

# Identification and some properties of Alfalfa mosaic alfamovirus isolated from naturally infected tomato crop

Irena Zitikaite,

Marija Samuitienė

*Institute of Botany,  
Žaliųjų Ežerų 49, LT-08406  
Vilnius, Lithuania*

*E-mail: irena.zitikaite@botanika.lt;  
marija.samuitiene@botanika.lt*

Alfalfa mosaic alfamovirus (AMV) causing disease in tomato (*Lycopersicon esculentum* Mill.) crop in Lithuania has been isolated and identified on the basis of determination of an experimental host range, symptom expression, morphological properties and a positive immunological test of the virus isolates. The primers designed on the basis of published sequences were applied for amplification of AMV RNA fragments in reverse transcription-polymerase chain reaction (RT-PCR) using host plants experimentally infected with AMV. The detection of AMV in tomato and inoculated test-plants was confirmed by the RT-PCR technique. Analysis of PCR products in polyacrylamide gel electrophoresis revealed amplification of approximately 620 bp (base pair) fragments which were in agreement with the size of the fragment expected from the sequence data.

**Key words:** tomato, Alfalfa mosaic alfamovirus, identification, ELISA, RT-PCR

## INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.), an annual plant of the family *Solanaceae* Juss., suffers from viral diseases which decrease the production of this crop. More than twenty different viruses have been reported as pathogenic to tomato. A number of viruses singly infecting this host are known to have potential to inflict severe economic losses. In nature, many diseases of great economic importance are caused by a mixture of different viruses affecting the host. Alfalfa mosaic virus (AMV) is the type species of the genus *Alfamovirus* and belongs to the family *Bromoviridae*. AMV has a tripartite single-stranded genome. RNAs 1, 2, 3 and subgenomic RNA4 are separately encapsidated into bacilliform particles which are 18 nm wide and have lengths characteristic of the RNA encapsidated (about 56, 43, 35 and 30 nm, respectively) [1]. The three genomic RNAs are not infective. Infection can start only in the presence of RNA4 or its translation product (CP). Viral particles of varying length are not distinguishable serologically. In addition, the virus preparations contain spheroidal particles each containing two copies of RNA 4. Virus particles contain 16–17% of RNA [2]. The thermal inactivation point for AMV is 50 to 70 °C (usually between 60 and 65 °C); the dilution end point is  $1 \times 10^{-3}$  to  $1 \times 10^{-4}$ ; and longevity *in vitro* is 1 to 4 days [3, 4].

AMV mostly infects herbaceous plants, but several woody species are included in the natural host range [5]. The experimental and natural host ranges include over 600 species in 70 families. AMV causes diseases in several vegetable crops, including pepper (*Capsicum annuum* L.), celery (*Apium graveolens* L.), bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.), lettuce (*Lactuca sativa* L.), tomato and eggplant (*Solanum melongena* L.). Natural infections caused by AMV are less frequent in

tomato than in pepper crops. It has been found naturally infecting potato (*S. tuberosum* L.) and alfalfa (*Medicago sativa* L.) [3, 6–8]. In Spain, AMV has been found naturally infecting alfalfa, pepper, tomato and common borage (*Borago officinalis* L.) [9]. The virus was tested in wild tuberous comfrey (*Symphytum tuberosum* L.) growing in the Botanical Garden of Bologna (Italy) [10]. Field plantings of cowpea (*Vigna unguiculata* (L.) Walp) and mung bean (*V. mungo* (L.) Hepper) in Iran were naturally infected by AMV, and seed yield was reduced by about 10% [11]. In USA, AMV was isolated from naturally infected tumble pigweed (*Amaranthus albus* L.) and chickpea (*Cicer arietinum* L.) [12]. The occurrence of AMV in yellow lupin (*Lupinus luteus* L.) in Poland was described [13]. Symptoms may be masked. In many species, bright yellow mottle or mosaic is common. Severe necrosis may also occur. At least 15 aphid species are known to transmit the virus in the stylet-borne or non-persistent manner. In nature, AMV is preserved and transmitted with the seed of alfalfa, pepper and weeds (*Datura stramonium* L., *Solanum nigrum* L., *Chenopodium quinoa* Willd. and possibly *Melilotus* sp.) [14]. In Lithuania, AMV was detected in leguminous (alfalfa, clover, lupin) crops [15] and in phloxes [16].

