How Does Vector Bemisia Tabaci use Visual and Olfactory Cues in Orientation to A Virus-Infected Host Plant?

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Abstract

Cucurbit chlorotic yellows virus (CCYV) has caused serious damage to melon crops in many countries in recent years. It is exclusively transmitted by the notorious pest *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in a semi-persistent manner. Previous studies have shown that both persistently and non-persistently transmitted viruses can manipulate orientation and performance of vector insects through changing host phenotype to facilitate virus spread. However, as a semi-persistently transmitted virus, how CCYV affect vector *B. tabaci* in locating host plants by altering physiological traits of host plants is still unknown. In this study, we investigated *B. tabaci* visual and olfactory preference between healthy and CCYV-infected host plant *Cucumis sativus*. Volatile profiles of healthy and CCYV-infected *C. sativus* plants were analyzed by using gas chromatography-mass spectrometry (GC-MS). Initially, it was found that vector insects preferred to settle down CCYV-infected *Cucumis sativus* seedlings in free choice. The concentrations of total volatiles and terpenes in cucumber plants were notably decreased after CCYV infection, and especially, the concentrations of α-pinene, β-ocimene, α-farnesene, and nonanal, responsible for olfactory attraction of *B. tabaci*, dramatically reduced in CCYV-infected plants. Subsequently, we investigated the visual preference of *B. tabaci* to CCYV-infected and healthy host, and found that *B. tabaci* adults showed significant preference to CCYV-infected host. CCYV induced yellowing symptoms in host leaves may explain the visual preference of *B. tabaci* adults to infected hosts. This study indicated that visual cues could play a vital role in vector insects locating virus-infected host plants.

Introduction

More than 80 percent of plant viruses are dependent on vectors for spread, so virus transmission is closely related to behaviors and biological traits of vector insects (Hohn 2007). Transmission modes of plant viruses by vector insects are classified into circulative persistently transmitted, non-circulative persistently transmitted, semi-persistently transmitted and non-persistently transmitted according to binding sites and retention period of virions within vector insects as well as time for acquisition and inoculation from and to plants (Dader et al. 2017). More and more studies indicated that plant viruses could manipulate vector insects’ behaviors directly and/or indirectly and thereby affect interactions between vector insects and plants (He et al. 2015; Ingwell et al. 2012; Wang et al. 2020; Wan et al. 2020; Eigenbrode et al. 2002). For example, viruses influence vector orientation and feeding behaviors indirectly by changing the color, morphology, odor and quality of host plants. Also, plant viruses can cause direct effect on vector insects when carrying virions, such as on longevity, fecundity, feeding or olfactory behaviors. Wan et al. (2020) found that mating behavior of thrips adults lasts significantly longer when thrips carrying tomato spot wilt virus (TSWV). Feeding behaviors of vector insects recorded by electrical penetration graph (EPG) found that the CCYV and TYLCV (tomato yellow leaf curl virus) affected the phloem ingestion of vector insects, which directly promote the virus transmission efficiency (Lu et al. 2017; He et al. 2015). Virus-infected host plants generally attract vector insects by changing host phenotypes, like plant volatiles, nutrition quality, color (Liu et al. 2019; Fereres et al. 2016; Chen et al. 2017; Mwando et al. 2018). Chen et al. (2017) indicated that TYLCV greatly reduced host volatiles (e.g., o-
xylene) to attract vector insects to feed and lay eggs. Wang et al. (2019) found mikania micrantha wilt virus (MMWV) notably changed volatile profiles of host plants and vector insects preferred to settle down on virus-infected host plants.

However, some studies indicated that viruses with various transmission manners induced different plant phytochemical changes. As for non-persistently transmitted viruses, vector insects could acquire virions during short brief ingestion, and virions could be lost with salivary secretion (Ng et al. 2006). Manck et al. (2010) showed that cucumber mosaic virus (CMV), a non-persistent transmitted virus, reduced the palatability of host plants to vector aphids. After short probing on CMV-infected host, vector insects would carry virions and transfer to new hosts. On the contrary, persistently transmitted viruses require vector insects continuously feeding on virus-infected host for virion acquisition (Su et al. 2015; Jhan et al. 2019; Moeini et al. 2020). Thus, persistently transmitted viruses enhance the host plant quality in order to attract vector insects for long-term feeding on virus-infected plants. CCYV, as a semi-persistently transmitted virus, how it manipulates vector insect behaviors through changing host phenotypes is still unknown.

