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Presence and molecular characterization of cucumber mosaic virus on safflower in Serbia

Dragana Milošević[1]* · Maja Ignjatov[1] · Ana Marjanović Jeromela[1] · Zorica Nikolić[1] · Gordana Tamindžić[1] · Dragana Miljaković[1] · Ivana Stanković[2]

[1]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia
[2]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080, Belgrade-Zemun, Serbia

Summary: Safflower (*Carthamus tinctorius* L.) is an important oilseed crop belonging to the family Asteraceae. A total of 46 safflower samples were collected from Srbobran locality (South Bačka District) in Serbia in 2015 and analysed for the presence of cucumber mosaic virus (CMV), alfalfa mosaic virus (AMV), and lettuce mosaic virus (LMV), using commercial double-antibody sandwich (DAS)-ELISA kits. Both viruses, CMV and AMV, were detected serologically in the collected samples. None of the analysed samples was found to be positive for LMV. The presence of CMV was further confirmed by mechanical transmission to test the plants including *Chenopodium quinoa, C. amaranticolor*, *Nicotiana glutinosa,* and *Datura stramonium* as well as *C. tinctorius*, confirming the infectious nature of the disease. Molecular detection of CMV was performed by amplification of a 871 bp fragment in all the tested samples, using the specific primers CMVCPfwd/CMVCPrev that amplify the entire coat protein (CP) gene and part of 3'- and 5'-UTRs of CMV RNA 3. The RT-PCR products derived from the isolates 290Saff and 294Saff were sequenced (MH577791 and MH577792, respectively) and compared with the CMV sequences available in GenBank. Phylogenetic analysis based on CP gene sequences showed clustering of the selected isolates into three subgroups: IA, IB and II. Serbian CMV isolates found in safflower belong to subgroup II. To our knowledge, this is the first report on CMV infection of safflower in Serbia, which has the potential to cause substantial damage to safflower production and pose a threat to other economic crops grown in Serbia.
Key words: cucumber mosaic virus, DAS-ELISA, RT-PCR, safflower

Introduction

Safflower (*Carthamus tinctorius* L., family Asteraceae) has been grown for centuries, primarily for its colourful petals, and used as a food colouring, flavouring agent, for vegetable oil production and for textile dye preparation (Esendal, 2001; Ekin, 2005). Considerable interest for the use of safflower as forage has recently occurred (Landau et al., 2005). Safflower has been receiving a lot of publicity, not so much for its colourful petals, but because it is regarded as one of the most important sources of vegetable oils. Safflower seeds contain nearly 35-50% oil, 15-20% protein and 35-45% hull fraction (Dobrinoiu et al., 2011).

As a result of breeding in the Institute of Field and Vegetable Crops (IFVCNS) in Novi Sad, Serbia, a collection of several genotypes of safflower has been formed and so far two varieties NS Lana and NS Una have been registered in the Republic of Serbia. Moreover, the demand for safflower breeding and its commercial establishment comes as a result of changed production conditions which include long drought periods during the summer season, when safflower, similarly to sunflower, produces higher yields than most other crops. As support to the breeding programs, research has been conducted in order to examine the presence of disease causal agents and their effect on yield and quality of safflower seeds.

Safflower is attacked by many diseases caused by fungi, bacteria, viruses, and suffers from physiological disorders due to abiotic stresses (Patil et al., 1993). Safflower is a natural host of cucumber mosaic virus (CMV), alfalfa mosaic virus (AMV), lettuce mosaic virus (LMV) (Klisiewiez, 1965,1966; Tomas, 1981), turnip

Corresponding author:
dragana.milosevic@ifvcns.ns.ac.rs

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mosaic virus (Klisiewiez, 1983), and tobacco streak virus (TSV) (Chander Rao et al., 2003).

