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Original article

Identification of *Tomato black ring virus* from tomato plants grown in greenhouses in Saudi Arabia

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A B S T R A C T

A survey was conducted in Al-Kharj governorate, Riyadh region to identify viruses causing variety of virus-like symptoms on tomato plants. A total of 135 samples were collected from symptomatic tomato plants. Symptoms included mottling, deformation, necrosis of leaves and fruits. Eighteen viruses were tested by DAS-ELISA. *Tomato black ring virus* (TBRV) was the virus of concern as it was not detected in Saudi Arabia before and was detected in 52.6% of the collected samples in this study. RT-PCR was used to confirm detection of TBRV and to sequence the amplified products to determine molecular characteristics of this virus. In the host range test study that was performed using a purified isolate of TBRV, sixteen out of the twenty two tested plants showed symptoms. *Brassica oleracea* was not infected by this virus. Gel electrophoreses (2% agarose) yielded fragments of 978 bp of coat protein gene of TBRV. Nucleotide sequences of purified RT-PCR products for three TBRV Saudi isolates were deposited in the GenBank with the following accession numbers MT274656, MT274657, and MT274658. These isolates of TBRV indicated a close Phylogenetic relationship of (99–100%) among themselves and with five isolates from Poland (95–98%) but a distant relationship of 85% with isolates from England and Lithuania deposited in the GenBank. This is the first report for detection and molecular characterization of TBRV infecting tomato plants in Saudi Arabia.
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1. Introduction

The first report of *Tomato black ring virus* (TBRV) on tomato plants was in the United Kingdom by Smith in1946 (Smith, 1946). TBRV belongs to the subgroup B of Genus *Nepovirus*, Subfamily *Comovirinae*, family *Secoviridae*, order *Picornavirales* (Digiaro et al., 2015; Dunez and Le Gall, 2011). Different strains of this virus cause ring spot diseases of bean, sugar beet, lettuce, raspberry, and strawberry, yellow vein of celery, 'bouquet,' pseudo-aucuba of potato, shoot-stunting of peach, one type of mosaic of Robinia pseudoacacia and unnamed diseases of leek and onion, in addition to the black ring of tomato (CABI and EPPO, 1999).

TBRV has three isometric particles (B, M, and T) with a diameter of 26 nm which contain 44%, 32%, and 0% nucleic acid, respectively. TBRV genome consists of two single-stranded RNAs of about 7400nt (RNA1) and 4600nt (RNA2) in length, respectively, carrying a small covalently attached VPg protein at 5′ ends and a poly A tail at their 3′ ends. Each genomic RNA translated to polyprotein, which is cleaved into mature functional proteins by the RNA1-encoded protease. RNA1 is responsible for viral replication and polyprotein processing, while RNA2 carry coat protein and movement genes (Digiaro et al., 2015; Jończyk et al., 2004b; Mayo and Robinson, 1996).

The first report of a mosaic disease on tomato plants in Saudi Arabia was based only on symptoms in 1957 (Talhouk, 1957). After that, different viruses were detected and identified on tomato using serological and/or molecular methods. The identified viruses included *Tobacco mosaic virus* (TMV), *Tomato yellow leaf* curl *virus* (TYLCV), *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Tomato mosaic virus* (ToMV), *Tomato spotted wilt virus* (TSWV), *Potato virus X* (PVX), and *Tomato leaf* curl *virus* (TLCV) (Al-Shahwan et al., 1997; Al-Shahwan, 2003). *Tomato chlorosis virus* (ToCV) and *Tomato leaf* curl *Sudan virus* (ToLCSDV), which are recently

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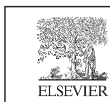

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identified in Saudi Arabia (Al-Saleh et al., 2014a; 2014b; Sohrab et al., 2016). Also TBRV was recently reported in Saudi Arabia (Al-Shahwan et al., 2020).

The objectives of this study are to conduct a survey in the Al-Kharj Governorate, Saudi Arabia, to determine the occurrence of TBRV in greenhouse tomatoes, to determine the biological, serological and molecular characteristics of TBRV in Saudi Arabia, and to determine the phylogenetic relationships among the Saudi isolates and isolates reported in other countries.