CCYV (genus *Crinivirus*, family *Closteroviridae*) mainly damages cucurbit plants such as *Cucumis melo*, *C. sativus* etc, resulting in yellowing symptoms on leaves. CCYV has been reported in many Asian and some American countries and seriously decreased crop production (Okuda et al. 2010; Huang et al. 2010; Gu et al. 2011; Hernandez et al. 2021). CCYV is transmitted by the vector insects *B. tabaci*. This species of whitefly is a species complex consisting of at least 34 biotypes (cryptic species), and it transmits more than 200 plant viruses (Chi et al. 2020; Henrique et al. 2019; Chen et al. 2019) with the host range of more than 600 plant species. Now, Q biotype (MED) and B biotype (MEAM1) are two cryptic species of *B. tabaci* dominating in China (Wu et al. 2002; Teng et al. 2010; Chu et al. 2010).

Vision and olfaction play vital roles in locating host plants by herbivore insects. When herbivore insects select host plants in a short-range, vision is used to locate host plant (Song et al. 2018). As is well known, plant viruses can interfere host metabolites and induce symptoms of host plants. Those changes may alter vector foraging behavior indirectly. Studies on effects of CCYV on interactions of vector insects and host plant would help to understand transmission mechanisms of CCYV and other *B. tabaci*-borne viruses, and may contribute to implementation of new strategies for control of plant viruses and their insect vectors.

### Methods And Materials

**Plants and Insects** Cucumber plants *C. sativus* (var. Bojie-107) were cultivated in plastic pots (diameter = 10 cm, height = 12 cm), with 1 plant in each pot, and were maintained in a greenhouse with photoperiod Light: Dark = 16:8, temperature $27 \pm 3 \, ^\circ\text{C}$, relative humidity: $70 \pm 5\%$. Seedlings with 3–4 true leaves were used for experiments.

Colony of *B. tabaci* Q biotype (cryptic species Mediterranean) was maintained on healthy cucumber plants. The genetic purity of *B. tabaci* Q biotype was monitored according to Li et al. (2016). Transferring
Non-viruliferous *B. tabaci* adults were transferred onto CCYV-infected cucumber plants for a 3-d acquisition-access period (AAP). Then, one hundred viruliferous adults of *B. tabaci* were transferred into the a clip cages and kept on the leaves of healthy plants for 3 days for inoculation, and then the insects were removed from CCYV-infected plants. The eggs and nymphs were removed with a brush. After 20 days, the plants were used for experiments. Before the experiments, the virus infection status of plants was detected by RT-PCR (Zang et al. 2005).

**RNA Extraction and Synthesis of cDNA** Total RNA was extracted from 100 mg plant leaves with Trizol reagent according to the manufacturer's instructions (Takara Bio, Shiga, Japan). Total RNA was treated with RNase-free DNase I for 2 min at 42°C to remove residual DNA. The RNA concentration was determined by microspectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 µg) was used with PrimerScript RT reagent kit (Takara) for reverse transcription according to the instructions.

**Virus Titer Determination in Plants** Primers were designed by Primer 5.0 software according to CCYV coat protein coding sequences (the forward primer: 5′-GCGACCATCATCTACAGGCA-3′, nucleotide positions 548–567; the reverse primer: 5′-CCGACTTGTTCTTTCAGAGC-3′; nucleotide positions 679–699). The qRT-PCR assays were performed using TB Green *Premix Ex Taq*™ (Takara, Code No. RR820A). Reactions were carried out in a total volume of 20 µL: 10 µL of TB Green *Premix Ex Taq*™, 1 µL cDNA or plasmid dilutions, 0.8 µL of each primer (the forward primer and the reverse primer), 0.4 µL of ROX Reference Dye II and 7 µL dd H2O. Amplification reactions were performed as follows: 94°C for 2 min; 40 cycles of 94°C for 15 s, 60°C for 20 s, 72°C for 20 s (Li et al. 2016), for making standard curve, serial 10-fold dilutions of plasmid (1.49 × 10^3 to 1.49 × 10^9 copies/µL) were used as templates for real-time RT-PCR, and the standard curve equation is \( Y = -3.222 \log X + 35.909, \ R^2 = 1 \). Finally, virulence titers were calculated by standard curve.

**Impacts of CCYV on *B. tabaci* Host Selection Preference**

**Bi-choice Assay** CCYV-infected and healthy *C. sativus* seedlings were placed alternately in insect cages (60 cm × 60 cm × 60 cm), and 100 viruliferous and non-viruliferous *B. tabaci* adults, after 30 min starvation, released into the middle area of the cage. The number of *B. tabaci* adults on each plant were counted at intervals of 1 hr, 3 hr, 5 hr and 24 hr. Four replicates were conducted in each group.

