

**Palestinian Isolate of Tomato Yellow Leaf Curl Virus: Capsid and Nucleic Acid Retention in *Bemisia tabaci*, Transmission, and Field Study of Virus Association with the Vector and Non-Vector Insects**

دراسة العزلة الفلسطينية لفيرس تجعد واصفرار اوراق البندورة من حيث انتقالها وديمومة احتفاظ الذبابة البيضاء بالغلاف البروتيني والحامض النووي لها واجراء دراسة حقلية حول اتحاد الفيروس بالحشرات الناقلة وغير الناقلة

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**I- Abstract**

Studying the retention of nucleic acid of the Palestinian isolate of the tomato yellow leaf curl virus in the whitefly vector, *Bemisia tabaci*, by using the polymerase chain reaction (PCR) showed, on one hand, that the viral DNA was retained for at least 24 days after the 48-hours of acquisition access on TYLCV-infected tomato plants. On the other hand, enzyme-linked immunosorbent assays (ELISA) revealed that the virus's antigen persisted for only eleven days, which is much shorter than the retention of the virus's genome. This feature proved that there is a close relationship between the capsid's retention in the whitefly vector and the virus's transmissibility which remained for eleven days. In addition, *In Vitro* studies proved that the non-vector insects including whitefly,

*Trialiorodes vaporariorum*, and aphids, *Myzus persicae*, *Aphis gossypii* and *Macrosiphum pisi* can also acquire both the viral DNA and the capsid, when given 48-hour acquisition access on infected tomato. Furthermore, studying the viruliferous *B. tabaci* collected from tomato fields in Al-Far'a region revealed high association of both the viral DNA and the capsid compared with those collected from eggplants near tomato fields which showed low association. This research also proved that the above mentioned non-vector insects pointed out above have the ability to acquire the virus under field conditions with various rates. Therefore, the maximum acquisition of the viral DNA recorded in *T. vaporariorum* collected from Al-Far'a region was found to be 50%.

**Key words:** Tomato yellow leaf curl virus, *Bemisia tabaci*, TYLCV-genome, TYLCV-capsid

## II- الملخص

عند استخدام فحص البوليميريز لدراسة فترة بقاء الحامض النووي للعزلة الفلسطينية لفيروس تجعد واصفرار أوراق البندورة داخل الذبابة البيضاء، تبين أن هذا الحامض يبقى عالقا لمدة تصل الى ٢٤ يوما بسبب تغذي الذبابة لمدة ٤٨ ساعة على نبات البندورة المصاب بالفيروس. كذلك تبين من خلال فحص الاليزا أن الغلاف البروتيني للفيروس يتم الاحتفاظ به داخل الحشرة فقط لمدة ١١ يوما، الأمر الذي يبين وجود علاقة وثيقة بين فترة احتفاظ الحشرة بالغلاف البروتيني للفيروس وقدرتها على نقل الفيروس للنبات السليم والتي تستمر لمدة ١١ يوما. اضافة لذلك بينت الفحوصات نفسها أن ذبابة البيوت الزجاجية *(Trialiorodes vaporariorum)* ومن الدراق الأخضر *(Myzus persicae)* ومن البطيخ *(Aphis gossypii)* ومن البازلاء *(Macrosiphum pisi)* تستطيع اكتساب كل من الحامض النووي والغلاف البروتيني للفيروس بعد تغذيتها على النبات المصاب لمدة ٤٨ ساعة مع العلم أن هذه الحشرة هي غير ناقلة لفيروس تجعد واصفرار أوراق البندورة. ومن ناحية

أخرى، أثبتت الدراسة الحقلية أن الذبابة البيضاء التي جمعت من حقول البندورة المزروعة بمنطقة الفارعة، كانت تحمل الحامض النووي والغلاف البروتيني للفيروس بنسب عالية وصلت إلى ٩٥% للحامض النووي و ٩٠% للغلاف البروتيني. إضافة لما سبق أثبتت الدراسة أيضا أن الحشرات غير الناقلة لهذا الفيروس والسابق ذكرها تستطيع أن تكتسب الفيروس تحت ظروف الحقل وبنسب متفاوتة وصل أعلاها إلى ٥٠% في ذبابة البيوت الزجاجية.