2. Material and methods

2.1. Sample collection and ELISA test

Hundred thirty-three samples showing virus like symptoms which included: mottling, mosaic, leaf curling, malformation, blisters, necrotic lesions, black streaks, yellowing of the young emerging leaves, vein chlorosis, flower abortion, death of sepals, fruit deformation, fruit spots, and fruit cracking (Fig. 1) were collected from greenhouse grown tomato plants in different seven locations in Al-Kharj Governorate (six samples from Alhazim, twenty-nine from Addubaiyah, forty-four from Alshadidah, seven from Alyamamh, twenty-two from Nissah, six from Alsahba, and nineteen from Alhayathem), Riyadh region, during four seasons starting fall 2015. These samples were tested for the occurrence of TBRV and seventeen other viruses normally known to infect tomato namely *Alfalfa mosaic virus* (AMV), *Beet curly top virus* (BCTV), *Cucumber mosaic virus* (CMV), *Peanut stunt virus* (PSV), *Pepino mosaic virus* (PepMV), *Potato leafroll virus* (PLRV), *Potato Y virus* (PVY), *Tobacco etch virus* (TEV), *Tobacco mosaic virus* (TMV), *Tomato aspermy virus* (TAV), *Tomato black ring virus* (TBRV), *Tomato bushy stunt virus* (TBSV), *Tomato chlorotic spot virus* (TCSV), *Tomato mosaic virus* (ToMV), *Tomato ringspot virus* (ToRSV), *Tomato spotted wilt virus* (TSWV), *Tomato yellow leaf* curl *virus* (TYLCV), and *Tomato yellow ring virus* (TYRV) by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) using specific kits obtained from AC-Diagnostic Inc. (USA). In addition to, two samples were collected from greenhouse grown tomato from Al-Hasa governorate, and were checked for TBRV only

2.2. TBRV single lesion isolate preparation

The TBRV isolate was biologically purified through a single local lesion technique repeated two times on *Chenopodium amaranticolor* Coste & Reyn plants (Kahn and Monroe, 1963; Kuhn, 1964). After that, a single local lesion was taken and used to prepare inoculation sap, then greenhouse grown *Nicotiana tabacum* plant was mechanically inoculated after being dusted with 400 mish carborundum for propagation and maintenance and kept in the greenhouse (25–28 °C). DAS-ELISA test was used to confirm the infection of *N. tabacum* with the purified TBRV isolate.

2.3. Host range test

Twenty-two plant species belonging to different botanical families were used; which are: *Brassica oleracea, C. album, C. amaranticolor, C. glaucum, C. quinoa, Citrullus lanatus, Cucumis melo, C. sativus, Cucurbita pepo* (zucchini), *C. pepo* (pumpkin), *Datura stramonium, Gomphrena globosa, Lagenaria siceraria, Phaseolus vulgaris, Pisum sativum, N. occidentalis, N. tabacum, N. glutinosa, Solanum lycobersicum, S. melongena, S. nigrum*, and *S. tuberosum* (Brunt et al., 1996). Mechanical inoculation was performed according to a procedure adopted by Hill (1984).