**Olfactory Preference to Plant Extracts** Healthy and CCYV-infected plant leaves were ground into powder with liquid nitrogen. Two grams of plant leaf powder were soaked in 10 mL 80% methanol solution for 24 hours, and the extracts were used for Y-shaped tube test. Standard chemicals (purity > 99%) were diluted with paraffin oil with paraffin oil used as control.

Two hundred microliters of plant extracts or diluted chemicals were added onto the 3 × 2 cm filter paper, and the two pieces of filter paper were separately placed into two bottles connected with a Y-shaped tube olfactometer. The olfactometer consist of one main tube and two arms (glass tubes, each with 10 cm long and 2 cm internal diameter) with 60° angle between the two arms. Flow of the odor was blown into...
the tube at 0.3 L/min for 10 minutes before insects were released into the end of the main arm. An adult was tested each time, and behavioral selection responses were observed. When an insect crossed 1 / 3 of an arm within 3 min, it was regarded as an efficient choice. Twenty adults were tested in each group with 3 replicates.

**Collection and Analysis of Plant Volatiles** Volatiles from aerial parts of infected and healthy host plants were collected by using aeration sampling system as described by Mwando et al. (2018). The plant was contained in an oven plastic bag (50 cm × 55 cm) and connected to an air-generating device with an inlet flow rate of 0.3L/min. Volatiles were collected by passing the outlet air through a PoraPak Q (60 mg, mesh 50–80, supelco, Bellefonte, PA, USA) filter at the rate of 0.1 L/min. Before use, PoraPak Q filters were washed sequentially with hexane, acetone and diethyl ether for three times, and dried by a stream of nitrogen gas. After 8hr collection, the filters were eluted with 500 µL hexane and then stored at -80 ℃ for analysis. Collection of volatiles were conducted from 10:00 p.m- 6:00 a.m. Collections for each group of plants were repeated for five times.

Collected volatiles samples were analyzed using gas chromatographic-mass spectrometry (GC-MS) (Agilent 7890B couple 5977, Agilent Technologies, USA), equipped with a non-polar HP-5 MS column (30 m × 0.25 mm, 0.25 µm film). One microliter sample was injected into GC system with helium as a carrier gas at a flow rate of 1.0 ml/min. The temperature program was 40℃ for 2 min, raising up to 180 ℃ at the rate of 5 ℃/min, finally up to 250 ℃ in the rate of 15 ℃/min. Chemical spectra were recorded at 70 eV in the electron impact (EI) ionization mode. Substances were identified by comparing with mass spectral data library (NIST 14.0) and Masshunter working station. Typical chemicals were identified by comparing the retention time and mass spectrogram with standard chemicals. The standard chemical nonylacetate was diluted with hexane to 6 concentrations, and the external standard curve was established according to the different concentrations and peak areas ($R^2 = 0.9976$). The concentration of chemicals were calculated by the standard curve.

**Whitefly** *B. tabaci* **Visual Preference Assay** One hundred adult insects, after starved for 30 min, were released into the center of the release point in the glass box (Fig. 1). Plants infected by CCYV for 20 days and healthy plants were placed on both sides of the lower area. Because of the airtight glass plates and tubes, air can’t circulate within the device. The number of insects in both tubes were counted after 1 hr. Experiments were repeated seven times. There is no air flow within the device, and we exchanged positions of two groups of plants every time. Thus, the environmental influence could be eliminated.

**Statistical Analysis** SPSS 22.0 was used to analyze the differences between the treatment and the control group. The significant levels were set as $P < 0.05$ (**”), $P < 0.01$ (**”), and $P < 0.001$ (*****). For data analysis of *B. tabaci* behavioral response tests, the number of *B. tabaci* was transferred into a percentage for difference analysis (Tamiru et al. 2011).

**Results**
**Virus Titers Determination in Plants** The virus titers varied significantly at different stages. At 10 days after CCYV infection, the amount of coat protein gene molecules was $1.40 \times 10^2$ copies, was $2.94 \times 10^6$ copies at 20 days, virus titer was $2.33 \times 10^6$ copies at 30 days, while nothing was found in healthy cucumber leaves (Fig. 2). With the increase of virulence titers, the yellowing symptoms got more obvious.

**B. tabaci bi-choice Assay** Whitefly *B. tabaci* bi-choice assay showed that, after releasing insects for 3 hr, the percentages of *B. tabaci* selecting CCYV-infected plants were significantly higher than that for healthy plants (Fig. 3), and both viruliferous and non-viruliferous *B. tabaci* adults preferred to settle down CCYV-infected host plants. This result implies the CCYV didn't affect vector's preference to virus-infected plants.

