Natural occurrence and phylogeny of *Tomato yellow leaf curl virus* on *Malva parviflora* and *Melilotus indicus* from Iraq

M. Al-Waeli[1,2], A. Dizadji[1], G.H. Mossahebi[1], A. Ahangaran[3]

[1]Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran
[2]Department of Plant Protection, Faculty of Agriculture, Basrah University, Iraq
[3]Plant Protection Organization, Tehran, Iran
* Corresponding author email: adizaji@ut.ac.ir

Abstract: *Tomato yellow leaf curl virus* (TYLCV) is one of the most important worldwide viruses causing severe disease in tomato cultures worldwide. During 2015-2016, a total of 110 leaf samples from asymptomatic and symptomatic weed species were collected from tomato fields in central and southern Iraq. To assess the TYLCV incidence, double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was carried out using a commercial kit, which revealed the presence of the virus in 10 out of 110 (9.1%) of the samples. Subsequently, a DNA fragment of 789 bp, covering TYLCV V1 gene, was amplified in five out of 10 ELISA positive samples of *Malva parviflora* and *Melilotus indicus* by PCR. After cloning and sequencing, multiple alignment of the nucleotide sequence of amplicons and other TYLCV isolates showed the highest identity of 99% with TYLCV-KISR strain2 (KJ830841) from Kuwait and TYLCV-Florida (AY530931) from USA isolates. No evidence for the presence of a second genomic component in the five Iraqi isolates was observed by PCR using different sets of primers specific for DNA-B or satellite DNA β. In phylogenetic analysis our five isolates were categorized in both groups I and II. There is not any separation between isolates according to host plant species or geographical origin. To our knowledge, this is the first report of TYLCV on both weed species in Iraq. Weeds serve as bridges to maintain virus and vector populations through the year. More effective quarantine programs are needed to avoid further expansion of TYLCV in Iraq.

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Introduction

Tomato yellow leaf curl virus (TYLCV), is a member of the genus *Begomovirus*, family *Geminiviridae*, causes severe losses to tomato cultures in tropical and subtropical regions worldwide (Czosnek & Laterrot 1997). TYLCV is transmitted in a persistent manner, with the whitefly *Bemisia tabaci* (Ghanim *et al*., 1998). Earlier studies have supported its limited distribution but more recent research shows it has been spreading rapidly and widely all around the world especially in the new world (Shirazi *et al*., 2014). TYLCV can infect tomato and several other cultivated crops, e.g. *Phaseolus vulgaris*, *Solanum melongena*, *Cucurbita moschata* and *Capsicum annum* (Gharsallah Chouchane *et al.*, 2007; Abou-Jawdah *et al*. 1999; Navas-Castillo *et al*., 1999; Reina *et al*., 1999; Kim *et al*., 2011), ornamentals, e. g. *Eustoma grandiflorum* and *Petunia hybrid* (Díaz-pendón *et al*., 2010; Kil *et al*., 2014a) herbs, e.g. *Mercurialis ambigua* and *Artemisia annua* (Ding *et al*., 2008; Sanchez-Campos *et al*; 2000) and weeds e.g. *Solanum luteum (*Sanchez-Campos *et al*., 2000; Cui *et al*., 2004;), *Cleome viscosa*, *Croton lobatus* (Salati *et al*., 2002), *Lamium amplexicaule* (Kil *et al*., 2014b), *Malva* sp. and *Melilotus officinalis* (Shirazi *et al*., 2014), *Datura stramonium* and *Solanum nigrum* (Cohen *et al*., 1995; Mansour & Al-Musa, 1992). Association of TYLCV with uncultivated plants, as an alternative hosts, is important because these plants play an important role in perpetuation and spread of the virus. *B. tabaci* biotype B is highly polyphagous, with high reproduction rate on numerous crops, weeds and non-cultivated plants, so it is believed that this biotype plays an important role in emergence of tomato-infecting begomoviruses by horizontal transfer of indigenous viruses (Castillo-Urquiza *et al*., 2008).