### III- Introduction

Tomato yellow leaf curl virus (TYLCV) is the most devastating viral disease affecting tomato (*Lycopersicon esculentum* Mill) cultivation in several Mediterranean countries (Pico *et. al.*, 1996, p. 151-196, Czosnek *et. al.*, 1990, p. 1-6). The virus is considered to be the most serious viral disease complex of cultivated tomato crops in tropical and warm temperate regions of the world, where losses up to 100% are incurred (Moriones *et. al.* 2000, p. 123–134, Czosnek *et. al.*, 2001, p. 291–322). TYLCV was first reported in Israel in the 1960s (Pico *et. al.* 1996, p. 291–322). In Palestinian territories, Sawalha (2009a) studied the virus occurrence on solanaceous hosts and the distribution of the virus natural reservoirs in summer season in North West bank. In addition, the virus incidence in tomato fields grown in the northern regions of west bank was also studied (Sawalha 2009c). In Jordan, the disease is responsible for severe crop damage, especially for tomato grown during the fall seasons in the Jordan Valley (Al-Musa, 1982, p. 361-363).

Weakening of the whole tomato plant, flower and fruit abscission caused by the viral infection result in serious yield losses. These losses are particularly severe when the infection occurs before flowering. Since

fruits often do not attain the appropriate commercial size, size reduction of fruits may also cause a decrease in quality (Pico *et. al.*, 1996, p. 291–322).

The disease, which is caused by a monopartite Gemini virus (Fauquet *et. al.* 2003, p. 405-421, Czosnek *et. al.* 1988, p. 1-6) is readily transmitted by a whitefly vector, *Bemisia tabaci* (Genn) (Kashina *et al.*, 2003, p. 188–199, Brown and Czosnek, 2002, p. 65–100). The virus is transmitted by the vector in a persistent and circulative manner by which the vector remains inoculative for several days after it acquires the virus (Pico *et. al.* 1996, p. 291–322, Oetting and Yunis, 2004, p. 68-69). The virus is the first reported whitefly-transmitted Gemini-virus possessing a single genomic component, but it is unusually heterogeneous and some isolates have two genomic components (Rochester *et. al.*, 1994, p. 477-485).

Since TYLCV is a very serious disease affecting tomato cultivation in Palestine (Sawalha, unpublished data), this research describes the relationship between the Palestinian isolate of TYLCV with its vector, *B. tabaci*, and provides additional information in this regard by studying the retention of both genomic DNA and the capsid of the virus in the vector under lab and field conditions using the methods of polymerase chain reaction (PCR) and the enzyme-linked immunosorbent assays (ELISA), respectively. In addition, this research also demonstrates the possibility of virus acquisition by a non-vector whitefly, *Trialeurodes vaporariorum* and aphids including *Muzus persicae* Sulzer, *Aphis gossypii* Glover and *Macrosiphum pisi* Kalt. under lab and field conditions. In addition, the

likelihood and feasibility of using both PCR and ELISA tests for monitoring the viruliferous whiteflies under field conditions is also evaluated.

#### **IV- Materials and Methods**

##### **Virus Source**

Samples of young stems were collected from tomato plants showing TYLCV symptoms of yellowing and upward leaf curling in Al-Far'a region (Tobas district). Scions obtained from the collected samples were cleft grafted onto rootstocks of healthy tomato plants during the 3<sup>rd</sup> or 4<sup>th</sup> true leaf stage. The grafts were then held firm with plastic clips (Marsic and Osvald, 2004, p. 243-249). The inoculated plants were grown in glasshouse under insect-free conditions. The virus was identified based on virus transmissibility, host range, PCR and serological tests as discussed below.

