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**Co-Transmission of *Pepper huasteco yellow vein virus* and
Pepper golden mosaic virus in Chili Pepper by *Bemisia tabaci* (Genn.)**

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Abstract: The capacity of simultaneously acquiring and transmitting *Pepper huasteco yellow vein virus* (PHYVV) and *Pepper golden mosaic virus* (PepGMV) geminiviruses by their whitefly vector (*Bemisia tabaci* Gennadius) to pepper plants *Capsicum annuum* was investigated. Information was obtained relating to the effect of factors involved in the virus-plant interaction such as whiteflies density by plant, Acquisition Access Periods (AAP), Inoculation Access Periods (IAP) and source of viral infected plants. Transmission trials indicated that a single virus-infested whitefly can transmit PHYVV and PepGMV to pepper plants. It was demonstrated that an AAP of 1 h was sufficient for acquisition of PHYVV and PepGMV by the vector. A minimum IAP of 48 h was required for the vector to transmit these geminiviruses to the plant. We also determined that one whitefly was capable of acquiring and transmitting both geminiviruses simultaneously. It appears that PHYVV helps in the dispersion of PepGMV inside the plant. Several possible explanations of our results are discussed.

Key words: Whitefly, geminivirus, vector transmission, pepper, virus-vector interaction

INTRODUCTION

The whitefly *Bemisia tabaci* Gennadius is a pest of many important crops in Mexico (Avila-Valdez and Hinojosa-Reyes, 2000). This species affects crops such as pepper, tomato, celery, sweet potato, spinach, bean, potato, tobacco, cassava, carrot, crucifers and cucurbits. Whiteflies belong to the order Hemiptera, which includes 1200 species of 140 genera. These insects affect crops and weeds in different ways either they are herbaceous or ligneous. The agricultural problems related to whiteflies have increased due to factors such as: the transportation of infected material with insects, changes in weather conditions, intensification of agricultural practices, indiscriminate use of insecticides

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and development of insecticide resistance (Cohen, 1990). The whitefly *B. tabaci* was first reported in Mexico in Southern Tamaulipas in 1986, associated with the pepper disease named rizado amarillo del chile (Brown *et al.*, 1989; Garzón-Tiznado *et al.*, 1989), in which geminiviruses were subsequently found to be involved (Garzón-Tiznado *et al.*, 1993; Garzón-Tiznado, 1998; Torres-Pacheco *et al.*, 1996). Geminiviruses belong to the Geminiviridae family. They have twined particle morphology and circular, single stranded DNA genome, with either monopartite or bipartite genomic organization (Hanley-Bowdoin *et al.*, 1999). The Geminiviridae include four genera (Mastrevirus, Curtovirus, Topocovirus and Begomovirus), based on genomic structure, host range and type of insect vector. Mastrevirus and Curtovirus have monopartite genomes and are transmitted by leafhoppers. The Mastreviruses infect monocots and Curtoviruses infect dicots. Topocovirus includes all variants of the *Tomato pseudo curly top virus* (TPCTV). Finally, Begomoviruses, containing a bipartite genome, are whitefly (*Bemisia tabaci*)-transmitted and infect dicotyledonous plants (Soto and Gilbertson, 2003).

The first reported geminivirus-associated disease of pepper crops was the rizado amarillo del chile caused by Begomoviruses including *Pepper huasteco virus* (PHV, now called *Pepper huasteco yellow vein virus* or PHYVV) and *Texas pepper virus* (TPV, now called *Pepper golden mosaic virus* or PepGMV), that were associated with the disease (Garzón-Tiznado *et al.*, 1993; Torres-Pacheco, 1997). In Mexico, pepper is the one of the most important horticultural crops. In different pepper producing regions, both PHYVV and PepGMV have been detected in mixed or single infections (Torres-Pacheco *et al.*, 1996; Vera-Aguado, 2000).

