Characterization of cucumber mosaic virus and its satellite RNAs associated with tomato lethal necrosis in Serbia

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Abstract A four-year survey (2012–2015) was carried out to examine the genetic diversity of cucumber mosaic virus (CMV) isolates infecting tomato, as well as the presence and diversity of their satellite RNAs (satRNAs), collecting a total of 226 samples throughout the most important growing regions in Serbia. Besides CMV-like symptoms, the collected samples also exhibited more severe symptoms, such as systemic necrosis of leaves, branches and stems, accompanied by fruit malformation and necrosis. In a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), the presence of CMV was detected in approximately one quarter of the tested samples. Identification of CMV was confirmed by conventional reverse transcription-polymerase chain reaction (RT-PCR) and sequencing of the coat protein (CP) gene of a group of 11 selected Serbian CMV isolates. Phylogenetic analysis of the CMV CP sequences of these selected isolates revealed their heterogeneity, as they fell into two different subgroups, IA and II. An additional RT-PCR analysis of CMV positive isolates using satellite RNAs specific primers detected the presence of satRNAs in eight samples. Sequence and phylogenetic analyses showed that Serbian CMV satRNAs variants were very heterogeneous, belonging to necrogenic and non-necrogenic variants. Necrogenic variants were divided into two groups, B and B1, containing a characteristic ‘necrogenic consensus’ sequence at the 3′ end of the RNA. A necrotic phenotype co-determined by satRNAs was expressed in inoculated tomato plants.

Keywords Tomato · Cucumber mosaic virus subgroups · satRNA variants · Phylogenetic analysis · Molecular detection

Introduction

Tomato, one of the most valuable vegetable crops, is affected by more than 146 viruses, several of which represent a serious threat to successful tomato production (Seekeyewa 2006; Hanssen et al. 2010; Xu et al. 2017). The number of viruses infecting tomato is constantly increasing, probably due to technological advancement in virus detection, global trade and climate changes, resulting in very significant yield losses especially in developing countries (Bani et al. 2006; Hanssen et al. 2010; Xu et al. 2017). In Serbia, some viruses and virus-like symptoms are present in almost all tomato
crops and the tomato virome so far consists of six viruses, including cucumber mosaic virus (CMV), potato virus Y (PVY), alfalfa mosaic virus (AMV), tomato spotted wilt tospovirus (TSWV), tomato mosaic virus (ToMV) and tobacco mosaic virus (TMV), and PVY and CMV are the most frequent and widespread (Nikolić et al. 2018).

CMV belongs to the genus *Cucumovirus* (family *Bromoviridae*) (Bujarski et al. 2019) and it is one of the most important viruses of many vegetable and ornamental plants. It has been reported to infect over 1300 species in 500 genera and at least 100 families, including some economically important vegetable crops, such as tomato (García-Arenal and Palukaitis 2008). CMV is primarily spread by numerous aphid species in a non-persistent manner, but it may be transmitted mechanically or by *Cuscuta* plants, while it is seed-transmitted in some hosts (Palukaitis et al. 1992; García-Arenal and Palukaitis 2008).

CMV has spherical particles of 29 nm in diameter and a tripartite genome composed of single stranded RNAs (RNA 1, RNA 2 and RNA 3). RNA 1 and RNA 2 encode two proteins (1a and 2a, respectively), which are components of the replicase complex. The bicistronic RNA 2 also encodes protein 2b, a viral RNA silencing suppressor, which is also involved in viral local and systemic spread and symptom expression. RNA 3 is bicistronic and encodes 3a and 3b proteins corresponding to the movement protein (MP) and coat protein (CP), respectively (Palukaitis et al. 1992; Roossinck 2002; Palukaitis and García-Arenal 2003; Jacquemond 2012).