##### **Collection and Rearing of *B. Tabaci***

Adult whiteflies, *B. tabaci*, were collected from eggplant fields grown in Al-Far'a region. The colonies of the insects were reared in muslin-covered woody cages containing TYLCV-immune plants including eggplants, cauliflower and pumpkin. The cages were housed in an insectary glasshouse at a temperature of about 29 °C (Jetter *et. al.* 2001, p. 1-60, Gerling, 1990, p. 57-112). The first generation of the insects was obtained by transferring the pupae into other cages containing the TYLCV-non-hosts as mentioned above. Whitefly rearing

on TYLCV-immune plants continued for 25 days (Gerling, 1990, p. 57-112).

#### **TYLCV Acquisition by *B. Tabaci***

The adult whiteflies that emerged during the same 48 hours were collected, and random samples were checked for TYLCV using both ELISA and PCR as mentioned below. After assuring the nonviruliferous case of the whiteflies, they were caged with TYLCV-infected tomato plants. The insects were then given a 48-hour acquisition feeding period then transferred and reared on pumpkin plants for the rest of their lives.

#### ***B. Tabaci* Transmission of TYLCV**

Forty viruliferous whitefly samples were transferred daily from pumpkin plants into cages of healthy tomato plants and were given a 48-hour inoculation feeding period before they were sprayed with an insecticide. The symptoms of TYLCV were recorded and then the plants were tested by PCR for virus infection as discussed below.

### **Sampling of Viruliferous *B. Tabaci* for Lab Testing**

Similarly, samples of thirty insects were collected daily from pumpkin plants as described above and stored at  $-20^{\circ}\text{C}$  until the end of the experiments. Five insects from the collected samples were kept in 0.5 ml eppendorf tubes for DNA analysis while the rest were put in Falcon tubes for capsid analysis.

### **Retention of the Viral DNA in *B. Tabaci***

PCR-based method was employed to check the presence of the viral DNA genome in the insects. The procedure of the PCR-based method was conducted as described by Navot *et. al.* (1992, p. 1199-1202), Sawalha (2009b, p. 28) and Tortora *et. al.* (2002, p. 254-255). The sequence of TYLCV specific primers (from 5' to 3') were P1V, ATACTTGGACACCTAATGGC, nucleotides (nt) 61-80, and P4C, TGGACATCT AGACCTAAG, nt. 2054-2071. The sequence of primer P1V corresponds to the viron positive strand whereas primer P4C is complementary to the viron strand. The reaction was run for 35 cycles using the PTC-100 Peltier thermal cycler. The amplified PCR products were electrophoresed for 90 minutes and then photographed.

### **Retention of the Viral Capsid in *B. Tabaci***

The triple antibody enzyme linked Immunosorbent assay (TAS-ELISA) was carried out as described by Sawalha *et. al.* (2000, p. 339) and Pico *et. al.* (1999, p. 1006-1012). The whiteflies were extracted at a proportion of 1/10 (w/v) as suggested by Adgen Ltd (Scotland UK)

through personal communication. The antibodies were cross-absorbed overnight with acetone-washed non-viruliferous whiteflies.

In addition, each ELISA plate contains control samples as follows: six negative control samples of extract obtained from whiteflies reared on healthy pumpkin plants for at least two generations, four positive control samples of extracts obtained from whiteflies fed for 48 hours on TYLCV-infected tomato plants, and three samples of the buffer used in whitefly extraction.