CMV belongs to the genus *Cucumovirus* in the family *Bromoviridae* and causes significant damage to many agricultural crops including vegetables, ornamentals, and legumes resulting in devastating yield losses (Palukaitis & Garcia-Arenal, 2003). CMV is transmitted by numerous of aphid species, notably *Aphis gossypii* and *Myzus persicae*, in a non-persistent manner as well as by seed of several hosts (Francki et al., 1979). The genome of CMV comprises three single-stranded genomic RNAs (RNA 1, 2, 3) and two subgenomic mRNA (RNA 4, RNA4A) (Palukaitis et al., 1992). According to serological relationships, nucleic acid hybridization data, and molecular analyses of the genomic RNAs, CMV isolates are divided into subgroups I and II (Anderson et al., 1995; Palukaitis & Garcia-Arenal, 2003). Furthermore, subgroup I is divided into two subgroups IA and IB with 92% to 95% nucleotide homology (Palukaitis et al., 1992; García-Arenal & Palukaitis, 2008)

Considering the frequent presence of CMV on various plant species in Serbia, after the first detection of AMV infecting safflower at Rimski Šančevi in Serbia (Milošević et al., 2015a), a survey was conducted in order to establish the presence and distribution of safflower viruses in Serbia, as well as to determine the genetic relationship of Serbian safflower CMV isolates with isolates from other parts of the world.

Material and Methods

Sample collection

During 2015 the survey was carried out in order to determine the occurrence and distribution of viruses infecting safflower at the oil species collection. After visual inspection, a total of 46 samples of symptomatic safflower plants were collected from the localities at Srbobran (South Bačka District) in the Province of Vojvodina. The samples of safflower plants comprised of leaves which exhibited the typical symptoms of viral infection, such as mosaic infection, chlorotic mottling and leaf deformation. Samples were transported and stored at 4°C until testing by ELISA or stored at -20°C until RNA extraction and RT-PCR.

Double-antibody sandwich enzyme linked immunosorbent assay (DAS)-ELISA

Collected samples were serologically tested on the presence of CMV, AMV and LMV utilizing double-antibody sandwich (DAS)-ELISA kits using polyclonal antisera from Loewe Biochemica GmbH (Germany) according to the manufacturer's instructions. Extracts from fresh leaves were ground in an extraction buffer at a ratio of 1:10 (w/v). After addition of the substrate (1 mg/ml of p-nitrophenyl phosphate, Sigma-Aldrich, USA), the plates were incubated at room temperature for 2 hours and the extinction was measured at 405 nm (A405) using an ELISA plate reader (Multiscan Ascent, Finland). Commercial positive and negative controls and extracts from healthy safflower tissue were included in each test. Samples were considered to be positive when the absorbance values were at least two times higher than the negative controls.

Mechanical transmission

Crude sap extracted from the leaves of two serologically positive samples (290Saff and 294Saff) using 0.01 M phosphate buffer (pH 7) was mechanically inoculated onto five plants each of *Chenopodium quinoa, C. amaranticolor*, *Nicotiana glutinosa,* and *Datura stramonium* as well as *Carthamus tinctorius*. All test plants were inoculated at the 3 to 4 true-leaf stage. The inoculated plants were grown in glasshouse conditions and symptoms on inoculated plants were recorded in 18-20 days after inoculation. All inoculated plants were assayed by DAS-ELISA test to confirm CMV presence.

Reverse transcription polymerase chain reaction (RT-PCR)

Conventional RT-PCR was conducted for further confirmation of the serological findings. Total RNAs from the leaf tissue were extracted from 100 mg by the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, and used as template in RT-PCR. RT-PCR was performed using the One-Step RT-PCR Kit (Qiagen, Germany) using CMV specific primer pair CMVCPfwd/CMVCPrev (Milojević et al., 2012) which amplifies an 871-bp fragment of the entire coat protein (CP) gene.

