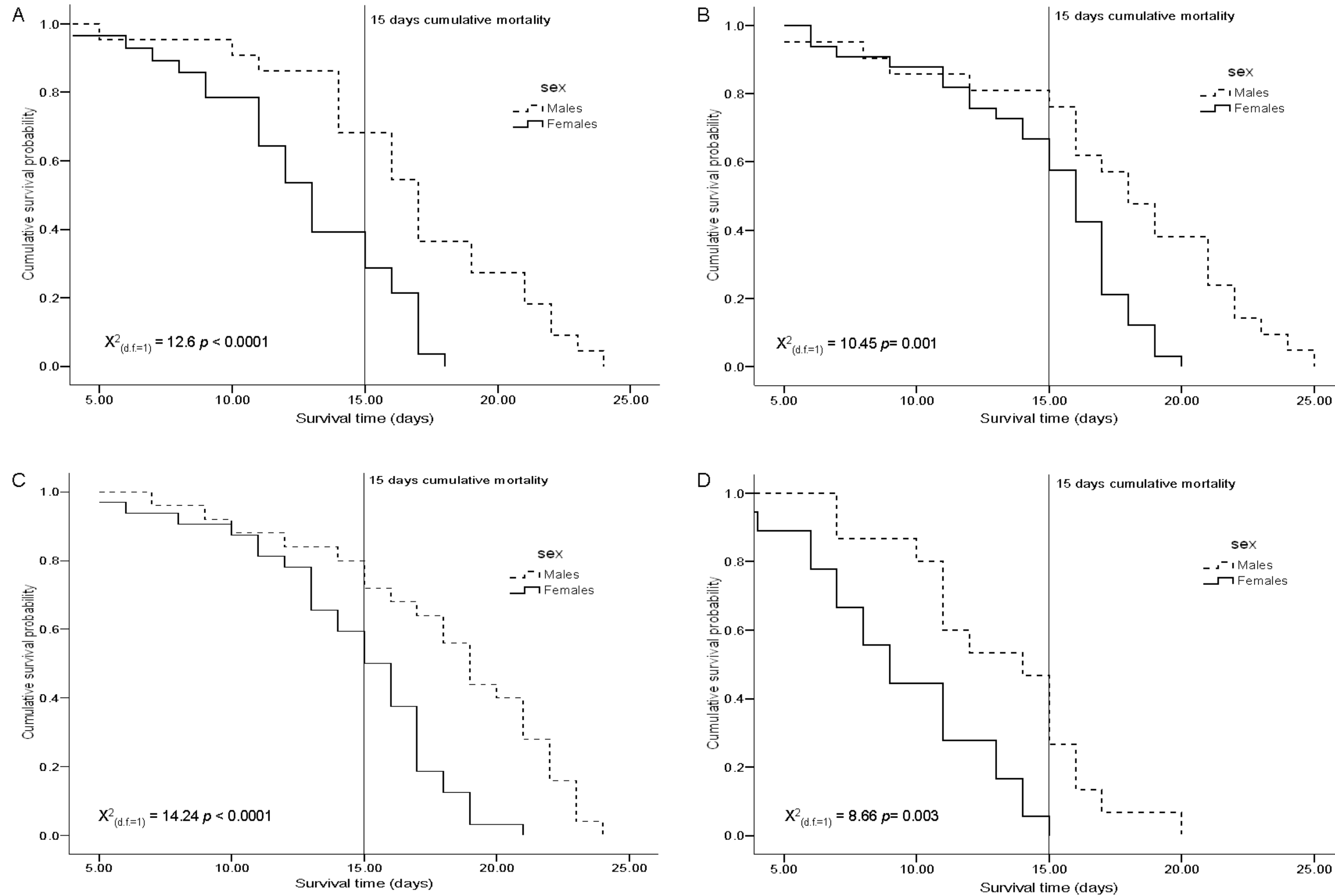

Figure 3.2: Comparison between male and female *F. occidentalis* survival time and 15 days mean cumulative mortality within each treatment. Letters A, B, C & D represent the treatments T1, T2, T3 & T4 respectively. Kaplan-Meier Survival; Means and medians compared between males and females per treatment using Log rank at significance level of $P = 0.05$.

3.4.2 Development time

The difference in development time between males and females of *F. occidentalis* was evaluated in each treatment, and a comparison was also made between the exposed and the unexposed treatments. Significant effects were obtained for sex and treatments, however, there was no interaction between the two factors ($F_{3,239} = 2.24$, $P = 0.0843$). The results show that males had significantly shorter development time compared to females ($F_{1,239} = 166.22$, $P < 0.0001$), regardless of the treatment (Fig. 3.3). Additionally, some males were observed to emerge 1 to 2 days earlier than females. When comparing the treatments exposed and non-exposed to TSWV, the development time of both males and females in the exposed treatments was significantly shorter, (on average females = 9.02 days and males = 7.89 days) compared to their un-exposed counterparts (on average females = 10.42 days and males = 8.82 days) ($F_{3,239} = 31.41$, $P < 0.0001$), again no discrepancy between the different TSWV isolates in the exposed treatments could be found.

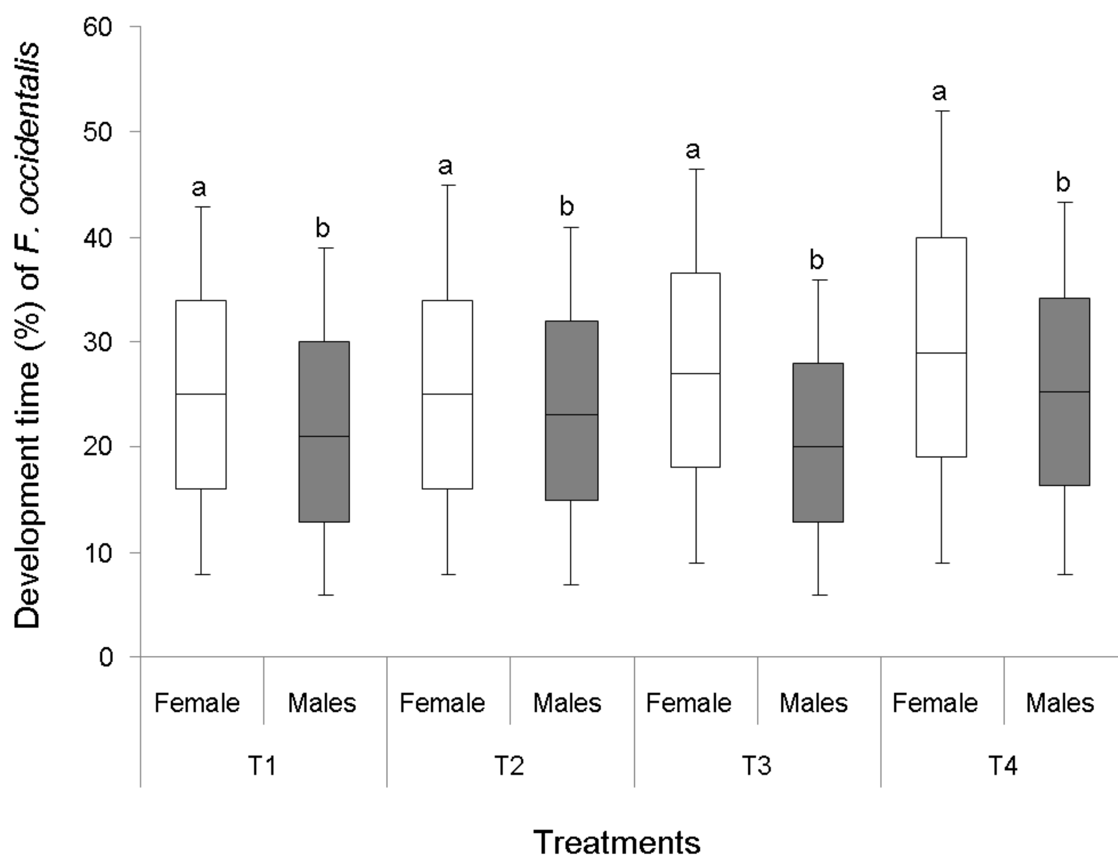

Figure 3.3: Box and whisker plot of development rate in days, comparison between males and females per treatment (T1, T2, T3 & T4). Box plots with different letters indicate significant difference. (ANOVA; Means compared using Tukey test, $P = 0.05$).

3.4.3 Feeding intensity Manual scoring of scars from male or female *F. occidentalis*

A difference in feeding intensity (the measure of scars caused by an individual *F. occidentalis* on a leaf during feeding) between males and females was quite obvious by visual observation. Females produced much more extensive scars in comparison to males with more frequent small punctures (Fig. 3.1). Statistical analysis of estimated leaf disk area covered by scars (manual scoring - Table 3.1) after an individual feeding for the given time points (2 and 5 days), revealed significant differences between females and males, with females causing significantly more intense injuries on the leaf disk during feeding than males ($F_{1,239} = 324.81$, $P < 0.001$) (Fig. 3.1). This was replicated in all the four treatments, regardless of whether *F. occidentalis* adults were exposed or unexposed to TSWV in their larval stages (Fig. 3.4). Further comparison between the exposed and unexposed treatments, a significant difference was observed (Table 3.2). Males and females exposed to TSWV, regardless of the isolate, fed slightly more intensive compared to their unexposed counterparts. Also, the longer an individual stayed on the leaf disk, the more significant was the damage caused (Fig. 3.4; Table 3.2).

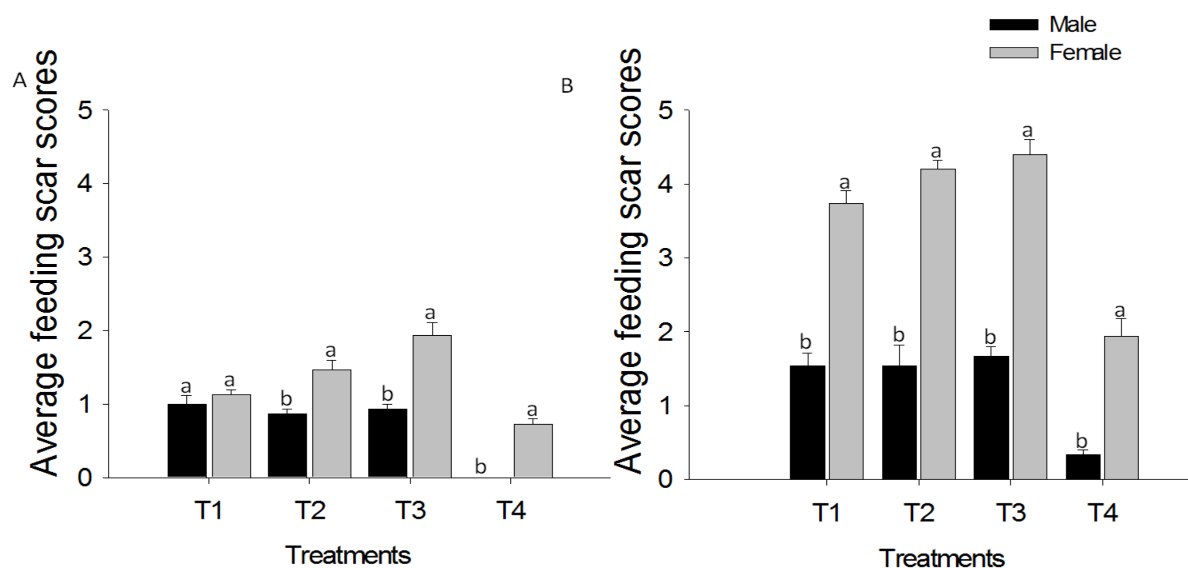

Figure 3.4: Comparison of percentage leaf area damaged between males and females; figure A, shows after 2 days feeding period, and B shows after 5 days feeding period for *F. occidentalis*, per treatment (T1, T2, T3 & T4). Bars with different letters indicate significant difference. (ANOVA; Means compared using Tukey test, $P = 0.05$ N = 15).

Table 3.2: Tukey test results for feeding intensity test. Comparison of feeding scars produced by males and females *F. occidentalis*, either exposed or unexposed to TSWV, for a period of 2 and 5 days. Significant difference between the sexes at $P = 0.05$. ns non-significant, * $P <0.05$, ** $P <0.01$, *** $P <0.001$.

Feeding intensity test			
Source	**DF**	**mean of sq**	**Pr>F**
Treatment	3	26.18	***
Sex	1	127.60	***
Days	1	119.00	***
Treatment*Sex	3	1.84	**
Treatment*Days	3	2.79	***
Sex*Days	1	42.50	***
Treatment*Sex*Days	3	1.20	*

3.4.4 Comparison of Transmission Efficiency between Males and Females

Successful transmission of TSWV by *F. occidentalis* was determined using the intensity of virus load detected per leaf disk fed on by individual adults that had been exposed to TSWV in their early larval stages. This was valued in terms of optical densities obtained from ELISA readings minus the minimum threshold (the average value of the non-infected controls, plus three times their standard deviation). Females had the highest percentage of transmitters compared to males (Table 3.3). However, comparison of the virus load detected per leaf disks showed that males had the highest median values compared to females (Fig. 3.5). Statistical evaluation using ANOVA showed males having significantly higher transmission efficiency than females ($P = 0.0478$), in terms of the intensity of virus load detected per leaf disk. The variation within the range of the analyzed values was observed to be wide in both cases, especially above the threshold, with females having the widest range (Table 3.3).

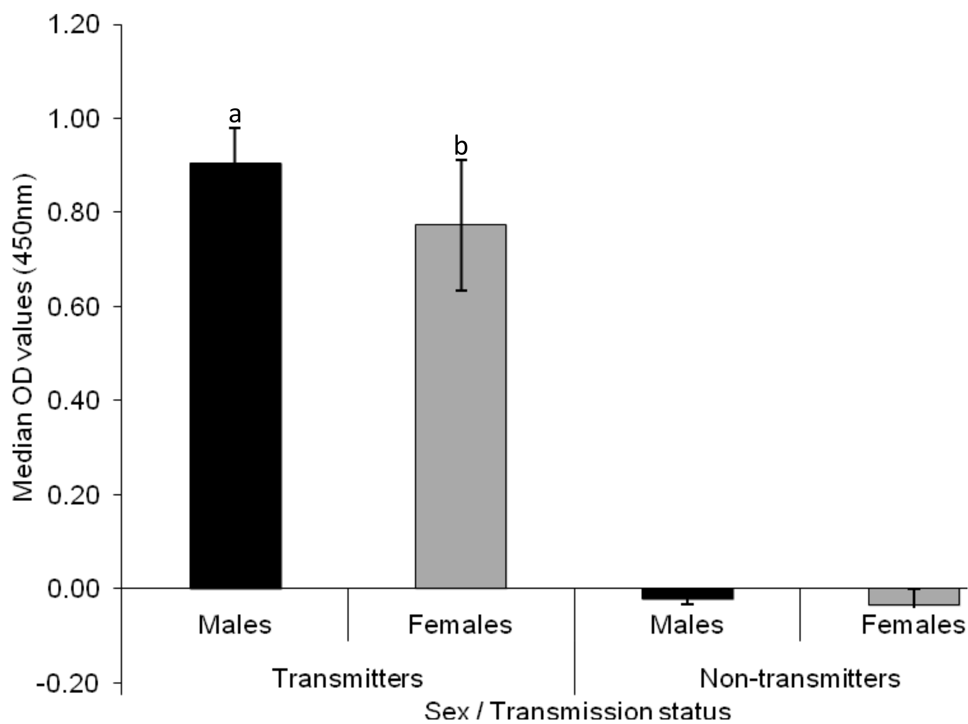

Figure 3.5: Transmission efficiency results from Amplified ELISA evaluation at Optical Density (OD) value of 450 nm, comparison of median values of the detected virus load per leaf disks between males and females *F. occidentalis*. X-axis = Threshold (the average value of the negative controls plus 3 times their standard deviation), Means compared at significance level of $P = 0.05$.

Table 3.3: Analysis of the transmission efficiency between males and females *F. occidentalis* individuals after 36 hours inoculative feeding on leaf disks. Comparison made between the sexes on; percentage transmission, average OD values, median and range comparison between the transmitters and the non-transmitters. Different letters within the mean and the median column indicate significant difference at $P = 0.05$. The average value of the non-infected controls, plus three times their standard deviation made the minimum threshold for the OD values

Transmission efficiency analysis				
Sex /Status	**% transmission (Total) per gender**	**OD Values (minus the Minimum threshold) (450 nm)**		
		Mean OD	**Median**	**Range**
Male Transmitters	31%	1.02 a	0.9037 a	0.50- 1.76
Female Transmitters	40%	0.82 b	0.773 b	0.29-1.58
Male Non-transmitters	18%	-0.043	-0.022	-0.003-0.148 (-)
FemalesNon-transmitters	11%	-0.11	-0.036	-0.0015-0.327 (-)

3.5 Discussion

This study on the influence of vector gender in the virus-vector-plant interaction, an attempt to understanding the intraspecific variation in the transmission of TSWV by adult *F. occidentalis*, was prompted by several reports which have shown that the efficiency of transmission of TSWV is highly variable within *F. occidentalis* populations. This variability in transmission efficiency may result from different abilities between sexes of *F. occidentalis* to acquire and transmit the tospoviruses (van de Wetering *et al.*, 1998; van de Wetering *et al.*, 1999b; Rotenberg *et al.*, 2009). Another reason could be that vector competence is genetically determined as reported for other thrips species (Halaweh and Poehling, 2009; Cabrera-La Rosa and Kennedy, 2007). The differences between males and females in terms of feeding behaviour and scar production (Sakurai *et al.*, 1998; van de Wetering *et al.*, 1999b) have also been reported to play a role in the transmission variability as well as influencing virus spread, hence the ratio of males and females in terms of the composition of the vector population, is quite important (Inoue and Sakurai, 2006). Furthermore, by variation of specific allele frequencies in a population due to the haploid-diploid inheritance system (Crespi *et al.*, 1991; Moritz, 1997), phenotypic variation (vector competence) in subsequent generation can be expected (Gillings *et al.*, 1995; van de Wetering *et al.*, 1999a).

Our results show that males of *F. occidentalis* had significantly longer survival time compared to females regardless of the treatment, i.e. whether exposed or unexposed to TSWV. This was confirmed by higher mortality in females compared to males. This observation is not common in insect populations, since very often males have shorter life span compared to females because they are only “needed” for insemination of the females. However, this could be as a result of our special experimental conditions, as males and females were reared individually, and thus the males did not spend their energy on searching and inseminating the females, whereas females invested in intensive reproduction, and therefore the female body was subjected to higher stress compared to males. Additionally, males were also observed to emerge 1 to 2 days earlier in comparison to females, contributing to their longer survival period as adults. This characteristic is wide spread among insects, and is referred to as “protandry” (Rhonda R. Snook, 2014; Alcock and Thornhill, 2014), a mechanism which increases the encounter rate with females and therefore reproductive success (Wiklund and Fagerström, 1977; Singer, 1982; Wiklund and Christer Solbreck, 1982). Terry and Schneider (1993) reported that males of *F. occidentalis* inseminated several virgin females in a period of 1 or 2 hours after female eclosion, and there after females have periods of several days in which they reject males in their attempt to copulate, reducing their mating chances as adults. This suggests that males are facing an

intensive competition with co-specific males "fighting" for receptive females, possibly accompanied by a higher dispersal activity (Mateus *et al.*, 2003).

We also observed that males of *F. occidentalis* were able to copulate with the last stages of female pupae (results not shown), hence the early emergence of males before females give them even more advantage as they experience absolutely no "resistance" by the females during mating process. Moreover, (Bryan and Smith, 1956) observed that females of *F. occidentalis* are relatively inactive immediately before oviposition, therefore giving the males another "window" to mate with less resistance from the females. This together with the early mating could also explain the always higher number of females in a population compared to males, since only a few females will "escape" insemination and develop as reproductive virgins producing only male offspring (Higgins and Myers, 1992). However, up to now, there have been no quantitative studies in *F. occidentalis* in this regard. Generally, independent from the gender effects, we could also confirm our earlier findings (Ogada *et al.*, 2013), which we reported a significant increase in longevity and lower mortality of *F. occidentalis* exposed to TSWV compared to the unexposed ones. A triggered immune response has been suggested to be responsible for the increased fitness after exposure to TSWV (de Medeiros *et al.*, 2004). These mutualistic effects of microorganism and their host are also described for other herbivorous insects. For instance, there are reports on enhanced internal defence against toxic compounds and parasites as a result of the presence of mutualistic gut bacteria, as indicated by Dillon *et al.* (2000) about symbionts that eliminate pathogenic fungi from the gut of the desert locust. Erwinia species in the gut of *F. occidentalis* have been described to improve host longevity (de Vries *et al.*, 2001, 2004), this could also be related to the improved immunity effect of the symbiotic relationship, as this gut bacterium is acquired and transmitted via feeding on the host plant by the *F. occidentalis* larvae.

Further comparison of the development rate between the two sexes in all the four treatments clearly showed a significant difference between males and females, with males having a shorter development period compared to females. Subsequently, exposing *F. occidentalis* to TSWV caused a significant reduction in the development time for both males and females compared to their unexposed counterparts. This is in line with the findings of Stumpf and Kennedy (2007) who also reported a reduction in development time in TSWV infected males compared to the uninfected. However, in their study the effect was not the same for female *F. occidentalis*, which contradicts our findings as well as earlier findings (Belliure *et al.*, 2005) which report an increase in development rate in both males and females exposed to TSWV compared to the unexposed ones.

Based on visual observations of feeding behaviour we could show that individual females fed more intensively producing significantly numerous scars to the plant material compared to their male counterparts in all the treatments and at all time periods of observation (2 and 5 days). This agrees with findings by Stafford *et al.* (2011), Kindt *et al.* (2006) and van de Wetering *et al.* (1998), using electrical penetration graph (EPG) recording of feeding intensity and image analysis of feeding scars. van de Wetering *et al.* (1998) pointed out that scar production and TSWV transmission are not correlated. Previous studies (Broadbent and Allen, 1995) also reported negative correlations between scar productions and TSWV transmission. Based on the resulting damages and the quantification by EPG, the feeding behaviour of *F. occidentalis* could be classified as either penetrative or shallow feeding (Stafford *et al.*, 2011; van de Wetering *et al.*, 1998; Kindt *et al.*, 2006; Sakimura, 1962). Feeding on mesophyll cells, resulting in an extreme plasmolysis or complete disappearance of cells is categorized as penetrative feeding, which leads to a silvery, scarred appearance of the affected tissue as a result of replacement of the empty cells with air. On the other hand, shallow feeding is primarily restricted to epidermal tissues or to a few adjacent mesophyll cells. This involves thrips making a number of feeding probes, which result in tiny silvery patches, but without obvious scarring of the plant (Chisholm and Lewis, 1984). From our observations during the experiments, males could be classified as shallow feeders and females more as penetrative feeders, based on their feeding styles as seen in Fig. 3.1. A good condition of the cells is a prerequisite in the cell to cell movement of the virus within the host plant, supporting the initial establishment and the spread of the virus to other cells, and thus the damage caused by the females during feeding may arrest the spread of this virus to the neighbouring cells (van de Wetering *et al.*, 1998) lowering distribution and establishment efficiency. This could be the potential explanation for the significantly low virus titer we found per leaf disk fed on by individual females compared to males in the transmission efficiency study. The difference in feeding intensity can be attributed to the low mobility and high consumption rate of females needed for the biomass for reproduction (eggs) as well as their large body sizes compared to males (van de Wetering *et al.*, 1998). The clear differences in feeding behaviour between males and females of *F. occidentalis*, and the negative correlation to the transmission of TSWV is a crucial indication of the importance of considering the sex-ratio of thrips in the prediction and evaluation of TSWV dynamics. Moreover, Rotenberg *et al.* (2009) reported a significantly higher virus titer in individual females than in males, but higher transmission efficiency in males than females. With this they concluded that successful virus transmission is dependent on other factors apart from the absolute virus titer in the insect. Furthermore, previous studies on the effect of TSWV on feeding behaviour indicated that

TSWV infected males fed more than uninfected males (Stafford *et al.*, 2011). The frequencies of feeding increased by up to threefold, as well as the number of non-ingestion probes, therefore increasing the probability of TSWV inoculation. Our results also show the same trend when we compare both males and females of *F. occidentalis* from all the exposed treatments to their unexposed counterparts.

A split sex ratio in the population as a result of haplodiploidy (Crespi *et al.*, 1991; Moritz, 1997) could be a major factor for the instability in the transmission efficiency of TSWV in *F. occidentalis* population, since the ratio varies between males and females per generation. The existing biases among adult sex ratios could also be a function of other factors such as differences between the sexes in their dispersal, distribution in response to host quality, and longevity (Reitz, 2009) among others. These biological and ecological attributes of *F. occidentalis* make it one of the most significant and difficult to manage pest in the world and thus supporting TSWV epidemic.

From this study, we could conclude that male bias in the transmission of TSWV by *F. occidentalis* can be attributed to their ability to survive longer, their shorter development time, greater efficiency in virus transmission due to non-ingestion probing in their feeding behaviour, and their higher mobility, compared to the females. These are important attributes of thrips behaviour controlling efficacy of virus transmission and ratio of virus competent genotypes in populations.

4 Manipulation of *Frankliniella occidentalis* by *Tomato spotted wilt virus* (Tospovirus) via the host plant nutrients to enhance its transmission and spread

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4.1 Abstract

Earlier studies have shown that *Tomato spotted wilt virus* (TSWV) influences the biology, performance and behavioural patterns of its vector *Frankliniella occidentalis*. In this study, using *Capsicum annuum* as the host plant, we aimed to determine the manipulation of *F. occidentalis* by TSWV through switching of the diet (+ or - TSWV) during vector's development. Behavioural patterns, fitness as well as vector performance were evaluated. The specific parameters investigated included; longevity/survival, fecundity, development time, feeding and preferential behaviour. *F. occidentalis* were reared on either TSWV infected (exposed) or healthy leaves (non-exposed) throughout their larval stages. The emerging adults were then individually transferred to either healthy or TSWV infected leaf disks. This resulted into four treatments; consisting of exposed or non-exposed thrips reared on either infected or healthy leaf disks as adults. All *F. occidentalis* exposed to TSWV in their larval stages had shorter development time regardless of the adults' diet. While the ones that were later reared on healthy leaf disks as adults recorded the highest longevity and reproduction rate. Furthermore, adults of *F. occidentalis* that were exposed to TSWV in their larval stages showed preference towards healthy leaf disks (-TSWV), while the non-exposed significantly preferred the infected leaf disks (+TSWV). These are further indications that TSWV modifies the nutritional content of its host plants, which influences vector's biology and preferential behaviour, in favour of its multiplication and dispersal. The findings offer additional explanation to the often aggressive spread of the virus in crop stands.

Key words: *Frankliniella occidentalis*, Tospovirus, *Tomato spotted wilt virus*, Vector manipulation hypothesis.

4.2 Introduction

Tomato spotted wilt virus (TSWV) is a member of the genus *Tospovirus*, the only plant infecting genus in the family *Bunyaviridae* (van de Wetering *et al.*, 1999b; Whitfield *et al.*, 2005). TSWV has a wide host range and worldwide distribution (Sherwood *et al.*, 2000; EPPO/CABI, 1997). This virus can lead to major economic losses on tomato, lettuce, pepper, papaya, eggplant, French beans, artichokes, broad beans, celery and different ornamental plants (Roselló *et al.*, 1996). It is transmitted exclusively by thrips, with *Frankliniella occidentalis* (Pergande): commonly known as western flower thrips, being the most efficient vector due to its equally large host range and worldwide distribution (Wijkamp *et al.*, 1995; Nagata and Peters, 2001; Whitfield *et al.*, 2005).

F. occidentalis acquires the virus during feeding by sucking cell contents from infected plants. Transmission of TSWV is in a persistent and propagative manner (Wijkamp *et al.*, 1996; Maris, 2004), and virus replication within the vector is essential for high transmission efficacy. The virus is passed from the midgut lumen through the midgut cell wall and finally to the salivary glands via cell to cell movement (Ullman *et al.*, 1992; Whitfield *et al.*, 2005). Presence of non-structural proteins (NSs) and viral inclusions in thrips body tissues and the primary salivary glands are indications of replication of the virus in the vector (Ullman *et al.*, 1992, 1993, 1995; Wijkamp and Peters, 1993). Mainly adults (and partly the second instars) can transmit the virus, but only if acquired in the early larval stages. The transmitting adults can vector the virus throughout their lifetime with the highest efficacy immediately after emergence and thereafter, at a slightly lower but steady rate (Wijkamp *et al.*, 1996; Chatzivassiliou *et al.*, 1999).

