MOLECULAR DIAGNOSIS AND DETERMINE EFFECT OF CULTIVARS, DATES OF PLANTING AND GEOGRAPHIC LOCATIONS IN PERCENTAGE AND SEVERITY INFECTION OF *BIGEMINIVIRIDAE TOMATO YELLOW LEAF CURL VIRUS* (*TYLCV*) ON TOMATO IN FIVE DESERT AREAS OF IRAQ.

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Key words: molecular diagnosis, geographic, *bigeminiviridae tomato yellow leaf curl virus*, locations, infection

ABSRACT:

A survey of *Bigeminiviridae Tomato yellow leaf curl* virus (TYLCV) in five desert areas of Iraq (Basra, Kut, Najaf, Baghdad and Saladin) to molecularly diagnoses and determine the proportion and severity of the virus on common and hybrid tomato varieties, as well as dates of appearance of the virus. *Bigeminiviridae Tomato yellow leaf curl* virus (TYLCV) was molecularly diagnosed for the first time in Saladin by Polymerase Chain Reaction (PCR), as well as the symptoms caused by virus on host and transport by *Bemisia tabaci* . the result showed the efficiency of molecular diagnosis in very precise detection of the virus ,where a virus appeared for each primer used in molecular diagnosis .Two primers were used in the diagnosis of the virus of different sizes which indicates the presence of virus in Plants taken from fields and artificial infection plants . The highest increase in percentage and severity of infection in province of Basra, which amounted to 96, 4 and 30,3 for normal and hybrid varieties at beginning of August respectively, and amounted to 4,55 and 5,2 at beginning of October respectively, and was lowest in the province of Saladin . The study showed that the date of cultivation at beginning of August was highest in proportion and severity of injury compared to second date of cultivation at beginning of October.

التشخيص الجزيئي وتحديد تأثير الأصناف ومواعيد ومواقع الزراعة في نسبة وشدة الاصابة بفيروس تجعد واصفرار اوراق الطماطة *Bigeminiviridae Tomato yellow leaf curl virus* (*TYLCV*) في خمسة مناطق صحراوية من العراق

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الكلمات المفتاحية: التشخيص الجزيئي، ومواقع الزراعة، فيروس تجعد واصفرار اوراق الطماطة، وشدة الاصابة.

الخلاصة:

اجري مسح لفيروس تجعد واصفرار أوراق الطماطة (TYLCV) *Bigeminiviridae* في خمس مناطق صحراوية في العراق (البصرة، الكوت، النجف، بغداد، صلاح الدين) لغرض التشخيص الجزيئي وتحديد نسبة وشدة الاصابة بالفيروس على أصناف الطماطة الشائعة والهجينه، وكذلك تحديد مدد ظهور الفيروس. شخص فيروس (TYLCV) لأول مرة في صلاح الدين بواسطة اختبار تفاعل البلمرة المتسلسل (PCR) ، بالإضافة إلى الأعراض التي يسببها الفيروس على المضيف والنقل بواسطة *Bemisia tabaci*. أظهرت النتائج كفاءة التشخيص الجزيئي في الكشف الدقيق

جدا للفيروس، حيث ظهرت الحزم المتعلقة بالفايروس لكل بادىء استخدم في التشخيص الجزيئي. تم استخدام اثنين من البوادىء في تشخيص الفيروس من مختلف الأحجام مما يدل على وجود الفيروس في النباتات المأخوذة من الحقول والنباتات التي اجري لها العدوى الصناعية. ظهرت أعلى زيادة في نسبة وشدة الاصابة في محافظة البصرة ، والتي وصلت إلى 96 %، 4 و 30 %، 3 للأصناف العادية والهجينة في بداية شهر اب على التوالي، ووصلت إلى 55% ، 4 و 5% ، 2 في بداية شهر تشرين الاول على التوالي ، وكان أدناها في محافظة صلاح الدين. أوضحت الدراسة أن تاريخ الزراعة في بداية شهر اب كان الأعلى في نسبة الإصابة وشدتها مقارنة بالتاريخ الثاني للزراعة في بداية شهر تشرين الاول.

