



## Occurrence and molecular characterization of alfalfa mosaic virus in eggplant in Serbia

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Received 3 February 2021; Accepted 23 March 2021

### ABSTRACT

In the 2018–19 growing season, a total of 51 leaves of eggplant plants grown under field conditions were collected randomly from nine private gardens at four different localities in the Province of Vojvodina. Eggplants with nearly 40% of plants showing bright yellow to white mosaic or mottling of leaves were found throughout the inspected fields (gardens). The collected samples were analyzed for the presence of alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV) and potato virus Y (PVY) using commercial double-antibody sandwich (DAS)-ELISA kits. Serological analysis of eggplant samples revealed the presence of AMV in 80.39% collected samples. None of the analyzed samples was positive for CMV and PVY. The virus was successfully mechanically transmitted to test plants including *Nicotiana benthamiana*, *Chenopodium quinoa*, *C. amaranticolor*, as well as eggplant seedlings, confirming the infectious nature of the disease. The presence of AMV in eggplants was further verified by reverse transcription–polymerase chain reaction (RT-PCR) and sequencing, using the primers CP AMV1 and CP AMV2 that amplify part of the coat protein (CP) gene. The phylogenetic analysis showed that Serbian AMV isolates grouped into a separate well-supported group together with AMV isolates from Italy, Croatia and previously characterized isolates from Serbia. To our knowledge, this is the first report of AMV infection of eggplant in Serbia.

**Keywords:** Eggplant, alfalfa mosaic virus, DAS-ELISA, RT-PCR, phylogenetic analysis.

### ИЗВОД

Током 2018. и 2019. године, прикупљен је 51 узорак биљака плавог патлиџана из девет усева са четири различита локалитета гајења ове биљне врсте у Војводини. Током прегледа, на близу 40% биљака плавог патлиџана могли су се уочити симптоми у виду жућкастобеличастих пега распоређених по читавој површини лиске. Прикупљени узорци анализирани су на присуство вируса мозаика луцерке (alfalfa mosaic virus, AMV), вируса мозаика краставца (cucumber mosaic virus, CMV) и Y вируса кромпира (potato virus Y, PVY) коришћењем комерцијално доступних китова за DAS-ELISA тест. Серолошким анализама утврђено је присуство AMV у 80,39% прикупљених узорака. Присуство CMV и PVY није доказано ни у једном од тестираних узорака. Вирус је успешно пренет механичким инокулацијама тест биљака *Nicotiana benthamiana*, *Chenopodium quinoa*, *C. amaranticolor*, као и на клијанце плавог патлиџана, чиме је потврђена инфективна природа обољења. Присуство AMV у биљкама плавог патлиџана је даље потврђено применом RT-PCR методе (реверзна транскрипција праћена ланчаном реакцијом полимеразе) и секвенционирањем одабраних изолата коришћењем специфичних прајмера CP AMV1 и CP AMV2, који омогућавају умножавање гена за протеин омотача. Филогенетска анализа показала је груписање српских AMV изолата у јасно одвојену групу заједно са AMV изолатима из Италије, Хрватске и претходно окарактерисаним изолатима из Србије. Према нашим сазнањима, ово је први извештај о присуству вируса мозаика луцерке на плавом патлиџану у Србији.

**Кључне речи:** плави патлиџан, вирус мозаика луцерке, DAS-ELISA, RT-PCR, филогенетска анализа.

### 1. Introduction

Eggplant (*Solanum melongena* L., fam. Solanaceae) is a popular vegetable in Serbia. It is cultivated on small family farms and provides a good source of income for farmers with limited resources. Besides, the increasing number of sunny days in our country provides excellent quality of this vegetable. Eggplant is grown in open fields during summer and in plastic and glass greenhouses during winter (Al-Ani et al., 2011).

Because of its nutritional value, high fiber content and very low levels of saturated fatty acids, it is presumed that the demand for eggplant will increase, and that therefore the production should be increased as well (Sowinska and Krygier, 2013). China was by far the largest producer of eggplants, accounting for over 62% of world production, while the total Serbian eggplant production in 2017 was 2,041 t (FAO, 2017).