Viruses are reported to modify the behaviour of their vectors with both direct and plant-mediated indirect effects, consequently enhancing their transmission. As the vectors are mobile and can show preferences depending on host plant infection status, virus spread within or between crop stands depends on the motivation of the vector for dispersal as well as its host selection behaviour (preferences), therefore, plant-mediated indirect virus manipulation of the vector biology and behaviour could change transmission and disease dynamics (Fereres and Moreno, 2009; Jeger *et al.*, 2004). Such mutual effects are reported as basically important in plant virus ecology and epidemiology (Jones, 2014), and examples involving different groups of herbivorous and virus vectoring insects are already published. For instance, a study by Ingwell *et al.* (2012) reported that acquisition of a plant virus (*Barley yellow dwarf virus*) can directly alter host plant selection behaviour of its vector the cereal aphid (*Rhadopalosiphum padi*). This induced preferential behaviour can increase the virus spread, as non-infected aphids

preferred to feed on infected plants while infected aphids preferred the non-infected plants. Also the exposure of *F. occidentalis* to TSWV has been reported to influence the vector's behaviour as well as fitness, for example; increased feeding rate, higher survival, shorter development time and reduced fecundity (Sakimura, 1963; Robb, 1989; Wijkamp *et al.*, 1996; Maris *et al.*, 2004; Stafford *et al.*, 2011; Ogada *et al.*, 2013). Moreover, some studies have demonstrated a triggered immune response in the vector after exposure to the virus (de Medeiros *et al.*, 2004), but energy costs due to virus replication which led to a kind of trade-off between vector longevity and performance in terms of reproduction (Ogada *et al.*, 2013). Contradicting results reported on the effects of tospoviruses on survival, reproduction, development and feeding of thrips, may have arised from the complex patterns of interaction within the triangle TSWV-plant-vector which could be influenced by several factors such as the suitability of the host plant, virus isolate, vector gender, environmental factors, genetic constitution of individual thrips, feeding behaviour and also the interactions between these factors (Stumpf and Kennedy, 2005, 2007; Ogada *et al.*, 2013) . Furthermore, infection of host plants with TSWV can be of an advantage to *F. occidentalis* due to lowered plant defense potential against the feeding vector, resulting in longer survival due to the readily available nutrients (Belliure *et al.*, 2005, 2008, 2010; Blua *et al.*, 1994; Awmack and Leather, 2002; Ogada *et al.*, 2013). As a result, TSWV not only benefits from thrips hosting and providing the resources for its propagation, but "actively" improves vector efficacy for its own dispersal to a new host plant.

The aim of this study therefore, was to determine TSWV manipulation of *F. occidentalis*, by subjecting *F. occidentalis* to a diet switch (+ or - TSWV) during its development. Then evaluating performance and behavioural changes, focusing on development time, survival (longevity and mortality), reproduction and preferential behaviour, with the purpose of elucidating the importance of these virus induced vector's life processes in the multiplication and dispersal of the virus in a crop stand, and hence developing precise predictive models for the disease as well as control strategies of both the virus and the vector.

4.3 Materials and Methods

4.3.1 Host plant

The host plant used for all the experiments with *F. occidentalis* and for TSWV propagation, was 3-4 weeks old *Capsicum annuum* L. (Solanaceae), which is a preferred and important host for *F. occidentalis* and susceptible to TSWV by thrips transmission as well as mechanical inoculation. *Nicotiana benthamiana* L. (Solanaceae) (3-4 leaf stage) served as a reservoir plant for TSWV maintenance, only because it is easy to handle for mechanical inoculation. The plants

were maintained at green house conditions (28-30 °C and 70-80% relative humidity (RH)). *Phaseolus vulgaris* L. plants and pods were used for the maintenance of *F. occidentalis* in stock culture and synchronized rearing respectively.

4.3.2 Thrips culture

Isolate of *F. occidentalis* (F.o 2) was obtained from Wageningen University, the Netherlands. A virus free stock culture was established on bean plants (*P. vulgaris*) at 2-3 leaf stage in a thrips proof cage maintained in climate chamber conditions. For synchronized rearing adults from the stock culture were kept on bean pods supplemented with a commercial honey-bee pollen mixture (Naturprodukte-mv.de; Naturprodukte Lembcke, Faulenrost, Germany) in glass jars, closed on top with 64µm thrips-proof nylon net. The bean pods were replaced at an interval of one day, and the harvested pods with eggs transferred to new glass jars for the emergence of larvae. *F. occidentalis* used in all the experiments were from the synchronized rearing. Both the stock culture and the synchronized rearing were maintained in separate climate cabins completely isolated from any other sources of thrips at 25±2 °C, 60-70% (RH) and 16:8h Light: Dark (L: D) photoperiods.

4.3.3 *Tomato spotted wilt virus* isolates and mechanical inoculation

The TSWV isolate (TSWV-N12) was also obtained from Wageningen University, the Netherlands, and mechanically inoculated onto 3 weeks old *C. annuum* as a host plant, as well as on *N. benthamiana* as a reservoir plant. For mechanical inoculation of TSWV, protocol developed by Mandal *et al.* (2008) was used. It involves a chilled inoculum consisting of: infected leaf sap in 0.1 M phosphate buffer, 0.2% sodium sulphite and 0.01M 2-mercaptoethanol and 1% each of Celite 545 and Carborundum 320 grit. Application to the host plant was done using a soft finger rubbing technique. After a period of 10-14 days incubation, the first visual symptoms appeared and samples of these plants were tested using a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to confirm successful infection by TSWV. The infected plants were maintained at 28-30 °C (greenhouse condition) and served as inoculum source for further series of mechanical inoculation.

4.3.4 TSWV acquisition by *F. occidentalis*

For TSWV acquisition by *F. occidentalis*, infected leaflet of *C. annuum* was placed in a petri dish (Ø 15 cm) with a gypsum ($CaSo_4$ and charcoal,9:1 ratio) layered base. The gypsum layer was then moistened with distilled water followed by placing a filter paper on top to absorb excess water. Afterwards, healthy or infected leaflet was placed on the filter paper and newly hatched larvae were blown softly onto the leaflet for an acquisition access period (AAP) until

pupation. The lid of each petri dish had three equally spaced Ø 12 mm punched holes covered with thrips proof 64 µm nylon mesh for ventilation. The sides of the petri dishes were additionally sealed with Parafilm M® (Pechiney Plastic Packaging, Inc., USA) to avoid any escape of *F. occidentalis*. 4-6 days after AAP (before adults' emergence), pupae in their last stages were transferred individually onto new *C. annuum* leaf disks (Ø 17 mm). The leaf disks were either virus free (healthy) or infected with TSWV-N12. This method is referred to as the "individual leaf disk assay". Successful acquisition by *F. occidentalis* was checked by testing 5 pupae from each treatment (exposed and non-exposed) using Amplified DAS-ELISA, which is an improved form of DAS-ELISA in terms of sensitivity (by amplifying the signals at least 10-fold more) using ELISA Amplification kit from Invitrogen Life Technologies GmbH (Cat. No. 19589-019).

4.3.5 General setup

Two *F. occidentalis* cohorts were used: The term 'exposed', referring to those that fed on TSWV infected leaflet throughout their larval period only, and 'non-exposed', referring to those that fed on healthy leaflet. This disregarded the adult exposure, as it was only used to distinguish the cohorts as the focus was on the diet combinations during the vector developmental. The virus status of the source plants was controlled by DAS ELISA. The resulting adults were later reared on either virus infected or healthy leaf disks, and the below mentioned parameters evaluated. Leaf disk assay was used in all the experiments.

The following treatments of *F. occidentalis* were used (larvae /adults diet combinations):

1. Non-exposed larvae / adults reared on healthy leaf disks (C-C)
2. Non-exposed larvae / adults reared on TSWV-N12 infected leaf disks (C-N12)
3. Larvae exposed to TSWV-N12 / adults reared on healthy leaf disks (N12-C)
4. Larvae exposed to TSWV-N12 / adults reared on TSWV-N12 infected leaf disks (N12-N12)

Leaf disks for adult maintenance were changed at one day intervals. All the experiments were carried out in climate chamber conditions: 25±2 °C, 60-70% (RH) and 16:8 h Light: Dark (L: D) photoperiod.

4.3.6 Life parameters of *F. occidentalis*

4.3.6.1 Development time

Ten 1day old L1 larvae were randomly selected for each treatment described above. Development time was recorded from L1 to adult emergence for each individual per treatment. This experiment was repeated two times.

4.3.6.2 Longevity and mortality

Ten individuals for each treatment combination were controlled daily and leaf disks changed. Daily mortality was recorded throughout the adult life time, but only 15 days recording was used to calculate cumulative mortality, because beyond this time mortality was not considered to be as a result of treatment effects only. At the same time, longevity, referring to the total number of days an individual lived, was ascertained. The experiment was repeated 3 times.

4.3.6.3 Fecundity

Five virgin females from each treatment combination were left to lay eggs on their respective leaf disks. The daily replaced leaf disks were kept for four days for L1 emergence. The number of emerging offspring was recorded to determine the daily realized fecundity per individual. Total fecundity was also evaluated for each individual female during its lifetime. This experiment was repeated two times.

4.3.6.4 Preferential behaviour

Gypsum petri dishes of Ø 18 cm were prepared by covering the moist bottom with filter paper divided virtually into four equal compartments. For each petri dish, leaf disks punched out of infected plants (TSWV-N12) and non-infected plants (C) were arranged equidistant to the center (Fig. 4.1). Twenty adults of *F. occidentalis*, either exposed or non-exposed to TSWV-N12, were released at the center of each petri dish, then the petri dishes were covered using thrips proof (64 µm) nylon mesh lid, and sealed with parafilm to avoid any escape. After 24 hours, the thrips were anaesthetized using CO_2, and the number of thrips per leaf disk counted. The individuals that were not settled on any leaf disk compartment but were within the petri dish were recorded as ‘Out’. The experiment was repeated three times.

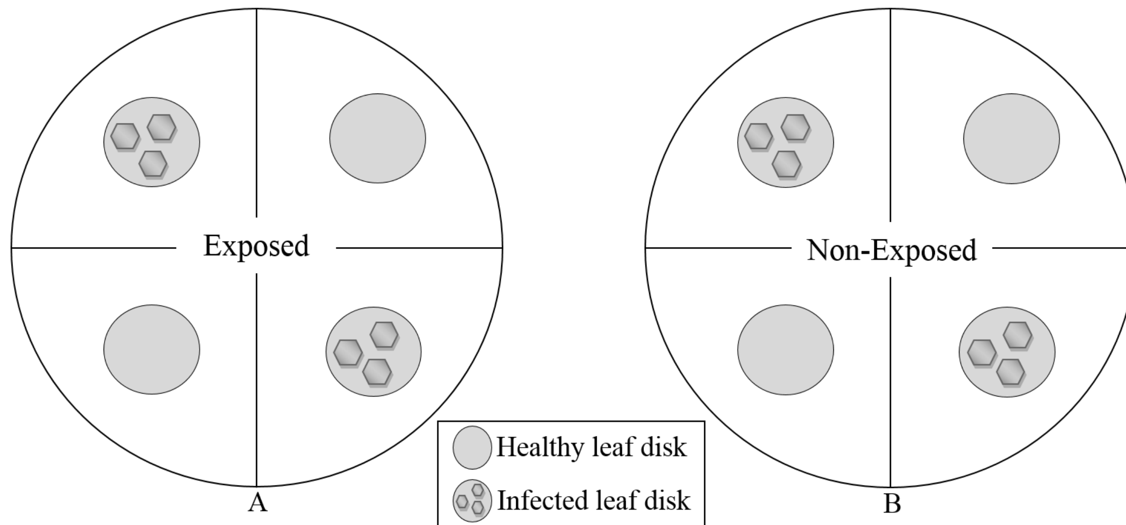

Figure 4.1: Example of leaf disks arrangement in petri dish arena for preference tests. (A) Exposed and (B) non-exposed *F. occidentalis* adults, released at the center of each petri dish containing two virus infected and two healthy leaf disks.

4.3.7 Statistical analysis

Different measurements within each treatment repeats were tested for normal distribution (Shapiro-Wilk normality test at P>0.05). The average value obtained from each repetition in the experiments (response variables): longevity, mortality, total lifetime fecundity, development time and preferential behaviour, were analyzed statistically using two-way analysis of variance (ANOVA, with explanatory variables (factors) being leaf disk virus status (infected vs healthy) and *F. occidentalis* virus status (exposed vs non-exposed). When the interaction between the two factors (second order interaction) was significant, the Sidak correction method (α=0.05) was used to avoid Type I error, followed by a pairwise multiple comparison procedure using Holm-Sidak method in SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA). In all the analysis, level of significance was evaluated at P = 0.05.

4.4 Results

4.4.1 Development time

The results of this experiment revealed a significant difference in development time between TSWV-N12 exposed and non-exposed *F. occidentalis*. All treatments with larval development on healthy plants (non-exposed) differed significantly from the other treatments (exposed) and showed longer development time regardless of the status of the leaf disk they were transferred to as adults; healthy leaf disks (C-C), or infected leaf disks (C-N12) leaf disks ($F_{(1,4)} = 40.33$, P

= 0.003). Additionally, there was no significant difference in development time within the treatment combinations of non-exposed (C-C and C-N12), as well as exposed *F. occidentalis* (N12-C and N12-N12) (Holm-Sidak method, P <0.05). We found no significant interaction between the treatments and the *F. occidentalis*' exposure status ($F_{(1,4)} = 0.333$, $P = 0.595$) (Fig. 4.2).

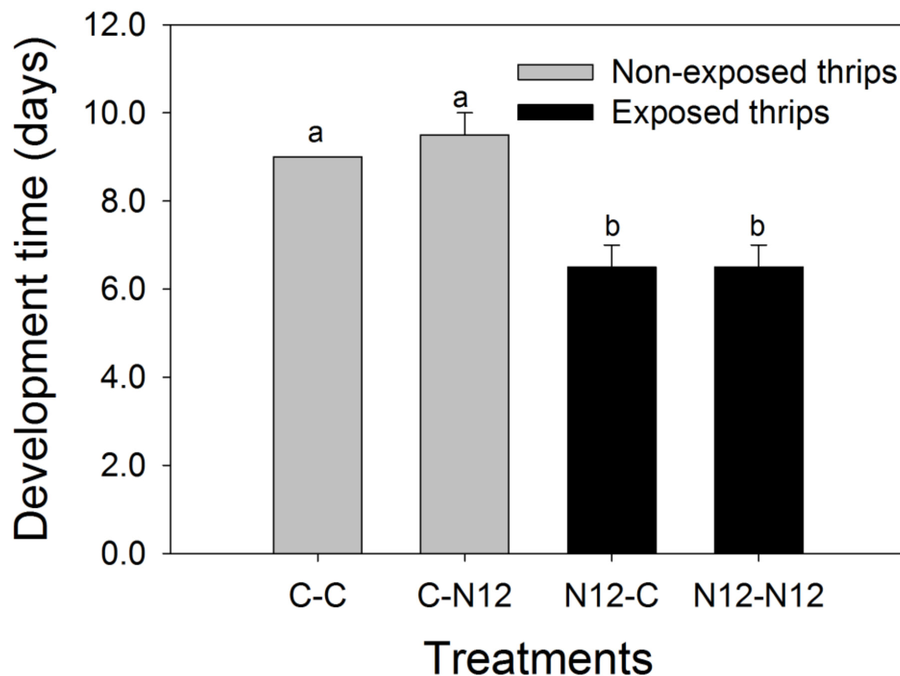

Figure 4.2: Comparison of development time between *F. occidentalis* treatments. That is: Non-exposed reared on healthy leaf disk (C-C), Non-exposed reared on infected leaf disk (C-N12), Exposed reared on healthy leaf disk (N12-C), and Exposed reared on infected leaf disk (N12-N12). Different letters indicate significant differences (Two-way ANOVA, Holm-sidak, P = 0.05, n = 20).

4.4.2 Longevity and survival

Statistical analysis of lifetime longevity of individuals in each treatment revealed significant differences between the exposed and the non-exposed *F. occidentalis* ($F_{(1,8)} = 16.067$, P = 0.004). Longevity of *F. occidentalis* exposed to TSWV-N12 in their larval stages and reared on healthy leaf disks as adults (N12-C) was significantly higher compared to: those that were later reared on TSWV-N12 infected leaf disks (N12-N12), and to the non-exposed individuals that were later reared on healthy leaf disks (Fig. 4.3). The non-exposed *F. occidentalis* lived significantly longer when reared on healthy leaf disks (C-C) as adults compared to the ones that were later reared on TSWV-12 infected leaf disks (C-N12). However, there was no significant difference between the non-exposed and the exposed *F. occidentalis* reared on TSWV-N12 infected leaf disks; C-N12 and N12-N12. There was a significant difference between the mean

values of different treatment combinations ($F_{(1,8)} = 30.761$, $P < 0.001$), but no interaction between the treatment combinations and the *F. occidentalis* exposure status ($F_{(1,8)} = 0.946$, $P = 0.359$).

Also the 15 days cumulative mortality analysis corroborated the longevity results, showing significantly different mortalities in the treatment combinations ($F_{(1,4)} = 16$, $P = 0.0285$). *F. occidentalis* larvae exposed to TSWV-N12 and reared on healthy leaf disks as adult (N12-C), had significantly lower mortality compared to exposed *F. occidentalis* reared on infected leaf disks (N12-N12). There was no significant difference between the exposed *F. occidentalis* reared on infected leaf disks (N12-N12) and non-exposed *F. occidentalis* reared on infected (C-N12). Additionally, there was no significant difference between the non-exposed *F. occidentalis* reared on either infected (C-N12) or non-infected leaf disks (C-C). There was no statistically significant interaction between the treatment combinations and the *F. occidentalis* exposure status ($F_{(1,4)} = 0.53$, $P = 0.488$) (Fig. 4.4).

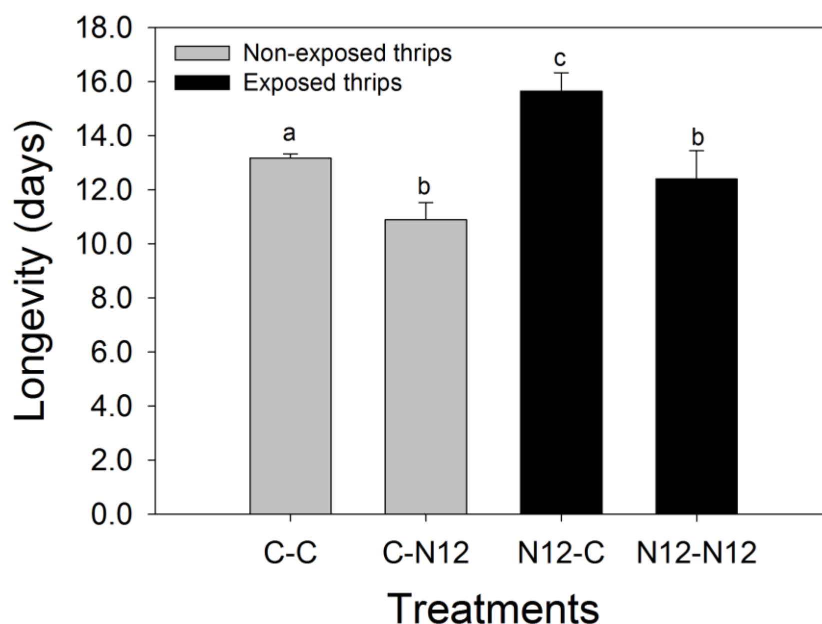

Figure 4.3: Comparison of longevity between four *F. occidentalis* treatments combinations. Non-exposed reared on healthy leaf disk (C-C), Non-exposed reared on infected leaf disk (C-N12), Exposed reared on healthy leaf disk (N12-C), and Exposed reared on infected leaf disk (N12-N12). Different letters indicate significant differences (Two-way ANOVA, Holm-sidak, $P = 0.05$, n = 30).

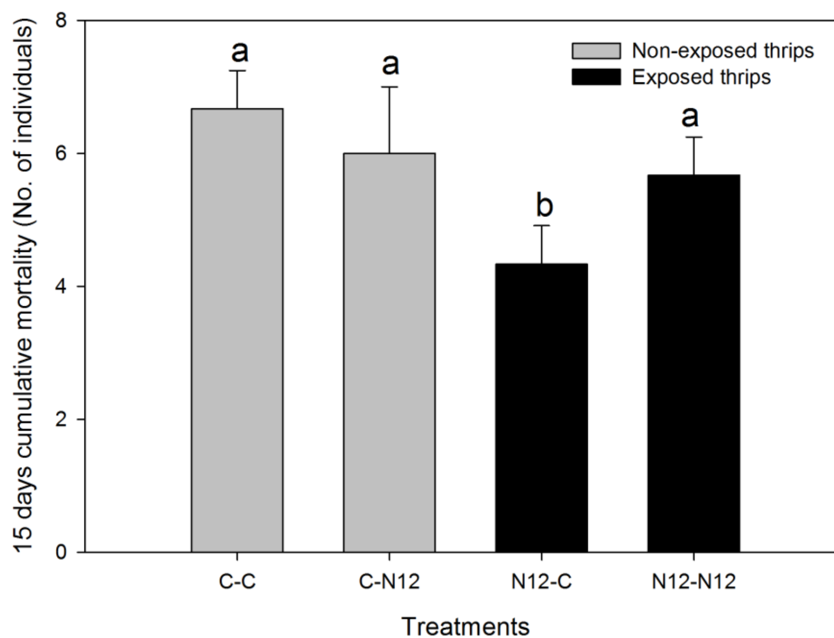

Figure 4.4: Comparison of 15 days cumulative mortality between *F. occidentalis* treatments combinations. Non-exposed reared on healthy leaf disk (C-C), Non-exposed reared on infected leaf disk (C-N12), Exposed reared on healthy leaf disk (N12-C), and Exposed reared on infected leaf disk (N12-N12). Different letters indicate significant differences (Two-way ANOVA, Holm-sidak, $P = 0.05$, n = 30).

4.4.3 Fecundity

Total fecundity throughout the lifetime of individual females was significantly higher in the *F. occidentalis* exposed to TSWV-N12 compared to the non-exposed ($F_{(1,4)} = 673.293$, $P < 0.001$). Also there was significant difference between the treatment combinations ($F_{(1,4)} = 90.962$, $P < 0.001$). Non-exposed *F. occidentalis* larvae that were later reared on healthy leaf disks as adults (C-C) had higher fecundity compared to their counterparts that were later reared on infected leaf disks (C-N12). Also, exposed *F. occidentalis* reared on healthy leaf disks (N12-C) showed significantly higher fecundity than the ones reared on TSWV-N12 infected leaf disk (N12-N12). Additionally, fecundity of exposed *F. occidentalis* was significantly higher when reared on TSWV-N12 infected leaf disks as adult (N12-N12) compared to the non-exposed reared on infected leaf disks (C-N12). Also the fecundity of exposed *F. occidentalis* reared on healthy leaf disks was significantly higher compared to the non-exposed reared on healthy leaf disks (C-C) (Fig. 4.5). The interaction between the treatment combinations and the exposure status of *F. occidentalis* was statistically significant ($F_{(1,4)} = 19.069$, $P = 0.012$).

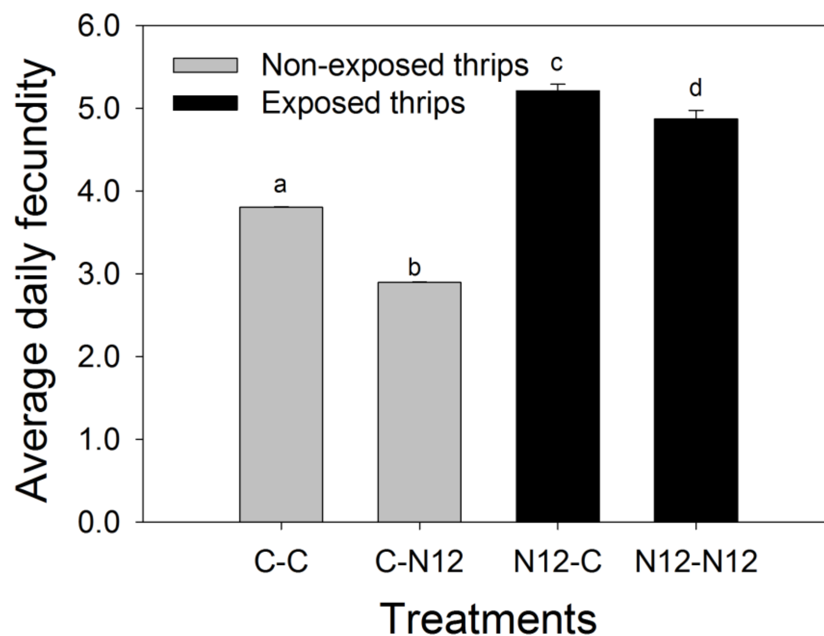

Figure 4.5: Comparison of daily fecundity between *F. occidentalis* treatments combinations. Non-exposed reared on healthy leaf disk (C-C), Non-exposed reared on infected leaf disk (C-N12), Exposed reared on healthy leaf disk (N12-C), and Exposed reared on infected leaf disk (N12-N12). Different letters indicate significant differences (Two-way ANOVA, Holm-sidak, $P = 0.05$, n = 10).

4.4.4 Preferential behaviour

The virus status of the leaf disks significantly influenced the preferential behaviour of *F. occidentalis* adults ($F_{(2,12)} = 18.523$, $P < 0.001$) regardless of their exposure status which had no significant influence ($F_{(1,12)} = 0.000$, P = 1.000). The adults *F. occidentalis* that were not exposed to TSWV as larvae clearly preferred TSWV infected leaf disks (N12) over the non-infected (C), with significantly high numbers being recovered from the infected leaf disks (TSWV) than from the healthy leaf disks (C) or 'Out'. The numbers that were 'Out' were significantly lower than the ones recovered from the healthy (C) or infected leaf disks (N12) (Fig. 4.6). Concurrently, the amount of *F. occidentalis* adults exposed to TSWV in their larvae that were recovered on healthy leaf disks (C) were higher than on the TSWV-N12 infected leaf disks (N12) though the difference was not significant. However, the difference between the numbers recovered on healthy leaf disks (C) and the ones that were not on any leaf disk ('Out') was significant. Despite higher numbers of exposed *F. occidentalis* being recovered on the infected leaf disks (N12) compared to the ones that were 'Out', the difference was not significant (Fig. 4.6). Interaction between the exposure status of *F. occidentalis* (exposed or non-exposed) and the infection status of the leaf disks (healthy or infected) was statistically significant ($F_{(2,12)} = 8.862$, $P = 0.004$) (Fig. 4.6).

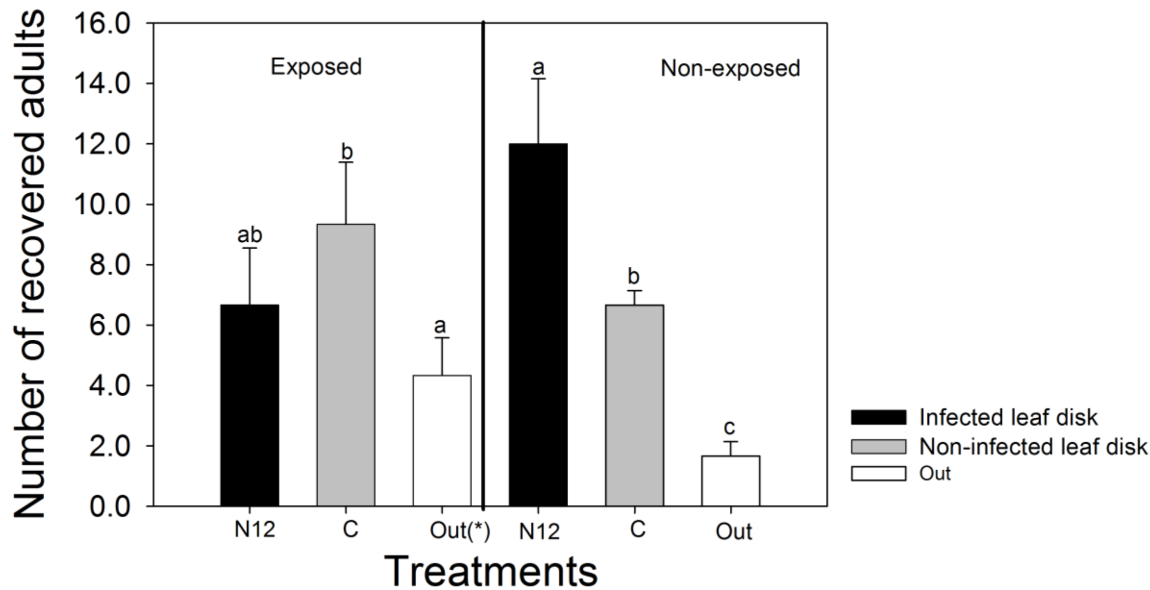

Figure 4.6: Comparison of preferential behaviour of exposed and non-exposed *F. occidentalis* towards TSWV infected (N12) leaf disks. 'Out' refers to thrips that either died or were not found in the designated compartments of the test leaf disks. Different letters indicate significant differences (Two-way ANOVA, Holm-sidak, $P = 0.05$, n = 60).

