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The Presence of Cucumber Mosaic Virus in Pot Marigold (*Calendula officinalis* L.) in Serbia

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Summary: During 2014 a total of 67 pot marigold samples from five different localities in the Province in Vojvodina were collected and analysed for the presence of *Cucumber mosaic virus* (CMV) and *Impatiens necrotic spot virus* (INSV) using commercial double-antibody sandwich (DAS)-ELISA kits. CMV was detected serologically in all inspected localities in 67.16% collected samples. None of the analysed samples was positive for INSV. The virus was successfully mechanically transmitted to test plants including *Chenopodium amaranticolor*, *C. quinoa*, *Datura stramonium*, *Nicotiana tabacum* 'Samsun' and *N. glutinosa*, as well as pot marigold seedlings, confirming the infectious nature of the disease. The presence of CMV in pot marigold plants was further verified by RT-PCR and sequencing, using the specific primers CMV CPfwd/CMVCPrev that amplify coat protein (CP) gene. Phylogenetic analysis based on the CP gene sequences showed clustering of the selected isolates into three subgroups, IA, IB and II, and Serbian CMV isolates from pot marigold belong to subgroup II.
Keywords: *Calendula*, coat protein gene, cucumber mosaic virus, Impatiens necrotic spot virus, isolates, marigold, RT-PCR, sequencing

Introduction

Serbia is situated in the region suitable for the cultivation of medicinal plants owing to the favourable climate, soil and unpolluted environment. According to unofficial estimates, approximately 3,500 ha of medicinal plants are grown and the most significant production area is in the Province of Vojvodina, Serbia.

Calendula officinalis L. (family Asteraceae), commonly known as pot marigold, is a flowering perennial ornamental, rich in essential oils, that is commonly grown in gardens or as potted plant all over Europe. The flower is normally used as food additive to confer both colour and flavour to foods (Blumenthal 2000, Hamburger et al. 2003). Marigold is also widely used in traditional and homeopathic medicine as infusions and ointments prepared with its petals. It exhibits several therapeutic activities, such as anti-inflammatory, anti-tumorogenic and cytotoxic activities (Kalvatchev et al. 1997, Ramos et al. 1998, Blumenthal 2000, Hamburger et al. 2003).

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Some of the marigold plants developed viral-disease-like symptoms consisting of yellowing, mosaic, vein chlorosis and mild curling. The infected plants were stunted, yielding a small number of twisted and deformed flowers. Growth reduction was exhibited by the production of small leaves clustering around the main stem. The severely infected plants tended to cease growth showing necrosis at the top. According to previous data, the two major viruses of marigold are *Cucumber mosaic virus* (CMV) and *Impatiens necrotic spot virus* (INSV) (Hanson et al. 1951, Ghotbi 2013).

The most common viral disease of pot marigold is described as "chlorotic mottle" and it is caused by CMV. As a type species of *Cucumovirus* genus, it is reported to infect 1287 plant species in 518 genera belonging to 100 families (Edwardson & Christie 1997). It was described for the first time in America in 1916, as a cucumber and squash disease agent (Francki et al. 1979). Since then, CMV has become a widespread virus, though it is predominantly found in temperate regions with the most favourable conditions for the growth of its vectors - aphids. CMV is transmitted in a non-persistent manner by more than 80 aphid species, and the most efficient CMV vectors are *Myzus persicae* and *Aphis gossypii* (Garcia-Arenal & Palukaitis 2008).

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Considering the frequent presence of CMV on various plant species in Serbia (Petrović et al. 2010, Stanković et al. 2011, Vučurović et al. 2011, Vučurović et al. 2012, Milojević et al. 2013), after the first detection of CMV infecting pot marigold (Milošević et al. 2015), a survey was conducted in the main pot marigold producing areas in the Province of Vojvodina in order to detect the presence and distribution of CMV and to determine the genetic relationship of Serbian pot marigold CMV isolates with those from other parts of the world.

Materials and Methods

Survey and sample collection

During 2014, a total of 67 samples of symptomatic pot marigold plants were randomly collected from five crops at five different localities of South Bačka (Srbobran, Đurđevo), Srem (Ruma) and Central Banat districts (Zrenjanin, Žitište) in the Province of Vojvodina. Samples of pot marigold plants comprised of leaves which exhibited typical symptoms of viral infection, such as 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.