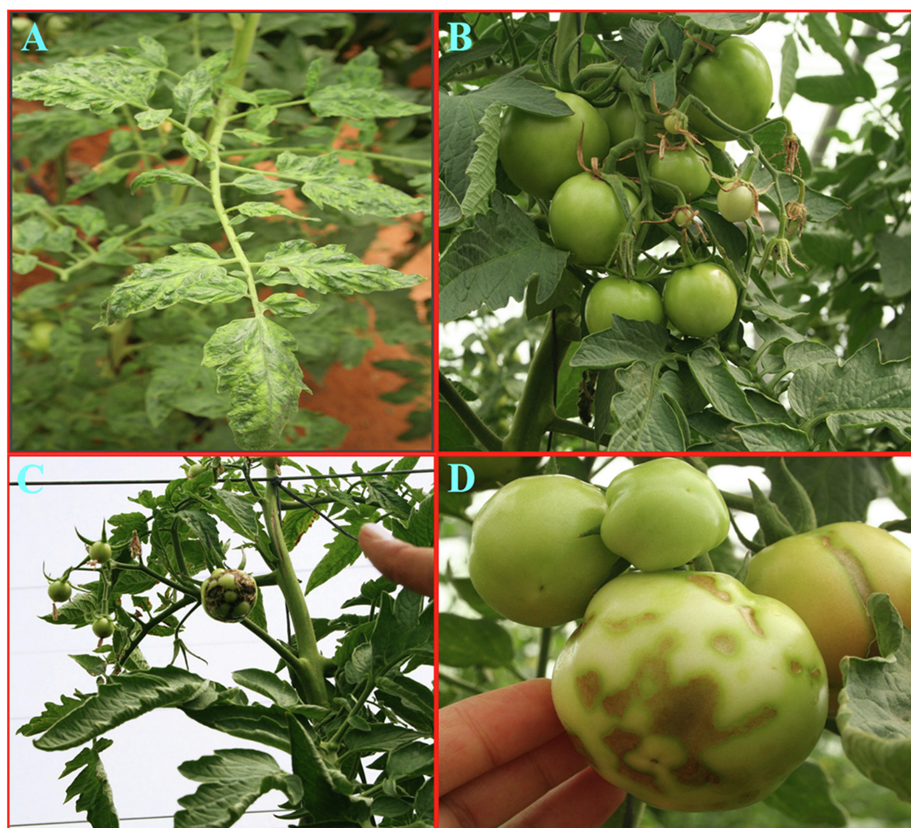

Fig. 1. Some of the virus-like symptoms on greenhouse grown tomato plants observed during sampling. A) Mottling on leaves, B) Necrosis of sepals, C) Fruit deformation, and D) Necrotic blotch on fruits.

Five grams of *N. tabacum* (propagative host) leaves were grounded in a mortar and pestle (infected with a single isolate of TBRV), in fifteen ml of 10 mM Potassium Phosphate Buffer, pH 7. Sterile cheesecloth was used to filter the homogenate. Four replicates were used in this experiment, control plants were treated with buffer only. All plants were kept in the greenhouse at a temperature between 25 and 27 °C. Data for symptoms expression were recorded after three weeks postinoculation. The experiment was conducted two times, and DAS-ELISA was used to check TBRV infection with all tested plants (Hill, 1984).

2.4. RT-PCR, sequencing and sequence analysis

Total RNA extractions were carried out from nine selected positively DAS-ELISA samples, a total RNA isolation kit (Jena Bioscience, Jena, Germany) was used for this purpose. A pair of primers were designed and used to amplify a fragment of 978 bp in the CP region, F-TBRV: 5′-GCAAACCAACGCTCTATGTTGT-3′, and R-TBRV: 5′-AGAGCCAAACTGGAATGGTAGG-3′.

Two-step RT-PCR protocol was used. First-strand complementary DNA (cDNA) was synthesized with specific R-TBRV primer in 20 µl volume using MultiScribe Reverse Transcriptase (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems™, USA) according to the manufacturer's instructions. The subsequent PCR in 25 µl volume was performed using one µl RT product as a template cDNA. Amplification was done by using the PCR Master Mix kit from Promega Corporation (Cat no. 75050, Part# 9PIM750) following the manufacturer's instructions. PCR cycling conditions comprised 2 min of denaturation at 95 °C, then 35 cycles of 95 °C for 30 sec, 65 °C for 45 sec, and 72 °C for 1 min, followed by final cycle at 72 °C for 5 min. Amplified PCR products were then visualized on 1% Agarose Gel as the target DNA size was about 978 bp. The gel was stained with ethidium bromide. PCR products were stored at −20 °C for further use. FavorPrep GEL/PCR Purification mini Kit from FAVORGEN BIOTECH CORP. www.favorgen.com was used to purify the PCR products. Purified DNA for the three selected isolates (two from Al-Kharj K9, K41 and one from Alhasa H1 along with specific primers were sent to the Macrogene company, South Korea (Macrogen 10F, 254, Beotkkot-ro, Geumcheon-gu, Seoul, South Korea) for two-directional sequencing. Sequences from the bidirectional sequencing were blasted on the NCBI BLAST program for nucleic acids, and the sequences were identified and compared with other published TBRV sequences on the NCBI website. Sequences were aligned, cleaned by the BioEdit program. These sequences were imported to Lasergene 7 program to check identity percentage of the Saudi isolates of TBRV with each other, and with ten published TBRV isolates on the NCBI website, and then the homology tree was constructed.

