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Original Article

Genetic diversity and infectivity analysis of tomato yellow leaf curl virus Oman and its associated betasatellite

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Abstract

Tomato yellow leaf curl virus-Oman (TYLCV-OM), a variant of the Tomato yellow leaf curl virus-Iran (TYLCV-IR) strain, was identified in 2005 as the cause of tomato yellow leaf curl disease (TYLCD) in Oman and is associated with a betasatellite namely as Tomato leaf curl betasatellite (ToLCB). Surveys were carried out from three diverse Governorates of Oman to investigate the correlation between the betasatellite and the virus. The visual assessment and scoring of infected tomato plants in the field revealed that the association of betasatellite with the disease was highest in Sharqia at 77%, followed by Dakhlia at41% and lowest in Batinah at30% . Ten isolates from three distinct regions of Oman were analyzed: two from Al Batinah, two from A'Dakhliah, and six from A'Sharquiah. All TYLCV-OM isolates were identified as variants of the 2005 isolates Al Batinah. However, a new recombinant form of TYLCV-OM, which could impact its virulence or spread, was identified in the Al Batinah region. Mutations observed in the Al Batinah isolates of TYLCV-OM coincided with recombination events involving ToLCOMV. Examination of the intergenic regions (IRs) of TYLCV-OM and ToLCOMV indicated that recombination occurred within the IR. Specifically, TYLCV-OM acquired a segment spanning coordinates 1 to 132 nt from ToLCOMV, which may influence its genetic diversity. The implications of these findings for the evolutionary dynamics of the begomovirus complex associated with yellow leaf curl disease are discussed. Inoculation of infectious construct of TYLCV-OM alone or with ToLCB resulted in severe leaf curl symptoms but leaf yellowing was more pronounced in the presence of ToLCB. Real-time quantitative data showed that TYLCV-OM was accumulated to higher level in the presence of betasatellite.

Keywords: Begomovirus, Whitefly borne virus, TYLCV-IR, Mutation, Recombination, Infectivity analysis

1. Introduction

Begomoviruses, belonging to the genus Begomovirus in the family *Geminiviridae*, are characterized by their circular, single-stranded DNA (ssDNA). These viruses, which pose significant threats to vital crops, typically present as incomplete icosahedral particles and are transmitted in a circulative, persistent manner by the whitefly Bemisia tabaci [1]. The majority of begomoviruses are monopartite, although bipartite forms are more common in New World begomoviruses. These bipartite viruses consist of DNA-A and DNA-B molecules, each approximately 2600–2800 nt in size. DNA-A encodes proteins essential for various viral functions, including DNA replication (AC1 or Rep and AC3 or REn), transcriptional activation (AC2 or TrAP), and genome encapsidation (AV1 or CP). On the other hand, genes on DNA-B (BV1 and BC1) facilitate systemic movement of the viral genome within infected plants. These genes are separated by an intergenic region (IR) of around 300 nucleotides, containing motifs for genome replication and transcription, including the origin of replication. Monopartite begomoviruses, resembling DNA A, consist of a single genomic component. Begomoviruses employ two mechanisms for replication: rolling circle replication (RCR) and recombination-dependent replication (RDR). RDR is utilized by viruses to repair damaged or incomplete DNA and generate recombinant variants [2-5].

Begomoviruses enhance their evolutionary flexibility and adaptability to local adaptation through the utilization of gene flow, facilitated by recombination [6, 7]. Genetic exchange has significantly contributed to the diversity and evolutionary dynamics of begomoviruses [8, 9]. Recombination among begomoviruses, it helps in adaptation to heterogeneous environments required by plant viruses. For successful infections, they need to invade different tissues and organs [10]. Environment, insect vector population and host plant population can change with time and with the introduction of new insecticides and resistant cultivars[11, 12]. For that reason, the adaptation of viruses is required for survival. The adaptive capacity of plant viruses is provided by mutation and genetic exchange and component capture [13]. Viruses evolve quickly through genetic exchange of different viruses via recombination or re-assortment [14]. The recombination of viruses can lead to important phenotypic effects, promoting viral adaptation

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and emergence of virulent viruses and different host range [15]. Hence, it is essential to comprehend the function and mechanisms linked with recombination to understand the phenomenon of emergence of novel viruses and enhance control strategies [14].

There are several examples where monopartite begomoviruses have captured components to enhance their virulence and host range. For example, monopartite begomoviruses are often associated with betasatellite/ alphasatellites to overcome host defense or enhance host range. The acquisition of DNA B by monopartite viruses has been documented in cases such as Pepper leaf curl Lahore virus, Sri Lanka cassava mosaic virus, and Tomato leaf curl Gujarat virus. Variations in evolutionary rates between DNA A and DNA B have led to the hypothesis that DNA B may function as a satellite that has adapted to various begomoviruses.

