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STUDY OF THE DISTBUTION OF TOMATO BROWN RUGOSE FRUIT VIRUS (ToBRFV) IN SOUTHERN BULGARIA

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Abstract

The aim of the study is to analyze and investigate viral pathogens on tomatoes and peppers to detect Tomato Brown Rugose Fruit Virus (ToBRFV) in seeds and plant samples. The only hosts of Tomato brown rugose fruit virus are tomato (Solanum lycopersicum L.) and pepper (Capsicum annuum L.). The spread of ToBRFV carries major risks in tomato and pepper cultivation which are important crops grown in Europe. The symptoms resemble those of other viral infections, such as Tomato Mosaic virus, Pepino Mosaic virus and others found in Bulgaria. To achieve the aim of the study and establish ToBRFV over the two-year period, laboratory analyses were performed on more than 28 seed samples of tomates and peppers, imported or in movement within the EC. About 47 plant samples from greenhouses, with the origin of the plants from other countries, were also analyzed. Samples were tested using the ELISA method for five viruses. As a result of this mass screening, the ToBRFV virus was detected on tomato seeds in Bulgaria for the first time. On the recommendation by the phytosanitary authorities, the infected seeds and the tomato plants produced from them were destroyed in order to prevent and limit the future spread of Tomato Brown Rugose Fruit Virus on the territory of Bulgaria.

Key words*: ToBRFV, ELISA method, spread, destruction, Bulgaria.*

INTRODUCTION

The virus was first detected in 2014-2015 in Israel (EPPO, 2017) and Jordan (Salem et al., 2016), where the incidence in some areas reached 100%. Over the next few years, due to its ability to overcome the resistance of Tm-2/Tm-22 (and Tm-1) resistance genes in tomatoes (Luria et al., 2017), it spread rapidly in production sites. In 2018-2019 it was reported and detected in Italy (Sicily, Panno et al., 2020), Germany (Menzel et al., 2019), Turkey (Fidan et al., 2019), Greece and the Netherlands (EPPO, 2019), where measures were taken to eradicate the infestation. Tomato brown streak virus belongs to the Tobamovirus group and has the characteristic features of this group, such as high stability and infectivity. Mechanical transmission of the virus is highly pronounced, as in tobacco mosaic (TMV), (Kovacevsky, 1970) tomato mosaic (ToMV) (Jones et al., 2014) and other viruses of this group (Broadbent, 1976; Hollings and Huttinga, 1976). The virus is transmitted contactly via contaminated tools, hands, clothing, from plant to plant, infected seeds and planting material, and persists in plant debris and contaminated soil for months to several years (Dombrovsky and Smith, 2017; EPPO, 2020). Tomato brown streak virus (ToBRFV) has no known natural vector (Adams et al., 2016), but possible transmission of tobamoviruses by bumblebees (*Bombus terrestris*) (Levitzky et al., 2019) and birds was reported (Peters et al., 2012). The main hosts of Tomato brown rugose fruit virus are tomatoes (*Solanum lycopersicum* L.) and peppers (*Capsicum annuum* L.) (Luria et al., 2017; Cambrón-Crisantos et al., 2018; Fidan, 2020). ToBRFV symptoms are broad-spectrum and appear on leaves, petioles and fruit. On leaves, symptoms appear first on the young upper part of the plant, such as surface malformation, chlorosis and mosaic with clear chlorotic pale spots. Subsequently, the nervature and leaf petura are deformed, wrinkled and distinct discoloration (browning) is observed, and eventually the plants die. Brown necrotic lesions, similar to ToMV and TMV, are observed on leaf petioles of infected plants, and fruits ripenen unevenly, expressed by the appearance of red, brown and green areas at the same time, symptoms similar to PepMV (Alkowni et al., 2019). With peppers, foliar symptoms include deformation, yellowing and mosaic. Fruits are deformed, with yellow or brown areas or green stripes, plants suffer from slow growth and often die (Luria et al., 2017). The spread of ToBRFV

carries great risks when growing tomatoes and peppers which are important crops grown in Bulgaria. On the basis of the EU Decision 2019/1615 a program was established for the monitoring of vegetable crops for quarantine pests and for monitoring and sampling in the production of tomatoes and peppers. Reports of newly discovered and eradicated outbreaks of tomato brown streak virus in neighboring countries (Fidan et al., 2019; EPPO, 2019) and on our territory (EPPO, 2021) stimulated us to investigate the occurrence of this virus in regions with traditions in the production of tomatoes and peppers in Bulgaria.