4.5 Discussion

In this study, several parameters of *F. occidentalis* biology and behaviour were evaluated with respect to the influence of TSWV, by assuming that TSWV induced changes in the nutritional quality of the vector's diet, which was varied during larvae and adult developmental stages. The results of development time study showed that all the *F. occidentalis* (from L1 onwards) that were initially exposed to TSWV had significantly shorter development time than the non-exposed. This may be due to the alleged physiological reactions within the vector and the host plant as a result of the replicating virus. This supports research by Ogada *et al.* (2013) and Maris *et al.* (2004) who reported shorter development time of *F. occidentalis* exposed to TSWV compared to the non-exposed. Furthermore, Awmack and Leather (2002) concluded that plants infected with virus have higher amino acid contents which are especially important for development during the larval period; therefore, we can extend this conclusion to our findings. On the other hand, Wijkamp *et al.* (1996) did not find any influence of TSWV on the development time of *F. occidentalis*; however, they used different virus strains and host plants in their experiments. The importance of diet uptake or quality during the larval period in defining development time is affirmed by the observation that after transfer of the non-feeding pupa to either infected or healthy leaf disks for further development, there were no additional

effects on development time. Therefore, the observed differences could only be related to the vector nutrition during the larval stages.

We also found a significantly higher longevity of individuals exposed to TSWV in their larval stages and later transferred to healthy leaf disk as adults. Ogada *et al.* (2013) also reported similar results, where except for the TSWV isolate, the experimental conditions, host plant and *F. occidentalis* strain were the same. However, this was not the case when the exposed individuals were transferred onto TSWV infected leaf disks as adults. These results show the important influences of the host plant health status (+ or - TSWV), on *F. occidentalis* especially with regards longevity. In general, plants primary metabolism which include amino acids, carbohydrates, sugar, lipids and water content, can be changed due to attack by a pathogen or insect. Nutrients available to the insects, and as a result their survival, can be relatively affected by these changes in the primary metabolism (Stout *et al.*, 2006). It has been reported that plants infected with virus have a higher content of free amino acids, carbohydrates and starch (Blua *et al.*, 1994; Awmack and Leather, 2002). Therefore, improvement of *F. occidentalis* longevity after exposure to TSWV could be due to alteration in plant primary metabolism. Furthermore, a triggered immune system in the vector as a result of exposure to the virus has also been reported, which involves transcriptional up-regulation of antimicrobial peptides (de Medeiros *et al.*, 2004). This, we could also presume to be responsible for the observed increase in longevity. Additionally, it can be speculated that virus infection may initiate a negative cross-talk between the metabolic signalling pathways within the plant defence systems against attack by the virus (Salicylic acid pathway (SA)) and the vector (Jasmonic acid pathway (JA)). Up regulation of SA, a typical defence reaction to pathogen infection can result in down regulation of JA, which triggers defence against herbivores (Belliure *et al.*, 2010). Therefore, increase in longevity and thus survival of the vector could be linked to the compromised plant defence against thrips attack, and thus easy access to nutrients by the vector (Belliure *et al.*, 2005, 2008; Abe *et al.*, 2012; Nachappa *et al.*, 2013) during larval development. However, for the exposed *F. occidentalis* individuals that spent their entire lives on TSWV infected leaf disk, the longevity was significantly lower compared to those that were later reared on healthy leaf disks as adults. This result could be explained as a negative effect of TSWV on the longevity of *F. occidentalis*. We assume that the replicating virus, and the continued acquisition of the virus, increased the virus load within the vector, overwhelming the initially triggered defence system leading to pathological effects. Additionally, Stumpf and Kennedy (2005) hypothesised a compromised gut function of the vector; as a result of the virus infection, leading to the observed adverse effects on fitness and performance. These assumptions are comparable to the results

reported by De Angelis *et al.* (1993) and Wijkamp *et al.* (1996), where the thrips spent their entire lives or a significant part of it on infected plants. The positive effect was only found when *F. occidentalis* were exposed to TSWV for AAP throughout their larval development until pupation, but later spent the rest of their adult lives on healthy leaf disks.

Reproduction rate was also significantly higher in treatments where *F. occidentalis* were exposed to TSWV, regardless of the leaf disk status they were reared on as adults. This could be due to the already mentioned improved nutritional condition in the infected plants. A research done by Nachappa *et al.* (2013) showed that infection of tomato plants with TSWV increased the fecundity of two spotted spider mites by 30%. Differences in fecundity could also be as a result of differences in host plant acceptance or discrimination behaviour of *F. occidentalis*, especially in the case of the non-exposed individuals that were transferred on infected leaf disks. Earlier studies have reported varying results on fecundity of *F. occidentalis* whether thrips were exposed to TSWV or not. Wijkamp *et al.* (1996) reported no significant difference between TSWV exposed and non-exposed *F. occidentalis*, however the host plant for their experiments was not a convenient for *F. occidentalis*, and the thrips spent their entire life time on infected or non-infected leaf disks. The study by Ogada *et al.* (2013) reported a negative effect of TSWV on *F. occidentali*s fecundity, which they attributed to the replicating virus in the vector system leading to competition for resources, which are highly needed during reproduction period. The larvae were reared on infected leaflets for AAP and later pupae were transferred onto healthy leaf disks for egg laying. Additionally, Awmack and Leather (2002) suggested that during adult stage there is an influence of nutritional status of the host plant on the rate of fecundity. These authors concluded that poor quality of host plant could alter the oviposition behaviour of herbivorous insects by reducing the number of eggs deposited per plant to avoid food shortage for the offspring. Therefore, the difference in experimental set ups and the host plants quality (Stumpf and Kennedy, 2005), could have led to the reported differences in fecundity.

Exposure or non-exposure to TSWV greatly influenced the preferential behaviour of *F. occidentalis* towards either infected or non-infected host plant. This result can be explained by an evolutionary mechanism that leads to enhancement of acquisition and transmission of TSWV as shown also for other host-virus-vector systems. Differences in colour between the virus infected and healthy plants could be a reason for herbivorous insect to be attracted to the infected plants. In our experiments we observed yellowing of the infected leaves compared to the non-infected ones. Döring *et al.* (2009) demonstrated that for instance aphids, are more attracted to the infected plants with virus due to colour changes as a result of virus infection.

Ogada *et al.* (2013) also showed that non-exposed *F. occidentalis* preferred infected leaf disks to non-infected ones. Similarly, Maris *et al.* (2004) demonstrated that significantly higher numbers of *F. occidentalis* were recovered on plants infected with TSWV in choice experiments compared to the non-infected ones. However, in our study only the non-exposed *F. occidentalis* preferred the infected leaf disks, and thus the colour changed influence. The exposed *F. occidentalis* significantly preferred the non-infected hence “normal coloured” leaf disks, we are therefore cautious to deduce any colour triggered preference mechanism for *F. occidentalis*. Thus, the observed switch in preferential behaviour of the TSWV exposed *F. occidentalis* towards the non-infected host plant could be linked to what Ingwell *et al.* (2012) referred to as ‘Vector Manipulation Hypothesis’, whereby the virus manipulates its vector in such a way to promote its dispersal between host plants. Preference of the non-exposed *F. occidentalis* towards the infected plants increases the probability of acquisition that would lead to transmission, since they will lay eggs on the infected plants, while preference of the exposed *F. occidentalis* for non-infected plants enhances the transmission probability (Ingwell *et al.*, 2012). The restlessness of the virus carrying *F. occidentalis* could also be seen during the preferential behaviour study, as high numbers were recovered outside the choice arenas after the 24 hours’ time period, in case of the set up where the exposed individuals were released, compared to the set up with the non-exposed. Several studies have shown that the exposure to plant viruses can change the preferential behaviour of the vector. For instance, acquisition of *Barley yellow dwarf virus* (BYDV) and *Potato leaf-roll virus* (PLRV) can alter the preferential behaviour of their vectors, *Rhopalosiphum padi* and *Myzus persicae*, respectively (Eigenbrode *et al.*, 2002, Jiménez-Martínez *et al.*, 2004; Medina-Ortega *et al.*, 2009; Werner *et al.*, 2009; Bosque-Pérez and Eigenbrode, 2011). Additionally, it has been shown that apart from the virus load of the host plant, other factors such as host plant resistance or susceptibility can influence thrips preferential behaviour (Maris *et al.*, 2004).

In conclusion, we found strong indications that the proposed hypothesis of ‘Vector Manipulation’ by Ingwell *et al.* (2012) is relevant for the studied system *C. annuum*-TSWV- *F. occidentalis*, as the discussed influences: both in the vector developmental parameters and preferential behaviour, could lead to a predictable enhancement of acquisition and transmission of TSWV.

5 Predictive models for *Tomato spotted wilt virus* spread dynamics, considering *Frankliniella occidentalis* specific life processes as influenced by the virus

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5.1 Abstract

Several models have been developed on predictive epidemics of arthropod vectored plant viruses in an attempt to bring understanding to the complex but specific relationship between the three cornered pathosystem (virus, vector and host plant), as well as their interactions with the environment. A large body of studies mainly focuses on weather based models as management tool for monitoring pests and diseases, with very few incorporating the contribution of vector's life processes in the disease dynamics, which is an essential aspect when mitigating virus incidences in a crop stand. In this study, we hypothesized that the multiplication and spread of *Tomato spotted wilt virus* (TSWV) in a crop stand is strongly related to its influences on *Frankliniella occidentalis* preferential behavior and life expectancy. Model dynamics of important aspects in disease development within TSWV-*F. occidentalis*-host plant interactions were developed, focusing on *F. occidentalis*' life processes as influenced by TSWV. The results show that the influence of TSWV on *F. occidentalis* preferential behaviour leads to an estimated increase in relative acquisition rate of the virus, and up to 33% increase in transmission rate to healthy plants. Also, increased life expectancy, which relates to improved fitness, is dependent on the virus induced preferential behaviour, consequently promoting multiplication and spread of the virus in a crop stand. The development of vector–based models could further help in elucidating the role of tri-trophic interactions in agricultural disease systems. Use of the model to examine the components of the disease process could also boost our understanding on how specific epidemiological characteristics interact to cause diseases in crops. With this level of understanding we can efficiently develop more precise control strategies for the virus and the vector.

Key words: *Frankliniella occidentalis*, *Tospovirus*, *Tomato spotted wilt virus*, Predictive models, Modelling, Preferential behaviour, Vector Manipulation Hypothesis.

5.2 Introduction

Tomato spotted wilt virus (TSWV) belongs to the genus *Tospovirus*, the only plant infecting genus in the family *Bunyaviridae* (Whitfield *et al.*, 2005; van de Wetering *et al.*, 1999b). TSWV is transmitted exclusively by thrips, with *Frankliniella occidentalis* (Western flower thrips) being the most efficient vector due to its equally large host range (Whitfield *et al.*, 2005; Wijkamp *et al.*, 1995; Nagata and Peters, 2001). *Tospoviruses* and their complex virus–vector interaction were ranked second in a recent survey by plant virologists based on the perceived economic and scientific importance (Scholthof *et al.*, 2011).

Transmission of TSWV by *F. occidentalis* occurs in a propagative and persistent manner (Whitfield *et al.*, 2005; Sherwood *et al.*, 2000). Only adults (and partly the second instars) can transmit the virus, but only when the virus is acquired at the early larval stage of the thrips' life cycle (Nagata, Inoue-Nagata, *et al.*, 1999b; Chatzivassiliou *et al.*, 2002; Ullman *et al.*, 1992; Wijkamp and Peters, 1993; van de Wetering *et al.*, 1996), and after the virus has replicated and circulated inside the host midgut (Wijkamp, van Lent, *et al.*, 1993; Ullman *et al.*, 1993). An increase in the viral protein in the thrips vector during their development is an indication of TSWV replication in the host (Nagata, Inoue-Nagata, *et al.*, 1999b; Nagata *et al.*, 2002; Wijkamp *et al.*, 1995; van de Wetering *et al.*, 1996). TSWV initially infects the midgut, replicating first in the epithelium and then in the muscle fibres surrounding the foregut and the midgut of the developing larvae. The virus then spreads to the salivary glands where it also replicates to achieve a high virus titer in the saliva and from which it is then transmitted to the plants during feeding (Nagata, Inoue-Nagata, *et al.*, 1999b; Nagata *et al.*, 2002; Moritz *et al.*, 2004; Hogenhout *et al.*, 2008; Ullman *et al.*, 1995,Ullman *et al.*, 1997). TSWV acquisition by second larval stage or by adults support infections in the midgut tissues, but the virus does not spread to the salivary glands therefore cannot be transmitted to plants (de Assis Filho *et al.*, 2002, 2004; Stafford-Banks *et al.*, 2014).

Virus replication in the tissues of its thrips host suggests the likelihood for pathological effects that could affect the fitness and performance of the vector. However, consistent demonstration of such effects has proven difficult due to the complex virus-vector-host plant interaction (Stumpf and Kennedy, 2007). Moreover, studies are now available showing varying effects between vector and pathogen on development rate, survival, reproduction rate and preferential behaviour of the vector (Maris *et al.*, 2004; Ogada *et al.*, 2013; Shrestha *et al.*, 2012; Robb, 1989; Sakimura, 1963; Wijkamp *et al.*, 1996; Stafford *et al.*, 2011), which can further influence the transmission and spread of the virus. However, it has proven difficult to predict these

observed influences in vector and plant populations due to the complexity of *Tospovirus*-vector-host plant interaction. Mathematical models can therefore help to quantify the virus propagation dynamics in a crop stand as influenced by virus induced vector life's processes.

Nevertheless, despite both TSWV and *F. occidentalis* being great threats to the global economy, very little effort has been made to come up with a wholesome model specifically forecasting TSWV epidemiology considering the complex and mutualistic interactions in the triangle virus-vector-host plant. According to a review paper by Jones et al (2010), by the year 2010 there were only 2 published papers on general predictive models for TSWV epidemics involving thrips vector (Olatinwo *et al.*, 2008; Yudin *et al.*, 1990). These papers together with several modelling studies that followed, mainly focused on the importance of weather conditions in the mentioned interaction (de Wolf and Isard, 2007; Magarey *et al.*, 2007), with only a few of them incorporating the aspects of the vector in varying ways. For example, influence of weather factors on: vector's population dynamics (Morsello and Kennedy, 2009; Morsello *et al.*, 2010), vector abundance (Madden *et al.*, 1990), vector activities (Chappell *et al.*, 2013) and vector species diversity (Madden *et al.*, 1983). Initial work on developing models from an arthropod-centred perspective rather than the usual plant-centred perspective has been reported (Jeger *et al.*, 2009). Most recently, Jeger et al. (2015) published a first study which focused on the contribution of thrips based parameters such as thrips age at virus acquisition and transmission, incorporated into a general predictive model of transmission characteristics within TSWV-thrips interaction.

We therefore presume that the application of a more specific vector–centred model (*F. occidentalis*-TSWV) incorporating virus induced vector life processes influencing the disease epidemic development, would provide better understanding to the often aggressive spread of TSWV in crop stands, and also allow more precise forecasting for efficient management strategies for both *F. occidentalis* and TSWV.

5.3 Methodology

5.3.1 Study system

Several examples of behavioural changes in herbivores serving as vectors as a result of hosting (or infection by) the relevant pathogens have been reported (Shalileh *et al.*, 2016; Ingwell *et al.*, 2012; Thomas *et al.*, 2005; Carmo-sousa *et al.*, 2014; Rajabaskar *et al.*, 2014; Roosien *et al.*, 2013). Most often such changes favour the replication and/or spread of the pathogen. For instance, TSWV benefits by modifying the behaviour and fitness, as well as improving the host plant quality for its vector (Ogada *et al.*, 2013; Shalileh *et al.*, 2016). Other studies have related the improved fitness of the viruliferous (virus carrying) thrips to triggered immunity (de Medeiros *et al.*, 2004), as the insect lack an adaptive immune system (Irving *et al.*, 2001). Together with the virus induced preferential behaviour, these ensure longer survival of the infected vector (Ogada *et al.*, 2013) and thus promoting multiplication and spread of the virus, which indicates a mutualistic relationship. Furthermore, exposure of *F. occidentalis* to TSWV has been reported to cause significant reduction in development time, which has been alleged to be due to biochemical reactions within the vector as a result of the replicating virus (Ogada *et al.*, 2013; Shalileh *et al.*, 2016). These reported viral influences on the vector are independent of the age at acquisition, the transmission potential of the vector, or the virus isolate (de Assis Filho *et al.*, 2002, 2004; Ogada *et al.*, 2013). Several studies have analysed the characteristics of these epidemiological consequences of persistent-propagative transmission (Jeger *et al.*, 1998; Madden *et al.*, 2000). However, they do not consider the specific virus manipulated life processes on the vector, as well as the lack of trans-ovarian (vertical) transmission of TSWV; meaning that each generation must re-acquire the virus for the disease epidemic to continue (Nagata, Inoue-Nagata, *et al.*, 1999b; Wijkamp *et al.*, 1995; van de Wetering *et al.*, 1996). Even though larvae 2 (L2) and the adults can ingest TSWV particles, they are not able to transmit the virus due to the role of a tissue barrier from the midgut muscle cells to salivary glands (Moritz *et al.*, 2004; Ohnishi *et al.*, 2001; Ullman *et al.*, 1992; de Assis Filho *et al.*, 2004). This interdependency between vector life-stage and virus transmission involves multiple infection and dissemination barriers related to the developmental changes (life cycle) of thrips vectors (Nagata, Inoue-Nagata, *et al.*, 1999b; Kritzman *et al.*, 2002; Naidu *et al.*, 2008).

This paper aims to provide a specific analysis of TSWV disease dynamics in a model framework consisting of specific transmission characteristics in the *Tospovirus*-thrips interaction, focusing on the influences of the TSWV on *F. occidentalis* biology in terms of life expectancy (survival) and preferential behaviour.

For model development assumptions, we refer to the general approaches reported in most of the plant viruses' predictive models (Jeger *et al.*, 1998, 2009, 2015; Chappell *et al.*, 2013), with additional transmission characteristics focusing on the *F. occidentalis* developmental as well as behavioural aspects as influenced by TSWV (Ogada *et al.*, 2013; Shalileh *et al.*, 2016).

5.3.2 Model description and development

The influence of TSWV on *F. occidentalis*' preferential behaviour patterns, development time, survival, as well as the key aspects of the vector lifecycle such as developmental stages, forms the basis of the hereby reported model. We consider two scenarios for the host plant; TSWV presence (infected, I) or absence (healthy, H). The basis of the assumptions is that TSWV presence influences the transmission intensity of *F. occidentalis* by its influence on adults' preferential behaviour and survival, which further affects the disease incidence in a crop stand.

F. occidentalis adults status can be described as **Healthy** ($\boldsymbol{A_N}$); if it is not exposed to TSWV infected plant by feeding, **Infected** ($\boldsymbol{A_I}$); if the vector fed on infected host plant but at a less crucial stage for acquisition in view of transmission, that is the L2 ($\boldsymbol{L2_N}$) and/or the adult stage ($\boldsymbol{A_N}$), or **Transmitter** ($\mathbf{A_T}$); if the vector fed on infected host plants at the Larvae 1 (L1) stage which is essential for later transmission by adults. $\mathbf{A_T}$ is the only group of adults that are able to transmit the virus.

Due to the high numbers of *F. occidentalis* classes and the interactions between them, we chose to use a deterministic model consisting of differential equation systems.

The model describing the disease and the vector dynamics was developed based on the following assumptions;

- Virus transmission to host plants is dependent on the presence of transmitter vectors and healthy host plant.
- Acquisition of the virus by the vector is by feeding on an infected host plant.
- The feeding stages of the vector are L1, L2 and adult; pre-pupa and pupa stages do not feed.
- Only L1 that emerge on infected host plants and acquire the virus can become transmitters as adults.
- L2 and adults are able to acquire the virus and become infected but not transmitters.
- Larval stages (L1 and L2) are mobile but restricted to the plant where they hatched from eggs due to lack of wings.
- Pre-pupae and pupae stages are immobile, do not feed and spend their entire time in the growth media i.e. not on the plant.

- The virus replicates inside the vector and is transmitted transstadially.
- Adults are the only stage that can move from plant to plant, therefore, essential for the virus transmission and thus spread.
- Transovarial transmission of the virus is not possible.
- Exposure of the vector to the virus leads to improved fitness, only when feeding on healthy host plant after the exposure.
- Preferential behaviour of the adults to the host plant is influenced by their status i.e. viruliferous adults (infected or transmitters) prefer healthy host plants, while the virus-free (healthy) prefer infected host plants.

5.3.3 Parameter estimation

Parameter estimation was based on recent findings which indicated a form of mutualism between TSWV and its vector *F. occidentalis* (Ogada *et al.*, 2013; Shalileh *et al.*, 2016). Development and behaviour of *F. occidentalis* (exposed or non-exposed to TSWV) on host plants with different health status (+/- TSWV) is reported to be different. As TSWV infection on the host plants influences the vector biology by reducing the development time, increasing survival and changing the preferential behaviour of the vector, only if the virus exposed vectors were later reared on healthy host plant as adults (Shalileh *et al.*, 2016, Ogada *et al.*, 2013). Therefore, the life cycle of the vector will vary accordingly (Table 5.1). All compartments of the models are summarized in Table 5.2 with their corresponding descriptions. Initial values of the vector population and virus abundance were estimated (Table 5.2) to evaluate the interaction.

Table 5.1: Development time values of different life stages of *F. occidentalis* growing on host plants with different TSWV status (infected or healthy host plants).

Developmental stages and time of *F. occidentalis* on healthy host plants (Zitter and Daughtrey, 1989; Robb *et al.*, 1988)		**Development time on TSWV infected host plants** (Ogada *et al.*, 2013)
Egg→L1 (Emergence)	2-4 days	2-3days
L1→L2	1-2 days	1day
L2→Pre-pupa	2-4 days	2days
Pre-pupa→Pupa	1-2 days	1day
Pupa→Adult	1-3 days	1-2days
Egg→Adult	14-21days	9-10days
Adult lifetime	30-45 days	42-51days

Table 5.2: List of model compartments, symbols and their descriptions, with hypothetical initial values.

Symbol	Description	Initial values
H	Fraction of healthy plants	791/800
I	Fraction of infected plants	9/800
E_H	Amount of eggs in healthy plants	6000
E_T	Amount of eggs in infected plants	108
$L1_H$	Amount of L1 in healthy plants	5760
$L1_T$	Amount of L1 in infected plants	99
$L2_H$	Amount of L2 in healthy plants	3000
$L2_I$	Amount of L2 emerging in newly infected plants	0
$L2_T$	Amount of L2 in infected plants	45
PP_N	Amount of non-infected pre-pupae from healthy plants	0
PP_I	Amount of infected pre-pupae from infected plants	0
PP_T	Amount of pre-pupae from infected plants that will become transmitters	0
P_N	Amount of non-infected pupae from healthy plants	0
P_I	Amount of infected pupae from infected plants	0
P_T	Amount of transmitter pupae from infected plants	0
A_N	Amount of non-infected *F. occidentalis* adults	1200
A_I	Amount of infected *F. occidentalis* adults	310
A_T	Amount of transmitter *F. occidentalis* adults	600

5.3.4 Model dynamics

A flowchart illustration of selected influences of TSWV on *F. occidentalis* is given below (Fig. 5.1), indicating all developmental classes of the model. A comparison is made between *F. occidentalis* that fed on virus infected (+TSWV) and on healthy (-TSWV) host plants, in terms of TSWV influences on their life expectancy as well as preferential behaviour. There are three groups of adults as a result of the exposure status; Healthy, Transmitters and Infected adults, already described above.

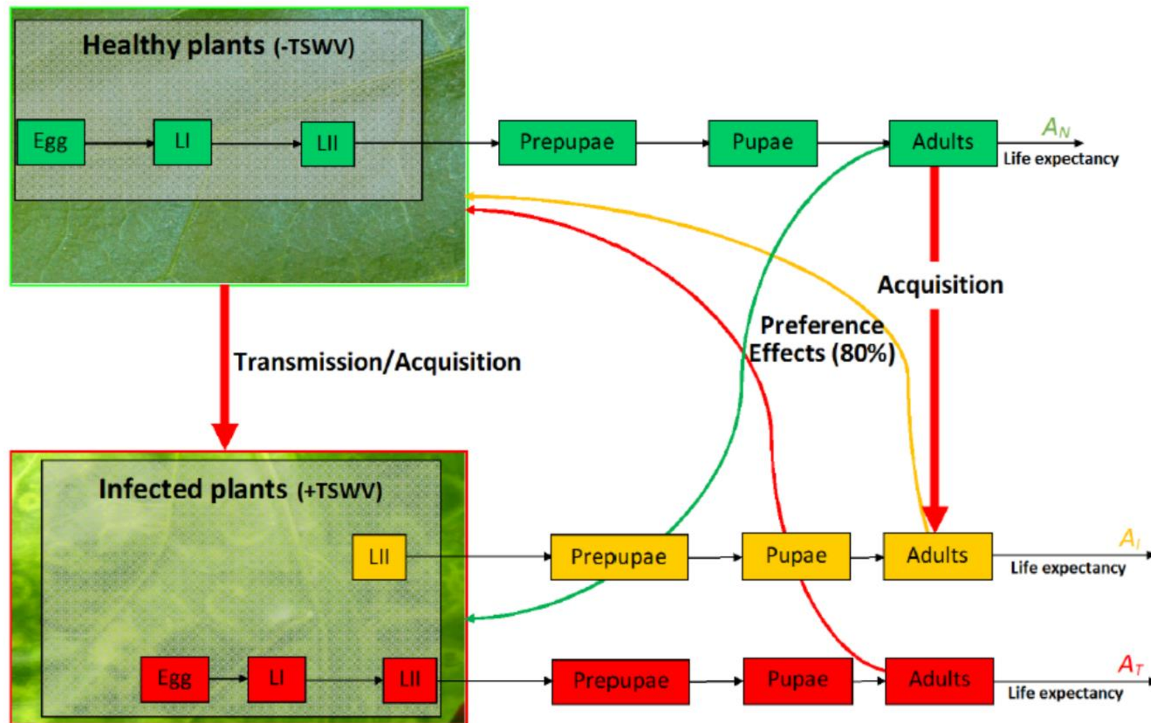

Figure 5.1: Flowchart describing TSWV–*F. occidentalis* interaction dynamics. The three coloured pathways represent exposure status of *F. occidentalis* to TSWV; Green ➔ Healthy, Orange ➔ Infected and Red ➔ Transmitter individuals. The green, yellow, and red arrows indicate the virus induced preferential behaviour of adults, with a preferential factor of 80%.

5.3.5 Description of developmental classes dynamics

5.3.5.1 Eggs

Eggs are either laid on TSWV infected or healthy host plants based on the preferential behaviour of the adults, which is influenced by their infection status. Since transovarial (vertical) transmission is not possible, all eggs are considered healthy, even if they are laid on infected host plant. The assumption of preferential behaviour is based on our recent study with *F. occidentalis* and TSWV (Shalileh *et al.*, 2016). The amount of eggs laid on healthy plants is determined by the number of adults in each category and their infection status, and is modelled by:

$$\mu(p_{IH} * A_I + p_{NH} * A_N) * H$$

where $p_{IH} = 0.8$ and $p_{NH} = 0.2$, are preferential factors effects on the infected and non-infected adult *F. occidentalis* to feed on healthy plants. μ is the average number of eggs laid per adult per day. The total amount of eggs laid on infected plants per day is given by:

$$\mu\big((1 - p_{IH}) * A_I + (1 - p_{NH}) * A_N + (1 - p_{TH}) * A_T\big) * I$$

When transmitter adults lay eggs on healthy plants, we assume that they would feed on the plant transmitting the virus, and thus changing the status of the plant. The complete dynamics of eggs is given below.

In healthy plants:

$$\frac{dE_H}{dt} = \mu(p_{IH}A_I + p_{NH}A_N)H - \lambda_T H E_H - (\eta_{EH} + \tau_{EH})E_H \quad (1)$$

In infected plants:

$$\frac{dE_T}{dt} = \mu\big((1 - p_{IH})A_I + (1 - p_{NH})A_N + (1 - p_{IH})A_T\big)I + \lambda_T H E_H - (\eta_{ET} + \tau_{ET})E_T \quad (2)$$

where, $\frac{1}{\eta_i}, i = EH, ET$ is the average number of days required for eggs to emerge in healthy and infected plants (3 days and 2.5 days) respectively. $\tau_i = 20\%$, is the estimated fraction of eggs destroyed per day.

The parameter λ_T which models the transmission rate of the infection to plants is defined by:

$$\lambda_T = p_{TH}\beta_I \frac{A_T}{A_T + A_I + A_N} \quad (3)$$

β_I is the contact rate that leads to infection and $p_{TH} = 0.8$, is the preferential factor of the transmitter adult *F. occidentalis* to feed on healthy plants.

