Title: Understanding the interactions among the crop plants, a virus, insect vector whiteflies and their endosymbionts

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Abstract

Interactions among the four biotic agents - the host plants, a virus, insect vector whiteflies and their bacterial endosymbionts was investigated in this study. The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is an important crop pest that is infected by several endosymbiotic bacteria that can play varying roles on their hosts. Both whiteflies and endosymbionts are affected by the host plants they feed on, which in turn affect the fourth agent – the viruses, which are transmitted by the whiteflies. We investigated these interactions on five different host plants – eggplant, tomato, beans, okra and cotton - using a single whitefly species Asia 1 infected with three different bacteria *Portiera*, *Wolbachia* and *Arsenophonus*. *B. tabaci* Asia 1 transmits the *Tomato leaf curl Bangalore virus* (ToLCBV) effectively, which was the virus used in the study. We found host plants having a significant impact on whitefly growth and development. Eggplant was most favourable, while okra and tomato were least preferred. The endosymbiont *Wolbachia* was significantly affected by feeding of *B. tabaci* on different host plants while *Portiera* and *Arsenophonus* were unaffected. When whiteflies fed on ToLCBV-infected tomato plants, the concentration of *Arsenophonus* increased significantly while other endosymbionts remain unchanged. Understanding these interactions will help in managing both whiteflies and viral diseases.

Keywords: whitefly, tomato leaf curl virus, *Arsenophonus*, *Wolbachia*, qRT-PCR

Introduction

The whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is a pest of many crops across the tropics, sub-tropics and temperate regions of the world (Oliveira et al. 2001; Bradshaw et al. 2019). Due to its small size (1-2 mm), rapid multiplication rate and resistance to many insecticides, the *B. tabaci* population increase rapidly on several crop plants including beans, cassava, cotton, okra, potato, tomato, and other crops across the world. They feed on over 600 plant species globally, causing some of the highest damages recorded for any crop pests (Greathead 1986; Secker et al. 1998; Attique et al. 2003). However, by far the most serious damage is caused by the transmission of many economically important plant viruses (currently >300 species) belonging to five virus genera: *Begomovirus*, *Carlavirus*, *Crinivirus*, *Ipomovirus* and *Torradovirus* from different virus families (Navas-Castillo et al. 2011). Over 200 species of begomoviruses are exclusively transmitted by *B. tabaci* adults which is thus the largest group vectored by *B. tabaci* (Guo et al. 2015).

Extensive economic losses caused by diseases of whitefly-transmitted viruses have threatened food security and poverty alleviation efforts globally with losses ranging from 20 to 100% (Basu 1995; Czosnek et al. 2001; Dasgupta et al. 2003; Cathrin and Ghanim 2014). *Tomato leaf curl Bangalore virus* (ToLCBV) (*Begomovirus*, *Geminiviridae*) is one of the most prominent viruses infecting tomato transmitted by *B. tabaci*. Some of the reasons for the emergence and establishment of whitefly-transmitted diseases include genetic changes in the virus through mutation and recombination, genetic attributes of vector population coupled with the polyphagous nature of *B. tabaci* and long-distance movement of plant material or vectors through trade. The International Union for the Conservation of Nature and Natural Resources (IUCN) has listed *B. tabaci* among the top 100 world's worst invasive species. Therefore, it is highly regulated species in many countries and regions including Australia, China, USA as well as in the EU and Africa (Boykin and De Barro 2015).

