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Abstract

In this study we analysed the ability of individual thrips to transmit *Tomato spotted wilt virus* (TSWV) in a population of *Frankliniella occidentalis* over their lifespan as adults (about 10 days). In three experiments a total of 636 thrips were individually tested for their transmission capacity through leaf disc assays using four inoculation access periods (IAPs). Almost half of the transmitting thrips maintained the capacity to infect leaf discs in each of the four IAPs, confirming the persistent propagative nature of the transmission modality. Nevertheless, a relevant number of thrips (9.25% of transmitter thrips) was able to transmit in the early phases of their adult life (for the first two IAPs), but did not transmit the virus for the remainder of their lifetime. We compared the virus titer of these individuals at the end of the fourth IAP with that of individuals that maintained transmission ability in the four IAPs and showed a statistically significant difference. This difference could be evidence for recovery from TSWV infection in individual thrips.

Keywords

Thrips; tospovirus; transmission; viral quantification.

Introduction

Tomato spotted wilt tospovirus (TSWV), the type member of the genus *Tospovirus* (Fauquet et al., 2005) (family Bunyaviridae), is one of the most devastating plant viruses infecting many horticultural

crops, as well as weeds worldwide (Goldbach & Peters, 1996; Prins & Goldbach, 1998; Pappu et al., 2009). Tospoviruses are exclusively transmitted by thrips belonging to the family Thripidae (order Thysanoptera) (Whitfield et al., 2005); currently 13 thrips species of the five genera *Frankliniella*, *Thrips*, *Scirtothrips*, *Ceratothripoides* and *Dictyothrips* are recognized as tospovirus vectors (Jones, 2005; Premachandra et al., 2005; Whitfield et al., 2005; Ohnishi et al., 2006; Ciuffo et al., 2010).

The TSWV–thrips interaction is peculiar because the tospoviruses may originally have been insect-infecting viruses that adapted secondarily to plant infection (Goldbach & Peters, 1996). Rapid co-evolution between thrips and tospoviruses is shown by the variability of different thrips populations in their efficiency in transmitting different TSWV isolates or tospovirus species (Nagata et al., 2004; Whitfield et al., 2005). Several hypotheses have been formulated to explain the variability in transmission ability; genetically, such variability was recently linked to an inheritable recessive trait under genetic control in the case of TSWV and *Thrips tabaci* Lindeman (Cabrera-La Rosa & Kennedy, 2007). In the thrips–virus relationship, a factor to be considered is the specific effects of virus infection on the fitness of the thrips host: these probably vary according to the specific thrips–virus combination, and remain unclear, with many studies giving contradictory results (Wijkamp et al., 1996; Ullman et al., 1997; Maris et al., 2004; Belliure et al., 2005; Stumpf & Kennedy, 2007). Nevertheless, at the molecular level, TSWV replication is likely to lead to a complex of responses in the insect body that may affect its fitness through pathogenesis (Goldbach & Peters, 1996; Wijkamp et al., 1996; Belliure et al., 2005; Stumpf & Kennedy, 2007). This feature allows the virus–vector relationship to be thought of as that of a virus causing an infection in the insect vector that may or may not be termed ‘disease’ according to each species-specific thrips–virus interaction. Insects have the capacity to activate their innate defence mechanism against a variety of pathogens through various molecular and cellular mechanisms (Strand, 2008); indeed, their cellular immune response to fungi, bacteria and protozoa is well known (Barillas-Mury et al., 2000; Irving et al., 2001; Hoffmann & Reichhart, 2002). In contrast, their response to viral infections remains relatively poorly understood (Li et al., 2002; Roignant et al., 2003; Bangham et al., 2006; Strand, 2008; Gerardo et al., 2010; McNeil et al., 2010). In the specific case of virus infection of the insect vector, TSWV infection of *Frankliniella occidentalis* (Pergande) induced the differential expression of several genes that are characteristically initiated as part of the insect defence response to pathogens, providing evidence that thrips mount an immune response to TSWV (de Medeiros et al., 2004; Rotenberg & Whitfield, 2010).

The persistent propagative infection of *F. occidentalis* indicates the potential for life-long infection, although a number of previous studies have shown that transmission efficiency decreases with age (van de Wetering et al., 1999; Whitfield et al., 2008). Nevertheless, transmission efficiency could be modified by factors other than age, such as the attack of a parasitic nematode (Sims et al.,

2009) or the specific onset of a defence response, which could conflict with the maintenance of high virus titer inside the insect, which was shown to be a prerequisite for efficient transmission (Rotenberg et al., 2009). In addition, recently, virus infection was shown to modify the feeding behaviour of its thrips vector, resulting in more efficient virus transmission of male populations (Stafford et al., 2011).

