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The spreading of *Alfalfa mosaic virus* in lavandin in Croatia

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SUMMARY

A survey was conducted in 2012 and 2013 to detect the presence and distribution of *Alfalfa mosaic virus* (AMV) in lavandin crops growing in continental parts of Croatia. A total of 73 lavandin samples from six crops in different localities were collected and analyzed for the presence of AMV and *Cucumber mosaic virus* (CMV) using commercial double-antibody sandwich (DAS)-ELISA kits. AMV was detected serologically in 62 samples collected at three different localities, and none of the samples tested positive for CMV. For further analyses, six selected samples of naturally infected lavandin plants originating from different localities were mechanically transmitted to test plants: *Chenopodium quinoa, C. amaranticolor, Nicotiana benthamiana* and *Ocimum basilicum*, confirming the infectious nature of the disease. Molecular detection was performed by amplification of a 751 bp fragment in all tested samples, using the specific primers CP AMV1/CP AMV2 that amplify the part of the coat protein (CP) gene and 3'-UTR. The RT-PCR products derived from the isolates 371-13 and 373-13 were sequenced (KJ504107 and KJ504108, respectively) and compared with the AMV sequences available in GenBank. CP sequence analysis, conducted using the MEGA5 software, revealed that the isolate 371-13 had the highest nucleotide identity of 99.5% (100% amino acid identity) with an isolate from Argentina originating from *Medicago sativa* (KC881010), while the sequence of isolate 373-13 had the highest identity with an Italian AMV isolate from *Lavandula stoechas* (FN667967) of 98.6% (99% amino acid identity). Phylogenetic analysis revealed the clustering of selected isolates into four molecular groups and the lavandin AMV isolates from Croatia grouped into two distinct groups, implying a significant variability within the AMV lavandin population.

Keywords: *Alfalfa mosaic virus*; Lavandin; Molecular detection; Phylogenetic analysis; Croatia

INTRODUCTION

Numerous species, hybrids and cultivars of the genus *Lavandula*, commonly known as lavender, have been cultivated on a large scale in some South European countries for the extraction of essential oils or as ornamentals in gardens and landscapes. Lavender essential oils are widely used in aromatherapy, medicine, pharmacy, perfume and cosmetics industry (Hui et al. 2010; Herman et al., 2013; Soltani et al., 2013) ranking lavender among globally important crops (Weiss, 1996).

Lavender is widely grown throughout the Mediterranean, in Australia and the United States. The Adriatic coast in Croatia is traditionally associated with lavender cultivation. Unfortunately, the coastal lavandin-growing regions have significantly shrunk after natural disasters such as fire, especially on Hvar Island, which is famous for its lavender production, but also because of a shift in producers' focus to other crops (Vrandečić, personal communication). On the other hand, commercial cultivation of lavender has increased since 2005 in continental parts of Croatia which are characterized by significant seasonal temperature differences, i.e. usually warm to very hot summers and cold winters. Excellent adaptability to different weather conditions has recommended lavandin (*Lavandula* × *intermedia* Emeric ex Loiseleur) to be widely cultivated in continental climate.

Data on viruses infecting plants of the genus *Lavandula* are scarce but *Alfalfa mosaic virus* (AMV) and *Cucumber mosaic virus* (CMV) have been reported infecting lavender plants. The most common viral disease of lavender is described as "yellow mosaic" and it is caused by AMV (Marchoux & Rougier, 1974; Giunchedi & de Ferrer, 1977; Martínez-Priego et al., 2004; Kobyłko et al., 2008; Parrella et al., 2010). The virus has been reported in Italy, on *Lavandula latifolia* x *L. angustifolia* (Giunchedi & de Ferrer, 1977) and *L. stoechas* L. (Parrella et al., 2010), in Spain, on *L. officinalis* (Martínez-Priego et al., 2004), and in France, on *L. hybrida* (Marchoux & Rougier, 1974). Lavender plants infected with AMV exhibit yellow leaf and stem spotting, yellow mottling, calico mosaic, leaf curling and shortening of internodes, giving the plants a bushy appearance. The flowering of infected plants does not change in the number, size or color of flowers but lavender plants infected with AMV have lower essential oil production quality through deteriorated composition, i.e. changed concentrations of some of the main essential oil components (Bellardi et al., 2006; Bruni et al., 2006).