Serological detection

Serological testing was performed utilizing double-antibody sandwich (DAS)-ELISA kits with commercial antisera specific for detection of CMV and INSV (Loewe Biochemica, Germany), following the manufacturer's instructions. Plant tissue samples were ground in extraction buffer (1:10 wt/vol). After incubation with p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) at room temperature for 2 h in the dark, absorbance at 405 nm was measured with an ELISA microplate reader (Multiskan Ascent, Finland). Commercial positive and negative controls and extracts from healthy pot marigold tissue were included in each test. Samples were considered as positive if the mean absorbance value at 405 nm was at least twice that of the negative control.

Mechanical transmission

Five plants of each *Chenopodium quinoa, C. amaranticolor*, *Nicotiana tabacum* 'Samsun', *N. glutinosa,* and *Datura stramonium* as well as *Calendula officinalis* seedlings were mechanically inoculated with sap from the leaves of five serologically positive samples, one sample from each locality, using 0.01 M phosphate buffer (pH 7). The test plants were inoculated at the 2-3 true-leaf stage and maintained under greenhouse conditions for symptoms to develop over a period of up to four weeks post-inoculation. All inoculated plants were assayed by DAS-ELISA to confirm CMV presence.

RT-PCR detection

Presence of CMV in pot marigold plants was further confirmed by conventional reverse transcription (RT)-PCR. Total RNAs were extracted from 100 mg leaf tissue by the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and used as template in RT-PCR. RT-PCR was carried out using the One-Step RT-PCR Kit (Qiagen) using CMV-specific primer pair, CMVCPfwd and 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 1 µl extracted RNA in a final volume of 25 µl. Amplifications were performed in a thermal cycler (Eppendorf, Germany) under the following programs: reverse transcription at 50°C for 30 min and an initial PCR denaturation step at 95°C for 15 min, followed by 35 cycles consisting of a denaturation step of 30 s at 94°C, primer annealing for 30 s at 58°C, and extension for 30 s at 72°C. The final extension was performed at 72°C for 10 min. Total RNAs obtained from a Serbian CMV isolate from pepper (GenBank Accession No. KC288146) and a healthy pot marigold plant served as the positive and the negative control, respectively. The amplification fragments were determined using electrophoresis on 1% agarose gel containing ethidium bromide (0.5 µg/mL). The expected size of the amplified fragments was estimated by comparison with O´RangeRuler™ 100 bp DNA Ladder, ready-to-use (Fermentas, Lithuania). The agarose gel was visualised in UV transilluminator, and the images were captured with DOC PRINT system (Vilbert Lourmat, USA).

Sequencing and phylogenetic analysis

Products of a predicted size, obtained in RT-PCR assays from isolates, 231Cal originating from Srbobran locality were sequenced directly after the purification with QIAquick PCR Purification Kit (Qiagen). Additionally, a previously identified pot marigold CMV isolate 232Mrg (Milošević et al. 2015) was also included in the investigation.

Sequence of the Serbian CMV isolate was compared with the previously reported CMV 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.

A phylogenetic tree was constructed using 40 CP sequences of CMV isolates retrieved from GenBank (Table 1) and those CMV CP sequences generated in this study and using the Maximum Parsimony (MP) method in MEGA5 software. The reliability of the obtained tree was evaluated using the bootstrap method based on 1000 replicates, and bootstrap values <50% were omitted. An isolate of *Peanut stunt virus* (Acc. No. U15730) was used as the outgroup sequence (Table 1). 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. CMV isolates with coat protein sequences used in the phylogenetic analysis