5.3.5.2 Larvae 1 (L1)

L1 emerging from eggs laid on the infected plants will automatically feed on the infected plants, and since this is a crucial stage for acquisition in view of transmission by adults, these L1 are referred to as transmitters. While the L1 that emerged from the healthy host plants will feed on the healthy plants and thus are referred to as healthy. However, in case of eggs laid by the transmitter adults on the healthy plants as a result of the preferential behaviour, there is a possibility of TSWV transmission to the healthy plants during egg laying, assuming that they fed on the plants, therefore changing the status of healthy plants to TSWV infected, and thus, the L1 emerging and feeding on these newly infected host plants also become transmitters. The dynamics of this population is given by;

In healthy plants:

$$\frac{dL1_H}{dt} = \eta_{EH}E_H - \lambda_T H\, L1_H - (\eta_{L1H} + \tau_{L1H})L1_H \quad (4)$$

In infected plants:

$$\frac{dL1_T}{dt} = \eta_{ET}E_T + \lambda_T H\, L1_H - (\eta_{L1T} + \tau_{L1T})L1_T \tag{5}$$

where, $\frac{1}{\eta_i}, i = L1H, L1T$ is the average number of days required for L1 to develop to L2 in healthy and infected plants respectively (1.5 days, 1 day). $\tau_{L1H} = 20\%$ and $\tau_{L1T} = 10\%$ are estimated daily death rates of L1.

5.3.5.3 Larvae 2 (L2)

The transmitter L1 develop to transmitter L2 on the TSWV infected host plants, and the healthy L1 to healthy L2 on the healthy host plants. However, in case of transmission of TSWV to the healthy plants by the transmitter adults, the resulting L2 will feed on the now TSWV infected plants, acquire the virus, and become infected. Nevertheless, transmission by adults resulting from this group is not possible. Some literatures suggest that the early stages of L2 can successfully acquire the virus for transmission at adult stage; however, this has not been adequately validated.

The dynamics of this population is given by;

In healthy plants:

$$\frac{dL2_H}{dt} = \eta_{L1H}L1_H - \lambda_T H\, L2_H - (\eta_{L2H} + \tau_{L2H})L2_H \tag{6}$$

In infected plants:

$$\frac{dL2_I}{dt} = \lambda_T H\, L2_H - (\eta_{L2I} + \tau_{L2I})L2_I \tag{7}$$

$$\frac{dL2_T}{dt} = \eta_{L1T}L1_T - (\eta_{L2T} + \tau_{L2T})L2_T \tag{8}$$

where, $\frac{1}{\eta_i}, i = L2H, L2I, L2T$ are average numbers of days required for L2 to develop to prepupae in healthy and infected plants (3 days, 2 days). $\tau_{L2H} = 20\%, \tau_{L2I} = 10\%$ and $\tau_{L2T} = 10\%$ are estimated daily death rates of L2.

5.3.5.4 Prepupae and pupae

Since these two stages are immobile, do not feed and are primarily in the growth media, i.e. outside the plant, their status is solely dependent on the status of the preceding L2. That is, healthy, infected and transmitter L2 will result to similar categories for both pupal stages. We subdivided each of these classes into three subclasses depending on their status.

The prepupa population dynamics is given as:

$$\frac{dPP_N}{dt} = \eta_{L2H}L2_H - (\eta_{PPN} + \tau_{PPN})PP_N \tag{9}$$

$$\frac{dPP_I}{dt} = \eta_{L2I}L2_I - (\eta_{PPI} + \tau_{PPI})PP_I \tag{10}$$

$$\frac{dPP_T}{dt} = \eta_{L2T}L2_T - (\eta_{PPT} + \tau_{PPT})PP_T \tag{11}$$

The pupa population dynamics is given as:

$$\frac{dP_N}{dt} = \eta_{PPN}PP_N - (\eta_{PN} + \tau_{PN})P_N \tag{12}$$

$$\frac{dP_I}{dt} = \eta_{PPI}PP_I - (\eta_{PI} + \tau_{PI})P_I \tag{13}$$

$$\frac{dP_T}{dt} = \eta_{PPT}PP_T - (\eta_{PT} + \tau_{PT})P_T \tag{14}$$

where, $\frac{1}{\eta_i}, i = PPN, PPI, PPT;\ PN, PI, PT$ are the average number of days required for development from prepupa to pupa (1.5 days, 1 day and 1 day); and from pupa to adult, for the healthy, infected and transmitter status respectively (2 days, 1.5 days and 1.5 days). $\tau_{PPN} = \tau_{PN} = 20\%$ and $\tau_{PPI} = \tau_{PPT} = \tau_{PI} = \tau_{PT} = 10\%$ are estimated daily death rates of pupae and prepupae.

5.3.5.5 Adults

Adults are the most important stage in the transmission cycle, as they are mobile and can fly from one plant to the other, therefore, are responsible for the spread of TSWV in a crop stand. The status of the adults is also dependent on the pupae status, which results into the three adults' categories; Healthy, Infected and Transmitters. Unlike the pupal stages, the adults are mobile and feed. Healthy adults can feed on TSWV infected host plants and acquire the virus, which leads to a change in their status from healthy to infected adults, but not transmitters due to the developmental barriers already mentioned. Furthermore, the influence of TSWV on the preferential behaviour can be seen clearly in this stage, due to their ability to fly. And as we already reported, the viruliferous adults: infected and transmitters, show preference towards healthy over the TSWV infected host plants, while the virus free adults prefer TSWV infected over healthy host plants (Shalileh *et al.*, 2016; Ogada *et al.*, 2013). These consequently influence the amount of eggs laid on either infected or healthy host plants, and thus the status of the resulting *F. occidentalis* adults in the population, which further influence the spread of TSWV.

The adults' population dynamics is given as follows:

$$\frac{dA_N}{dt} = \eta_{PN}P_N - \beta_A IA_N - \tau_{AN}A_N \tag{15}$$

$$\frac{dA_I}{dt} = \eta_{PI}P_I + \beta_A I A_N - \tau_{AI}A_I \quad (16)$$

$$\frac{dA_T}{dt} = \eta_{PT}P_T - \tau_{AT}A_T \quad (17)$$

where, $\frac{1}{\tau_i}, i = AN, AT$ and AI are adults' life expectancy depending on the fraction of healthy plants available, and is modelled as:

$$\frac{1}{\tau_{AN}} = 37.5, \frac{1}{\tau_{AT}} = \frac{1}{\tau_{AI}} = 37.5 + 10.5(2H - 1)$$

where, H is the fraction of healthy plants. This assumption is based on the fact that life expectancy of adults exposed to TSWV is higher on healthy plants than on infected plants.

Virus acquisition by healthy *F. occidentalis* adults is modelled as;

$$(1 - p_{NH})\beta_A I\, A_N,$$

where, $(1 - p_{NH})$ is the preference towards infected plants, and β_A is the contact rate between the insect and infected plant.

5.3.6 Simulations

Numerical simulations of the model were performed using the modeling software Vensim PLE (**Ventana Systems, Inc.,** Harvard, USA) with a Runge-Kutta 4 scheme. Different scenarios were compared to evaluate the impact of TSWV influence on preferential behaviour and life expectancy of *F. occidentalis*, on the propagation and spread dynamics of the virus in a plant population. The maximum evaluation time was set at 100 days (after TSWV introduction in a crop stand) and the step sizes were fixed to minimum (0.0078125 days) to avoid any stiffness of the scheme.

5.4 Results

5.4.1 Influence of TSWV induced preferential behaviour of *F. occidentalis* on the TSWV-vector-host plant interaction dynamics

The considered dynamics for the interaction were based on reports that exposure of *F. ccidentalis* to TSWV leads to a change in their preferential behaviour. Non-exposed *F. occidentalis* (healthy) prefer TSWV infected host plants; while the TSWV exposed ones significantly prefer healthy host plants.

5.4.1.1 Disease spread dynamics (Acquisition/ Transmission)

In terms of TSWV spread dynamics, *F. occidentalis* relative acquisition rate and virus transmission rate to healthy host plants were modelled in the presence and absence of virus induced preferential behaviours of the vector. The results of the relative acquisition rate show that the preferential behaviour effect was beneficial above the threshold, and only up to the first 30 days after TSWV introduction into a crop stand. Thereafter, the influence of preference on acquisition is limited (Fig. 5.2). On the other hand, the rate of TSWV transmission by *F. occidentalis* is greatly influenced by the preferential behaviour of the vector. With the highest influence occuring in the first approximately 63 days after TSWV introduction into a crop stand. Thereafter, the influence of preference is limited due to the reducing fraction of the healthy plants (Fig. 5.3).

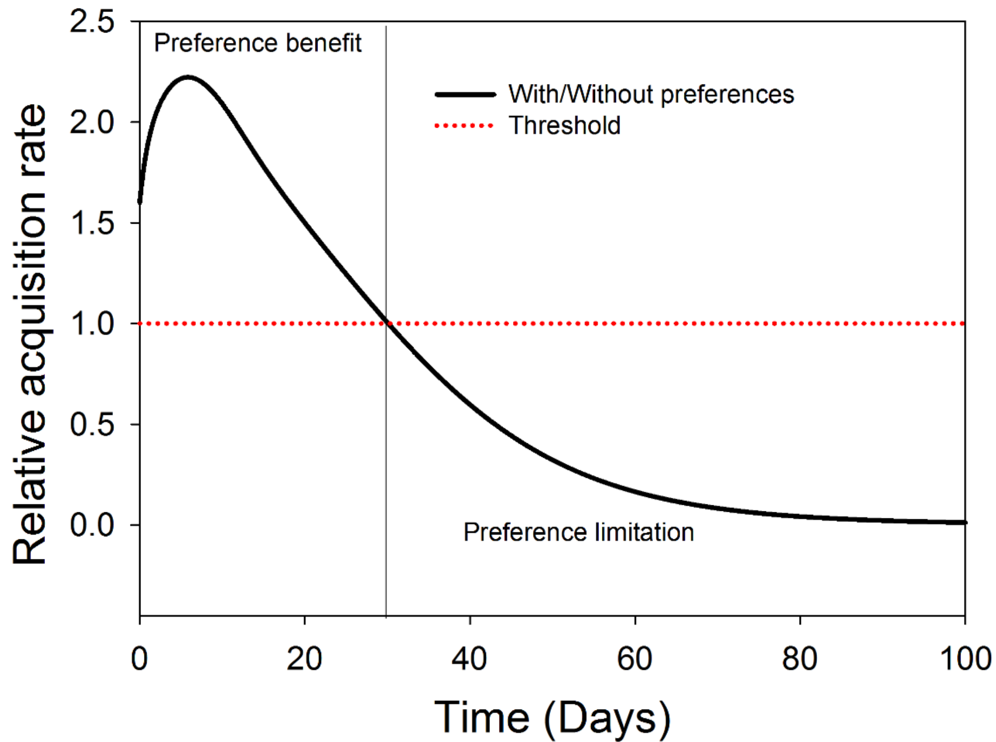

Figure 5.2: Relative acquisition rate of TSWV by *F. occidentalis* in the presence of TSWV induced preferential behaviour. The dotted line represents the threshold indicating when *F. occidentalis* preferences has no effect on the TSWV acquisition rate, and the vertical line represents the time at which the threshold is reached.

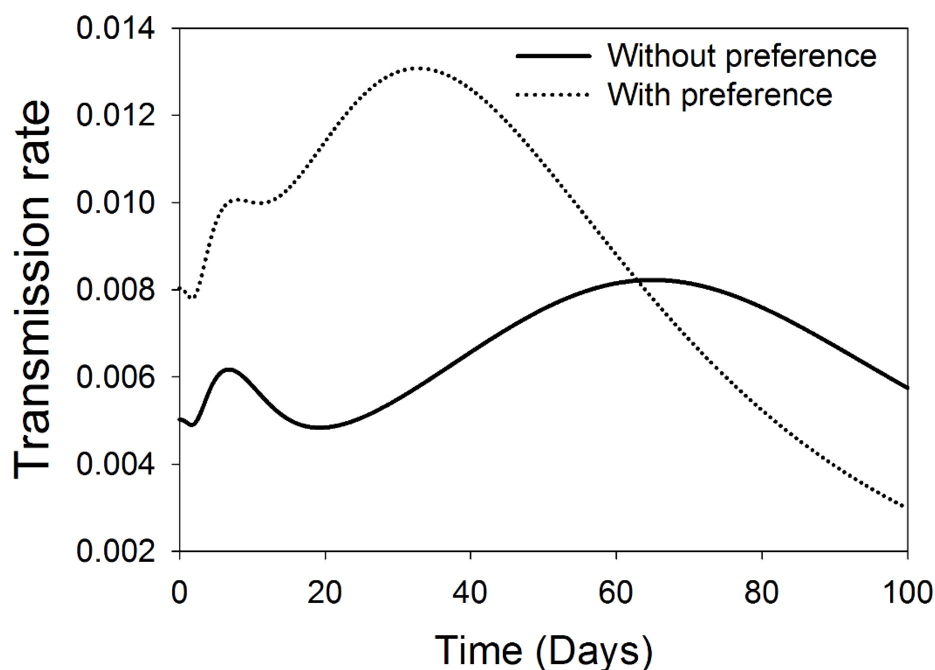

Figure 5.3: Comparison of effect of TSWV induced preferential behaviour on the transmission rate of *F. occidentalis*. The solid and the dotted lines represent the dynamics of the transmission rate of TSWV to host plants in the absence and in the presence of preference effects respectively.

5.4.1.2 Host plants abundance dynamics

It can be observed that the influence of the virus induced vectors' preference in the first 100 days of TSWV introduction in a crop stand accelerates the transmission of the disease to healthy plants. The model estimates that preferences of viruliferous vectors (infected and transmitters) to healthy plants may increase the transmission rate by up to 33.3%. The consequence can lead to up to 30% decrease in the fraction of healthy plants (Fig. 5.4A). On the other hand, the influence of *F. occidentalis* preferential behaviour on the fraction of the TSWV infected host plants over time indicates a steady rise of the infected plants. Thus, the TSWV induced preferential behaviour benefits the virus in terms of spread (Fig. 5.4B).

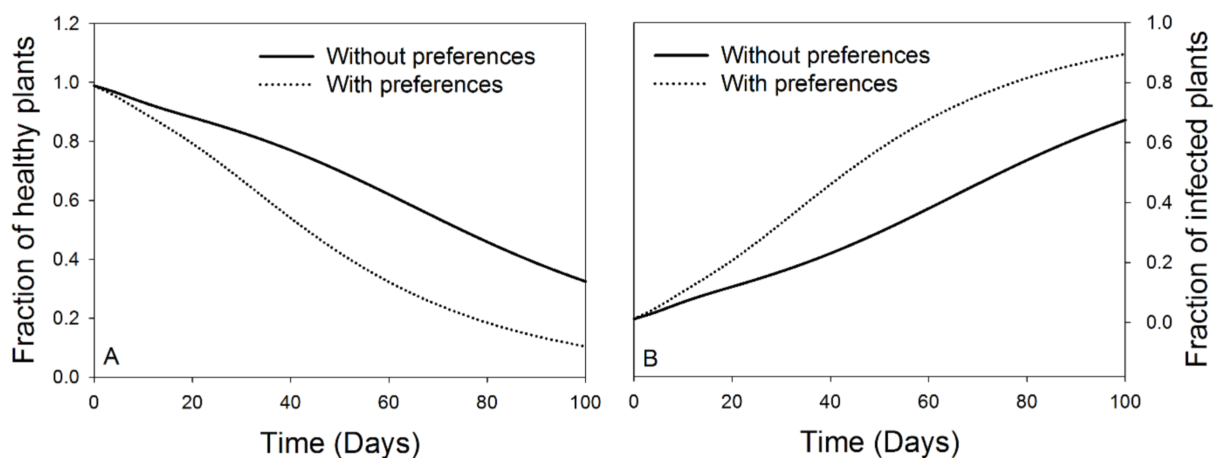

Figure 5.4: Influence of virus induced preferential behaviour of *F. occidentalis* on host plant abundance. A. Represent fraction of healthy plants overtime. B. Represent fraction of infected plants overtime.

5.4.1.3 Vector abundance dynamics model – Adults

Since adult is the crucial stage for TSWV spread due to its mobility and host plant choice behaviour, it was the only stage in *F. occidentalis*' life cycle considered; in terms of vector abundance, for the analysis of the influence of TSWV induced preferential behaviour in the first 100 days of the virus introduction in a crop population. This was based on our listed assumptions. In the absence of preference, the population of the transmitter adults shows exponential rise compared to the healthy and the infected adults (Fig. 5.5A). By introducing TSWV induced preferential behaviour to the model, the transmitters population increases exponentially 80 days after the virus introduction in the crop stand (Fig. 5.5B). A comparison of the adults' abundance ratios shows clearly that the benefit of virus induced vector preference is only available within the first 22 and 40 days after the introduction of TSWV in the crop

stand, only for the infected and transmitter adults respectively, but not for the healthy adults' population, after these days the influence of preference is limited (Fig. 5.6).

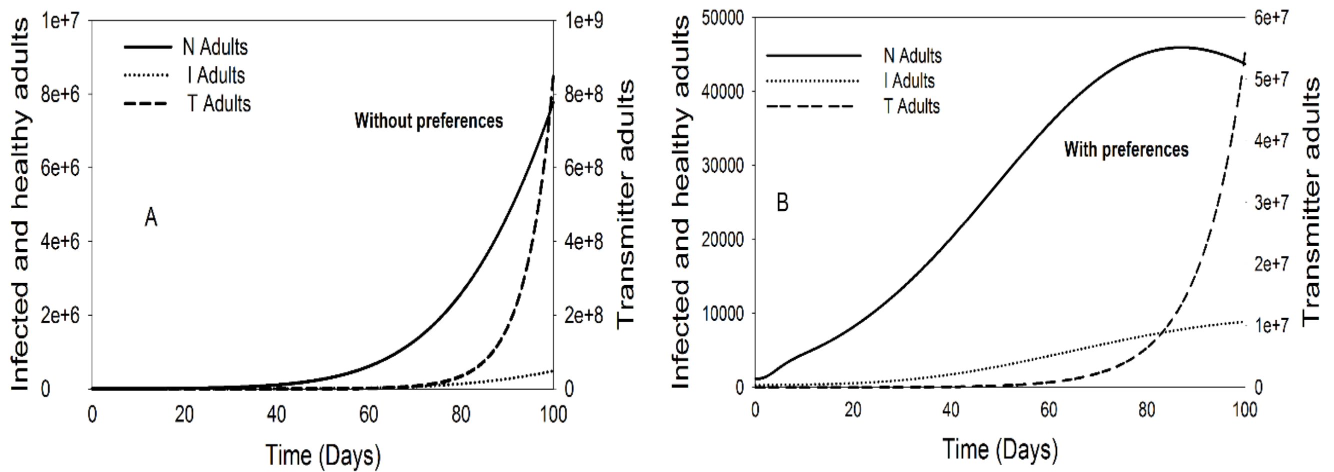

Figure 5.5: Population dynamics of adult *F. occidentalis* in the presence and absence of preference effects. A. Represents the adult population dynamics without the influence of TSWV induced preferential behaviour. B. Represents the adult population dynamics with the influence of TSWV induced preferential behaviour. The initials N, I, and T, refer to healthy, infected and transmitters respectively.

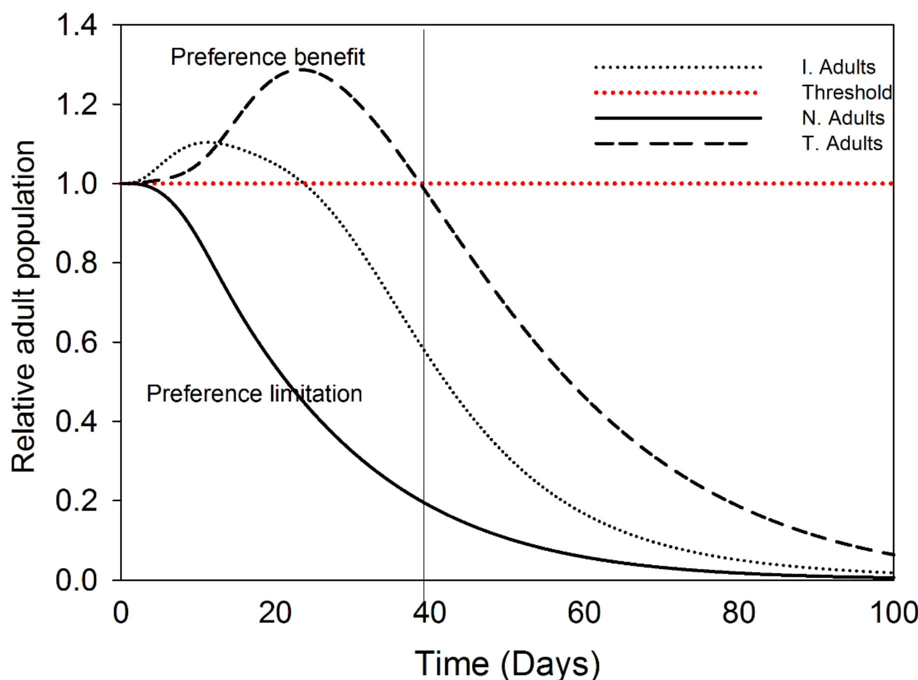

Figure 5.6: Relative population dynamics of *F. occidentalis* adults in the presence and absence of preference effects. Population dynamics of *F. occidentalis* adults' in the presence relative to the absence of TSWV induced preferential behaviour. The initials N, I, and T, refer to healthy, infected and transmitters respectively.

5.4.2 TSWV induced effects on *F. occidentalis* life expectancy in TSWV-vector-host plant interaction dynamics

The influence of TSWV on *F. occidentalis* development time and survival was modelled in terms of adults' life expectancy, and its contribution to the different aspects of disease development.

5.4.2.1 Disease spread dynamics (Acquisition/ Transmission)

Looking at the acquisition ratio of TSWV with respect to life expectancy of the adult *F. occidentalis* in the presence or absence of virus effects, the benefits of the virus influences are observed above the threshold and only in the first 58 days after TSWV introduction into a crop population. Thereafter the influence is limited (Fig. 5.7). For the transmission rate to healthy plants in the presence of TSWV effects, the benefits of *F. occidentalis* improved life expectancy are observed only in the first 46 days after virus introduction, there after the effects are limited. The opposite applies for situation without virus effects (Fig. 5.8).

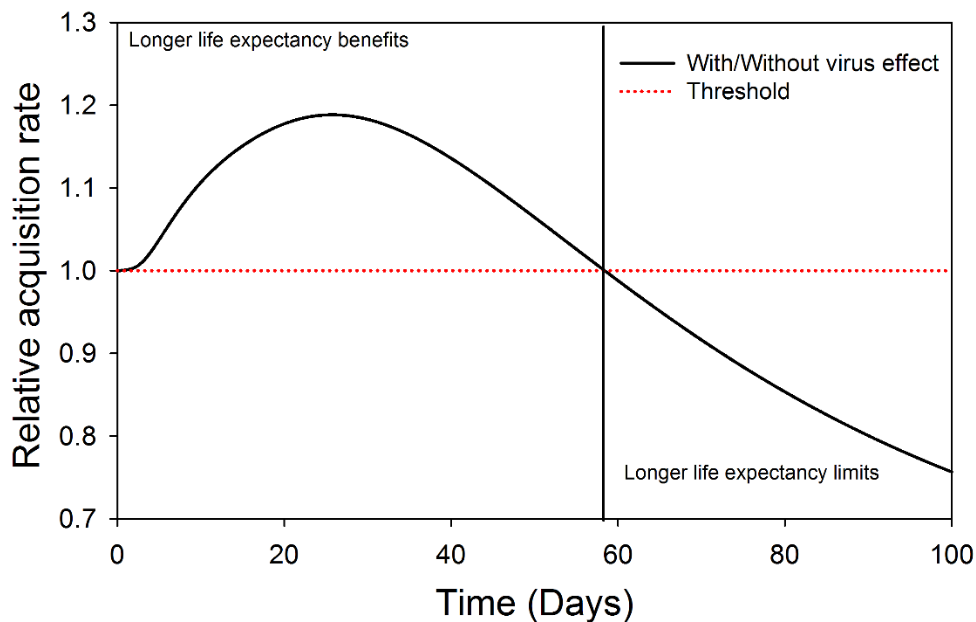

Figure 5.7: Relative acquisition rate dynamics of *F. occidentalis*, when TSWV has an effect with respect to no effect on *F. occidentalis* life expectancy. The dotted line represents the threshold; indicating when TSWV effect on *F. occidentalis* life expectancy does not influence the acquisition rate, and the vertical line represents the time at which the threshold is reached.

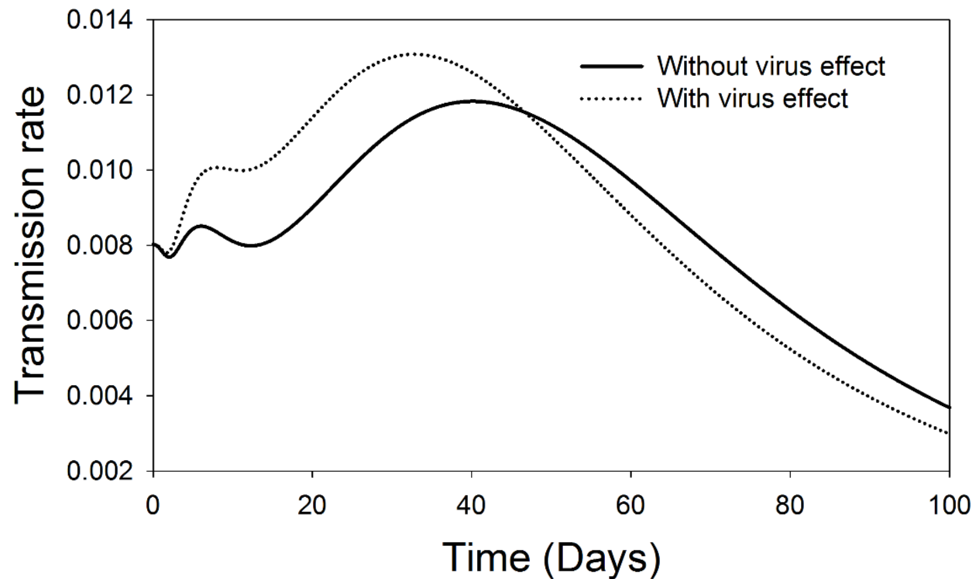

Figure 5.8: Comparison of the TSWV induced effect on *F. occidentalis* life expectancy on the transmission rate of TSWV. The solid line represents the transmission rate of TSWV to plants in the absence of TSWV induced effect on life expectancy over time, and the dotted line represents transmission rate in the presence of TSWV induced effect on life expectancy.

5.4.2.2 Host plants abundance dynamics

The influence of life expectancy of TSWV infected *F. occidentalis* on host plants abundance both in the presence or absence of TSWV effects results in a steady decline in the fraction of healthy host plants in a given population, with the presence of TSWV effect resulting in slightly lower fractions of healthy plants compared to the absence of TSWV effects (Fig. 5.9A). While for the infected host plants, there is a steady rise, for both with or without TSWV effects. However, inclusion of the virus effects in the model slightly increases the fraction of infected host plants compared to neglecting it (Fig. 5.9B).

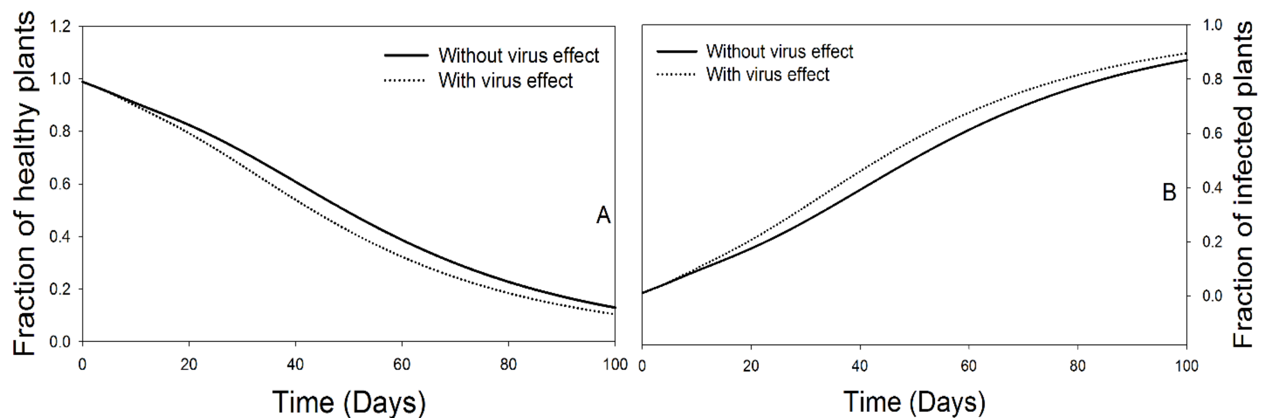

Figure 5.9: Influence of TSWV induced effect on *F. occidentalis* life expectancy on host plants abundance over time. A. Represents the fraction of healthy plants, and B. Represents the fraction of infected plants. Dotted and solid lines represent the dynamics of the fraction of plants, 'with' and 'without' TSWV induced effects on *F. occidentalis* life expectancy respectively.