About 44 genetic groups/cryptic species of *B. tabaci* are known to exist in the world on different hosts (Kanakala and Ghanim 2019). These species have variable behaviour with respect to feeding, development (biology) and transmission of viruses (Walling 2000; Elsayed 2011). Several hosts are not equally suitable for the development of all whitefly species as they may have toxins or lack essential nutrients (Denno and Roderick 1992; Baldwin and Preston 1999; Elsayed 2011). Some hosts may favour by supplying enriched nutrients to the pest that can result in an outbreak (Brodbeck and Strong 1987). In contrast, a few hosts may lack the essential nutrients that play a vital role in the development of the herbivore and that resulted in low/slow population build-up (Shah 2017). For compensating such effects, insects depend on bacterial symbionts for obtaining nutrients that are limited or lacking in the diet, thereby, improve their physiology and invasive abilities (Buchner 1965; Schwemmler 1989; Oliver et al. 2003; Scarborough et al. 2005; Feldhaar 2011; Su et al. 2013). *B. tabaci* has one of the highest number of endosymbiotic infections, with eight different vertically transmitted bacteria reported so far (Gueguen et al. 2010; Bing et al. 2013; Marubayashi et al. 2014). The prevalence of the symbionts varies among whitefly populations, geographical regions and host plants with a great diversity within the bacterial communities (Khatun et al. 2019). Primary endosymbionts, *Portiera aleyrodidarum* and seven secondary endosymbionts, *Cardinium*, *Arsenophonus*, *Rickettsia*, *Wolbachia*, *Hamiltonella*, *Fritschea* and *Hemipteriphilus* are known to infect *B. tabaci.* Some of these are involved in both symbiotic and antagonistic association with their host (Bing et al. 2013; Hashmi et al. 2018). The symbiotic associations include helping the whiteflies with host adoptability (Tsuchida et al. 2004), increased reproduction (Engelstadter & Hurst, 2009), stress tolerance, genetic diversity or gene flow and thus evolution (Charlat et al. 2009; Ferrari and Vavre 2011; Bing et al. 2013; Tajebe et al. 2014; Chung et al., 2017; Ghosh et al. 2018).

During the process of symbiosis many proteins including the heat shock proteins (HSPs) and chaperons are secreted by endosymbionts inside the vector (van den Heuvel et al. 1994). They are known to help in adaptation to the stress of the insect host but can also be exploited by the plant viruses inside the vector to protect themselves from insect immune reactions (Oliver et al. 2003; Gutierrez 2013). Plant viruses interact with HSPs produced by the endosymbiotic bacteria for the safe circulative translocation in the vector and thereby successful transmission into new host and further spread (Gorovits et al. 2013). For example, a 63-kDa GroEL protein produced by the *Hamiltonella* in *B. tabaci* protects *Tomato yellow leaf curl virus* (TYLCV) during its passage through the haemolymph (Morin et al. 1999). However, these interactions are very specific between the bacterial strain, and the virus and the whitefly species involved. Environment and geographical location also

appear to play a significant role in these interactions but these are yet to be defined conclusively. Information on the bacteria interacting with the safe passage of ToLCBV in the indigenous and the most predominant whitefly, ASIA I from South India are unknown. The aim of this study was therefore to understand these complex insect host-endosymbiotic bacteria-virus interactions on the popular vegetables such as broad beans, eggplant, okra, tomato as well cotton. We investigated the effect of feeding of whiteflies on these crops by Asia I species' biology and development, and the dynamics of endosymbionts and their effect on ToLCBV transmission.

Materials and methods

Whitefly and virus cultures: A colony of whitefly *B. tabaci* Asia I species was originally collected from Coimbatore, India and maintained on eggplants with temperature 27± 3°C, 60% relative humidity and L12:D12 in the quarantine laboratories of the Natural Resources Institute (NRI), University of Greenwich, UK. The ToLCBV-infected tomato scions collected from the ICAR-Indian Institute of Horticultural Research fields (13°08'03.2"N 77°29'33.1"E), Hessarghatta Lake, Bengaluru, India were grafted on to healthy tomato seedling maintained in the quarantine glasshouse at NRI. Total DNA was extracted separately from 10 female adult whiteflies using the Chelex method (Walsh et al. 1991) with slight modifications (Ghosh et al. 2015). Presence of symbionts confirmed by PCR amplification of 16S or 23S rDNA using genus-specific primers (Ghosh et al. 2015).

