



## Pepino mosaic virus, a new threat for Serbia's tomatoes

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### Abstract

**Aim of study:** To report the occurrence of Pepino mosaic virus (PepMV) on tomato in Serbia and to genetically characterize Serbian PepMV isolates.

**Area of study:** Tomato samples showing virus-like symptoms were collected in the Bogojevce locality (Jablanica District, Serbia).

**Material and methods:** Collected tomato samples were assayed by DAS-ELISA using antisera against eight economically important or quarantine tomato viruses. Three selected isolates of naturally infected tomato plants were mechanically transmitted to tomato 'Novosadski jabučar' seedlings. For confirmation of PepMV infection, RT-PCR was performed using specific primers PepMV TGB F/PepMV UTR R. Maximum-likelihood phylogenetic tree was constructed with 47 complete CP gene sequences of PepMV to determine the genetic relationship of Serbian PepMV isolates with those from other parts of the world.

**Main results:** The results of DAS-ELISA indicated the presence of PepMV in all tested samples. Mechanically inoculated 'Novosadski jabučar' seedlings expressed yellow spots and light and dark green patches, bubbling, and curled leaves. All tested tomato plants were RT-PCR positive for the presence of PepMV. The CP sequence analysis revealed that the Serbian PepMV isolates were completely identical among themselves and shared the highest nucleotide identity of 95.1% (99.2% aa identity) with isolate from Spain (FJ263341). Phylogenetic analysis showed clustering of the Serbian PepMV isolates into CH2 strain, but they formed separate subgroup within CH2 strain.

**Research highlights:** This is the first data of the presence of PepMV in protected tomato production in Serbia. Considering increased incidence and rapid spread in Europe, the presence of PepMV on tomato could therefore represent serious threat to this valuable crop in Serbia.

**Additional key words:** ELISA test; molecular detection; CP gene; phylogenetic analysis.

**Abbreviations used:** aa (aminoacid); AMV (Alfalfa mosaic virus); CMV (Cucumber mosaic virus); CP (coat protein); DAS-ELISA (double antibody sandwich enzyme linked immunosorbent assay); nt (nucleotide); PepMV (Pepino mosaic virus); PVY (Potato virus Y); RT-PCR (reverse transcription polymerase chain reaction); TMV (Tobacco mosaic virus); ToMV (Tomato mosaic virus); TSWV (Tomato spotted wilt orthotospovirus); TYLCV (Tomato yellow leaf curl virus).

**Authors' contributions:** IS, AV, KZ, BP and DR collected samples and carried out the experiment. IS, AV and IV contributed to data analysis. IS wrote the paper. BK supervised the work and critically revised the manuscript. All authors read and approved the final article.

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## Introduction

Pepino mosaic virus (PepMV; genus Potexvirus; family Alphaflexiviridae) is an emerging virus causing devastating yield losses in tomato crops worldwide (Ling, 2007; Gómez *et al.*, 2012a; Hanssen & Lapidot, 2012). PepMV

was described for the first time infecting pepino in Peru (Jones *et al.*, 1980), but first infection of tomato was detected in the main tomato growing areas in the Netherlands and Great Britain (Wright & Mumford, 1999; van der Vlugt, 2000) in the late 1990s. Thereafter, the virus has spread rapidly in the tomato crops and was discovered in many

countries in Europe, Asia, and North America (Mumford & Metcalfe, 2001; French *et al.*, 2001; Aguilar *et al.*, 2002; Cotillon *et al.*, 2002; Ling, 2007; Hanssen & Thomma, 2010; Gómez *et al.*, 2012a,b). PepMV is currently included in the European Plant Protection Organization A2 list (EPPO, 2016) as the causative agent of an important disease in protected tomato crops, resulting in huge yield and market value losses (Spence *et al.*, 2006).

PepMV has flexuous rod-like particles about 500 nm, that contain a single-stranded, positive-sense RNA genome of about 6.4 kb, flanked by 5' and 3' untranslated regions (UTRs) with 5' cap and a 3' poly(A) tail. Virus genome consists of five open reading frames (ORFs): ORF1 encoding replication-associated proteins, including the viral RNA-dependent RNA polymerase (RdRp); ORF2-ORF4 encoding the triple gene block (TGB) proteins TGB1, TGB2 and TGB3 involved in virus movement; and the ORF5 encoding the coat protein (CP) (Mumford & Metcalfe, 2001; Aguilar *et al.*, 2002; Pagán *et al.*, 2006). Based on the sequence analysis and biological characteristics, five different PepMV strains have been recognized so far: LP (the original Peruvian strain), EU (European strain), US1 (American strain), CH2 (Chilean strain), and PES (the new Peruvian strain infecting wild tomatoes) (Hanssen & Thomma, 2010; Moreno-Pérez *et al.*, 2014; Agüero *et al.*, 2018). Moreover, mixed infections with different strains, as well as recombination among them were also detected (Pagán *et al.*, 2006; Hanssen *et al.*, 2008; Pospieszny *et al.*, 2008; Hasiów-Jaroszewska *et al.*, 2010b). EU and CH2 are the most common in Europe, but currently isolates belonging to CH2 strain have spread rapidly and become predominant (Hanssen *et al.*, 2008; Gómez *et al.*, 2009, 2012b). PepMV efficiently spreads mechanically from plant to plant, while long distance spread of the virus is through contaminated seeds or infected transplants (Córdoba-Sellés *et al.*, 2007; Hanssen *et al.*, 2010).