Many viruses belonging to *Tymovirus*, *Potyvirus*, *Cucumovirus*, *Tobamovirus* and *Carlavirus* genera were

reported to infect eggplant (Sastry, 1982; Vyanjane and Mali, 1984; Zhou et al., 2015; Hančinský et al., 2020), which can cause significant damage due to plant stunting, crinkling, and mottling accompanied by leaf and fruit abnormalities. The viruses that commonly occur in eggplant either in field or greenhouse cultivation are cucumber mosaic virus, CMV (Crescenzi et al., 1994; Bagewadi et al., 2015), potato virus Y, PVY (Sastry et al., 1974; Sadeghi et al., 2008), as well as alfalfa mosaic virus, AMV (Kemp and Troup, 1977; Al-Ani et al., 2011). Although AMV has been described as being able to infect eggplant, it is considered to be of minor economic importance to eggplant (Marchoux and Rougier, 1974; Al-Shahwan and Abdulla, 1998). However, in recent surveys of eggplant, AMV was found to be the most widespread in the crop (Ozdemir et al., 2011) and was also often detected in eggplant crops grown adjacent to the alfalfa fields (Al-Shahwan et al., 2017).

AMV, the type member of the genus Alfamovirus in the family Bromoviridae, is one of the most widespread viruses and has a wide host range with over 400 hosts belonging to more than 50 botanical families (Hiruki and Hampton, 1990). Although differences in the host range between the different AMV strains have been observed, there is no restriction in host range for a particular AMV strain. In this way, the same AMV strain can infect several plant species belonging to different families (Price, 1940; Schmelzer, 1962). The infection with AMV caused a broad range of symptoms including bright yellow calico mosaic and leaf lesions with chlorosis mostly damaging the chloroplast tissues (Balasubramaniam et al., 2014). The virus is transmitted to a wide host range by several species of aphids in a non-persistent manner, but it can also be spread mechanically (Fleysh et al., 2001; Winterman and Natwick, 2012). Furthermore, AMV is seed-transmitted in some hosts (Bol, 2010).

AMV is a multipartite virus, whose genome is composed of three positive-sense ssRNA molecules (RNA1, RNA2 and RNA3) and a subgenomic RNA4. The genomic nucleic acid is not infectious without the capsid. RNA 1 and 2 encode the viral replicase proteins P1 and P2, respectively, while RNA 3 is bicistronic and codes for the movement protein (MP) (Erny et al., 1992) and the coat protein (CP), which is translated from the subgenomic messenger, RNA 4 (Tenllado and Bol, 2000; Chen and Olsthoorn, 2010).

Although virus-like symptoms on eggplant plants have been observed for years, no investigation was conducted to resolve the etiology of the disease and its distribution in the country. Therefore, the occurrence, incidence and prevalence of eggplant viruses in Serbia are unknown today. Since virus-like symptoms have been increasingly noticed in Serbia over the last few years, the first survey on the presence of eggplant was conducted.

The main objective of this study carried from 2018 to 2019 was to identify the virus or viruses causing symptoms observed in the Province of Vojvodina. Another objective was to give the first insight into the occurrence and distribution of the determined causal agent by sampling and serological testing of symptomatic eggplants. Additionally, the study focused on the molecular characterization and phylogenetic analysis of virus isolates obtained from diseased eggplants.

## 2. Materials and methods

### 2.1. Plant materials and serological identification

During 2018 and 2019, the survey and sample collection were conducted in order to determine viruses associated with eggplant crops in four locations of the Vojvodina Province (Đurđevo, Čonoplja, Bačka Palanka, and Rimski Šančevi). After visual inspection of nine private gardens in which eggplant was grown in the open field, a total of 51 samples were randomly collected. Leaf samples taken from different parts of each plant were transported to the laboratory and stored at -20°C until processed.