3. Results

3.1. ELISA results

The available eighteen different ELISA kits mentioned in the material and methods section were used to detect the following viruses: AMV, BCTV, CMV, PSV, PepMV, PLRV, PVY, TEV, TMV, TAV, TBRV, TBSV, TCSV, ToMV, ToRSV, TSWV, TYLCV, and TYRV.

The obtained ELISA results showed that all these viruses have been detected in the collected samples in Al-Kharj governorates, Saudi Arabia except PLRV, TBSV, and TSWV. Results showed that 59.4% (79 / 133) of tomato samples (symptomatic samples) were infected with at least one of the detected viruses. Sixty nine out of hundred thirty three samples (51.9%) were found positive to TBRV and out of seventy- nine (87.3%) of DAS-ELISA positively samples were found positive to TBRV, 65.2% were singly infected with TBRV. Whereas 18% of all samples were have mixed infection with at least one virus (Table 1). The collected two samples from Al-Hasa were checked by DAS-ELISA for TBRV only and were positive.

3.2. Host range

The host range experiments were done with a pure singly local lesion TBRV isolate (MT174657). Symptoms were observed on sixteen host plants out of the 22 tested plant species. The observed symptoms were local necrotic lesions (*C. amaranticolor* and *Datura stramonium*), Chlorotic local spots (*C glaucum*, and *C. album*), Chlorotic spots develop to systemic chlorosis (*C. quinoa*), mottling (*N. glutinosa*, and *C. pepo*), systemic ring spots and mosaic (*N. tabacum, and G. globosa*), mosaic (*S. nigrum*, and *P. vulgaris*), stunting and cup-shaped leaf (*S. tuberosum*), mottling and marginal chlorosis (*C. sativus*), mottling and leaf deformation (*S. lycopersicum*), and systemic necrosis on *C. melo* (Fig. 2). Six plant species did not show symptoms. These were *B. oleracea, C. lanatus, C. pepo* (Pumpkin), *L. siceraria, P. sativum*, and *S. melongena*. Five of these plant species (*C. lanatus, L. siceraria, C. pepo* (Pumpkin), *P. sativum*, and *S. melongena*) were found to be symptomless carrier as they were positive to DAS-ELISA. *B. oleracea* was the only plant species among all host range plants that did not show symptoms and negative to ELISA. It was found that twenty-one out of twenty-two plant species were positive to TBRV by DAS-ELISA (Table 2).

3.3. RT-PCR, sequencing and sequence analysis

cDNA products give smears with all samples, confirming that cDNA was synthesized. Amplification of cDNA gave PCR products, and specific bands were visible with all samples, and their comparison with 50 bp Bioline HyperLadder DNA marker indicated that

Table 1
Detected viruses in greenhouse tomato samples collected from different locations in Al Kharj governorate, Riyadh region.

Locations	Positive DAS-ELISA results																
	TBRV	AMV	BCTV	CMV	PepMV	PSV	PVY	TAV	TEV	TCSV	TMV	ToMV	ToRSV	TYLCV	TYRV	Mixed infection	TBRV single infection
Alhazim	6	1	1	1	0	2	1	1	0	2	4	2	0	0	4	6	0
Addubaiyah	14	0	0	0	6	0	0	0	0	2	5	3	0	3	0	7	7
Alshadidah	29	0	0	0	1	1	0	0	1	2	0	0	2	1	0	4	25
Alyamamh	3	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	2
Nissah	4	0	2	2	0	0	2	2	0	0	0	0	0	0	0	2	2
Assahba	6	0	0	0	0	0	0	1	0	0	2	0	0	0	0	2	4
Alhayathem	7	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2	5
Total	69	2	5	3	7	3	3	4	1	7	11	5	3	4	4	24	45
Percentages	51.9	1.5	3.8	2.3	5.3	2.3	2.3	3.0	0.8	5.3	8.3	3.8	2.3	3.0	3.0	18	65.2