Virus	Isolate[a]	Country	Host	Gen Bank Acc. No.
CMV[b]	**231Cal**	**Serbia**	***Calendula officinalis***	**KP765696**
CMV	**232Mrg**	**Serbia**	***Calendula officinalis***	**KP034963**
CMV	MAD99/4	Spain	*Cucurbita pepo*	AJ829770
CMV	MAD96/1	Spain	*Cucumis melo*	AJ829768
CMV	I17F	France	/	X16386
CMV	MAD99/1	Spain	*Cucumis melo*	AJ829776
CMV	Ban	Israel	*Musa* sp.	U43888
CMV	702-07	Serbia	*Nicotiana tabacum*	GQ340670
CMV	CMV-P6	USA	*Nicotiana tabacum*	D10545
CMV	207	Australia	*Lycopersicon esculentum*	AJ585517
CMV	151-08	Serbia	*Cucurbita pepo*	HM065509
CMV	115-08	Serbia	*Cucurbita pepo*	HM065510
CMV	CMV-FC	USA	*Nicotiana tabacum*	D10544
CMV	PR36	USA	/	M98500
CMV	-	Colombia	*Musa* sp.	U32859
CMV	P1	China	/	AJ006988
CMV	KM	Japan	*Cucumis melo*	AB004780
CMV	Cas	Poland	*Lilium* sp.	DQ018286
CMV	RB	China	*Phaseolus vulgaris*	AJ006990
CMV	113	USA	/	AF523340
CMV	NT9	Taiwan	/	D28780
CMV	Trk7	Hungary	*Trifolium repens*	L15336
CMV	C7-2	Japan	/	D42079
CMV	M-48	Taiwan	/	D49496
CMV	K	USA	/	AF127977
CMV	ABI	Korea	*Gladiolus* sp.	L36525
CMV	Tfn	Italy	*Lycopersicon esculentum*	Y16926
CMV	Ixora	USA	*Ixora* sp.	U20219
CMV	Oahu	Hawaii	*Musa* sp.	U31220
CMV	S	USA	*Cucurbita pepo*	AF063610
CMV	Q	Australia	*Capsicum annuum*	M21464
CMV	LS	USA	*Lactuca sativa*	AF127976
CMV	Simp2	Poland	*Lilium* sp.	FJ621495
CMV	M2	Japan	/	AB006813
CMV	Kin	United Kingdom	/	Z12818
CMV	Ri-8	Spain	*Lycopersicon esculentum*	AM183119
CMV	Pl-1	Spain	*Lycopersicon esculentum*	AM183116
CMV	SD	China	*Nicotiana tabacum*	AB008777
CMV	Tsh	China	*Lycopersicon esculentum*	EF202597
CMV	TN	Japan	*Lycopersicon esculentum*	AB176847
CMV	Ns	Hungary	*Nicotiana glutinosa*	AJ511990
CMV	Rs	Hungary	*Raphanus sativus*	AJ517802
PSV[c]	ER	India	*Vigna unguiculata*	U15730

[a] - All data are from GenBank; [b] - Isolates originating from pot marigold from Serbia; [c] – *Peanut stunt virus* sequence used as outgroup.

Results and Discussion

Virus detection and symptomatology in the field

During the visual inspection of pot marigold fields in 2014, similar symptoms were observed in all inspected localities with disease incidence ranging from 15 to 50%. Mosaic was the most frequent symptoms, varying from mild to strong, as well as various chlorotic streaks, mild leaf deformation, and more or less pronounced blistering. The observed symptoms were typical of CMV infection, as described by many authors (Hanson et al. 1951, Sang & Varma 1975, Naqvi et al. 1981, Rahaman & Rao 1992).

Serological analysis of pot marigold samples, revealed the presence of CMV in all inspected localities in the Province of Vojvodina. The presence of CMV was detected serologically in 67.16% tested samples and all were negative for INSV (Table 2). After the first detection of CMV in 7 out of 10 tested pot marigold samples originating from the Đurđevo locality, the virus was detected serologically in additional 38 pot marigold samples collected from four different localities: Srbobran, Ruma, Zrenjanin, and Žitište. The highest incidence of CMV was in the Đurđevo locality (70% tested samples positive), as well as in the Srbobran locality, where the presence of the virus was confirmed in 7 out of 15 tested samples (46.66%). In the locality of Zrenjanin the presence of CMV was detected in 35.71% tested samples, while in Ruma locality presence of the virus was confirmed in 23.08% tested samples. In Žitište locality the presence of the virus was detected only in 3 out of 15 pot marigold samples.

The natural occurrence of CMV in pot marigold was reported by Hanson et al. (1951), Joshi & Dubey (1972) and Sang & Varma (1975). As a new CMV host in Serbia, pot marigold represents a potential virus reservoir and additional source of inoculum to recognize the known host range and prevalence of CMV in weed hosts.

Mechanical transmission

All five selected samples of naturally infected pot marigold plants, one from each locality, that were serologically positive for CMV, were successfully transmitted mechanically to the test plants. All inoculated plants produced the symptoms, which is in correlation with earlier descriptions (Singh et al. 1999, Iqbal et al. 2011) from 5 to 10 days post-inoculation. All five mechanically inoculated *Chenopodium amaranticolor* and *C. quinoa* reacted uniformly showing small local lesions, while systemic mosaic and leaf malformations were observed on *Nicotiana tabacum* 'Samsun' and *N. glutinosa*, 5-6 and 10 days post-inoculation, respectively. Also, the virus was successfully mechanically transmitted to *C. officinalis* that reacted with symptoms identical to those observed on the original host plants. Serbian CMV isolate from pot marigold were not able to infect *D. stramonium* plants, though this plant is reported as experimental host plant of CMV (Iqbal et al. 2011). Test plants were assayed by DAS-ELISA and all inoculated plants of each species tested positive for CMV.