Based on immunological and/or molecular analysis, CMV isolates are generally classified in two main subgroups named subgroup I and II. Subgroup I isolates are further divided into subgroups IA and IB. While isolates in subgroups IA and II are found all over the world, isolates in subgroup IB are thought to be of East Asian origin with a few exceptions, such as several isolates from the Mediterranean region, California, Brazil and Australia (Palukaitis et al. 1992; Palukaitis and García-Arenal 2003; Jacquemond 2012; Giakountis et al. 2018).

CMV is a helper virus of a small linear single-stranded satellite RNA (satRNA) that depends on CMV for replication, encapsidation and transmission. Until now, a set of more than 180 CMV satRNA sequence variants, belonging to 65 CMV isolates have been described, providing insight into their high genetic diversity worldwide (García-Arenal and Roossinck 2019). The first class of CMV satRNAs comprises variants with sequences of 332–334 nucleotides (nt) and these variants are not associated with geographical origin, the strain of CMV or the host plant, while the second class of CMV satRNAs has longer sequences (386–405 nt) associated with limited distribution and with CMV isolates belonging to subgroup IB (García-Arenal and Roossinck 2019). CMV satRNAs are involved in modulation of symptom expression in CMV-infected plants. Symptom modification of CMV by satRNAs depends on the virus strain, satRNA and the host plant (Palukaitis and García-Arenal 2003). Some CMV satRNAs may not modulate symptoms, most of them attenuate symptoms induced by CMV infection, while other satRNA variants induce lethal necrosis, stunting, or a bright yellow chlorosis on tomato plants (Collmer and Howell 1992; García-Arenal and Palukaitis 1999; Simon et al. 2004). CMV satRNA variants can be classified in two main groups, necrogenic and non-necrogenic, based on their ability to induce either systemic necrosis or chlorotic or mosaic symptoms on tomato (Devic et al. 1990).

Regardless of the fact that CMV is commonly encountered as one of the most important viruses for tomato production in Serbia (Nikolić et al. 2012; Nikolić et al. 2018), no information is available on its diversity and population structure. Moreover, symptoms of systemic necrosis and severe fruit malformation of some CMV-infected tomatoes have attracted our attention due to possible infections with both CMV and satRNAs. Considering an assumption that those severe symptoms are caused by isolates of CMV bearing satRNAs, the aim of this study carried from 2012 to 2015 was to confirm the presence of CMV satRNAs in Serbia, and to give the first insight into its distribution, genetic relationships and diversity. In addition, the study also focused on determining the subgrouping affiliation of CMV isolates originating from tomato on the basis of phylogenetic analysis of the CP gene. Results of those analyses will enable us to better anticipate the impact of CMV satRNAs emergence on Serbian tomato production, as well as the production of other susceptible crops.
Material and methods

Sampling and isolate collection

An intensive survey was conducted from 2012 to 2015 in order to collect plants showing any symptom suggestive of virus infection (Nikolić 2018; Nikolić et al. 2018). A total of 226 tomato samples showing symptoms resembling those of CMV infection or showing lethal necrosis were selected and tested for the presence of CMV. To find those infected only with CMV, samples were tested by double-antibody sandwich (DAS)-ELISA test using commercial diagnostic kits (Bioreba, AG, Reinach, Switzerland) against CMV and other most common tomato viruses: PVY, TSWV, AMV, pepino mosaic virus (PepMV), TMV, ToMV, potato virus X (PVX), tomato yellow leaf curl virus (TYLCV), and tomato ringspot virus (ToRSV).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs of 55 CMV positive isolates were extracted from 100 mg of frozen symptomatic leaves or fruits using a cetyltrimethylammonium bromide (CTAB) protocol (Bekesiova et al. 1999) and subjected to RT-PCR. RT-PCR was performed using the One-Step RT-PCR Kit (Qiagen, Hilden, Germany) and CMV CPfwd/CPrev specific primers (Milojević et al. 2012), previously described to amplify the sequence of coat protein (CP) gene (Table 1). Total RNAs of Serbian CMV isolates from cucurbits (GenBank Accession Number HM065509) and healthy tomato plants served as positive and negative controls, respectively.