Introduction:

Tomato plants *Solauinm lycoperrsici* is cultivated throughout the year in Iraq under open and protected agriculture, and comes first in Iraq (Matloob, 1980). The cultivated areas in Iraq amounted to 22,532 hectares with a production rate of 12,507 kg / ha (Central Bureau of Statistics, 2016). The cultivation of tomato crop has expanded in recent years in desert areas of Iraq, which is reflected in increase in spread of diseases,and infected with many fungal, bacterial and viral diseases. *Tomato Yellow Leaf Curl Virus* is considered one of the most important viral diseases which reduces crops yield and a determining factor for growing tomatoes in some desert areas.The expansion of crop cultivation has been accompanied by the spread of many diseases which have become economically important over time. The virus is most important viral diseases affecting crop and is a reduced factor for agriculture in the southern and central regions of Iraq (Alfahad, 1999, Hammadi and Rifai, 2005).

Bigeminiviridae Tomato yellow leaf curl **virus (TYLCV)** belongs to the Geminiviridae family and Begomovirus genus (Malla et.al, 1997).There are approximately 6 types and 15 strains of **TYLCV** (Czosneky, 2007).This virus has a narrow family extent where it also infects some economical crops. The physical properties of **TYLCV** is single DNA and twin molecules with a length of 30 Nanometers and diameter of 20 Nanometers (Varma and Malathi, 2003).

Many methods have beer relied on to diagnose the viruses, the first of which was the studying of symptoms on the detecting plants .This is considered one of the main methods which have been relied on to diagnose and detect plant viruses and its strains in the first stages of studying plant viruses till now (Verma et.al.,2006; Ghaly et.al.,2006). Serological tests have been adopted ,and these are simple tests ,highly specialized and precise in diagnosing viruses and depends on the interaction of antibodies with its antigens .The most important of these test is Elisa test ,who is considered the first to use this technique in diagnosing plant viruses (Adams and Clark ,1997).A newly used technique is PCR which is one of the rapid ,reliable ,dependable and sensitive methods in diagnosing plant viruses compared to other tests (Menzel et,al.,2002).It has become possible to use this technique for detecting very low concentrations of viruses in plant structure and has still been one of the most **reliable** and **precise** techniques for detecting viruses and specifying its strains (Verma et.al,2006).

Determining the timing of onset of disease and rate of infection are important factors in the resistance of diseases, especially viral diseases, by knowing the movement of the disease and avoid agriculture in times of peak and spread of viruses (Gul-sekr and others, 2015). Determination of severity of virus through design of disease scale is one of factors that assess severity of disease and losses resulting from it and give decisions necessary for prevention or control of disease and degree of resistance or tolerance of varieties to be selected in agriculture (Bhyan et.al, 2007).

Due to absence of studies in molecular diagnosis and an integrated study to determine the dates of TYLCV and the correlation with the proportion and severity

of infection in several desert areas of Iraq and importance of this disease economically and rapid spreading, which is supported by outbreak population of white fly ***B. tabaci*** decide to conduct this research according to the following axes:

1- Field survey to detect the presence of *TYLCV* in several desert areas of Iraq
2- Molecular diagnosis of *TYLCV*.
3- Determine dates of onset of *TYLCV* injury.
4- Measuring percentage and severity of *TYLCV* infection on common and hybrid tomato varieties.

Materials and methods:

Sample collection. In five provinces of Iraq (Basra, Kut, Najaf, Baghdad and Saladin), a total of 200 leaf samples from tomato plants showing TYLCV symptoms (leaf curling, yellowing and stunting) were collected from 50 fields in various locations. The samples were divided into common and hybrid tomato varieties. The process was repeated on two dates of planting in early August and November. Samples were either processed immediately or kept at -20℃ prior to analysis.

