Preference of aphid *Myzus persicae* (Hemiptera: Aphididae) for tobacco plants at specific stages of *Potato virus Y* infection

Jinyan Liu[1], Yingjie Liu[1], Philip Donkersley[2], Yonghao Dong[1], Xi Chen[1], Yun Zang[1], Pengjun Xu[1, 3], Guangwei Ren[1, 3]

[1]Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao 266101, China

[2] Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK

[3] Corresponding author:

Pengjun Xu, Mailing address: Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, 266101, P.R. China; Phone: +86-532-88701012; Fax: +86-532-88701032; E-mail: xupengjun@163.com.

Guangwei Ren, Mailing address: Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, 266101, P.R. China; Phone: +86-532-88701012; Fax: +86-532-88701032; E-mail: renguangwei@caas.cn.

Abstract *Potato virus Y* (PVY) is a common pathogen affecting agricultural production worldwide, and is mainly transmitted by *Myzus persicae* in a non-persistent manner. Insect-borne plant viruses can modify the abundance, performance, and behavior of their vectors by altering host plant features; however, most studies have overlooked the fact that the dynamic progression of virus infection in plants can have variable effects on their vectors. We addressed this point in the present study by dividing the PVY infection process in tobacco into three stages (early state, steady state and late state) according to viral copy number, and then compared the variational effects of PVY-infected tobacco (*Nicotiana tabacum*) plants on host selection and feeding behavior of *M. persicae*. A Y-shaped olfactory apparatus and electrical penetration graph (EPG) method were used to evaluate host selection and feeding behavior, respectively. Interestingly, we found that PVY-infected plants at the steady state of infection attracted more aphids than healthy plants, whereas no differences were observed for those at the early and late states. In terms of feeding behavior, intracellular punctures which are closely related to PVY acquisition and transmission were more abundant on PVY-infected tobacco plants at the early and steady states of infection than in non-infected plants. These results indicate that PVY-infected host plants can alter the host selection and feeding behavior of aphids in a stage-dependent manner manner, which is an important consideration when studying the interactions among host plants, virus, and insect vectors.

Introduction

As a member of the family *Potyviridae*, *Potato virus Y* (PVY) has been a persistent problem for decades in solanaceous crops production worldwide [1]. PVY infection negatively impacts crop quality and reduces overall yields, resulting in significant economic losses [2]. PVY is mainly transmitted by aphids-e.g., green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), which is distributed worldwide and is highly polyphagous, and can damage plants both directly by feeding on their vascular bundles and indirectly by transmitting pathogenic viruses such as PVY and *Cucumber mosaic virus* (CMV; *Cucumovirus*, *Bromoviridae*) [3]. As one of the most common and efficient vectors, *M. persicae* transmits PVY in a non-persistent manner during brief probes of the plant epidermis, making it very difficult to control [4].

To control insect-transmitted plant viruses, it is critical to clarify their effects on the feeding behavior and dispersal of insect vectors [5]. The transmission of insect-borne plant pathogens depends on the abundance and behavior of their vectors [6]. The behavior of insect vectors can be altered by plant pathogens; for instance, the frequency and nature of the virus-vector interaction can be modified to enhance disease transmission, and it could be altered either indirectly through the virus-infected host plant or directly after pathogen acquisition by the vector [7-9]. Several plant viruses are known to infect their insect vectors, for instance, *Tomato spotted wilt virus* (*Tospovirus*, *Bunyaviridae*)-viruliferous male *Frankliniella occidentalis* (Pergande) showed threefold higher frequency and duration of phloem sap feeding than non-viruliferous males, a behavior that may increase virus transmission efficiency [10]. Many viruses modify the behavior and performance of vectors to indirectly optimize their transmission. For non-persistently transmitted viruses such as CMV, efficient transmission to a new host usually depends on virus acquisition during aphid feeding and swift vector dispersal from infected plants [11]. *M. persicae* and *Aphis gossypii* are initially attracted to CMV-infected plants in response to virus-induced volatiles but are subsequently dispersed, preferentially settling on non-infected plants since these are superior reproductive hosts [12]. Many studies have reported that non-persistent viruses induce changes in the host plant to

enhance vector attraction, but this can reduce vector fitness on the host plant, thereby decreasing vector performance and promoting its rapid spread [13, 14].

