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Investigation of Tomato Ringspot Virus (ToRSV) by Real-Time TaqMan RT-PCR in Hakkari Province, Turkey

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ABSTRACT

Tomato ringspot virus (ToRSV) belongs to the Nepovirus genus in the family *Secoviridae*. It has a wide host range and is listed as a quarantine virus in Turkey. In this study, 80 leaf samples were collected from tomato, pepper, cucumber and grapevine cultivation sites located in three different parts of Hakkari province: Şemdinli, Çukurca and Center districts. Real-time TaqMan reverse transcription-polymerase chain reaction (RT-PCR) method was used for the detection of the virus. Amplification was carried out in reaction mix including QuantiNova Probe RT-PCR kit (Qiagen, Germany) using primers and TaqMan probe based on 3'-UTR (untranslated region) of virus, which amplified a 182 bp product of the genome. ToRSV was detected in 13 of the 80 samples and threshold cycle (CT) values ranged from 23.9 to 37.4. It was found that 16.25% of the samples collected from the districts of Hakkari province were found to be infected with ToRSV whereas no ToRSV was detected in the samples collected from the center of the city. The virus was detected on pepper and cucumber samples in Çukurca district, and it was also detected in tomato, pepper, cucumber and grapevine samples in Şemdinli district. To our knowledge, this study is the first report of molecular detection of ToRSV by real-time TaqMan RT-PCR in Turkey.

Keywords: ToRSV, Molecular detection, Tomato, Pepper, Cucumber, Grapevine, Turkey

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1. Introduction

ToRSV (tomato ringspot virus, genus Nepovirus, subgroup C, family *Secoviridae*) is a bipartite single-stranded, positive sense RNA virus (Sanfaçon et al. 2006; 2009). ToRSV primarily infects perennial plants such as tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana tabacum*), grapevine (*Vitis vinifera*), blueberry (*Vaccinium corymbosum*), strawberry (*Fragaria vesca*), geranium (*Pelargonium domesticum*), raspberry (*Rubus idaeus*), blackberry (*Rubus fruticosus, Rubus sp*), walnut (*Juglans regia*) and ornamental plants and causing diseases that results in great economic losses. Experimental host diversity of ToRSV is also very high and about 35 families are susceptible to this virus (Samuitiene et al. 2003; OEPP/EPPO 2005; Fuchs et al. 2010; Sneideris et al. 2012; Tzanetakis & Martin 2013; Zindovic et al. 2014). The most typical symptom of ToRSV infection in plants is the presence of annular spots on the leaves. It has also other conspicuous symptoms in fruit trees and grapevines. In the grapevines, the virus manifests itself especially with necrotic pitting, spongy phloem tissue, fall of fruit, the rosette formation of leaves, ring spots on the leaves and general decrease in yield (OEPP/EPPO 2013). In infected plants, the effect of the virus can be seen as, pale yellow and pale green spots on the leaves that develop along the major side veins or the main vein of the leaves and causing systemic chlorotic or necrotic ring stains and deformation as well as inhibition of the fruit growth. In certain cases the virus does not show any visible symptoms, being usually characterized by a decrease in the yield. ToRSV is transmitted by natural ways, such as seeds, transplantation, pollen, vegetative organs and different species of *Xiphinema* (Bitterlin et al. 1987; Pinkerton et al. 2008).

The objective of this research is to determine the presence of ToRSV in tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), cucumber (*Cucumis sativus*) and grapevine (*Vitis vinifera*) samples,collected from three different districts of Hakkari province, by real-time TaqMan RT-PCR method.

2. Material and Methods

2.1. Field surveys and sample collection

In early autumn of 2014 and summer of 2015, 80 leaf samples of tomato, pepper, cucumber and grapevine plants were collected from Çukurca, Şemdinli and Center districts of Hakkari province (Durankaya, Kırıkdağ, Üzümcü, Çimenli, Geçitli

villages in the Center; Narlı, Geçimli, Kayalı villages in Çukurca; Bağlar, Şapatan, Güzelkonak, Yukarıyokuş, Balova villages in Şemdinli district). The samples were collected from various plant species based on the presence of suspicious viral symptoms at the time of sampling, such as necrosis, chlorosis, mosaic, and ring stains and transported to the laboratory in cool conditions and stored at 4 °C until tested.

2.2. Preparation of primers and total nucleic acid extraction

A pair of primers and probe were synthesized to amplify the 182-bp region in 3'-UTR of ToRSV RNA1 genome and used at the real-time TaqMan RT-PCR method (Table 1). RNA extraction from leaf samples of tomato, pepper, cucumber and grapevine were conducted by using the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) as specified in the manufacturer's protocol. Total nucleic acids were extracted from 80 samples and tested for the presence of ToRSV along with positive and negative controls.