Molecular detection, identification and phylogenetic analysis

The results of serological analyses of CMV presence in pot marigold crops in Serbia was further confirmed by molecular RT-PCR method using specific primers CMVCPfwd and CPrev which amplify fragment covering the entire coat protein (CP) gene and part of 3'- and 5'-UTRs. These primers successfully detected the presence of CMV in all tested samples and amplified cDNA fragments of predicted size. One clear band of 871 bp was visible in all tested samples as well as in positive control. No amplicon was recorded when extract from the healthy pot marigold plant was used as template in the RT-PCR assay.

Table 2. Presence and incidence of *Cucumber mosaic virus* in pot marigold during 2014

Locality	District	Tested samples	Positive samples
Đurđevo	South Bačka	10	7 (70%)[a]
Srbobran	South Bačka	15	7 (46.66%)
Ruma	Srem	13	3 (23.08%)
Zrenjanin	Central Banat	14	5 (35.71%)
Žitište	Central Banat	15	3 (20%)
Total		67	25 (37.31%)

[a] - Number of infected samples (% infected samples calculated over the total number of tested samples).

After purification, the RT-PCR product derived from the isolate 231Cal was directly sequenced in both directions using the same primer pair as in RT-PCR and deposited in GenBank (GenBank Accession No. KP765696). Sequence analysis of CP gene, conducted with MEGA5 software, revealed 99.5% nt identity (100% aa identity) between the two Serbian CMV isolates from pot marigold. Multiple sequence alignment of the CP open reading frame showed that the Serbian isolate 231Cal shared the highest nucleotide identity of 99.5% (100% amino acid identity) with six CMV isolates from Poland (EU191027, DQ018292), Italy (FN257306), Iran (KC763473), China (EF202597), and Serbia (KC414925).

A Maximum parsimony tree (Figure 1) reconstructed using the partial sequences of the coat protein gene revealed that CMV isolates determined in this study and selected sequences of 40 previously characterized CMV isolates retrieved from GenBank database clustered into two groups supported with high bootstrap values (100%). Group I was further divided into two subgroups IA and IB. The division of isolates into three subgroups (IA, IB and II) is in accordance with the previous reports (Palukaitis & Zaitlin 1997, Roossinck et al. 1999). Genetic diversity among two molecular groups of isolates was 0.151±0.083, whereas within each group and subgroups were: 0.010±0.004 (IA), 0.036±0.015 (IB) and 0.012±0.005 (II). Subgroup IA contained 19 isolates from different parts of the world, Europe (Spain, Hungary, Serbia, Poland and France), Asia (Japan, China and Israel), America (USA and Colombia) and Australia. Subgroup IB contained 12 isolates from Europe (Spain and Italy), Asia (Japan, China, Taiwan and Korea), and America (USA and Hawaii). In subgroup II nine isolates classified from Europe (Hungary and Poland), Asia (Japan and China), Africa (South Africa), North America (USA), UK, Australia as well as two Serbian CMV isolates from pot marigold.