The aim of the present study is to analyze and investigate tomato and pepper viral pathogens with the aim of identifying tomato fruit brown streak virus (ToBRFV) in seeds and plant samples in regions with a tradition of vegetable production, such as Southern Bulgaria. The analysis of the obtained data will serve to prepare recommendations for the improvement of the currently applied tomato and pepper cultivation technology in greenhouses and outdoors and will affect the increase of producers' incomes.

MATERIALS AND METHODS

A total of 75 samples were analyzed during the serological tests at the Forestry University in Sofia, of which 42 plant, 5 fruit and 28 seed samples originated from Southern Bulgaria. Samples arrived at the laboratory after random visual inspections of plants with symptoms of virus infection or were sent by farmers to assess seed health or problems in greenhouse production from the study areas. 8 samples were analyzed in 2021 and 67 samples in 2022. The leaf and fruit samples with and without symptoms were homogenized 1:20 (w/v) in PBS extraction buffer. Seed samples were soaked at 4°C for 4 h in 1:25 (w/v) extraction buffer, followed by homogenization. In the present study, we used enzyme-linked immunosorbent assay (ELISA) as the most reliable, rapid, highly specific and cost-effective method (Clark and Adams, 1977). DAS-ELISA serological test kits containing antibody (IgG) and alkaline phosphatase conjugate (IgG-AP) for ToBRFV and PepMV detection were from (Loewe, Germany), TMV/ToMV (Sediag SAS, France, Agdia, USA), TSWV and CMV (Loewe, Germany and Bioreba AG, Switzerland) and Loewe controls developed based on existing scientific publications and standards (Luria et al., 2017; Panno et al., 2020; Ling, 2007; De Àvila , 1992; Palukaitis et al., 1992; Albrechtsen, 2006; Blystad et al., 2015; EPPO, 2015). ELISA extraction buffer (pH 7.4); Carbonate buffer (Coating BF, pH 9.6); Conjugate buffer (Conjugate BF, pH 7.4); Diethanol amine buffer (DEA BF, pH 9.8); PBS-T wash buffer (pH 7.4). Antibodies and conjugates at dilutions recommended by the manufacturers were aplied. The reaction results were read using an ELISA reader (spectrophotometer) at a wavelength of λ 405 nm and repeated for correction at λ 495 nm. As a positive reaction, we consider all results showing more than 2 times the values of optical density (OD) readings of the negative control.

RESULTS AND DISCUSSIONS

During the first year of the study, 39 laboratory analyses were performed and on 8 samples and virus infection was detected - in 3 samples from the region of the city of Kardzhali on peppers with TSWV infection and in two samples from the region of the city of Plovdiv from samples of tomatoes with TSWV and pepper plants with CMV infection. After performing the serological laboratory analysis, ToBRFV, TMV, ToMV and PepMV was not detected in 2021 (Table 1).

In 2022, we expanded the survey areas and included those in which there are already reports from the NPPO. Visual inspections in greenhouses revealed signs of virus infection similar to ToBRFV and other viruses, which was confirmed by laboratory diagnostics (Figure 1).

Figure 1. Symptoms of ToBRFV (A) and tomato plants Pink ID variety (B) (Blagoevrgad, Zh. Avramov)

Table 1. Results of laboratory analyzes for ToBRFV, TMV/ToMV, PepMV, TSWV, CMV by the years, host plant, their origin and virus infection detected