While recombination events can potentially occur across the genome, they frequently involve the intergenic region. The intergenic region (IR) is a crucial non-coding segment in all begomovirus genomes, spanning approximately 300 nucleotides, and is vital for replication and gene expression regulation [16]. Positioned between the two virion-sense genes (V1 and V2) and the four complementary-sense open reading frames (ORFs) of monopartite begomoviruses (C1, C2, C3, and C4), the IR exhibits high similarity between DNA A and DNA B of the same virus species. It encompasses numerous motifs essential for gene expression regulation and replication, earning it the designation of the "common region[17, 18]. It contains iterons or Rep binding motifs which are reiterated sequences recognized by the Rep for binding to initiate RCR. Iterons or cis-acting elements consist of 5-8nt sequences and are repeated directly or inversely 2 to 4 times and situated at changeable distances from the conserved stem loop structure [19, 20]. It is the promoter of the virion and complementary sense ORFs [21]. IR includes a region that contains sequences capable of forming a hairpin loop structure [22]. The structure contains a conserved nonanucleotide sequence 5'-TAATATTAC-3'[23, 24], which contains the recognition site for Rep protein and act as origin of replication (ori) [25, 26]. It contains modular cis-acting elements, including TATA and CA motifs, which play pivotal roles in initiating the transcription of AV1/ AV2 and AC1/AC2, respectively [27, 28].

This work was conducted to study the mutation and recombination in TYLCV-OM effect on the sequence of intergenic region of TYLCV-OM. TYLCV-OM belongs to a group of closely related begomoviruses that emerged in the Middle East and Eastern Mediterranean region and has spread to different parts of the world and is often associated with the dominance of B-biotype of Bemisia tabaci. The region encompassing Iran and its neighboring countries is the center of the diversity of TYLCV.

2. Materials and methods

2.1. Sample collection and DNA extraction

Leaf samples displaying typical symptoms of Tomato Yellow Leaf Curl Disease (TYLCD), indicative of infection by TYLCV, were collected from Al Batinah, and from Al Sharquia and Al Dakhlia during November and December 2011. Total nucleic acids were extracted from these leaf samples utilizing a modified CTAB protocol [29].

2.2. Virus amplification, cloning and sequencing

The circular DNA molecules present in the extracted DNA samples were amplified using rolling-circle amplification (RCA) with TempliPhi DNA amplification kits, following the manufacturer's guidelines (GE Healthcare Life Sciences, NJ, Illinois, USA). The RCA product was then digested using a unique *Pst*I cutter enzyme to generate monomers, which were then coned into into a PUC19 vector to facilitate further analysis. Purification of putative full-length clones was performed using a GeneJET Plasmid Miniprep Kit (Fermentas), followed by sequencing conducted commercially (Macrogen, South Korea).

2.3. Sequence analysis

The sequences were assembled using SeqMan, which is a component of the Lasergene sequence analysis software package (DNA Star Inc., Madison, WI, USA). Sequence similarity researches were conducted by comparing the sequences to other TYLCV sequences available in the database using BLAST. Multiple sequence alignments were carried out using the Clustal V program within the Molecular Evolutionary Genetic Analysis (MEGA) 5.05 version for Mac. A phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei (1993). The Neighbor-Joining algorithm of Clustal V was used to visualize, manipulate, and print the tree using TreeView [30].

2.4. Construction of TYLCV-OM infectious clone

A partial tandem repeat (PTR) of begomovirus clone T16 (DQ644565.1) was prepared for Agrobacterium-mediated inoculation in binary vector pCAMBIA-1301. A fragment of ~1.9kb or 0.7-mer with intergenic region was released from pUC19-TYLCOMV construct using PstI and EcoRI restriction enzymes. This fragment was ligated into *Pst*I/*Eco*RI site of the binary pCAMBIA-1301 vector to produce pCAM-TYLCOMVPE. A monomer of TYLCOMV was released from pUC19-TYLCOMV by digestion with *Pst*I and was ligated head to tail into pCAM-T YLCOMVPE linearized by digestion with *Pst*I to produce pCAM-TYLCOMVPTR. The orientation of the 1.7-mer PTR was confirmed by the digestion with EcoRI to produce a genome-length fragment. Likewise, to generate the infectious construct for the betasatellite clone of TYLCV-OMB Tb-1 (Accession No. HE800544.1), the clone was digested with BamHI and *Xba*I enzymes to release a fragment of approximately 1089 bp. Subsequently, this fragment was cloned into the binary vector pCAMBIA-1301. The resulting partial clone was then linearized using *Xba*I and ligated with the full-length betasatellite component, which was also released with *Xba*I. This process yielded a partial direct repeat construct.