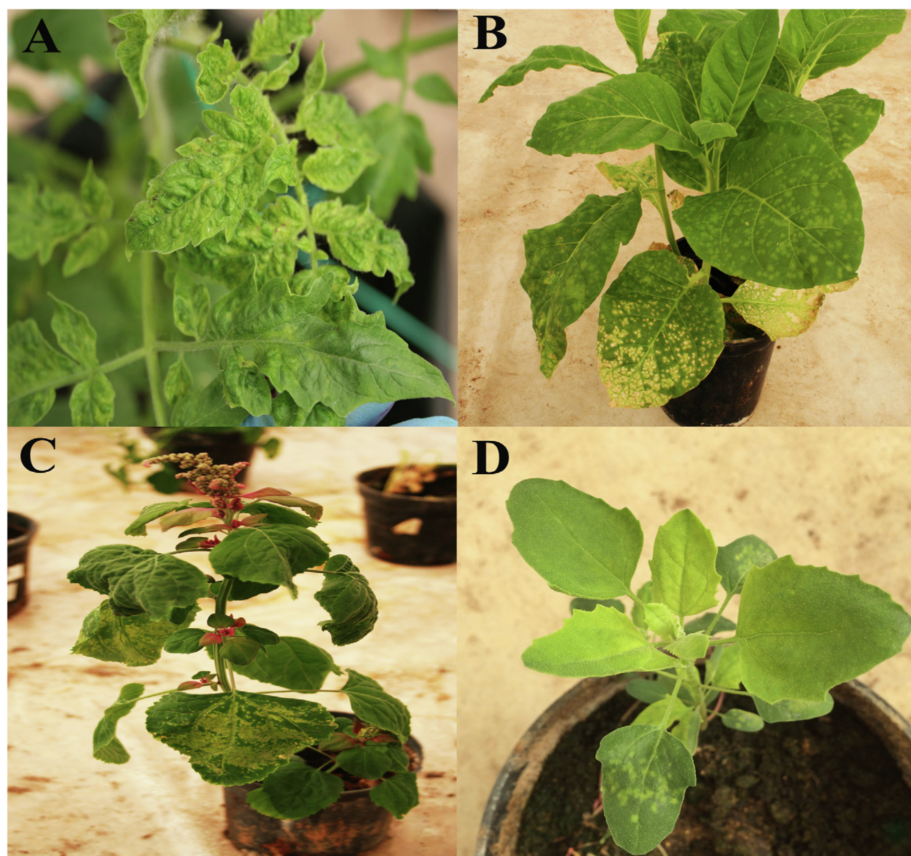

Fig. 2. The observed symptoms of TBRV on some host range tested plant species: A) Mottling and blisters on *S. lycobersicum*, B) Systemic Ring spots on *N. tabacum*, C) Necrotic spots on *C. amaranticolor*, and D) Chlorotic spots symptoms on *C. quinoa.*

the size of the specific bands was approximately 978 bp, confirming the presence of TBRV in these samples (Fig. 3).

Sequences of the three Saudi TBRV isolates were submitted to GenBank and assigned the following accession numbers: MT274656 from Al-Hasa, while MT274657, and MT274658 from Al-Kharj. Phylogenetic analyses for the coat protein gene sequence of Saudi and GenBank isolates of TBRV confirmed the percentage similarity between them (Fig. 4). Percentage identity data between all TBRV isolates revealed that all the Saudi isolates were closely related (99–100%), and these isolates were also closely related to isolates from Poland (MG458221 and KX977561) with a percentage of nucleotide similarity of 98–99% (Hasiów-Jaroszewska et al., 2018; Zarzyńska-Nowak et al., 2017), and distantly related to isolates from Lithuania (KF678369, and KF678370) and England (X80831.1) (Le Gall et al., 1995; Šneideris and Staniulis, 2014) (Table 3).

Table 2
Symptoms expression of host range plants three weeks after mechanical inoculation with a pure isolate of TBRV under greenhouse conditions, and their response to DAS-ELISA.