The genetic requirements on the part of the tospoviruses during insect infection are fairly well known, despite the limitation of the lack of a reverse genetic system for tospoviruses. The initial uptake of the virus in the midgut epithelial cells is thought to occur through receptor-based endocytosis, for which indirect evidence has previously been reported (Bandla et al., 1998; Kikkert et al., 1998; de Medeiros et al., 2000). Genetic and biochemical evidence revealed the involvement of glycoproteins (Sin et al., 2005; Naidu et al., 2008; Whitfield et al., 2008) as the viral determinant in this recognition process. Furthermore, a role in insect infection was also hypothesised for TSWV non-structural protein (NSs), the suppressor of gene silencing in plants (Takeda et al., 2002; Bucher et al., 2003; Lokesh et al., 2010), as well as the determinant involved in overcoming the Tsw resistance gene in pepper plants (Margaria et al., 2007): in fact, this protein is abundant in the salivary gland tissue of infected thrips, supporting an important role for this protein in vector transmission (Goldbach & Peters, 1996). Moreover, the ability of NSs to suppress silencing was also shown in tick cells (Garcia et al., 2006), suggesting that NSs could also interfere with silencing-based defence mechanisms in insect cells (Ding et al., 2004; Li & Ding, 2005), although possible interference with other anti-viral insect defence pathways cannot be ruled out.

Our current understanding of the anti-viral insect immune system is incomplete, particularly in the tospovirus–thrips interaction, and very little is known about how it may dynamically affect the virus titer during the lifespan of the adult insect. We therefore set up an experimental system to follow the efficiency of transmission for each individual thrips during approximately 10 days of their adult life through four successive inoculation access periods (IAPs). At the same time, we examined the titer of viral RNA at the end of the fourth IAP, in groups of individuals that differed in transmission efficiency during their lifespan. In particular, we showed that virus RNA titer was much lower in individuals who lost the capacity for virus transmission in the last two IAPs, when compared to individuals that maintained the efficiency of transmission for all four consecutive IAPs.

Materials and methods

Virus isolation and maintenance

The TSWV strain p202/3WT isolate from Sicily (Margaria et al., 2007) used in the experiment was derived from symptomatic leaf discs of *Datura stramonium* L. infected by *F. occidentalis*, kept under

liquid nitrogen and only transmitted twice by mechanical sap inoculation, in order to decrease the possibility of generating non-transmissible or low transmissible viral mutants. This isolate was mechanically inoculated on *D. stramonium*, at the two or three true leaf stage, using extraction buffer (50 mM phosphate buffer, pH 7, containing 1 mM Na-EDTA, 5 mM Na-DIECA and 5 mM Na-thioglycolate) for homogenization. Plants used for transmission experiments were grown in an insect-proof glasshouse at 20–25°C.

TSWV transmission trials

Laboratory transmission trials were carried out with leaf disc assays (Wijkamp et al., 1995; Tedeschi et al., 2001) using populations of *F. occidentalis* from north-western Italy. Thrips collected in the fields (in order to maintain some of the natural variability present in natural thrips populations) were reared on pollen and green bean pods, both as a food source and green bean pods as a oviposition site in gauze-covered glass jars, with corrugated cardboard on the bottom to provide pupation sites. Large-scale rearing was conducted in growth chambers at 25±1°C, 65±5% r.h. and 16:8 L:D (Tedeschi et al., 2001). In order to obtain larvae, several green bean pods were introduced into glass jars and left for 2 days to allow oviposition. Then pods were individually isolated in glass tubes and egg hatching was monitored; after hatching, larvae no older than 2–3 h were placed in a cage (Tashiro, 1967) on infected D. stramonium leaves. The systemically infected leaves were previously checked by double antibody sandwich–enzyme linked immunosorbent assay (DAS–ELISA) for TSWV, as previously described (Ciuffo et al., 2008). After 48 h the larvae were transferred to other cages on green bean pods to complete their development to adults.

After determining the sex, 1-day-old adult thrips were individually tested for virus transmission in plastic tubes (1.5 mL) using a leaf disc (12 mm diameter) of *D. stramonium*. Each thrips was allowed four inoculation access periods (IAPs) of 48 h each. After 72 h floating on water in 24-well plates, the leaf discs were analysed by DAS–ELISA with specific antiserum against TSWV. Each disc was homogenised with 0.5 mL of PBS-Tween containing 2% of PVP. Samples were considered positive if absorbance values were at least three times those of healthy controls. During the IAPs, adult thrips mortality was checked every 12 h. The transmission experiment was repeated three times.