The percentage and severity of infection of plant samples imported into the laboratory were calculated according to the following law:

$$\text{Disease incidence} = \frac{\text{No. of infected plants}}{\text{Total no. of plant assessed}} \times 100$$

As for the severity of the infection, we have relied on the diseases index developed by Alfahad and ASalmani (2015) in terms of the symptoms and on Al-Rifai and others (2007) to degrees of disease :(0 = healthy plant, 1 = few deformities in leaves, 2 = wrinkles with little yellowing in leaves, 3 = wrinkle more with yellowing of leaves, 4 = wrinkle and yellowing in leaves)

Severity of infection (%) = [sum (class frequency × score of rating class)]/ [(total number of plants) × (maximal disease index)] × 100

Molecular diagnosis of *Tomato yellow leaf curl* virus (TYLCV):

Buffers used in isolating the DNA:

First Extraction Buffer: extraction buffer is composed of:

1.4 molar Na cl, 100 mili molar Tris-Hcl, 20 mili molar Na_2 EDTA, 2% CTAB Sterilized with autoclave (Maniatis et.al. 2001).

50 Milliliters were prepared with a weight of gram of CTAB ,then 14 milliliters of Na cl were added (5 molars)prepared from dissolving 7.30 gm of salt in 25ml of distilled water ,and then adding 5 ml of Na_2 EDTA solution with a concentration of 0.4 molar prepared with a weight of 3.72 gm and dissolved in 20 ml of distilled water .The PH was adjusted to 8 by using 1 molar of NaOH solution .Then ,the proportion was increased to 25 ml by distilled water .Finally ,5ml of Tris-Hcl solution was added with a weight of 3.02 and dissolved in 7.5ml of distilled water , the PH was adjusted to 8 by using 1 molar of Hcl solution .Then ,the proportion was increased to 100ml by distilled water .After that ,the extraction buffer proportion was completed to 50 ml and PH was adjusted to 8 using 1 molar of NaOH solution, and sterilized with autoclave at temperature of 121c˚,under the pressure of 1bar for a period of 15 minutes.

Washing buffer:

100 ml washing buffer was prepared by dissolving 0.136 gm of Ammonium acetate in 76 ml of absolute ethanol, and the proportion was completed to 100 ml by distilled water.

Chloroform buffer: Isoamyle Alcohol (1:24):

100ml of this buffer was prepared by mixing 96 ml of chloroform with 4 ml of Isoamyle Alcohol, and kept in a tight and dim bottle at a temperature of 4C˚.

The method of (Weigand et.al., 1993) was followed:

1-1gm leaves for each sample of the studied plants is weighed after washing with distilled and sterilized water, cut to pieces, and an appropriate amount (immersed) of liquid nitrogen is added in a casserole dish, then grinded till it becomes white powder in as much as possible.
2-The powder is placed in glass tubes and 5ml of extraction buffer kept in a water bath at temperature of 65 C˚ is added the tubes are incubated in the vibrating water bath at the same temperature for a period of 60-90 minutes.
3-After that ,the tubes are left so that it acquires room temperature and then 4 ml of chloroform buffer :isoamyle alcohol (1:24) is added for each tube with continuous stirring for a period of 15 minutes.
4-Then, the tubes are transferred to a centrifuge and the mixture is discarded with a speed of 4000 cycle /minute for a period of 15 minutes.
5- After the discarding period is complete, the upper water layer is lifted by a micropipette of the capacity 1ml to another sterilized tube, and the previous proportion itself of chloroform buffer is added and discorded again with the same speed.
6-The upper water layer is lifted by a micropipette, placed in new sterilized tubes, 5ml of cooled isopropyl alcohol is added, and mixed by calm stirring until it becomes a white mass representing DNA strings.
7-The DNA strings are pulled by a glass rod with a hooked end and placed in another tube containing 4ml of washing buffer and left for 20 minutes.
8-Raised by the rod to sterilized tubes containing 200-400 microliter of TE Buffer ,and moved from time to time until we gain a complete dissolving of DNA ,and then the stock samples are kept at a temperature of 20C˚to be used in upcoming experiments.

