BIOLOGIJA. 2006. Nr. 2. P. 63–67

Detection and characterization of a new nepovirus isolated from *Lycopersicon esculentum* Mill. crop in Lithuania

I. Zitikaitė[1], E. Survilienė[2],

Z. Jančys[1]

[1]Institute of Botany, Žaliųjų ežerų 49, LT-08406 Vilnius, Lithuania
E-mail: izitika@botanika.lt; zenonas@botanika.lt

[2] Lithuanian Institute of Horticulture, Babtai, LT-54333 Kaunas district, Lithuania
E-mail: e.surviliene@lsdi.lt

In 2004, samples of diseased tomato were found in a private greenhouse of Vilnius district. Naturally infected tomato plants hade symptoms suggestive of virus infection. The symptoms of infection were exhibited as the general interveinal-yellowing of leaves, bright mottling, reduced leaf size and stunted plant growth. These tomato samples were analysed using transmission electron microscopy (EM), serology, test plants and reverse transcription-polymerase chain reaction (RT-PCR). The identification of the virus was based on the results of determined experimental host range, symptomatology, positive double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) tests, morphology of virus particles (about 30 nm in diameter) and a specific amplification fragment size (420 bp) of virus RNA in RT-PCR. The obtained results indicated that this virus had properties characteristic of *Arabis mosaic nepovirus* (ArMV). This is the first report of tomato as a natural host for ArMV in Lithuania.

Key words: tomato, *Arabis mosaic virus,* detection, ELISA, RT-PCR

INTRODUCTION

Tomatoes (*Lycopersicon esculentum* Mill.) are important commercially grown vegetable crop. They are susceptible to a great number of viruses. About twenty different viruses have been described on affected tomato crops worldwide [1–3]. Over the last 15 years, nine different viruses of seven different genera have been isolated and identified in tomato plants in Lithuania. Some of them have been well characterized, whereas others have been characterized only in part. In the course of survey of virus diseases of tomatoes, a virus with isometric particles was detected by EM investigation and mechanical transmission from tomato plants which showed a mild interveinal-yellowing of leaves, bright mottling and reduced leaf size. Preliminary observations indicated that this virus, which was referred to as isolates 0408 and 0422, had the properties resembling those of nepoviruses [4]. *Tomato ringspot virus* (ToRSV), one of the nepoviruses, was isolated from naturally infected tomato plants expressing symptoms specific of this virus on leaves. This virus induced typical severe symptoms on the main diagnostic plant species: necrotic local ringspots and systemic mottling, and apical deformation [5]. The host range of nepoviruses is wide. Using modern diagnostic tools, more important crops were established as new hosts for these viruses. The properties the virus from tomato plants investigated by us corresponded to *Arabis mosaic virus* (ArMV) from the genus *Nepovirus* [6]. ArMV has a bipartite genome with two RNA species. The virus particles are isometric and about 30 nm in diameter [4]. Several nematodes of the family *Longidoridae* have been suspected of transmitting ArMV, but only the evidence for *Xiphinema diversicaudatum* is adequate [7, 8]. ArMV has also been reported from sugarbeet, celery, cucumber, cucurbita, carrot, horseradish and lettuce plants [1]. A number of other cultivated and wild species have been reported as hosts. This virus was described as an agent of virus disease infecting *Crocus* L. plants in Lithuania [9]).

The aim of this study was to present data on the occurrence and identification of a new virus disease agent from tomato plants in Lithuania, based on the results of host range and morphology of virus particles, immunological properties of antigens, and adapt a sensitive molecular method for specific detection of the virus in infected test plants by the RT-PCR technique.