Detection and quantification of viral RNA in thrips

Adult thrips alive at the end of the fourth IAP were separated into non-transmitters and transmitters. Transmitter thrips were then categorised into three classes: (a) transmitting only in the first or second IAP and unable to transmit in the third and fourth (1/4 IAP); (b) always transmitting (4/4 IAPs); (c) intermediate cases including transmitting in one (third or fourth), two or three IAPs (others). To verify

and compare the trend in viral titer in thrips, adults belonging to different classes for each transmission experiment were chosen for q-RT-PCR and processed at the end of the fourth IAP (only surviving individuals).

Total RNA was extracted from samples of 2–5 thrips following the procedure described in Boonham et al. (2002) with minor modifications. Five hundred µL of Trizol reagent (Invitrogen, Carlsbad, CA) were added for purification in plastic tubes (2 mL) containing the insects, as described in Mason et al. (2003). The contents were ground in sterile mortars with liquid nitrogen, and incubated at 25°C for 5 min. They were then transferred into plastic tubes and after adding 100 µL of chloroform, vortexed for 5 s, incubated at 25°C for 3 min and centrifuged at 12 100 *g* for 10 min in a microcentrifuge (Eppendorf 5402, Hamburg, Germany). The supernatant (250 µL) was transferred into another tube, along with 250 µL of isopropanol 99% and 1 µL of 1 mg mL^{-1} glycogen, and incubated on ice for 10 min. After centrifugation at 12 100 *g* for 10 min, the pellet was washed with 500 µL of 70% ethanol, and centrifuged at 12 100 *g* for 1 min; after removing the ethanol, the pellet was centrifuged at 12 100 *g* for 1 min, dried in a vacuum chamber (Speed-Vac Concentrator, Savant, NY, USA), re-suspended in 50 µL of water and centrifuged at 11 000 *g* for 5 s.

Reverse transcription from RNA extracts was carried out using random primers and the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Viral quantification was carried out by RT-PCR, using the following primers and TaqMan® probes: TSWV-CP-17F, TSWV-CP-100R, TSWV-CP-73T, WFTRNA-25F, WFT-RNA-93R-C and WFT-48T (Boonham et al., 2002). Real-time PCR reactions were carried out using a TaqManTM gene expression Master mix (Applied Biosystems) following the protocol and amplification conditions suggested by the manufacturer in a Step One Plus™ Real-Time PCR System (Applied Biosystems).

The resulting data were analysed using Step One™ software version 2.0 (Applied Biosystems). Relative quantification of TSWV RNA expression was carried out by comparative C_t estimation ($\Delta\Delta C_t$) (Livak & Schmittgen, 2001). Adults reared on healthy *D. stramonium* in laboratory conditions were used as a negative control during RNA extraction and RT-PCR amplification. In one experiment, absolute quantification of viral RNA was carried out using a dilution curve of a plasmid containing the coding region of the TSWV N gene (Margaria et al., 2007) quantified by fluorimetry.

Statistical analysis

The effect of the gender of thrips on the virus transmission efficiency, and the effect of virus infection on thrips mortality were analysed as a general linear model with a binary distribution and logit link considering a randomised block design where each thrips represents a statistical unit.

Also the effect of the gender of thrips and trial on their specific association to the different transmission classes was modelled as a general linear model with a binary distribution and logit link analysing a randomised block design. Analysis was run in two different and independent steps: the first step tested 1 IAP against the pooled group 4 IAPs+others; the second step tested 4 IAPs against others.

To compare virus titer in individual thrips, the significance of differences between the relative expression ratios of samples belonging to the different classes was calculated using the Kruskal–Wallis non-parametric test ($P \leq 0.05$).

All statistical analyses were performed using SPSS version 17.0 (SPSS, Chicago, IL, USA).

Results

Efficiency of TSWV transmission by *F. occidentalis*

Overall, 477 out of 636 *F. occidentalis* adults tested in leaf disc assays in the three transmission trials were able to inoculate TSWV, showing a transmission efficiency of 75.7% (Table 1). The population tested in the three experiments comprised 462 females (72.6%) and 174 males (27.4%), showing a transmission rate of 73.2% and 79.9%, respectively, that resulted to be not statistically significant (Table 1; sex: Wald $\chi^2 = 3.579$, $P = 0.059$; trial: Wald $\chi^2 = 9.794$, $P = 0.007$). In addition, rates of discoloured leaf discs as evidence of thrips feeding were usually lower in males than in females (data not shown). During the four IAPs, mortality was 13.8% for transmitters and 23.3% for non-transmitters (Table 2), and resulted to be significant (transmitter/non-transmitter: Wald $\chi^2 = 5.504$, $P = 0.019$; trial: Wald $\chi^2 = 15.403$, $P = 0.000$).