The rizado amarillo del chile was the first reported disease associated with mixed infections by geminiviruses and thus provide an interesting model to study acquisition and transmission of both viruses by *B. tabaci*. Some research has been carried out in México in order to understand the pepper-geminivirus interaction (Mendez-Lozano *et al.*, 2003). However, nothing is known about the process regarding the role of the vector in the acquisition and transmission of both geminiviruses (Hunter *et al.*, 1998). Some data suggests that the transmission of geminiviruses is carried out in a persistent and circulative manner. A period of latency of approximately 6 to 12 h was required before the transmission event of *Squash leaf curl virus* (SLCV) (Rosell *et al.*, 1999, 2003). It has been speculated that geminiviruses do not replicate inside the whitefly since there is no transovarial step. Although recent reports of transmission in the case of *Tomato yellow leaf curl virus* (TYLCV) in a population of *B. tabaci* suggested otherwise (Ghanim *et al.*, 1997). Knowledge about the biology of acquisition and transmission of geminiviruses by whiteflies is needed in order to improve the control of geminivirus diseases. The goal of this work was to determine the effect of some factors in the single and simultaneous PHYVV and PepGMV transmission by whiteflies.

MATERIALS AND METHODS

Establishment of a Whitefly (*B. tabaci* Genn.) Colony

A whitefly *B. tabaci* (biotype B) colony was established on Tobacco (*Nicotiana tabacum* cv. Xanthi) and was put into rearing boxes (60×35×30 cm). To ensure that whiteflies were virus-free, the insects were reared during five alternate generational steps using different host plants as following: potato, cotton, bean, tomato and finally tobacco. Detection of geminiviruses within the vectors and the host plants was carried out by Polymerase Chain Reaction (PCR) as reported previously (Ascencio-Ibañez *et al.*, 2002; Rojas *et al.*, 1993). This study was carried out between March 2006 and August 2007.

Source of Geminivirus-Infected Plants

For the studies of virus acquisition and transmission, pepper plants (*Capsicum annuum* c.v. Sonora Anaheim) were inoculated by a biolistic procedure (750 psi and gap distance of 2 cm) using a concentration of 2.5 µg of each viral DNA component (Anaya-López *et al.*, 2003). These viral

components were cloned in the plasmid Blue Script (SK +) and digested with Eco RI for A component of both PHYVV and PepGMV; Bam HI for PHYVV B component and Hind III for PepGMV B component prior to inoculation of the plants. Plants were incubated under greenhouse conditions and used in transmission trials of 15 days after inoculation (dai).

Virus-Vector Transmission Relationships

The whiteflies were placed individually in contact with the virus-source plants or host plants. During the Acquisition Access Periods (AAP) or Inoculation Access Period (IAP), the whiteflies remained inside a structure that consisted of a plastic container measuring 11 cm in diameter and 15 cm height. The insects were withdrawn once the corresponding process was complete. The plants were then incubated in a growth chamber for 5 days. Conditions within the growth chamber were maintained at a temperature of 24°C and relative humidity of 70% during the transmission experiments. Four replicates of five plants (twenty plants per treatment) were evaluated. The number of vectors varied only in the test trials. All of the other trials were carried out with one whitefly per plant. Experimental plants were arranged inside the growth chambers in completely randomized positions. To determine the minimum number of *B. tabaci* adults required to transmit PHYVV and PepGMV, whiteflies were allowed to feed on virus-infected plants for a 48 h Acquisition Access Period (AAP) and then allocated in groups of 1 and 2 vectors/plant for a 48 h Inoculation Access Period (IAP). The AAP required for the transmission of PHYVV and PepGMV was determined by feeding *B. tabaci* adults on infected plants for periods of 1, 3, 6, 12, 24 and 48 h. The tests were carried out with one whitefly by plant for a 48 h IAP. The IAP required for the transmission of PHYVV and PepGMV was determined by feeding *B. tabaci* adults on infected plants for 24 h AAP. The tests were carried out with one whitefly by healthy plant for a 12, 24 and 48 h IAP. Finally, in the mixed infection assays, the analysis consisted of five different kinds of virus source: (1) three plants inoculated only with PHYVV, (2) three plants inoculated only PepGMV, (3) three plants harbouring both geminiviruses, (4) three plants: one of them harbouring only PHYVV, the second harbouring only PepGMV and the third with the mixture and (5) two plants: One carrying only PHYVV and another with PepGMV only. The tests were carried out with one whitefly per plant.