TYLCV symptoms are variable in different host plants, while some cultivated and/ or weed hosts are symptomless. Infected tomato and likely cucumber plants may show stunting, yellowing and upward leaf curling (Glick *et al*., 2009, Shirazi *et al*., 2014), while infected cultivated plants, e.g. eggplant and pumpkin and weeds, e.g. *Malva* sp. and *Melilotus officinalis* are symptomless (Kim *et al*., 2011, Shirazi *et al*., 2014). Rate of mutation and recombination among TYLCV isolates plays an important role in biological and genetic virus features including genetic variation, disease symptoms or even infection of new hosts (Owor *et al*., 2007; Monci *et al*., 2002; Varsani *et al*., 2008). So far, eight strains/variants of TYLCV have

been characterized, including TYLCV-IL from Israel and Iran, TYLCV-Mld from Israel, TYLCV-IR, TYLCV-Bou and TYLCV-Ker from Iran, TYLCV-Gez from Sudan, TYLCV-OM from Oman and Iran, and TYLCV- KISR from Kuwait (Bananej *et al*., 2004; Idris & Brown, 2005; Khan *et al*., 2008; Lefeuvre *et al.*, 2010; Pakniat *et al*., 2010; Al-Ali *et al*., 2015).

TYLCV has a worldwide distribution, and several studies have detected this virus in the Middle East, e.g. Iran (Azizi *et al*., 2008), Saudi Arabia (Ajlan *et al*., 2007), Egypt (Nakhla *et al*., 1993), Oman (Khan *et al.*, 2008), Lebanon (Abou-Jawdah *et al*., 1999), Jordan (Anfoka *et al*., 2005), Israel(Cohen and Harpaz, 1964), Turkey (Navot *et al.*,1989) and Yemen (Bedford *et al.*,1994) . TYLCV has been reported in Iraq by Makkouk (1978) and recently, a complete genome sequence of a TYLCV isolate has been reported from tomato plants of Iraq (Al-Kuwaiti *et al.*, 2013). Nowadays, there is no published report about the association of begomovirus on weeds from Iraq. Present study aimed to determine the occurrence of TYLCV in certain important weeds of tomato fields and phylogenetic status of five isolates originated from wild hosts based on V1 (coat protein, CP) gene.

Materials and Methods

Sampling

During 2015-2016, a total of 110 leaf samples from asymptomatic and symptomatic weed plants, including *Trifolium* sp, *Amaranthus*. sp, *Sonchus oleraceus* , *Malva parviflora* , *Melilotus indicus* showing leaf curling, yellowing and stunting were collected from tomato fields located in five provinces of the central and southern Iraq. The fresh samples were immediately placed in plastic bags, all the samples (leaves, stems) were divided into two groups and placed separately into new plastic bags one of which was processed with 2-5 days for serological and the second kept at -20ºC prior to analysis (Table 3).

DAS-ELISA

Collected symptomatic and symptomless weed samples were investigated for the presence of TYLCV by using a complete commercial kit (Bioreba, AG, Switzerland) in double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) in accordance with the manufacturer's instructions The absorbance of each well was measured at 405 nm (OD_{405}) by ELISA-reader (Hiperion MPR4+, Germany). Samples were considered positive only if the absorbance value was more than three times that of the negative control.

DNA Extraction and PCR Amplification

The CTAB method (Lodhi *et al*., 1994) was used as an efficient option for total DNA extraction from TYLCV ELISA positive leaf tissue samples and homologous control (healthy) plants. The genomic DNA was used as a template to amplify a 789 bp fragment of TYLCV genome, covering the full length of V1-CP, using a degenerate primer pair, V1 (CP) forward/reverse primers (Table 1, Kim *et al*., 2011). PCR was carried out for 35 cycles consisting of 95°C three min, 95°C one min, 52°C one min, 72°C one min and the final extension time was 72°C for 10 min. The primer pairs (PBL1v2040, PCRc1) and (Beta01, Beta02) Table (1) have been used for detection DNA-B and satellite DNA β, respectively. Finally, the reaction products were analyzed by electrophoresis on 1% agarose gels in a TBE buffer, followed by ethidium bromide gel staining (1 μg· ml^{-1}) for 20 min and photographing with White/Ultraviolet transilluminator, UVP (Orme Technologies, Cambridge,. UK).