Possible presence of satRNAs in total RNA extracts of all CMV positive samples was determined using CMVsat-fwd/CMVsat-rev (Škorić et al. 1996) (Table 1). Leaf tissue from healthy tomato plants and RNase-free water were included as negative controls in each RT-PCR reaction.

The RT-PCRs were performed in a T-1 Thermocycler (Biometra) in 25 μl final volume containing 400 μM of each of the four dNTPs, 0.6 μM of viral sense and complementary sense primers, 5 μl of 5x Qiagen OneStep RT-PCR Buffer, 1 μl of RT-PCR enzyme mix, 1 μl extracted RNAs, and 14 μl RNase-Free Water. The reverse transcription was performed at 50 °C for 30 min, followed by an initial PCR denaturation step at 95 °C for 15 min, a three-step cycle (denaturation, annealing and extension), applying conditions and the number of cycles depending on the used primers (Table 1), and a final extension at 72 °C for 10 min. PCR products were separated using electrophoresis on 1% agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator.

For further molecular characterization, 11 positive samples with single CMV infection were selected, based on field symptoms and origin (Table 2).

Sequencing and phylogenetic analysis

RT-PCR products for the 11 selected isolates were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced in both directions on an automated sequencer (Macrogen, Korea) using the same

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**Table 1** Primers used for cucumber mosaic virus (CMV) and CMV satRNAs detection

| Virus | Primer pair | Sequence (5’ to 3’) | Cycling (temperature/time) for primer pairs | Amplicon size (bp) | Reference |
|-------|-------------|----------------------|---------------------------------------------|-------------------|-----------|
|       | CMVCPfwd   | CAT GGATGCTT CTCCRCGAG | Denaturation 94 °C/60 s | 871 | Milojević et al. 2012 |
|       | CMVCPrev   | CGTAAAGCTGGATGG ACAACC | Annealing 52 °C/60 s | |
| CMV   |             |                       | Extension 72 °C/60 s | 35 | |
|       | CMVsat-fwd | AAGGATCCGGGTCC TGBDDDDGAATG AAGGATCGGTITTTG TTTGWTRAGAAAT TGGCYRGAG | No. of cycles | 355 | Škorić et al. 1996 |
| CMV   |             |                       | Denaturation 94 °C/60 s | 2 | |
| satRNAs | CMVsat-rev |                       | Annealing 42 °C/60 s | |
|       |             |                       | Extension 72 °C/60 s | 35 | |
primers as in the RT-PCR procedure. All sequences generated in this study were deposited in the GenBank database and assigned accession numbers (Table 2). The sequences were compared with each other, as well as with previously reported isolates available in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) 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).

Phylogenetic trees were constructed using the maximum likelihood algorithm implemented in MEGAX based on CP sequences of CMV isolates, as well as sequences of satRNAs variants, trimmed to the length of the shortest fragment, i.e. 558 and 258 bp, respectively. The best-fitting model of nucleotide (nt) substitution was investigated using the MODELTEST implemented in MEGAX and Kimura 2-parameter model Gamma distributed (K2 + G) was selected for both sets of sequences. The reliability of the obtained tree was evaluated using the bootstrap method based on 1000 replicates, and bootstrap values <50% were omitted. Intra- and inter-group diversity values were calculated as the average genetic distance using K2 + G model for each tree. An isolate of peanut stunt virus (PSV; Acc. No. EU570238) was used as the outgroup sequence for construction of CMV phylogenetic tree.

Bioassay

Crude sap extracted from all CMV positive samples bearing satRNAs was used to inoculate mechanically five Solanum lycopersicum ‘Novosadski jabučar’ plants using 0.01 M phosphate buffer (pH 7). Mechanical transmission was performed when test plants were at the 2 to 3 true-leaf stage. All inoculated plants were maintained in greenhouse conditions to allow symptom development for up to four weeks after inoculation.