Considering the importance of lavender, the increasing spread of AMV on various types of lavender in the Mediterranean, and common presence of many aphids that are vectors of the virus, AMV is potentially a limiting factor for successful production of lavender in Croatia. After the first detection of AMV infecting *L.* x *intermedia* (Vrandečić et al., 2013), a survey was conducted in the main lavandin-producing areas of continental Croatia in order to detect the presence and distribution of AMV and possible presence of CMV, and to determine the genetic relationship of Croatian lavandin AMV isolates with those from other parts of the world.

MATERIAL AND METHODS

Survey and sample collection

During 2012 and 2013, a survey was carried out in order to determine the presence and distribution of AMV in lavandin crops in continental parts of Croatia. After visual inspection of six lavandin crops at three different localities (Banovo Brdo, Belišće and Vinkovci) in Slavonija and Baranja Counties, a total of 73 samples of symptomatic plants were randomly collected. Samples prepared of leaves from different parts of each plant were placed in plastic bags, and stored at 4°C until testing by enzyme-linked immunosorbent assay (ELISA) or stored at -20°C until RNA extraction.

Serological detection

Serological testing was performed by double-antibody sandwich (DAS)-ELISA utilizing commercial antisera (Bioreba AG, Reinach, Switzerland): AMV and CMV, 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, St. Louis, MO) at room temperature for 2 h in the dark, absorbance at 405 nm was measured with an ELISA microplate reader (DASsrl, Italy) and samples were considered positive if the absorbance value was higher than twice the absorbance of the negative control. Commercial positive and negative controls and extracts from healthy lavandin tissue were included in each ELISA test.

Mechanical transmission

Crude sap extracted from the leaves of six serologically positive samples, two samples from each locality, using 0.01 M phosphate buffer (pH 7), was mechanically inoculated onto five plants each of *Chenopodium quinoa*, *C. amaranticolor*, *Nicotiana benthamiana* and *Ocimum basilicum*. 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 after inoculation. All inoculated plants were assayed by DAS-ELISA test to confirm AMV presence.

RT-PCR detection

For further confirmation of ELISA-positive results, six selected samples were tested by conventional reverse transcription (RT)-PCR assay. Total RNAs were extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed using the One-Step RT-PCR Kit (Qiagen) with the AMV specific primer pair CP AMV1 and CP AMV2 (Finetti-Sialer et al., 1997), yielding a 751-bp fragment corresponding to the partial coat protein (CP) gene and 3'-UTR.

The RT-PCR reaction mixture included 400 µM each of the four dNTPs, 0.6 µM of the viral sense and complementary sense primer, and 1 µl extracted RNA in a final volume of 25 µl. RT-PCR reaction was performed in a thermal cycler (Biometra, T-1 Thermocycler) following the protocol: 30 min at 50°C for reverse transcription, 15 min at 95°C for initial PCR activation/denaturation, followed by 35 cycles of 1 min at 95°C for denaturation, 1 min at 49°C for annealing, 2 min at 72°C for primer extension and 10 min at 72°C for final extension. Amplified products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under a UV transilluminator. Total RNAs obtained from a Serbian AMV isolate from tobacco (GenBank Accession No. FJ527749) and a healthy lavandin plant served as the positive and the negative control, respectively.

Sequencing and phylogenetic analysis

After purification (QIAquick PCR Purification Kit, Qiagen) the amplified products obtained from two selected isolates, 371-13 and 373-13 originating from different localities (Belišće and Vinkovci, respectively), were directly sequenced in both directions using the same primer pair as in RT-PCR and deposited in GenBank. Additionally, a previously identified AMV isolate 70-12 originating from Croatian lavandin (Vrandečić et al., 2013) was also included in the investigation (Table 1).

Sequences of the Croatian AMV isolates generated in this study were compared with AMV sequences available in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) 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 the AMV CP sequences generated in this study, one already published (Vrandečić et al. 2013) and 26 CP sequences of AMV retrieved from GenBank (Table 1) by the Neighbor-Joining algorithm implemented in MEGA5. The best-fitting model of nt substitution was investigated using the MODELTEST implemented in MEGA5, and the Kimura 2-parameter model Gamma distributed (K+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 K+G.