Binary vector constructs were introduced into Agrobacterium tumefaciens strain AGL1 cells using electroporation. Inoculation was performed by cultivating A. tumefaciens cultures in LB medium supplemented with carbenicillin (100 mg/ml) and kanamycin (50 mg/ml) at 28°C for 48 hours until the optical density (OD600) reached 0.6–1.0. Following cultivation, the cells were harvested by centrifugation at 4400 rpm for 15 minutes at 22°C and resuspended in a solution containing 10 mM MgCl2 and 100 mM acetosyringone. After 3 hours of incubation, the cells were infiltrated into the undersides

Table 1. Genome features of tomato yellow leaf curl virus-OM.

Clone	Isolate Description	Accession Number	Size (nt)	Position of genes					
				V1	V2	C1	C2	C3	C4
DT1	OM:DT1:11	JN604487	2767	302-1078	142-492	1551-2615	1220-1627	1075-1479	2165-2458
DT2	OM:DT2:11	JN604488	2767	302-1078	142-492	1551-2615	1220-1627	1075-1479	2165-2458
KW1	OM:KW1:11	JN604484	2777	295-1071	135-485	1521-2609	1213-1620	1068-1472	2159-2452
KW2	OM:KW2:11	JN604485	2776	259-1071	135-485	1535-2608	1213-1620	1068-1472	2158-2451
KW3	OM:KW3:11	JN604486	2767	302-1078	142-492	1551-2615	1220-1627	1075-1479	2165-2458
T-38	OM:T-38:11	HE819245	2774	308-1084	148-498	1557-2621	1226-1633	1081-1485	2171-2446
KB18-7	OM:KB18-7:11	HE819240	2778	308-1084	148-498	1557-2621	1226-1633	1081-1485	2171-2446
Bidbid	OM:Bidbid:11	HE819243	2779	259-1071	135-485	1535-2611	1213-1620	1068-1472	2158-2451
NZ-74	OM:NZ-74:11	HE819242	2763	289-1065	129-479	1538-2602	1207-1614	1062-1466	2152-2445
Bidiya	OM:Bidiya:11	HE819241	2766	301-1077	141-491	1550-2614	1219-1626	1074-1478	2164-2457
Tom 30	OM: Soh:12	KF229721	2763	289-1065	129-479	1538-2602	1207-1614	1062-1466	2152-2445
Tom 36	OM:Li1:12	KF229722	2763	289-1065	129-479	1538-2602	1207-1614	1062-1466	2152-2445
Tom 46	OM:Br3:12	KF229723	2791	309-1085	149-499	1558-2622	1227-1634	1082-1486	2172-2465
Tom 47	OM:Br5:13	KF229724	2790	309-1085	149-499	1558-2622	1227-1634	1082-1486	2172-2465
Tom 48	OM:Br4:13	KF229725	2790	309-1085	149-499	1558-2622	1227-1634	1082-1486	2172-2465
Tom 49	OM:Br6:13	KF229726	2784	302-1078	149-492	1542-2615	1220-1627	1075-1479	2165-2458

of young leaves of 3–4-week-old *Nicotiana benthamiana* and tomato var. *Pusa ruby* plants using a 5 ml syringe, following previously described methods [31]. The plants were maintained in an insect-free glasshouse at 26°C. The presence of the virus and the betasatellite in inoculated plants was confirmed by PCR using primers FDCP-382/RDCP-1038 and beta01/beta02, respectively.

2.5. Southern blot analysis

DNA samples were electrophoresed on 1% agarose gels in 0.5X TAE buffer and transferred to nylon membranes (Roche GmbH, Germany). The virus titer was determined by amplifying the CP gene of the Tom-16 clone (Acc. No. JN604484) using the FDCP-382/RDCP-1038 primer pair (Table 1) and labeling it with digoxigenin with a PCR DIG Probe Synthesis Kit (Roche GmbH, Germany) according to the manufacturer's instructions. For betasatellite detection, a 1089-bp *BamH*I and *Xba*I fragment of a betasatellite clone (Acc. No. HE800544.1) was gel purified and labeled with digoxigenin using a DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche GmbH, Germany).