Results

Detection of CMV and CMV satRNAs, and isolate selection for molecular characterization

Results of initial DAS-ELISA analysis using antisera against the most common tomato viruses showed that CMV was only detected in single infection in all of 55 out of 226 tested samples (24.34%). None of the tested samples reacted with PepMV, TYLCV, ToRSV and PVX antiserum, but single or mixed infections with PVY, TSWV, AMV, TMV and ToMV were detected in 171 (75.66%) of the collected samples (data not shown).

Table 2 Geographic origin, and sequences of cucumber mosaic virus (CMV) isolates and their satellite (sat) RNAs

| Isolate | Geographic origin | Year | Symptoms | GenBank accession number of CP gene | CMV subgroup | Presence/ type of satRNA | GenBank accession number of satRNAs |
|---------|------------------|------|----------|-----------------------------------|-------------|--------------------------|-------------------------------------|
| AR1-12  | Aradac           | 2012 | severe fruit malformation and necrosis, systemic necrosis of leaves, stems and branches | KT270572 | IA                       | +/nc                  | KM358138                            |
| AR2-12  |                  |      |          | MH032569 | IA                       | +/nc                  | KM358139                            |
| 137-13  | Ub               | 2013 | mosaic and filiformism | MH032570 | II                    | –                     | /                                   |
| 367-14  | Porodin          | 2014 | severe fruit malformation and necrosis, systemic necrosis of leaves, stems and branches | MH032571 | II                    | +/nc                  | KP719209                            |
| 169-2p-15 | VelikoVojlovce   | 2015 | mosaic, leaf deformation, filiformism and fruit mottling | MH032572 | IA                    | +/nnc                 | MH032565                            |
| 170-2p-15 |                 |      |          | MH032573 | IA                    | +/nnc                 | MH032566                            |
| 177p-15 |                  |      |          | MH032575 | IA                    | –                     | /                                   |
| 180p-15 |                  |      |          | MH032576 | II                    | +/nnc                 | MH032568                            |
| 174p-15 | Cekavica         |      |          | MH032574 | IA                    | +/nnc                 | MH032567                            |
| 190-15  | Togočevec        |      | mosaic and leaf deformation | MH032577 | IA                    | –                     | /                                   |
| 253-15  |                  |      |          | MN656189 | IA                    | +/nnc                 | MF964233                            |

aNecrogenic satRNAs; bNon-necrogenic satRNAs
ELISA results were confirmed using the CMVCpfwd/CMVCPrev specific primers, and CMV was detected in all 55 ELISA-positive samples.

All 55 CMV isolates, in which virus presence was confirmed by serological and molecular methods, were tested for the presence of CMV satRNAs. The primer pair CMVsat-fwd/rev was able to amplify satRNA sequences associated to eight out of the 55 tested CMV isolates, giving amplicons of an expected approximate size of 355 bp (Table 2).

For further CMV molecular characterization, a total of 11 selected CMV isolates were chosen based on their origin and field symptoms (Table 2, Fig. 1). In addition to the eight CMV isolates in which the presence of satRNAs was detected, three more CMV isolates without satRNAs and originating from different locations were included in the phylogenetic analysis in order to gain a deeper insight into the genetic structure of CMV population. Two isolates (190-15 and 253-15) induced mosaic and leaf deformation (Fig. 1a), one isolate (137-13) induced mosaic and reduced leaf lamina to filiformism (Fig. 1b), five isolates (169-2p-15, 170-2p-15, 174p-15, 177p-15, and 180p-15) mosaic, leaf deformation, filiformism and fruit mottling, while three isolates (AR1-12, AR2-12, and 367-14) induced severe fruit malformation and necrosis (Fig. 1c), as well as systemic necrosis of leaves, stems and branches (Fig. 1d).