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

Isolate*	Geographical origin	Host	GenBank Acc. No.
70-12	Croatia	lavandin	JX996119
371-13	Croatia	lavandin	KJ504107
373-13	Croatia	lavandin	KJ504108
196-08	Serbia	tobacco	FJ527749
VRU	England	garden lupin	AF015716
15/64	England	garden lupin	AF015717
S	England	lucerne	X00819
425 Medison	USA	clover	K02703
425 Leiden	USA	clover	L00162
126-A	Italy	*purslane*	AJ130704
195-AN	Italy	tomato	AJ130705
F-430	Italy	bean	AJ130706
Danza	Italy	tomato	Y09110
Lye-80	France	tomato	AJ130703
Caa-1	France	pepper	AJ130707
Dac-16	France	carrot	AJ130708
Lyh-1	France	wild tomato	AJ130709
Ca375	Canada	potato	DQ314749
Ca175	Canada	potato	DQ314750
Ca399	Canada	potato	DQ314751
Ca400	Canada	potato	DQ314752
Ca401	Canada	potato	DQ314753
Ca508	Canada	potato	DQ314754
Ca518	Canada	potato	DQ314755
Ca616	Canada	potato	DQ314756
N20	Australia	/**	AF332998
NZ34	New Zealand	pea	AF215664
KR1	Korea	potato	AF294432
KR2	Korea	potato	AF294433

* - All data are from GenBank;
** - Host plant is not known

RESULTS

Virus detection and symptomatology in the field

During the visual inspection of lavandin fields in 2012 and 2013, various foliar symptoms were observed in all inspected localities with disease incidence ranging from 10 to 40%. Plants infected early in the growing season showed severe symptoms including stunting, bright yellow calico mosaic, yellow mottling and leaf distortion, while those infected at later stages of growth exhibited only mild mosaic symptoms.

Serological analysis of lavandin samples, revealed the presence of AMV in all inspected localities in Croatia (Table 2). None of the samples analyzed was positive for CMV. After the first detection of AMV in 2012, its presence was confirmed also in 2013 in 47 lavandin samples collected from three commercial lavandin crops in two different localities: Belišće and Vinkovci. The highest incidence of AMV was in the locality Banovo Brdo (100% samples tested positive) as well as in the locality Belišće, where virus presence was proved in both inspected crops, i.e. in 35 out of 38 tested samples (92.11%). The presence of AMV in the locality Vinkovci was detected in one of the three examined lavandin crops, and in 60% of the tested samples.

Host range

Virus isolates from naturally infected lavandin plants, two from each locality, including the isolate described by Vrandečić et al. (2013), were successfully transmitted mechanically to the test plants. All five mechanically inoculated *C. quinoa* and *C. amaranticolor* plants reacted uniformly, showing local chlorotic spots accompanied by mosaic, while the infected *N. benthamiana* and *O. basilicum* developed bright yellow mosaic, 6-8 and 12-14 days post-inoculation, respectively. The test plants were assayed by DAS-ELISA and all inoculated plants of each species tested positive for AMV.

Molecular detection and identification

The specific primers CP AMV1 and CP AMV2 successfully detected the presence of AMV in all tested samples from lavandin crops in Croatia by amplifying the part of the CP gene and 3'-UTR and obtaining fragments of predicted size. One clear band of 751 bp was visible in all tested samples as well as in positive control. No amplification product was observed when extract from the healthy lavandin plant was used as template in the RT-PCR assay.

After purification, the RT-PCR product derived from the isolates 371-13 and 373-13 were directly sequenced in both directions using the same primer pair as in RT-PCR and deposited in GenBank (KJ504107 and KJ504108, respectively). The sequence of the Croatian AMV isolates exhibited nucleotide identities ranging from 97.3% to 98.4%. Multiple sequence alignment of the CP open reading frame revealed 98.4% identity between the isolates 70-12 and 373-13. The sequences of these two isolates differed from each other at ten nucleotide positions, which cause two amino acid substitutions (99.0% aa identity). Isolate 371-13 differed from isolates 70-12 and 373-13 at 13 (97.9% nt identity) and 17 (97.3% nt identity) nucleotide positions, respectively. These differences were predicted to cause three (98.6% aa identity) amino acid substitutions in each case. Isolate 371-13 had the highest nucleotide homology of 99.5% (100% aa identity) with an isolate from Argentina originating from *Medicago sativa* (KC881010), while the sequence of isolate 373-13 showed the highest nucleotide identity with an Italian AMV isolate from *Lavandula stoechas* (FN667967) of 98.6% (99% amino acid identity).