Detection of ToLCBV in tomato plants was carried out by visual inspection of symptoms expressed and PCR. Total DNA was extracted from the tomato leaf samples using CTAB method (Lodhi et al., 1994; Maruthi et al., 2002). ToLCBV detection by PCR was with newly designed primers

(Forward 5'- GTCAGCAATCTGCCAACGAC-3', Reverse 5'–GTGTTGGATTGCCAGTCCCT-3') to amplify 431 bp size product of the virus C1 gene. Primers were developed using complete genome sequences of tomato leaf curl viruses available in the National Centre for Biotechnology Information (NCBI) nucleotide database (ACC No. AY754812.1). Amplification of the C1 gene was carried out in 15 µl volumes containing 1 µl DNA lysate (1:5 dilution) as template, 10 µM of each primer, 2 mM of dNTPs, 1.5 x DreamTaq Green buffer and 0.15 unit DreamTaq Green DNA polymerase (ThermoFisher Scientific Ltd., UK). Amplification cycle consisted of 95 °C for 1 min followed by 35 cycles of 94 °C for 30s, annealing of 60 °C for 15s and 72 °C for 50s and final extension for 7 min at 72 °C. PCR products were visualised on 1% agarose gels containing Sybrsafe staining solution (Invitrogen Biotechnology, USA). The PCR product was purified using a PCR Purification kit (ThermoFisher Scientific) and sent to sequencing by Eurofins Genomics, Germany. Four PCR products were sequenced and the consensus sequences were submitted to NCBI (accession number MN752118). **Investigating the effect of host plants on whitefly biology**

The freshly emerged whitefly adults (2 days old) were collected from the core colony maintained on eggplants and 20 pairs (20 female and 20 male). They were transferred on to eggplant var. Black beauty (used as a control), okra var. Clemson's spineless, broad bean var. Sutton dwarf and tomato var. Money maker and cotton (an African cultivar) for feeding and egg laying. Ten plants were inoculated for each host and on each plant insects were released on two fully opened leaves using clip cages. A total of 200 pair of adults per host were confined for up to 5 days . The number of adults alive were counted on the 5^{th} day, and up to 20 were collected in 90% ethanol for understanding the dynamics of endosymbionts (see below). The remaining female adults were released on to ToLCBV-infected tomato plant for virus acquisition . The fecundity of *B. tabaci* on each

plant was recorded and the number of nymphs survived and adults emerged was also recorded up to 37 days after releasing the adults.

Investigating the effect of host plants on endosymbionts

Total nucleic acid was extracted from ten whitefly females separately from each host using 20% Chelex method (Ghosh et al. 2015). Presence of endosymbionts in whiteflies was detected and quantified by the real-time PCR (qPCR) using 2X DyNAmo Flash SYBR green PCR kit (Thermo Scientific, UK) (Ghosh et al. 2015). Amplification of *Wolbachia, Arsenophonus* and *Portiera* was performed in 10 µl reactions using their gene-specific primers with the whitefly β actin as a reference gene (Table 1). The mean normalised relative quantities of each endosymbiont was calculated by comparing the quantification cycle (Cq) value of respective endosymbionts to that of the whitefly β actin gene (reference gene) according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The mean relative quantities of different endosymbionts from each host-plant was analysed using two-way ANOVA after log transformation by comparing to the Cq values (relative expressions) of endosymbionts in whiteflies on the control eggplants.

Investigating the effects of host plants on ToLCBV transmission

Several 100 adult whiteflies were collected from the core colony and released on ToLCBV-infected tomato plants for acquiring the virus for 24 h. Single viruliferous whitefly was then collected and released on to each healthy tomato plant (n=10) of 3-4 leaf stage in a clip cage for 48h for virus inoculation. The remaining whiteflies were collected in 90% ethanol and stored in -20^{o}C for understanding the effect of ToLCBV on endosymbiont composition. A total of 10 plants were inoculated for each treatment and each inoculated plant was maintained up to 35 days for virus establishment. Disease symptoms were recorded by visual observation and by virus detection by PCR as described above.

Investigating the effect of ToLCBV on endosymbionts

To investigate if *B. tabaci* feeding on ToLCBV-infected plants has affected endosymbiont composition, total DNA was extracted from whitefly adults fed on diseased tomato plants. The aforementioned methods were followed for the quantification of endosymbionts using qPCR. The analysed Cq values of whitefly endosymbionts from healthy tomato plants were used as control.

Results

Characterisation of whitefly, virus and endosymbionts

Amplification of 23S rDNA gene of *Arsenophonus* revealed about 60% infection rate in the core colony. Detection of *wsp* gene of *Wolbachia* and 16S rDNA gene of *Rickettsia* showed 90% and 30% infection rates, respectively (Figure 1). Likewise, amplification of the replication gene (C1) of ToLCBV in virus-infected plants was (431 bp) (Acc. No. MN752118) also as expected (Figure 2).