Estimating the concentration and purity of DNA:

The concentration and purity of DNA for the extraction was estimated by using the Nano drop device by taking 1 microliter of DNA stock samples after the mixture has been discarded in the micro centrifuge device for five seconds to ensure the dropping of liquid drops present on the wall, and then placed in the designated place in the device, and taking the reading of concentration and purity which appears on the computer monitor of the device (Maniatis et.al., 2001).

Buffers used in the expulsion process:

1-Tris Borate EDTA (TBE) Buffer with a power of 10X.
This buffer consists of:
0.89 molar Tris- base, 0.89 molar boric acid, 0.02 molar, 8 PH Na_2EDTA
200 ml of TBE Buffer of 10X was prepared by taking 21.6 gm of Tris- base with 11 gm of Boric acid and dissolving it into 160 ml of distilled water, then adding 10 ml of Na_2EDTA buffer with a concentration of 0.4 molar and the PH is adjusted to 7.8, and after that the proportion is completed to 200ml in distilled water and sterilized by

autoclave .At each use, it is diluted by adding 9 parts of distilled water to 1 TBEX.
2-Ethidium bromide stain:
It was prepared with a concentration of 10 mg /ml, by dissolving 100 mg of stain powder in 10 ml of distilled water and kept in a sterilized bottle at temperature of 4C° until it is used .And then, 40 microliters/liter of distilled water are added.
The method of preparing Agarose gel and the process of gel electrophoresis for DNA:
The method of Maniatis et.al. (2001) was adopted.
The Agarose gel is prepared with a concentration of(1%) from Agarose powder in 50 ml of 10 TBEX in a microwave for two minutes and then left to reach a temperature of 50-60 C°.The gel buffer is poured in the special tray of the gel electrophoresis device after adjusting the special comb to make holes at one of the ends of the gel .pouring is conducted quietly to avoid making bubbles , and if they are made , we should remove it by using a pipette and then the gel is left to be solid.
1-After the gel becomes solid, the comb is lifted and a tray is placed in the tray of the gel electrophoresis that contains an enough amount to cover the bearing holes of the 1XTBE buffer.
2-The samples are bearded in the gel holes and the marker in special holes on one of the sides of the gel.
3-The gel electrophoresis device is operated by passing electrical current with a voltage difference of 3 volt/cm and after adjusting the poles since the gel must be placed from the side of the samples (comb) at the negative pole .The direction of electrophoresis of the samples towards the positive pole till the reaching of the samples before the end .The process takes (1-3) hours.
4-Transferring the gel to a tray containing an Aithedeomebromide with a concentration of (0.5) mg for each 1 ml of distilled water, and then it is left for half an hour with continuous shaking by a shaker device.
5-The gel is photo by the E-graph device.
Doubling the DNA segments related to TYLCV virus by using the PCR technique
Materials and Buffer used: (Aziz et.al. 2008)
1-premix, supplied by pioneer company
2-DNA Template
3-specific primers (table 1), supplied by Reagent Company
4-Sterilized distilled water.

Table-1: represents the primers used in detecting the virus

Bundle size	Correlation temperature of primer	Final concentration	Sequence	Name of primer	NO
406bp	52 C°	20 picomol/ microliter	5′-TTGACTGGTGAATCTCTTCCT-′3 5′-CACCAATGAGAAGGACAAGA′-3	18sf 18sr	2
670bp	52 C°	20 picomol/ microliter	5′-CGCCCGTCTCGAAGGTTC-′3 5′-GCCATATACAATAACAAGGC-′ 3	TYLCV-F TYLCV-R	2

1-The DNA concentration in the studied samples was adjusted with a concentration of 50 Nano gm /microliter for each sample Table (2).
2-The tubes containing the interaction mixture are prepared with a capacity of 0.2ml and placed in ice until the additives clarified in the table

Table-2: Represents ingredients reaction PCR

Ingredients	Final concentration	Proportion of one sample/microliter
Sterilized distilled water	-	32
Primer	20 picomol	2
DNA Template	50 Nano gm /microliter	2
Premix	-	2

Automated scanning is implemented at temperature 94C° for 4 minutes followed by 25 cycle of scanning at 94C° for one minute , and the correlation of the primer at 52C° for one minute , and elongation at 72 C° for and final elongation at 72 C° for 10 minutes
4-After the ending of the program period inside the thermo cycle device, the samples are taken and the doubling of DNA out puts are detected by the electrophoresis device.