Table 1. Characteristics of used primers in this study

Primer name	Genome part	Expected size	Nucleotide sequence 5'-3'	Reference
V1 (CP) Forward (EcoRI) V1 (CP) Reverse (EcoRI)	DNA-A	789 bp	GAATTCATGTCGAAGCGWCCA GAATTCTTAATTTKRTAYTGAATCATAGAA	(Kim *et al*., 2011).
PBL1v2040 PCRc1	DNA-B	600 bp	GCCTCTGCAGCARTGRTCKATCTTCATACA CTAGCTGCAGCATATTTACRARWATGCCA	(Rojas *et al*., 1993)
Beta01 Beta02	Beta-Satellite	1350 bp	GGTACCACTACGCTACGCAGCAGCC GGTACCTACCCTCCCAGGGGTACAC	(Briddon *et al*., 2002).

PCR-Cloning and Sequencing

The amplified fragment of the viral genome in the predicted size was excised from the agarose gel and cleaned with a GeneAll extraction kit (GeneAll Kit, Korea) according to the manufacturer's protocol. The extracted PCR products were ligated into a pJET 1.2/blunt cloning vector (CloneJET PCR Cloning Kit; Thermo Fisher Scientific, USA) and transformed into *Escherichia coli* strain DH5α by using heat shock. The transformants were selected on a Luria–Bertani (LB) plate supplemented with 50 $mg.ml^{-1}$ ampicillin, grown in LB liquid media and used for plasmid isolation using standard alkaline lysis protocol (Sambrook *et al*. 1989). The presence of the insert was verified by PCR amplification using the vector universal primer pairs of pJET1.2 F/R. Recombinant plasmids containing the target insert were sent to a commercial sequencing laboratory (Macrogen Inc., South Korea) to determine their nucleotide sequences, bidirectionally. The nucleotide sequence data determined in this study have

been deposited in the NCBI GenBank database under the accession numbers shown in Table 2.

Table 2. Characteristics of TYLCV isolates used in phylogenetic analysis in this study