In order to study thrips transmission ability during their lifespan, only adult thrips alive at the end of the fourth IAP (i.e. 10 days after adult emergence) were considered. The combinations of transmission capability within the populations during the four IAPs in our experiment are shown graphically in Fig. 1A. Overall, 9.3% thrips were transmitters only in the first or second IAP, whereas 48.7% were transmitters in all four IAPs; furthermore, the total percentages of transmitter thrips in each IAP did not differ significantly and varied from 72% (first IAP) to 80% (third IAP) (ANOVA: $F_{3,8} = 0.478$, $P = 0.706$, n = 3) (Fig. 1A). Male transmitters showed longer persistence in their transmission ability: in fact, the percentage of thrips inoculating TSWV in all four IAPs was much higher for males than for females (Fig. 1B).

To detect possible evidence of loss of TSWV virus titer in individual insects losing the ability to transmit the virus, the transmission patterns observed for each individual were separated into three categories: (a) thrips transmitting only in the first or second IAP and unable to transmit in the third and fourth (1/4 IAP), (b) thrips always transmitting (4/4 IAPs), and (c) intermediate cases (here defined as 'others'). Of the transmitter thrips in 1/4 IAP (first or second), 9.2% were female and 9.5% male, whereas those in 4/4 IAPs were 44.1% female and 61.9% male (Table 3). The first step in the comparison [1/4 IAP (first or second) against the pooled group 4/4 IAPs+others], no statistical differences were found in transmission efficiency between males and females (sex: Wald $\chi^2 = 0.025$, $P = 0.874$; trial: Wald $\chi^2 = 11.042$, $P = 0.004$). The second step in the comparison (4/4 IAPs against others) showed a sex effect (sex: Wald $\chi^2 = 8.860$, $P = 0.003$; trial: Wald $\chi^2 = 25.085$, $P = 0.000$). Males resulted to be more efficient in transmitting the virus multiple times as also reported in Fig. 1B.

TSWV detection by q-RT-PCR

For virus quantification, thrips samples included both sexes. In the three transmission trials the C_t value (amplification cycle number at which fluorescence was emitted) was 24–35 for all tested samples (the C_t for healthy thrips samples being 35). As a rough example of the variability between different groups of samples, for one of the experiments we calculated the absolute amount of viral RNA detected in groups of four insects chosen according to their transmission pattern during the four IAPs using a standard dilution curve of plasmid DNA as the reference for quantification (not shown): in the samples comprising individuals from 1/4 IAP (first or second) the amount of RNA for each sample varied between 0.2 and 24.9 fg, whereas in the samples including individuals from 4/4 IAPs the amount of RNA ranged between 13.6 fg and 1.6 pg.

A more extensive analysis that would take into account an internal host expression standard was carried out by applying the relative expression quantification method calculated with the $\Delta\Delta C_t$, arbitrarily choosing as the standard reference sample (RQ = 1) one of the samples of individuals that stopped transmitting the virus in the last two IAPs (1/4 IAP, first or second). In the first trial, the average RQ values of transmitters in 1/4 IAP (first or second) and in 4/4 IAPs were 0.8 and 14.8, respectively (Table 4). Indeed, at the end of their lifespan virus RNA concentration was significantly higher in thrips transmitting in 4/4 IAPs than in those transmitting in 1/4 IAP (Fig. 2A), which showed the lowest RNA virus titer at the end of the four passages. There was no virus accumulation in healthy thrips included as controls. In the second experiment, the virus concentration was again much higher in thrips transmitting in 4/4 IAPs than in those transmitting in 1/4 IAP (first or second) (Fig. 2B and Table 4). In the third trial, RQ values were higher in thrips transmitting in 4/4 IAPs than in those

transmitting in 1/4 IAP and in other cases (Fig. 2C and Table 4), confirming the results of the other two trials. Given the limited number of thrips that lost the capacity to transmit after the first and second IAPs, in this last experiment we also included thrips transmitting during the third IAP, but not the fourth IAP. Overall indeed, these groups of insects showed a much lower viral RNA titer when compared to thrips that maintained virus transmission up to the fourth IAP (Fig. 2).

Statistical analysis confirmed a highly significant difference between virus concentration in thrips that transmitted in at least one of the first three IAPs but not the fourth IAP (possibly partially 'recovering'), and thrips that maintained their transmission ability in 4/4 IAPs (Kruskal–Wallis test: df = 1, $\chi^2 = 5.333$, $P = 0.021$, n=4 in the first experiment; df = 1, $\chi^2 = 4.500$, $P = 0.034$, n=3–4 in the second experiment; df = 1, $\chi^2 = 5.333$, $P = 0.021$, n = 4 in the third experiment).

Discussion

The population of *F. occidentalis* used in our experiments proved to transmit TSWV efficiently, and to be fit for laboratory conditions; in fact, overall mortality was low during the transmission trials. Moreover, we found significant differences in mortality rates between transmitter and non-transmitter thrips, indeed the mortality rate was lower in transmitter thrips than in non-trasmitter ones suggesting a beneficial virus effect on insect survival, as reported previously (Belliure et al., 2005; Stumpf & Kennedy, 2007). However, the specific effects of virus infection on the fitness of the thrips host should be further investigated to take in account the vast array of factors affecting the biological traits of the vector thrips.