#### **Non-Specific Acquisition of TYLCV by Non-Vector Insects**

Adult whiteflies of *T. vaporariorum* and aphids of *M. persicae*, *A. gossypii* and *M. pisi* were collected from cucumber, squash and eggplant fields in Al-Far'a region. The whiteflies were identified according to Gerling (1990, p. 57-112) then reared on pumpkin for several generations. Similarly, the aphids were identified according to Oetting and Yunis (2004, p. 68-69) and reared on cucumber, eggplants, pepper and squash for several generations. Adult whiteflies and aphids were selected randomly and given 48 hour acquisition access on TYLCV-infected tomato, then removed and stored at -20 °C for laboratory analysis. Both PCR and ELISA tests were used as shown above to check the acquisition of the viral DNA genome and capsid respectively.

#### **Detection of Viral DNA and Capsid from *B. Tabaci in Vivo***

Samples of *B. tabaci* were collected in the middle of July 2003 from tomato growing sites in Al-Far'a region. The samples were collected

using aspirator as follows: 100 samples from tomato fields, 100 samples from natural flora beside tomato fields, and 100 samples from eggplant fields near tomato fields. The samples were then transferred to the lab and analyzed as indicated above for the viral DNA and capsid using PCR and ELISA respectively.

### **Detection of Viral DNA and Capsid from Non-Vector Insects *in Vivo***

Insect samples were collected from natural flora growing beside tomato fields in Al-Far'a region using a handheld vacuum insect sucker (vacuum sampling). The samples were identified, and 100 samples were selected randomly from each type of *T. vaporariorum*, *M. persicae*, *A. gossypii* and *M. pisi*. The samples were analyzed for the DNA of the virus using PCR as pointed above.

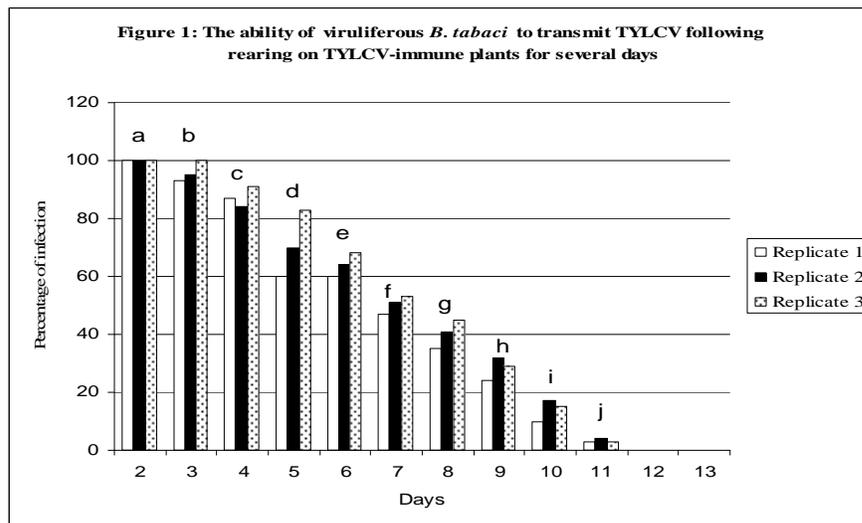
### **Statistical Analysis**

Analysis of the data was conducted using the Two-Sample Tests of Proportions according to Lind *et. al.* (2005, p. 262-263). The results were analysed using a level of significance when  $\alpha = 0.05$ . Statistical analysis was done based on Dr. Elias Dabeet's recommendations, Department of Mathematics & Statistics, Faculty of Arts & Sciences, Arab American University of Jenin.