Isolates	Accession no.	Country	Reference
IQ:Ba-5	MF429941	Iraq: Basrah	This study
IQ:Na-10	MF429947	Iraq: Najaf	This study
IQ:Dq-7	MF429931	Iraq: Dhi-Qar	This study
IQ:Dq-11	MF429932	Iraq :Dhi-Qar	This study
IQ:Dq-14	MF429933	Iraq: Dhi-Qar	This study
IQ:Dq-1	MF429928	Iraq: Dhi-Qar	(Al-Waeli *et al*., unpublished)
IQ:Dq-2	MF429929	Iraq: Dhi-Qar	(Al-Waeli *et al*., unpublished)
IQ:Dq-6	MF429930	Iraq: Dhi-Qar	(Al-Waeli *et al*., unpublished)
IQ:Ka-4	MF429934	Iraq: Karbala	(Al-Waeli *et al*., unpublished)
IQ:Ka-5	MF429935	Iraq: Karbala	(Al-Waeli *et al*., unpublished)
IQ:Ka-42	MF429936	Iraq: Karbala	(Al-Waeli *et al*., unpublished)
IQ:Ba-Kh2	MF429937	Iraq: Basrah	(Al-Waeli *et al*., unpublished)
IQ:Ba-Kh6	MF429938	Iraq: Basrah	(Al-Waeli *et al*., unpublished)
IQ:Ba-Sf2	MF429939	Iraq: Basrah	(Al-Waeli *et al*., unpublished)
IQ:Ba-Sf11	MF429940	Iraq: Basrah	(Al-Waeli *et al*., unpublished)
IQ:Ba-91	MF429942	Iraq: Basrah	(Al-Waeli *et al*., unpublished)
IQ:Ba-100	MF429943	Iraq: Basrah	(Al-Waeli *et al*., unpublished)
IQ:Ba-Zu53	MF429944	Iraq: Basrah	(Al-Waeli *et al*., unpublished)
IQ:Ba-Zu82	MF429945	Iraq: Basrah	(Al-Waeli *et al*., unpublished)
IQ:Na-4	MF429946	Iraq: Najaf	(Al-Waeli *et al*., unpublished)
IQ:Na-32	MF429948	Iraq: Najaf	(Al-Waeli *et al*., unpublished)
TYLCV- isolate-Iraq	JQ354991	Iraq: Baghdad	(Al-Kuwaiti *et al.,* 2013)
SS11-IL	JQ867092	China	GenBank
CN:SH-IL	GU434144	China	GenBank
Egypt-IL	AY594174	Egypt	(Hosseinzadeh *et al.,* 2014)
Iran-IR	AJ132711	Iran	(Hosseinzadeh *et al.,* 2014)
Kahnooj-Ker	EU635776	Iran	(Hosseinzadeh *et al.,* 2014)
Iran-Ir2	EU085423	Iran	(Hosseinzadeh *et al.,* 2014)
TOB-OM	KT990213	Iran	GenBank
Genaveh-BOU	GU076454	Iran	(Hosseinzadeh *et al.,* 2014)
Jiroft-OM	GU076453	Iran	(Hosseinzadeh *et al.,* 2014)
Iran-IL	GU076446	Iran	(Hosseinzadeh *et al.,* 2014)
Jordan-IL	GQ861426	Jordan	(Lefeuvre *et al*., 2010)
cucumber-IL	EF433426	Jordan	(Anfoka *et al*., 2009)
Jordan-IL	EF054893	Jordan	(Lefeuvre *et al*., 2010)
Jordan-Mld	EF158044	Jordan	(Lefeuvre *et al*., 2010)
Homra-IL	JX444575	Jordan	GenBank
KISR-4	KR108214	Kuwait	(Al-Ali *et al*.,2015)
KISR-2	KJ830841	Kuwait	(Al-Ali *et al*.,2015)
LBa4-Mld	EF185318	Lebanon	(Lefeuvre et *al*., 2010)
Ra3-IL	EF051116	Lebanon	(Lefeuvre *et al*., 2010)
Moroccan-IL	EF060196	Morocco	GenBank
Netherlands-IL	FJ439569	Netherlands	(Lefeuvre et al., 2010)
MU_MU3-NC6-IL	KX347169	New Zealand	GenBank
KIRT14-IL	KX347164	New Zealand	GenBank
Tom96-OM	LN680631	Oman	GenBank
Tom92-OM	LN680630	Oman	GenBank
Tm-8-OM	HG969282	Oman	GenBank
Tm-3-Om	HG941651	Oman	GenBank
Spain7297-Mld	AF071228	Spain	(Lefeuvre *et al*., 2010)
Arizona-IL	EF210554	USA	(Lefeuvre *et al*., 2010)
Florida-IL	AY530931	USA	(Lefeuvre *et al*., 2010)
TYLCMLV	FM212663	Cameroon	GenBank

Phylogenetic Analysis

The contigs were assembled using DNASTAR (Lasergene, Wisconsin, USA), then obtained nucleotide sequences were compared with available related sequences by online Blastn software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For sequence comparisons, the determined nucleotide sequences were aligned with those of reference TYLCV isolates obtained from the NCBI database (Table 2) using Muscle within the MEGA 6 sequence analysis package and default parameters (Tamura *et al*., 2013). Phylogenetic analysis was performed with MEGA6 based on maximum likelihood using the Tamura three-parameter nucleotide substitution model (Tamura, 1992) with gamma distributed rates amongst sites (T92+G). Stability of the phylogenetic tree was estimated by bootstrap analysis of 1000 replicates. *Tomato yellow leaf curl Mali virus* (TYLCMV) which belongs to the *Begomovirus* was used as an outgroup in this analysis.