Detecting the doubling out puts:

The molecular weights of the doubling of DNA output inside the Thermo cycle are estimated by conducting electrophoresis processes on the Agarose gel using the volumetric guide.

Result and Discussion:

First: Isolating and diagnosing TYLCV on Tomato
1-symptoms:
There appeared on tomato plants affected with TYLCV symptoms after 2-3 weeks from infection represented by atrophy (lack of growth, dwarf ,yellowing and curling of new leaves , and the small leaves are very small in size and curled up wards and down wards giving a cup shape .These symptoms agree with what has been mentioned by many researchers concerning the infection symptoms of this virus (Shawkat,1982; Awath,2005; Al-Waely ,2006).

Image-1: Representing infection symptoms of *TYLCV* and healthy tomato plant.

2-Diagnosing *TYLCV* using PCR Technology

A-Results of isolating DNA:

The DNA was isolated from the young leaves of tomato plants in a successful manner according to the Weigand et.al. (1993) method in which CTAB was used .Thus, appropriate quantities of DNA were obtained with purity ranging between (2.04-2.06) with a concentration of (54.1-216.4).Each of the purity and concentration were estimated using the Nano drop device .The dilutions of DNA samples were adjusted to obtain a concentration of 50 Nano gram /microliter which is the appropriate concentration to conduct the PCR interactions.

The process of isolating DNA from plants is relatively more difficult than in other organisms as a result of the presence of a thick wall surrounding the cellular membrane .In addition ,some plants consist of a large quantity of phenolic materials and poly saccharides which are considered pollutants since they precipitate with the DNA giving a liquid of high viscosity and are considered inhibitors of PCR interactions(Adams and Do,1997).To get rid of these substances , a dilution of the extracted DNA has been conducted to reduce the percentage of inhibiting saccharides (Pandey et.al.,1996).

The Weigand et.al. (1993)method is considered one of the most the efficient and successful methods in many laboratories for isolating DNA from plants with high molecular weight and purity and appropriate for the techniques of Genetic engineering .Thus , from the first step with breaking the hard and strong plant cell walls using the manual powder with the presence of liquid nitrogen , the low temperatures (-196 C˚)operate on stopping the activity of nuclear enzymes which are released once the cell walls are broken .

The process of exposing the mixture to a temperature of 65C˚ directly after it was -0C˚ with the presence of CTAB facilitates the process of moulting proteins from the DNA so that it is replaced by CTAB and becomes part of the structure of the DNA by composing CTAB-Nucleic acid complex to protect the DNA in the aqueous phase and not being precipitated with the other ingredients with in the following discard steps.

As far EDTA which enters in to the extraction buffer , it works as a grapple factor since it pulls the magnesium ions which are necessary for the effectiveness of nuclear enzymes which work on decomposition of DNA which inhibits the work of such enzymes (Wilson and Walker,2004).In order to purity the DNA from the rest of cell ingredients , chloroform is used as an organic solvent which gets rid of the CTAB material and keeps the DNA in the aqueous phase (Sambrook et.al,.1989).To remove the remnants of proteins and saccharides related to DNA ,the discard period speed is increased after adding chloroform ,and to precipitate the DNA add cooled isopropyl alcohol so that the DNA appears in the form of a white dense mass transferred by a glass tube containing the washing buffer which consists of Ammonium acetate and ethanol to wash the DNA from the suspending materials .Thus , we obtain a snow –white DNA and is dissolved in TE and kept at a temperature of (-20C˚) until it is used (Manitalis et.al.,2001).