## V- Results

### B. *Tabaci* Transmission of TYLCV

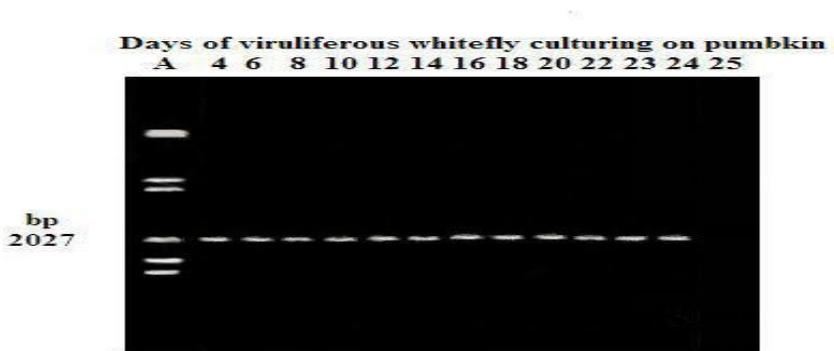
Observing the symptoms of TYLCV on tomato plants showed that the whiteflies were able to transmit the virus for 11 days after they were given a 48 hour access feeding period on infected tomato plants. Infected plants are severely stunted. Shoots become erect. Leaflets are reduced in size, curl upwards and become distorted. Flowers wither, and plants set very few fruit. Continuous reduction in the transmission efficiency was achieved as time progressed until the minimum value 11 days after virus acquisition (Fig. 1)



\* Values marked with the same letter do not differ significantly according to Two-Sample Tests of Proportions ( $\alpha = 0.05$ )

### Retention of the Viral DNA in *B. Tabaci*

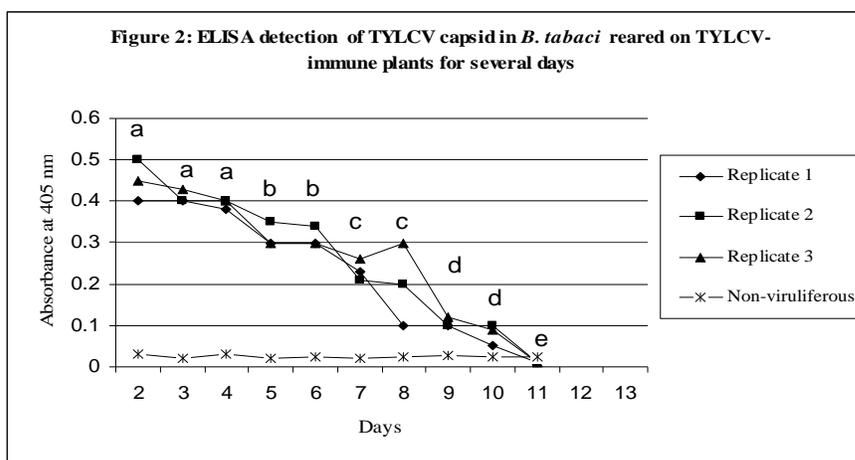
The retention of the viral DNA in *Bemisia tabaci* was investigated for at least four weeks. Therefore, it was concluded that the viral DNA was readily detected in the whitefly for up to at least 24 days after 48 hours of acquisition access. PCR signals were strong as appeared on electrophoresis gel starting from the first day after removal from the virus source until collecting the last sample 24 days later. No DNA amplification occurred when PCR was conducted for the nucleic acid extract from the whiteflies reared on healthy pumpkin plants. The fragment size of the amplified PCR product was typical to that obtained by TYLCV amplification (Plate 1).



**Plate (1):** Gel electrophoreses of the PCR products obtained by amplification of TYLCV DNA from *B. tabaci* cultured on pumpkin plants. Samples were taken from the culture at different periods. Lane A: *Lambda Hind III* DNA size marker. Lanes 4-25: Days of viruliferous whitefly culturing on pumpkin. Primers are P1V/P4C.