Despite the fact that PepMV has expanded rapidly in Europe in recent years and has become a major pathogen of tomato production in Mediterranean basin (Gómez *et al.*, 2012a; Hanssen & Lapidot, 2012), the presence of the virus has not yet been recorded in Serbia (Nikolic, 2018; Nikolic *et al.*, 2018). This paper describes the first finding of PepMV in Serbia and provides information on partial molecular characterization of the isolates.

## Material and methods

### Sampling and serological detection

In July 2019, during a survey to determine the presence of tomato viruses in Serbia, virus-like symptoms, including yellow angular spots accompanied by leaves necrosis and distortion, as well as fruit discoloration were observed on tomato 'Runner' grown in two separate plastic tunnels in

the Bogojevce locality (Jablanica District, Serbia). Before sampling, disease incidence was estimated in each crop by counting plants exhibiting virus-like symptoms in a random batch of 100 plants in four replicates. Symptomatic plants were sampled and assayed by double-antibody sandwich (DAS)-ELISA test using commercial polyclonal antisera (Bioreba AG, Reinach, Switzerland) against eight economically important or quarantine tomato viruses, including: Cucumber mosaic virus (CMV), Potato virus Y (PVY), Tomato spotted wilt orthotospovirus (TSWV), Alfalfa mosaic virus (AMV), Pepino mosaic virus (PepMV), Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), and Tomato yellow leaf curl virus (TYLCV). After incubation with *p*-nitrophenyl phosphate (Sigma-Aldrich, USA) at room temperature for 1-2 h in the dark, absorbance at 405 nm was measured with an ELISA microplate reader (DAS srl, Italy). Samples were considered positive if the mean absorbance value at 405 nm was two-fold higher than the mean of the negative control. Commercial positive and negative controls (Bioreba AG, Switzerland) were included in each test.

### Biological assay

Mechanical transmission of PepMV to test plants was performed using three ELISA-positive samples. Symptomatic leaves were grounded in 0.01 M phosphate buffer (pH 7) and mechanically inoculated on five plants of tomato seedlings (*Solanum lycopersicum* 'Novosadski jabučar') per each sample at the 2-3 true-leaf stage. The test plants were kept under greenhouse conditions up to four weeks post-inoculation for symptoms development. Upper leaves of all inoculated plants were assayed by DAS-ELISA to confirm PepMV presence.

### RNA extraction and RT-PCR assay

Serological findings were verified with reverse transcription (RT)-PCR assay. Total RNAs from all naturally infected tomato plants were extracted using a cetyltrimethylammonium bromide (CTAB) protocol (Bekesiova *et al.*, 1999) and subjected to reverse transcription (RT)-PCR assay. RT-PCR was performed with the One-Step RT-PCR Kit (Qiagen GmbH, Germany) using PepMV-specific primer pair, PepMV TGB F and PepMV UTR R (Mumford & Metcalfe, 2001), which amplifies an 844-bp fragment of the entire CP gene. Leaf tissue from healthy tomato plants and RNase-free water were included as negative controls in each RT-PCR reaction.

The RT-PCR reaction mixture included 5 µL of 5x Qiagen OneStep RT-PCR Buffer, 1 µL of dNTP mix, 1.5 µL of the viral sense and complementary sense primers (10 µM), 1 µL of RT-PCR enzyme mix (Omniscript Reverse

Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA Polymerase), 1  $\mu$ L of extracted RNA and 14  $\mu$ L RNase-free water resulting in a final volume of 25  $\mu$ L. The reaction was performed using a thermal cycler (Biometra, T-1 Thermocycler), as follows: reverse transcription at 50°C for 30 min and an initial PCR denaturation step at 95°C for 15 min, followed by 35 cycles of three steps (94°C for 30 s, 57°C for 45 s, and 68°C for 1 min); and the final elongation was performed at 68°C for 10 min. The amplified products were determined using electrophoresis on 1% agarose gel containing ethidium bromide.