MATERIALS AND METHODS

The samples of diseased tomato leaves and fruits for investigation were found in a private greenhouse of Vilnius district. The tomato plants with expressed visual virus disease symptoms were investigated for virus

presence. The experimental work was carried out at the greenhouse and Plant Virus Laboratory of Institute of Botany. The virus was identified by symptoms of test plants inoculated with two isolates from tomato [1, 4, 6]. In order to differentiate the virus isolates and identify the virus, test plants of *Aizoaceae* Rudolphi, *Amaranthaceae* Juss., *Asteraceae* Dum., *Chenopodiaceae* Vent., *Cucurbitaceae* Juss., *Fabaceae* Lindl. and *Solanaceae* Juss. families were used.

The specimens for mechanical inoculation were prepared by homogenising the tissue of affected tomato samples in 0.1 M sodium phosphate buffer, pH 7.0 (1:3 wt/vol), containing as a stabilising agent 0.02% 2-mercaptho-ethanol or 0,01 M sodium diethyldithiocarbamate (Na DIECA). The inoculations were performed with the aid of carborundum powder as an abrasive. The symptom expression was studied on several plant species (Table). Symptom development on indicator hosts was recorded every two days during the 12 days following inoculation and twice a week in the subsequent 30 days. The possibility of symptomless infection was checked by EM or back-inoculation to healthy test plants.

Presence of virus particles and their morphology was determined by investigation of negatively stained with 2% uranyl acetate dip preparations using a transmission JEOL JEM-100S electron microscope (EM) at instrumental magnification of 25000^{x} [10, 11].

Serological detection and confirmation of virus presence in tested samples of tomato and experimentally infected test plants were conducted by the ELISA direct double antibody sandwich technique as described [12], using polyclonal antiserum to ArMV produced by Loewe Biochemica GmbH (Germany). DAS-ELISA test was carried out at the Laboratory of State Plant Protection Service. Enzyme-substrate reactions were measured at 405 nm using a Multiscan ELISA reader. The ELISA readings were banked on buffer controls, and a sample was considered positive when its A_{405nm} value exceeded three

Table 1. **Reaction of herbaceous hosts to infection by isolates 0408 and 0422 from tomato**

No	Herbaceous hosts	Diagnostical symptoms
1	*Amaranthus caudatus* L.	0
2	*A. paniculatus* L.	0
3	*A. retroflexus* L.	0
4	*Atriplex hortensis* L.	0
5	*Beta vulgaris var. saccharifera* Alet.	S: ChlSp
6	*Capsicum annuum* L.	0
7	*Celosia argentea* f. *cristata* (L.) Kuntze	L: NL
8	*Chenopodium album* L.	L: ChlL; S: YSp
9	*C. amaranticolor* Coste et Reyn	L: ChlL; S: VC
10	*C. ambrosioides* L.	L: NL; S: LeDis
11	*C. foetidum* Schrad.	0
12	*C. quinoa* Willd.	L: ChlL; S: Stunt, TDis
13	*Cucumis sativus* L. Trakų pagerinti', 'Krukiai'	S: VC, ChlSp
14	*Datura stramonium* L.	L: WL; S: ChlSp
15	*Glycine max* (L.) Merr.	0
16	*Gomphrena globosa* L.	L: NL; S: difMo
17	*Lycopersicon esculentum* Mill. 'Rutuliai', 'Svara', 'Balčiai', 'Ryčiai'	S: VC, M, Mo, TDis, Stunt
18	*Nicandra physalodes* (L.) Gaertn.	S: difChlMo, VN
19	*Nicotiana alata* Link et Otto	0
20	*N. debneyi* Domin.	L: NL; S: M, Mo, VB, LeDis
21	*N. glutinosa* L.	L: NL; S: VC, Mo
22	*N. occidentalis* L.	L: NL
23	*N. rustica* L.	L: NL; S: ChlSp
24	*N. sylvestris* Speg et Comes	L: NL
25	*N. tabacum* L. 'White Burley'	L: NL; S: ChlSp, Stunt
26	*N. tabacum* L. 'Samsun'	S: ChlSp
27	*Ocimum basilicum* L.	0
28	*Petunia hybrida* Vilm.	S: ChlRiSp
29	*Phaseolus vulgaris* L. 'Bataaf'	L: ChlL; S: difChlSp
30	*Tetragonia expansa* Murr.	L: ChlL; S: VC, Mo
31	*Vigna unquiculata* (L.) Walp.	S: VC, ChlMo
32	*Zinnia elegans* Jacq.	0