### Retention of the Viral Capsid in *B. Tabaci*

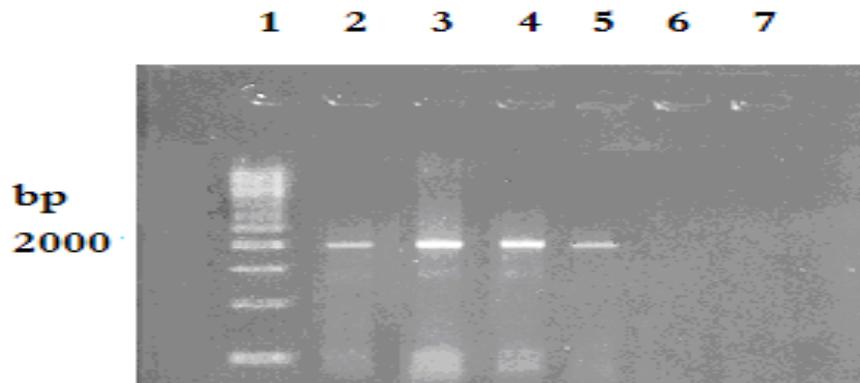
After conducting the ELISA test and investigating the viral capsid's retention of TYLCV, the results showed that the capsid of TYLCV was detected two days later in the viruliferous whiteflies reared on pumpkin after removal from the virus source. In addition, quantitative measuring of ELISA plates at 405nm revealed that the amount of virus antigen decreases continually by time as the insect feeds on TYLCV immune plants. Therefore, the ELISA readings achieved the maximum value two days after removing the insect from the virus source, then the reading values started decreasing until the eleventh day when they became very close to the reading values obtained by the non-viruliferous whiteflies (Fig:2).



\* Values marked with the same letter do not differ significantly according to Two-Sample Tests of Proportions ( $\alpha = 0.05$ ).

### Non-Specific Acquisition of TYLCV by Non-Vector Insects

The results of the PCR-based method proved that the adult *T. vaporariorum* and the adult aphids including *M. persicae*, *A. gossypii* and *M. pisi* could acquire the viral DNA non-specifically since the extracts of these insects contained DNA that annealed with TYLCV-specific primers. Also, the number of DNA bands formed in gel electrophoreses elucidated that such insects acquire the virus in a high rate hence most of the tested insects were found carriers for the viral DNA genome (Plate 2).

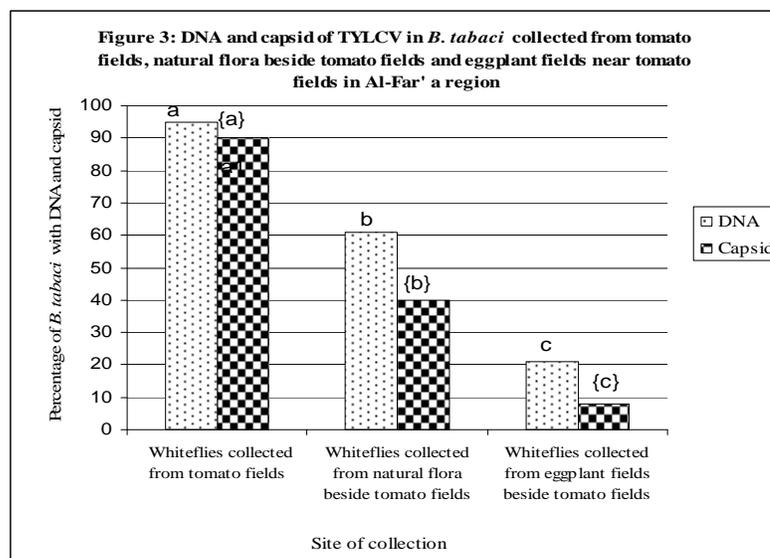


**Plate (2):** Gel electrophoreses of PCR amplified TYLCV genome from non-vector insects fed on TYLCV-infected tomato plants (lanes 2-5). Lane 2: *A. gossypii*, lane: 3: *T. vaporariorum*, lane 4: *M. persicae*, lane 5: *M. pisi*. Lanes 6 & 7: Insect mixture fed on TYLCV-free tomato plants. Lane 1: 500 base DNA size marker. Primers are P1V/P4C.

Furthermore, serological assays revealed that these insects could also acquire the virus antigen after the 48 hour acquisition feeding period on the virus source plants as indicated by ELISA.