Detection of PHYVV and PepGMV

Detection of PHYVV and PepGMV in the plant and in the insect was carried out using PCR with specific primers for each virus according to Anaya-López *et al.* (2003). The amplified DNA fragments were analyzed by electrophoresis in agarose gels (1%, 80 volts, 1 h). The measure of response in these trials was evaluated as the grade of the severity of the infection with the scale reported by Godínez-Hernández *et al.* (2001).

DNA Extractions

Plant DNA extractions were carried out according to Dellaporta *et al.* (1983) and for insect DNA extractions we utilized DNeasy Tissue kits (QIAGEN, Valencia C.A., USA). The whiteflies were collected from the plants and maintained at -70°C for subsequent individual DNA extraction. Disruption of the insect was carried out in an electric mixer with 180 µL of phosphates buffer pH 7.0 and then homogenized.

Statistical Analysis

Data were subjected to analysis of variance using the general linear models according to SAS methods. Statistical differences between treatments were analyzed by Tukey's method (Montgomery, 1991).

RESULTS

Detection of Virus-Free Whiteflies

The initial collection of adult whiteflies was carried out in a confined box containing 65-day-old potato plants. Thus, the whiteflies were then transferred successively to five plants each of the following crops: Bean, cotton, tomato and tobacco. Geminivirus detection was carried out in order to confirm the virus-free state of the whiteflies used in the transmission experiments. No geminivirus was detected in the first generation (Fig. 1). We did not detect geminiviruses in any of the successive host plants used in the different generations handled with the vector. In the case of potato, we detected geminivirus, but never within the vector.

Virus Transmission Studies

One or two whiteflies per plant were used to determine the minimum number of whiteflies to transmit PHYVV and PepGMV. The results suggested that one individual whitefly was sufficient to transmit geminiviruses and cause infection. In Fig. 2, the DNA amplification results indicated viral

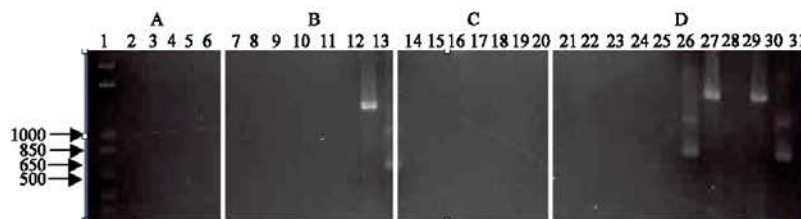


Fig. 1: Detection of Geminiviruses. Products of PCR obtained of extracts of DNA of Whitefly (WF) and of plants used through several generation transfers. Panel A, lane (1) Molecular marker 1 kb Leader, lanes (2-6) PCR of 1st-5th generation of WF with the primers of Rojas (≈ 1156 pb); Panel B, lanes (7-11) PCR of 1st-5th generation of WF with the primers of MOT-CP (≈ 650 pb); lane (12) PCR of the initial population of WF with the primers of Rojas; lane (13) PCR, of the initial population of WF with the primers of MOT-CP; Panel C, lanes (14-20) PCR using the primers of Rojas in plants used to produce the 1st one to the fifth generation of WF, bean, cotton, cotton, tomato and tobacco, respectively; Panel D, lanes (21-25) PCR using the primers of MOT-CP in plants used to produce the 1st one to the fifth generation of WF, bean, cotton, cotton, tomato and tobacco, respectively; lane 26 PCR with the primers MOT-CP of the potato plant of where was carried out the initial collection of WF, lane 27 PCR with the primers Rojas of the potato plant of where was carried out the initial collection of WF; lane (28) PCR with the primers of Rojas of a viral free pepper plant; lane (29) PCR with the primers of Rojas of viral source plant (positive control); lane (30) PCR with the primers of MOT-CP of a viral source plant (positive control) and lane (31) PCR with the primers of MOT-CP of a viral free pepper plant (negative control)