Genetic diversity of CMV isolates

All 11 selected isolates were successfully sequenced and submitted to the GenBank database (Table 2). Multiple nucleotide and deduced amino acid sequence comparison implicated variability in the CMV population in tomato in Serbia. Concerning similarity, Serbian CMV isolates originating from tomato could be classified into two quite distinct groups. The first group comprised eight isolates (AR1–12, AR2–12, 169-2p-15, 170-2p-15, 177p-15, 174p-15, 190–15, and 253–15) with nt identities of 91.2%–100% (84.6%–100% aa identities), while the second group included only three isolates (137–13, 367–14, and 180p-15) with nt identities of 98.6%–99.6% (98.9%–100% aa identities). The CP sequences of these two groups of Serbian CMV isolates shared nt identities of 98.1%–95.8%. BLAST results revealed that CP sequences in the first group of the Serbian CMV isolates showed the highest nt identity of 93.46–99.39% with those from different geographical areas belonging to CMV subgroup I, while isolates from the second group shared the highest nt identity of 99.29%–100% with GenBank isolates belonging to CMV subgroup II.

A maximum likelihood tree based on partial sequences (558 nt) of the CP gene revealed that the CMV isolates determined in this study and selected sequences of 45 previously characterized CMV isolates retrieved from the GenBank database (Supplementary Table S1) clustered into two main subgroups I and II supported by high bootstrap values (93 and 92) and an overall level of nucleotide diversity of 0.143 ± 0.014 (Fig. 2). Subgroup I of the CMV isolates was divided into further two subgroups, IA and IB. Genetic diversity among the three subgroups of the CP gene isolates ranged from 0.07 ± 0.008 to 0.342 ± 0.031, whereas diversity within each group was: 0.023 ± 0.002 (IA), 0.057 ± 0.006 (IB), and 0.012 ± 0.002 (II). The Serbian CMV isolates grouped into two subgroups: IA (eight isolates) and II (three isolates).

Genetic structure of CMV satRNAs population

The identities of the obtained amplicons were confirmed by sequencing all eight variants of CMV satRNAs. The Serbian CMV satRNA sequences determined in this study were submitted to GenBank (Table 2). Direct sequencing of amplified products generated high-quality sequence data which were successfully used for the further sequence analyses.

In the BLAST search analysis, the sequences of Serbian CMV satRNA variants showed over 94% sequence identity with the most similar CMV satRNA sequences from other geographical areas of the world. Sequence comparison indicated variability in the Serbian CMV satRNA population in tomato. Serbian satRNAs shared 83.6%–99.2% identities. Serbian CMV satRNAs were classified into two distinct groups based on sequence similarities. The first group consisted of five variants (169-2p-15-satRNA, 170-2p-15-satRNA, 174p-15-satRNA, 180p-15-satRNA, and 253-15-satRNA) with nt identities of 98.1%–100%, while the second group was more divergent and included only three variants (AR1–12-satRNA, AR2–12-satRNA, and 367–14-satRNA) with nt identities of 90.1%–95.8%. Two CMV satRNAs (AR1–12-satRNA and AR2–12-satRNA), collected from the same field in 2012, had lower sequence homology than the homology
between AR1–12-satRNA and 367–14-satRNA collected two years later.

The association of satRNAs with CMV was detected in plants with or without systemic necrosis. The ‘necrogenic consensus’ sequence: GA-GCUAAGGC UUA...UGCUAUGCUGAU (Devic et al. 1990; Fisher 2013) was confirmed in three variants (AR1–12-satRNA, AR2–12-satRNA and 367–14-satRNA) causing systemic necrosis. The variant 367–14-satRNA had the characteristic ‘necrogenic consensus’, while sequences of the two other variants differed at several positions of ‘necrogenic consensus’ (Fig. 3). Nucleotide changes were found at position 309 (G to A) of both variants, at positions 323 and 328 (U to C) of the variant AR1–12-satRNA, and 319 (C to G) of the variant AR2–12-satRNA. This functional domain of tomato necrosis was not found in the sequences 169-2p-15-satRNA, 170-2p-15-satRNA, 174p-15-satRNA, 180p-15-satRNA and 253–15-satRNA. Additionally, the core or expanded chlorosis-induction domain, located at the 3′ end of the satRNA (Zhang et al. 1994; Fisher 2013), was not found in these five variants.