Table 1- List of primers and probe used for detecting ToRSV

Primer/Probe		*Sequence*	*Target gene and position*	*Reference*
ToRSV-UTR (Forward primer)	F	5'-GAATGGTTCCCAGCCACTT-3'	3' –UTR 7686-7704 bp of RNA1	Tang et al. 2014
ToRSV-UTR (Reverse primer)	R	5'-AGTCTCAACTTAACATACCAC-3'	3' –UTR 7847-7867 bp of RNA1	
ToRSV-UTR (Probe)	P	FAM-5'-AGGATCGC-TACTCCTCCGTCAAC-3'-BHQ-1	prob –7746–7768 bp	

2.3. The real-time TaqMan RT-PCR

The 3'-UTR sequence of RNA1 genome of ToRSV was amplified by real-time TaqMan RT-PCR method. Positive control was used to obtain high accuracy and optimization in real-time TaqMan RT-PCR. The plant sample (the original host: *Pelargonium* sp) obtained from Leibniz-Institut DSMZ German Collection of Microorganisms and Cell Cultures (Germany) was used as positive control. Nuclease-free water was used as negative control. The total RT-PCR reaction mix was prepared by using QuantiFast Probe PCR (Qiagen, Germany) and it consisted of 1 µl forward primer (0.3 µM), 1 µl reverse primer (0.3 µM), 1 µl prob (0.5 µM), 10 µl 2xProbe RT-PCR Mix, 0.2 µl QuantiFast RT Mix, 4.4 µl $MgCI_2$ (5.5 µM) and 1.4 µl RNase-free water. 5 µl of RNA isolated from leaf samples was added to the mix, amounting a total of 20 µl. For amplification, complementary DNA (cDNA) was synthesized at 50 °C for 10 min, initial denaturation was conducted at 95 °C for 5 min and amplification step were performed in a total of 40 cycles at 95 °C for 15 min, and at 60 °C for 45 sec. Real time RT-PCR analyses were performed using Rotor-Gene Q (Qiagen, Germany) and Rotor Gene Q Series Software (version 2.3.1).

3. Results and Discussion

3.1. Field observation

Field surveys were conducted in Çukurca, Şemdinli and Center districts of Hakkari province during the 2014-2015 growing season. It was observed during the surveys that vegetable farming is generally done without the use of pesticide in these areas and so that plants are susceptible to viral and other infectious agents such as bacteria, fungi etc. The samples were collected in accordance with the common symptoms that are known to be caused by ToRSV on tomato, pepper, cucumber and grapevine (Figure 1). The plants showing no apparent known symptoms were also sampled for control. Eighty samples were collected from 13 villages in study area (Table 2).

Figure 1- Symptomatic plants collected in the field survey from Hakkari province: a) Tomato b) Grapevine c) Pepper d) Cucumber

Table 2- List of plant samples collected from Hakkari province for real-time TaqMan RT-PCR analyses