Year/No	Culture	Sample	Region	ToBRFV	TMV / ToMV	PepMV	TSWV	CMV	Results +	Virus +
2021/1	Pepper	Fruits	Kardzali	1	2	1	1	1	1	TSWV
2021/2	Pepper	Plant	Plovdiv	1	2	1	1	1	1	CMV
2021/3	Tomato	Plant	Plovdiv	1	2	1				
2021/4	Tomato	Fruits	Plovdiv	1	2	1	1	1	1	TSWV
2021/5	Pepper	Plant	Plovdiv	1	2			1		
2021/6	Tomato	Plant	Vratsa	1	2	1	1			
2021/7	Tomato	Plant	Vratsa	1	2	1	1			
2021/8	Pepper	Seeds	Pazardzhik	1		1		1		
2022/9	Tomato	Seeds	Plovdiv	1	1	1				
2022/10	Tomato	Seeds	Plovdiv	1	1	1				
2022/11	Tomato	Seeds	Plovdiv	1		1				
2022/12	Tomato	Seeds	Plovdiv	1		1		1		
2022/13	Tomato	Seeds	Plovdiv	1		1		1		
2022/14	Tomato	Seeds	Plovdiv	1		1		1		
2022/15	Tomato	Seeds	Plovdiv	1		1				
2022/16	Pepper	Seeds	Plovdiv	1		1		1		
2022/17	Pepper	Seeds	Plovdiv	1		1				
2022/18	Pepper	Seeds	Plovdiv	1		1				
2022/19	Pepper	Seeds	Plovdiv	1		1				
2022/20	Pepper	Seeds	Plovdiv	1		1		1		
2022/21	Pepper	Seeds	Plovdiv	1		1		1		
2022/22	Tomato	Plant	Blagoevgrad	1	2	1	1	1		
2022/23	Tomato	Plant	Blagoevgrad	1	2	1	1	1	1	ToBRFV
2022/24	Tomato	Plant	Blagoevgrad	1	2	1	1	1		
2022/25	Tomato	Plant	Kyustendil	1	2	1	1	1		
2022/26	Tomato	Plant	Kyustendil	1	2	1	1	1		
2022/27	Tomato	Plant	Kyustendil	1		1	1			
2022/28	Tomato	Plant	Kyustendil	1		1	1			
2022/29	Tomato	Plant	Kyustendil	1	2	1	1	1		
2022/30	Tomato	Plant	Kyustendil	1	2	1	1	1		
2022/31	Tomato	Plant	Kyustendil	1	2	1	1			
2022/32	Tomato	Plant	Kyustendil	1	2	1	1			
2022/33	Tomato	Plant	Kyustendil	1		1	1	1		
2022/34	Tomato	Plant	Kyustendil	1		1	1	1		
2022/35	Pepper	Plant	Kyustendil	1		1	1	1		
2022/36	Pepper	Plant	Kyustendil	1		1	1			
2022/37	Pepper	Plant	Kyustendil	1		1	1			
2022/38	Pepper	Plant	Kyustendil	1		1	1			
2022/39	Pepper	Plant	Kyustendil	1		1	1			
2022/40	Pepper	Plant	Kyustendil	1		1	1			
2022/41	Pepper	Plant	Kyustendil	1		1	1			
2022/42	Tomato	Plant	Vratsa	1	2	1	1	1	1	ToBRFV
2022/43	Tomato	Fruits	Vratsa	1	2	1	1	1	1	ToBRFV
2022/44	Pepper	Plant	Vratsa	1	2	1	1	1	1	ToBRFV
2022/45	Tomato	Plant	Smolyan	1	2	1	1	1	1	ToBRFV
2022/46	Tomato	Plant	Vratsa	1	2	1	1	1		
2022/47	Tomato	Plant	Vratsa	1	2	1	1			
2022/48	Tomato	Plant	Vratsa	1	2	1	1			
2022/49	Tomato	Plant	Vratsa	1	2	1	1			
2022/50	Tomato	Plant	Pazardzhik	4		4	1	3	1	ToBRFV
2022/51	Tomato	Plant	Blagoevgrad	1	2	1	1	1	1	ToBRFV
2022/52	Tomato	Fruits	Blagoevgrad	1	2		1	1	1	ToBRFV
2022/53	Tomato	Fruits	Blagoevgrad	1	2	1	1	1	1	TSWV
2022/54	Tomato	Plant	Smolyan	1	2	1	1	1	1	ToBRFV
2022/58	Pepper	Plant	Pazardzhik	1		1	1	1		
2022/59	Tomato	Plant	Smolyan	1				1		
2022/60	Tomato	Plant	Kyustendil	1	2	1	1	1	1	ToMV
2022/61	Tomato	Seeds	Plovdiv	1		1				
2022/62	Tomato	Seeds	Plovdiv	1		1				
2022/63	Tomato	Seeds	Plovdiv	1		1				
2022/64	Tomato	Seeds	Plovdiv	1		1				
2022/65	Tomato	Seeds	Plovdiv	1		1				