**Phylogenetic analysis of CMV satRNA**

A maximum likelihood tree revealed that CMV satRNA variants determined in this study and selected sequences of 43 previously characterized CMV satRNA variants retrieved from the GenBank database (Supplementary Table S2) clustered into two major groups of CMV satRNA variants, necrogenic and non-necrogenic, showing genetic diversity between them of 0.200 ± 0.027 (Fig. 4). Necrogenic CMV satRNA variants are further divided into groups B and B1 (0.117 ± 0.022), and non-necrogenic into groups A and C (0.307 ± 0.047). The genetic diversity among the four groups of satRNA variants ranged from 0.117 ± 0.022 to 0.373 ± 0.059, while diversity within each group was: 0.036 ± 0.005 (B), 0.013 ± 0.006 (B1), 0.063 ± 0.010 (A) and 0.106 ± 0.019 (C). Overall, genetic diversity among the Serbian satRNA variants and the 43 satRNA variants included in phylogenetic analysis was 0.149 ± 0.016. The clustering of 51 CMV satRNA variants into four different groups was supported by high bootstrap values and high nucleotide homology among sequences within...
each group. Three of the Serbian CMV satRNA variants clustered into necrogenic variants, but in different groups. AR1–12-satRNA and 367–14-satRNA variants belong in group B, while the variant AR2–12.satRNA was separated from them into group B1. Phylogenetic analysis showed the clustering of the variants 169-2p-15-satRNA, 170-2p-15-satRNA, 174p-15-satRNA, 180p-15-satRNA and 253–15-satRNA into a distant

Fig. 2 Maximum likelihood tree based on nucleotide sequences of 56 CMV CP gene isolates using Polish PSV isolate as the outgroup sequence. Phylogram was generated with MEGAX using Kimura 2-parameter model Gamma distributed. Bootstrap analysis was performed with 1000 replicates and bootstrap values (>50%) are shown next to relevant branches. Scale bars: substitutions per site. The Serbian CMV isolates are bolded and underlined.
In this study, necrogenic, as well as non-necrogenic CMV satRNAs were associated with different CMV subgroups, IA and II (Table 2, Fig. 2).

**Biological assay**

All CMV isolates containing satRNAs were successfully transmitted mechanically from naturally infected tomato plants to *Solanum lycopersicum* ‘Novosadski jabučar’. The test plants mechanically inoculated by isolates with non-necrogenic CMV satRNAs (169-2p-15-satRNA, 170-2p-15-satRNA, 174p-15-satRNA, 180p-15-satRNA, and 253–15-satRNA) developed only mosaic with or without filiform type symptoms, while the tomato plants infected with isolates associated with necrogenic CMV satRNAs, i.e. AR1–12-satRNA and AR2–12-satRNA, exhibited top necrosis 10–14 and 367–14-satRNA 15–20 days post-inoculation. Symptoms developed on inoculated tomato plants resembled those of natural infection.

**Discussion**

CMV is one of the most common viruses in many vegetable and field crops, as well as ornamentals in Serbia (Stanković et al. 2011; Vučurović et al. 2012; Petrović et al. 2010; Milojević et al. 2013, 2014; Milošević et al. 2015; Milojević et al. 2016). As all these crops are potential virus reservoirs, CMV has been recognized as one of the most frequent and prevalent viruses in Serbian tomato crops (Nikolić et al. 2018).