Our study aimed to detect the variability in transmission ability during an adult thrips lifespan for each individual within a population competent for transmission: we showed that more than 70% of transmitter thrips (i.e. thrips transmitting in at least one IAP) were already able to transmit by the first IAP, and that 48.7% were able to transmit in all four IAPs (Fig. 1). However, the transmission behaviour differed significantly in relation to thrips gender. Although the percentage of transmitter thrips was not significantly different between males and females in our experiments (Table 1), we could observe that such difference was statistically significant in the last IAPs (Table 3), showing a tendency of males to transmit for longer time; males were previously shown to transmit more frequently than females, according to Rotenberg et al. (2009). The ability to transmit multiple times is known to be related to the feeding behaviour (van de Wetering et al., 1999). In particular, infected males show a greater probing activity resulting in more punctured plant cells, often leaving them suitable for virus replication (Stafford et al., 2011), as confirmed by lower rate of discoloured leaf discs produced by males in our study. Most of the adult thrips were infected and infectious throughout their lifespan, providing further confirmation that TSWV is transmitted by insect vectors in a

persistent-propagative manner (Ullman et al., 1997; Whitfield et al., 2005). The infected adults of *F. occidentalis* continued to transmit during the four IAPs with no statistically significant difference between periods, showing the absence of significant differences in transmission efficiency in relation to age, as hypothesized in other studies (van de Wetering et al., 1999; Whitfield et al., 2008; Rotenberg et al., 2009).

The virus titer in thrips that always transmitted virus (4/4 IAPs) was significantly higher than in thrips transmitting only in the first or second IAP; therefore we can confirm the correlation between high virus titer and virus transmission, as observed in previous studies (van de Wetering et al., 1999; Rotenberg et al., 2009). However, among transmitter thrips there were about 9% of individuals transmitting only in the first or second IAP, and in these thrips, the virus titer at the end of their life was significantly lower than that of thrips maintaining the competence for transmission up to the fourth IAP. The same lower accumulation was observed in thrips transmitting at some point in the first three IAPs but not in the fourth (third experiment, Fig. 2 and Table 4). We were unable to directly check the amount of virus for these groups of thrips at the end of the first, second or third IAP, when they were able to transmit, since RNA extraction is destructive, and therefore the necessary evaluation of transmission in the subsequent IAPs would have been impossible; nevertheless, given previous studies that associated ability to transmit virus only to individuals with high virus titer, we can hypothesise that these thrips possessed initially a high virus titer that enabled transmission and only later in their lifespan, their virus titer decreased, correlating with absence of transmission after the first two IAPs.

We observed the absence of a statistically significant correlation between loss of transmission capacity and vector ageing, whereas in a small subset of individuals, loss of transmission appeared to be linked to what is generally defined as 'recovery', with partial clearance of the virus titer (given the very low RNA virus amounts found in some of the samples, close to the detection limit). To our knowledge, this is the first time that indirect evidence has been provided for such an occurrence in virus–insect vector interactions. Our work does not elucidate the molecular or histological mechanism of this loss of vector ability linked to a decrease in virus titer: as a working hypothesis, we envision the possibility that some *F. occidentalis* individuals that immediately lost the viral titer might have activated an anti-viral defence response allowing their recovery.

Some authors hypothesised that the insect immune response could affect viral replication and consequently be responsible for the low virus titer in thrips (de Medeiros et al., 2004). Thrips could mount an immune response to TSWV, as a reaction to the fact that virus replication could affect their fitness through pathogenesis (Goldbach and Peters, 1996; Wijkamp et al., 1996; Belliure et al., 2005; Stumpf & Kennedy, 2007). Indeed the evolutionary and adaptive implications of the insect–virus

relationship have been discussed previously (Cotter et al., 2004). Further indirect evidence that TSWV triggers the innate defence mechanism in insects is the presence of a number of individuals (not specifically addressed in our study) that began transmitting the virus with a consistent delay during their adult life. Insect anti-viral defence mechanism could be more or less efficient in individual thrips. In this case it would be an individually specific response active in a population genetically suited for virus transmission.

