Impacts of Cucurbit Chlorotic Yellows Virus (CCYV) on Biological Characteristics of Its Vector *Bemisia Tabaci*

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Research

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Abstract

Background: It is known that plant viruses, to facilitate their transmission, can change the phenotypes and defense pathways of the host plants and thereby the performance of their vectors. Cucurbit chlorotic yellows virus (CCYV), a newly reported virus occurring on cucurbit plants and many other plant species, is transmitted specifically by B and Q biotypes of tobacco whitefly, *Bemisia tabaci* (Gennadius), in a semipersistent manner. This study evaluated the direct and indirect effects of CCYV on *B. tabaci* performance to better understand the plant-virus-vector interaction in terms of its impacts on the biological characteristics of its vector.

Methods: In this study, by using CCYV-*B. tabaci*-cucumber as the model, we investigated whether or how a semipersistent plant virus impacts the biology of its whitefly vectors directly and/or indirectly. Virion titer, body size, life table parameters, survival rate of nymphs and adults, reproduction capacity of both adult sexes as well as sex ratio were compared between whiteflies on CCYV-infected plants and ones on healthy plants.

Results: CCYV virions were detectable in nymphs from 1st to 4th instar and adults of *B. tabaci* with different titers. Female nymph duration and female adult longevity greatly extended on CCYV-infected plants, but male nymph duration and male adult longevity were not significantly influenced. In addition, on CCYV-infected plants, the body length and oviposition of adult *B. tabaci* increased, but the egg hatching rate and survival rate of different stages of the whiteflies were not affected. Most interestingly, the sex ratio (female:male) significantly increased up to 66.40% in whitefly populations on CCYV-infected plants, while the female ratio remained about 50.53% on healthy plants.

Conclusions: These results indicated that CCYV can significantly impact the biological characteristics of its vector *B. tabaci* through the host plants. It is speculated that CCYV and *B. tabaci* have established a typical mutualist relationship mediated by host plants.

Introduction

The plant viruses have developed very specific relationships with insect vectors in the long course of coevolution. Approximately 80% of the plant viruses depend on insect vectors for transmission (other vectors can be fungi, mites and nematodes, etc) [1, 2]. More and more researches have proved that plant viruses can regulate the growth, mating, immunity, feeding, reproduction and other behaviors of vector insects, and thus affect the spread of the viruses. Studies have shown that after carrying tomato yellow leaf curl virus (TYLCV), the development of ovaries and fecundity were significantly changed and the feeding behaviors were promoted in *B. tabaci* [3–5]. The activities of protective enzymes and detoxifying metabolic enzymes in brown planthopper (BPH) *Nilaparvata lugens* and white-backed planthopper (WBPH) *Sogatella furcifera* were significantly increased by vectoring rice black streaked dwarf virus (RBSDV), indicating that the viruses may change the metabolic process and affect the immune system of their vectors [6, 7].The nymphal development and adult longevity of *S. furcifera* carrying southern rice

black-streaked dwarf virus (SRBSDV) were significantly extended, the females laid fewer eggs after feeding on the infected rice plants, but there was no effect of the virus on the development or longevity of brown planthopper [8–10]. The nymphs of *Laodelphax striatellus* were significantly prolonged after being infected by rice stripe virus (RSV), and the egg development were impaired and the incubation rate dropped significantly, however, the weight of female gain and phloem ingestion time during feeding also increased [11–13]. After carrying tomato spotted wilt orthotospovirus (TSWV), *Frankliniella occidentalis* significantly extended its developmental period and mating time, and produced more progeny, most of which were males with stronger virulent ability, thus improving the ability of virus transmission [14]. more offspring were produced in *F. occidentalis* on the TSWV-infected plants. The incubation period was significantly shortened and pupated faster on virus-infected plants. These results show a mutualistic relationship between *F. occidentalis* and TSWV [15]. Thus, different transmission types of viruses have different effects on the vectors.

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is one of the most important agricultural pests and the most efficient vectors for the transmission of plant viruses in the world [16]. According to statistics, *B. tabaci* can transmit 212 viruses from 5 families and 5 genera [17–19], and some of these viruses cause serious damage and economic losses to agricultural production.