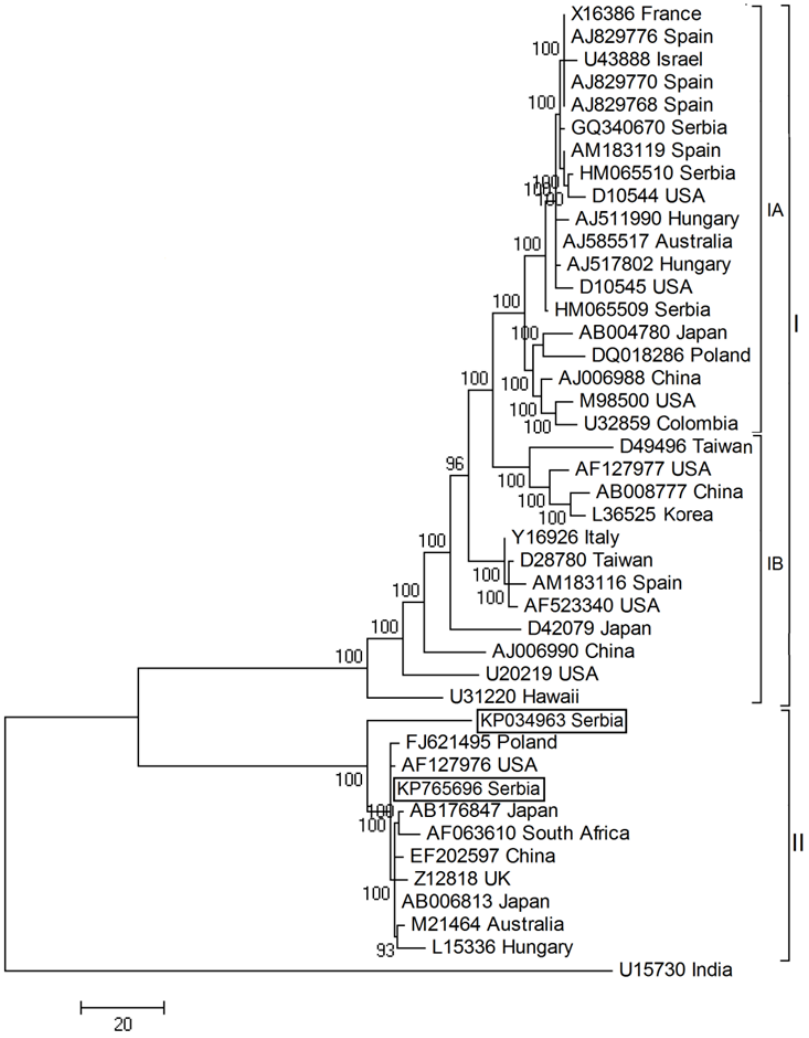

Figure 1. Maximum parsimony tree of was constructed based on nucleotide sequences of partial CP sequence of 42 CMV isolates. Phylogram was generated with MEGA5 using bootstrap analysis with 1000 replicates and bootstrap values (>50%) are shown next to relevant branches. The tree was rooted with *Peanut stunt virus* (U15730). The two Serbian isolates from pot marigold are framed.

Considering that pot marigold has been traditionally grown in Serbia, as one of the most significant medicinal plants, the occurrence of CMV can represent a limiting factor for its successful production. As CMV can often be found on various crops in Serbia (Krstić et al. 2002, Dukić et al. 2004, Vučurović et al. 2009, Milojević et al. 2013), then due to its easy transmitting in a non-persistent manner by aphids, and a wide circle of hosts (Garcia-Arenal & Palukaitis 2008), constant monitoring of CMV status and presence in our country is necessary.

Conclusions

After previously reported outbreak of CMV on pot marigold as a new host in Serbia for the first time, in this study the virus was detected in four additional localities in the Province of Vojvodina using DAS-ELISA tests, bio assay, and molecular detection by RT-PCR with specific primers. Sequencing of CP gene of selected isolates from pot marigold and suitable phylogenetic analysis showed the position of Serbian CMV isolate from pot marigold in CMV population worldwide. Due to great damages caused by CMV worldwide, determination of variability within population of CMV in pot marigold crops, but also establishing relationships with the isolates originating from other hosts in Serbia will contribute to the clarification of some unknown epidemiological aspects and discovery of plants significant for viral conservation, with the general purpose to design and implement efficient control and prevent the possible introduction of new viral strains in our country through intensive international exchange of plant material.

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Prisustvo virusa mozaika krastavca na nevenu (*Calendula officinalis* L.) u Srbiji

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Sažetak: Tokom 2014. godine, sa pet različitih lokaliteta gajenja nevena u Vojvodini sakupljeno je ukupno 67 uzorka koji su serološki testirani na prisustvo virusa mozaika krastavca (*Cucumber mosaic virus,* CMV) i virusa nekrotične pegavosti impatiensa (*Impatiens necrotic spot virus*, INSV), korišćenjem komercijalno dostupnih kitova za DAS-ELISA test. Prisustvo CMV dokazano je na svih pet pregledanih lokaliteta i to u 67,16% prikupljenih uzoraka, dok prisustvo INSV nije dokazano ni u jednom od testiranih uzoraka. Virus je uspešno prenet mehaničkim inokulacijama test biljaka *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana tabacum* 'Samsun' i *N. glutinosa*, kao i na sejance nevena, čime je potvrđena infektivna priroda oboljenja. Prisustvo CMV u biljkama nevena potvrđeno je primenom RT-PCR metode i sekvencioniranjem uz korišćenje specifičnih prajmera CMVCPfwd/ CMVCPrev koji omogućavaju umnožavanje gena za protein omotača (CP gena). Filogenetska analiza na osnovu sekvence CP gena pokazala je grupisanje izolata u tri podgrupe, IA, IB i II, a izolati CMV iz nevena iz Srbije grupišu se u podgrupu II.

Ključne reči: *Calendula*, gen za protein omotača, izolati, neven, RT-PCR, sekvencioniranje, virus mozaika krastavca, virus nekrotične pegavosti impatiensa