2.6. Real-time quantitative PCR for quantification of viral molecules in inoculated plants

To quantify viral molecules in inoculated plants exhibiting typical TYLCV-OM symptoms, Quantitative PCR (qPCR) was performed using SYBR Green dye (IQ SYBR Green Supermix by BIO-RAD, USA). A primer set based on the nucleotide sequences of the TYLCV-OM CP gene was designed: QF (5' TAAAAGGCGCACTAATGGGTAGACCGTAGA 3') targeting nucleotides 102-131, and QR (5' GGCGATAACCACCTTCCCG 3') targeting nucleotides 251-233, to amplify a 150 bp product specific to TYLCV-OM.

2.7. Statistical analysis

Statistical analysis for this study was conducted using quantitative real-time PCR data to compare viral DNA levels between plants inoculated with TYLCV-OM alone and those co-inoculated with TYLCV-OM and the betasatellite. The data were analyzed using a t-test to determine the significance of differences in viral DNA accumulation, with a p-value of less than 0.05 considered statistically significant. Error bars in the graphs represent standard deviations, and different letters above the bars indicate significant differences between groups.

3. Results

3.1 TYLCB enhances symptoms of TYLCV-OM and higher levels of virus accumulate in the presence of betasatellite

During the 2011 survey in Al Batinah, Al Sharqiyah, and A'Dakhliyah, typical TYLCV symptoms were observed on tomato plants. These symptoms included upward curling of leaflet margins, reduced leaflet area, yellowing of young leaves, stunting, and flower abortion in early infections. In peppers, symptoms included upward curling of leaf margins, chlorosis of the intervein and leaf margins, vein thickening, and severe yellowing.

Full genomic components of TYLCV were produced using rolling circle amplification followed by digestion with *Pst*I. The components of ten isolates were cloned and sequenced. The full-length viral DNA clones ranged from 2763 to 2790 base pairs (Table 1). Ten clones were fully sequenced and sequences were assembled. The full-length clones were found to have six ORFs. Two ORFs, V1 and V2, were situated on the sense strand of the virus, while four ORFs, C1, C2, C3, and C4, were positioned on the complementary strand.

3.2. Phylogenetic analysis

Full DNA sequence of TYLCV-OM isolates were phylogenetically compared with each other. Al Batinah isolates of 2011 made a separate clade with an identity of 98.3%. The isolates of Al Batinah 2012, three isolates of AlBatinah 2013 and the isolate of Nizwa (A'Dakhliah) cluster together in the second clade with an identity of more than 96.5%. The isolates of A'Sharquia and the isolate of Bidbid (A'Dakhliah) cluster together in the third clade (Figure 1). The isolates of Al Sharquia and the isolate of Bidbid of A'Dakhliah are separated into two variants; the first variant includes (KW1, KW2 and Bidbid) and the second includes (KW3 and Bidiyah) with nucleotide identity of less than 93.6% between the variants and more than 95.1% within each variant. The identity between Al Dakhliah isolates is 92.8% which makes them to be separated into two variants. However, Al Batinah isolates of 2011 shared 98.5% nucleotide sequence identity, and less than 93.8% identity with the isolates of Al Sharquia and A'Dakhliah (Table 2).

Phylogenetically analysis of the intergenic regions (IR) revealed it as the source of variation among the isolates. The nucleotide identity of IR between Al Batinah isolates of 2011 is 100% and shared an identity of 85.8% with ToLCOMV (HE819239). However, the identity between Al Batinah isolates of TYLCV-OM of 2011 and other TYLCV-OM isolates is less than 74.8%. The identity of IR of A'Dakhliah isolates is 95.2% (Table 2).

3.3. Mutation and recombination analysis in the IR region

The analysis of IR of TYLCV-OM isolates of 2005, 2011, 2012 and 2013 from Al Batinah, A'Dakhliah and A'Sharquiah revealed an occurrence of mutations followed by recombination with the 2005 ToLCOMV isolate, as shown in Figure 2. The comparisons of isolates of 2011, 2012 and 2013 with the isolate of TYLCV-OM (OM-Alb12

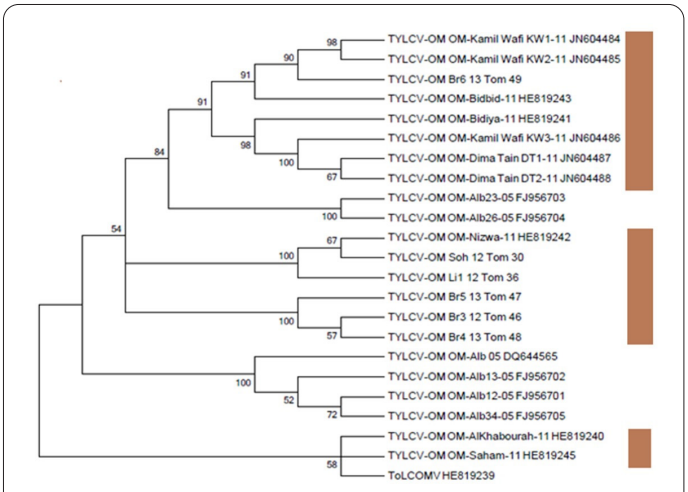

Fig. 1. Phylogenetic dendrograms based upon alignments of full genome of TYLCV-OM from Al Batinah governorates during 2005, 2011, 2012 and 2013, and Al Sharquia and A'Dakhliah during 2011.