PVY and *M. persicae* have been well studied as an ideal model for investigating the relationship between plant viruses and their insect vectors. It was reported that *M. persicae* is initially unable to discriminate between infected and healthy hosts [15, 4], but later develop a preference for the former after prolonged feeding; several similar studies have demonstrated that aphids prefer infected host plants [16]. It is thought that PVY chemically mediates insect–plant interactions by activating the salicylate pathway and decreasing plant resistance to aphid vectors [17], which could enhance aphid fecundity and density on PVY-infected plants [18]. However, none of these studies examined the dynamic relationship between PVY-infected host plants and aphid behavior at different stages of the virus infection process. To this end, the present study investigated whether the stage of PVY infection of tobacco plant affects the host selection and feeding behavior of aphids.

Materials and Methods

Aphid Colonies, Host Plants and Virus Culture

M. persicae colonies were established from a single parthenogenetic female collected at Jimo Experimental Station (36.4454°N, 120.5862°W) in China. The colonies were maintained on healthy tobacco plants (*Nicotiana tabacum* 'K326') in climate chambers at 25℃ with 65% relative humidity on a 14:10-h light/dark cycle. Infectious clones of PVY (PVY-N605) were provided by the plant virus laboratory of Shandong Agricultural University.

Virus Detection and Quantification

Tobacco plants at the seven or eight true leaf stage were inoculated with plant tissue homogenate infected with PVY by mechanical friction. The plant tissue was ground with 10 times the equivalent volume of 0.1 M potassium phosphate buffer (pH 7.4) on ice. Carborundum powder was then added and the mixture was applied to the surface of tobacco leaves using cotton swabs [19]. Control plants were mock-inoculated in the

same manner with healthy tobacco plant tissue. To detect the presence of PVY in tobacco plants, specific primers amplifying a 535-bp fragment (PVY-F1/PVY-R1) were designed according to the genomic sequence of the infectious PVY clone that was used. The cycling parameters were as follows: 95 ℃ for 4 min, and 35 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s, and 72 ℃ for 30 s. In the preliminary experiment, we inoculated 10 tobacco plants by friction, and all of them were successfully infected (i.e., infection efficiency = 100%).

TaqMan real-time quantitative PCR using a standard curve was carried out to quantify PVY copy number. A sufficient number of tobacco plants were inoculated with PVY at one time and leaves collected from different parts of the plant were quantified 3, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, and 24 days after PVY inoculation. Each plant was sampled only once. Total RNA extracted from plant leaves of known weight was prepared and the cDNA templates were obtained as previously described [20]. Fragments containing the primers and probes of PVY were amplified with the primers PVY-F /PVY-R using a program consisting of 35 cycles of 94 ℃ for 30 s, 56 ℃ for 30 s, and 72 ℃ for 30 s. To generate the standard curve [21], PCR products were cloned into the pEASY-T vector (TransGen, Beijing, China); quantitative PCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction contained the following: 2× reverse transcriptase (RT)-PCR buffer (10 μl), forward and reverse primers (0.4 μl each, 20 pmol/μl), TaqMan probe (0.8 μl, 40 pmol/μl), 50× RT-PCR enzyme mix (0.2 μl), DNA template (2 μl), and 6.2 μl double-distilled (dd)H_2O. As the negative control for viral DNA extraction, cDNA from virus-free tobacco plants was used as the template; the no-template control was ddH_2O. Thermal cycling conditions were 95 ℃ for 30 s; 40 cycles of 95 ℃ for 5 s; and 60 ℃ for 34 s. Each sample was analyzed in triplicate. Primer and probe sequences are shown in Table S1.

Host Selection by Aphids

The Y-shaped olfactory apparatus was used to evaluate host choice by aphids (PVY-infected vs. healthy plants) as previously described [22]. Briefly, the instrument

was placed in a dark box and plants subjected to different treatments were individually placed in the flavor source bottle. A single apterous aphid (starved for 2 h) was placed in a Y-shaped base arm for observation. A selective reaction was noted when the aphid passed the halfway point of the treatment or control arm; when no obvious selection trend was observed after 5 min, it was regarded as no choice. The airflow velocity was 200 ml/min/arm. According to the results of virus quantification, we chose tobacco plants inoculated with PVY for 5, 12, and 24 days respectively as well as healthy plants of the same ages to test the selection behavior of aphids. Three replicates (33 or 34 apterous aphids per replicate) of each group were analyzed, and a total of 100 aphids were tested in each group with each aphid was tested once. The two arms of the olfactory instrument were exchanged after 10 aphids had been tested, and the tobacco plants and "Y" tube were replaced after 33 or 34 aphids had been tested. Each plant was used only once. The "Y" tube was cleaned with alcohol and dried before use.