The RT-PCR reaction mixture included 400 µM each of the four dNTPs, 1 µl of RT-PCR enzyme mix, 0.6 µM each primer and 1µl extracted RNA in a final volume of 25 µl. Amplifications were performed in a thermal cycler (Eppendorf, Germany). Reverse transcription was performed at 50°C for 30 min, followed by an initial PCR denaturation step at 95°C for 15 min, and 35 cycles of denaturation on 94°C for 60 s, annealing on 52°C for 60 s, and extension on 72°C for 60 s; and a final extension at 72°C for 10 min. The Serbian isolate of CMV (GenBank Accession No. KC288146) from pepper and a healthy safflower plant were used as the positive and the negative control, respectively.

RT-PCR products were separated using electrophoresis on 1% agarose gel containing ethidium bromide (0.5g/mL). The expected sizes of the amplified fragments were estimated by comparison with O´RangeRuler™ 100 bp DNA Ladder, ready-to-use (Fermentas, Lithuania). The agarose gel was visualised in a UV transilluminator, and the images were captured with the DOC PRINT system (Vilber Lourmat, USA).

Sequencing and phylogenetic analysis

The amplified products from the selected isolates were sequenced in both directions (ABI 3730XL

Automatic Sequencer, Macrogen, Korea), using the same primers as in RT-PCR directly after purification, with a QIA quick PCR Purification Kit (Qiagen, Germany). The obtained sequences of the Serbian CMV isolates were compared with the previously reported isolates available in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/), using the ClustalW program (Thompson et al., 1994) and MEGA5 software (Tamura et al., 2011). A p-distance model was applied for nucleotide (nt) and deduced amino acid (aa) sequence analyses.

Phylogenetic tree was created using 40 CP sequences of CMV isolates (Table 1) which were retrieved from GenBank and two CMV sequences generated in this study by the use of Neighbour-Joining method implemented in MEGA5 software. The reliability of the obtained tree was evaluated using the bootstrap analysis with 1000 replicates, and bootstrap values <50% were collapsed. Intra- and inter-group diversity values were calculated as the average genetic distance using Kimura 2-parameter model Gamma distributed (K2+G) which was chosen as the best-fitting model of nt substitution.

Table 1. CP gene sequences of *Cucumber mosaic virus* isolates used in the phylogenetic analysis

Virus	Isolate	Country	Host	Gen Bank Acc. No.
CMV[a]	**290Saff**	**Serbia**	***Carthamus tinctorius***	**MH577791**
CMV[a]	**294Saff**	**Serbia**	***Carthamus tinctorius***	**MH577792**
CMV	702-07	Serbia	*Nicotiana tabacum*	GQ340670
CMV	151-08	Serbia	*Cucurbita pepo* 'Olinka'	HM065509
CMV	115-08	Serbia	*Cucurbita pepo* 'Olinka'	HM065510
CMV	232Mrg	Serbia	*Calendula officinalis*	KP034963
CMV	MAD99/4	Spain	*Cucurbita pepo*	AJ829770
CMV	MAD96/1	Spain	*Cucumis melo*	AJ829768
CMV	I17F	France	/	Y18137
CMV	207	Australia	*Solanum lycopersicum*	AJ585517
CMV	Q	Australia	*Capsicum annuum*	M21464
CMV	Fny	USA	*Cucumis melo*	D10538
CMV	CMV-P6	USA	*Nicotiana tabacum*	D10545
CMV	CMV-FC	USA	*Nicotiana tabacum*	D10544
CMV	PR36	USA	/	M98500
CMV	Ixora	USA	*Ixora sp.*	U20219
CMV	K	USA	/	AF127977
CMV	S	USA	*Cucurbita pepo*	AF063610
CMV	/	Colombia	*Musa* sp.	U32859
CMV	Cas	Poland	*Lilium* sp.	DQ018286
CMV	Simp2	Poland	*Lilium* sp.	FJ621495
CMV	Ly2	Korea	*Lilium longiflorum*	AJ296154
CMV	ABI	Korea	*Gladiolus sp.*	L36525
CMV	M-48	Taiwan	/	D49496
CMV	Oahu	Hawaii	*Musa* sp.	U31220
CMV	KM	Japan	*Cucumis melo*	AB004780
CMV	D8	Japan	*Raphanus sativus*	AB004781
CMV	Y	Japan	*Nicotiana tabacum*	D12499
CMV	Pepo	Japan	*Cucurbita pepo*	AF103991
CMV	C7-2	Japan	/	D42079
CMV	42CM	Japan	*Cucumis sativus*	AB368498
CMV	M2	Japan	/	AB006813
CMV	Kin	United Kingdom	/	Z12818
CMV	P1	China	/	AJ006988
CMV	Phy	China	/	DQ412732
CMV	Cah1	China	*Canna* sp.	FJ268746
CMV	CTL	China	*Brassica chinensis*	EF213025
CMV	Cb7	China	*Solanum lycopersicum*	EF216867
CMV	Tsh	China	*Solanum lycopersicum*	EF202597
CMV	CS	China	*Arachis hypogaea*	AY429437
CMV	Ca	China	*Arachis hypogaea*	AY429432
CMV	Vir	Italy	*Capsicum annuum*	HE962480
PSV[b]	ER	India	*Vigna unguiculata*	U15730