5.4.2.3 Vector abundance dynamics model – Adults

Regarding the population dynamics of the three vector cohorts, the influence of life expectancy was modelled based on adults' mortality in each cohort, both with and without virus effects (Fig. 5.10). For the healthy adults (Fig. 5.10A), an influence of the presence of virus effects on life expectancy with respect to mortality is observed 40 days after virus introduction in a crop stand, resulting in reduced mortality compared to the absence of virus effects. For the infected adults (Fig. 5.10B), the influence is minimal with a small difference being observed 80 days after TSWV introduction. While for the transmitter adults (Fig. 5.10C), the influence is observed 60 days after virus introduction, with significantly higher mortality in the presence of virus effects compared to without virus effects.

When modelling the virus influences on life expectancy with respect to the relative mortality rate (Fig. 5.10D), for both infected and transmitter adults' cohorts, the rate increases steadily at the same pace in their respective populations. However, for the non-infected adults there is no influence on the rate of mortality, as the only expected influence would be in the presence of virus effects, but since the status of the non-infected adults change to infected adults when they come in contact with the virus, the virus effects do not affect the healthy population in terms of the relative mortality rate, and thus equals to the threshold.

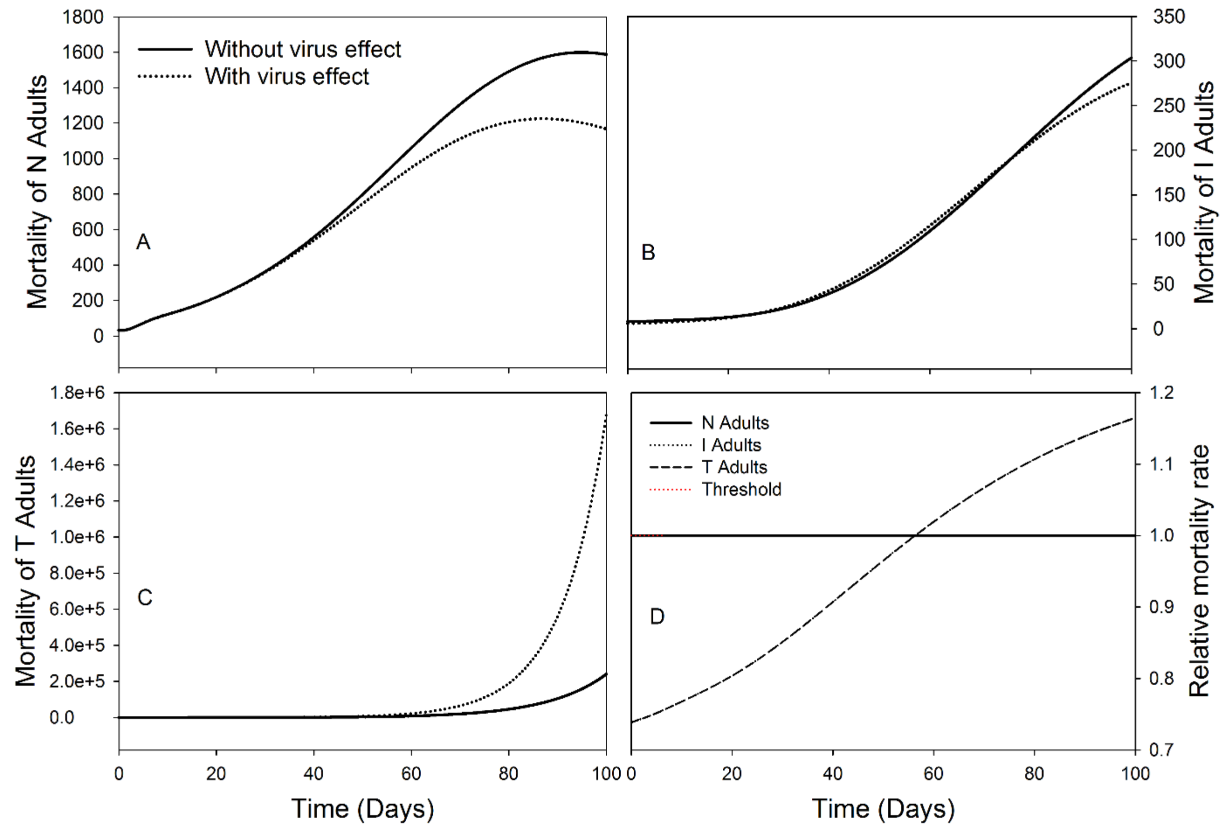

Figure 5.10: Dynamics of adults' life expectancy as influenced by 'with' or 'without' virus effects on *F. occidentalis* mortality over time. A. On healthy adults, B. On infected adults, C. On transmitter adults and D. Represent the relative mortality rate over time for each adult cohort under the TSWV induced effect on *F. occidentalis* life expectancy. The initials N, I, and T, refer to healthy, infected and transmitters respectively.

5.4.2.4 Relative adults' population dynamics model

Evaluation of the relative adults' population dynamics (with/without virus effects on life expectancy) of different vector cohorts in the first 100 days after TSWV introduction in a crop population shows a positive virus effect on the dynamics of transmitter adults' population with a steady rise over time observed above the threshold. For the infected adults' population, there is also a positive effect of the virus, however the rise is minimal, only slightly above the threshold, and the effects are limited to 60 days after virus introduction (Fig. 5.11). For the healthy adults' population, there are no virus effects observed in the first 40 days after virus introduction, but thereafter, the effects are negative.

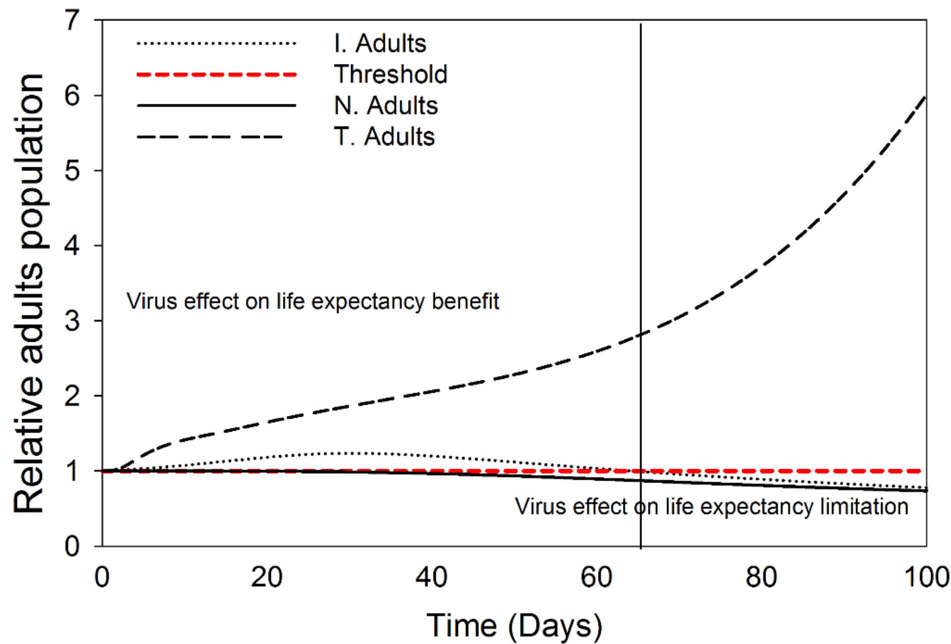

Figure 5.11: Relative *F. occidentalis* adult population; 'with' with respect to 'without' TSWV effect, on *F. occidentalis* life expectancy. The red dashed line represents the threshold indicating when TSWV effect on *F. occidentalis* life expectancy does not influence the adult population and the vertical line represents the time at which the threshold is reached. The initials N, I, and T, refer to healthy, infected and transmitters respectively.

5.5 Discussion

Since TSWV transmission is dependent on the vector biology and behaviour, both direct and indirect host plant mediated virus effects on the vector are crucial for the prediction of the virus epidemics. The hereby reported modelling of disease spread dynamics which involves the virus acquisition and transmission rate by the vector, is linked to the observed dynamics of the host plants and vectors abundance in relation to the vectors' life expectancy and preferential behaviour as influenced by the virus. With the introduction of TSWV in a crop population, the fraction of the healthy plants is expected to diminish over time due to the progressive transmission of the virus; this means a change in the host plant status from healthy to infected, and thus, an increase in the ratio of infected plants. Considering the reported TSWV induced preferential behaviour of *F. occidentalis* (Stafford *et al.*, 2011; Shalileh *et al.*, 2016; Bautista *et al.*, 1995), the non-infected adults tend to choose the infected plants over the healthy plants both for feeding and egg laying, leading to more eggs laid in the infected plants resulting in a population outburst of transmitter adults in the following generation, and thus, further TSWV spread to the next available healthy plant. This explains the high populations of the transmitter

adults observed over time after the introduction of the virus. The observed steady increase in the fraction of the infected host plants is expected to reach a plateau after a certain time period (beyond the 100 days), this is because the fraction of the healthy host plants is decreasing at the same rate, and therefore the influence of preference is limited, which also limits further transmission. Such vector-modified behaviour as a result of virus influences is a common trait among the *Tospoviruses*. This phenomenon has also been reported in other virus-vector systems, for example the cereal aphids *Rhopalosiphum padi* and *Myzus persicae*, after acquisition of *Barley yellow dwarf virus* (BYDV) and *Potato leafroll virus* (PLRV) respectively (Ingwell *et al.*, 2012; Medina-Ortega *et al.*, 2009; Bosque-Pérez and Eigenbrode, 2011; Werner *et al.*, 2009; Jiménez-Martínez *et al.*, 2004; Eigenbrode *et al.*, 2002), and also in whiteflies *Bemisia tabaci* after acquisition of *Tomato yellow leaf curl virus* (TYLCV) (Moreno-Delafuente *et al.*, 2013). Several other factors besides virus manipulation, have also been reported to contribute to the observed behavioural changes on the vector. For instance, differences in colour and odour between infected and non-infected host plant have been linked to this behaviour. Aphids and thrips are reported to be more attracted to host plants infected with virus due to colour change (yellowing) and odour cues (Döring *et al.*, 2009; Maris *et al.*, 2004; Ogada *et al.*, 2013). However, in all these reports the insects had no prior exposure to the virus and were considered healthy, which represents only one path of our model where virus free adults that developed on healthy plant are attracted to the infected host plants. For the other two paths of the model; the infected and the transmitter adults, colour and odour cues do not seem to play any role, therefore, the only explanation would be the manipulation of the vector by the virus to enhance its transmission and spread, a mechanism which could be linked to an evolutionary interaction between plant viruses and their vectors (Ingwell *et al.*, 2012).

The observed increase in life expectancy of *F. occidentalis* in response to TSWV also showed some influences on the disease spread dynamics. In the relative acquisition and the transmission rates of the vector, the virus effects on life expectancy are obviously positive primarily in the early stages of the disease development, but thereafter tending towards negative. This relates back to the TSWV induced preferential behaviour (Ogada *et al.*, 2013; Shalileh *et al.*, 2016), and its dependent on the health status of the host plant. In the adults' abundance model, the mortality of the healthy adults follows a normal sigmoid function with or without virus effects. The observed slight reduction with virus effect could be attributed to the reduced population of healthy individuals due to adults switching status, i.e. from healthy to infected, as a result of contact (acquisition) with a virus infected host plant, and is again a consequence of the virus induced preferential behaviour (Ogada *et al.*, 2013; Shalileh *et al.*, 2016). For the infected

adults, the mortality is expected to increase steadily with or without virus effects. However, after 80 days there is a slight reduction in mortality due to virus effects: as the virus induced preferential behaviour manipulates the infected adults to choose healthy host plants, thus reducing mortality. The transmitter adults experience an exponential rise in mortality with virus effects after 60 days of disease progression over time. This is because the fraction of the healthy plants is reducing due to the increasing rate of transmission as a result of the rising numbers of the transmitter adults, leading to an increase in the fraction of the infected plants, and thus an equal increase in mortality due to limited preferential effects. The increase in life expectancy for the viruliferous paths compared to the healthy path of the vector as displayed in this model, is consequently dependent on the virus induced preferential behaviour of the vector, and the fraction of the healthy host plants (Shalileh *et al.*, 2016). The increased fitness of *F. occidentalis* exposed to TSWV has been associated with triggered immunity (de Medeiros *et al.*, 2004), as the insect lack an adaptive immune system (Irving *et al.*, 2001). This explains the observed increase in survival of the viruliferous populations on healthy host plants (Ogada *et al.*, 2013).

A phenomenon termed “Vector Manipulation Hypothesis” by Ingwell et al. (2012), was the motivation of our study. It describes an evolutionary mechanism in plant pathogen–vector interactions that promote the multiplication of the pathogen and transmission to new host plants. Therefore, incorporating virus manipulated vector’s life processes in the predictive models would solve the problem of over or under estimation, as it is evident that this aspect is important for a realistic description of disease incidences by predictive models (Chappell *et al.*, 2013). A more wholistic expansion of our model would be incorporation into a detailed weather based model, as well as inclusion of various aspects in the *Tospovirus*-vector-hostplant interaction like; vector gender, individual vector genetics, among others.

In conclusion, the dynamics of the complex and specific TSWV-*F. occidentalis*-host plant interaction, with focus on virus induced and host plant-mediated effects on the vector’s life processes, is a new contribution to the currency of the predictive models. A thorough understanding of the vector’s life processes as influenced by the virus, and their contribution to the virus-vector-hostplant interaction, is critical for a more precise prediction of TSWV epidemics.

6 Differential proteomics analysis of *Frankliniella occidentalis* immune response after infection with *Tomato spotted wilt virus* (Tospovirus)

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6.1 Abstract

Tomato spotted wilt virus (TSWV) is mainly vectored by *Frankliniella occidentalis* Pergande, and it potentially activates the vector's immune response. However, molecular background of the altered immune response is not clearly understood. Therefore, using a proteomic approach, we investigated the immune pathways that are activated in *F. occidentalis* larvae after 24 hours exposure to TSWV. Two-dimensional isoelectric focusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-IEF/SDS/PAGE) was used combined with mass spectrometry (MS) to identify proteins that were differentially expressed upon viral infection. High numbers of proteins were abundantly expressed in *F. occidentalis* exposed to TSWV (73%) compared to the non-exposed (27%), with the majority functionally linked to the innate immune system such as: signaling, stress response, defense response, translation, cellular lipids and nucleotide metabolism. Key proteins included: 70 kDa Heat Shock Proteins, Ubiquitin and Dermcidin, among others, indicative of a responsive pattern of the vector's innate immune system to viral infection.

Key words: Innate immunity; *Frankliniella occidentalis;* proteomics; *Tomato spotted wilt virus*; 2D-IEF SDS/PAGE, mass spectrometry

6.2 Introduction

Tomato spotted wilt virus (TSWV) is a type species of the genus *Tospovirus*, the only plant infecting genus in the family *Bunyaviridae* (Whitfield *et al.*, 2005; van de Wetering *et al.*, 1999b), which mainly consists of animal infecting viruses vectored by arthropods, e.g. ticks and mosquitoes (Nichol *et al.*, 2005b). Although TSWV is transmitted exclusively by multiple species of thrips (Ullman *et al.*, 1997), *Frankliniella occidentalis* (Pergande), order *Thysanoptera*: *Thripidae*, is the main and the most efficient vector (Riley and Pappu, 2004; Wijkamp *et al.*, 1995; Whitfield *et al.*, 2005), making this interaction very specific. The specificity is governed by receptor based endocytosis allegedly involving a 94 kDa and a 50 kDa surface receptor proteins reported in *F. occidentalis* and linked to the recognition of TSWV infection (de Medeiros *et al.*, 2000; Kikkert *et al.*, 1998; Bandla *et al.*, 1998). Evidence exists of involvement of viral glycoproteins as determinants in the recognition process in the vector's midgut (Naidu *et al.*, 2008; Whitfield *et al.*, 2008). Both TSWV and *F. occidentalis* are of great economic importance due to their worldwide distribution, broad host range, and challenges in their control strategies (Whitfield *et al.*, 2005; Mumford *et al.*, 1996a; Sherwood *et al.*, 2000; Parrella *et al.*, 2003; Scholthof *et al.*, 2011).

The transmission of TSWV is in a persistent and propagative manner mainly by adult *F. occidentalis* (Ullman *et al.*, 1993; Sherwood *et al.*, 2000; Whitfield *et al.*, 2005), but only if the adults acquired the virus during their early larval stages (Ullman *et al.*, 1992; Wijkamp and Peters, 1993; van de Wetering *et al.*, 1996; Moritz *et al.*, 2004; Whitfield *et al.*, 2005). After replicating and circulating inside the host midgut, the virus is passed via cell to cell movement from the midgut lumen through the midgut cell wall into the haemocoel and finally to the salivary glands where it replicates further during thrips development (Whitfield *et al.*, 2005; Nagata, Inoue-Nagata, *et al.*, 1999a; Nagata *et al.*, 2002; Ullman *et al.*, 1993). Therefore, for successful transmission of the virus to occur, infection and multiplication of the virus in the salivary glands is mandatory (Wijkamp *et al.*, 1995; van de Wetering *et al.*, 1996; Nagata, Inoue-Nagata, *et al.*, 1999a; Nagata *et al.*, 2002). An increase in the viral non-structural proteins (NSs) and inclusion bodies in thrips body tissues and the primary salivary glands soon after the uptake of the virus has been reported, confirming the replication of the virus in the vector (Ullman *et al.*, 1992, 1993, 1995, 1997; Moritz *et al.*, 2004).

Replication of TSWV in *F. occidentalis* suggests a likelihood for pathological effects as well as changes in the vector's physiology which may influence the performance and fitness negatively

(Stumpf and Kennedy, 2007; Nault, 1994). However, it has proven difficult to confirm such effects due to the complexity of the virus-vector-host plant interaction (Thomas *et al.*, 2005). Conversely, recent studies have reported improved fitness of *F. occidentalis* as a result of feeding on TSWV infected host plants (Stafford *et al.*, 2011; Shrestha *et al.*, 2012; Ogada *et al.*, 2013; Shalileh *et al.*, 2016), assuming a triggered immune response to TSWV infection (de Medeiros *et al.*, 2004).

Small RNA pathways are reported to be responsible for the primary antiviral defense response in invertebrates immunity (Kemp *et al.*, 2013). Three small RNAs which play important antiviral roles in invertebrates have been identified in *Diptera*, model *Drosophila*: microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs) (Ding, 2010), which only differ on how they are processed (Bernstein *et al.*, 2001; Lee *et al.*, 2003; Denli *et al.*, 2004; Brennecke *et al.*, 2007). The response of the siRNA pathway is triggered by the presence of double stranded RNA (dsRNA), which is a byproduct of replication or gene expression common in nearly all viruses (Bernstein *et al.*, 2001). Similarly in vertebrates, the innate immune response to viral infection is triggered by the presence of dsRNA, however, the response is mediated by interferon (IFN) rather than RNAi (Schoggins and Rice, 2011). Conversely, to overcome the small RNA-directed immune response of the host, viruses have evolved methods to suppress the RNAi in their vectors which is considered as an example of evolutionary process between pathogens and their host (Dawkins and Krebs, 1979). In TSWV genome, the small RNA segment is reported to encode NSs which function as suppressors of RNA silencing (Bucher *et al.*, 2003; Lokesh *et al.*, 2010; Goldbach and Peters, 1996; Ding *et al.*, 2004; Li and Ding, 2005; Garcia *et al.*, 2006). The expression of these suppressors of RNA silencing in the vector might be a possible explanation for the mechanism of the non-lethal establishment and maintenance of persistently transmitted RNA viruses in invertebrates (O'Neal *et al.*, 2014), but this is still widely debated and remains poorly understood (Goic *et al.*, 2013).

Unlike vertebrates, the invertebrates solely rely on the innate immune system for defense against microbial infections and stresses since they lack an adaptive immune system (Barillas-Mury *et al.*, 2000; Hoffmann *et al.*, 1999; Irving *et al.*, 2001). Activation of the innate immune system, in both mammals and insects, involves receptor based recognition of the invading micro-organism by a germline-encoded pattern recognition receptors (PRRs), which include Toll-like receptors (TLRs) and cytoplasmic receptors, which recognize distinct microbial components known as pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs is

followed by direct activation of several overlapping and unique genes which initiate the expression of various antimicrobial peptides (AMP) as well as a variety of proteolytic cascades by the activation of the extra- and intracellular signaling cascades (Akira *et al.*, 2006; Hoffmann *et al.*, 1999; Barillas-Mury *et al.*, 2000). TLRs are the most studied of the innate immune receptors which detect conserved pathogen molecules, thereby forming the first line of the cellular defense. Additionally, the invertebrates' innate immune response to viral infection has been found to be very specific (O'Neal *et al.*, 2014). Recently, Goic *et al.* (2013) suggested a possibility of memory to prior exposure, despite lacking the antibody driven adaptive immunity found in vertebrates. Most studies on the invertebrate innate immune system have mainly been performed on *Drosophila* as the model organism, focusing on microbial infection, making it a reference point with regards to the available information and sequence databases (Barillas-Mury *et al.*, 2000; Hoffmann *et al.*, 1999).

Since the mechanisms involved in the persistent non-lethal maintenance of the RNA viruses in invertebrate vectors such as thrips are not well understood, and immune responses responsible for the virus triggered developmental and survival effects still being debated upon (Shalileh *et al.*, 2016; Ogada *et al.*, 2013; de Medeiros *et al.*, 2004), we initiated a pilot study to investigate the immune response of *F. occidentalis* to infection with TSWV. *F. occidentalis* larvae (L1) were exposed to feed on TSWV infected *Capsicum annuum* L. leaves, and after a 24 hours acquisition access period, they were collected and subjected to a proteomic analysis to identify differentially expressed proteins. We hypothesized that defense related proteins are activated in *F. occidentalis* in response to TSWV infection.

6.3 Materials and Methods

6.3.1 TSWV isolate

TSWV isolate (TSWV-N12) was obtained from the laboratory of virology, Wageningen University, the Netherlands. The isolate was maintained on 3 weeks old *C. annuum* as a host plant by mechanical inoculation according to a protocol developed by Mandal et al. (2008), which comprises a chilled inoculum of infected leaf sap in 0.2% sodium sulfite, 0.1 M phosphate buffer, 0.01 M 2-mercaptoethanol and 1% each of celite 545 and carborundum 320 grit. Soft finger rubbing technique was used to apply the inoculum onto the host plant. The first visual symptoms appeared after 10-14 days incubation period, and samples of these plants were tested using a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to confirm successful infection by TSWV. The infected plants were maintained at 28-30 °C

(greenhouse conditions) and served as inoculum source for further series of mechanical inoculation, as well as virus source for acquisition by *F. occidentalis* in the experiment.

6.3.2 *F. occidentalis* culture

F. occidentalis (F.o. 2), was also acquired from the laboratory of virology, Wageningen University, the Netherlands. A virus free stock culture was established on bean plants (*Phaseolus vulgaris* L.) at 2-3 leaf stage in a thrips proof cage maintained at 25±2 °C, 60-70% (RH) and 16:8h (L: D) (climate chamber conditions). To ensure availability of populations of the same age at any one time, a synchronized rearing was developed using adults from the stock culture which were fed on bean pods supplemented with a commercial honey-bee pollen mixture (Naturprodukte-mv.de; Naturprodukte Lembcke, Faulenrost, Germany) in glass jars, closed on top with 64µm thrips-proof nylon net. The bean pods were replaced at an interval of one day, and the harvested pods with eggs transferred to new glass jars for the emergence of L1 larvae. *F. occidentalis* used in this experiment were of the same age (<12 hours old) from the synchronized rearing. Both the stock culture and the synchronized rearing were maintained in separate climate cabins completely isolated from any other sources of thrips.

6.3.3 TSWV acquisition by *F. occidentalis* and sample preparation

At least 300 of < 12 hours old *F. occidentalis* larvae (L1) were introduced on *C. annuum* leaflets that were either healthy or heavily infected with TSWV (confirmed by DAS-ELISA), by softly blowing them off the bean pods. The leaflets were individually placed in a 15 cm Ø gypsum ($CaSO_4$ and charcoal 9:1 ratio) layered petri dish. The gypsum layer was then moistened with distilled water followed by placing a filter paper on top to absorb excess water. Virus infected or healthy leaflets were then placed on the filter paper, where the newly hatched L1 larvae were introduced for an acquisition access period (AAP) of 24 hours. The lid of each petri dish had three equally spaced 12 mm Ø punched holes covered with thrips proof 64 µm nylon mesh for ventilation. The petri dishes were additionally sealed with Parafilm M® (Pechiney Plastic Packaging, Inc., USA) to avoid any escape of *F. occidentalis*. After the 24 hours AAP, the larvae were carefully collected into a 2ml Eppendorf tube for each treatment (covering ≈ 3 mm of the Eppendorf tube base, i.e ≈ 250 L1 larvae) using a fine paint brush, then shock frozen in liquid nitrogen. The frozen materials were then pulverized into fine powder using a bead mill pulverizer machine. This was repeated 3 times for biological replicates.

6.3.4 Protein isolation: Phenol extraction method

Total soluble proteins were extracted according to Colditz et al. (2004). The pulverized insect materials were homogenized in extraction buffer (700 mM sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCl, 2% (v/v) β-mercaptoethanol and 2 mM PMSF, pH adjusted to 8.0). Then, saturated phenol (pH 6.6/7.9; Amresco, Solon, USA) was added. This was followed by several rounds of centrifugation before the proteins were precipitated using 0.1 mM ammonium acetate in methanol and then incubated at −20 °C overnight. The solution was again centrifuged, then the pellet was re-suspended in 80 % acetone solution, followed by final centrifugation. The resulting pellet was air dried at room temperature and then weighed.

6.3.5 Two-dimensional isoelectric focusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-IEF/SDS-PAGE)

From the resulting protein pellet, 5 mg were re-suspended in rehydration buffer (8 M Urea, 2 M thiourea, 2% (w/v) CHAPS, a trace of bromophenol blue, ddH_2O, 100 mM DTT, 12 µl/ml DeStreak-reagent, 0.5% (v/v) IPG-buffer pH 3-11 NL, GE Healthcare, Freiburg, Germany) and directly loaded onto an IPG strip (18 cm, pH 3–11 non-linear (NL), GE Healthcare, Freiburg, Germany). Isoelectric focusing was performed as described in Mihr and Braun (2003). The IPG strips were equilibrated twice for 15 min each time, first by immersing them in equilibration buffer (6 M urea, 50 mM Tris–HCl (pH 8.8), 30% glycerol and 2% SDS) with DTT (1%, w/v) to reduce the cysteine residues, and then in equilibration buffer with iodoacetamide 4% (w/v), for informal alkylation of cysteine residues. For the second gel dimensions, the equilibrated strips were shortly rinsed in tricine gel buffer (3 M Tris-HCl, pH 8.45, 0.3% SDS). The IPG strips were then carefully fixed horizontally onto 12% acrylamide SDS gels. The gel run was performed for 18 h at 30 mA per gel using the Bio-Rad Protean II XL gel system (Biorad, München, Germany). The two-dimensional gel electrophoresis for TSWV exposed and non-exposed *F. occidentalis* were repeated at least three times for the 3 biological replicates.

6.3.6 Gel staining procedure

Fixing of all the 2D gels was done using 10% (v/v) acetic acid in 15% (v/v) ethanol for 2 hours, then stained overnight with Coomassie blue CBB G-250 (Merck, Darmstadt, Germany) as described by Neuhoff et al. (1985; 1990). The gels were then de-stained several times using ddH_2O before being scanned on a UMAX Power Look III Scanner (UMAX Technologies, Fremont, CA, U.S.A.).

6.3.7 Quantitative gel analysis of the differentially expressed proteins

The gel scans were evaluated using Delta2D software, version 4.3 (Decodon, Greifswald, Germany) as described in Berth et al. (2007) and Lorenz et al. (2014). Three replicates per treatment (*F. occidentalis* exposed or non-exposed to TSWV) were used (Fig. 6.1A). Spot detection was done automatically with minor manual corrections of obvious gel disturbances. Delta2D software was used for in-gel normalization of the overlays of three replicate gels per treatment (Fig. 6.1B). Significant variation in spot volumes between the exposed and the non-exposed *F. occidentalis* gels was determined using a Student's t.-test (p-value ≤ 0.05) based on the normalized relative spot volume. Spots differing in volume were color coded according to their respective treatment (Fig. 6.1C). A true difference in protein abundance was considered only if the variation in spot volumes between the compared treatments had a factor ≥ 1.5. Protein spots with significant variation in volume were subsequently identified by mass spectrometry.