All collected samples were tested using the double-antibody sandwich (DAS)-ELISA kits utilizing commercial antisera against the most common eggplant viruses: alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), and potato virus Y (PVY) (Loewe Biochemica, Germany), following the manufacturer's protocol. Plant tissue samples were ground in extraction buffer (1:10 w/v). After incubation with p-nitrophenyl phosphate (Sigma-Aldrich, USA) at room temperature for 2 h in the dark, absorbance at 405 nm (A405) was measured with an ELISA microplate reader (Multiscan Ascent, Finland) and samples were considered positive if the average optical density (OD) was two-fold higher than the average OD of the negative control. Commercial positive and negative controls and extracts from healthy eggplant leaves were included in each ELISA test.

### 2.2. Mechanical transmission

Crude sap extracted from one ELISA-positive sample of nine serologically positive samples, one sample from each location, was used to inoculate mechanically five healthy *Solanum melongena* seedlings as well as five plants of each of the species *Nicotiana benthamiana*, *Chenopodium quinoa*, and *C. amaranticolor*, using 0.01 M phosphate buffer (pH 7). All inoculated plants were maintained under greenhouse conditions to allow symptom development for 15–25 days after inoculation. All inoculated plants were assayed by DAS-ELISA test to confirm AMV presence.

### 2.3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs were extracted from 100 mg leaves of all ELISA positive samples using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. RT-PCR was performed with the One-Step RT-PCR Kit (Qiagen GmbH, Germany) using the AMV specific primer pair, CP AMV1 and CP AMV2 (Finetti-Sialer et al., 1997), which amplify a 751bp fragment corresponding to the partial coat protein (CP) gene and 3'-UTR.

The RT-PCR reaction mixture included 5 µl of 5x Qiagen OneStep RT-PCR Buffer, 400 µM of each of the four dNTPs, 0.6 µM of the viral sense and complementary sense primer, and 1 µl extracted RNA in the final volume of 25 µl. The RT-PCR reaction was performed in a thermal cycler (Eppendorf, Germany) following the protocol: 30 min at 50°C for reverse

transcription, 15 min at 95°C for initial PCR denaturation, followed by 35 cycles for 1 min at 95°C for denaturation, 1 min at 49°C for annealing, 2 min at 72°C for primer extension, and a final extension at 72°C for 10 min. The amplified products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide (0.5g/mL) and visualized using a UV light with Bio-print cx4 (Vilber Lourmat, Germany). The Serbian isolate of AMV (GenBank Accession No. KP034961) from safflower and a healthy eggplant were used as the positive and the negative control, respectively.

#### 2.4. Nucleotide Sequence and Phylogenetic Analysis

RT-PCR products of the two selected isolates, 37Sm and 198Sm collected in 2018 and 2019, respectively, were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced in both directions on an automated sequencer (Macrogen, Korea) using the same primers as those used for amplification. The obtained nucleotide sequences were deposited in the GenBank database and assigned accession numbers. Partial CP gene sequences of Serbian AMV isolates were compared with each other, as well as with AMV sequences available in the GenBank database using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the Clustal W program (Thompson et al., 1994) implemented in MEGA7 software (Kumar et al., 2016). A p-distance model was applied for nucleotide (nt) and deduced amino acid (aa) sequence analyses.

A phylogenetic tree based on the partial CP sequences of two AMV isolates obtained in this study and 21 CP sequences of AMV retrieved from the GenBank was constructed by the neighbor-joining (NJ) algorithm implemented in MEGA7. The best-fitting model of nt substitution was investigated using the MODELTEST implemented in MEGA7, and the Kimura 2-parameter model Gamma distributed (K2+G) was chosen. 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.