Table 2. Percentage nucleotide sequence identities complete genome of Tomato yellow leaf curl virus-Oman from different Governorates.

Governorates	Number of Isolates	% of Identity with Alb 12	% of Identity with Alb 23
Batinah 11	2	95.6-96.5	93.3
Batinah 12	3	96.2-97.3	94.0-94.3
Batinah 13	2	95.6	93.1-93.0
Batinah 13	1	89.8	89.2
Sharq 11	6	91.3-93.8	93.0-90.9
Dakhliah (Nizwa) 11	1	97.1	94.2
Dakhliah (Bidbid) 11	1	93.1	92.0

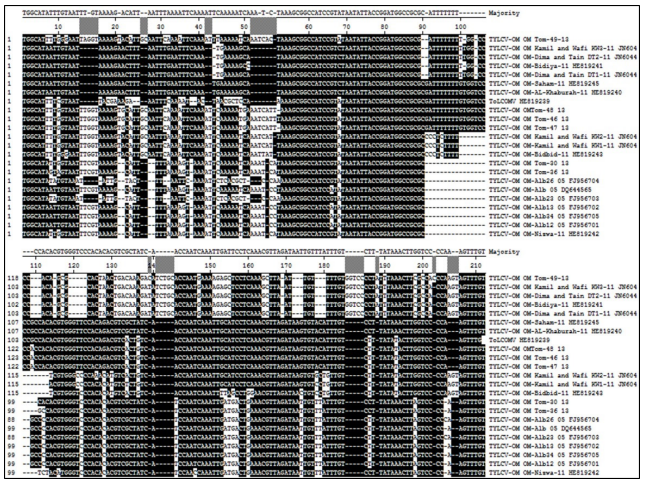

Fig. 2. Intergenic region (IR) organization of TYLCV-OM and ToLCV-OM.

[FJ956701]) of 2005, show different kinds of mutation and recombination with ToLCOMV [HE819239]. TYLCV-OM (OM-Nizwa-11 [HE819242] has a slight mutation, GC was inserted in the coordinates 109-110 and TCT was substituted by CCC in the coordinates 111-113. The isolates of Bidbid of A'Dakhliah [HE819243] and Al Kamil and Alwafi of A'Sharquiah (KW1 [JN604484] and KW2 [JN604485]) of 2011 were found to have three types of mutation; addition, deletion and substitution. In the sequence of these isolates TA, GCAA, CCCTCTTTT and AGT were found to be inserted in the coordinates 2719-2720, 2725-2728, 1-9 and 116-119 respectively. CCCC was found to be deleted from the coordinates 20-24 of the sequence. Substitution of CCT by ATT and GATGA by TTAGC was recorded in the coordinates 2754-2756 and 63-67 respectively. The isolates Al Batinah (Tom 30 [KF229721] and Tom 36 [KF229722]) of 2012 were found to have no mutation or recombination. The isolates of Al Batinah (Tom 46 [KF229723]) of 2012 and (Tom 47 [KF229724] and Tom 48 [KF229725]) of 2013 were found to have an insertion of TG and GCAA in the coordinates 2719-2720 and 2725-2728 respectively. In the same isolates, the substitution of AA by TT and ATCCT by TCATT were recorded in the coordinates 2705-2706 and 2751-2755, respectively. The isolates of Saham [HE819245] and Al Khaburah [HE819240] have deletions of TTCGT, AT and AAATCC in the coordinates 2714-2718, 2740-2741 and 2749-2754 respectively. These two isolates have the insertion of AA, AA and C in the coordinates 2719-2720, 2725-2726 and 2736 respectively. In the sequence of these isolates, the substitution of A by T, G by A, TT by AG, C by G and T by G was found in the coordinates 2722, 2730, 2734-2735, 2743 and 2747 respectively. In addition to the mutation, a significant segment spanning coordinates 1-132 (132 nt) was found to be donated by ToLCOMV [HE819239]. The isolates of A'Sharquiah (DT2 [JN604488], KW3 [JN604486], DT1 [JN604487] and Bidiya [HE819241]) have deletions of TTCGT, AT, AAATCC, GGTCC, G, AAT and TTA in the coordinates 2714-2718, 2740-2741, 2749-2754, 28-32, 79, 82-84, and 88-90 respectively. These two isolates have the insertion of AA, AA, C, ATTTTTTTTGGCCCC, TCTGC, GGTCC and AGT in the coordinates 2719-2720, 2725-2726, 2736, 1-15, 51-55, 96-100 and 117-119 respectively. In the sequence of these isolates, the substitution of A by T, G by A, TT by AG, CA by TG and T by G and AC by TA, GTCGCTATC- by TGACAAAGAT, TTGATGA by AGAGCTC and CG by GC was found in the coordinates 2722, 2730, 2734-2735, 2743-2744 and 2747 and 35-36, 41-49, 61-67 and 74-76 respectively. The isolate of Al Batinah (Tom 49 [KF229726]) has deletions of G, AAT and TTA in the coordinates 79, 82-84, and 88-90 respectively. This isolate has the insertion of GCAA, ATTTTTTTTGGCCCC, TCTGC, GGTCC and AGT in the coordinates 2725-2729, 1-15, 51-55, 96-100 and 117-119 respectively. In the sequence of this isolate, the substitution of AA by TT, T by G, TC by AG, AC by TA, GTCGCTATC- by TGACAAAGAT, TTGATGA by AGAGCTC and CG by GC was found in the coordinates 2705-2706, 35-36, 41-49, 61-67 and 74-76 respectively.