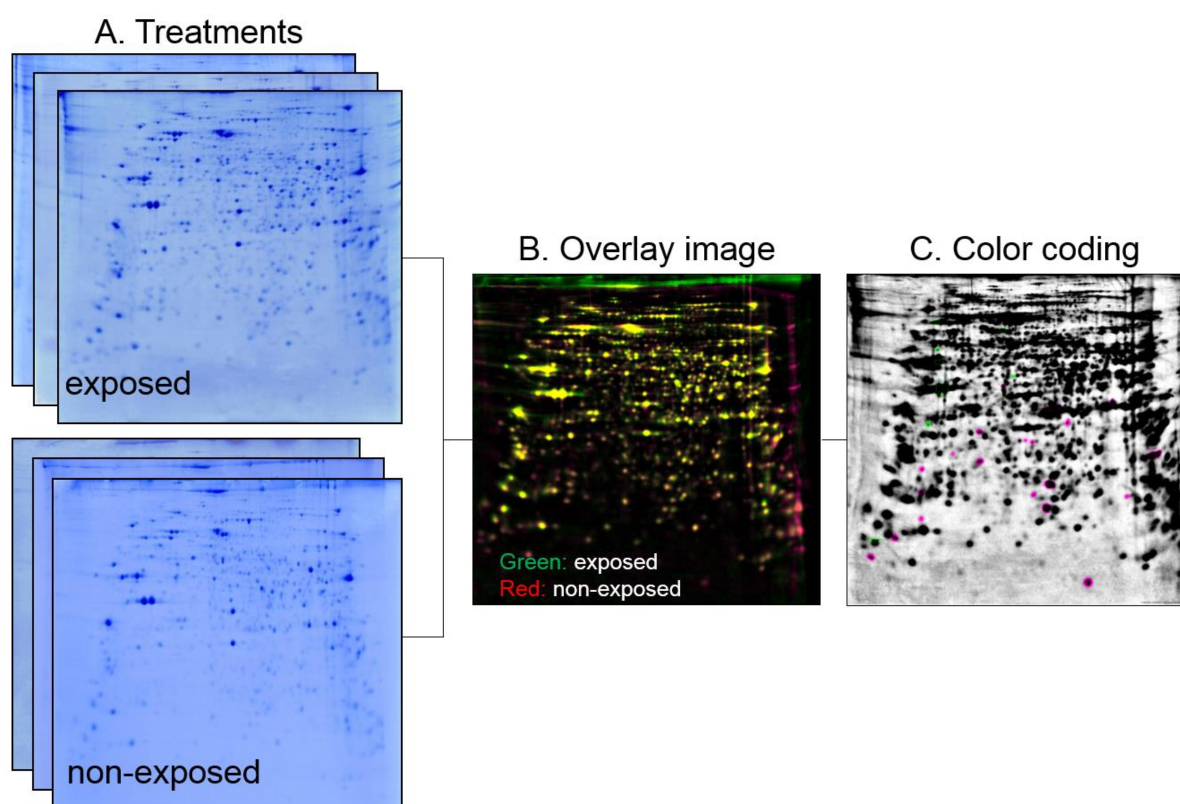

Figure 6.1: Quantitative gel analyses using Delta2D software. A. Exposed and non-exposed treatment replicates (3 replicates each), B. Overlay gel image of the two treatments in Delta2D, and C. Color coding gel image of the differentially expressed protein spots in the exposed (red spots) and the non-exposed (green spots) treatments.

6.3.8 Protein identification by mass spectrometry

Analyses of the differentially expressed proteins between treatments were based on proteins identified by mass spectrometry (MS). Tryptic digestion and MS analysis were performed according to Klodmann et al. (2010) using the EASY-nLC System (Proxeon) coupled to a MicroTOF-Q II mass spectrometer (Bruker Daltonics, Bremen, Germany). Protein identification was carried out using the MASCOT search algorithm (www.matrixscience.com) against the (i) Ensembl Metazoa (www.metazoa.ensembl.org), (ii) Flybase (www.flybase.org), (iii) SwissProt (www.uniprot.org) and (iv) NCBInr (www.ncbi.nig.gov) databases.

A schematic of the experimental workflow is shown in Fig. 6.2.

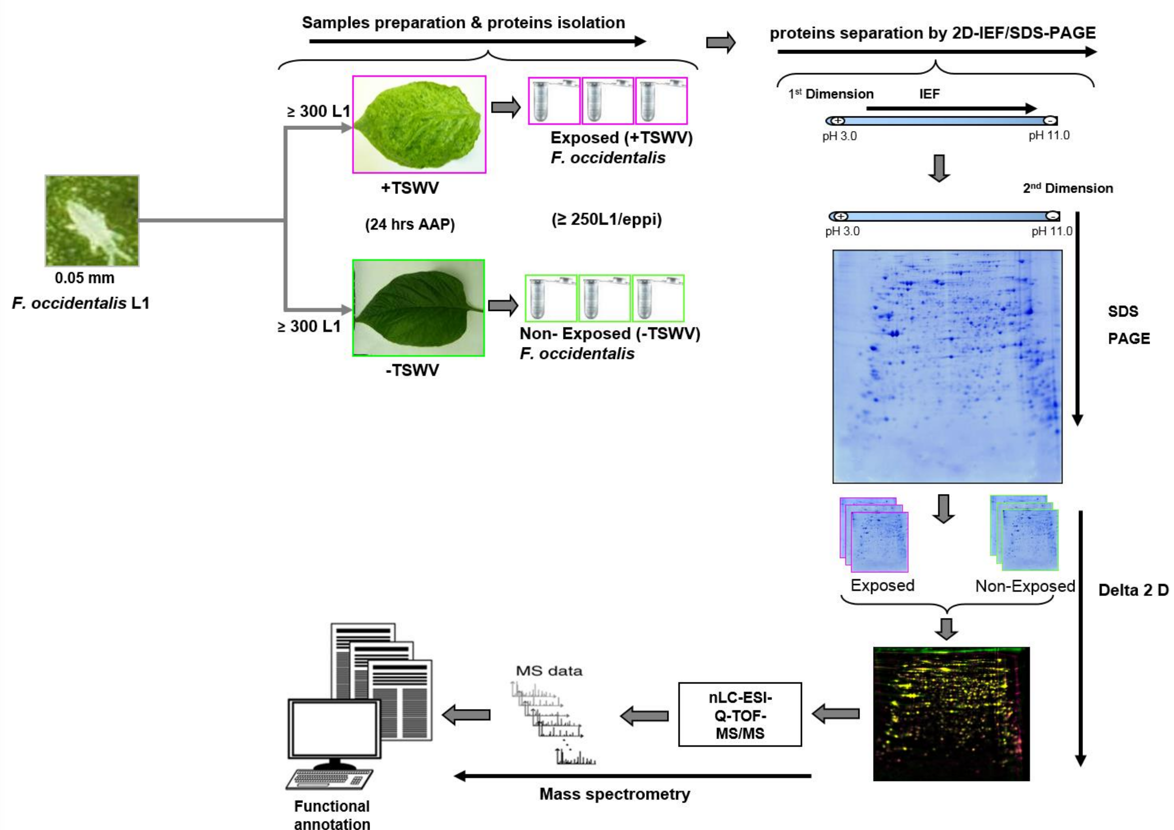

Figure 6.2: Schematic of the experimental workflow for the proteomics analysis of the differentially expressed proteins in *F. occidentalis* L1 after 24-hours exposure to TSWV.

6.4 Results

6.4.1 Changes in the proteome of *F. occidentalis* L1 after 24-hours exposure to TSWV

From the Delta2D evaluation, 901 spots were detected, but only 30 showed significant variation in volume according to a p-value limit of ≤ 0.05 based on the normalized relative spot volume (Fig. 6.3). Out of the 30 spots, 22 increased in volume in response to TSWV (significantly

increased in the exposed treatment) (Table 6.1), while 8 decreased in volume (significantly increased in the non-exposed treatment) (Table 6.2). Proteins in spots that significantly changed in volume (Fig. 6.3) were analysed by mass spectrometry. Not all the differentially expressed spots were successfully characterised, due to the limited data bases as well as some contamination with keratin. Nevertheless, names and physiological background of the identified proteins are given in Table 6.3. Furthermore, an interactive gel-map is available online (www.gelmap.de/1290 password: FoProt) which summarizes Fig. 6.3 and Table 6.3.

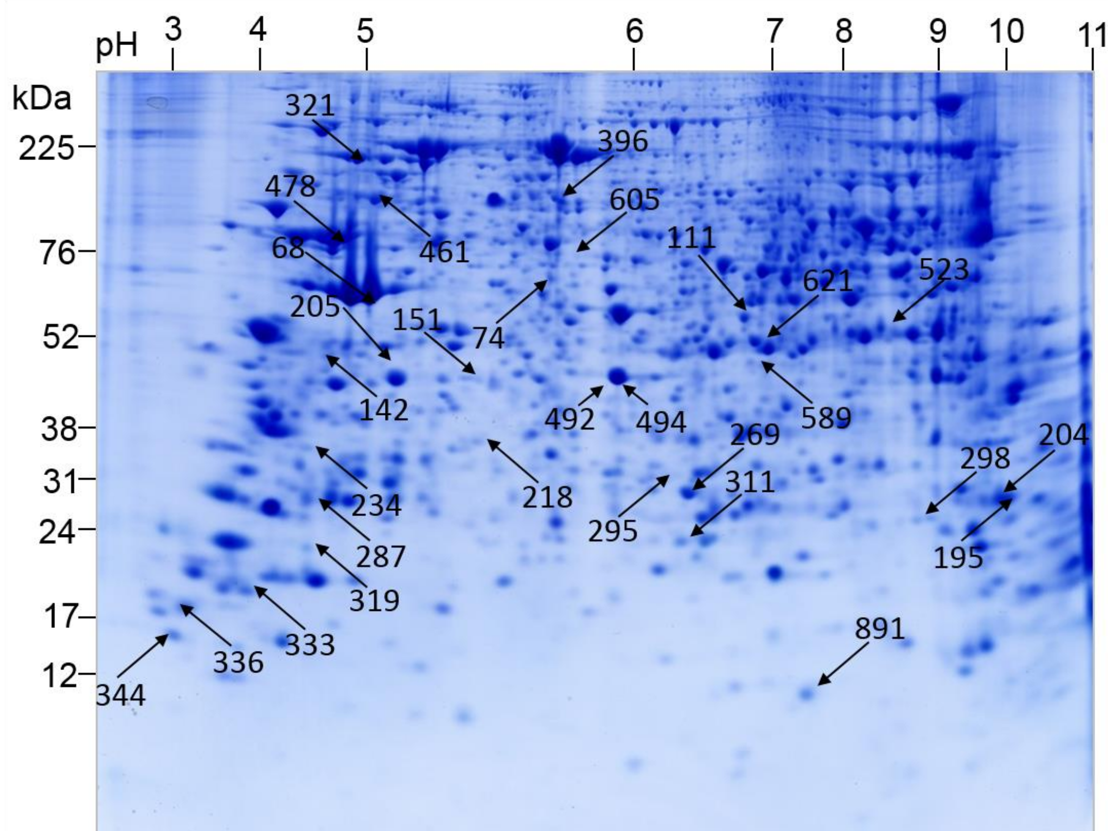

Figure 6.3: Two-dimensional reference gel map for *F. occidentalis* proteome. A 3-11 non-linear immobilized pH gradient (IPG) strip was used to separate total soluble proteins by isoelectric focusing (pIs are given above the 2D gel), followed by their separation according to molecular mass by SDS-PAGE (the masses of standard proteins are given to the left of the 2D gel). Coomassie brilliant blue G250 was used to stain the gels. The arrows indicate spots which are significantly altered in volume, and the numbers represent spot IDs (www.gelmap.de/1290 password: FoProt).

Table 6.1: Means of normalized relative spot volumes and coefficient of variation for the proteins increased in abundance in *F. occidentalis* after 24 hours exposure to TSWV.

Mean normalized volume of group 'Exposed'	Coefficient of variation of group 'Exposed'	Mean normalized volume of group 'Non-exposed '	Coefficient of variation of group 'Non-exposed'	Ratio of mean normalized volume 'Non-exposed' / mean normalized volume 'Exposed'	t-Test of mean normalized volume 'Non-exposed' / mean normalized volume 'Exposed'	Spots ID
0.175	7.769	0.115	0.637	0.657	0.003	**311**
0.057	8.051	0.037	17.183	0.651	0.023	**621**
0.301	2.831	0.194	1.192	0.643	0.000	**269**
0.167	10.682	0.102	6.991	0.611	0.009	**204**
0.034	13.461	0.021	5.329	0.604	0.016	**589**
0.021	0.612	0.013	30.211	0.587	0.030	**319**
0.113	14.094	0.062	14.129	0.545	0.0160	**195**
0.029	5.639	0.016	29.497	0.540	0.018	**74**
0.074	20.712	0.040	5.072	0.534	0.034	**287**
0.014	5.778	0.007	16.674	0.531	0.003	**205**
0.129	19.470	0.068	14.109	0.523	0.032	**523**
0.075	18.104	0.038	19.434	0.502	0.027	**333**
0.029	6.121	0.014	38.013	0.484	0.020	**234**
0.044	11.184	0.021	8.603	0.472	0.003	**111**
0.134	6.327	0.051	61.951	0.381	0.023	**891**
0.066	14.095	0.025	49.919	0.375	0.019	**218**
0.056	8.214	0.020	31.137	0.363	0.003	**151**
0.033	13.690	0.010	12.416	0.310	0.002	**295**
0.075	20.786	0.023	43.378	0.305	0.016	**344**
0.022	15.907	0.006	56.374	0.288	0.011	**494**
0.015	6.366	0.004	90.328	0.286	0.019	**492**
0.029	21.616	0.001	117.720	0.030	0.003	**298**

Table 6.2: Means of normalized relative spot volumes and coefficient of variation for the proteins of decreased abundance in *F. occidentalis* after 24 hours exposure to TSWV.

Mean normalized volume of group 'Exposed'	Coefficient of variation of group 'Exposed'	Mean normalized volume of group 'Non-exposed'	Coefficient of variation of group 'Non-exposed'	Ratio of mean normalized volume 'Non-exposed' / mean normalized volume 'Exposed'	t-Test of mean normalized volume 'Non-exposed' / mean normalized volume 'Exposed'	Spots ID
0.042	19.732	0.160	18.726	3.792	0.006	**336**
0.022	19.487	0.043	20.868	1.997	0.037	**142**
0.013	7.894	0.023	11.650	1.774	0.008	**461**
0.144	3.329	0.252	21.757	1.754	0.050	**321**
0.335	11.531	0.559	10.955	1.671	0.012	**478**
0.146	4.941	0.231	5.661	1.591	0.001	**396**
0.009	8.502	0.014	10.765	1.537	0.015	**605**
1.835	7.969	2.775	14.279	1.512	0.035	**68**

6.4.2 Proteins identification

Most proteins were identified via protein sequences of other insects, which included: *Drosophila* spp (64%), *Anopheles gambiae* (5%), *Ceratitis capitata* (9%), *Lysiphlebus testaceipes* (5%), *Sarcophaga peregrine* (5%), *Bactrocera dorsalis* (9%) and *Vespula vulgaris* (5%), as well as other metazoan sequences (Fig. 6.4). Sequence identity within exons on average was above 90%. Hence, the protein sequences of the metazoans used were fitting for the identification of *F. occidentalis* proteins. The identified proteins represented homologs of the corresponding proteins in *F. occidentalis* (which does not exclude that they might have differing physiological roles).

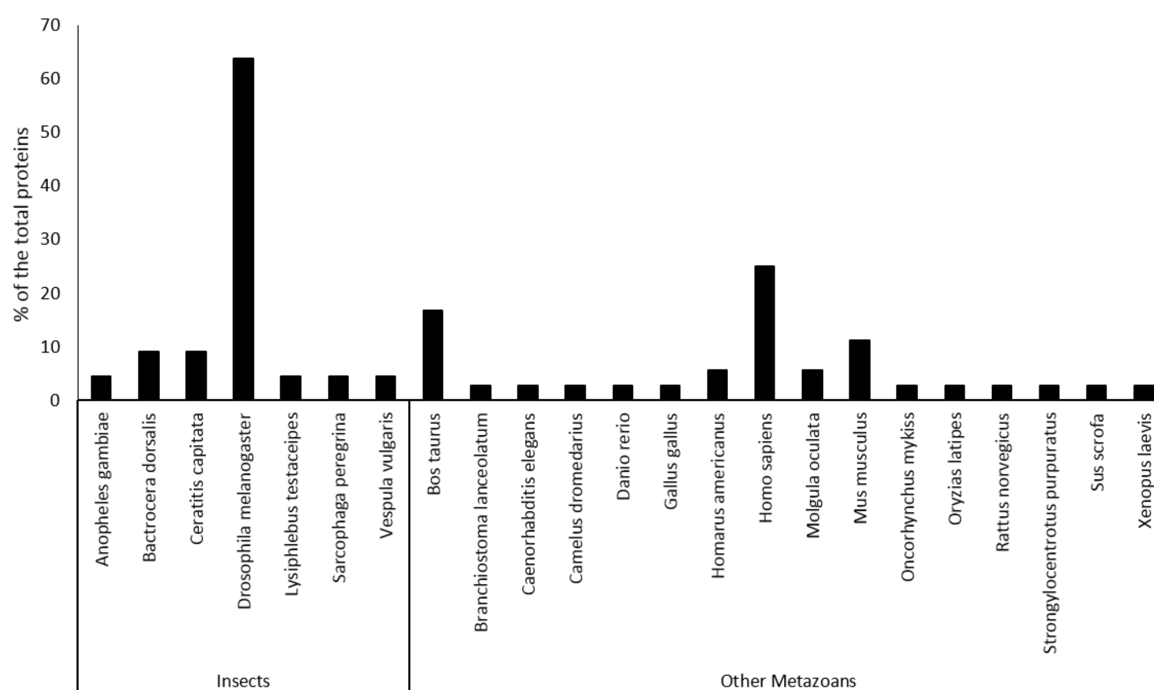

Figure 6.4: *F. occidentalis* proteins identified from other insects and metazoans protein databases.

6.4.3 Functional classification of identified proteins

Identified proteins were classified according to their functions based on various databases (Table 6.3). 26% of the proteins that were found to be significantly increased in abundance in response to the virus were identified to be involved in translation, 15% are involved in insect defense response, 13% in signaling pathways, another 13% in anatomical development, 9% in cellular lipid metabolic processes ($NADP^+$), 8% are structural proteins, another 8% are involved in stress response, 5% in nucleotide metabolism and 3% are actin binding proteins (Fig. 6.5). On the

other hand, 63% of the proteins that were significantly decreased in abundance are structural proteins, 17% are involved in energy metabolism, 8% in amino acid metabolism and 4% are involved in protein processing, sugar metabolism and cell cycle each (Fig. 6.6).

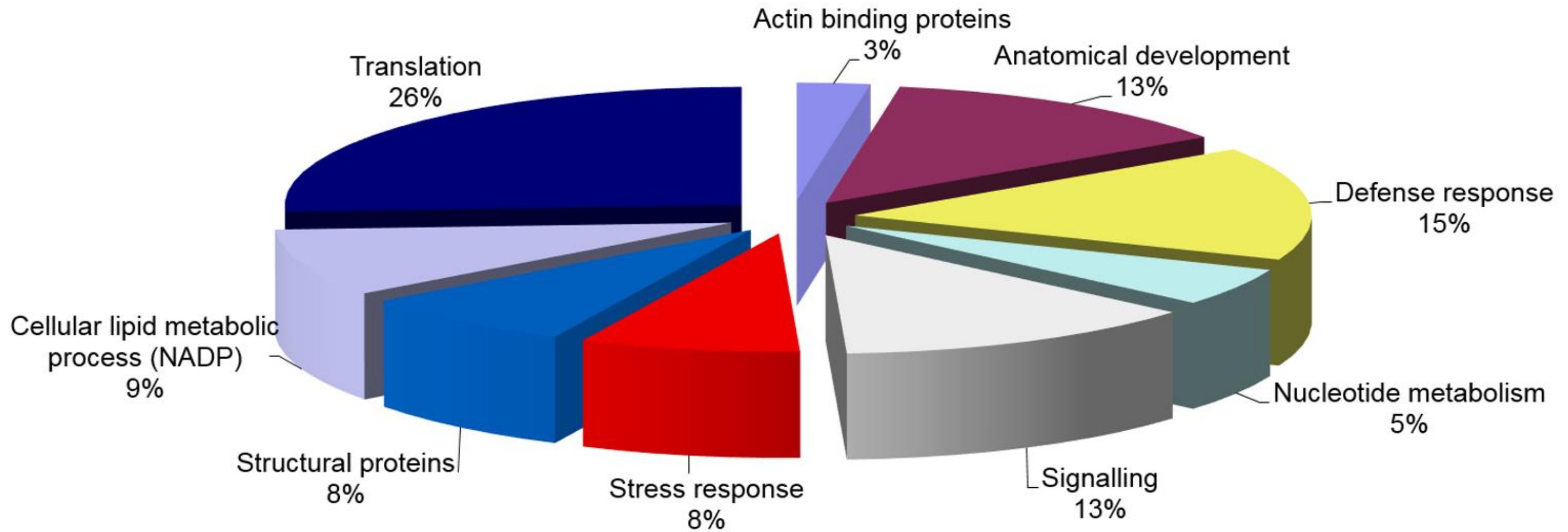

Figure 6.5: Functional classification of proteins of increased abundance in *F. occidentalis* L1 after 24 hours exposure to TSWV.

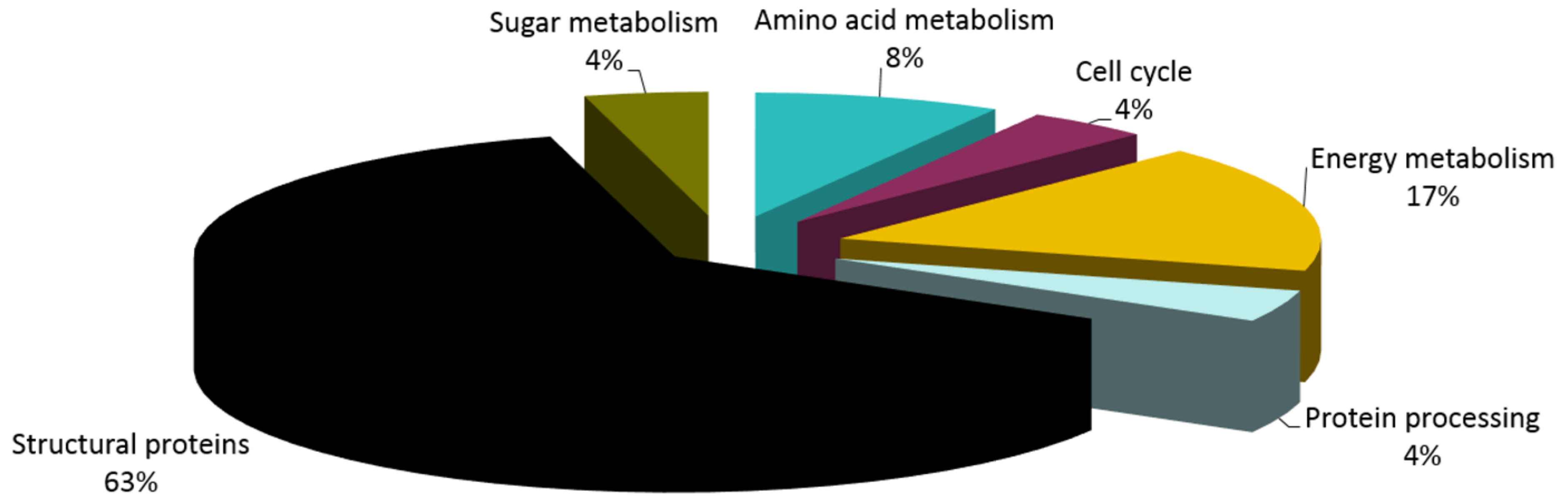

Figure 6.6: Functional classification of proteins of decreased abundance in *F. occidentalis* L1 after 24 hours exposure to TSWV.

Table 6.3: Protein identifications from spots with changed volumes between the TSWV exposed and the non-exposed (24 hours) *F. occidentalis* L1. Student's t-test (p-value ≤ 0.05) was used to determine significant changes in spot volume between the treatments based on normalized relative spot volumes. Changes in spot volume ≥ 1.5 were considered to represent alterations. MASCOT search algorithm was used for protein identification (www.matrixscience.com) against the (i) Ensembl Metazoa (www.metazoa.ensembl.org), (ii) Flybase (www.flybase.org), (iii) SwissProt (www.uniprot.org), and (iv) NCBInr (www.ncbi.nig.gov) databases. A) Proteins of increased abundance in *F. occidentalis* after the 24-hour exposure to TSWV, and B) Proteins of decreased abundance in *F. occidentalis* after the 24-hour exposure to TSWV (www.gelmap.de/1290 password: FoProt).