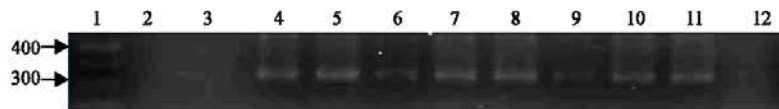


Fig. 2: Detection of PHYVV in plants infected with only one whitefly. PCR with the primers 240 and 241, lane (1) Molecular marker 1 kb Ladder; lane (2-3) Negative Control: not infective whitefly; lanes (4-7) plants inoculated with a single WF; lane (8-10) plants inoculated with two WF and lane (11) plant infected with PHYVV (positive control); lane (12) virus-free pepper plant (negative control)

Table 1: Effect of Acquisition Access Period (AAP) in the feeding on PHYVV-induced symptom severity and incidence of infection by a single whitefly

Acquisition Access Period (AAP) (h)	Symptom severity	Incidence*	PHYVV detection in plant**
48	4±0.30 ^a	18/20±1.0 ^a	Positive
24	3±0.17 ^{ab}	16/20±0.8 ^{ab}	Positive
12	3±0.19 ^{ab}	16/20±0.7 ^{ab}	Positive
6	3±0.27 ^{ab}	17/20±1.0 ^a	Positive
3	2±0.22 ^{bb}	18/20±0.7 ^a	Positive
1	2±0.17 ^{bb}	18/20±0.9 ^a	Positive

The values are medium±standard deviation; 4 repetitions by treatment (p>0.05), *: No. of the plants infected by treatment
 **: PHYVV detection was carried out using the primers 240 and 241 (1)

Table 2: Effect of the different Inoculation Access Periods (IAP) in the feeding on PHYVV-induced symptom severity and incidence of infection by a single whitefly

Inoculation Access Period (IAP) (h)	Symptom severity	Incidence*	PHYVV detection in plant**
48	4±0.02	18/20±0.89	Positive
24	0±0.00	0/20±0.00	Negative
12	0±0.00	0/20±0.00	Negative

The values are medium±standard deviation; 4 repetitions by treatment (p>0.05), *: No. of the plants infected by treatment
 **: PHYVV detection was carried out using the primers 240 and 241 (1)

Table 3: Effect of the different Acquisition Access Period (AAP) in the feeding on PepGMV-induced symptom severity and incidence of infection by a single whitefly

Acquisition Access Period (AAP) (h)	Symptom severity	Incidence*	PepGMV detection in plant**
48	7±0.06 ^a	19/20±a	Positive
24	6±0.09 ^{ab}	16/20±a	Positive
12	5±0.09 ^b	18/20±a	Positive
6	5±0.05 ^b	15/20±b	Positive
3	5±0.07 ^b	16/20±a	Positive
1	5±0.08 ^b	14/20±b	Positive

The values are medium±standard deviation; 4 repetitions by treatment (p>0.05), *: No. of the plants infected by treatment
 **: PepGMV detection was carried out using the primers JM23 and JM 24 (1)