### Sequencing and phylogenetic analyses

The amplified products derived from the three selected isolates were purified by QIAquick PCR Purification Kit (Qiagen) and sequenced in both directions using the same primer pair as in RT-PCR (Macrogen, The Netherlands). Obtained sequences were deposited in GenBank. Sequences of the Serbian isolates were aligned and compared with each other by calculating nucleotide (nt) and deduced amino acid (aa) identities using the ClustalW program (Thompson *et al.*, 1994) and MEGAX software (Kumar *et al.*, 2018), as well as with the previously reported PepMV isolates available in the GenBank using the similarity search tool BLAST.

A phylogenetic tree was constructed using 44 complete CP gene sequences of PepMV retrieved from GenBank (Table 1) and those generated in this study, using the Maximum-likelihood method implemented in MEGAX. Robustness of the generated phylogenetic relationships was assessed by subjecting the data set to 1,000 bootstrap replicates, and bootstrap values <50% were omitted. Intra- and inter-group diversity values were calculated as the average genetic distance using Tamura-Nei's model (TN93+I) which was chosen as the best-fitting model of nt substitution.

## Results and discussion

### Symptoms in the field and PepMV detection using DAS-ELISA

During the visual inspection of open-field and protected tomato crops in 2019, symptoms resembling those caused by PepMV were observed in two 'Runner' tomato crops grown in plastic tunnels in the Bogojevce locality. Tomato plants showed irregular chlorotic or light yellow lesions which enlarged and coalesced and were accompanied with necrosis and scorching of infected leaves. Disease symptom incidence was estimated at 80%. Serological analysis revealed the presence of PepMV in all 15

tested tomato samples. All samples were negative for the presence of CMV, PVY, TSWV, AMV, TMV, ToMV, and TYLCV. PepMV induces a wide range of symptoms, depending on the tomato cultivar, virus strain, and environmental conditions including light and temperature (Spence *et al.*, 2006; Hanssen & Thomma, 2010). The most common leaf symptoms consist of yellow angular spots, mosaic, scorching, and deformation of infected leaves (Hanssen & Thomma, 2010). Some of these symptoms were observed at the Bogojevce locality, but the typical PepMV symptom in the form of fruit discoloration, labeled as marbling (Mumford & Metcalfe, 2001; Spence *et al.*, 2006; Hanssen *et al.*, 2008) was less frequently noticed and recorded only on a small number of fruits (Figs. 1a, 1b, and 1c).

### Mechanical transmission

PepMV isolates from three randomly selected symptomatic samples were successfully mechanically transmitted onto the tomato 'Novosadski jabučar' seedlings. After 15 days, all inoculated plants had developed characteristic PepMV symptoms (Hanssen & Thomma, 2010) including yellow spots (Fig. 1d) and light-dark green leaf mosaic, bubbling, and curled leaves. Test plants were analyzed using DAS-ELISA and all inoculated plants tested positive for PepMV.

### Molecular detection and phylogeny

The presence of PepMV in tomato plants was further confirmed by RT-PCR. Gel electrophoresis detected a single band of the 844 bp in all ELISA-positive samples. No amplification products were recorded in negative controls. The RT-PCR products obtained from three selected isolates, 192-19, 193-19-2, and 193-19-3, were successfully sequenced and submitted to the GenBank (MN656186, MN656187, and MN656188, respectively). Direct sequencing of amplified products generated high-quality sequences data which were successfully used for the sequence analyses. Multiple sequence alignment of the CP gene showed that the Serbian PepMV isolates were completely identical among themselves (100% nucleotide and amino acid identity) and shared the highest nucleotide identity of 95.1% (99.2% aa identity) with the Spanish isolate (FJ263341) originating from tomato.