Effect of host plants on whitefly biology

The five host plants had varying effects on the fecundity and biology of the Asia I *B. tabaci* species. Out of the 20 adults released per plant, the highest number survived on eggplant (10.5±0.49 adults/plant), beans (10.5±0.88 adults/plant) followed by cotton (7.5±0.85 adults/plant) after five days. Okra (0.15±0.08 adults/plant) supported least number of adults while significant mortality was seen on tomato (4.2±1.31 adults/plant) ($F=46.73$; $P<0.0001$) (Figure 3A). Maximum fecundity (egg laying) was observed on beans

(99.94±12.17 eggs/plant), followed by eggplant (97.05±12.64). Least number of eggs were found on tomato (0.5 ±0.24 eggs /plant) (F=25.76; P<0.0001) (Figure 3B). Highest number of nymphs emerged into adults on eggplant (52.6±7.70adults/plant), followed by cotton (17.77 ±4.14) but a few on beans (12.00±3.46), and okra (10.15±1.13) and none on tomato (0 ± 0) (Figure 3C; F 14.82, P<0.0001).

Understanding the effect of host plants on endosymbionts

The concentration of *Wolbachia*, *Arsenophonus* and *Portiera* differed significantly in whiteflies fed on all hosts except beans compared to the control eggplants (Cq=1). Among hosts, okra (p=0.0362), cotton (p= 0.000802) and tomato (p=0.0001) had significantly negative effect on endosymbiont population in the whiteflies (Table 2). Concentration of *Wolbachia* was significantly reduced in whiteflies fed on cotton (Cq = 0.30; p = 0.0001), okra (Cq= 0.64; p = 0.002910) and tomato (Cq = 0.36; p = 0.000127) compared to eggplant. Whiteflies fed on tomato had significantly lower quantities of primary endosymbiont, *Portiera* (p = 0.0224) compared to control eggplants. No effect on *Arsenophonus* was observed in the whiteflies fed on any plants (Figure 4 A, B, C). RT -qPCR amplification of *Rickettsia* was unusual and hence this bacteria was not further investigated in host plant and transmission experiment.

Understanding the effect of host plants on virus transmission

B. tabaci fed on cotton, eggplant and tomato (prior to ToLCBV acquisition) transmitted ToLCBV to 90% of the healthy tomato plants, while those fed on beans achieved 70% transmission rate. Transmission was not possible by the whiteflies fed on okra as all adults died by the 5th day after release. Feeding of whiteflies on different host plants prior to virus acquisition on tomato thus did not significantly affect ToLCBV transmission.

Understanding the effect of virus on endosymbionts

The concentration of *Wolbachia* significantly reduced in *B. tabaci* fed on cotton (p=0.000154), eggplant (p=0.000711), beans (p=0.0240) and healthy tomato (p=0.000160). Only the concentration of *Arsenophonus* was increased in the whiteflies initially fed on healthy tomato plants followed by feeding on ToLCBV-infected tomato (Cq=12.53; p = 0.00968) (Figure 5A, B, C).

Discussion

Host plants play crucial role on the development and survival of whiteflies. We found that the survival of *B. tabaci* adults was significantly influenced by the change in host plants. The survival of Asia I *B. tabaci* on eggplants was understandably not affected as the core colony was also maintained on the same host. Beans and cotton also favoured whitefly adult survival, but the nymphal development was subsequently affected. Tomato and okra were least supportive for adult survival, oviposition, nymphal and adult development. A sudden shift in feeding on different host plants can result in sudden changes in dietary constituents. Plant nutrition has a significant impact on herbivore survival rates, longevity and reproduction (Bi et al. 2001). Feeding on different host plants having variable levels of essential amino acids and sugars (Butler and Henneberry 1985; Klingauf 1987; Chapman 1988; Enkegaard 1993; Hu et al. 2010). For example, Biotype Q ues more free amino acids from the phloem sap of tomato plant than Biotype B (Guo et al. 2019) due to its better adaptability to tomato host. Presence of inhibitory compounds such as secondary metabolites could also affect *B. tabaci* performance on okra (tannins, alakaloids, flavonoids, phenols, terpeniods) and tomato (chlorogenic acids) (Howe and Jander 2008; Elsayed 2011; Thomson 2011; Vince and Zoltan 2011; War et al. 2012; Kim et al. 2014; Abobaker et al.