Phylogenetic analysis

A Neighbor-Joining analysis based on partial sequence of the CP gene revealed that the AMV isolates acquired in this study and the selected sequences of 26 previously characterized AMV isolates retrieved from the GenBank database clustered into four molecular groups regardless

Table 2. Presence and incidence of *Alfalfa mosaic virus* in lavandin crops in Croatia in 2012 and 2013

Year	Locality	Crop type	Genotype	No. of inspected crops	No. of tested samples	No. of crops infected with AMV	Positive samples
2012	Banovo Brdo	Comercial nursery	Budrovka	1	15	1	15 (100%)*
2013	Belišće	Comercial crop	Budrovka	2	38	2	35 (92.11%)
	Vinkovci	Comercial crop	Grosso	3	20	1	12 (60%)
Total				6	73	4	62 (84.93%)

* - Number of infected samples (% of infected samples calculated over the total number of tested samples)

of their geographic origin or plant host (Figure 1). Genetic diversity among the four molecular groups of isolates ranged from 0.026±0.004 to 0.062±0.009, while diversity within each group was: 0.021±0.004 (I), 0.015±0.004 (II), 0.020±0.003 (III), and 0.022±0.003 (IV). The first molecular group contained four isolates from France and two isolates from England, while only three isolates from Australia, USA, and New Zealand formed the second group. In molecular group III, two isolates from Korea and one each from the USA, Italy, England, and Canada were clustered, and the Croatian AMV isolate 371-13 was in that group. The other two AMV isolates from Croatia originating from lavandin (70-12 and 373-13) grouped into molecular group IV together with seven isolates from Canada, three isolates from Italy, and one isolate from Serbia.