Cucurbit chlorotic yellows virus (CCYV) (genus Crinivirus, family Closteroviridae), as an emergent plant virus, was firstly identified in melon (*Cucumis melo*) in Japan in 2004 [20]. Composed of two single-stranded RNA, and transmitted specifically by *B. tabaci* in a semipersistent manner [21, 22]. CCYV can systematically infect melon plants such as watermelon, luffa, pumpkin and non-melon plants such as beet, quinoa, datura, and *Nicotiana benthamiana* [20], causing chlorotic leaf spots and complete yellowing of leaves, which seriously affected the yield and quality of melons [21]. In our previous study, we found that CCYV had direct and indirect effects on the feeding behavior of *B. tabaci*, and the degree of influence depends on the biological type and sex of the insects [23, 24]. Although *B. tabaci* nymphs do not play roles in transmitting the virus, viral accumulations in the body indeed affect the growth and development of the nymphs, and thus affect the vitaliy and ability of the adults to transmit the virus. However, there have been no reports about the effect of CCYV on the biological characteristics of *B. tabaci*. The interaction between plant viruses and vector insects is the result of evolution by natural selection. Different types of viruses may have different influences on plant and on vector insects. In this study, the effects of CCYV on the growth, development, reproduction and other biological characteristics of *B. tabaci* were studied in order to provide theoretical basis for the in-depth study of the interaction between *B. tabaci* and CCYV and its mechanism, and to provide a new idea for the implementation of virus prevention and control strategies.

Materials And Methods

The plants and insects

The colony of *B. tabaci* Mediterranean (MED, Q biotype) was maintained on cucumber plants (*Cucumis sativus* L.cv. Bojie-107) in cages (60 cm × 60 cm × 80 cm) in the greenhouse at 28 ± 1℃, L:D = 16 h:8 h and 75 ± 1% relative humidity. The genetic purity of *B. tabaci* Q biotype cultures was monitored every 3 generations using the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) technique combined with the sequencing of *mtCO1* gene [25]. To obtain CCYV-infected plant cultures, cucumber plants at 2 true-leaf stage were inoculated with *Agrobacterium tumefaciens*-mediated CCYV clones [26]. Plants of cucumber were kept under above-mentioned conditions.

Detection of CCYV virions

Total RNA of individual whiteflies or infected cucumber plants was extracted using TRIzol® Reagent (Invitrogen Carlsbad, CA, USA) following the manufacturer's instructions. RNA concentration and purity were measured in a NanoDrop™ spectrophotomer (Thermo Scientific Wilmington, DE, USA) and stored at -80℃ for subsequent analysis. Total RNA (1 μg) from each sample was reverse transcribed to generate the first-strand cDNA using the PrimeScript® RT reagent Kit (Takara, Dalian, China).

Primers were designed based on coding sequences of CCYV coat protein (CP) by using primer premier 5 software and the nucleotide sequence in GenBank (Accession No: HM581658.1). The primers used are shown in Table 1. Subsequent primer-blast searches showed that they had a high specificity towards CCYV. PCR products were connected with pMD18-T Vector to construct standard recombinant plasmid. Six gradients (3.40×10^3-3.40×10^8 copies/μL) of standard recombinant plasmids were set up as a template for real-time qRT-PCR, with three replicates for each concentration, meanwhile blank control and negative control were set up. Amplification reactions were performed as follows: 94℃ for 2 min, 40 cycles of 94℃ for 15 s, 60℃ for 20 s, 72℃ for 20 s. According to the standard curve automatically generated by the instrument, the correlation coefficient R^2 = 0.9984, the amplification efficiency E = 95%, and the standard curve equation is Y=-3.3396lg$X$ + 27.8480 (Figure S1). *Ct* value of each sample was detected by qRT-PCR, and CCYV virus contents in cucumber plants or *B. tabaci* were calculated.