3.4. Infectivity of TYLCV-OM and TYLCV-OMB

The initial symptoms of TYLCV-OM and TYLCV-OM/TYLCV-OMB infection appeared in *N. benthamiana* plants at 15 days post inoculation (dpi), progressively worsening until 21 dpi. In tomato plants, symptoms of TYLCV-OM and TYLCV-OM/TYLCV-OMB infection were first observed at 23 dpi, becoming severe by 28 dpi. In *N. benthamiana*, symptoms included upward leaf curling, vein thickening, yellowing, leaf crumpling, interveinal yellowing, reduced leaf size in young leaves, and plant stunting (Figure 3, panel B). Symptoms in tomato plants primarily manifested as pronounced interveinal chlorosis, particularly along leaf edges, with minimal leaf curling or crumpling (Figure 3, panel E). Older symptomatic leaves also exhibited necrosis around the veins. Betasatellite was detected in 40% of field-collected tomato plants from the Dakhlia region, while 30% of samples tested positive for betasatellite in the Al-Batinah region. The highest percentage 76% betasatellite was found in the Sgarqia region while no TYLCV isolates were identified in the Dhofar region. The interaction of TYLCV-OMB [isolate Tb-1] with TYLCV-OM [isolate Tom-16] was investigated.

In tomato plants co-inoculated with TYLCV-OM/

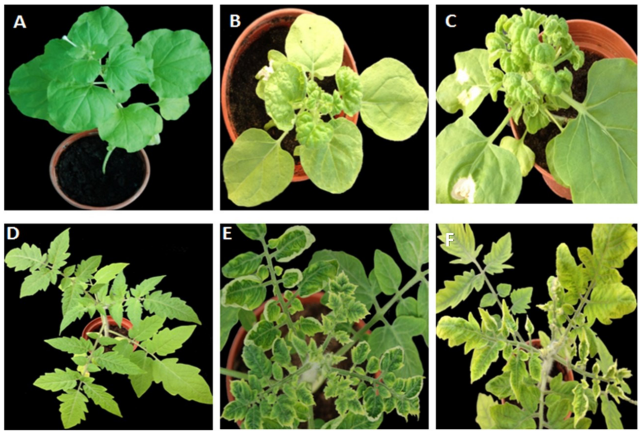

Fig. 3. Tomato and *N. benthamiana* plants exhibiting symptoms of TYLCV-OM infection. Mock-inoculated *N. benthamiana* (A) and tomato (D) plants. *N. benthamiana* (B) and tomato (E) plants inoculated with TYLCV-OM. *N. benthamiana* (C) and tomato (F) plants inoculated with TYLCV-OM and TYLCV-OMB. Images were captured at 30 days post inoculation (dpi).

TYLCV-OMB, increased yellowing and interveinal chlorosis, coupled with an overall reduction in young leaf size, were observed. The presence of betasatellite notably altered symptom expression in tomato plants, with less interveinal yellowing, leaf crumpling, and downward leaf rolling (Figure 3, panel F). Similarly, in *N. benthamiana*, symptoms resembled those induced by the virus alone, with the notable difference of increased leaf crumpling in the presence of betasatellite (Figure 3, panel C).