### 3. Results and discussion

**Table 1.**

Presence and incidence of alfalfa mosaic virus in eggplant in Serbia in 2018 and 2019

Year	Locality	No. of inspected crops	No. of tested samples	Positive samples on AMV
2018	Čonoplja	2	9	7 (77.78%) <sup>a</sup>
	Đurđevo	1	9	9 (100%)
	Bačka Palanka	1	7	4 (57.14%)
2019	Čonoplja	2	10	10 (100%)
	Đurđevo	2	10	9 (90%)
	Rimski Šančevi	1	6	3 (50%)
Total		9	51	41 (80.39%)

a – Number of infected samples (% of infected samples calculated over the total number of tested samples)

In 2018, the virus was identified in 20 out of 25 (80%) tested samples. The highest incidence of AMV was in the Đurđevo locality, where virus presence was confirmed in 100% of tested samples. In the Čonoplja locality, AMV was detected in 77.78%, while the virus

#### 3.1. Virus detection and symptomatology in the field

The occurrence, incidence and prevalence of eggplant viruses in Serbia are unknown today. However, during the visual inspection of eggplant fields in 2018 and 2019, virus-like symptoms were observed in all inspected localities. Symptoms including distinct bright yellow to white mosaic or mottling (calico) appeared first on the oldest leaves, which later merged in yellow parts of leaves or whole leaves (Figure 1). Also, fruits were reduced in number and size. Characteristic symptoms on diseased host plants such as bright yellow calico mosaic were often associated with AMV (Al-Ani et al., 2011; Al-Saleh and Amer, 2013).



**Figure 1.** Symptom of alfalfa mosaic virus on eggplant

Over a two-year period, using a serological method, the presence of AMV was detected in 80.39% samples, while PVY or CMV was not detected in any of the 51 collected and tested eggplant samples. Also, the virus was detected in all inspected localities in the Province of Vojvodina (Table 1).

was confirmed in 57.14% of the tested samples in the Bačka Palanka locality.

In 2019, the virus was noticed in 22 out of 26 (84.62%) tested samples and the highest incidence of AMV was in the Čonoplja locality (100% tested samples

positive). In the Đurđevo locality, the presence of the virus was confirmed in 9 out of 10 tested samples (90%), while in the Rimski Šančevi locality, the virus was detected only in 3 out of 6 tested samples.

It has been reported that eggplant is infected by many viruses under open-field and plastic and glass greenhouse conditions (Sastry et al., 1974; Brunt et al., 1996; Rakib et al., 2011). The natural occurrence of AMV in eggplant was reported in France (Marchoux and Rougier, 1974), Ontario (Kemp and Troup, 1977), Italy (Ozdemir et al., 2011), and Saudi Arabia (Al-Shahwan et al., 2017). The virus has a broad host range and is non-persistently transmitted by many aphid species, and data on the virus occurrence in various crops all over the world show that AMV is constantly present, and in some years in a high percentage, which can significantly threaten the successful production (Jones et al., 2014; Chatzivassiliou et al., 2004). Furthermore, recent studies in Serbia have shown that AMV population is well established in tobacco (Stanković et al., 2011), tomato (Krstić et al., 2007; Nikolić et al., 2018), pepper (Petrović et al., 2010), alfalfa (Bulajić et al., 2010), and safflower (Milošević et al., 2015), which might be the reason for the increased incidence and prevalence in the eggplant.

### 3.2. Host range

Virus isolates from naturally infected *Solanum melongena*, one from each locality, were successfully transmitted mechanically to the test plants. All inoculated plants produced symptoms, which is consistent with earlier descriptions (El-Abhar et al., 2018; Al-Saleh and Amer, 2013), 15 to 20 days post-inoculation. The mechanically inoculated *Chenopodium quinoa* and *C. amaranticolor* plants reacted uniformly, showing chlorotic local lesions, while the infected *Nicotiana benthamiana* developed bright yellow mosaic, 8–10 and 15–20 days post-inoculation, respectively. Also, the virus was successfully mechanically transmitted to *S. melongena*, which exhibited symptoms identical to those observed on the original host plants. Test plants were assayed by DAS-ELISA and all inoculated plants of each species tested positive for AMV.