### Detection of Viral DNA and Capsid from *B. Tabaci* in Vivo

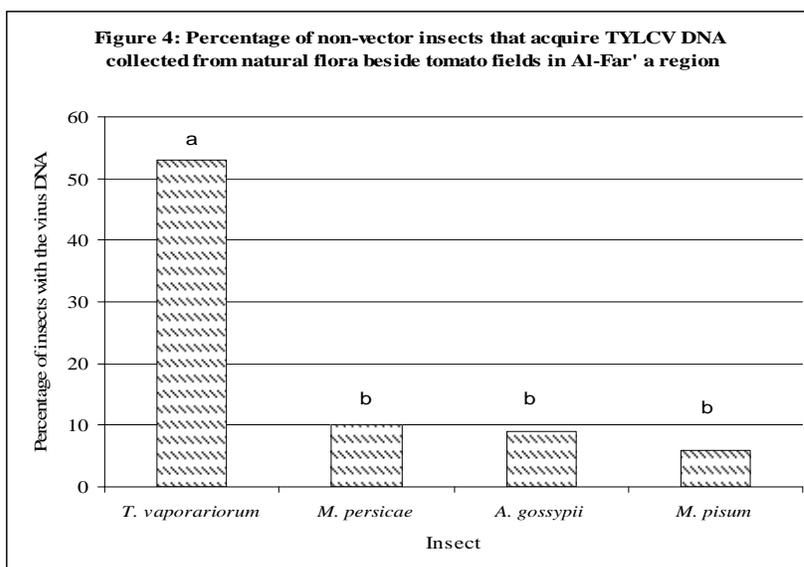
PCR and ELISA showed that the maximum viral occurrence occurs in the whiteflies collected from tomato fields followed by whiteflies collected from natural flora beside tomato fields, and finally the whiteflies collected from eggplants near tomato fields. The viral DNA and capsid occurrence collected from tomato fields, natural flora, and eggplants was found to be 95/90, 61/40 and 20/8 respectively. The occurrence of viral DNA was greater than the occurrence of capsid for all samples (Fig 3).



- \* Values marked with the same letter do not differ significantly according to Two-Sample Tests of Proportions ( $\alpha = 0.05$ ). DNA columns were compared with each other and the same was done for the capsid columns.

### Detection of Viral DNA and Capsid from Non-Vector insects *in Vivo*

The PCR tests revealed that the viral DNA associated with all insect samples collected from natural flora beside tomato fields in Al-Far'a region. The maximum occurrence of DNA was obtained from *T. vaporariorum* (53%) followed by *M. persicae* (10%), *A. gossypii* (9%) and *M. pisi* (6%) (Fig 4).



\* Values marked with the same letter do not differ significantly according to Two-Sample Tests of Proportions ( $\alpha = 0.05$ ).

## VI- Discussion

This research, based on the study of the retention of the capsid and nucleic acid of the Palestinian isolate of TYLCV in the *B. tabaci*, revealed that the virus's nucleic acid persisted much longer than that of the virus's capsid. Hence, the complete cessation of the virus capsid in the whitefly vector was achieved eleven days after insect removal from the virus source, whereas the retention of the nucleic acid wasn't achieved until at least 24 days later. In addition, the results of whitefly transmissibility showed that the whitefly vector, *B. tabaci*, can inoculate TYLCV until twelve days after they were given the 24 hour acquisition access on the virus infected plants. Therefore, the results suggest a close association between the retention of the virus capsid in the whitefly vector and its ability to transmit and inoculate the virus to assay plants. Consequently, Rubinstein and Czosnek (1997, p. 2683–2689) reported that the viral nucleic acid of the Israeli isolate is retained in the vectors during their entire adult life after 48 hours acquisition access which is far beyond the ability of the insect to disperse the virus. In the same country, Pico *et. al.* (1996, p. 151-196) reported that the whiteflies can stay inoculative for 20 days after 48 hours acquisition but the virus is not retained through the life span of the insect. With regard to other countries, similar results were obtained by other researchers. In Jordan, Mansour and Al-Musa (1992, p. 122-125) reported that the whiteflies stay inoculative for the Jordanian isolate of TYLCV for 11 days after 48 hours acquisition feeding period on virus-infected plants. On the other hand, Caciagli *et. al.* (1995, p. 163-170) found that the Sardinian isolate of the same virus persists in the whitefly vector for just 8 days after