Results

Serological and Molecular Detection of TYLCV

Based on DAS-ELISA results, 10 out of 110 (9.1%) weed samples, collected from tomato cultivated fields of five different provinces of Iraq, reacted positively with TYLCV specific antibodies. The ELISA- positive samples include eight (three asymptomatic and five symptomatic) *M. parviflora* and two asymptomatic *M. indicus*. No ELISA positive samples were found in weed plants collected from Karbala (Table 3). Subsequently, the expected PCR product size of 789 bp was amplified in three malva samples showing leaf curling and stunting symptoms (Fig 1) and one symptomless malva and melilotus samples, while no amplicons were produced from extracts of healthy malva and melilotus plants. No evidence for the presence of a second genomic component in the five Iraqi isolates was observed by PCR using different sets of primers specific for DNA-B or satellite DNA β.

Table 3 Characteristics of collected weed samples (location, species and the number of TYLCV-positives in ELISA and PCR)

Location	No. of sampled weed species							ELISA positive	PCR positive	TYLCV infected species
	Total	*Trifolium* sp.	*Amaranthus* sp	*Sonchus oleraceus*	*Malva parviflora*	*Melilotus indicus*	Others			
Karbala	5	-	1	-	3	1	-	0	0	-
Babel	6	1	-	2	2	-	1	1	0	*Malva parviflora*
Najaf	12	2	-	1	5	2	2	2	1	*Malva parviflora*
Dhi-Qar	41	3	4	6	9	5	14	5	3	*Malva parviflora*
Basrah	46	4	5	7	4	9	17	2	1	*Melilotus indicus*
Total	110	10	10	16	23	17	34	10	5	-

Figure 1: Symptomatic TYLCV infected *Malva parviflora*, showing stunting and leaf curling.