A) Proteins of increased abundance in *F. occidentalis* after the 24-hour exposure to TSWV

ID	Accession	Name	Organism	MM calc [kDa]	pI calc	MASCOT Score	Sequence Coverage (%)
			Structural protein				
74	ARP2_DROME	Actin-related protein 2	*Drosophila melanogaster*	44.7	5.8	293	16
74	ACT2_MOLOC	Actin, muscle-type	*Molgula oculata*	42.2	5	275	17
74	ACT1_BACDO	Actin, indirect flight muscle	*Bactrocera dorsalis*	41.7	5.2	267	21
			Anatomical development				
589	HORN_HUMAN	Hornerin	*Homo sapiens*	282.2	10.4	39	1
333	HAND2_DANRE	Heart- and neural crest derivatives	*Danio rerio*	23.2	9.7	41	3
205	CATL_SARPE	Cathepsin L	*Sarcophaga peregrina*	37.8	6	47	4
333	IDGF3_DROME	Chitinase-like protein	*Drosophila melanogaster*	49.2	7.8	36	1
74	HDX_HUMAN	Highly divergent homeobox (DNA Binding)	*Homo sapiens*	77.2	5.5	43	2
			Translation				
111	RLA0_CERCA	60S acidic ribosomal protein P0	*Ceratitis capitata*	33.9	7.6	224	10
111	RLA0L_HUMAN	60S acidic ribosomal protein P0-like	*Homo sapiens*	34.3	5.3	112	6
111	RLA0_CAEEL	60S acidic ribosomal protein P0	*Caenorhabditis elegans*	33.8	6.3	69	6
111	SYEP_DROME	Bifunctional glutamate/proline--tRNA ligase	*Drosophila melanogaster*	189.3	9.4	42	1
195	RL12_BOVIN	60S ribosomal protein L12	*Bos taurus*	17.8	10.3	56	5
204	RL12_BOVIN	60S ribosomal protein L12	*Bos taurus*	17.8	10.3	53	5
204	EIF3J_DROVI	Eukaryotic translation initiation factor 3, subunit J	*Drosophila virilis*	27	4.6	41	4
287	IF5A1_BOVIN	Eukaryotic translation initiation factor 5A-1,	*Bos taurus*	16.8	4.9	49	5
523	RL5_LYSTE	60S ribosomal protein	*Lysiphlebus testaceipes*	34.4	10.1	48	5

621	RLA0_CERCA	60S acidic ribosomal protein	*Ceratitis capitata*	33.9	7.6	150	8
			Stress response				
74	HSP7D_DROME	Heat shock 70 kDa protein	*Drosophila melanogaster*	71.1	5.2	317	11
74	HSP7C_DROME	Heat shock 70 kDa protein	*Drosophila melanogaster*	72.2	5.1	273	6
74	HSP7E_DROME	Heat shock 70 kDa protein	*Drosophila melanogaster*	74	6	209	8
			Signaling				
151	FIBA_CHICK	Fibrinogen alpha chain	*Gallus gallus*	82.4	5.6	39	1
195	AKAP3_MOUSE	A-kinase anchor protein 3	*Mus musculus*	95.5	5.9	40	1
195	UBIQ_CAMDR	Ubiquitin	*Camelus dromedarius*	8.6	7.6	83	24
621	VCIP1_MOUSE	Valosin-containing interacting protein 135 (VCIP135)	*Mus musculus*	134.4	6.8	42	1
621	GBLP_DROME	Guanine nucleotide-binding subunit beta-like	*Drosophila melanogaster*	35.6	7.9	53	3
			Defense response				
298	REV1_HUMAN	DNA repair protein REV1	*Homo sapiens*	138.2	9.5	39	0
74	HUGAB_VESVU	Inactive hyaluronidase B	*Vespula vulgaris*	40	9.8	38	2
195	DCD_HUMAN	Dermcidin	*Homo sapiens*	11.3	6.1	44	10
195	DCD_HUMAN	Dermcidin	*Homo sapiens*	11.3	6.1	37	10
311	UBP36_HUMAN	Ubiquitin carboxyl-terminal hydrolase 36	*Homo sapiens*	122.6	10.4	52	1
311	HP1_DROME	Heterochromatin protein 1	*Drosophila melanogaster*	23.2	4.9	36	4
			Nucleotide metabolism				
523	GMPR_ASCSU	GMP reductase	*Ascaris suum*	39.2	6.8	76	3
621	GBLP_DROME	Guanine nucleotide-binding subunit beta-like	*Drosophila melanogaster*	35.6	7.9	53	3
			Cellular lipid metabolic process (NADP)				
621	ALD1_MOUSE	Aldose reductase-related protein 1	*Mus musculus*	36	6.9	56	3
			Actin binding protein				
621	CORO7_RAT	Coronin-7 (Fragment)	*Rattus norvegicus*	48.9	5.3	41	2

B) Proteins of decreased abundance in *F. occidentalis* after the 24-hour exposure to TSWV

ID	Accession	Name	Organism	MM calc [kDa]	pI calc	MASCOT Score	Sequence Coverage (%)
			Structural protein				
68	ACT5_BACDO	Actin-5, muscle-specific	*Bactrocera dorsalis*	41.7	5.2	1332	50

68	ACT2_MOLOC	Actin, muscle-type	*Molgula oculata*	42.2	5	1308	51
68	ACTB_BOVIN	Actin, cytoplasmic 1	*Bos taurus*	41.7	5.2	1251	49
68	ACTB_ORYLA	Actin, cytoplasmic 1	*Oryzias latipes*	41.7	5.2	1199	49
68	ACTG_ANSAN	Actin, cytoplasmic 2	*Anser anser*	41.9	5.3	1192	47
68	ACTA_STRPU	Actin, cytoskeletal 1A	*Strongylocentrotus purpuratus*	41.8	5.2	1153	42
68	ACTC_BRALA	Actin, cytoplasmic	*Branchiostoma lanceolatum*	41.7	5.1	1144	39
68	ACTBL_HUMAN	Beta-actin-like protein 2	*Homo sapiens*	42	5.3	499	22
396	HORN_HUMAN	Hornerin	*Homo sapiens*	282.2	10.4	85	2
478a	TBAT_ONCMY	Tubulin alpha chain, testis-specific	*Oncorhynchus mykiss*	50	4.8	1155	42
478a	TBA1_DROME	Tubulin alpha-1 chain	*Drosophila melanogaster*	49.9	4.9	1151	42
478a	TBA3_HOMAM	Tubulin alpha-3 chain	*Homarus americanus*	50	4.8	806	28
478a	TBA1_HOMAM	Tubulin alpha-1 chain	*Homarus americanus*	50	4.9	776	25
478a	TBB_PIG	Tubulin beta chain	*Sus scrofa*	49.8	4.6	275	14
142	ACTA_BOVIN	Actin, aortic smooth muscle	*Bos taurus*	42	5.1	47	3
			Protein processing				
321	GRP78_BOVIN	78 kDa glucose-regulated protein	*Bos taurus*	72.4	4.9	243	9
			Sugar metabolism				
605	ENO_DROME	Enolase	*Drosophila melanogaster*	54.3	9.4	186	11
			Energy metabolism				
68	ACTBL_HUMAN	Beta-actin-like protein 2	*Homo sapiens*	42	5.3	499	22
478a	ATPB_DROME	ATP synthase subunit beta, mitochondrial	*Drosophila melanogaster*	54.1	5	705	33
478a	VATB_DROME	V-type proton ATPase subunit B	*Drosophila melanogaster*	54.5	5.1	52	2
461	ATPB_DROME	ATP synthase subunit beta, mitochondrial	*Drosophila melanogaster*	54.1	5	844	33
			Amino acid metabolism				
605	SAHH_ANOGA	Adenosylhomocysteinase	*Anopheles gambiae*	47.6	5.5	82	6
605	SAHHA_XENLA	Adenosylhomocysteinase A	*Xenopus laevis*	47.7	6	53	6
			Cell cycle				
605	CDC45_MOUSE	Cell division control protein 45 homolog	*Mus musculus*	65.3	5.1	48	1

6.5 Discussion and conclusion

During virus recognition and infection process, key proteins are involved which have to be considered when discussing the vectors innate immune response to viral infections. By clustering the proteins that were abundantly increased in *F. occidentalis* after exposure to TSWV, 9 functional groups were determined which include: stress response, signaling, defense response, translation, structural, anatomical development, actin binding, and proteins involved in cellular lipid and nucleotide metabolism. Most of these groups were found to be directly associated with the innate immune system, which therefore form the focus of our discussion.

The primary immune components in invertebrates are mainly found within the hemolymph and are involved in virus recognition as well as initiation of defense responses (Christensen *et al.*, 1989; Paskewitz and Christensen, 1996). Fibrinogen alpha chain protein was significantly increased in abundance in *F. occidentalis* exposed to TSWV. This protein is involved in signal transduction resulting in blood coagulation and melanization in vertebrates by formation of an insoluble fibrin matrix. Fibrin is one of the primary components of blood clot (hemostasis). This reaction is well studied in mammals, however, whether and how a comparable coagulation process in insect hemolymph is involved in host defense is not yet affirmed, and its investigation has proven to be extremely difficult (Cerenius *et al.*, 2010; Lavine and Strand, 2002; Hoffmann and Reichhart, 2002). Studies on *Helicoverpa zea* larvae infected with *Autographa californica polyhedrovirus*, reported that the circulating haemocytes encapsulated and killed the invading pathogen (Washburn *et al.*, 1996). Other reports have shown the involvement of serine protease and lipase, extracted from digestive juice of *Bombyx mori,* in antiviral signaling against *Bombyx mori polyhedrovirus* (Nakazawa *et al.*, 2004; Ponnuvel *et al.*, 2003). Additionally, several insect viruses from the family *Baculoviridae* are responsible for the induction of apoptosis during infection, limiting virus replication in the host (Clarke and Clem, 2003). Also, inhibition of virus survival in *F. occidentalis* hemolymph has been reported and linked to the potential barriers to virus replication within the hemolymph. Thus, the mechanism of TSWV movement within the vector's body remains unknown (Nagata, Inoue-Nagata, *et al.*, 1999a; Whitfield *et al.*, 2005).

The Fibrinogen alpha chain protein that was significantly increased in abundance in *F. occidentalis* exposed to TSWV, is a ligand for the Toll-like receptor 4 (TLR4), which recognizes PAMPS expressed by the virus and mediates the production of cytokines necessary for the development of effective immunity, by initiating signal transduction events induced by the pathogen, leading to blood coagulation and melanization reported in vertebrates (Cerenius *et al.*, 2010; Lavine and Strand, 2002; Hoffmann and Reichhart, 2002). TLR4 and TLR2 (see below)

have been reported to detect several viral glycoproteins (Akira *et al.*, 2006). For instance, in mammals, since the TLRs are known PRRs, *Vesicular stomatitis virus* glycoprotein (VSV-G) was previously reported to induce antiviral response dependent on TLR4 signaling (ligands include several viral proteins) (Georgel *et al.*, 2007) which activates the immune system. Additionally, the envelope protein of mouse *Mammary tumor virus* (MMTV) activates TLR4 (Burzyn *et al.*, 2004). Viruses or viral components such as *Human cytomegalovirus* (CHMV), *Herpes simplex virus* 1 (HSV-1) and *Measles virus* (MV) hemagglutinin protein have been reported to activate TLR2 (Bieback *et al.*, 2002; Compton *et al.*, 2003). Therefore, we can conclude that the stimulation of increased proteins which function as ligands for the TLR2 (Hsp 70, see below) and TLR4 (Fibrinogen alpha chain protein) in *F. occidentalis*, is triggered by TSWV infection, which is enveloped with glycoproteins, and thus, an antiviral response in the host innate immune system.

Effector pathways have also been linked to the antiviral innate immunity in mammalian TLRs. These include autophagy, which refers to natural catabolic process dependent on the cytosolic lysosome, and is constitutively competent to destroy infectious viruses and essential viral components that link viral detection to the signaling of the antiviral innate immunity (Richetta *et al.*, 2013; Mizushima, 2007). It is also reported to be crucial for antiviral defense in insects (Shelly *et al.*, 2009). Additionally, autophagy plays a house keeping role in maintaining the integrity of intracellular organelles and proteins as well as eliminating intracellular pathogens (Glick *et al.*, 2010). Generally, it is thought to be a non-selective degradation system, unlike the ubiquitin-proteasome system (see below), which specifically recognizes only ubiquitinated proteins for proteasomal degradation. The three types of autophagy pathways reported are: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), which all take part in the immune response (Mizushima, 2007). CMA is very specific and complex, involving recognition by the 70kDA heat shock cognate protein (Hsp70)-containing complex (Česena *et al.*, 2012; Bandyopadhyay *et al.*, 2008). The pathway is triggered upon viral recognition independent of viral replication. In *Drosophila*, it is triggered upon recognition of the VSV-G (Nakamoto *et al.*, 2012). We presume an antiviral autophagy dependent on the Toll signaling pathways in *F. occidentalis* in response to exposure to TSWV, as the stress response proteins that were increased in abundance were the Hsp 70. These proteins act as chaperones which enable the cell to cope with the harmful aggregation of denatured proteins due to stress. They are associated with the CMA which is reported to be active at all times in different tissues in mammals and almost all cell types in culture studies, but highly triggered in response to stresses and variation in nutritional status of the cell (Cuervo *et al.*, 1995).

Hsp 70 is a ligand to the TLR2, whose function is to recognize PAMPS that are expressed on pathogens, e.g. the viral glycoprotein, and mediates the production of cytokines which are necessary for the development of effective immunity (Waltenbaugh *et al.*, 2008). Cytokines are expressed abundantly in the peripheral haemocytes, and are responsible for the mediation of the host response to pathogen intrusion via stimulation of nuclear factor Kappa-light-chain-enhancer of activated B cells (NF-κB) (Hayden *et al.*, 2006; Doyle and Neill, 2006). NF-κB is a protein complex which belongs to the category of "rapid-acting" primary transcription factors, as it is found in almost all animal cell types in an inactive state and do not require new protein synthesis in order to become activated. This makes it one of the primary responders to harmful cellular stimuli. In vertebrates, NF-κB regulates various aspects of the innate and adaptive immune systems, by controlling cytokines production, transcription of antimicrobial effectors as well as genes that regulate cell survival, differentiation and proliferation (Hayden *et al.*, 2006). It is also involved in cellular response to stress, bacterial or viral antigens, cytokines, free radicles, among others (Gilmore, 2006; Perkins, 2007). In insects, the activation of NF-κB is triggered by the Toll pathway (which evolved independently in insects and mammals) and by the Imd (immune deficiency) pathway (Waterhouse *et al.*, 2007). The finding that the stimulation of TLRs, which are known PRRs, leads to the activation of NF-κB, brings further understanding on how different pathogens activate NF-κB (Hayden *et al.*, 2006).

The processing of the NF-κB is mediated by the ubiquitin-proteasome pathway which involves selective degradation of their C-terminal region containing ankyrin repeat motifs of the NF-κB inhibitor (IκB) (Karin and Ben-neriah, 2000). The IκB degradation is triggered in response to stimuli, such as those transduced in TLR activation, which potentiate NF-κB dimer activation in a non-canonical pathway (Basak *et al.*, 2007; Lo *et al.*, 2006). This process of NF-κB activation occurs solely via the activation of an IκB Kinase (IKK), which phosphorylates serine residues in the IκB regulatory domain. The phosphorylated IκB inhibitor molecules are then modified by a process called ubiquitination, leading to their degradation by a cell structure called proteasome, thus freeing NF-κB to enter the nucleus where it triggers the expression of specific genes leading to immune response (Nelson *et al.*, 2004; Livolsi *et al.*, 2001).

Proteins linked to the ubiquitin-proteasome pathway were also significantly increased in *F. occidentalis* after exposure to TSWV: Ubiquitin, Valosin-containing interacting protein 135 (VCIP135) and Ubiquitin carboxyl-terminal hydrolase 36. Ubiquitin, an 8.5 kDa regulatory protein, found in almost all tissues of eukaryotic organisms, was the most significantly increased protein in terms of abundance after *F. occidentalis* exposure to TSWV (24 fold). It forms the

main component of the ubiquitin-proteasome pathway which involves several sequential ATP-dependent enzymatic cascades (Wilkinson *et al.*, 2005; Komander and Rape, 2012; Kimura and Tanaka, 2010). Ubiquitination or ubiquitilation refers to the addition of ubiquitin to a substrate protein, which may affect the protein in many ways, like signaling for its degradation via the proteasome, affect its cellular location and activity, as well as prevent or promote protein-protein interactions (Glickman and Ciechanover, 2002; Mukhopadhyay and Riezman, 2007; Papers *et al.*, 2003). Only poly-ubiquitination at a defined lysine molecule is linked to the degradation by the proteasome, while the mono-ubiquitinations are involved in the regulation of processes such as translation, inflammation, endocytic trafficking and DNA repair (Mcdowell and Philpott, 2013). Additionally, unanchored-polyubiquitin has also distinct roles such as activation of protein kinases, and signaling. However, the other proteins, VCIP135 and Ubiquitin carboxyl-terminal hydrolase 36, are involved in the de-ubiquitination process in the ubiquitin-proteasome pathway, which leads to the activation of ubiquitin by cleaving of the inactively expressed form of ubiquitin (Turcu *et al.*, 2009).

A-kinase anchor protein 3 was also significantly increased in *F. occidentalis* after exposure to TSWV. It functions as a regulator of protein kinase A signaling cascade, as well as a transmembrane receptor protein of serine/threonine kinase signaling pathway. The pathway involves a series of molecular signals initiated by the binding of an extracellular ligand to a receptor on the surface of the target cell regulating programmed cell death (apoptosis), cell differentiation, among others, by phosphorylating the OH group of serine or threonine (Vijayaraghavan *et al.*, 1999).

Another immune related protein that was significantly increased in *F. occidentalis* in response to TSWV infection is Dermcidin (10 fold). It has antimicrobial activities and promotes natural cell survival under severe stress (Zeth, 2013). The proposed mechanism for the antimicrobial activities includes an ion gradient decoupling across biological membranes, and proteolytic activity (Song *et al.*, 2013). Heterochromatin protein 1, a stress response protein, was also increased in abundance. Its main function is the repression of transcription by altering the structure of chromatin, also referred to as chromatin silencing e.g. by conversion of large regions of DNA into an inaccessible state often called heterochromatin (Vermaak and Malik, 2009). Additionally, DNA repair protein REV1 and Inactive hyaluronidase protein, both of which are linked to the host defense response, were increased abundantly in response to TSWV infection. Inactive hyaluronidase proteins are usually triggered in response to the invasion by foreign bodies or injury, leading to restriction of damage to the tissue attacked or prevention/recovery

from the infection, while DNA repair protein REV1 protein is required for the induction of mutations as a form of immune reaction in response to physical and chemical agents (UniProtKB/Swiss-Prot, 2015).

Increase in abundance of the structural proteins and those involved in anatomical development, in response to viral infection, could be associated with the rapid growth and development reported in *F. occidentalis* after exposure to TSWV (Belliure *et al.*, 2005, 2008; Ogada *et al.*, 2013; Shalileh *et al.*, 2016). Additionally, the proteins associated with translation that were increased abundantly in response to TSWV could be linked to the replicating virus within the vector (Stumpf and Kennedy, 2007; Nault, 1994). For instance, Eukaryotic translation initiation factor 5A-1, one of the abundantly increased translation protein in this study, is an mRNA-binding protein, involved in the regulation of mRNA turnover by controlling the actin dynamics and cell cycle progression (RNA decay). It is also involved in stress response and maintenance of cell wall integrity. Additionally, it regulates TNF-alpha-mediated apoptosis (UniProtKB/Swiss-Prot, 2015). Moreover, the proteins involved in cellular lipids and nucleotides metabolic processes that were abundantly increased in *F. occidentalis* after exposure to TSWV, could also be associated with the replicating virus in the vector (Stumpf and Kennedy, 2007; Nault, 1994). For example, Guanine nucleotide-binding subunit beta-like protein, which is involved in nucleotide metabolism, functions by recruiting, assembling and/or regulating of a variety of signaling molecules. It is also involved in the positive regulation of viral genome replication by the host, which is initiated in response to viral infection. This selective mRNA translation opens up a target for the development of broad antiviral intervention in the host (Majzoub *et al.*, 2014).

It is important to state that these differentially expressed proteins are naturally present and are involved in basal multifaceted roles in the invertebrate physiology, but they are only activated significantly in response to stress or pathogen attack (Badillo-Vargas *et al.*, 2012). Thus, the use of L1s of *F. occidentalis* which were not exposed to TSWV, enabled us to clearly identify the differentially expressed proteins in response to viral infection. The proteins that were decreased in abundance in TSWV exposed *F. occidentalis* (which were the ones increased in abundance in the non-exposed treatment), were clearly those proteins which are basically involved in the normal anatomical growth and development of *F. occidentalis*.

Despite being able to see clearly the involvement *F. occidentalis* immune related proteins in response to viral infection, the use of other Metazoan databases in the functional description of the differentially expressed protein offers only basic information. Availability and use of *F.*

occidentalis transcriptome database related to the innate immune system would enable a more specific and detailed analysis.

In conclusion, the hereby presented results indicate that the exposure of *F. occidentalis* to TSWV triggers increased production of certain proteins which are associated with the innate immune response of the vector to viral infection. This substantiates the research findings that have reported improved fitness in *F. occidentalis* exposed to TSWV, and thus, improving our understanding to the complex virus-vector-host plant interaction.

7 General discussion and conclusions

The hereby reported study was launched to unravel the complex and specific interplay between TSWV, *F. occidentalis* and their shared host plant *C. annuum*, by focusing on the contribution of specific factors to the reported variability in the transmission efficiency of the virus within a vector population. Vector's genetics, gender and life processes as influenced by the virus were evaluated at individual level to determine their contribution to this interaction.

The first part of this study (Chapter 2) gives insights into the basic mechanism of inheritance of the trait vector competence in *F. occidentalis* in the transmission of TSWV. Determination of individual genotypes, the distribution of alleles related to the trait vector competence in filial generation, as well as calculation of the expected change in allele frequencies in populations were the focus of this study. This is, to our knowledge, the first attempt to determine the contribution of individual vector genetics to *F. occidentalis*-TSWV interaction. Several studies have postulated that the variability in transmission efficacy observed in thrips populations is due to differences in sexes (van de Wetering *et al.*, 1999a), or in genotypes, which can be separated for instance by random amplification of polymorphic DNA (RAPD) analysis (Gillings *et al.*, 1995), indicating that the trait vector competence is manifested in the genome and is variable. Cabrera-La Rosa and Kennedy, (2007) hypothesized that vector competence trait is recessively inheritable, in the case of TSWV and *T. tabaci* Lindeman. Furthermore, Halaweh and Poehling, (2009) reported in their preliminary crossing experiments with *Ceratothriopoides claratris* (Shumsher) (Thysanoptera: Thripidae) vectoring *Capsicum Chlorosis Virus* (CaCV), that males can only inherit the trait vector competence from their mothers. Additionally, under inbreeding conditions in an isolated colony the ratio of competent versus non-competent individuals strongly declined with increasing homozygosity, suggesting also that vector competence in *C. claratris* is controlled by a recessive allele (Halaweh and Poehling, 2009). These observations formed the basis of this study, in which we aimed to determine whether the same mechanism of inheritance found in *C. clarathris* and *T. tabaci* is also applicable for the *F. occidentalis*-TSWV relationship. Using leaf disk assays, we were able to control individual virgins' reproduction as well the random coupling events. However, due to technical reasons; with regards to the incubation time of the inoculated leaf disks before testing with Amplified DAS-ELISA (Appendix 2.1), it was not possible to determine individual genotypes prior to crossing or virgin reproduction experiments. Which led to the uneven distribution of individuals among the genotypes studied. A future development of an efficient

virus detection technique would be a great improvement for the set-up. Nevetheless, from our results we were able to show that the inheritance of the trait vector competence in *F. occidentalis* is in a haplodiploid pattern, and by following the trait in the filial generation in the basic crossing experiments, we were able to determine that the trait is linked to a recessive allele, since it was only expressed in females who were homozygous recessive for the trait. Since the aim of this study was to determine the contribution of these findings to the reported variability in the transmission efficiency, we analysed and compared the allele frequency between males and females. The results showed that males had the highest frequency for the competent allele compared to females, hence the allele frequency was different between the sexes. At the same time, calculation of the expected allele frequencies overtime showed an estimated increase in homozygosity towards the competent (recessive) allele. This however, contradicts earlier studies which reported an increase in homozygozity of the non-competent allele (Halaweh and Poehling, 2009; Cabrera-La Rosa and Kennedy, 2007) because of the assumed inbreeding effects. Haplodiploid insects are reported to have lower levels of heterozygosity compared to the diploid insects, and hence lower levels of genetic variability (Crespi *et al.*, 1991), with females having twice as many copies of alleles compared to males, which is an important factor in the determination of the overall allelic frequencies in a population (Hedrick and Parker, 1997). Therefore, our findings are important contribution for the understanding of the reported instability in transmission efficiency within vector populations. Additionally, these findings are fundamental for future molecular research; especially for the development of molecular markers for the trait vector competence, which would be very helpful for relating phenotypic (bioassays) and genetic findings.

Chapter 3 of this thesis aimed to determine the contribution of gender differences in *F. occidentalis* to the reported variability in their transmission efficiency of TSWV within a population. Differences between male and female *F. occidentalis* in their abilities to acquire and transmit TSWV have been shown in various studies (van de Wetering *et al.*, 1998; van de Wetering *et al.*, 1999b; Rotenberg *et al.*, 2009), and were linked to gender specificity in feeding behaviour, with males making more puntures but less intensive ingestion than females. As a result males transfer the virus with higher efficiency, because they leave behind more none lethally punctured cells, which are prerequisites for the replication and spread of the virus within the host plant (van de Wetering *et al.*, 1998; Stafford *et al.*, 2011). Our results clearly corroborate these findings.

Stafford *et al.* (2011) reported a difference in virus load between males and females as a result of difference in body sizes, with females having the highest virus load because of their larger body sizes compared to males. Additionally, in our study we evaluated the biological difference between males and females in terms of development time and survival. We found that males had a shorter development time and higher survival rate compared to females, regardless of being exposed on non-exposed to TSWV. This, together with high transmission efficiency, and the fact that males have the highest frequency of the competent allele, renders males the significant gender in terms of virus transmission within a vector population. Therefore, the ratio of males to females in a vector population is quite important when trying to model the overall vector potential of a given or developing population (Inoue and Sakurai, 2006).

Since TSWV is transmitted in a persistent and propagative manner by *F. occidentalis*, it replicates and spreads throughout the body tissues (German *et al.*, 1992; Ullman *et al.*, 1992; Hogenhout *et al.*, 2008), leading potentially to behavioural as well as physiological changes in the vector which can influence their survival and performance (Belliure *et al.*, 2005, 2008). Contradicting as well as consistent results have been reported on the influences of TSWV on the vector‘s biology in terms of survival, reproduction, development time and feeding behaviours. For example, negative effects on survival of *F. occidentalis* after exposure to TSWV (Sakimura, 1963; Robb, 1989). However, some results have showed improvement in survival/fitness which has been linked to a triggered immune response to viral infection (Ogada *et al.*, 2013; de Medeiros *et al.*, 2004; Irving *et al.*, 2001). Moritz *et al.* (2004) found no influence on survival of *F. occidentalis* when reared on either TSWV infected or non-infected plant, but a reduction of development time of *F. occidentalis* reared on infected host plants which we also observed in our earlier study (Ogada *et al.*, 2013) as well as in our results reported in Chapter 4. In the studies where improved survival/fitness were reported, the vector was reared on healthly plants after exposure to the virus. However,The experimental set ups by Sakimura (1963), Robb (1989) and Moritz *et al.* (2004) were such that the vector spent their entire lives on the infected host plant, which resulted on the negative or no influences on the vector survival/fitness. Many factors such as, the virus isolates, host plant species and age, among others, could have contributed to the reported contradicting results. For example, longevity and survival of *F. occidentalis* have been reported to be highly dependent on the physiological properties of the host plant with respect to intensity and the progression of virus infection (Froissart *et al.*, 2010). Our study on the mutualistic relationship between the vector and the virus (Chapter 4), corroborates former observations of increased survival of *F.*

occidentalis after exposure to TWVS (Ogada *et al.*, 2013; de Medeiros *et al.*, 2004; Irving *et al.*, 2001), however, this effect is only significant when adult *F. occidentalis* are reared on healthy (virus free) leaf disks and not on TSWV infected leaf disks, after exposure to the virus. For *F. occidentalis* to benefit from the improved fitness, TSWV manipulates their preferential behavior towards the host plants, resulting in a switch in preference after exposure to TSWV, with the virus carrying *F. occidentalis* preferring to feed on healthy host plants instead of TSWV infected ones. This is an evolutionary mechanism which has also been reported in other virus-vector systems, for example the cereal aphid *Rhadopalosiphum padi* and the geen peach aphid *Myzus persicae*, after acquisition of *Barley yellow dwarf virus* (BYDV) and *Potato leafroll virus* (PLRV) respectively (Ingwell *et al.*, 2012; Medina-Ortega *et al.*, 2009, Bosque-Pérez and Eigenbrode, 2011; Werner *et al.*, 2009; Jiménez-Martínez *et al.*, 2004; Eigenbrode *et al.*, 2002). Also the whitefly *Bemisia tabaci* is manipulated in the same way after acquisition of *Tomato yellow leaf curl virus* (TYLCV) (Moreno-Delafuente *et al.*, 2013).

The 'Vector Manipulation Hypothesis', formulated by Ingwell *et al.* (2012) to refer to the a bove described phenomenon, alledged that the virus manipulates the vectors biology and behavior to favour its multiplication and spread in a crop stand. The importance of this hypothesis was affirmed in our study by the development of predictive models for the disease dynamics (Chapter 5), which showed how these virus-induced influences on the vector's life processes contributed significantly to the reported rapid spread of the virus in a crop population after the initial transmission. In addition, these models are important contribution to closing the existing gap of reliability and uncertainties in TSWV epidemiology prediction. Most of the available predictive models for TSWV epidemics are weather based (Jones *et al.*, 2010; Olatinwo *et al.*, 2008; de Wolf and Isard, 2007; Magarey *et al.*, 2007), with only very few that have included the vector related aspects (Chappell *et al.*, 2013; Jeger *et al.*, 2015). As a consequence, these models often over or underestimate the severity of disease dynamics. With the evidence of TSWV influences on *F. occidentalis* biology in terms of fitness, performance and behavior, in the work presented in Chapter 5, we developed the first predictive model that is based on the influences of the virus induced vector's life processes controlling substantially the dynamics of the disease spread. This basic approach is essential for future improvement of the precision of predictive models, however we concede that there is still room for incorporation of other factors like vector gender, individual vector genetics, weather variables, land topography, cropping systems, environmental factors among others to optimize modeling of the

disease dynamics. Additionally, we can suggest the rationale of this mathematical model for the prediction of the disease dynamics of other mentioned virus-vector systems.

In Chapter 6, the study was based on findings presented in Chapter 4, as well as our earlier studies (Ogada *et al.*, 2013), where improved fitness of *F. occidentalis* after exposure to TSWV was reported. Which corroborates the work done by de Medeiros *et al.* (2004) who reported a triggered immune response in *F. occidentalis* in response to TSWV infection, using subtractive cDNA libraries to probe *F. occientalis* DNA macroarrays, and then evaluated the activated genes related to the vector's immune system. For a more detailed study of the vectors immune response to viral infection, we used a proteomics approach to evaluate the differentially expressed proteins in *F. occientalis* in response to TSWV infection. The proteins in *F. occientalis* that increased in abundance after 24 hours exposure, were mainly functionally associated with the innate immune system of the vector. These included: stress response, translation, signaling and defense response proteins, which are associated with varous innate immune pathways in the vector, for example, signal transduction, ubiquitin-proteosome, authopagy pathways, among others. However, a drawback in these data is the lack of *F. occidentalis* sequences in the data bases used, which limited our analysis to only other available Metazoans data bases. Hence, our results are just but "a tip of an iceberg" regarding the possible arrays of defense related proteins that are activated in response to viral infection, since we have to consider that there is no 100% homology with the available data bases. Nevertheless, it is clear from our findings that the immune related proteins in *F. occientalis* are triggered in response to TSWV infection. We suggest a more improved analysis in future, but only after *F. occientalis* sequence data base is developed and made available.