The present study was initiated to investigate the disease of tomato crop caused by possible AMV, to identify the causal agent and to establish some properties (host range, symptoms, morphology of virions) of this virus.

## MATERIALS AND METHODS

Samples of tomato leaf tissue and fruits for investigation were collected at private gardens in Vilnius district. Twelve tomato plant samples expressing virus disease symptoms have been investigated for virus presence. The diagnostic study of the pathogen

was done at the greenhouse of Plant Virus Laboratory of Institute of Botany using standard methods for mechanically transmitted viruses. The virus was identified by test-plant reaction to inoculation with two isolates (Nos. 0311 and 0313) according to [3, 6, 17]. In order to differentiate virus isolates from diseased tomato samples and identify the virus, test-plants of *Aizoaceae* Rudolphi, *Amaranthaceae* Juss., *Chenopodiaceae* Vent., *Cucurbitaceae* Juss., *Fabaceae* Lindl and *Solanaceae* Juss. families were used (Table).

Specimens for mechanical inoculation were prepared by homogenizing leaf tissues of affected tomato and test-plants in 0.1 M sodium phosphate buffer, pH 7.0–7.2 (1 : 3 wt/vol), containing as stabilising agents 0.02% 2-mercapthoethanol or 0.01 M sodium diethyldithiocarbamate (Na DIECA). The inoculations were performed with the aid of carborundum powder as an abrasive [18]. Symptom development on indicator hosts were recorded every 2 days during 12 days after inocula-

tion and twice a week in the subsequent 30 days. The possibility of symptomless infection was checked electron microscopically (EM) or by back-inoculation to healthy test-plants.

Presence of virus particles and their morphology was determined by investigation of negatively stained (with 3% uranyl acetate) dip preparations using the JEOL JEM-100S transmission electron microscope at the instrumental magnification of 25000<sup>x</sup> [18–20].

To detect AMV in infected indicator plants, the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used [21]. Serological confirmation of virus presence in test samples of experimentally infected plants was conducted using the commercially prepared polyclonal antiserum to AMV (DSMZ Plant Virus Collection, Germany). Enzyme-substrate reactions were measured at 405 nm using an ELISA reader (Labsystem Multiskan RC). The ELISA readings were banked on buffer controls, and a sample was considered positive when its

Table. Experimental host range of Alfalfa mosaic virus isolated from tomato samples

Test-plant	Reaction	
	Local	Systemic
<b>Aizoaceae Rudolphi</b>		
<i>Tetragonia expansa</i>	–	(Leaf distortion)
<b>Amaranthaceae Juss.</b>		
<i>Amaranthus retroflexus</i>	Necrotic lesions	–
<i>Celosia argentea f. cristata</i>	–	–
<i>Gomphrena globosa</i>	Necrotic lesions	Mild mosaic, leaf distortion
<b>Chenopodiaceae Vent.</b>		
<i>Atriplex hortensis</i>	–	(Mild yellowing)
<i>Chenopodium amaranticolor</i>	Chlorotic spots	Mild mottling
<i>C. ambrosioides</i>	Necrotic lesions	–
<b>Cucurbitaceae Juss.</b>		
<i>Cucumis sativus</i>	Chlorotic spots	Vein clearing, mild mosaic
<i>Cucurbita pepo</i>	–	–
<b>Fabaceae Lindl.</b>		
<i>Phaseolus vulgaris</i>	Necrotic lesions	(Mild mottling)
<i>Pisum sativum</i>	Black necrotic lesions	(Stunting)
<i>Vicia faba</i>	Small necrotic lesions	–
<b>Solanaceae Juss.</b>		
<i>Capsicum annum</i>	–	Mild mosaic
<i>Datura stramonium</i>	–	Bright green mottling
<i>Lycopersicon esculentum</i>	–	Yellow mottling
<i>Nicandra physalodes</i>	Necrotic spots	Chlorosis, yellow mosaic
<i>Nicotiana alata</i>	–	(Interveinal chlorosis)
<i>N. clevelandii</i>	–	Chlorotic mottling
<i>N. debneyi</i>	Necrotic spots	Vein clearing, leaf distortion
<i>N. glutinosa</i>	Necrotic lesions	Mild mosaic
<i>N. rustica</i>	Necrotic lesions	Top necrosis
<i>N. tabacum</i>	(Necrotic lesions)	Vein clearing, mild mottling

(–) – infection occurred not in each case.