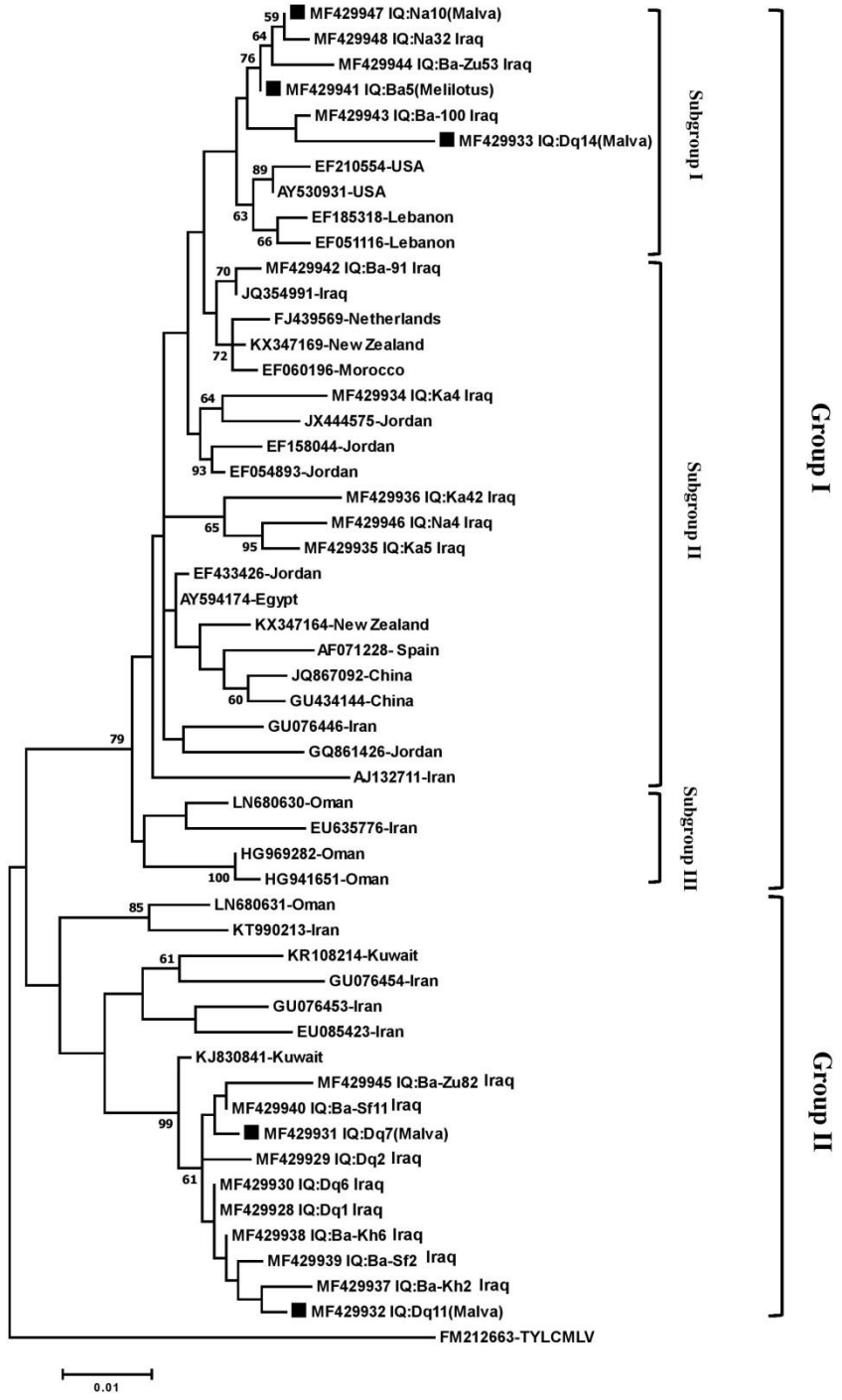

Figure 2: Phylogenetic tree based on nucleotide sequence of TYLCV ORF V1 (CP) constructed using MEGA 6 with 1000 bootstrap replications. Only bootstrap values greater than 60% are shown. The tree was rooted with the CP nucleotide sequences of *Tomato yellow leaf curl Mali virus* (TYLCMV) as an out-group species

Phylogenetic Analysis

BLAST analysis and sequence comparisons showed that TYLCV isolates in this study (IQ:Dq-7, IQ:Dq-11, IQ:Dq-14, IQ:Na-10 and IQ:Ba-5) shared the highest CP gene nucleotide identity of 96-99% with (GU076454) and (KJ830841) ,respectively. Phylogenetic analysis of V1-CP nucleotide sequence showed that all isolates were divided into two major groups, with three subgroups in group I (Fig 2). According to the phylogenetic tree, our five isolates were categorized in both groups I (subgroup I) and II. Two IQ:Na-10, IQ:Dq-14 (originated from *M. parviflora*) and IQ:Ba-5 (originated from *M. indicus*) grouped into subgroup I, while two other isolates (IQ:Dq-11 and IQ:Dq-7, originated from *M. parviflora*) were categorized in group II. In addition, we used 16 Iraqi isolates which had been deposited in GeneBank during our previous research (Al-Waeli *et a*l., unpublished).

Discussion

Tomatoes are grown in whole Iraq, both as open field and greenhouse crops and the average productivity of tomatoes has been steadily rising over time. So that Basrah governorate is also identified as an "efficient tomato cluster in Iraq" (USAID, 2007). Various weed species are commonly associated with tomato fields in Iraq. During the present survey several symptomatic and asymptomatic weed species, colonized by *B. tabaci*, were collected from tomato fields and screened to determine the presence of TYLCV. Based on serological tests results, 10 out of 110 weed samples showed positive reaction with TYLCV specific antibodies, while infection of only five samples including four *M. parviflora* and one *M. indicus* was confirmed by PCR. To our knowledge, this is the first report of TYLCV on both weed species in Iraq. Inconsistent results of serological and molecular detection could be partly due to serological relationships among several *Begomovirus* species, which was reported before (Rishi, 2004). In accordance with our previous results which showed only 13.9% of TYLCV infection in tomato fields of Iraqi central and southern regions (Al-Waeli *et al*., unpublished), a low incidence of TYLCV in tomato associated weeds (five out of 110) determined in current study, compared with that of determined in Dominican Republic (Salati *et al*., 2002) and Iran (Shirazi *et al*., 2014). Low rate of TYLCV detection in weed plants may be partly due to low titer of the virus and inefficiency of normal PCR, which was described before (Salati *et al*., 2002).