### 3.3. Molecular detection and phylogeny

Further confirmation of AMV was carried out using RT-PCR. The presence of AMV in all ELISA-positive samples was successfully confirmed by RT-PCR using the specific primers CP AMV1 and CP AMV2, which amplified part of the CP gene and 3'-UTR and obtained fragments of the expected size of 751 bp. No reaction was recorded in negative controls.

**Table 2.**

CP gene sequences of alfalfa mosaic virus isolates used in the phylogenetic analysis

GenBank Accession Number*	Country	Isolate	Host
MW369445	Serbia	37Sm	<i>Solanum melongena</i>
MW369446	Serbia	198Sm	<i>Solanum melongena</i>
KF147805	Serbia	258-11	<i>Solanum lycopersicum</i>
FJ527749	Serbia	196-08	<i>Nicotiana tabacum</i>
KC182567	Serbia	P-10-10	<i>Capsicum sativum</i>
KP034961	Serbia	292Saff	<i>Carthamus tinctorius</i>
EU925642	Serbia	100-08	<i>Syringa vulgaris</i>
JX996119	Croatia	70-12	<i>Lavandula x intermedia</i>
KJ504107	Croatia	371-13	<i>Lavandula x intermedia</i>
JX112757	Australia	EW	<i>Medicago sativa</i>
JX112758	Australia	Aq	<i>Medicago sativa</i>
JX112759	Australia	HU	<i>Medicago sativa</i>
KF487082	Saudi Arabia	Jehan1	<i>Medicago sativa</i>
KF487087	Saudi Arabia	Jehan6	<i>Medicago sativa</i>
FJ858265	Brazil	-**	<i>Medicago sativa</i>
JQ673587	Iran	Ke.Ba.Al	<i>Medicago sativa</i>
HQ332380	Turkey	DA-05	<i>Medicago sativa</i>
KX458466	Saudi Arabia	9_AMV	<i>Solanum tuberosum</i>
KX458467	Saudi Arabia	10_AMV	<i>Solanum tuberosum</i>
KX458468	Saudi Arabia	11_AMV	<i>Solanum tuberosum</i>
KX458470	Saudi Arabia	13_AMV	<i>Solanum tuberosum</i>
KX458471	Saudi Arabia	14_AMV	<i>Solanum tuberosum</i>
KX458472	Saudi Arabia	15_AMV	<i>Solanum tuberosum</i>
DQ314751	Canada	Ca399	<i>Solanum tuberosum</i>
DQ314752	Canada	Ca400	<i>Solanum tuberosum</i>
DQ314753	Canada	Ca401	<i>Solanum tuberosum</i>
Y09110	Italy	Danza	<i>Lycopersicon esculentum</i>
FR854391	Italy	Tef-1	<i>Teucrium fruticans</i>

\* - All data are from the GenBank, \*\* - Name of isolate is not known

After purification, the RT-PCR products derived from the selected isolates 37Sm and 198Sm were directly sequenced in both directions using the same primer pair as the one used in RT-PCR and were

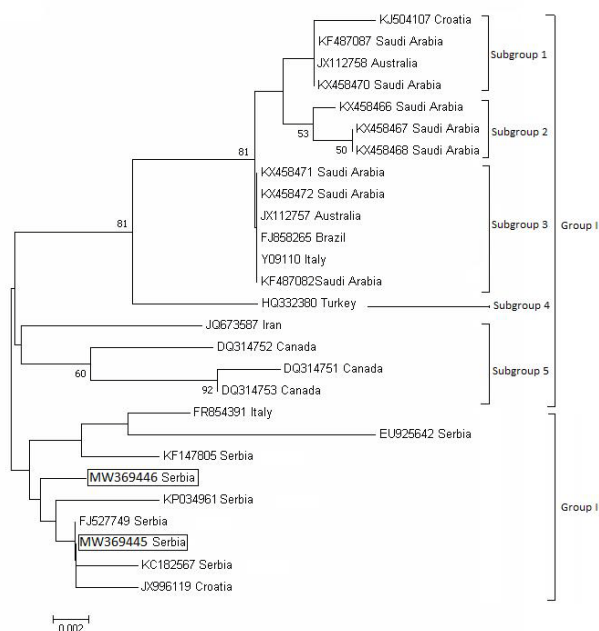
submitted to the GenBank and assigned accession numbers (GenBank Accession Nos. MW369445 and MW369446, respectively). Multiple sequence alignment of the CP gene, conducted with MEGA7

software, showed that the Serbian AMV isolates originating from eggplant were similar to each other (99.7% at the nucleotide level (98.9% amino acid identity) and shared the highest nucleotide identity of 100% (100% aa identity) with the Chinese isolate BJF1 (MN846749).