Table 1
PCR primers for CCYV detection

Primers	Positions	Sequence (5'-3')	Size (bp)
CCYV-F	548–567	GCGACCATCATCTACAGGCA	152
CCYV-R	679–699	CCGACTTGTTCCTTTCAGAGC	

Impacts of CCYV on biology of B. tabaci

Thirty pairs of male and female whitefly adults were respectively placed with a clip cage on a healthy or CCYV-infected cucumber plant for each treatment. After 24 hours, adults were removed from plants, and 30 eggs on each leaf were marked under the super-depth microscope (Keycence digital microscope VHX-600E) and other eggs were removed. Observations were taken every day under the super-depth

microscope until all eggs hatched and 1st -instar nymphs were fixed. Locations of the nymphs were marked. The egg hatching rates (P_0), survival rates (P_n) and duration of each instar nymphs, and sex ratio (P) of newly emerging adults were calculated with the following equations:

$$P_0 = \frac{N_1}{K} \times 100\% \quad (1)$$

$$P_n = \frac{N_{n+1}}{N_n} \times 100\% \quad (2)$$

$$P = \frac{M}{N_5} \times 100\% \quad (3)$$

Where N_1 is the number of 1st -instar nymphs; K is a constant of 30; P_n is the survival rates of each instar nymphs (n = 1, 2, 3, 4); M is the number of males or females and N_5 is the number of adults. Sizes of each individual of adults were measured. Four replicates were used for each treatment.

In another set of experiments, a couple of adults were placed with a clip cage on a leaf of healthy or CCYV-infected cucumber plant. The insects were moved to a new plant every 24 hours. Eggs on all leaves were counted, and dead male adults were replaced with new males until the female adults died. Ovipositional capacity and adult longevity were calculated.

Data statistics

IBM SPSS Statistics 21.0 was used to conduct data analyses. Comparisons in body size, oviposition, adult longevity, sex ratio as well as nymph duration of each instar of insects on healthy and CCYV-infected cucumber plants were made using Independent-Samples *t*-test; one-way ANOVA was used to analyze fertility rate and nymph survival rates among all instar nymphs. Significant differences were tested at the 0.05 or 0.01 level. If significant effects of CCYV on the above variables were found, the least significant difference Tukey's test was further used to compare the means between viruliferous and non-viruliferous *B. tabaci*. All data were expressed as Mean ± SE of three independent experiments.

Results

Detection of CCYV in cucumber

The cucumber plants at 2 true-leaf stage were inoculated with *Agrobacterium tumefaciens*-mediated CCYV clones. At 25 days post-infiltration, leaves of *C. sativus* plants agroinfiltrated developed yellowing symptoms, typical of CCYV infection in plants (Fig. 1A), whereas no symptoms were observed on healthy leaves. Analysis by RT-PCR using the primers is specific to the CP coding sequence (Table 1). All samples displayed amplification products of the expected sizes (Fig. 1B). The amplification products were sequenced, which verified CCYV infection. qRT-PCR was used to detect the copies of CCYV in healthy and

CCYV-infected *C. sativus*. The results showed that CCYV virions were only found in leaves of CCYV-infected *C. sativus* with 87114.56 copies, while no virus was found in healthy *C. sativus* (Fig. 1C).

Detection of CCYV in individual whiteflies

We used real-time qRT-PCR to detect CCYV virion numbers of individual *B tabaci* having fed on CCYV-infected cucumber plants for 3 d. The results showed that CCYV virions were detected in all instars of nymphs as well as adults of *B. tabaci*, with 21360.08 copies in adults, followed by 1424.54 copies in the 2nd -instar nymphs, and 112.34 copies in 4th -instar nymphs (Fig. 2).

Effects of CCYV on nymph duration and adult longevity of B. tabaci

B. tabaci nymph duration and adult longevity were shown in Fig. 3. The female and male nymphs of *B. tabaci* on healthy cucumber plants were 13.80 ± 0.27 d and 13.94 ± 0.34 d respectively. The developmental duration of female nymphs on CCYV-infected cucumber plants were 15.79 ± 0.31 d, and that of male nymphs was 14.36 ± 0.36 d. These results showed that CCYV significantly extended the developmental duration of female nymph ($P < 0.01$), but had no significant effect on duration of male nymph ($P = 0.391$) (Fig. 3A).

On healthy cucumber, the developmental duration of *B. tabaci* female adults were 12.32 ± 0.20 d, while that of male adults were 13.89 ± 0.22 d. And on CCYV-infected cucumber plants, longevity of the female adult *B. tabaci* was 13.87 ± 0.26 d, and that of male adults was 14.53 ± 0.36 d. The results showed that CCYV significantly extended the female adult longevity of *B. tabaci* ($P < 0.01$), but had no significant effect on longevity of male adult *B. tabaci* ($P = 0.136$) (Fig. 3B).