[a] - Isolates originating from safflower from Serbia; [b] - *Peanut stunt virus* sequence used as outgroup.

Research results

Virus detection and symptomatology in the field

During the visual inspection of the safflower field in 2015, mosaic was the most frequent symptom, varying from mild to severe, followed by chlorotic mottling and leaf deformations. In total, the CMV infection was detected in 32 of 46 (69.57%), while AMV was detected in 15 of 46 (32.61%) of serologically tested samples. Single infections were the most frequent infection type (67.39%) and CMV (52.17%) was the most common, while mixed infection was found in only 17.39% of the tested samples. All tested samples were negative for LMV. Examined plants with single CMV infection exhibited mild to severe mosaic infection on the leaves, leaf distortion, and growth reduction. Plants infected with AMV expressed bright yellow spots on the leaves. Mixed infections caused a variety of symptoms, such as more intensive symptoms of mosaic, yellowing and leaf malformations which were not of diagnostic value in preliminary evaluation.

CMV is a very common plant virus that infects many plant families and can cause significant economic losses in many fruit, vegetable, ornamental, and horticultural crops (Akhtar et al., 2010; Dubey et al., 2010; Jalender et al., 2015). Natural occurrence of CMV in safflower was reported by Fletcher (2001), and Ravinder et al. (1992). Chenulu et al. (1971) and Chauhan and Singh (1979) reported the seed borne nature of CMV in safflower. Because CMV is seed transmitted, safflower seeds should come from certified production, in order to prevent viral transmission through seeds and thus cause greater damage to the production. Also, as a perennial plant, safflower represents a potential virus reservoir and an additional source of inoculums, used in order to recognize the known host range and prevalence of CMV in weed hosts. This investigation provides the first information on the presence of CMV on safflower in Serbia.

Phylogenetic tree, based on 42 sequences of the CP genes of CMV isolates from different host plants, showed separation of the selected isolates into three