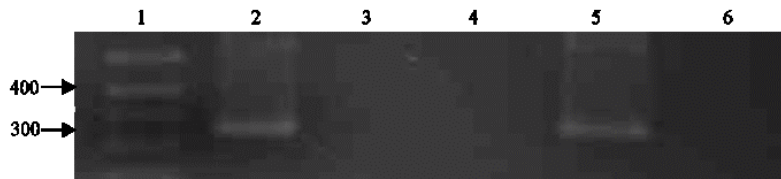


Fig. 3: Detection of PHYVV in plants exposed to different periods of transmission with the vector. Lane (1) Molecular marker 1 kb Ladder; transmission periods 2-4, (48, 24 and 12 h, respectively); 5, positive control and 6, negative control

presence when plants were inoculated with one virus-infested whitefly. The AAP required for the transmission of PHYVV indicated that 1 h was sufficient time for ingestion and PHYVV acquisition (Table 1). The longer the period of acquisition, the greater the severity of the disease. A lower incidence was observed for 12 and 24 h acquisition than for the rest of the trials.

Approximately 48 h was required for transmitting PHYVV after acquisition (Fig. 3). Inoculated plants had no viral symptoms and the virus was not detected in plants with 12 or 24 h of IAP (Table 2, Fig. 3). As in the case of PHYVV, PepGMV could also be ingested and acquired by the whitefly even in 1 h AAP. The previous result is inferred from the presence of the symptoms and the virus in the plant (Table 3). The longer the AAP, the greater the severity. Disease severity was greatest when the plants were inoculated with PepGMV. As for PHYVV, PepGMV could be transmitted after 48 h of IAP.

Table 4: Effect of different types of inoculum source plants on incidence* of mixed infection by whitefly

Treatments	PHYVV in plant	PepGMV in plant	PHYVV in whitefly	PepGMV in whitefly
1PHV (control)	18/20±2.34 ^a	0/20±0.00	18/20±1.56	0/20±0.00
1PepGMV (control)	0/20±0.00 ^c	17/20±1.67	0/20±0.00	20/20±0.00
3 PHV-PepGMV	19/20±2.17 ^a	18/20±1.78	19/20±2.24	19/20±1.00
1 PHV+1 PHV-PepGMV+1 PepGMV	12/20±1.98 ^b	0/20±0.05	20/20±0.00	11/20±1.28
1PHV+1 PepGMV,	13/20±2.10 ^b	2/20±0.07	20/20±0.00	4/20±2.10

The values are medium±standard deviation; 4 repetitions by treatment (p>0.05), *: No. of the plants infected by treatment
 **: PHYVV and PepGMV detection was carried out using the primers240 and 241 and JM23 and JM24, respectively (1)

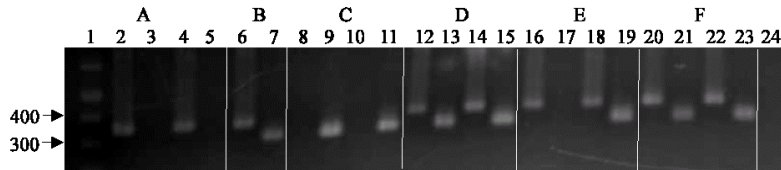


Fig. 4: Detection of PHYVV and PepGMV within plant and vector with mixed infections. Even lanes PHYVV and odd lanes PepGMV, the minor number in each Panel corresponds to the detection in the plant and the major number corresponds to the vector. Lane 1, Molecular marker 1 kb Ladder. Panel A: Inoculation with PHYVV; Panel B: lane 6 positive control of PHYVV and lane 7 positive control of PepGMV; Panel C: inoculation with PepGMV; Panel D: inoculated plants with PHYVV plus PepGMV; Panel E: treatment including inoculated plants as following: 1 plant with PHYVV, 1 plant with PHYVV plus PepGMV and 1 plants with PepGMV; Panel F: treatment with two inoculated plants: 1 with PepGMV and other with PHYVV. Lane 24: negative control. Size of DNA amplified is PHYVV: 350 pb and PepGMV 288 pb