$A_{405\text{nm}}$  value exceeded three times the mean value of the extracts from negative control (non-infected plants).

For detection of AMV by reverse transcription-polymerase chain reaction (RT-PCR) [22], leaf tissues of experimentally infected *Chenopodium amaranticolor* Coste et Reyn., *Atriplex hortensis* L. and *Nicotiana glutinosa* L. plants were used. Total RNA extraction was carried out according to the instruction of the Quick Prep total RNA extraction kit for the direct isolation of total RNA from most eukaryotic tissues or cells (Amersham Pharmacia Biotech, UK). Frozen tissue samples (25–50 mg) of experimentally infected test-plant tissues were grounded in liquid nitrogen and transferred to 1.5 ml microfuge tubes. 150  $\mu\text{l}$  of the extraction buffer was poured in the tube and 3  $\mu\text{l}$  of 14.3 M  $\beta$ -mercaptoethanol was added. The solution was mixed thoroughly to obtain a homogeneous suspension. 350  $\mu\text{l}$  of lithium chloride (LiCl) solution was added to the homogenized samples. The homogenization was continued by mixing the components. The tubes containing the suspension were placed on ice. 500  $\mu\text{l}$  of caesium trifluoroacetate (CsTFA) was dispensed into the homogenized samples and mixed well. The tubes were placed on ice for 10 min and later spinned for 15 min at 14 000 g. The RNA formed a pellet at the bottom of the microtubes. The proteins form a coat at the top of the tubes and DNA remains in the liquid phase. The protein coat and the liquid phase were carefully removed. RNA pellets were washed with three “kit” components: 75  $\mu\text{l}$  extraction buffer, 175  $\mu\text{l}$  LiCl solution and  $\mu\text{l}$  CsTFA solution. The tubes were vortexed several times to wash the RNA pellet. The samples were spinned in a microcentrifuge at 14 000 g for 5 min. The supernatants were discarded without disturbing the pellets. 1 ml of 70% ethanol was added to the samples and incubated at  $-20^\circ\text{C}$  for at least 2 hours. The samples were spinned in a microcentrifuge at 14 000 g for 5 min. The pellet was air-dried for 10–15 min keeping the tubes on ice. DEPC-treated (Diethyl Pyrocarbonate) water containing 1% of RNase inhibitor was added to the RNA pellets. The pellet was broken by pulse vortexing 5–10 times. The samples were incubated on ice for 15–30 min, followed by incubation at  $65^\circ\text{C}$  for 10 min and stored at  $-20^\circ\text{C}$ .

Two specific oligonucleotide primers of the coat protein gene of AMV were designed using published sequences for AMV [23] from the GenBank accession AMU12509. Nucleotide sequences of upstream primer AMVCP1 were: 5' – GCC TCT AGA ACC ATG AGT TCT TCA CAA – 3' and downstream primer AMVCP2: 5' – TCA ATG AGC ATC AAG ATA – 3'.

The dissolved RNA was used in experiments for AMV detection by RT-PCR. All PCR procedures were carried out in Eppendorf Mastercycler Personal.

For RNA denaturation, 0.4  $\mu\text{M}$  AMVCP2 primer was added to each sample of RNA solution, and the mixture was incubated 5 min at  $70^\circ\text{C}$  and 5 min at  $4^\circ\text{C}$ .

For the first strand cDNA synthesis, to the denaturated RNA solution a mixture containing 5x reaction buffer, 1% of RNase inhibitor, 10 mM deoxynucleoside triphosphate (dNTP) mixture and 10 Units of RevertAid™ M-MuLV reverse transcriptase (MBI Fermentas, Vilnius, Lithuania) were added. The first strand cDNA synthesis was carried out at  $37^\circ\text{C}$  for 60 min and  $70^\circ\text{C}$  for 10 min.

DNA amplification was performed in 55  $\mu\text{l}$  reaction mixtures containing each of the four dNTP at a concentration of 200  $\mu\text{M}$ ,

each primer at a concentration of 0.4  $\mu\text{M}$ , 10  $\times$  PCR buffer, 25 mM  $\text{MgCl}_2$  and 0.25 U of recombinant *Taq* polymerase (MBI Fermentas). PCR was carried out for 40 cycles using the following parameters: 1 min at  $94^\circ\text{C}$  (4 min for the first cycle), 2 min at  $52^\circ\text{C}$  and primers extension for 2 min (10 min in the final cycle) at  $72^\circ\text{C}$ .