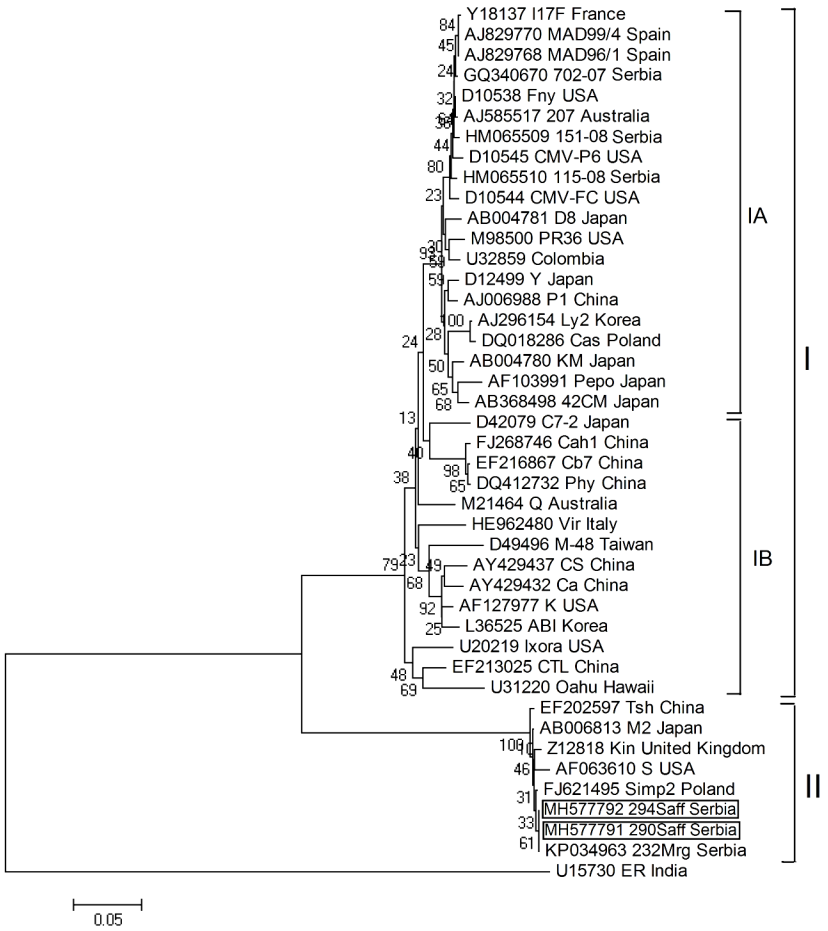

Figure 1. Phylogenetic analysis of cucumber mosaic virus using the neighbour-joining method. The phylogenetic tree was reconstructed based on nucleotide sequences of partial CP sequence of 42 CMV isolates. Phylogram was generated with MEGA5 using bootstrap analysis with 1000 replicates, while the bootstrap values (>50%) are shown next to relevant branches. Peanut stunt virus (U15730) was used as the outgroup. The two Serbian isolates from safflower are framed.

groups: IA, IB and II (Fig. 1), which is in accordance with the previous reports (Palukaitis and Zaitlin, 1997; Roossinck et al., 1999). Subgroup IA comprised CMV isolates from different parts of the world, such as Europe (Spain, Serbia, Poland and France), Asia (Japan, China and Korea), America (USA and Colombia) and Australia which 0.025±0.004 genetic diversity among the sequences of this molecular group. Subgroup IB included CMV isolates from Europe (Italy), Asia (Japan, China, Taiwan, and Korea), and America (USA and Hawaii) (0.069±0.006). Subgroup II contained the selected CMV isolates from safflower from Serbia, as well as the isolates from Asia (Japan and China), North America (USA) and Europe (UK, Poland, and Serbia) (0.007±0.002). Genetic diversity among three molecular groups was ranging from 0.071±0.007 to 0.287±0.021. In Serbia, CMV population in the safflower show to be different than CMV population in tobacco and pumpkins, where isolates of CMV belong to the IA subgroup (Stanković et al., 2011; Vučurović et al., 2012). Milošević et al. (2015b) reported the first finding of CMV isolates belonging to subgroup II (231Cal and 232Mrg), afterwards the isolates have spread and now can be detected in safflower.

Isolates from subgroup I are considered to be more dominant than the isolates from subgroup II, as they have a wider range of hosts and a higher prevalence (Roossinck et al., 1999; Tian et al., 2009), and therefore lead to greater damage in crops and greater economic losses. Higher prevalence of isolates from subgroup I cause more evident symptoms than the isolates from subgroup II, which is why isolates from subgroup II are harder to notice in the field (Xu et al., 1999; Tian et al., 2009).