Effects of CCYV on body length and oviposition of adult B. tabaci

As shown in Fig. 4A, the body length of *B. tabaci* female and male adults on healthy cucumber plants was 1066.30 ± 5.04 μm and 895.70 ± 4.13 μm, respectively. The body length of female and male *B. tabaci* was 1091.02 ± 4.05 μm and 913.52 ± 3.18 μm respectively when feeding on cucumber plants infected with CCYV. Independent Sample *t*-test showed that CCYV could significantly increase the body length of female and male adults ($P < 0.01$). Number of eggs laid by females on healthy plants were 105.03 ± 4.13, while and on the plants infected with CCYV, the number of oviposition of individual female adults were 125.22 ± 3.31 (Fig. 4B). Independent Sample *t*-test results showed that CCYV could significantly increase oviposition of female adults of *B. tabaci* ($P < 0.01$).

Effects of CCYV on hatching rate and nymphae survival rates of B. tabaci

The egg hatching rate and nymph survival rates of *B. tabaci* at various instars on healthy and virulent cucumbers were shown in Table 2. The egg hatching rate of *B. tabaci* on healthy cucumber plants was

91.10 ± 2.20%, and that on CCYV-infected cucumber plants was 85.53 ± 3.99%. Independent Sample *t*-test shown that CCYV had no significant effect on the egg hatching rate of *B. tabaci* ($P = 0.184$). The survival rates from 1st to 4th -instar nymphs on healthy cucumber plants were 98.25 ± 1.75%, 99.25 ± 0.75%, 100 ± 0.00% and 100 ± 0.00%, respectively, and the survival rates on CCYV-infected cucumber plants were 97.57 ± 1.59%, 96.00 ± 2.12%, 100 ± 0.00%, and 99.25 ± 0.75%, respectively. The nymph survival rates of *B. tabaci* on healthy cucumber plants were all higher than those on cucumber plants infected with CCYV, but the difference did not reach to a significant level ($P > 0.05$).

Table 2
Fertility rates and nymph survival rates of *B. tabaci* on healthy and CCYV-infected cucumber plants

Developmental stage	Healthy plants	CCYV -infected plants	Sig.
egg	91.10 ± 2.20a	85.53 ± 3.99a	0.184
1st -instar	98.25 ± 1.75a	97.57 ± 1.59a	0.831
2nd -instar	99.25 ± 0.75a	96.00 ± 2.12a	0.109
3rd -instar	100 ± 0.00a	100 ± 0.00a	—
4th -instar	100 ± 0.00a	99.25 ± 0.75a	0.109
egg + nymphs	88.84 ± 1.60a	79.52 ± 2.09a	0.184

Note: Values in the table show Mean±SE, and same letters in the same line indicate that survival rate of the *B. tabaci* on CCYV-infected and healthy cucumber plants was not significantly different at the 0.05 level.

Effect of CCYV on sex ratio of B. tabaci

The effect of CCYV on the sex ratio of *B. tabaci* is shown in Fig. 5. On the healthy cucumber plants, the ratio of female *B. tabaci* was 50.53%, but on the cucumber plants infected with CCYV, the ratio of female was 66.40%. *B. tabaci* had a higher percentage of females on the CCYV-infected cucumber plants ($P < 0.05$).

Discussion

Vector-borne pathogens can alter the phenotypes of their hosts and vectors in ways that influence the frequency and nature of interactions between them, with significant implications for the transmission and spread of diseases [27]. Previous studies have shown that plant viruses can affect the insect vectors, but the degrees of influence of different virus-vector combinations are not identical. There have been many reports on alteration of physiology, molecular biology or feeding behaviors in insect vectors by persistently transmitted plant viruses, for example, *Begomovirus* on *B. tabaci*, but few or no studies are available on impact of semipersistent viruses on vectors [3–5, 14]. In our present study, we reported the