**Phylogenetic analysis of Serbian CMV isolates**, based on CP gene sequences in this study, revealed that the CMV population in tomato is heterogeneous, indicating also its long-term presence and wide distribution across the country. This study also revealed a prevalence of isolates belonging to CMV subgroup IA, which is the largest CMV subgroup worldwide (Bonnet et al. 2005). The remaining Serbian CMV isolates analyzed in this study, were clustered with CMV isolates of subgroup II, which are found less frequently than those of subgroup IA. Probably, this may be in part because the number of isolates in CMV subgroup II is underestimated due to less pronounced symptoms (Xu et al. 1999; Tian et al. 2009). Tomato-infecting isolates of CMV subgroup II that infect tomato had not been detected in Serbia prior to this study. We have assumed that Serbian CMV isolates that belong to subgroup II had probably been introduced by infected seedling imports and then adapted to these new but favourable climatic conditions. New genetic variants of CMV may occur due to reassortment among subgroups IA, IB and II strains (Chen et al. 2007) or due to their recombination (Nouri et al. 2014). In most cases, the hybrid genotypes disappear from the population, but occasionally they can become abundant in the population (Bonnet et al. 2005). Therefore, attention should be paid not only to the spread of CMV subgroup II isolates in Serbia, but also to the emergency of new virus variants through genetic exchange.

This study contributes new information about the distribution of tomato lethal necrosis and its causal agent in European countries, in which the disease has gained in significance and poses a risk for tomato cultivation. This is the first report and preliminary molecular characterization
of CMV-associated satRNAs naturally infecting tomato in Serbia and causing total collapse and death of affected plants. Moreover, the obtained results revealed different phenotypes of CMV satRNAs, which were necrogenic or non-necrogenic for tomato crops in Serbia. Characterization of the selected eight CMV satRNA variants provided insight into their genetic diversity.

In this study, the analysis of plants showing symptoms closely resembling those described for CMV revealed the presence of this virus, but a huge number of symptomatic plants were in fact infected with tomato viruses other than CMV. However, a sudden outbreak of unusually severe necrotic symptoms in CMV-infected plants gained our attention in 2012, as well as in 2014. In both tomato necrosis outbreaks, the same necrosis syndrome was observed. Symptoms of systemic necrosis of tomato plants were very similar to those observed during earlier devastating epidemics of tomato necrosis.
in France, the Mediterranean basin and Japan (Kaper et al. 1976; Gallitelli et al. 1988; Kosaka et al. 1989; Jordá et al. 1992). The analysis of necrosis etiology revealed that severe symptoms of necrosis were induced by infection with CMV associated with satRNA of necrogenic phenotype, rather than by synergistic infection with different viruses. Necrogenic CMV satRNAs, regardless of whether they were supported by subgroup IA or II CMV isolates, provoked lethal necrosis, which suggested that CMV had no a determining effect leading to tomato necrosis, as reported by Escriu et al. (2000).

Different syndromes have been shown to be caused by different genotypes of CMV satRNA (García-Arenal and Roossinck 2019). In Italy and Spain, the presence of the small-size class of satRNAs was associated with syndrome of systemic lethal necrosis or stunted plants (Aranda et al. 1993; Grieco et al. 1997). Additionally, in Italy internal fruit necrosis caused by satRNAs of the larger-size class was reported (Crescenzi et al. 1993). According to García-Arenal and Roossinck (2019), Serbian necrogenic CMV satRNAs belong to the first small-size class of satRNAs and were associated with fruit necrosis. In addition to the necrogenic variants of CMV satRNA found in 2012 and 2014, non-necrogenic CMV satRNA variants were found in 2015 causing also severe effects of leaf deformation, filiformism and fruit mottling but without systemic necrosis. No attempt has been made here to detect mixed infections of necrogenic and non-necrogenic CMV satRNA variants, which may be frequent in a same plant. In those cases, non-necrogenic CMV satRNA variants could have been masked and their presence underestimated, as necrosis induction is frequently dominant over other symptom effects (Escriu et al. 2000).