So far many weed species have been reported as alternative hosts for TYLCV (Garcı´a-Andre´s *et al*., 2006; Papayiannis *et al*., 2011; Kil *et al*., 2014a), while some showing typical symptoms (Sánchez-Campos *et al*., 2000), however, many TYLCV-infected weeds remain asymptomatic and unnoticed. It is well known that weeds serves as alternative hosts for both TYLCV and its whitefly vector, *B. tabaci*, and act as bridges to maintain virus and vector populations through the year, in particular during a period free of tomato production. On the other hand, it was demonstrated that weeds species, e.g. *Solanum nigrum*, plays a role as a reservoir for virus recombination and increased diversity for tomato yellow leaf curl disease epidemics in Spain (Garcı´a-Andre´s *et al*., 2006).

According to Salati *et al*., (2002) and Shirazi *et al*., (2014) TYLCV-infected *Malva* sp. and *Melilotus officinalis* did not show any virus infection symptoms, likely, *M. indicus* plant infected with TYLCV was asymptomatic in current investigation, while three out of four TYLCV- infected *M. parviflora* showed severe leaf curling and stunting symptoms. Induced symptoms in TYLCV-free malva plants could be the result of other viruses presence.

In phylogenetic tree based on CP sequences, TYLCV isolates were categorized into two major groups, group I subdivided into three subgroups. In this tree studied Iraqi weed isolates fell into group I (subgroup I) and group II. Surprisingly, these isolates showed the closest relation with tomato isolates of the virus originated from Iraq (Al-Waeli *et al*., unpublished), clustered as separate clades in group I and II. There is not any separation between isolates according to host plant species or geographical origin. Contrariwise, Shirazi *et al*. (2014) and Garcı´a-Andre´s *et al.* (2007) found a correlation between geographical origin and host with phylogenetic positions. Three isolates (IQ:Na-10, IQ:Ba-5 and IQ:Dq-14), isolated from *M. parviflora* and *M. indicus*, created a sister group in close relationship with USA (TYLCV-IL strain) and Lebanese (TYLCV-IL and –Mld strains) isolates but distant from the other two isolates. IQ:Dq-11 and IQ:Dq-7 isolated from *M. parviflora* were grouped in a sister clade in group II with close relation with Kuwaiti (TYLCV-KISR strain) and Iranian (TYLCV- Ir2 and –OM strains) isolates. TYLCV isolates has been previously divided into two major groups based on CP sequences (Lefeuvre *et al*., 2010). Until now, eight TYLCV strains have been described (Lefeuvre *et al*., 2010; Al-Ali *et al*., 2015) worldwide. The greatest number of strains (five of the eight) has been found in Iran, the fact which marks Iran as the probable center of TYLCV diversity in the world (Lefeuvre *et al*., 2010).

In conclusion we report the natural infection of two weed species as the alternative host of TYLCV in Iraq, which increases the maintenance of the virus and

whitefly vector, the opportunity of recombination and also the genetic diversity. The strain of TYLCV isolates originated from weeds in this study could not be determined based on CP sequence, needs more attempts to obtain full genomic sequences and evaluate genetic diversity of Iraqi TYLCV isolates. Iraq have extensive trading links by sea and also shares a long borderline with Iran, the results of this research emphasize the establishment of an effective quarantine programs to avoid further expansion of TYLCV in Iraq.

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