effects of semi-persistent virus CCYV on the biological characteristics of the vector *B. tabaci*. Although the nymphs play no roles in virus transmission, their immobile stages (esp. 2nd to 4th instar) encounter the plant viruses when feeding on the plant. Nymphs can be affected, more or less, by virus particles taken with plant sap, and thereby may affect the status of the adults responsible for virus transmission. Therefore, this study comprehensively investigated the biological effects of CCYV on nymphs and adults of *B. tabaci*, with a view to fully obtaining the biological effects of the virus. The results indicated that all nymph instars can be infected with CCYV, and the virion titer amount varies with instars. The 2nd -instar has the highest virion titer amount (1424.54 copies) among the nymphs, followed by the 3rd -instar and the 1st -instar, and the 4th -instar (112.34 copies) has the lowest virion titer, which may be related to the behavior characteristics of each instar nymph. The 1st -instar nymphs have tentacles and feet and can crawl over a short distance to find a suitable feeding site and then settle down and start feeding. The tentacles and feet of the 2nd and 3rd -instar nymphs were degraded, and they had no crawling ability. They were fixed on the back of the leaves for feeding with the stylets [28]. The 4th -instar nymphs, also known as pseudo pupal stage, basically stopped feeding [29, 30], which may be the reason for the low virion titer of the 4th -instar nymphs. The virion titer of the adults was much greater than those of the nymphs, and the virion titer of the individual adult was up to 21360.08 copies. Adult *B. tabaci* is highly active and can even migrate over long distances with the assistance of air currents, becoming the main cause of the CCYV pandemic.

By comparing the development period of *B. tabaci*, it was found that CCYV could significantly extend the development period of female nymphs ($P < 0.01$) and the longevity of female adults ($P < 0.01$), but not significantly affect the development period of male nymphs ($P = 0.391$) and the longevity of male adults ($P = 0.136$). The influence of CCYV on the growth and development of females are much greater than that of males. This may be because females are larger than males and require more nutrients to reproduce, so females ingest more viruses than males, which in turn has a more significant impact on their growth and development. Longer development period and longevity means more possibility of virus transmission. Therefore, we speculate that females are more conducive to the transmission of CCYV virus than males.

Through a comparative analysis of the body length and oviposition of *B. tabaci*, we found that CCYV significantly increased the body length of female adults ($P < 0.01$) and male adults ($P < 0.01$), and increased the oviposition of individual female adult ($P < 0.01$). It may be due to an extended developmental period and a higher intake of nutrients. The size of insect is an important factor affecting population development potential and community structure and function [31–33]. Relevant studies have shown that, compared with smaller individuals within the same species, larger insects often have advantages in reproduction, flight, competition, stress resistance and other aspects, contributing to the improvement of population fitness [34].

CCYV significantly increased the proportion of female adult from 50.53% on healthy plants to 66.40% on CCYV-infected cucumber plants. There are two reproductive modes of *B tabaci*, including parthenogenesis and amphigenesis. The female offsprings of *B tabaci* are all developed from fertilized eggs, while the male offsprings may come from fertilized eggs and parthenogenesis [35]. The increase of

female proportion of whitefly may be due to the increase of body length caused by CCYV, which enables it to have comparative advantages in mating process and obtain more mating opportunities, so as to increase the proportion of female offspring by increasing the number of fertilized eggs, thus ensuring the reproduction of its offspring population.

Conclusions

In conclusion, our results confirmed that CCYV could manipulate the growth and development of its vector, *B. tabaci*. We found that CCYV had more effects on female than male in development duration by increasing duration of female nymphs and adults. Interestingly, CCYV could significantly increase the body length and oviposition of *B. tabaci* and the ratio of females became higher on cucumber plants infected with CCYV, which will undoubtedly increase the population fitness and beneficial to its population reproduction, thus, it is beneficial to the transmission of CCYV. These results clearly indicated that the biological characteristics of *B. tabaci* Q biotypes changed greatly when infected with CCYV, and the effect on females is much greater than on males. Based on the above results, we can infer that CCYV and *B. tabaci* have a typical mutualism relationship and play an important role in *B. tabaci* outbreak mechanism. In this paper, the effects of semi-persistent viruses on the biological characteristics of vectors are studied, which will enrich people's understanding of the plant-virus-vector interaction.