Insects have a well-developed innate response even though they are generally thought to lack an acquired immune system (Strand, 2008). A molecular framework for viral immunity has been identified in some insects, including a set of humoral and cellular defences that detect viral nucleic acids and activate the antiviral response (Gillespie et al., 1997; Li et al., 2002; Ponnuvel et al., 2003; Dostert et al., 2005; Irving et al., 2005; Wang et al., 2006; Campbell et al., 2008; Deddouche et al., 2008; Strand, 2008; Steinert & Levashina, 2011). Some defences are age-related, resulting in a more targeted response to the infected tissues in older insects (Eleftherianos et al., 2008; McNeil et al., 2010). Developmental resistance (between and within larval instars) was previously shown in a number of different lepidopteran–nuclear polyhedrosis virus systems (Engelhard & Volkman, 1995; McNeil et al., 2010). As an example, developmental resistance was shown to occur by comparing the pathogenesis of fourth-instar larvae immediately and 48 h after moulting in the case of the interaction between *Lymantria dispar* (L.) and *Lymantria dispar* multiple nucleopolyhedrovirus: the mechanism of such resistance is complex and relies on the role of a number of midgut barriers and systemic defences such as encapsulation and apoptosis (McNeil et al., 2010). Our study addressed developmentally regulated resistance, but it did so by addressing genetic variability among individuals within the same population, and only during their lifespan as adults. In our study ageing cannot explain all loss of transmission ability and the likely onset of the immune response, because the phenomenon was not gradually correlated with age, as some individuals lost transmission ability after the first or second IAP (Fig. 1), whereas others maintained a high virus titer until the end of the 10-day period of observation.

The molecular and biological knowledge of the TSWV–thrips relationship is poor, given the absence of a reverse genetic system for tospoviruses. As for the thrips anti-viral defence response, a normalized cDNA was recently constructed from first-instar larva of *F. occidentalis*, and 74 sequences were identified with putative homology to proteins associated with insect innate immunity including the antiviral pathway. This collection provides possible target genes that could be analysed in recovering thrips in order to check whether the ‘recovery’ we observed in some individual thrips is indeed the result of the upregulation of genes involved in the insect anti-viral response (Rotenberg & Whitfield, 2010).

A further element to be addressed is the recent evidence of modulation of the insect's innate response by mutualistic interaction with endosymbionts. The effects of gut bacteria on thrips are poorly understood (Arakaki et al., 2001; de Vries et al., 2004, 2008; Kumm & Moritz, 2007, 2008; Chanbusarakum & Ullman, 2009), but the cross talk between bacterial and virus resistance cannot be ruled out in the thrips–tospovirus system, since it was previously shown that the presence of endobacteria increases resistance to virus infection (Hedges et al., 2008; Teixeira et al., 2008; Moreira et al., 2009; Gerardo et al., 2010).

In further studies we will determine the specific-molecular framework correlated with the anti-viral defence response in recovered *F. occidentalis*, which could also be used to implement new control strategies blocking steps in the virus–vector interaction.

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Table 1 Total number of thrips analyzed in transmission leaf disc assays during the three experiments. Sex effect and trial effect were analyzed through General Linear Model with a binary distribution and logit link. (sex: Wald $\chi^2 = 3.579$, $P = 0.059$; trial: Wald $\chi^2 = 9.794$, $P = 0.007$).

Trial	Adults			Females			Males		
	No.	Transmitter		No.	Transmitter		No.	Transmitter	
		No.	%		No.	%		No.	%
First	286	214	74.8	219	167	76.3	67	47	70.1
Second	150	125	83.3	109	86	78.9	41	39	95.1
Third	200	138	69.0	134	85	63.4	66	53	80.3
Total	636	477	75.7	462	338	73.2	174	139	79.9

Table 2 Mortality of non-transmitter and transmitter thrips in the four IAPs during the three experiments. Virus infection effect and trial effect were analyzed through General Linear Model with a binary distribution and logit link (transmitter/non-transmitter: Wald $\chi^2 = 5.504$, $P = 0.019$; trial: Wald $\chi^2 = 15.403$, $P = 0.000$).

Trial	Adults	Not-transmitters			Transmitters		
	No.	No.	No. alive	% mortality	No.	No. alive	% mortality
First	286	72	50	30.6	214	177	17.3
Second	150	25	24	4.0	125	119	4.8
Third	200	62	48	22.6	138	115	16.7
Total	636	159	122	23.3	477	411	13.8

Table 3 TSWV transmission patterns of *Frankliniella occidentalis*, considering only individuals alive at the end of the fourth IAP in the three experiments. Sex effect and trial effect were analyzed through General Linear Model with a binary distribution and logit link. The first step compared 1 IAP against the pooled group 4 IAPs + others (sx: Wald $\chi^2 = 0.025$, P = 0.874; trial: Wald $\chi^2 = 11.042$, $P = 0.004$); the second step compared 4 IAPs against others (sex: Wald $\chi^2 = 8.860$, P = 0.003; trial: Wald $\chi^2 = 25.085$, $P = 0.000$).