Aphid Feeding Behavior

The electrical penetration graph (EPG) method was used to evaluate the feeding behavior of aphids between PVY- and mock-inoculated tobacco plants. Briefly, EPG waveforms were recorded using an eight-channel direct current EPG instrument (Wageningen University & Research, Wageningen, the Netherlands). In this experimental setup, aphids and plants formed an electrical circuit that was completed when the aphid inserted its stylet into the plant. A weak voltage was supplied to the circuit and any change in electrical properties was recorded as an EPG waveform that was correlated with the feeding activity of the aphid and site of stylet insertion into plant tissue. Apterous adult aphids of similar size were selected from healthy plants and an insect electrode was connected to their pronotum. After attachment of a 4-cm-long gold wire electrode (diameter 0.2 mm), the aphids were starved for 2 h prior to the experiment, then gently placed on the back of the third leaf of each plant. The recording time for each trial was 6 h. The waveform produced by one aphid on each plant was used as a replicate for a total of 15-20 replicates. The different

behavioral phases were manually labeled using software Stylet+ v01.23 software (EPG Systems, Wageningen, The Netherlands) as follows: potential drop (pd); non-penetration (np; i.e., aphid stylet is outside the plant); pathway phase (C; penetration into non-phloem tissue), derailed stylet activity (F), salivation into sieve elements (E1), and ingestion of phloem sap (E2). E1/E2 transition patterns were included in E2. Waveform patterns that were not terminated before the end of the experimental period (6h) were not excluded from the calculations. The above-mentioned waveforms were analyzed as previously described [23], and the following variables were measured and compared between non-infected and infected plants, including time to first probe, probing frequency and duration, time to phloem from the start of EPG, and duration of phloem sap ingestion, which was closely related to non-persistent virus propagation [24] and reflects the acceptability [25] of the food source to aphids.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics v.21 software (IBM, Armonk, NY, USA). Viral copy number was compared between different tobacco leaves by one-way analysis of variance. EPG parameters were calculated manually and individually for each aphid, and the mean and standard errors were determined using the EPG analysis Excel worksheet created for this study, and the data were analyzed with the Student's *t* test. All data were transformed if needed to meet assumptions of normality using log10(x+1) if needed. The chi-square test was used to evaluate differences between PVY-infected and healthy tobacco plants.

Results

Dynamics of PVY Copy Number in Tobacco Plants

We generated a standard curve for absolute quantification (Fig. S1). Using the neighboring leaf above the inoculated leaf, we quantified PVY copy number in the leaves of PVY-inoculated plants at different time points. PVY copy number differed significantly among samples depending on the time of virus inoculation (F = 52.748;

df = 6, 56; $P < 0.0001$) (Figure 1a): PVY copy number in tobacco infected by PVY within first 7 days was significantly increased, and was defined as early state; PVY copy number in tobacco infected by PVY between 8 and 18 days was stable, and this was defined as steady state; and PVY copy number in tobacco was declined with respect to the steady state 20 days after inoculation, which was defined as late state. We selected tobacco plants on day 10 after inoculation to examine the spatial distribution of the virus; the results indicated that PVY copy number was different in young and old leaves ($F = 7.819$; df =11, 24; $P < 0.0001$) (Figure 1b).

Host Selection by Aphids

The results showed that more aphids significantly selected PVY-infected tobacco plants 12 days after inoculation ($\chi^2 = 0.450$, $P = 0.006$). PVY-infected tobacco plants 5 days after inoculation were more likely to be selected, although the trend was not statistically significant ($\chi^2 = 7.515$, $P = 0.502$). There were no differences in aphid preference for PVY-infected vs. healthy plants 20 days after inoculation ($\chi^2 = 0.011$, $P = 0.917$) (Fig. 2).

Aphid Feeding Behavior

EPG variables describing *M. persicae* stylet penetration into virus-infected and healthy tobacco plant leaves are shown in Table 1. At different stages of the virus infection, aphids exhibited distinct behaviors related to cell puncture and phloem feeding behavior; in general, they showed a propensity for greater intracellular puncture rate on virus-infected plants at early and steady states of virus infection, as evidenced by the total number of pds and the number of pds during the time to phloem from the start of EPG (Table 1, variables 2 and 7).