2017; Zaynab et al. 2018). As a result, the fecundity of whiteflies was affected and hence less number of eggs laid on tomato and okra. Though fecundity of whitefly on beans was similar with eggplant, but during the course of development the nymphal emergence was significantly affected. A change in the host plant had thus significantly impacted *B. tabaci* Asia I survival and its development (Greenberg et al. 2009).

The qPCR results indicated the vital role of host plants on the dynamics of endosymbionts in *B. tabaci*. The dynamics of *Wolbachia* in *B. tabaci* after feeding on different hosts may be due to its probable involvement in host adaptation and insect nutrition (Figure 4A). *Wolbachia* is shown to supplement vitamin B to whiteflies that are lacking in the host and enhancing the fitness of whitefly i.e adjusting to new hosts (Hosokawa et al. 2010; Xue et al. 2012; Nikoha et al. 2014; Salem et al. 2014; Shah and Liu 2014). In the present study, reduction in *Wolbachia* in the *B. tabaci* fed on beans, cotton, okra and tomato may have led to failure in adjusting of whiteflies to these hosts.

Prior feeding of the Asia I *B. tabaci* on different host plants showed no significant effect on the transmission of ToLCBV, indicating that the source/origin of whiteflies has no effect on disease spread in the field. This is crucial information because the Asia I feed on 22 host plants, many of which are ubiquitous in the tropical agricultural systems of India (Ellango et al. 2015; Roopa et al. 2015). They provide a regular supply of whiteflies for the continued spread of ToLCBV. Removing the potential hosts of Asia I and not planting ToLCBV-susceptible tomato varieties near the bean or cotton crops is therefore important to prevent the rapid spread of the disease.

Only the concentration of *Arsenophonus* increased in whiteflies upon feeding on ToLCBV-infected plants. This was seen only in the Asia I species that fed on healthy tomato plants followed by ToLCBV-infected plants but not in the whiteflies that fed on other hosts. Increase in *Arsenophonus* levels in viruliferous *B. tabaci* Asia I confirms its potential interaction with the viruses (Gottlieb et al. 2010), and likely facilitating virus escaping the whitefly immune system and safe passage through the whitefly haemolymph. Many plant viruses interact with bacterial endosymbionts derived protein inside the vector for safe circulative translocation (Ohnesorge and Bejarano 2009; Gottlieb et al. 2010; Rana et al. 2012). TYLCV was known to interact with *Arsenophonus* derived protein for passing through its vector, *B. tabaci* (Rana et al. 2012). The GroEL-produced by *Hamiltonella* also known to interact with TYLCV in *B. tabaci* B genetic group and thus helps in successful passing of the virus through vector (Gottlieb et al. 2010). The results thus indicate that ToLCBV is interacting with *Arsenophonus* in whiteflies fed on ToLCBV-infected plants.These results, however, contrasting to the earlier reports that showed that TYLCV had no effect on the bacterial community associated with *B. tabaci* Middle East Asia Minor -1 (MEAM1) and *B. tabaci* Mediterranean (MED) genetic groups, perhaps there might be a difference due to the different genetic groups of *B. tabaci* or the different viruses involved (Liu et al. 2013). This study nevertheless identified the possible interaction between *Arsenophonus* and ToLCBV, which should be further investigated for greater understanding.

Conclusions

Host plants play a crucial role on endosymbiotic infections in *B. tabaci*. Feeding on different hosts has significant impact on growth and development of *B. tabaci* on tomato and okra, a negative effect on *Wolbachia* population but no effect on the transmission of ToLCBV. ToLCBV-infected tomato plants increased the

concentration of *Arsenophonus* in *B. tabaci* and thus indicating the latter's putative interaction in virus transmission. This also helps to investigate the potential of bacterial endosymbionts in disrupting virus and vector relationships and their by managing the spread of viral diseases.

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Compliance of ethical standard

Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

NRP and MNM designed the study. NRP executed the experiment and analysed the data. NRP and MNM drafted and reviewed the MS.

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