The resulting PCR products were analysed by electrophoresis in 5% polyacrylamide gel stained with ethidium bromide, and DNA bands were visualized using a UV transilluminator. DNA fragment size standard was PhiX174 RFI DNA Hae III digest (MBI Fermentas). Fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

## RESULTS AND DISCUSSION

Tomato plants naturally infected with the virus generally exhibited bright yellow mottle symptoms on interveinal regions of tomato leaves (Fig. 1 A) and on some fruits. Later, leaf yellowing was accompanied by leaflet deformation and plant stunting. For determination of experimental host range and the symptomatology of the causal agent, 12 leaf samples displaying these symptoms were collected from tomato plants. Two isolates (Nos. 0311 and 0313) were selected from the samples. The experimental host range of the virus isolates from tomato is listed in Table. The isolates produced similar symptoms in inoculated test-plant species. These isolates induced the following symptoms on the main diagnostic test-plant species:

*L. esculentum* – systemic mild yellow mottling and distortion of leaflets;

*Chenopodium amaranticolor* Coste et Reyn. – many local fine chlorotic spots or rings on leaves. Systemic infection in the form of mild mottling was observed;

*Cucumis sativus* L. – local small chlorotic spots, later vein clearing or mild mosaic with the development of top deformation of plants as systemic reaction;

*Datura stramonium* L. – bright green mild mottling of infected leaves (Fig. 1 B). *Phaseolus vulgaris* L. cv. 'Bataaf' – local necrotic lesions on leaves (Fig. 1 C). Systemic infection was not observed;

*Nicandra physalodes* (L.) Gaertn. – local necrotic spots and systemic interveinal chlorosis or yellow mosaic (Fig. 1 D);

*Nicotiana tabacum* L. cv. White Burley and Samsun – on inoculated leaves necrotic concentrically arranged, discontinuous ring patterns (occur not always). Systemic infection was in the form of slight vein clearing and mottling disappearing after some time, rarely malformation;

*N. debneyi* Domin. – irregular necrotic spots and systemic vein clearing with leaf size deformation (Fig. 1 E), plant stunting;

*Gomphrena globosa* L. – local severe necrotic lesions, systemic mild mosaic and distortion of leaflets (Fig. 1 F);

*Vicia faba* L. – local small necrotic lesions, which later covered the whole surface of the inoculated leaves, appeared 5–10 days after inoculation.

The virus infected a wide experimental host range. No significant differences in test-plant reaction between the two study isolates were determined. Symptoms induced by the Lithuanian AMV isolates from tomatoes on 17 diagnostic plant species were similar to those reported by other researchers for different