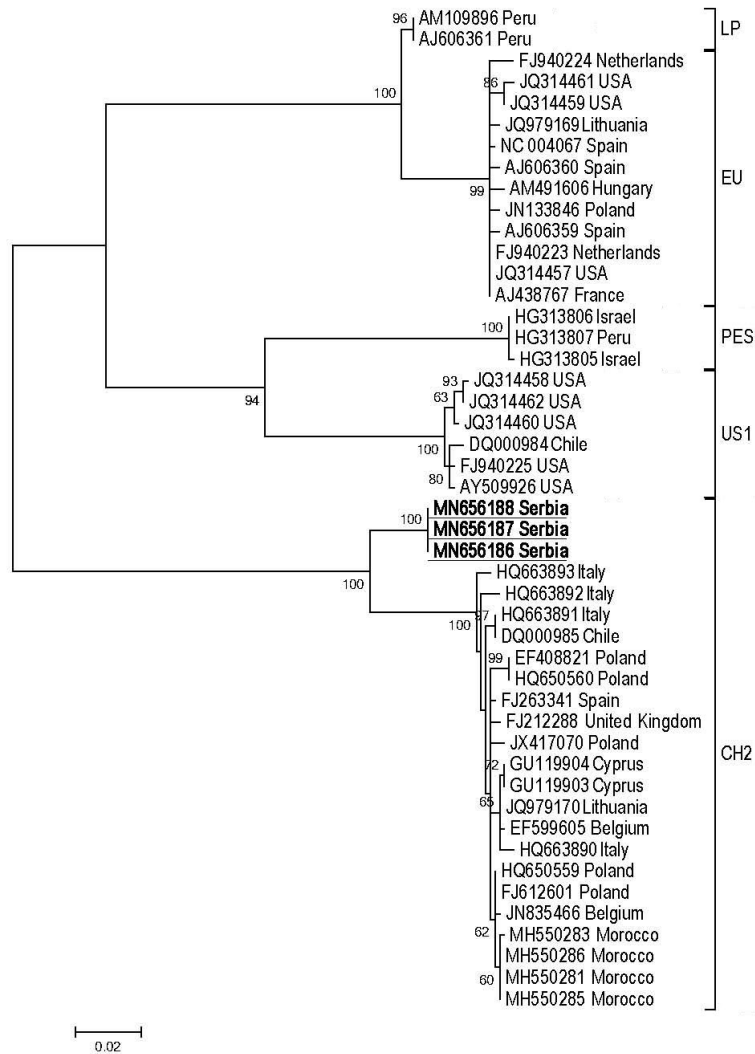
The sequences of the Serbian PepMV isolates were aligned with 44 CP sequences retrieved from GenBank, which represent the five known strains of PepMV and phylogenetic tree was constructed using Maximum-likelihood method. The phylogenetic analysis showed that the isolates were clustered into five groups (EU, LP, US1, CH2 and PES) (Fig. 2) as determined by previous reports

**Table 1.** Accession numbers and genotypes of Pepino mosaic virus isolates used in the phylogenetic analysis

Isolate name	Host plant	Country	GenBank accession number
PepMV-EU-France	<i>Solanum lycopersicum</i>	France	AJ438767
LE-2000	<i>Solanum lycopersicum</i>	Spain	AJ606359
LE-2002	<i>Solanum lycopersicum</i>	Spain	AJ606360
LP-2001	<i>Lycopersicon peruvianum</i>	Peru	AJ606361
SM.74	<i>Solanum muricatum</i>	Peru	AM109896
PepMV-H	<i>Solanum lycopersicum</i>	Hungary	AM491606
US1	<i>Solanum lycopersicum</i>	USA	AY509926
Ch1	<i>Solanum lycopersicum</i>	Chile	DQ000984
Ch2	<i>Solanum lycopersicum</i>	Chile	DQ000985
PepMV-PK	<i>Solanum lycopersicum</i>	Poland	EF408821
220606A1	<i>Solanum lycopersicum</i>	Belgium	EF599605
PepMV-UK	<i>Solanum lycopersicum</i>	United Kingdom	FJ212288
PMU06/16	<i>Solanum lycopersicum</i>	Spain	FJ263341
PepMV-Pa	<i>Solanum lycopersicum</i>	Poland	FJ612601
EU-tomato	<i>Solanum lycopersicum</i>	The Netherlands	FJ940223
DB1	<i>Solanum lycopersicum</i>	The Netherlands	FJ940224
US1	<i>Solanum lycopersicum</i>	USA	FJ940225
CY-PepMV-Parekklesia	<i>Solanum lycopersicum</i>	Cyprus	GU119903
CY-PepMV-Odou	<i>Solanum lycopersicum</i>	Cyprus	GU119904
Chi2.9	<i>Solanum pimpinellifolium</i>	Israel	HG313805
Tor9	<i>Solanum peruvianum</i>	Israel	HG313806
Yur1.5	<i>Solanum peruvianum</i>	Peru	HG313807
P19	<i>Solanum lycopersicum</i>	Poland	HQ650559
P22	<i>Solanum lycopersicum</i>	Poland	HQ650560
SAR09	<i>Solanum lycopersicum</i>	Italy	HQ663890
SIC1-09	<i>Solanum lycopersicum</i>	Italy	HQ663891
SIC2-09	<i>Solanum lycopersicum</i>	Italy	HQ663892
SAR01	<i>Solanum lycopersicum</i>	Italy	HQ663893
P11	<i>Solanum lycopersicum</i>	Poland	JN133846
CH2	<i>Solanum lycopersicum</i>	Belgium	JN835466
EU_CAHN8	<i>Solanum lycopersicum</i>	USA	JQ314457
US1_CAHN8	<i>Solanum lycopersicum</i>	USA	JQ314458
EU_EF09_58	<i>Solanum lycopersicum</i>	USA	JQ314459
US1_EF09_58	<i>Solanum lycopersicum</i>	USA	JQ314460
EU_EF09_60	<i>Solanum lycopersicum</i>	USA	JQ314461
US1_EF09_60	<i>Solanum lycopersicum</i>	USA	JQ314462
PepMV-NV	<i>Solanum lycopersicum</i>	Lithuania	JQ979169
PepMV-KK	<i>Solanum lycopersicum</i>	Lithuania	JQ979170
PepMV-P5	<i>Solanum lycopersicum</i>	Poland	JX417070
MA17-7a	<i>Solanum lycopersicum</i>	Morocco	MH550281
MA17-8a	<i>Solanum lycopersicum</i>	Morocco	MH550283
MA17-9a	<i>Solanum lycopersicum</i>	Morocco	MH550285
MA17-9b	<i>Solanum lycopersicum</i>	Morocco	MH550286
Sp-13	<i>Solanum lycopersicum</i>	Spain	NC_004067