Safflower is a very important raw material for industry, especially in medicine and food industry, and the presence of CMV can be a limiting factor for its successful production. Furthermore, as a new CMV host in Serbia, safflower has become a potential natural virus reservoir and an additional source of inoculum. Given that CMV can often appear on various plant species in Serbia, (Petrović et al., 2010; Stanković et al., 2011; Vučurović et al., 2012; Milojević et al., 2013, Milošević et al., 2015b; Nikolić et al., 2018) because of easy transmission in a non-persistent manner by aphids and a wide range of hosts (Garcia-Arenal & Palukaitis, 2008), constant monitoring is required regarding CMV status and their presence in our country.

Conclusion

This is the first report on the presence of CMV on safflower in Serbia, confirmed using DAS-ELISA tests, and molecular detection by RT-PCR with specific primers. Sequencing of CP gene of the selected isolates from safflower, as well as a suitable phylogenetic analysis, showed the position of Serbian CMV isolates from safflower in CMV population worldwide. Determination of variability within CMV populations found in different plant hosts in Serbia is expected to expand the knowledge of their epidemiology, with the final purpose of establishing and applying efficient control and preventing the introduction of new strains in our country caused by frequent international exchange of plant material.

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Prisustvo i molekularna karakterizacija virusa mozaika krastavca u usevu šafranike u Srbiji

Dragana Milošević · Maja Ignjatov · Ana Marjanović Jeromela · Zorica Nikolić · Gordana Tamindžić · Dragana Miljaković · Ivana Stanković

Sažetak: Šafranika (*Carthamus tinctorius* L.) predstavlja jednu od važnih uljanih kultura koja pripada familiji Asteraceae. Tokom 2015. godine, prikupljeno je 46 uzoraka biljaka šafranike poreklom iz Srbobrana koji su serološki testirani na prisustvo virusa mozaika krastavca (cucumber mosaic virus, CMV), virusa mozaika lucerke (alfalfa mosaic virus, AMV) i virusa mozaika salate (lettuce mosaic virus, LMV) korišćenjem komercijalnih kitova za DAS-ELISA test. U prikupljenim uzorcima dokazano je prisustvo CMV i AMV, dok prisustvo LMV nije dokazano ni u jednom od testiranih uzoraka. Prisustvo CMV je dalje potvrđeno mehaničkim inokulacijama test biljaka *Chenopodium quinoa*, *C. amaranticolor*, *Nicotiana glutinosa* i *Datura stramonium* kao i na *C. tinctorius*, čime je potvrđena infektivna priroda oboljenja. Molekularna detekcija CMV obavljena je amplifikacijom fragmenta dužine 871 bp kod svih ispitivanih izolata korišćenjem specifičnih prajmera CMVCPfwd/CMVCPrev koji omogućavaju umnožavanje celog CP gena kao i delove 5' i 3' UTR. U cilju dalje identifikacije, RT-PCR produkti izolata 290Saff i 294Saff su sekvencionisani (MH577791 i MH577792) i upoređeni sa CMV sekvencama dostupnim u GenBank bazi podataka. Filogenetska analiza na osnovu sekvence CP gena pokazala je grupisanje odabranih izolata u tri podgrupe, IA, IB i II. Izolati CMV iz šafranike poreklom iz Srbije grupisali su se u podgrupu II. Prema našim saznanjima, ovo je prvi izveštaj o prisustvu CMV na biljkama šafranike u Srbiji koji može da nanese veliku štetu u proizvodnji ove biljne vrste, a takođe predstavlja pretnju drugim ekonomski značajnim gajenim biljnim vrstama u Srbiji.

Ključne reči: DAS-ELISA, RT-PCR, šafranika, virus mozaika krastavca

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