**Chemical Analysis of Plant Volatiles** By analyzing the difference between healthy and CCYV-infected host plant volatiles, we found CCYV changed plant volatiles, with more and larger peaks in the healthy group (Fig. 4). The qualification and quantification analysis show CCYV-infected plants released less volatiles comparing with healthy plants (Table 1). Some chemicals in healthy plants, such as nonanal, α-farnesene etc, was not detected in CCYV-infected plant volatiles. Total concentration of volatiles and terpenes in CCYV-infected is remarkably lower than that in healthy plants (Fig. 5) ($P_{total} < 0.01; P_{terpenes} < 0.05$), especially monoterpens α-pinene and β-ocimene were notably decreased after CCYV infection (Fig. 5).
Table 1
Quantification of major volatile organic compounds from healthy and CCYV-infected *Cucumis sativus* seedlings*

| Volatiles compounds                      | Healthy (ng) | CCYV-infected (ng) | P value |
|------------------------------------------|--------------|--------------------|---------|
| 2-methyl-3-Pentanone                     | 1.3 ± 0.47   | 0.79 ± 0.04        | 0.130   |
| p-xylene                                 | 0.48 ± 0.08  | 0.43 ± 0.04        | 0.426   |
| α-pinene                                 | 0.34 ± 0.03  | 0.25 ± 0.02        | 0.012   |
| Benzaldehyde                             | 0.47 ± 0.17  | 0.22 ± 0.01        | 0.058   |
| 2,2,4,6,6-pentamethyl-Heptane            | 0.50 ± 0.08  | 0.30 ± 0.02        | 0.014   |
| D-Limonene                               | 1.35 ± 1.42  | 0.64 ± 0.36        | 0.443   |
| Benzyl alcohol                           | 0.30 ± 0.04  | nd                 |         |
| β-ocimene                                | 0.70 ± 0.16  | 0.25 ± 0.04        | 0.010   |
| Nonanal                                  | 0.26 ± 0.08  | nd                 |         |
| 1-butanol, 3,3-dimethyl-                 | 0.19 ± 0.01  | nd                 |         |
| 2,4,6-octatriene, 2,6-dimethyl-, (E,Z)-  | 0.40 ± 0.20  | 0.24 ± 0.05        | 0.234   |
| Methyl salicylate                        | 0.27 ± 0.15  | nd                 |         |
| Ethanone, 1-(4-ethylphenyl)-             | 0.43 ± 0.15  | 0.34 ± 0.07        | 0.386   |
| Dimethyl phthalate                       | 0.24 ± 0.03  | 0.21 ± 0.03        | 0.221   |
| α-farnesene                              | 0.19 ± 0.02  | nd                 |         |
| Dibutyl phthalate                        | 0.24 ± 0.01  | 0.21 ± 0.01        | 0.031   |

*The contents reflect the amount of volatiles released by host plants collected within 8 hours. An independent-sample *t* test was used to calculate difference of chemicals between CCYV-infected and healthy plants. Date = Mean ± Standard Error. The significance level was 0.05. ‘nd’ means not detected.

**Behavioral Responses of B. tabaci to Plant Extract and Standard Chemicals** B. tabaci showed olfactory preference to the extracts of healthy plants (Fig. 6A) (*P* < 0.05). Furthermore, the release of α-pinene, β-ocimene, nonanal and α-farnesene all attractive to vector B. tabaci, decreased after CCYV infection, (Fig. 6B) (*P*<farnesene < 0.05, *P*β-ocimene < 0.01, *P*nonanal < 0.001, *P*α-pinene < 0.001). It means that B. tabaci prefers the odor of healthy plants.

**Visual Preference to Infected and Healthy Plant Test** In visual selection test, the percentage of B. tabaci selecting CCYV-infected plant was significantly higher than that to healthy plants (Fig. 7) (*P* < 0.05). It indicates CCYV enhanced the visual preference of B. tabaci to CCYV-infected host plants.
Discussion

In this study, we found that CCYV infection improved vector insect *B. tabaci* the visual cues rather than olfactory cues in orientation to host plants. CCYV decreased volatiles emissions, especially the terpenes. The reduction of terpenes emission may contribute to protect vector insect from natural enemies. Since predators and parasitoids locate insects mainly depending on volatile terpenes (Shapiro et al. 2012), and high concentrations of terpenes would increase the possibility that insects being parasite by enemy insects. Additionally, Terpenes is a kind of anti-insect substances in plants, which commonly be reported that viruses can suppress plant defensive response to protect their vector insects (Patton et al. 2020; Abe et al. 2012; Wang et al. 2019). Luan et al. (2013) reported that virus reduced the terpene biosynthesis in host plants to protect vector. Thus, decreasing olfactory attraction can potentially protect vector insects.