Abbreviations

CCYV: cucurbit chlorotic yellows virus; TYLCV: tomato yellow leaf curl virus; RBSDV: rice black streaked dwarf virus; RSV: rice stripe virus; TSWV: tomato spotted wilt orthotospovirus; MED: Mediterranean; BPH: brown planthopper; WBPH: white-backed planthopper; qRT-PCR: quantitative reverse transcription polymerase chain reaction.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publication

All the authors consent to publish.

Availability of data and material

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Conceptualization, FY and JL; methodology, HH and JL; software, HH and ZZ; validation, FY and JL; formal analysis, HH; investigation, HH, ZZ, XT and DS; resources, FY; data curation, HH, XT and DS; writing—original draft preparation, HH; writing—review and editing, all authors; supervision, FY; project administration, JL; funding acquisition, FY and JL. All authors read and approved the final manuscript.

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Figures

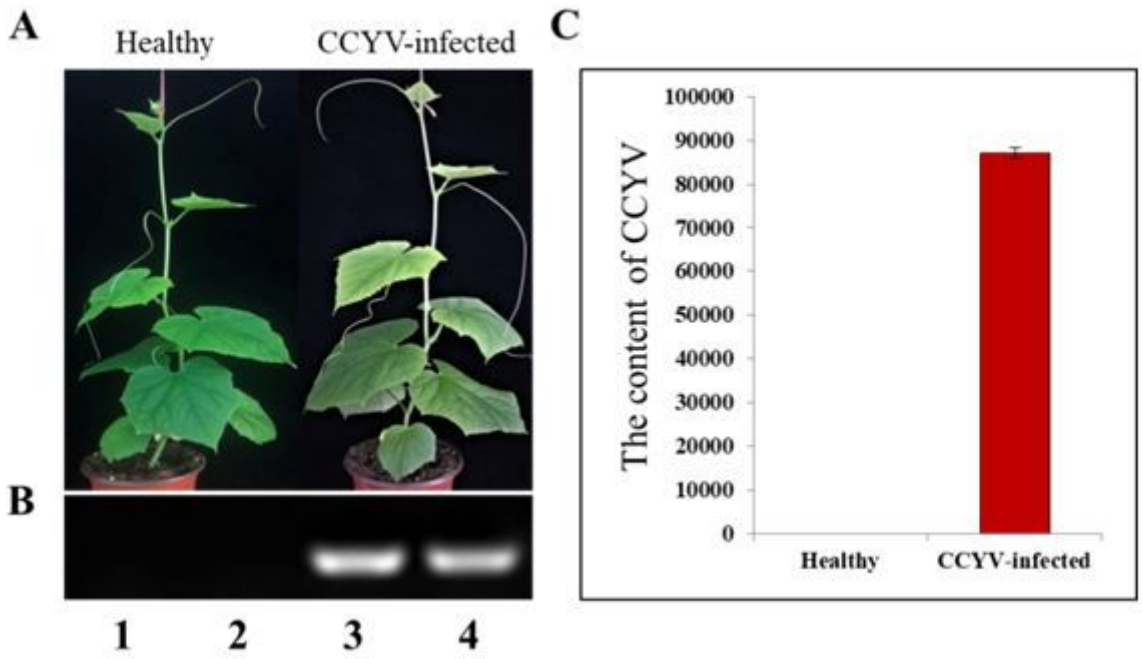

Figure 1

Determination of CCYV infection after agroinoculation in C. sativus. (A). Symptoms displayed on the systemic leaves of C. sativus at 25 days post-agroinoculation. Yellowing was observed on the systemic leaves of C. sativus. (B). RT-PCR detection of CCYV in the systemic leaves of C. sativus. Lane 1 and lane 2, healthy C. sativus; Lane 3 and lane 4, CCYV-infected C. sativus. (C). qRT-PCR detection the copies of CCYV in healthy and CCYV-infected C. sativus.