Although convergent evolution, which has been proved for CMV satRNAs, is viewed as an important problem for phylogenetic analyses of molecular data (Simon et al. 2004), several CMV satRNA phylogenetic analyses have been reported (Aranda et al. 1997; Grieco et al. 1997; Alonso-Prados et al. 1998). Phylogenetic analysis performed in this study showed that clustering of the compared set of 51 satRNA variants was neither correlated to their geographic or host origin nor their year of collection, as it was also reported by Alonso-Prados et al. (1998). Our analysis showed that the CMV satRNA variants are clearly divided into two major groups, depending on whether they induced or not necrosis in tomato plants. Necrogenic variants of satRNAs are further divided into two groups (B and B1), while non-necrogenic variants also fell into two groups (A and C). Moreover, the phylogenetic tree revealed that two necrogenic satRNAs from the same field clustered within different necrogenic groups (B and B1), while satRNAs originating from different years clustered into the same necrogenic group B. Similarly, Aranda et al. (1993) found that CMV satRNAs from the same year were not closer related to each other than they were to variants from different years. All Serbian CMV satRNAs, which clustered into the group of necrogenic variants, contained a characteristic ‘necrogenic consensus’ sequence at the 3′ end of the RNA, as reported for the known necrosis-inducing CMV satRNAs (Devic et al. 1990). CMV satRNA variants originating from tomato plants expressing leaf and fruit mottling, as well as leaf deformation and filiformism, fell into a different and distant A group of non-necrogenic CMV satRNA variants. Finding of necrosis-inducing domains (Devic et al. 1990) in Serbian CMV satRNA sequences and their comparison to sequences specific for necrogenic and non-necrogenic satRNA variants (Escriu et al. 2000) support this phylogenetic clustering. Sequence diversity and phylogenetic analysis showed high variation among tomato-originating satRNA variants in Serbia. Even though only eight Serbian CMV satRNAs were analyzed, they were found to exhibit high genetic divergence, even those isolated from a single field. High genetic variability of CMV satRNAs has been reported, resulting in very heterogeneous natural populations even more than populations of its helper virus itself, as previously reported (Aranda et al. 1993; Grieco et al. 1997; Alonso-Prados et al. 1998; García-Arenal et al. 2000). CMV satRNAs are able to vary under both experimental (Kurath and Palukaitis 1990; Alvarez et al. 2003) and field conditions (Kurath and Palukaitis 1989; Aranda et al. 1993).

In general, tomato systemic lethal necrosis has been reported as an epidemic in progress, affecting most tomato-growing regions in France, Italy, Greece, Spain and Croatia (Kaper et al. 1976; Gallitelli et al. 1988; Bem 1989; Jordá et al. 1992; Škorić et al. 1996). In Serbia, the situation of tomato necrosis was quite different. Since its first appearance at a single location in 2012, another outbreak was observed in 2014, again in just one location. In both fields, situated in important tomato-growing areas, a high disease incidence of lethal necrosis syndrome was noticed. It is not clear why the tomato necrosis outbreak that emerged in 2012 did not spread to other tomato crops in the region or extend over the following years, but rather reappeared in 2014 in an
isolated field, whence it again failed to spread. Successful widespread lethal necrosis symptoms in the fields might depend on high aphid population density. The spread of tomato necrosis in adjacent fields was not observed, probably due to successful and on-time eradication of infected fields.

Considering that CMV satRNAs spread epidemically in the CMV population (Alonso-Prados et al. 1998), and once the spreading starts, satRNA-free CMV variants may acquire satRNAs by over-infection of plants with satRNA-supporting isolates (Ohki et al. 1989), the isolated outbreaks of necrogenic CMV satRNA could pose a risk of possible epidemics in Serbia, under favorable conditions, primarily when aphid population density is high. Further epidemiological investigations are needed to identify and understand the factors affecting the emergence, severity and consequences of the disease caused by CMV plus necrogenic satRNAs in Serbia.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Human and animal studies** We confirm that in this research any human and/or animals participant was not used and there is no any disagreement with informed consent.

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