Plant volatiles act as a signal for herbivore insects in locating their host at long distance, but not a dominant role (Fereres et al. 2016; Wang et al. 2019). Herbivore insects integrate visual, olfactory and even gustatory cues when they are making the foraging decisions. *B. tabaci* tends to choose infected host by visual cues partially because of the CCYV-induced yellowing symptoms. Since color changes greatly affect insects locating host plants. *B. tabaci* was sensitive to yellow-blue color (500-580nm), especially yellow color is more attractive than the others (Mound et al. 1962; Prokopy et al. 1983). Viruses increased the visual attraction of host plant have been revealed in tomato chlorosis virus (ToCV)/tomato severe rugose virus (TSRV), tomato yellow leaf curl virus (TYLCV) (Fereres et al. 2016; Johnston et al. 2020). The three viruses above are all transmitted by *B. tabaci* and cause yellowing and chlorosis symptoms. Those evidences support our hypothesis that the CCYV induced symptoms could be responsible for the visual preference of *B. tabaci* to virus-infected host plants.

This study also highlights the importance of visual cues for insects with weak flying ability when selecting host plants. Virus induced symptoms of host plant, like morph or color changes, should be taken into consideration when studying how virus alter vector insect behaviors indirectly. In this study, we integrated visual and olfactory responses of vector insects to infected and healthy host plants, and our results may help understand how a semi-persistently transmitted virus manipulate vector behaviors indirectly. Yet, in this process, we are still interested in how CCYV cause such effects, to be more specific, which CCYV encoded proteins play roles in impacting the preference of *B. tabaci* to infected host, and how CCYV interact with host factors to attract vector insects.

Declarations

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**Conflicts of interest** The authors declare no conflict of interest.

**Availability of data and material** All data generated or analyzed during this study are included in this published article.
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Code availability Not applicable.

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Figures

Figure 1

The device for B. tabaci visual preference assay. The front view (A) and top view (B) of the visual selection device. The device is made of colorless glass (60 cm × 45 cm × 20 cm).
Figure 2

Virus titers (A) and symptom (B) of CCYV in plants at different stage Virus titers in healthy cucumber leaves and CCYV-infected leaves on 10, 20 and 30 days were detected by qRT-PCR. Columns show mean ± standard error. The X-axis shows different stages and Y-axis represents the copies of the CCYV coat protein gene. One-way ANOVA was used to analyze the difference (P < 0.05), different letters means that the virus titers are significantly different.
Behavioral response of non-viruliferous (A) and viruliferous (B) B. tabaci to CCYV-infected plant and healthy plant. This experiment was conducted in an insect cage (60 cm × 60 cm × 60 cm), and released B. tabaci in the center of two plants. Counting the number of B. tabaci for 4 times during 24 hours. The red dot represents the percentage of B. tabaci prefer to CCYV-infected hosts in each group, while the green dot represents the percentage of B. tabaci prefer to healthy hosts in each group. ‘*’ means P < 0.05, ‘**’ means P < 0.01, ‘***’ means P < 0.001. Independent-sample t test was used to analyze the difference (P < 0.05).
Figure 4

Gas Chromatographic profiles of head space volatiles from healthy (A) and CCYV-infected (B) Cucumis sativus seedlings. The black line (above) represents healthy group and the red line (below) represents the CCYV-infected group. 1: α-pinene, 2: β-ocimene, 3: nonanal, 4: α-farnesene.
Figure 5

Total amounts of volatiles and terpenes analysis from Non-infected and CCYV-infected Cucumis sativus seedlings. Columns show Mean ± Standard Error. ‘*’ means P < 0.05, ‘**’ means P < 0.01, independent-sample t test was used to calculate the difference.
Figure 6

Olfactory behavioral responses of B. tabaci to Cucumis sativus leaf extract (A) and standard chemicals (B). The olfactory preference assay to infected and healthy plant extracts (A) and to standard chemicals (B) were conducted by Y-shaped tube. Columns show Mean ± Standard Error (A). Each point represents one sample data and different colors represent different group (B). Twenty adults were test in each group, the number of B. tabaci was transferred into percentage for analyzing difference according to independent-sample t test. Three replicates were conducted. ‘*’ means P < 0.05, ‘**’ means P < 0.01, ‘***’ means P < 0.001.
Figure 7

Visual selection preference of B. tabaci between CCYV-infected and healthy plants Data = Mean ± Standard Error. One hundred adult insects were tested and seven replicates were carried out. The number of B. tabaci was transferred into percentage for analyzing difference by independent-sample t test (*’ < 0.05).