In conclusion, we are certain that the sequence of studies presented here provide a significant contribution in the understanding of the complex but specific interaction between TSWV, *F. occidentalis* and their shared host plants. Every chapter addresses selected factors of importance that influence this triangle relationship, showing that many factors as well as their interactions contribute either positively or negatively to the TSWV-*F. occidentalis*-host plant interaction. These complexicities of the involved controlling factors can now explain the pronounced variability in transmission efficiency of different *F. occidentalis* populations in natural systems. Therefore, we are convinced that these findings on vector's biology, behavour and genetics, as well as the virus manipulated influences on the vector's life process, can improve the development of precise management strategies for both the virus and the vector.

8 Outlook

In addition to the presented crossing experiments and evaluation of allele frequencies related to *F. occidentalis* vector competence in the transmition TSWV, a necessary and an important inclusion would be a population genetics study with *F. occidentalis* using Microsatellite based genotyping (also known as Short Sequence Repeat (SSR)) which was one of our additional research objectives. However, due to technical difficulties in DNA extraction from individual *F. occidentalis* using a DNeasy extraction kit, which resulted in too low amounts of DNA, we were forced to change our set ups, and could not achieve anaylsis of individuals' DNA, hence we sampled 6 individuals per extraction. Therefore, we were not able to clearly evaluate individual genotyping in a fragmented population over generations. Nevertheless, we developed a working SSR protocol, using M13 tailed forward primer (Appendix 4.1), using 8 polymorphic primers developed by Yang *et al.* (2012) (Appendix 4.2). Additionally, we were able to show that it is possible to perform direct PCR with individual *F. occidentalis* (Appendix 4.3), thus allowing the skipping of the individuals' DNA extraction step, this protocol could be further improved for better results. For efficient sequencing step with indivual PCRs, we recommend using directly labelled primers and combining all the available primers per PCR reaction mix with individual *F. occidentalis*. Together with the development of molecular markers, this approach will further improve the understanding into the molecular genetics of the vector's population with regards to the trait vector competence rounding off the objectives of the total study.

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Appendices

Appendix 1: DAS ELISA

DAS ELISA Buffers

Coating buffer (pH 9.6)

- 1.59 g Sodium Carbonate (Na2CO3)
- 2.93 g Sodium bicarbonate (NaHCO3)
- Dissolve in 900 ml A. dest, adjust pH to 9.6 with HCl and make upto 1 L
- Autoclave
- Store at room temperature

10x PBS (Phosphate Buffer Saline pH 7.4)

- 80 g Sodium Chloride (Nacl)
- 11.5 g dibasic Sodium Phosphate (Na2HPO4)
- 2 g monobasic Potassium Phosphate (KH2PO4)
- 2 g Potassium Chloride (KCl)
- Dissolve in 900 mL A.dest, adjust pH at 7.4 with NaoH or HCl and make upto 1L
- Autoclave
- Store at room temperature

PBS-T (PBS- Tween pH 7.4) = wash buffer

- 100 mL 10 X PBS
- 0.5 mL Tween 20
- Make upto 900 mL with A. dest, adjust pH to 7.4 with NaOH, or HCl and make upto 1 L
- No autoclaving
- Store at room temperature

PBS-TPO (PBS-T PVP egg albumin pH 7.4)

- 1g PVP (Polyvinylpyrrolidone, LOEWE)
- 0.1g egg albumin (LOEWE)
- Dissolve in 30 mL PBS – T, adjust pH to 7.4 with NaOH or HCl, make upto 50 mL
- No autoclaving
- Store in the refrigerator at 4 °C (for not more than 2 weeks)

Substrate buffer (pH 9.8)

- 9.7 mL diethanolamine
- 60 mL A.dest
- Adjust pH to 9.8 with HCl and make upto 100 mL with A.dest
- No autoclaving
- Store at room temperature

DAS ELISA Protocol

1. Dilute coating antibody (1st antibody IgG (LOEWE)) in coating buffer as recommended on the bottle label and add 100µl to the required number of wells for your test. (1:500 for TSWV test).
2. Wrap the plate tightly in cling film. Incubate the plate at 37°C for 4 hours.
3. Wash the plate five times with phosphate buffered saline + Tween 20 (0.05%) - PBST. To do this fill the wells of the plate with PBST and invert to remove the buffer. Repeat four times; pat the plate dry on paper towels.
4. Extract the samples by grinding 3 punches of leaf disks (using 1.5ml eppi.) of tissue with 1ml of PBS-TPO using a micro pistil. Centrifuge the plant concentrate at 10000 rpm for 3 min. and use the supernatant in the test.
5. Add 100µl of each sample, positive and negative control to the coated wells. All samples and controls are tested in duplicate.
6. Wrap the plate as described in (2) above and incubate at 4°C overnight (at least 16 hours).
7. Wash the plate as described in (3) above.
8. Dilute the antibody-enzyme (AP (LOEWE))) conjugate as recommended on the bottle label in conjugate buffer(PBS-TPO) and add 100µl to each test well (1:500 for TSWV test).
9. Wrap as in (2) above and incubate at 37°C for 4 hour.
10. Wash five times as described in (3) above.
11. Prepare the substrate just before use - add *p*NPP at 1mg/ml to substrate buffer
12. Add 100µl of prepared substrate to each test well.
13. Wrap the plate as in (2) above and incubate in the dark at room temperature for 1 hour or overnight for a good colour change.
14. Read the absorbance using a spectrophotometer at 405nm (for *p*NPP).

Appendix 2: AMPLIFIED DAS ELISA

Appendix 2.1: Amplified DAS ELISA- using ELISA amplification System(Kit) from Invitrogen (Cat.No. 19589-019)

The kit components included:

- Substrate (lyophilized NADPH)
- Amplifier (lyophilized amplifying enzymes (alcohol dehydrogenase and diaphorase))
- Positive control (streptavidin alkaline phosphatase conjugate)
    - With respective diluents for the substrate and the amplifier, which were reconstituted 10 min before use.

Addtitional requirement include preparation of:

- Tris-buffer saline (TBS) (pH 7.5)- the final washing buffer/positive control diluent: 0.05 M Tris-HCL (pH 7.5) and 0.15 M NaCl.
  To prepare add:
    - 1.51g of Tris base (MW 121.14)
    - 2.19g of sodium chloride (MW 58.5)
        - o Dissolve in 230 ml of deionized water
        - o Adjust pH to 7.5 with 2 M HCl
        - o Adjust the volume to 250 ml with deionized water
- Stop solution
  0.3 M H_2SO_4
  To prepare:
    - o _To 59 ml of deionized water, slowly add 1 ml of concentrated sulphuric acid (MW 98, specific gravity 1.84, purity 96 – 98%)
    - o **Caution:** When using sulphuric acid, exercise caution and follow manufacturer's recommendations. When making dilutions, **always add acid to water**.

ELISA Amplification System Protocol:

1. Perform a standard ELISA using alkaline phosphatase. Leave the last row of the microtiter plate empty at this stage for addition of the positive control.
2. Remove excess alkaline phosphatase by washing each well four times with 0.25 ml of TBS buffer (see above). Wet the positive control wells using this buffer. Remove the four wash from all wells just before you proceed with the next step.
3. Add the positive control dilutions to the last row of the microtiter plate.

4. Substrate: Add 50 µl of reconstituted substrate (prepared in advance) to each well, including the wells you have reserved for the positive control. Incubate for 15 min at 25 °C. To increase sensitivity, extend the length of incubation

   Amplifier: Add 50 µl of reconstituted amplifier (prepared in advance) to each well. Incubate for atleast 15 min at 25 °C, being careful not to contaminate wells.

   Add amplifier and substrate in same sequence.

5. Measure the color development using the assay method you have selected in advance.

   End point or stopped assay: stop color development after 15 min with 50 µl of 0.3 M H_2SO_4 per well. Add the stop reagent in the same sequence as the amplifier. Record the absorbance at 450 nm or 495 nm.

   Kinetic Read the absorbance at at 450 nm or 495 nm of each well every 3 min for 15 min.

6. To maintain the effectiveness of reconstituted substrate and amplifier for future use, store them immediately at 4 °C.

The principle of Amplified DAS ELISA

The “amplified DAS ELISA” is essentially the same as the usual DAS ELISA, until the substrate solution is added.

There is, however, one minor change. Namely, the RAM-AP is diluted 1000-fold rather than 500-fold to bring the background noises down to a reasonable level.

In principle, the colorimetric assay is in two steps.

First, NADP is converted to NAD by the alkaline phosphatase.

Second, a mixture of (1) a tetrazolium salt (INT), a diaphorase and alcohol dehydrogenase (ADH) is added. The pH for the second addition is 7.2. Since the alkaline phosphatase is basic, it effectively stops functioning after this second addition.

The NAD derived from the first step is a cofactor for ADH. As such, the NAD is reduced to NADH while ethanol is oxidised to acetaldehyde by the ADH (that is ADH transfers electrons from ethanol to NAD). Coupled to this reaction is that mediated by the diaphorase (a non specific electron transfer enzyme), which transfers electrons from NADH to INT. The result is regeneration of NAD, which can then function through another cycle (and hence yields amplification), and reduction of INT, which produces a soluble pigment with high extinction coefficient. The amount of INT ultimately reduced is a function of the concentration of NAD, which in turn is a function of the amount of alkaline phophatase in the ELISA well (Figure 1).

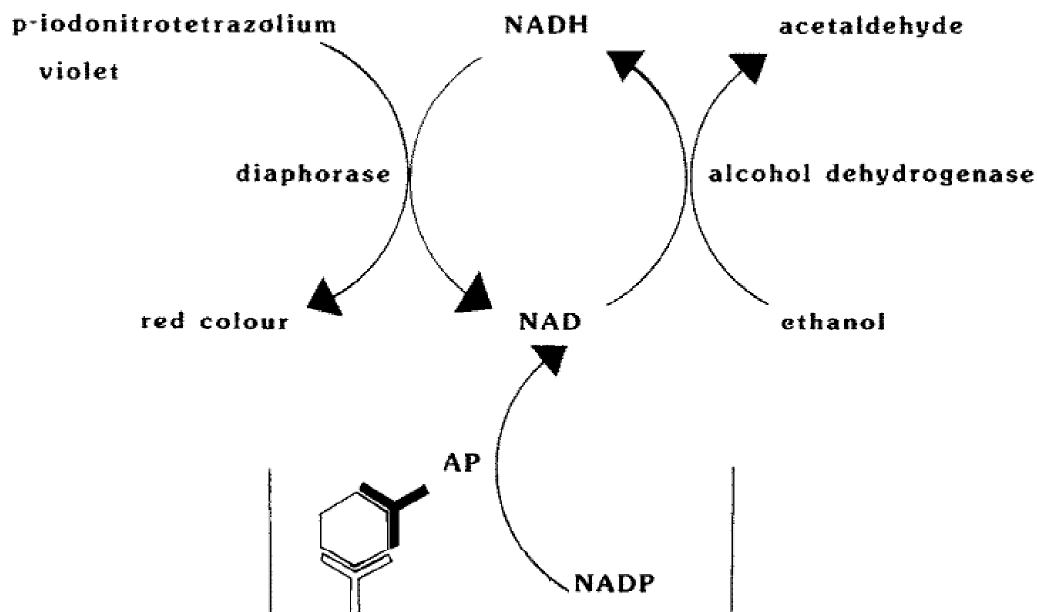

Figure 1: Schematic of the principle of alkaline phosphatase (AP) signal amplification

Appendix 2.2: Alternative Protocol-From Lab Mixed Reagents

1. After the ELISA plate is washed (following the antibody-AP incubation), the NADP substrate solution is added. It is prepared from stocks as follows:
   - 0.1 mM NADP (30 µl of a 20 mM NADP stock solution (at -20˚C) added to 6 ml of substrate buffer)
   - 50 µl of this first substrate solution is added to each well
2. After incubation for 30 minutes at room temperature, the second substrate solution is added, WITHOUT EMPTYING OUT THE FIRST. It is prepared from stock as follows:
   - 4.8 ml of 25 mM NaPi, pH 7,2
   - 1.2 ml of INT stock solution (-20 ˚C) (see below)
   - 150 µl of diaphorase stock solution (-20 ˚C)
   - 15 µl of alcohol dehydrogenase stock solution (4 ˚C)
   - 100 µl of this second substrate is added per well

   The quantities are for 1/2 96-well plate
3. Incubate for 30 min at room temperature, or until purple colour is sufficient. Then add 50 µl of 0.4 N HCL to stop the reaction.
4. Photogragh the plate immediately and assay in an ELISA reader as quickly as possible. After a short while, the reaction product begins to precipitate, and certainly after freezing and thawing it is likely to be precipitated.

The measuring wavelength for this ELISA should be at a bout 500 nm (492 nm is OK), while the reference wavelength should be at about 400 nm (414 nm is OK). Note that theses wavelengths are approximately the inverse of those used with normal substrate. Note that your ELISA reader is already programmed for subtracting the background of this ELISA substrate solution.

Reagent Sources

Diaphorase: Sigma, No. D-2381
NADP: Sigma,No. N-0505
Alcohol dehydrogenase: Sigma, No. A-3263
INT: Sigma, No. I-8377

Stock Solutions

NADP: 20mM in distilled water (15.3 mg/ml)*(Store in 30 µl aliquots at -20 ˚C)*

INT:

- 2.75 mM INT and 20% ethanol
- In 25 mM NaPi, pH 7.2
- *(Store in 1.2 ml aliquots at -20 ˚C)*

Substrate buffer (for NADP):

- 2.63 ml diethanolamine
- 0.10 g MgCl2 with 6 H_2O
- Adjust pH to 9.5 with HCl
- Bring to 500 ml with water
- *(Store at 4 ˚C)*

Diaphorase:

- 5mg/ml in 25 mM NaPi, pH 7.2
- *(Store in 150 µl aliquots at -20 ˚C)*

Alcohol dehydrogenase:5 mg/ml in:

- 50 mM tris-CL, pH 8.0
- 40% glycerol
- 0.1% sodium azide#10 mg/ml BSA
- *(Store in one aliquot at 4 ˚C—DO NOT FREEZE!)*

NaPi buffer:

- 25 mM Na_2HPO_4 - 1.42g in 400 ml H_2O
- 25 mM NaH_2PO_4 - 0.8625g in 250 ml H_2O
- Use NaH_2PO_4 to adjust Na_2HPO_4 to pH7.2

Appendix 3: Mechanical inoculation of TSWV on *C. annuum*

Figure 2: Mechanical inoculation of TSWV on *C. annuum* using soft finger rubbing technique.

Appendix 4: Population Genetics of *F. occidentalis* Using Microsatellites

Appendix 4.1: Microsatellites Protocol Using M13 tailed forward primer

The principle of M13-SSR-PCR is shown in Figure 3.

To consider:

Order the forward Primer with a M13-tail, i.e. add the sequence **GTAAAACGACGGCCAGT** to the 5'-end of the forward Primer. (Example RMS015: 5`**GTA AAA CGA CGG CCA GT** T AAT GTA GGC AGA TAT AAA GGA GT 3`)

The 700 or 800 IRD labelled M13-Primer (forward = uni) can be used. The concentration of the stock solution is 100 pmol/µL. The dilution is 1:40 (1 µL Primer + 39 µL H_2O) to obtain a working solution with a concentration of 2.5 pmol/µL. **Avoid exposure to light!!!** (Note: there are 10 µL aliquots of stock solution in the freezer. Please dilute these completely and make 40 µL aliquots. Finish these aliquots before new dilutions are made.)

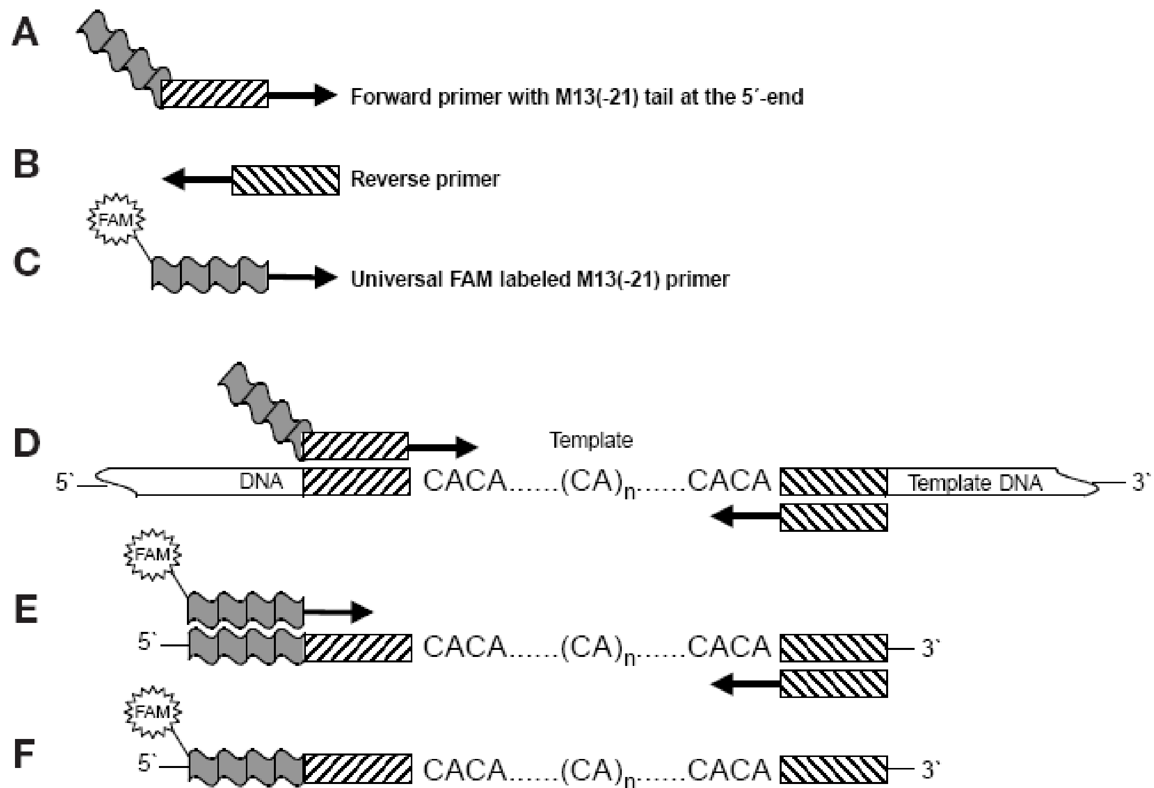

Figure 3: Principle of M13-SSR-PCR (from Schuelke, 2000)

M13-SSR-PCR Reaction mix

	Single SSR Reaction (10.0µl) in tubes	**Single SSR Reaction (20.0µl) in PCR plates**
H2O **[µl]**	**5.65**	**12.3**
10x Williams Buffer **[µl]**	**1**	**2**
dNTPs **(2mM) [µl]**	**0.75**	**1.5**
Taq-Polymerase DCS **(5U/µl) [µl]**	**0.1**	**0.2**
M13 tailed forward primer **(0.5pmol/µl** = 1:200) **[µl]**	**0.5**	**0.5**
M13 forward labelled (2.5pmol/µl = 1:40) **[µl]**	**0.5**	**0.5**
reverse primer **(5.0 pmol/µl** = 1:20) **[µl]**	**0.5**	**1.0**
Reaction Volume [µl]	**9.00**	**18.0**
DNA (10ng/µl) [µl]	**1.0**	**2.0**

To improve the specificity 0.75µl of Betain (5M) could be added in a single tube reaction (1.5µl for a plate).

The program

If using this system for SSCPs with the Licor Scanner increase the number of cycles to 30 or 35 in the first step and to 10 in the second.

Automate thermocycler program for PCR Amplication	
5 min	94 °C
17 to 25 cycles	
45 sec	94 °C
1 min	56-63 °C (choose suitable annealing temp. according to the specific primers used)
1 min	72°C
7 to 8 cycles	
30 sec	94°C
45 sec	52°C
1 min	72°C
1 cycle	
10 min	72°C

Add 100 µL formamide loading dye to each reaction and separate 0.5 µL on a SSR-acrylamide gel.

For SSCPs to be detected with the Licor Scanner apply 4µl of the PCR with 5µL SSCP-dye onto the MDE gel.

Note: *The reaction is very sensitive. Try first in 8-tube-stipes. Take twice the amount for one reaction in a microtiter plate.*

Literature: SCHUELKE M (2000) An economic method for the fluorescent labelling of PCR fragments. Nature Biotechnology 18, 233-234.

Appendix 4.2: Selected Primers from the study by Yang *et al.* (2012)

Locus	Genbank number (Sequence length)	Repeat motif	Primer sequence (5'-3')	Size range (bp)
WFT25	GT303588 (608bp)	(GA)7	F:CACCAGTCGCGTTCATTGA R:GCCTCCAGCAGCACAAGTA	96-149
WFT28	GT303349(784bp)	(TA)6	F:GGGCTTGAAATAATGTTCTG R:GTAAATAAATCAGTGGAGGGT	91-95
WFT51	GT310133(741bp)	(TG)8	F:GTACGCAGGAGAAGTAAATG R:ACAAATCCAGATGGCAACC	297-305
WFT66	GT305093(175bp)	(ACT)5	F:AACTTAGGAAGAAAGACTGTAGA R:TGTTTACGCACGCACGCAT	113-116
WFT87	GT303951(830bp)	(TG)5	F:GGTCTGAACTGTATGGGATG R:CAGGACCCTAGTATGTAAGAAA	259-277
WFT104	GT298583(602bp)	(GT)5	F:TCACGCAAGCTAACAGCCCT R:ACAAAGTTGCCTGCCTGAAT	150-159
WFT108	GT300460(686bp)	(AT)5	F:AGGATAGCTTGTTTTGTTGG R:CCATTTGTAACTAGCGTAGGA	135-140
WFT141	GT310355(612bp)	(GT)5	F:GCTTTTGCATACCTTGTCTTC R:GGTAAGGGCCGGTTTTGTT	174-183

Appendix 4.3: SSR results of direct PCR products of individual *F. occidentalis*

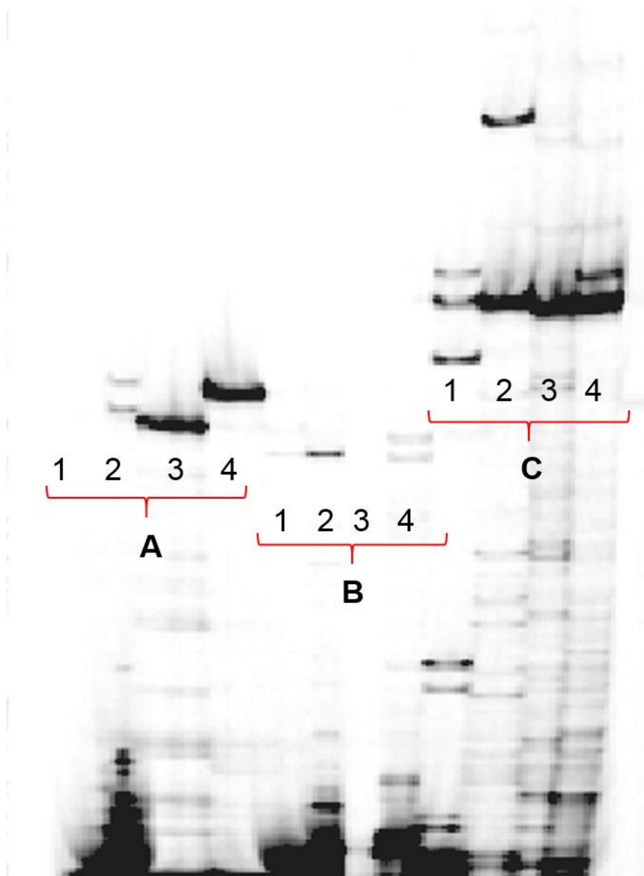

Figure 4: Direct PCR with individual *F.occidentalis*, 1-4 refers to individual PCR reaction of the individual thrips 1&2→ Individual females, 3&4→ Individual males. Using primers A. WFT104 (150-159bp), B. WFT108 (135-140bp) and C. WFT141 (174-183bp)

CURRICULUM VITAE

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Work Experience

2012- 2016:	Research Associate Gottfried Wilhelm Leibniz Universität Hannover, Institute of Plant Diseases and Plant Protection, Dept. Phytomedicine
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June-August 2004:	Internship- IAESTE (International Association for the Exchange of Students for Technical Experience) University of Reading (UK)

Workshops And Training

14th-16thMay, 2015:	Enhancing Risk-Index Driven Decision Tools for Managing Insect Transmitted Plant Pathogens. Asilomar, Pacific Grove, California USA

Project Grant/Fellowship Awards

2015:	Laurence Mound Travel Scholarship - The X International Symposium on Thysanoptera and Tospoviruses, Asilomar, California USA
2012-2016:	Deutsche Forschungsgemeinschaft (DFG) – PhD
2009-2011:	Deutscher Akademischer Austauschdienst (DAAD) – MSc.
2004:	British Council – BSc. Degree Internship at the University of Reading UK

LIST OF PUBLICATIONS

Peer Reviewed

P.A. Ogada, T. Debener and H.-M. Poehling (2016). The genetics of vector competence of western flower thrips (*Frankliniella occidentalis*) in *Tomato spotted wilt virus* transmission. *Ecology and Evolution (accepted).*

P.A. Ogada and H.M. Poehling (2015). Sex-specific influences of *Frankliniella occidentalis* (Western Flower Thrips) in the transmission of *Tomato spotted wilt virus* (*Tospovirus*). *Journal of Plant Diseases and Plant Protection* **122:** 264-274.

S. Shalileh, **P.A. Ogada**, D.P. Moualeu and H.M. Poehling (2016). Manipulation of *Frankliniella occidentalis* by Tomato spotted wilt virus (Tospovirus) via the host plant nutrients to enhance its transmission and spread. *Journal of Environmental Entomology* doi: 10.1093/ee/nvw102.

PA. Ogada, D.P. Moualeu and H.M. Poehling (2016). Predictive models for *Tomato spotted wilt virus* dispersal dynamics, considering *Frankliniella occidentalis* biology and preferential behaviour as influenced by the virus. *PloS One* **11** (5):1–20. doi:10.1371/journal.pone.0154533.

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P.A. Ogada, and H.M. Poehling (2016). Factors controlling virus-vector-host plant interactions: The model system *Frankliniella occidentalis* and *Tomato spotted wilt virus*. 48. Jahrestreffen des DPG-Arbeitskreises "Viruskrankheiten der Pflanzen", Gottfried Wilhelm Leibniz Universität Hannover, Germany. 7th-8th March, 2016, pp. 21 (*Keynote speech*).

P.A. Ogada, and H.M. Poehling (2015). Influence of vector gender of western flower thrips (*Frankliniella occidentalis*) in the transmission of *Tomato spotted wilt virus* (Tospovirus). In Mission possible: food for all through appropriate plant protection. The XVIII. International Plant Protection Congress (IPPC). Free University Berlin, Germany. 24th-27th August 2015, pp. 346 (*Poster presentation*).

P.A. Ogada, and H.M. Poehling (2015). The Genetics of Vector Competence of Western Flower Thrips (*Frankliniella occidentalis*) in *Tomato spotted wilt virus* Transmission. The Xth International Symposium on Thysanoptera & Tospoviruses (X^{th} ISTT). Asilomar, Pacific Grove, California USA. 16th- 20^{th}May, 2015, pp. 78 (*Oral Presentation*).

P.A. Ogada, E.G. Maiss and H.M. Poehling (2011). The Principle of Vector Competence of Western Flower Thrips (*Frankliniella occidentalis*) in the Transmission of *Tomato spotted wilt virus*. In International Research on Food Security, Natural Resource Management and Rural Development on the margin. Tropentag. University of Bonn, Germany. 12th-16th Sept, 2011, pp. 542 (*Poster Presentation*).

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DECLARATION

According to §6 (1) of the 'Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover' for the award of Dr. rer. hort.

I, Ogada Pamella Akoth, hereby declare that the work presented in this thesis, entitled:

"Factors controlling virus-vector-host plant interactions: The model system *Frankliniella occidentalis* and *Tomato spotted wilt virus*."

is my own original work, with no influence of a third party. All the used literatures, equipments and materials sources, as well as supporting institutions have been cited.
The thesis has not been and will not be submitted for any other university degree.

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Hannover, ………………………., Date,………………………….………

ERKLÄRUNG ZUR DISSERTATION

gemäß §6 (1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover für die Promotion zum Dr. rer. hort.

Hierdurch erkläre ich, Ogada Pamella Akoth, dass diese Dissertation mit dem Titel:

‘Factors controlling virus-vector-host plant interactions: The model system *Frankliniella occidentalis* and *Tomato spotted wilt virus*.’

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe. Meine wissenschaftliche Arbeit ist weder in Teilen noch in Gänze von Dritten gegen Entgelt oder sonstige Gegenleistung erworben oder vermittelt worden.

Die Dissertation wurde noch nicht als Prüfungsarbeit bei einer anderen Fakultät oder Universität eingereicht.

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Hannover, ………………………., Datum,…………………….………