Plant Species	Symptoms	DAS-ELISA Results
Chenopodium album	Chlorotic local spots	+
Chenopodium amaranticolor	Chlorotic and necrotic local spots	+
Chenopodium glaucum	Chlorotic local spots	+
Chenopodium quinoa	Chlorotic spots develop to systemic chlorosis	+
Datura stramonium	Necrotic local spots	+
Gomphrena globosa	Systemic Ring spots and mosaic	+
Nicotiana glutinosa	Local chlortic spots and Mottling	+
Nicotiana occidentalis	Tip necrosis	+
Nicotiana tabacum	Systemic Ring spots and mosaic	+
Solanum lycopersicum	Mottling, leaf deformation	+
Solanum nigrum	Mosaic	+
Solanum tuberosum	Stunting and cup shaped-like leaves	+
Phaseolus vulgaris	Mosaic	+
Cucumis sativus	Mottling and marginal chlorosis	+
Cucumis melo	Systemic necrosis	+
Cucurbita pepo (zucchini)	Mottling	+
Brassuca oleracea	No symptoms	–
Citrullus lanatus	No symptoms	+
Cucurbita pepo (Pumpkin)	No symptoms	+
Lagenaria siceraria	No symptoms	+
Pisum sativum	No symptoms	+
Solanum melongena	No symptoms	+

4. Discussion

Tomato, one of the most important vegetable crops worldwide suffers infection from several plant pathogens. Several viruses were reported to infect it (Brunt et al., 1990; Hanssen et al., 2010; Jones et al., 2014). Of the 15 viruses detected (out of 18 tested ones) in the samples collected in this study, TBRV was the virus of concern as it was detected for the first time in Saudi Arabia in this investigation and was the most frequently detected virus with a frequency of detection of 52.6%. Although TBRV was discovered on tomato more than six decades ago, scarce reports of its infections were published, and they were mostly on other

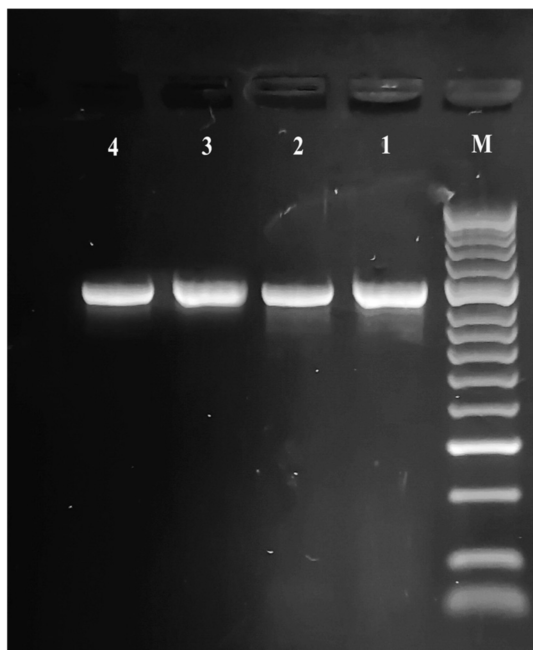

Fig. 3. Gel electrophoresis in 2% agarose showing RT-PCR products amplified from four tomato samples using specific primer pair designed to amplify 978 bp fragment of coat protein gene of TBRV. Representation of each well is written above the well. Lane M represents 50 bp Bioline HyperLadder DNA marker. 1, 2, 3 represent samples from Al-Kharj and 4 represent sample from Alhasa.

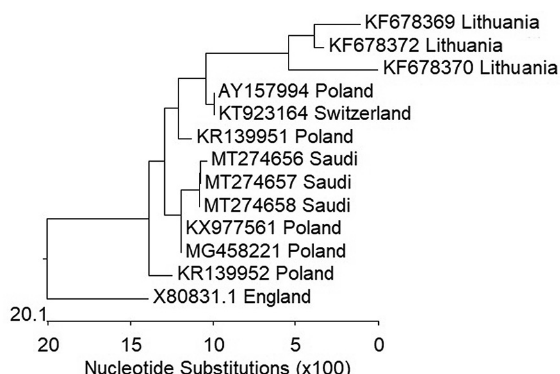

Fig. 4. Constructed homology tree based on multiple sequence alignment of the coat protein gene of Saudi isolates and ten other isolates of TBRV obtained from GenBank.

crops causing considerable disease incidence and yield losses (Gallitelli et al., 2005; Harrison, 1959; Hollings, 1965) due to its ability of transmission through several methods including mechanical transmission, transmission through seeds, pollen, and two species of nematodes namely *Longidorus attenuatus* and *L. elongatus* (Digiaro et al., 2015; Harrison, 1961; Pospieszny et al., 2020).