2022/66	Tomato	Seeds	Plovdiv	1		1				
2022/67	Tomato	Seeds	Plovdiv	1		1				
2022/68	Tomato	Seeds	Plovdiv	1		1		1		
2022/69	Tomato	Seeds	Plovdiv	1		1		1		
2022/70	Tomato	Seeds	Plovdiv	1		1				
2022/71	Tomato	Seeds	Plovdiv	1		1				
2022/72	Tomato	Seeds	Blagoevgrad	1		1		1	1	ToBRFV
2022/73	Tomato	Plant	Blagoevgrad	1	1	1	1	1		
2022/74	Tomato	Seeds	Plovdiv	1		1				
2022/75	Pepper	Seeds	Pazardzhik	1	1	1		1		
				75	62	72	41	41	15 Positive	

After carrying out 252 laboratory analyses of a total of 67 samples, 10 samples with a positive serological result for ToBRFV were found in 2022 (Table 1). In the region of Blagoevgrad, it was found in 4 samples of tomatoes in plant samples, seeds and fruit (Figure 2). In the Vratsa region, ToBRFV infection was confirmed in 2 samples of plant and fruit tomato and in 1 pepper plant sample. In the region of the city of Smolyan, infection by the virus was detected in 2 plant samples of tomatoes, and in the region of the city of Pazardzhik, a virus infection by ToBRV was detected. In 2 samples of tomato plants, TSWV infection originating from the region of the city of Blagoevgrad and ToMV originating from the region of the city of Kyustendil was detected (Figure 2). The results of laboratory analyses did not prove ToBRFV infection in the regions of the city of Plovdiv, Kardzhali and Kyustendil. The distribution of the results by culture, nature of the samples, surveyed areas, and the number of analyses performed for possible viral infection are shown in Table 2.

Table 2. Positive results of laboratory analyzes for ToBRFV, TMV/ToMV, TSWV, CMV and their origin.

Culture	Sample	No of samples	Region	ToBRFV	TMV / ToMV	PepMV	TSWV	CMV	Results +	Virus +
Pepper	Plants	11	Plovdiv	2	4	1	1	2	1	CMV
			Vratsa	1	2	1	1	1	1	ToBRFV
			Pazardzhik	1		1	1	1		
			Kyustendil	7		7	7			
	Seeds	8	Plovdiv	6		6		3		
			Pazardzhik	2	1	2		2		
	Fruits	1	Kardzali	1	2	1	1	1	1	TSWV
Tomato	Plants	31	Plovdiv	1	2	1				
			Vratsa	7	14	7	7	2	1	ToBRFV
			Pazardzhik	4		4	1	3	1	ToBRFV
			Smolyan	3	4	2	2	3	2	ToBRFV
			Blagoevgrad	5	9	5	5	5	2	ToBRFV
			Kyustendil	11	14	11	11	8	1	ToMV
	Seeds	20	Plovdiv	19	2	19		5		
			Blagoevgrad	1		1		1	1	ToBRFV
	Fruits	4	Plovdiv	1	2	1	1	1	1	TSWV
			Vratsa	1	2	1	1	1	1	ToBRFV
			Blagoevgrad	2	4	1	2	2	2	ToBRFV TSWV
Total amount		75 samples	7 regions	291 laboratory analizes					10 + 1 + 3 + 1 +	ToBRFV CMV TSWV ToMV

The result of one positive sample from the analyzes of 28 samples of tomato and pepper seeds confirmed the claims (Giesbers et al., 2021; EPPO, 2022) that the serological tests for these samples are not suitable for the detection of ToBRFV in seeds. Serological tests for ToBRFV

are currently adequate for the identification of the virus in plant samples and fruits, but should be accompanied by others to cover the full nature of the host samples tested. Reports of ToBRFV virus infection in 2022 were confirmed in the region of Smolyan and Pazardzhik and in two new regions of Vratsa and Blagoevgrad in southern Bulgaria, where farmers use imported seeds or seedlings originating from third countries or EU Member states.

Figure 2. Symptoms of infected tomatoes: A) and B) fruits with ToBRFV, Manusa variety; C) plant with ToBRFV, Pink ID variety; D) and E) TSWV on tomato fruits, Pink ID variety (Blagoevrgad region, Zh. Avramov)

CONCLUSIONS

The present study is the first scientific evidence of the spread of ToBRFV in southern Bulgaria. The visual symptoms are close to those of ToMV, TSWV and CMV on the host plants like tomato and pepper. To prove viral infection by ToBRFV in seed samples, the use of molecular identification methods is also necessary. Strictly compliance with phytosanitary measures by all farmers and the recommendations of NPPO to control of ToBRFV is necessary in order to limit the spread of the virus on the territory of Bulgaria.

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