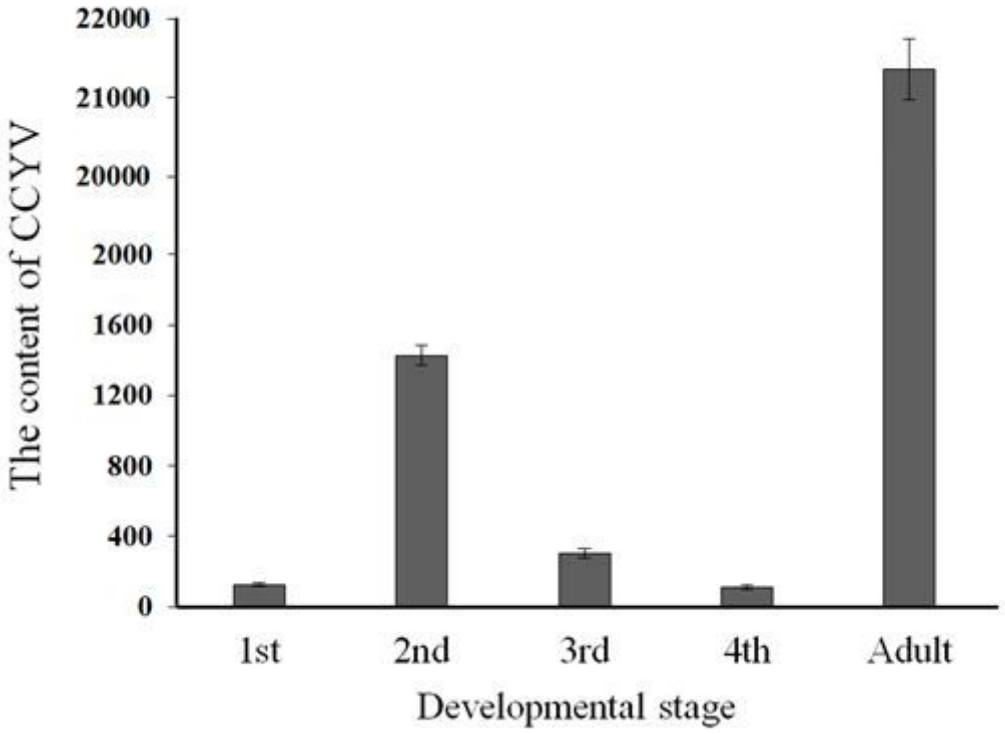

Figure 2

Virion copies of CCYV in individual whiteflies of B. tabaci.

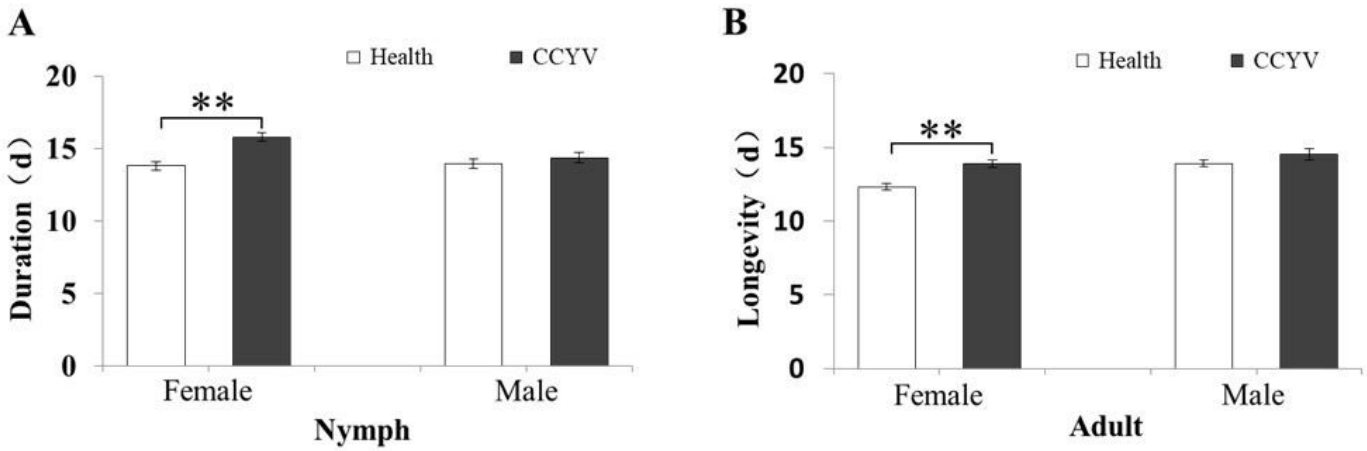

Figure 3

Impact of CCYV on the nymph duration (A) and adult longevity (B) of B. tabaci nymphs include stages from 1st-instar to 4th-instar. * indicate significance was at 0.05 level; ** indicate significance was at 0.01 level.