similar access feeding period. The above results showed that the virus-vector relationships of the Palestinian isolate differ among TYLCV isolates from Israel, Jordan and Sardinia. These differences could reflect the adaptation of the different TYLCV isolates to transmission by different whitefly populations in different geographical origins. Therefore, our finding proved that the Palestinian isolate of TYLCV has its own characteristics that differ from the Jordanian and Israeli isolates.

Similar results were reported by other researchers who studied other Gemini viruses. For example, Polston *et. al.* (1990, p. 850-856) reported that the correlation between the transmission ability of *B. tabaci* for the squash leaf curl Gemini virus (SLCV) and the detection of the virus antigen was better than the correlation between the virus transmission and the detection of the virus nucleic acid.

The difficulty to detect the virus capsid in the vector beyond eleven days after its removal from the virus source may be due to the attachment of the virus to the membrane of salivary glands of the insect which causes a blockage to part of the capsid that therefore lowers the possibility for binding with the specific antibodies (Cohen *et. al.*, 1989, p. 109-113).

The field studies showed that the occurrence of the viral DNA in *B. tabaci* is much greater than occurrence of the capsid. This may be explained according to our conclusion that the whiteflies do not lose the DNA; they rather retain it for long time and possibly even throughout their lifetime. The lower occurrence of the viral capsid compared with

the DNA was attributed to the gradual loss of the capsid by time as our results proved that the whiteflies did not retain it for more than 11 days.

The occurrence of both DNA and capsid in whiteflies collected from tomato fields may be attributed to high amounts of infected plants that provide ample viral source for the whiteflies. The lower occurrence of DNA and capsid in whiteflies collected from natural flora and eggplants was attributed to viral sources which became reduced as the whiteflies moved away from tomato fields to the nearby sites or even to the fields of the virus-immune plants as eggplants.

The results showed that the non-vector whiteflies, *T. vaporariorum*, and aphids, *M. persicae*, *A. gosypii* and *M. pisi* could also acquire both the DNA and the capsid of the virus after they were given a 48 hour acquisition access period. Similarly, Cohen *et. al.* (1989, p. 109-113) found that the *T. abutilouea* could acquire the antigen of the SLCV non-specifically at a higher rate compared to its vector *B. tabaci*. In addition, Polston *et. al.* (1990, p. 850-856) reported that the same virus (SLCV) could be acquired by the *T. vaporariorum*, which is a non-vector for the virus.

The high occurrence of viral DNA in non-vector insects feeding on natural flora beside tomato fields emphasized our results by proving their ability to acquire the virus nonspecifically. The maximum occurrence of viral DNA in *T. vaporariorum* compared with the other insects may be attributed to the activity of these whiteflies during the period of collection which enabled them to visit tomato fields regularly. The low

DNA acquisition by the tested aphid species was attributed to low flight activity of these insects.

Furthermore, the previously stated results indicate that PCR-based techniques could not be employed solely in studying the epidemiology of TYLCV by detecting the rate of inoculative vectors in the whitefly population because inoculativity remained for fewer days than the persistence of the DNA in the vector. Also, *T. vaporariorum*, which is a non-TYLCV vector, can acquire the virus non-specifically, and therefore hampers the epidemiological studies which depend on monitoring the virus in the whitefly vector. However, *T. vaporariorum*, *M. persicae*, *A. gossypii* and *M. pisi* that are common in the area of TYLCV can be used by epidemiologists and phyto-pathologists as indicator insects for the presence of TYLCV in the region.

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