The sequences of the Serbian AMV isolates were aligned with the selected sequences of 21 previously characterized AMV isolates retrieved from the GenBank database, and a phylogenetic tree was constructed using the neighbor-joining method. The phylogenetic analysis showed that the AMV isolates were clustered into two major groups (group I and II) (Fig. 2), as determined by a previous report (Alhudaib, 2019). Genetic diversity between groups I and II was  $0.024 \pm 0.006$ , while diversity within each group was:  $0.013 \pm 0.004$  (group I) and  $0.017 \pm 0.004$  (group II). Group I of the AMV isolates was divided into five subgroups, i.e. subgroups 1 to 5. Subgroup 1 included two isolates from Saudi Arabia, one from Croatia and one from Australia ( $0.002 \pm 0.002$ ); subgroup 2 was composed of three isolates from Saudi Arabia ( $0.002 \pm 0.002$ ); subgroup 3 included three isolates from Saudi Arabia, and one from each of the countries: Australia, Brazil and Italy ( $0.000 \pm 0.000$ ); and subgroup 5 comprised three isolates from Canada and one from Iran ( $0.019 \pm 0.005$ ). Subgroup 4 consisted of a single

isolate from Turkey. The mean distance among subgroups 1, 2, 3, 4 and 5 ranged from  $0.004 \pm 0.003$  to  $0.031 \pm 0.009$ .

The phylogenetic tree also revealed the clustering of Serbian (7), Croatian (1) and Italian (1) isolates in group II. The phylogenetic analysis placed AMV isolates originating from eggplant in Serbia into group II together with the other previously characterized AMV isolates from Serbia (Nikolić et al., 2018; Stanković et al., 2011; Milošević, 2013; Milošević et al., 2015). Similarly, it has been reported that all Canadian potato AMV isolates grouped into one of at least four different clusters (Xu and Nie, 2006). All isolates originating from Serbia regardless of their hosts or origin were grouped into the same group, indicating that in recent years there has been a local expansion of the AMV population in Serbia. On the other hand, all previously detected Serbian AMV isolates and isolates obtained in this study were closely related to isolate Tef-1 from Italy and isolate 70-12 from Croatia, suggesting that gene flow had occurred among these three countries. The high similarity level between isolates from different regions indicated that all isolates may have the same origin and that they are easily transmitted by infected seed, mechanical transmission or aphid transmission (Bol, 2008).



**Figure 2.** Neighbor-joining tree based on partial sequences of CP gene of 27 isolates of alfalfa mosaic virus. Phylogram was generated with MEGA7 using bootstrap analysis with 1000 replicates and bootstrap values (>50%) are shown next to relevant branches. The Serbian alfalfa mosaic virus isolates from eggplant are framed.

#### 4. Conclusions

The detection of AMV in all inspected localities, as well as the severity of symptoms and high disease incidence during 2018 and 2019, implied that the virus may be a serious constraint for the successful production of eggplant in Serbia. Due to great damage caused by AMV worldwide, the determination of variability within the population of AMV in eggplant crops and the establishment of relationships with the isolates originating from other hosts in Serbia will contribute to a better understanding of their

epidemiology as well as to the discovery of plants significant for viral conservation. Such knowledge will ensure effective control and prevent the introduction of new viral strains into our country through the international exchange of plant material.

#### Acknowledgment

This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant numbers: 451-03-9/2021-14/200032 and 451-03-9/2021-14/200116.

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