At a steady state of infection, aphids spent less time in the phloem of virus-infected as compared to healthy plants (Table 1, variable 15); additionally, during this period the number of np events was higher in the former (Table 1, variable 3). The total duration of intercellular pathway (C) events per aphid was relatively short in healthy plants (Table 1, variables 17 and 18), and the latency to the first

probing was greater in infected than in healthy plants at late state of virus infection (Table 1, variable 1). Throughout the infection process, F was relatively low in infected plants (Table 1, variable 16). However, there was no difference in the latency to initial probing of the phloem by analysis of variance. Likewise, there was no difference in the effective feeding time (E2 duration > 10 min) between aphids in the two groups of plants (Table 1, variable 12).

Discussion

The transmission of insect-borne plant virus depends upon the abundance and behavior of their vectors. Viruses employ sophisticated strategies to overcome the distance separating plants and penetrate the plant cell wall [26]. Our results demonstrate a mutualism between viruses and their vectors: virus-infected hosts attract more aphids and increase the number of intracellular punctures , which would in turn facilitates the spread of pathogenic viruses [27].

The performance and behavior of insect vectors altered by PVY directly or indirectly are not fully understood. It was demonstrated that PVY has a positive effect on aphid vector abundance and performance [17, 28]. However, most previous studies have examined the behavior and physiology of vector insects in host plants 2 to 3 weeks after inoculation [7, 28], without taking into consideration the dynamic nature of virus infection process. We addressed this in the present study based on the accumulation of PVY virus particles in tobacco plants.

Vector insects such as aphids respond to plant volatiles by means of highly sensitive antennal olfactory sensilla during host searching and selection [29-31]. Virus infection can induce changes in host plant cues that influence vector orientation, feeding, and dispersal [11, 32]. It was reported that aphids are more responsive to volatile cues on *Potato leafroll virus*-infected as compared to healthy plants [30]. Previous studies have shown that aphid vectors exhibit higher fecundity and population growth on PVY-infected as compared to non-infected plants [15, 16]. However, there were no reports on whether aphids can proactively distinguish the odors of PVY-infected and healthy tobacco. In our study, we observed a close

relationship between the time of virus infection process on host plants and attractiveness to aphids; PVY-infected tobacco plants preferentially attracted aphids at a steady level of virus infection, while the number of aphids chose healthy and PVY-infected tobacco plants at early or late states of virus infection was no significant difference. This indicates that aphids can perceive physiological cues resulting from virus infection during a specific stage of the infection process. While it took 3–8 days for the systemic spread of PVY, the virus only had a weak inductive effect on tobacco plants, especially on volatile emissions. However, as the virus infected the host plant over a prolonged period, host plant senescence and nutrition deteriorated significantly; consequently, virus-infected tobacco plants became relatively poor-quality hosts for aphids. We speculate that plants at late state of the virus infection exhibit enhanced dispersal of winged aphids, thereby promoting virus transmission.

The EPG method is widely used to monitor the feeding behavior of aphids [32], it allows the recording of signal waveforms corresponding to different probing activities as well as the position of the aphid stylet within plant tissues, which can provide valuable information on host acceptance and resistance mechanisms [33, 34]. We investigated the feeding behavior of aphids on tobacco plants with this technique; our results show that pd frequency on infected plants was increased in both early and steady states of the infection process. The most important stylet penetration parameter associated with enhanced acquisition of non-persistently transmitted viruses is the frequency of the pd waveform [35]. Aphids in this study showed a propensity towards PVY-infected plants, as evidenced by the higher number of intracellular punctures, which would ultimately increase virus transmission efficiency.

Virus-mediated changes in aphid feeding behavior via manipulation of plant olfactory and gustatory cues are a well-documented mechanism for enhancing acquisition and transmission efficiency [36]. At steady state of the virus infection, np events/transient and discontinuous stylet penetrations and intercellular pathway phase (C) were increased on PVY-infected tobacco plants, which is associated with host acceptance[25]. This indicates that the acceptance of PVY-infected plants by aphids was diminished; however, the effective feeding times of aphids on different host

plants which could impact aphid reproduction and development were similar. In addition, aphids on infected plants showed waveform F at a low frequency compared to healthy plants, indicating that PVY infection made penetration easier for aphids. In general, aphids preferred to probe on tobacco plants that were infected with PVY. At a late state of virus infection, there was no difference in stylet penetration frequency (pd/np) or phloem feeding behavior, probably because senescent tobacco plants regardless of infection status are unsuitable hosts for aphids.

In conclusion, the results of our study suggest that PVY-infected tobacco plants alter the host selection and feeding behavior of vector aphids in a stage-dependent manner. PVY-infected tobacco attracted more aphids at a steady state of the virus infection, leading to more intracellular punctures on the plants that would in turn enhance virus transmission efficiency. These findings not only provide insight into the interactions between host plants, virus, and insect vectors, but are important considerations when developing viral disease control strategies for crops.