3.5 Sothern blot analysis and quantification of viral molecules in inoculated plants

Southern blot analysis of DNA samples extracted from symptomatic N. benthamiana and tomato plants (Figure 4, panels A and B) revealed the presence of DNA forms typical of geminivirus replication. Viral DNA levels in plants inoculated with virus alone and those co-inoculated with virus and betasatellite were approximately equivalent (Figure 5, panels A and B).

Further quantitative real-time PCR analysis of inoculated samples demonstrated a slight increase in virus levels in the presence of betasatellite. Significant differences ($p<0.05$) were observed between plants inoculated with TYLCV-OM alone and those inoculated with betasatellite, with virus levels increasing by approximately 2×107 virus particles. The effect of quantitative real-time PCR data reveals that the simultaneous presence of TYLCV and its betasatellite, TYLCV+B altered the viral DNA profile in N. benthamiana and tomato plants. In Figure 5A we can also note that the relative viral DNA levels, represented by the (Tβ1- Tβ10) were higher in the plants co-infected with both the TYLCV and the betasatellite compared with the plants infected just by TYLCV. The differences are mentioned in letters (a and b) above the error bars, which depicts the increase in viral DNA which was found significant at 'p' less than 0.05. Specifically, the betasatellite increased the viral copy number of TYLCV twofold to show the role of betasatellite in viral replication. As would be expected, non-inoculated and non-constructed control plants were devoid of the viral DNA implying that the detection methods used for the virus were specific and effective.

Figure 5B represents the broader QPCR analysis of the individual plants. The plant samples were either inoculated with TYLCV named as sample (T1-T10) or with the combination of TYLCV+B labeled as (TP1-TP10). The samples T1 –T10 which was inoculated with TYLCV only had lower viral DNA value as compared with TP1–TP10 which was inoculated with TYLCV+B. The gradual increases that are observed in the viral accumulation in TYLCV+B also support the hypothesized claim of the betasatellite enhancing viral replication. Coincidently, in some samples we have observed cycles of growth and, therefore, a possible genetic or epigenetic regulation of the host's response to co-infection. Regarding the control samples, in both C1 and C2, the viral DNA level was always low thereby confirming the believability of the experiment developed.

By influencing the TYLCV replication and disease development, these results suggest that the betasatellite plays a key role in this system. When conspicuous maladies such as intensification of yellowing, chlorosis, and the curling of the leaves, the degree of viral DNA is also higher with N. benthamiana and tomato plants. This information will be relevant in the management of TYLCV and its associated diseases in crops as it demonstrates how TYLCV and its betasatellite interact.

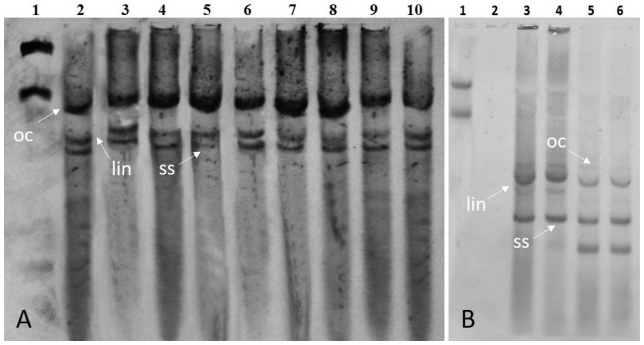

Fig. 4. Southern blot detection was performed to detect TYLCV-OM in agro-inoculated *N. benthamiana* and tomato plants. In panel A, samples included total DNA extracted from a field-infected tomato plant (lane 2), tomato plants inoculated with TYLCV-OM (lanes 3-6), and tomato plants co-inoculated with TYLCV-OM and TYLCV-OMB (lanes 7-10). In panel B, samples consisted of total DNA extracted from a mock-inoculated tobacco plant (lane 2), *N. benthamiana* plants inoculated with TYLCV-OM (lanes 3 and 4), and *N. benthamiana* plants co-inoculated with TYLCV-OM and TYLCV-OMB (lanes 5 and 6). Approximately equal amounts of total DNA (10 µg) were loaded in each case. Viral circular (oc), linear (lin), supercoiled (sc), and single-stranded (ss) DNA forms are indicated. Lane 1 in panels A and B contained 500 ng of TYLCV-OM plasmid clone hybridization controls.