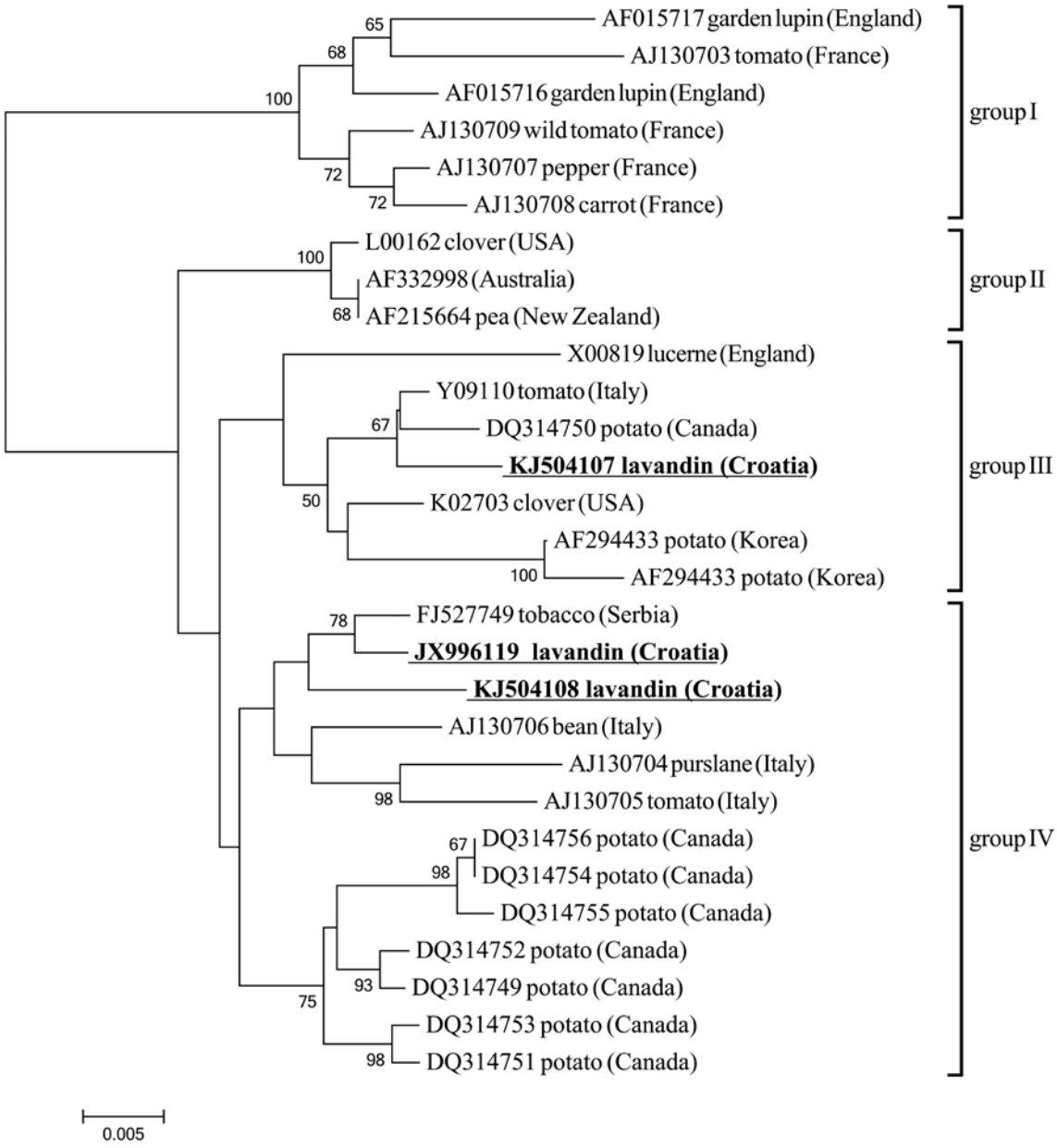

Figure 1. Neighbor-Joining tree based on partial sequences of the coat protein gene of 29 isolates of AMV. Phylogram was generated with MEGA5 using bootstrap analysis with 1000 replicates, and bootstrap values (>50%) are shown next to relevant branches. The AMV isolates from lavandin from Croatia are underlined and bolded

DISCUSSION

The popularity of lavandin and its essential oils reflects a trend of using natural products in medicine, pharmacy, cosmetics and other industries. That is why lavandin production is growing in terms of production areas and product value in Croatia and all over the world. The detection of AMV in 15 lavandin samples collected from a commercial nursery in 2012, as well as the severity of symptoms and high disease incidence, implied that the virus may represent a serious constraint for successful production of lavandin in Croatia (Vrandečić et al., 2013). As information about the variety of symptoms in lavandin, distribution of the virus, and above all its incidence was not available, a survey was conducted and lavandin production in continental parts of Croatia was inspected.

Regarding the previously reported outbreak of AMV in a lavandin commercial nursery in the locality Banovo Brdo (Vrandečić et al., 2013), the virus was detected serologically in this study in three additional commercial lavandin crops in two different localities in 2013. In all inspected lavandin fields, the incidence of virus infection was high, estimated to be up to 40%. AMV infection was confirmed in both inspected crops in the locality Belišće, i.e. in 92.11% of the tested samples, while virus presence in the locality Vinkovci was detected in 12 out of 20 lavandin samples collected from one inspected crop. In the Mediterranean region, AMV was first reported in *L. hybrida* in France (Marchoux & Rougier, 1974). Subsequently, the virus was reported infecting *L. latifolia* x *L. angustifolia* in Italy (Giunchedi & de Ferrer, 1977), then infecting *L. officinalis* in Spain (Martínez-Priego et al., 2004), and *L. stoechas* L. in Italy (Parrella et al., 2010). Although AMV was not detected in all inspected crops in Croatia, it was present in all localities included in this investigation with a significant field incidence. Taking into consideration the efficient spreading of AMV by several aphid vectors, such incidence and distribution imply that AMV could become a limiting factor for successful production of lavandin in Croatia.

AMV infection is often associated with characteristic symptoms on diseased host plants, such as bright yellow calico mosaic (Marchoux & Rougier, 1974; Giunchedi & de Ferrer, 1977; Martínez-Priego et al., 2004; Parrella et al., 2010), which was also observed on lavandin plants in Croatia. Additionally, lavandin plants infected early in the growing season showed stunting and severe yellow mottling accompanied with leaf distortion, while those infected at later stages of growth usually exhibited only mild mosaic.

The virus isolates from lavandin plants were able to induce symptoms on commonly used AMV test plants. All inoculated *C. quinoa* and *C. amaranticolor* plants produced symptoms typical of AMV, chlorotic spots on inoculated leaves accompanied with mosaic within 6-8 days post-inoculation (Parrella et al., 2010; Parrella et al., 2000). Infected plants of *N. benthamiana* and *O. basilicum* exhibited bright yellow mosaic 12-14 days post-inoculation, which is consistent with symptoms caused by AMV (Parrella et al., 2010; Wintermantel & Natwick, 2012). Mechanical transmission to the test plants confirmed the infectious nature of lavandin disease and the symptoms developing on selected test plants confirmed the biological identification of AMV.