Compliance with ethical standards

Funding This study was funded by the Agricultural Science and Technology Innovation Program (Grant No. ASTIP-TRIC04), and the Key Laboratory Project of China Tobacco Corporation (Grant No. 110201603011).

Conflicts of interest

The authors declare that they have no competing interests.

Ethical approval

No permit was required to study the virus infected plant.

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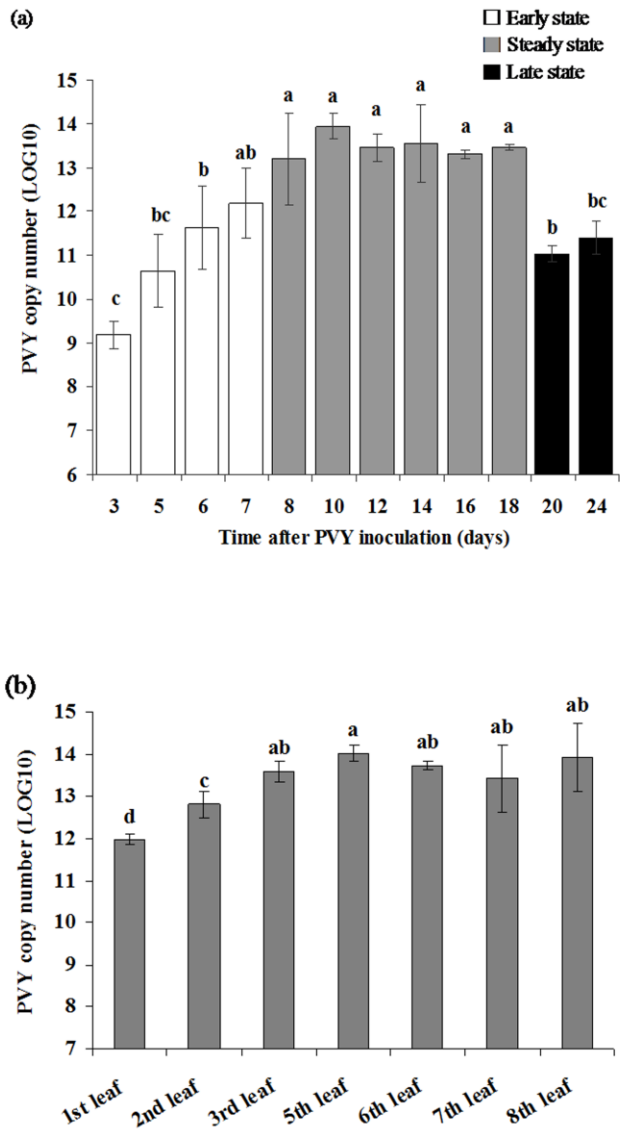

Figure 1. Viral quantification by TaqMan real-time PCR (One-way analysis of variance followed by the least significant difference test). **a** Viral particle content of tobacco plants at different times after inoculation with PVY. The 1st to 8th day after PVY infection was defined as early state; the 8th to 18th day after PVYinfection was defined as steady state; the time after 20 days of PVY infection was defined as late state. (F = 52.748; df = 6, 56; P < 0.0001) **b** Spatial pattern of changes in viral copy number in tobacco plants inoculated with PVY for 10 days. The first leaf is the youngest leaf. Values represent mean ± standard error (F = 7.819; df =11, 24; P < 0.0001).

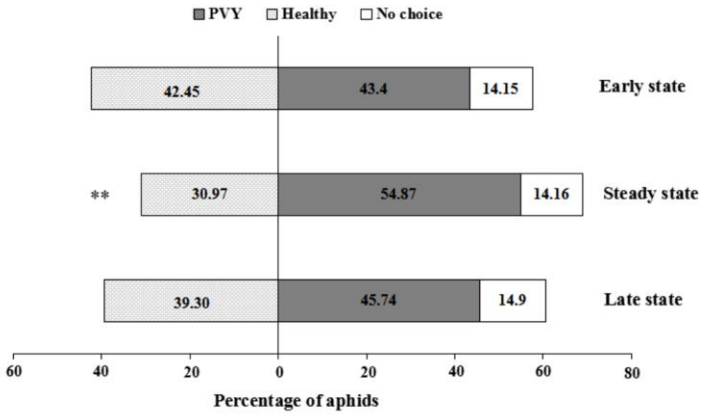

Figure 2. Preference of *M. persicae* for volatiles emitted by PVY-infected or non-infected tobacco plants. Different plants were used in this assay at different times post-inoculation. Early state: 5 days post-inoculation; steady state: 12 days post-inoculation; late state: 20 days post-inoculation. $^{**}P < 0.01$ (Student's *t* test).