Trial	No. transmitter thrips	Transmitter females				Transmitter males			
		No.	% 1 IAP (1st or 2nd)	% 4 IAPs	% others[a]	No.	% 1 IAP (1st or 2nd)	% 4 IAPs	% others[a]
First	177	147	12.2	33.3	54.4	30	20.0	46.7	33.3
Second	119	84	8.3	63.1	28.6	35	8.6	74.3	17.1
Third	115	75	4.0	44.0	52.0	40	2.5	62.5	35.0
Total	411	306	9.2	44.1	46.7	105	9.5	61.9	28.6

[a] thrips transmitted for 1/4 (3rd or 4th), 2/4 and 3/4 IAPs.

Table 4 TSWV quantification by q-RT-PCR in *Frankliniella occidentalis* adults alive at the end of the fourth IAP.

Trial	No. thrips per sample	Not transmitters		1/4 IAP (1st or 2nd)		4/4 IAPs		no 4th IAP[b]	
		No. samples	RQ values[a]	No. samples	RQ values[a]	No. samples	RQ values[a]	No. samples	RQ values[b]
First	3–4	2	0.03	4	0.83	4	14.78		
Second	4–5			3	0.34	4	6.90		
Third	2			1	1.00	4	86.21	3	3.00

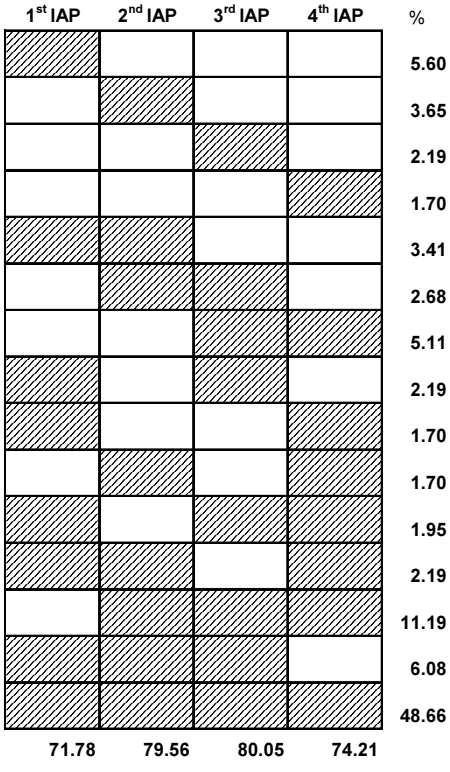
[a] in each experiment, we assigned RQ=1 to one sample belonging to the 1/4 IAP (1st or 2nd) group of thrips.
[b] thrips transmitted for 2/4 and 3/4 IAPs, but not at the 4th IAP.

Figure legends

Figure 1 Variation in transmission ability of transmitter thrips alive at the end of the fourth IAP (no. 411) during their lifespan: A) the combinations of transmission behaviour of thrips individually tested in the four consecutive IAPs; B) percentages of females and males inoculating TSWV to leaf disc in one, two, three or four of the four consecutive IAPs.

Figure 2 RQ values of samples of *Frankliniella occidentalis* from three distinct experiments (A, B and C). In each experiment adult thrips transmitting only in the first or second IAP (labelled as 1/4 IAP) or transmitting in all four IAPs (labelled as 4/4 IAPs) were analyzed by q-RT-PCR. In each experiment, one of the samples representing the 1/4 IAP groups (a different sample in each experiment) was arbitrarily assigned RQ=1. In the first experiment (A), thrips that did not transmit were also included (0/4), whereas in third experiment (C) a set of samples that transmitted in at least one of the first three IAPs, but not in the fourth IAP were included (labelled no 4^{th} IAP).

Figure 1


1st IAP
2nd IAP
3rd IAP
4th IAP
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5.60
3.65
2.19
1.70
3.41
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5.11
2.19
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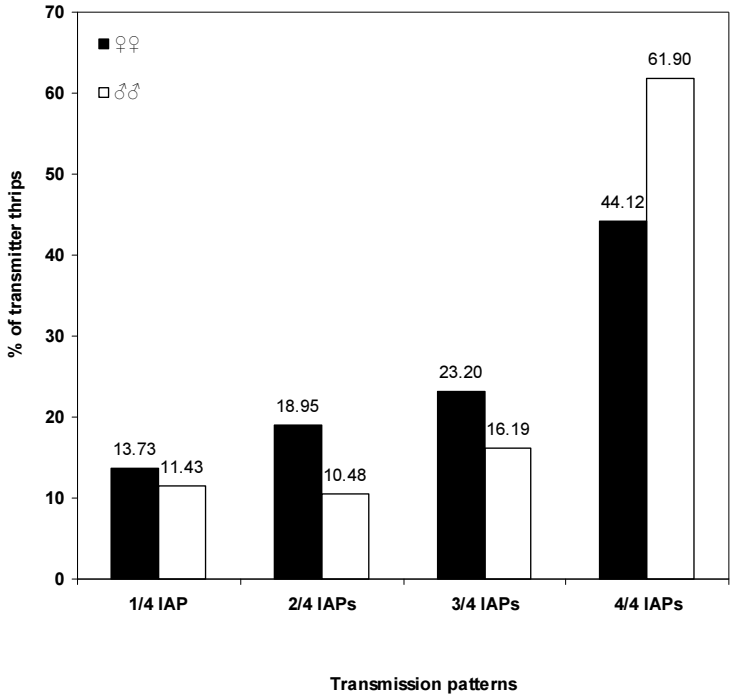
79.56
80.05
74.21