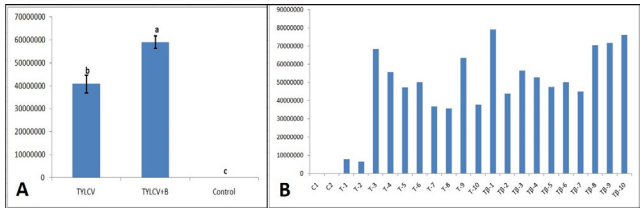

Fig. 5. A: The results are based on cumulative of ten samples for TYLCV and ToLCB; **B:** Represent the virus and betasatellite titer in individual samples.

4. Discussion

TYLCV-OM, identified as the isolate of TYLCV-IR associated with TYLCD in Oman in 2005 [32-34], was observed to be linked with TYLCD symptoms in tomatoes during our 2011 survey. These symptoms include upward curling of leaflet margins, reduced leaflet area, yellowing of young leaves, stunting, and early flower abortion. Our findings, along with those of the previous study by, provide evidence of TYLCV-OM's association with TYLCD in tomatoes during 2011.

The complete DNA sequences of ten isolates from three different governorates in Oman were cloned and sequenced. These genomes formed three distinct clades: the first clade comprised isolates from Al Batinah and Nizwa (A'Dakhliyah), the second clade included four isolates from A'Sharqiyah, and the third clade consisted of two isolates from A'Sharqiyah and one from Bidbid of A'Dakhliyah. The identity between the clades was less than 94%. Following the guidelines of the International Committee on Taxonomy of Viruses for begomovirus variant demarcation [35], these clades are designated as variants. Phylogenetic analysis of the intergenic region (IR) placed the 2011 Al Batinah isolates in a distinct clade compared to the 2005 Al Batinah isolates, with less than 74.8% nucleotide identity.. The alignment of IRs of all isolates of TYLCV-OM and ToLCOMV of Oman shows mutagenic and/or recombination effects on most of 2011 and 2013 isolates of TYLCV-OM. Firstly, a mutation in all the IR took place which produced an isolate that moved to A'Sharquia (DT2 [JN604488], KW3 [JN604486], DT1 [JN604487] and Bidiya [HE819241]). One of these isolates coexists with ToLCOMV [HE819239] in the same host plant and produced a recombinant isolate in which the recombination took place in 3' end IR which produced the isolates of Al Batinah 2011 (Saham [HE819245] and Al Khaburah [HE819240]). The recombinant isolate underwent another mutation in 5' IR and produced the isolates of Al Batinah of 2013 (Tom 46, 47 and 48). The mutated isolate of Al Batinah of 2011 underwent another mutation in 5' end IR and produced an isolate of Al Batinah of 2013 (Tom 49). However, some isolates of Al Batinah of 2005 moved as they are to A'Dakhliah (OM-Nizwa [HE819242] and Bidbid of A'Dakhliah [HE819243]) and A'Sharquiah (OM-KW1 [JN604484] and OM-KW2 [JN604485]) and were established there in 2011. Some isolates of Al Batinah from 2005 passed to 2013 (Tom 30 and 36). This result gave evidence that IR is the source of variation. Plant viruses have adaptive capacity through mutation and recombination [36]. It appears that the mutants were better adapted to the environmental conditions and hosts in Oman compared to the original TYLCV-OM. So selection may have taken place [36] and the original isolates were displaced by the new isolates. The mutant TYLCV-OM dominated Al Batinah Governorates and from there it spread to A'Sharquia and A'Dakhliah Governorates.

A few years later, mutant TYLCV-OM coexisted with a virus known as ToLCOMV in the same host, most probably tomato. ToLCOMV was registered to be associated with ToLCD of tomato in the AlBatinah region of Oman in 2005 [37]. In that host, a recombination-dependant replication (RDR) took place [27, 38]. As a result of RDR, recombination between TYLCV-OM and ToLCOMV took place and recombinant TYLCV-OM emerged. A fragment of the genome of TYLCV-OM situated between the coordinate 1nt to the coordinate 132nt was replaced by an equivalent fragment donated by ToLCOMV. The cross-over site is the IR, within the hot spot situated in the conserved stem-loop structure [1, 39]. Our findings suggest that the recombinant isolates of TYLCV-OM have either a higher accumulation rate in the host or have more opportunity to be transmitted by the vector. As a result, the original isolates were displaced by recombinant isolates of TYLCV-OM which dominated Al Batinah Governorates of Oman in 2011.

Declarations

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Consent for publication

Not applicable.

Competing interests

None of the authors declared any competing interest for this study.

Data availability

The data produced during the study are available in GenBank https://www.ncbi.nlm.nih.gov/nuccore/.

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