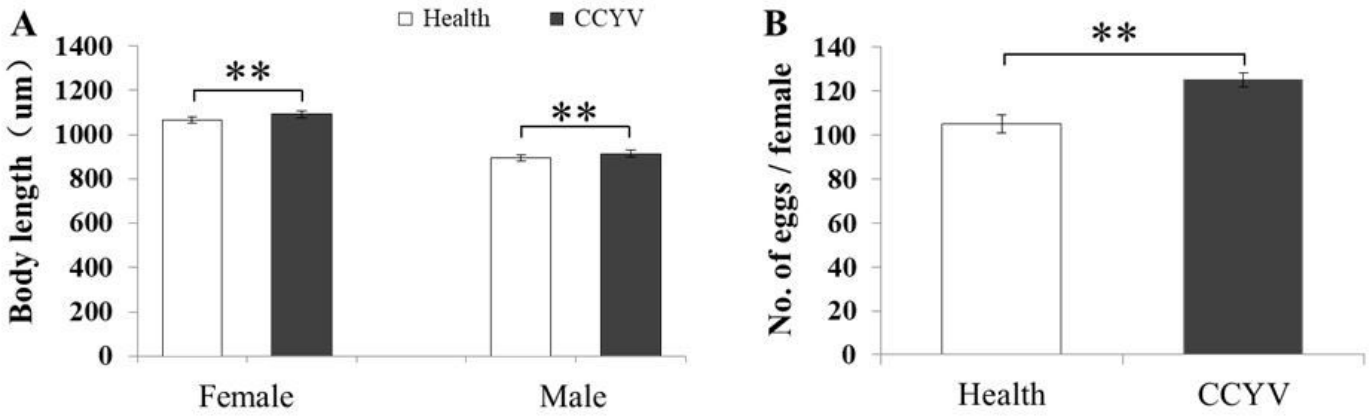

Figure 4

Impact of CCYV on the body length (A) and oviposition (B) of B. tabaci. * or ** indicate a statistically significant difference at p<0.05 or p<0.01.

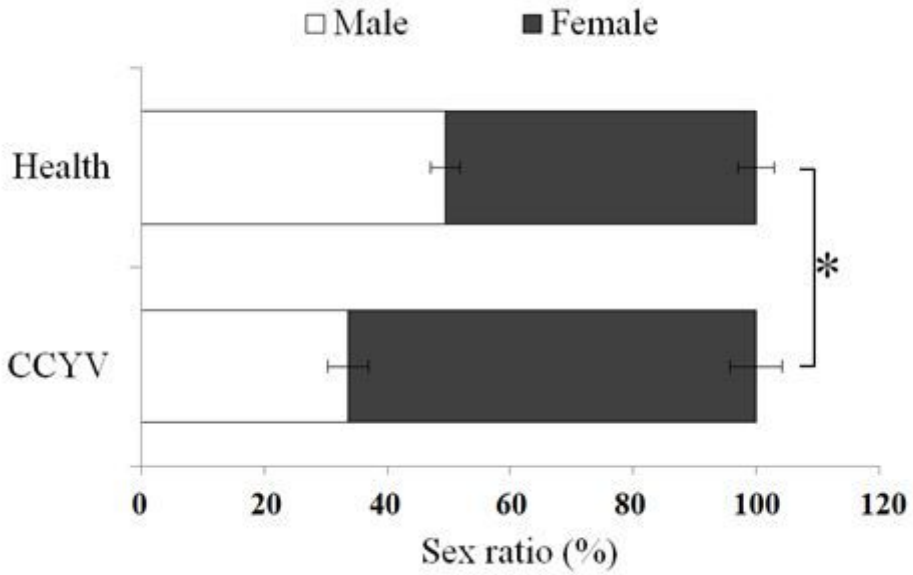

Figure 5

The sex ratio of B. tabaci on the healthy cucumbers and the cucumbers with CCYV. Note: * and ** indicate significant difference at level of P<0.05 and P<0.01 (Tukey's test), respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.ThestandardcurveofqRTPCR..pdf