B- Results of PCR interaction:

There are several factors, which must be adhered to obtain the best results, which are as follows:

1-The used DNA concentration

The concentration 50 Nano gram/microliter was used and is considered one of the best concentrations that is used to conduct the PCR interactions.

2-The used primer concentration

Several experiments were conducted with a concentration of 20 Pico mole and have given good results.

3-The organizing buffer and using nitrogenous base

Ready interaction ingredients (Premix PCR) were used and supplied by pioneer company.

The consist of fixed concentrations of magnesium ions and nitrogenous bases, polymer enzyme and inverse duplication enzyme.

4-Using the appropriate program in the thermo cycle device

The initial mounting was implemented at 94C˚for 4 minutes followed by 25 moulting cycles at 94C˚for 1 minute. The primer was connected at 52C˚for 1 minute and enlongment at 72C˚for 2 minutes and final enlongment at 72C˚for 10 minutes.

C-Electrophoresis results (Image 2):

The samples resulting from PCR interaction were electrophoresis a %1 concentration of Agarose since the bundles were clear .As for coloring (pigments) ,the concentration was 10 mg/1ml and gave good results

After adjusting all the conditions, the diagnostic experiments were conducted on DNA and the two primers showed different results as clarified in the (Image 2).

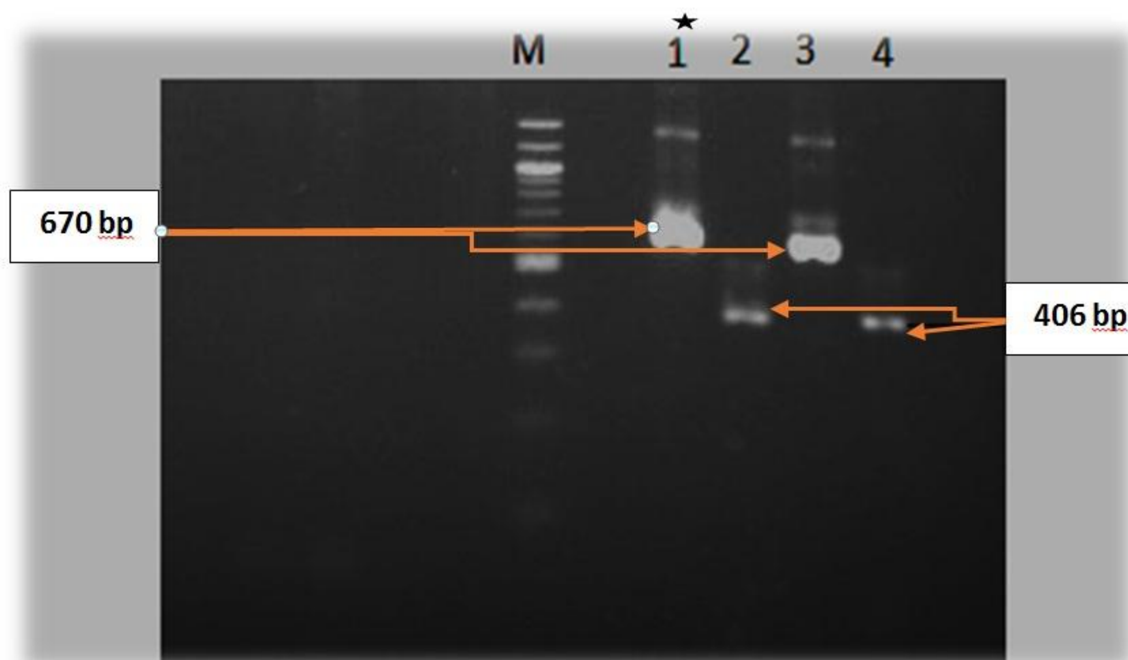

Image-2: Represents electrophoresis results on Agarose gel 1% for PCR interaction results of TYLCV virus.