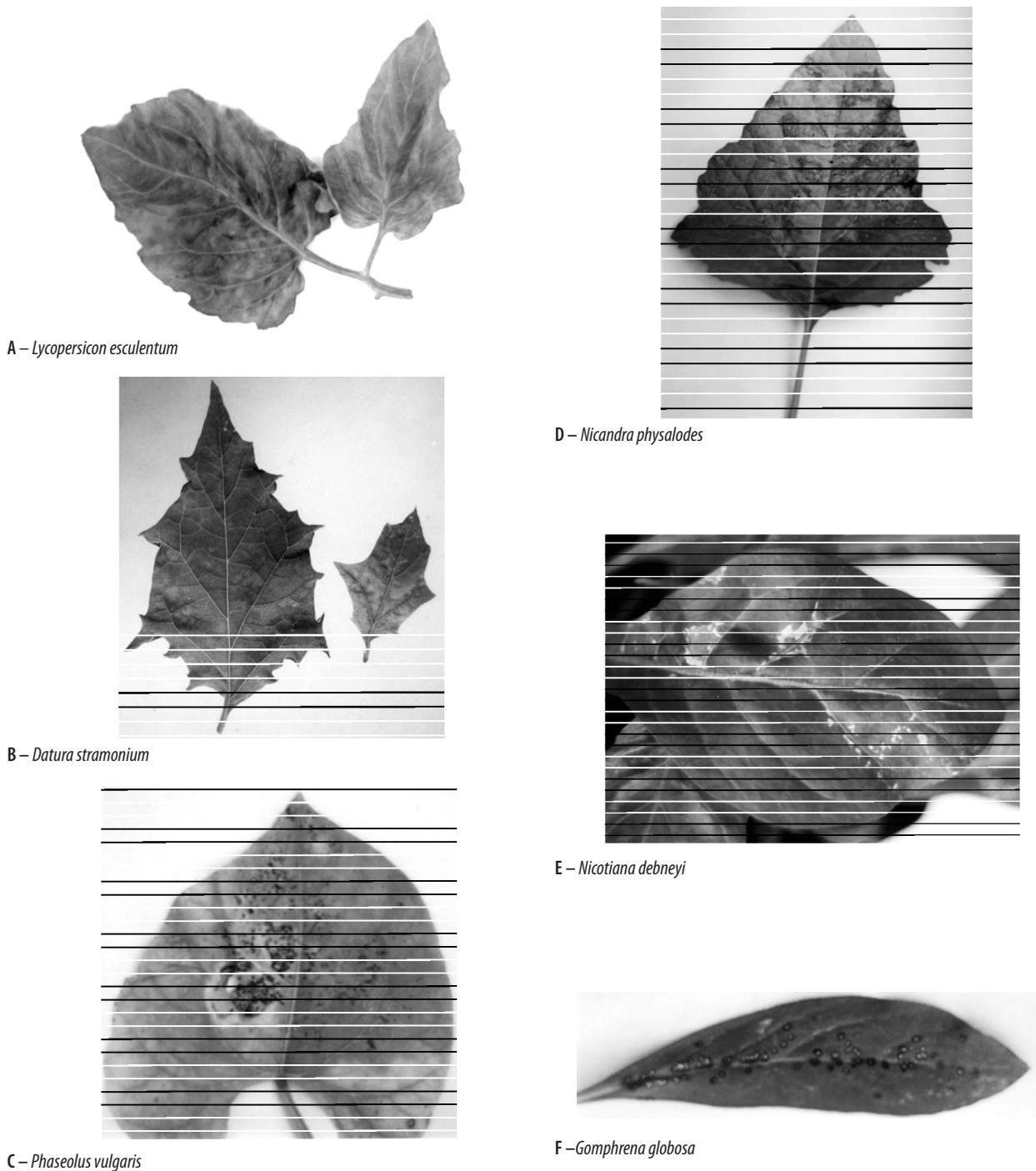


Fig. 1. Symptoms on tomato and test plants infected with AMV: A – *Lycopersicon esculentum*, B – *Datura stramonium*, C – *Phaseolus vulgaris*, D – *Nicandra physalodes*, E – *Nicotiana debneyi*, F – *Gomphrena globosa*

strains of the virus [4, 6, 17]. The other species of inoculated test-plants (*Tetragonia expansa* Murr., *Celosia argentea* f. *cristata* (L.) Kuntze, *Atriplex hortensis* L., *Cucurbita pepo* L., *N. alata* Link et Otto) remained symptomless, and no virus infection was revealed by EM.

EM examination of crude sap preparations of diseased tomato and test plant samples revealed characteristic bacilliform particles different in length (Fig. 2). The morphology of such particles is characteristic of AMV [6].

Positive serological reactions ( $A_{405\text{ nm}}$  values more than three times greater than those of the negative control – non-infected

plant) in DAS-ELISA using a commercially prepared antiserum against AMV were obtained with extracts of experimentally infected indicator species: *C. amaranticolor* (1.011), *G. globosa* (2.827), *D. stramonium* (1.243) (negative control – 0.505). Leaf extracts that had been positive to investigated virus by immunological test were mechanically rubbed onto leaves of *C. amaranticolor*, *G. globosa* and *D. stramonium* plants to confirm the authenticity of the virus.

For molecular confirmation of AMV detection in tomato by RT-PCR, Lithuanian isolates of AMV from tomato plants were used. The primer pair, designed on basis of published sequences,