No	*Host*	*Location*	*No*	*Host*	*Location*
1	Tomato	Çukurca-Narlı	41	Cucumber	Şemdinli-Balova
2	Tomato	Çukurca-Narlı	42	Grapevine	Şemdinli-Bağlar
3	Tomato	Çukurca- Narlı	43	Grapevine	Şemdinli-Bağlar
4	Tomato	Çukurca-Narlı	44	Grapevine	Şemdinli-Bağlar
5	Tomato	Çukurca-Geçimli	45	Grapevine	Şemdinli-Şapatan
6	Tomato	Çukurca-Geçimli	46	Grapevine	Şemdinli-Şapatan
7	Tomato	Çukurca-Kayalı	47	Grapevine	Şemdinli-Şapatan
8	Tomato	Çukurca-Kayalı	48	Grapevine	Şemdinli-Yukarıyokuş
9	Pepper	Çukurca-Narlı	49	Grapevine	Şemdinli-Yukarıyokuş
10	Pepper	Çukurca-Narlı	50	Grapevine	Şemdinli-Yukarıyokuş
11	Pepper	Çukurca-Geçimli	51	Grapevine	Şemdinli-Yukarıyokuş
12	Pepper	Çukurca- Geçimli	52	Grapevine	Şemdinli-Yukarıyokuş
13	Pepper	Çukurca-Narlı	53	Grapevine	Şemdinli-Yukarıyokuş
14	Pepper	Çukurca-Narlı	54	Grapevine	Şemdinli-Şapatan
15	Pepper	Çukurca-Narlı	55	Grapevine	Şemdinli-Şapatan
16	Pepper	Çukurca-Narlı	56	Grapevine	Şemdinli-Şapatan
17	Cucumber	Çukurca-Narlı	57	Cucumber	Şemdinli-Güzelkonak
18	Cucumber	Çukurca-Narlı	58	Cucumber	Şemdinli-Güzelkonak
19	Cucumber	Çukurca-Kayalı	59	Cucumber	Şemdinli-Güzelkonak
20	Cucumber	Çukurca-Kayalı	60	Tomato	Center-Durankaya
21	Tomato	Şemdinli-Balova	61	Tomato	Center-Durankaya
22	Tomato	Şemdinli-Balova	62	Tomato	Center-Durankaya
23	Tomato	Şemdinli-Güzelkonak	63	Tomato	Center-Durankaya
24	Tomato	Şemdinli-Güzelkonak	64	Tomato	Center-Durankaya
25	Tomato	Şemdinli-Güzelkonak	65	Tomato	Center-Durankaya
26	Tomato	Şemdinli-Yukarıyokuş	66	Tomato	Center-Durankaya
27	Tomato	Şemdinli-Yukarıyokuş	67	Pepper	Center-Durankaya
28	Tomato	Şemdinli-Yukarıyokuş	68	Pepper	Center-Durankaya
29	Pepper	Şemdinli-Güzelkonak	69	Pepper	Center-Durankaya
30	Pepper	Şemdinli-Güzelkonak	70	Pepper	Center-Durankaya
31	Pepper	Şemdinli-Güzelkonak	71	Tomato	Center-Üzümcü
32	Pepper	Şemdinli-Balova	72	Tomato	Center-Üzümcü
33	Pepper	Şemdinli-Balova	73	Grapevine	Çukurca-Narlı
34	Pepper	Şemdinli-Balova	74	Cucumber	Center-Kırıkdağ
35	Pepper	Şemdinli-Balova	75	Cucumber	Center-Kırıkdağ
36	Pepper	Şemdinli-Balova	76	Pepper	Center-Kırıkdağ
37	Cucumber	Şemdinli-Balova	77	Pepper	Center-Kırıkdağ
38	Cucumber	Şemdinli-Balova	78	Cucumber	Center-Çimenli
39	Cucumber	Şemdinli-Balova	79	Grapevine	Center-Çimenli
40	Cucumber	Şemdinli-Balova	80	Cucumber	Center-Geçitli

3.2. Molecular detection

The CT value of the positive control was 15.6. After determining the appropriate program for real-time TaqMan RT-PCR with the positive control, the procedure was applied to the other samples. After the tests for optimization, a total of 80 samples were evaluated by real-time TaqMan RT-PCR. The real-time TaqMan RT-PCR tests conclusively proved the presence of ToRSV in the province. Real-time TaqMan RT-PCR analysis of 80 samples collected in the field surveys revealed that 13 (16.25%) samples were infected with ToRSV. According to the real-time TaqMan RT-PCR results, CT value of ToRSV infected samples ranged from 23.88 to 37.41 (Table 3). Samples with CT value greater than 38 were ignored.

Table 3- CT (cycle threshold) values obtained from real-time TaqMan RT-PCR analyses of different plant samples collected from Hakkari province

The collected field	*Host*	*No of infected ToRSV the sample*	*CT value*
Çukurca-Geçimli	Pepper	12	33.49
Çukurca-Kayalı	Cucumber	20	35.99
Şemdinli-Balova	Tomato	21	32.66
Şemdinli-Balova	Tomato	22	34.09
Şemdinli-Güzelkonak	Tomato	24	30.9
Şemdinli-Güzelkonak	Tomato	25	37.29
Şemdinli-Yukarıyokuş	Tomato	26	33.49
Şemdinli-Yukarıyokuş	Tomato	27	37.41
Şemdinli-Yukarıyokuş	Tomato	28	32.48
Şemdinli-Balova	Pepper	34	34.96
Şemdinli-Balova	Pepper	35	37.04
Şemdinli-Balova	Cucumber	40	33.21
Şemdinli-Şapatan	Grapevine	54	23.88

The data obtained showed that ToRSV incidence was highest in Şemdinli district (28.20%) and lowest in Çukurca district (10%). ToRSV was detected in the tested tomato, pepper, cucumber and grapevine samples. None of the samples collected from Center were found to be infected with the ToRSV (Table 4). The results showed that ToRSV can be found in various cultivation sites in Hakkari province, but the virus is not wide spread in Hakkari province.