The goal of mixed infection assays was to determine if one whitefly was able to simultaneously ingest, acquire and transmit to PHYVV and PepGMV. The results suggest that simultaneous transmission was possible (Table 4, treatments 3 and 5; Fig. 4, panel D and F). In all cases, simultaneous infection by both geminiviruses was detected in the plants and vectors. In the case of treatments 4 and 5, it was observed that in the half of the vectors the presence of PepGMV was detected; however, it was not transmitted to any plant. In treatment 3, the same plant contained both viruses and both viruses were ingested, acquired and then transmitted in similar proportions. Whereas, in treatment 4 there was only one plant with both viruses and it was not able to infect any plants. Additionally, the virus was acquired by 50% of the vectors. In treatment 5, the proportion of insects was reduced even more than was detected in PepGMV. In this latter treatment there were no plants containing both geminiviruses.

DISCUSSION

The fact that no viruses were detected in the diverse host plants used from the first generation suggests that there was no transovarial transference of these geminiviruses. We found a single virulent whitefly was capable of causing the infection in the PHYVV-*C. annuum* c.v. Sonora Anaheim interaction. We cannot generalize this latter assertion because PHYVV differs from other geminiviruses in mechanic transmissibility (Garzón-Tiznado *et al.*, 1993) and perhaps it has developed an improved transmission mechanism mediated by the vector. The efficiency of geminivirus transmission has been previously illustrated in the whitefly-tomato-*Tomato yellow leaf curl virus* (TYLCV) interaction (Cubillo *et al.*, 1999). This data can be important for the management of the diseases that PHYVV causes in pepper crops. Previously, it had been reported that in tomato 0.3 geminivirus-infested whiteflies was sufficient to cause infection (Cubillo *et al.*, 1999). Present results suggested that a single vector is enough to cause the infection.

We observed that disease severity increased when the AAP was longer. This may be explained by longer acquisition times by the vector. A greater level of disease severity was observed here than previously reported (Anaya-López *et al.*, 2003). The fact that these viruses cannot be transmitted immediately supports the observation that PHYVV and PepGMV are persistent as other geminiviruses (Cohen *et al.*, 1983; Harrison, 1985). They are incorporated into the circulatory system of the insect where, perhaps, the virus undergoes some form of processing before it can be transmitted to the host plant.

The AAP treatments evaluated here contained either just one or both types of viruses in the source host plant with similar results observed for PHYVV and PepGMV. Nevertheless, when two host plants were used in the treatment, each one with only one virus reduced the efficiency of acquisition and transmission of PepGMV. It appears that preference in acquisition and transmission is given to PHYVV in pepper plants. Perhaps this is due to a greater affinity between PHYVV and pepper than PepGMV and the same host plant. Several reports indicated that in pepper, PHYVV was able to complement the functions of B component in PepGMV, but not vice versa (Torres-Pacheco, 1997; Mendez-Lozano *et al.*, 2003). Since the B component of the geminiviruses contributes to functions related to movement (Lazarowitz, 1992; Von Arnim and Stanley, 1992), it is conceivable that this component permits the greater ability of movement of PHYVV inside pepper plants than in the case of PepGMV. In fact, the vector injects the geminiviruses within the phloem when feeding (Cohen *et al.*, 1998; Freeman *et al.*, 2000). Thus, it is likely that a greater capacity of movement of PHYVV can also be observed in phloem tissue. According to our results, there is most likely a greater availability of factors that could interact with the movement of PHYVV in the different parts of the pepper plant. Thus, it could be explained, not only that it can be transmitted easier, but also that PHYVV could be acquired easier than PepGMV by the vector in pepper plants. In this manner, the mixed infection of PHYVV and PepGMV improves the acquisition and transmission of PepGMV and consequently, the dispersal of this virus in pepper crops and nature. Additionally, the coat protein of geminiviruses must play an important role in the transmission of both PHYVV and PepGMV as reported in other geminiviruses (Brown *et al.*, 1989; Cohen *et al.*, 1983). This study is an initial effort to understand the biology in the mixed transmission of geminiviruses by whiteflies in nature.

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