*The samples No (1) and No (3) represent primer 18SR/18SF, and sample No (2 and 4) represent primer TYLCVR/TYLCVF.

M-represents volumetric guide.

1-18SR/18SF represents primer with size 406bp.

2-TYLCV-R/TYLCV-F represents primer with size 670bp.

The first primer 18SR/18SF showed a correlation result with the virus genome a bundle of 406bp, and the second primer TYLCV-R/TYLCV-F with a bundle of 670bp, this result came similar to what Aziz et.al. (2008) reached and found in their study on screening of a variety of tomato plants infected with *TYLCV* in Iran.

The results of the statistical analysis (Table 3) showed that there were significant differences in the percentage and severity of infection of the studied

sites. Basra governorate exceeded the rest of the areas where the infection rate was (96, 30%) for the normal and hybrids varieties, respectively and severity of the injury reached (4, 3) respectively.

The results showed (Table 3) increase the proportion and severity of injury to the first date 1/8 compared to the second date 1/10.The rate of infection was (80.4, 22.2%) and (47.4, 13.6) for the first and second consecutive dates, and this applies to the severity of the injury as well.

The results of the trial (Table3) showed a high degree of tolerance to TYLCV infection, where proportions and severity of infection of hybrid varieties decreased compared to the common varieties in which incidence and severity of infection increased, especially on first date of cultivation.

Table-3: Effect of cultivars, dates of cultivation & locations in percentage & severity infection*of *Bigeminiviridae Tomato yellow leaf curl* virus (TYLCV) on tomato plants in Iraq.

Geographical location	Date of planting	Infection rate%		Severity of infection	
		Type of cultivar		Type of cultivar	
		common	hybrid	common	hybrid
Basrah	1-Aug	96 a	30 a	4 a	3a
	1-Oct	55 d	5 d	4 a	2b
Kut	1-Aug	80 b	23 b	3 b	2b
	1-Oct	49 d	3 d	3 b	2b
Najaf	1-Aug	82 b	28 a	4 a	3a
	1-Oct	51 d	3 d	3 b	2b
Baghdad	1-Aug	73 c	18 b	3 b	2b
	1-Oct	42 e	1 d	2 c	1c
Saladin	1-Aug	71 c	12c	2 c	1c
	1-Oct	40 e	1 d	2 c	1c
average	1-Aug	80.4 A	22.2 A	3.2 A	2.2 A
	1-Oct	47.4 B	2.6 B	2.8 B	1.6 B

* numbers that have similar small letters in one column or similar capital letters in same row are not significant differences according to Dunkin multipliers test at 5% .

Perhaps reason for increase in proportion and severity of injury in province of Basra compared to rest of provinces to existence of large areas and intensive cultivation of tomato crop as well as adjacent to countries where same crop is infected with same virus as Iran, Saudi Arabia and Kuwait, this is why resettlement of virus in region, Secondary families and not to use an integrated management of resistance and this is consistent with what indicated by Al-Fahad (1999) and Al-Dosari (2002) , and it is known that there is an impact of the geographical impact of the spread of diseases, and one of the reasons for this difference in soil type, where soil content of elements and water may indirectly affect the development of disease through its impact on the strength and speed of growth and then escape disease, this is close to what was said Cadman(1963), Czosnek and laterrot(1997) and Hussien et al.(2013) .

As for the dates of agriculture, it is known that early agriculture attracts vital vectors of viruses, including the white fly, because there are no other plants at the time, It is found on tomato crop, which affects the plants in high rates and early growth, this comes close to the extent reached by Crescenzi et al. (2004) and Rashid et al (2008).

Scientific institutions and seed production companies have targeted their research programs to combat this virus after causing large losses around the world, thus producing resistant and tolerant varieties, thus enhancing the reduction of its spread and the severity of the symptoms it causes (Eui-Joon et. al, 2016). This is evident from the results obtained in low percentage and severity of *TYLCV* infection in hybrids and in different areas under study. This result is applicable to Czosnek (2007) and Khalaf et.al, (2011).

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