A


♀♀
♂♂
% of transmitter thrips
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60
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40
30
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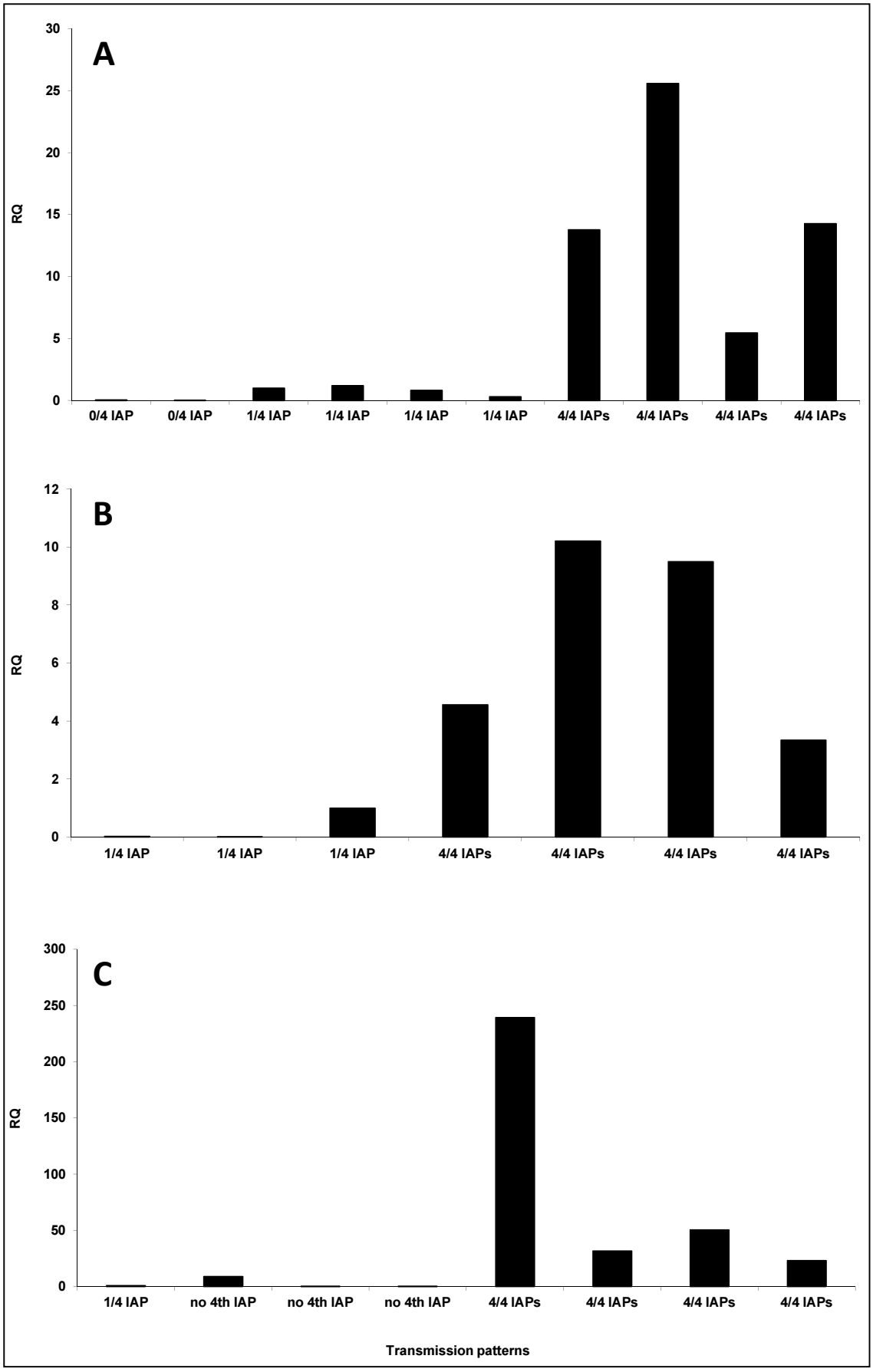
1/4 IAP
2/4 IAPs
3/4 IAPs
4/4 IAPs
Transmission patterns

B

Figure 2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Transmission patterns