Multiple nucleotide and deduced amino acid sequence comparisons implicated a variability in AMV populations in lavandin in Croatia. Two of three Croatian AMV isolates originating from lavandin were closely related in both nucleotide and amino acid levels in CP sequences, while the isolate 371-13 was more distantly related to them. The phylogenetic analysis based on the nucleotide sequence of the CP gene showed that the AMV isolates from Croatia and 26 other AMV isolates might be divided into four distinct molecular groups. Although a previous study had shown that AMV isolates from France and Italy fell into two distinct subgroups (Parrella et al., 2000), our phylogenetic data analysis of AMV strains are in agreement with those of Xu and Nie (2006) and Stanković et al. (2011), who found that AMV isolates may be divided into four distinct molecular groups. Two isolates originating from the Croatian localities Banovo Brdo and Vinkovci (70-12 and 373-13, respectively) clustered within the molecular group IV, but the isolate 371-13, originating from the locality Belišće, fell into a different molecular group (group III). Similarly, it has been reported that Canadian potato AMV isolates grouped in two different clusters (Xu & Nie, 2006). Our analysis also indicated that the Croatian AMV isolates did not share a recent common ancestor, representing two distinct lineages of AMV in Croatia.

Data collected in this investigation, showing AMV spreading into new production localities and its substantial incidence in lavandin in Croatia make one of the first reports on diseases of this valuable crop in the region. Determination of the variability within population of AMV in lavandin crops, but also the detection of relationships with isolates originating from other host plants in Croatia will contribute to our better understanding of the epidemiology of this pathogen, especially relating to virus reservoirs in nature and the way of introduction into lavandin crops. The obtaining of virus-free lavandin planting material and implementation of additional control measures in order to prevent further spreading of AMV, including a prevention of potential introduction of new destructive strains in Croatia due to intensive international exchange of plant material, will be of special attention in the future.

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Širenje virusa mozaika lucerke u usevu lavande u Hrvatskoj

REZIME

Tokom 2012. i 2013. godine sprovedena su istraživanja u cilju utvrđivanja prisustva i rasprostranjenosti virusa mozaika lucerke (*Alfalfa mosaic virus*, AMV) u usevu lavande u kontinentalnom delu Hrvatske. Sa šest lokaliteta gajenja ukupno je sakupljeno 73 uzorka lavande koji su serološki testirani na prisustvo AMV i virusa mozaika krastavca (*Cucumber mosaic virus*, CMV) korišćenjem komercijalno dostupnih kitova za DAS-ELISA test. Prisustvo AMV dokazano je u 62 uzorka lavande sakupljena sa tri različita lokaliteta, dok prisustvo CMV nije dokazano ni u jednom od testiranih uzoraka. Za dalja istraživanja odabrano je šest uzoraka prirodno zaraženih biljaka lavande poreklom iz različitih lokaliteta, koji su uspešno preneti mehaničkim inokulacijama na test biljke *Chenopodium quinoa, C. amaranticolor, Nicotiana benthamiana,* i *Ocimum basilicum*, čime je potvrđena infektivna priroda oboljenja. Molekularna detekcija obavljena je amplifikacijom fragmenta dužine 751 bp kod svih ispitivanih izolata korišćenjem specifičnih prajmera CP AMV1/CP AMV2 koji omogućavaju umnožavanje dela gena za proteinski omotač i 3' neprepisujućeg regiona. U cilju dalje identifikacije, RT-PCR produkti izolata 371-13 i 373-13 su sekvencionisani (KJ504107 i KJ504108) i upoređeni sa AMV sekvencama dostupnim u GenBank bazi podataka. Analizom sekvenci gena za protein omotača, korišćenjem MEGA5 softverskog paketa, najviši stepen nukleotidne sličnosti od 99.7% (100% aminokiselinska sličnost) izolat 371-13 pokazao je sa izolatom AMV iz Argentine poreklom iz *Medicago sativa* (KC881010), dok sekvenca izolata 373-13 najveću sličnost od 98.6% (99% aminokiselinska sličnost) deli sa italijanskim izolatom AMV iz *Lavandula stoechas* (FN667967). Filogenetska analiza pokazala je grupisanje izolata u četiri molekularne grupe, a izolati AMV iz lavande iz Hrvatske grupišu se u dve odvojene grupe, ukazujući na postojanje značajne varijabilnosti u populaciji AMV poreklom iz lavande.

Ključne reči: Virus mozaika lucerke; lavanda; molekularna detekcija; filogenetska analiza; Hrvatska