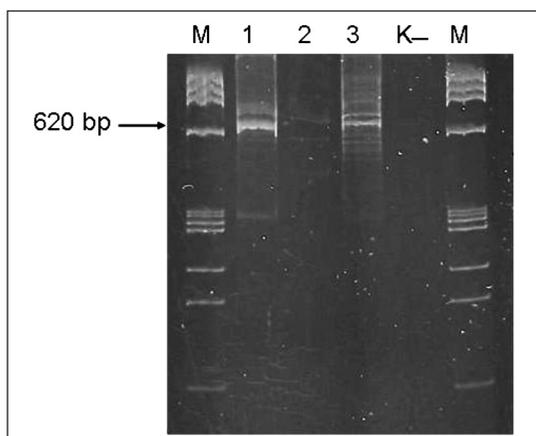


**Fig. 2.** Electron micrograph of AMV virions negatively stained with 3% uranyl acetate. The bar indicates 100 nm

successfully amplified AMV cDNA templates in RT-PCR using AMV isolates from tomato crops. A specific PCR product was obtained in two investigated plant samples but not with sample of *A. hortensis* and negative control (water). Specific bands were observed in gel electrophoretic analysis at the position corresponding to the expected size of the amplification products of approximately 620 bp genome fragment of AMV RNA (Fig. 3). The primer pair, designed on the basis of published sequences, successfully amplified AMV cDNA templates in RT-PCR and confirmed infection of AMV isolated from field-grown tomato samples found in Lithuania.

The results of biological, morphological, serological and molecular confirmation of this virus disease agent confirm that it is an AMV isolated from tomato plants. On the basis of the results of our experiments and literature data these virus isolates could be attributed to AMV from the genus *Alfamovirus*. This is the first report of AMV infection detected in a field-grown tomato crop in Lithuania.

Our study was conducted to provide information on the response of field-grown tomato crop to AMV infection. AMV is the only virus in the genus *Alfamovirus*; it has a wide host range among weed and crop plants [3, 4, 6]. It is seedborne in alfalfa, and essentially all alfalfa fields can be considered infec-



**Fig. 3.** 5% polyacrylamide gel electrophoresis of PCR products of amplified AMV samples and control. Lanes M, DNA Ladder: 1353, 1078, 872, 603, 310, 281; 271, 234, 194, 118, 72 bp; Lane 1, AMV infected *Chenopodium amaranticolor* tissue; Lane 2, AMV-inoculated *Atriplex hortensis* tissue; Lane 3, *Nicotiana glutinosa*; K-, water control.

ted. Alfalfa appears to be one of the most important reservoirs and the overwintering host of AMV. AMV infection in tomatoes generally occurs when tomatoes are grown near alfalfa fields. The virus is transmitted by several species of aphids, which can transmit AMV after having fed on the infected plants for only 1 second [14]. Spread from alfalfa to the surrounding crops via aphids is common. The virus is transmitted by pollen and grafting and not transmitted by contact among the plants [17].

## CONCLUSIONS

1. Alfalfa mosaic alfamovirus (AMV) has been detected and identified in symptomatic field-grown tomato samples found in Lithuania.

2. The identity of the detected virus disease agent with AMV has been confirmed by the methods based on test-plant reaction, the morphology of virions, DAS-ELISA and RT-PCR data.

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Irena Zitikaitė, Marija Samuitienė

#### **IŠ NATŪRALIAI UŽSIKRĖTUSIŲ POMIDORŲ IŠSKIRTO LIUCERNOS MOZAIKOS VIRUSO NUSTATYMAS IR KAI KURIOS SAVYBĖS**

##### *Santrauka*

Straipsnyje pateikiamas Lietuvoje pomidorus (*Lycopersicon esculentum* Mill.) pažeidusio alfamoviruso apibūdinimas. Pomidorai su virusinei infekcijai būdingais simptomais buvo aptikti privačiuose Vilniaus r. daržuose. Virusą pažeisti pomidorų lapai pasižymėjo neryškiu gelsvu margumu, difuziniu dėmėtumu, lapalakščių deformacija ir augimo sutrikimu. Iš pomidorų lapų ekstrakto išskirtas virusas buvo nustatytas naudojant augalus indikatorius, peršviečiamąjį elektroninį mikroskopą, imunofermentinį (DAS-ELISA) testą ir atvirkštinės transkripcijos-polimerazės grandininę reakciją. Pagal specifinius simptomus indikatoriniuose augaluose, platų šiam patogeniui jautrių augalų spektrą, virionų morfologiją (baciliški), teigiamas DAS-ELISA reakcijas ir viruso specifinio cDNR fragmento (apie 620 bp) amplifikaciją tiriuose lauko pomidoruose buvo atpažintas liucernos mozaikos virusas (Alfalfa mosaic alfamovirus, AMV). Tai yra pirmas pranešimas apie aptiktą AMV atviro grunto pomidoruose Lietuvoje.