Table 4- ToRSV infection rate in tomato, pepper, cucumber and grapevine samples collected from Hakkari province

		Collected Samples -ToRSV Infected Samples				*Avarage infection rate (%)*
Province	*District*	*Tomato*	*Pepper*	*Cucumber*	*Grapevine*	
Hakkari	Center	9-0	6-0	4-0	2-0	0
	Çukurca	8-0	8-1	4-1	0-0	10
	Şemdinli	8-7	11-2	5-1	15-1	28.2
Total		25-7	25-3	13-2	17-1	16.25

ToRSV is a virus with a very wide host range. The damage caused on plants by this virus has encouraged us to work on it. ToRSV spreading from North America to other parts of the world, is also reported from Netherlands, Chile, Australia, Iran (Samuitiene et al. 2003; Moini 2010; Sokhansanj et al. 2012; Rivera et al. 2016; Roberts et al. 2018).

Presence of ToRSV can be determined by biological indexing, serological and molecular methods. Mechanical inoculation to herbaceous plants is also applied and is known to be simple and reliable. On the other hand, biological indexing is a time-consuming method and it requires considerable experience, meaning that only a limited number of plants can be tested by use of this method. Enzyme Linked Immunosorbent Assay (ELISA) and Double-Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) can be used for serological diagnosis of ToRSV. Moini (2010) detected ToRSV by ELISA method in the leaf samples collected from apples in the north-east region of Iran. The genome of most plant viruses consists of RNA. Detection of the RNA sequence by PCR requires some changes. Prior to the application of PCR, RNA must have a reverse copy called cDNA. The RT-PCR is a very sensitive method and there may be inhibition problems in the samples. It should also be noted that the use of this method requires experienced researchers (OEPP/EPPO 2005). Detection of ToRSV by RT-PCR has been developed for multiple strains of ToRSV in both herbaceous and woody plants (Griesbach 1995). Msikita (2007) compared ELISA with RT-PCR methods for ToRSV detection and preferred RT-PCR with the identified appropriate primary sequence. Digiaro et al. (2007) studied the development of degenerate and specific primers for differential and simultaneous RT-PCR detection between subgroups A, B and C of grape infecting nepoviruses. They were designed

specifically for RNA-1 3'-UTR for grapevine and provided a source for studies on the determination of this factor in grapevine with obtained positive results. A real-time RT-PCR test has been developed for rapid and sensitive detection of ToRSV. Stewart et al. (2007) tested samples for ToRSV primarily by ELISA. Real-time RT-PCR detection of ToRSV was performed in host tissues and a comparison was made between real-time PCR and ELISA. It was concluded that the results obtained by real-time PCR were more sensitive than ELISA. It was also seen that the samples that did not show positive results by ELISA were positive when tested in much lower amounts by real-time RT-PCR. Osman et al. (2008) compared low-density sequences using real-time TaqMan PCR and RT-PCR in the detection of grapevine viruses and examined the reliability of the results for ToRSV. This was the first report on the use of low-density sequences in the detection of plant viruses. Tang et al. (2014) detected the presence of ToRSV on grapevine by targeting RNA-1 3'-UTR region by real-time Taqman RT-PCR. In terms of specificity, sensitivity and reliability in the detection of ToRSV, real-time TaqMan RT-PCR and other real-time RT-PCR methods were compared. The real-time TaqMan RT-PCR used in that study was designed for the highly conserved region of ToRSV 3'-UTR. The TaqMan real-time RT-PCR test showed that the method can be widely used in the overall detection of ToRSV over a wide range of hosts and it also served as a resource for the method used in our research.

4. Conclusions

The presence of ToRSV in Turkey has been reported in tomato, pepper, cucumber (Fidan 1995; Arlı-Sökmen & Şevik 2006), stone fruit (Azeri & Çiçek 1997), blackberry (Sertkaya 2010) and strawberry (Yeşilçöllü et al. 2011). The methods used in these studies were ELISA and RT-PCR. In the studies where the primary method was real-time RT-PCR, grapevine was preferred as a host for detection of ToRSV. In this study, we detected the presence of ToRSV in different hosts. Samples with CT values ≤38 were accepted infected with ToRSV. CT value of the positive control was found to be 15.6, but CT value of the other samples that were considered positive was higher. CT values increased as the density of the virus decreased in the samples. This may have stemmed from the evaluation of different hosts. In the survey, ToRSV was detected in tomato, pepper, cucumber and grapevine in Şemdinli district and in pepper and cucumber in Çukurca of Hakkari province. Of the 80 samples, 13 (16.25%) samples were found to be infected with ToRSV and it is good to note that areas infected with ToRSV are uncommon. ToRSV-infected plants are concentrated mostly in Şemdinli. It is noteworthy that the uncommon use of pesticides and the use of local seeds in the fields observed are widespread. ToRSV can be transmitted by mechanically, nematode vectors, seeds and pollen in some plants, therefore it will be appropriate to comply with the internal quarantine rules. Although there have been a number of attempts to identify the presence of ToRSV in Turkey, this study is the first report of molecular detection of ToRSV in different hosts by real-time TaqMan RT-PCR.

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