Two samples were collected from Al-Hasa Governorate in order to check the spread of TBRV in areas other than Al-Kharj. These samples reacted positively to TBRV, which suggests that this virus enters and spread in Saudi Arabia probably by imported seeds (Johansen et al., 1994; Lister and Murant, 1967; Pospieszny et al., 2020).

The wide host range of this virus and its ability to be transmitted in several ways, probably made it one of the important plant viruses. It is interesting that whereas this virus causes symptomatic infection on plants, it also sometimes causes symptomless infection on other plants (Rymelska et al., 2013). Five of the host range plants in this study were symptomlessly infected with this virus when tested by DAS-ELISA and were positive. All of them were previously reported as hosts for this virus (Bellardi and Pisi, 1985; Brunt et al., 1990; CABI, 2016; Rymelska et al., 2013). Also *B. oleracea* was negative to TBRV by DAS-ELISA which was not reported before as a host for this virus (Table 1).

The polymerase chain reaction (PCR) is a useful method used to detect different plant viruses (Digiaro et al., 2007). TBRV RNA2 has a single open reading frame that encodes polyprotein, which then were cleaved by protease to give the coat protein and movement protein (Demangeat et al., 1992; Digiaro et al., 2015; Jończyk et al., 2004b; Mayo and Robinson, 1996). Coat protein gene generally used in these types of studies as it was proved as a handy tool for plant virus characterization including *Nepoviruses* as they were used in this study (Hasiów-Jaroszewska et al., 2015; Šneideris and Staniulis, 2014).

The TBRV population is highly diverse, and the phylogenetic analysis based on the coat protein gene (CP) sequences of the Saudi isolates and other imported NCBI isolates revealed the presence of distinct groups with the nucleotide sequence variability of the CP gene ranging from 0 to 15% similar to what was reported earlier (Jończyk et al., 2004a; Pospieszny et al., 2020; Šneideris and Staniulis, 2014). CP gene nucleotide sequence comparisons show that all Saudi TBRV isolates are located in the same clade of the Polish isolates while it located in a different clade for England isolate and that are in a cluster in a different clade of Lithuanian isolates (Le Gall et al., 1995; Šneideris and Staniulis, 2014).

Table 3
Percentage identity between three Saudi isolates of *Tomato black ring virus* (TBRV) and isolates obtained from other countries deposited in the GeneBank.

Accession	Country	Host	Saudi Isolates		
			MT274656	MT274657	MT274658
MT274656	Saudi Arabia	*S. lycopersicum*	100	100	99
MT274657	Saudi Arabia	*S. lycopersicum*	100	100	99
MT274658	Saudi Arabia	*S. lycopersicum*	99	99	100
MG458221	Poland	*S. lycopersicum*	98	98	97
KX977561	Poland	*S. lycopersicum*	98	98	97
KR139951	Poland	*S. tuberosum*	96	96	95
KR139952	Poland	*Cucumis sativus*	95	95	94
KT923164	Switzerland	–	94	95	95
AY157994	Poland	*R. pseudoaccacia*	94	95	95
KF678372	Lithuania	*Clematis* sp.	88	88	88
KF678370	Lithuania	*Lamprocapnos spectabilis*	85	86	86
KF678369	Lithuania	*Hosta* sp.	85	85	85
X80831	England	*Daucus carota*	84	85	85

5. Conclusions

TBRV was detected for the first time in greenhouse tomato plants grown in Saudi Arabia. Some plant species react silently to TBRV infection without expressing any symptoms which may consider as a source of infection in the greenhouse. Since TBRV was detected in Al-Kharj Governorate in Riyadh Region, and in Al-Hasa Governorate in the Eastern Region the rest of the Kingdoms regions ought to be surveyed to determine the extent of this virus spread in the country. The suggested survey will be more useful if it also includes hosts other than tomato such as grape, potato, strawberry, and other hosts in the Kingdom of Saudi Arabia.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent was obtained from all individual participants included in the study.

Authors contributions

A. Al-Shudifat, I. Al-Shahwan, and M. Al-Saleh: samples collection and conducting the experiments. A. Al-Shudifat, O. Abdalla, and M. Amer: analysis and interpretation of results. A. Al-Shudifat, and I. Al-Shahwan, and O. Abdalla: draft manuscript preparation. All authors reviewed the results and approved the final version of the manuscript.

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