**Figure 1.** Symptoms of PepMV infection on tomato plants: (a) yellow mosaic; (b) necrosis and leaf decay; (c) fruit discoloration (marbling); (d) yellow spots on leaves after artificial inoculation.



**Figure 2.** Phylogenetic analysis based on the nucleotide sequences of the coat protein gene generated using Maximum-likelihood method by MEGAX and bootstrap values on the branches represent the percentages out of 1000 bootstrap replicates program. The Serbian isolates are bolded and underlined.

(Pospieszny *et al.*, 2008; Gómez *et al.*, 2012b; Moreno-Pérez *et al.*, 2014; Gómez-Aix *et al.*, 2019). Genetic diversity among the five strains ranged from  $0.032 \pm 0.006$  to  $0.217 \pm 0.014$ , while diversity within each strain was:  $0.000 \pm 0.000$  (LP),  $0.006 \pm 0.001$  (EU),  $0.001 \pm 0.001$  (PES),  $0.007 \pm 0.002$  (US1), and  $0.017 \pm 0.002$  (CH2). The level of genetic diversity among five strains was high enough in relation to the diversity within each strain supporting obvious phylogenetic split among PepMV strains. The Serbian PepMV isolates fell into CH2 group and a close phylogenetic relationship among them may suggest their common origin. However, phylogenetic analysis revealed that Serbian PepMV isolates formed separate subgroup within CH2 strain with high bootstrap support of 100% and genetic divergence of  $0.053 \pm 0.008$  from the other isolates of this group. Similarly, Alcaide *et al.* (2020) revealed two separate clades within CH2 strain found in Spain. For further insights into genetic diversity of Serbian PepMV isolates, more detailed molecular characterization of other genomic regions are needed. Isolates of the EU and CH2 strains are common in Europe (Pagán *et al.*, 2006; Hanssen *et al.*, 2008; Gómez *et al.*, 2009; Davino *et al.*, 2017), but isolates highly similar to those from PES as well as recombinant isolates between CH2 and EU were also detected in Spain (Pagán *et al.*, 2006). EU isolates initially spread in European tomato crops, while CH2 isolates spread epidemically later on, becoming predominant in many tomato growing areas (Hanssen *et al.*, 2008; Ling *et al.*, 2008; Gómez *et al.*, 2009; Hasiów-Jaroszewska *et al.*, 2010a; Gómez *et al.*, 2012b). Moreover, in recent years the spread of CH2 isolates has also been reported in new regions, including Greece (Efthimiou *et al.*, 2011), Italy (Tiberini *et al.*, 2011), South Africa (Carmichael *et al.*, 2011), Cyprus (Papayiannis *et al.*, 2012), and Lithuania (Zizyte *et al.*, 2013). This situation is comprehensible given that Hanssen *et al.* (2008) have proven that CH2 isolates spread more rapidly within tomato crop than EU isolates.

To our best knowledge, this is the first report of PepMV in Serbia. Tomato is an important and traditionally grown vegetable crop in Serbia and the presence of PepMV could represent an important constraint to its production since no resistant varieties are available. Phytosanitary measures were taken to eradicate the outbreak, but more detailed surveys should be carried out in order to prevent the potential spread of the virus in the area.

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