Table 1 Stylet penetration behaviors of *M. persicae* on healthy and PVY-infected plants

EPG variable	Tobacco plants (early state)			Tobacco plants (steady state）			Tobacco plants (late state）		
	virus-infected (*n*=23)	Healthy (*n*=19)	*P*	virus-infected (*n*=16)	Healthy (*n*=20)	*P*	virus-infected (*n*=15)	Healthy (*n*=13)	*P*
1 Time to 1st probe (min)	4.61±3.75	9.31±7.99	0.242	0.48±0.07[a]	0.60±0.09[a]	0.295	9.92±8.33*	4.35±4.70	0.034
2 Number of pd	153.68±37.04**	127.20±40.64	0.001	174.00±35.74**	105.881±22.90	<0.001	137.60±36.78	109.77±38.20	0.061
3 Number of np	27.15±17.97	22.10±13.37	0.160	1.39±0.48[a]**	1.03±0.67[a]	<0.001	20.14±14.05	15.92±9.54	0.374
4 Total duration of np (min)	57.01±32.49	57.54±26.77	0.466	51.94±25.72	34.09±26.94	0.061	47.60±33.40	44.01±34.68	0.808
5 Total duration of np / Total record time (%)	15.00±9.04	16.17±7.70	0.519	14.43±7.13	9.57±7.41	0.465	13.22±9.29	12.33±9.60	0.828
6 Time to phloem from the start of EPG (min)	83.78±48.82	92.54±64.10	0.124	1.75±0.07[a]	1.71±0.07[a]	0.676	67.61±50.24	96.48±83.39	0.076
7 Number of pd during the time to phloem from the start of EPG	53.69±38.07*	39.20±31.49	0.022	51.60±18.80**	25.65±11.62	<0.001	31.21±19.57	30.64±16.24	0.934
8 Number of E1	22.14±13.33	19.73±12.10	0.552	20.81±13.41	27.88±13.94	0.148	21.14±11.90	17.69±11.70	0.365
9 Total duration of E1 (min)	64.13±38.05	64.63±31.91	0.869	1.79±0.42[a]	1.89±0.49[a]	0.137	66.16±32.79	62.77±40.81	0.813
10 Total duration of E1 / Total record time (%)	17.98±10.66	18.51±9.63	0.487	18.09±6.87	25.26±13.12*	0.018	18.69±8.89	17.94±10.97	0.814
11 Number of E2	18.00±12.60	16.05±11.37	0.771	15.81±14.35	23.29±14.09	0.141	18.08±9.46	14.92±12.81	0.819
12 Continuous duration of E2 > 10min (times)	2.00±1.72	1.79±1.81	0.769	0.22±0.07[a]	0.22±0.07[a]	0.982	0.92±1.19	0.92±1.12	0.482
13 Total duration of E2 (min)	77.55±66.58	75.57±54.7	0.810	60.42±47.47	74.97±39.12	0.343	54.98±37.87	40.95±41.61	0.394
14 Total duration of E2 / Total record time (%)	19.67±15.02	20.24±11.37	0.728	16.78±13.18	21.62±11.81	0.250	15.27±10.52	11.52±11.48	0.528
15 Total duration of E1+E2 / Total record time (%)	37.89±15.81	38.54±17.92	0.899	34.83±13.45	46.84±16.25*	0.028	34.97±9.42	31.21±17.75	0.482
16 % of aphids showing waveform F(F>10 min) (%) [b]	30.43	52.63*	0.012	43.75	59.10	0.139	40.00	69.23**	0.005
17 Total duration of C (min)	148.92±43.98	132.05±36.43	0.176	157.13±39.45*	116.93±41.77	0.049	143.62±32.17*	102.53±41.30	0.05
18 Total duration of C / Total record time (%)	41.61±12.31	37.42±10.59	0.529	43.57±11.01	32.90±11.02	0.334	39.79 ± 9.00	29.14 ± 10.77	0.269

Data in the table represent mean ± SE. For the same row values marked by " * " indicate significant differences ($0.01 < P \leqslant 0.05$); " ** " indicate highly significant differences ($p \leqslant 0.01$).

[a] The data were transformed by log10 (x+1)

[b] Chi-square test