Abbreviations: L – local reaction, S – systemic reaction, VB – vein banding, VC – vein clearing, VN – vein necrosis, M – mosaic, Mo – mottling, Chl – chlorotic, RiSp – ring spotting, YSp – yellow spotting, dif – diffuse, Stunt – stunting, NL – necrotic lesions, LeDis – leaf deformation, TDis – top deformation, WL – white lesions, 0 – no infection.

times the mean value of the extracts from non-infected plants.

For virus detection isolated from tomato plants by RT-PCR the frozen plant material was used. For total RNA extraction, frozen tissues of plants inoculated with ArMV *Gomphrena gobosa* L., *Nicotiana glutinosa* L., and *N. debneyi* Domin. were used. Total plant RNA was extracted from healthy leaves of *N. debneyi*. RNA extraction was carried out according to the instruction of QuickPrep™ Total RNA Extraction Kit for the direct isolation of total RNA from most eukaryotic tissues or cells (Amersham Biosciences, UK). Frozen tissue samples were grounded in liquid nitrogen and transferred to microfuge tubes. 150 µl of the extraction buffer was poured in the tube and 3 µl of 14.3 M 2-mercaptoethanol was added. 350 µl of lithium chloride (LiCl) and 500 µl of caesium trifluoracetate (CsTFA) solutions were added to the homogenized samples. The RNA formed a pellet at the bottom of the microtubes. The protein coat and the liquid phase were carefully removed and proceeded to wash the total RNA pellets with three Kit components. The supernatants were discarded without disturbing the pellets. 1 ml of 70 % ethanol was added to the samples. Diethyl Pyrocarbonate (DEPC)-treated water containing 1 µl of RNAse inhibitor was added to the RNA pellets.

For molecular detection of ArMV in the study plants, the Protocol for diagnosis of the quarantine organism drafted by Val Harju (Central Science Laboratory, Saud Hutton, York YO41 1 LZ) Version Jan 2003 was used. A combination of short, specific primers and thermostable DNA polymerase were used to amplify the target sequence, through repeated cycles of denaturation, reannealing, and DNA synthesis at high temperatures [13].

Pellets of total RNA were resuspended in the solution containing 25 mM $MgCl_2$, 10 × PCR buffer, 4 mM dNTP mix, 1U/µl RNAse inhibitor, 2.5 U/µl RevertAid™ M-MuLV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania) and downstream primer AP2[2]. Reverse transcription ran in the following programme on the termal cycler: one cycle of: 15 min at 42 °C, 5 min at 99 °C and 5 min at 5 °C.

The primer pair used in RT-PCR was designed for ArMV: AP1[2] (5' – AAT ACC CCG GGT GTT ACA TCG – 3') and AP2[2] (5' – CAT TAA CTT AAG ATC AAG GAT TC – 3') (GenBank Accession No. # 55460, X81814, X81815) [14].

DNA amplification was performed in reaction mixtures containing sterile PCR water, 25 mM $MgCl_2$, Upstream primer AP1[2], 10 × PCR buffer, and recombinant *Taq* polymerase (MBI Fermentas) using Eppendorf Mastercycler Personal. PCRs were carried out for 23 cycles using the following parameters: 2 min at 55 °C (1.5 min at 94 °C for the first cycle – denaturation of template), 3 min at 72 °C, 1 min at 94 °C. Final annealing was carried out for 2 min at 55 °C and further extension for 10 min at 72 °C.

The resulting PCR products were analysed by electrophoresis through 5% polyacrylamide gel in 1 × TBE buffer, stained with ethidium bromide, and the amplified DNA fragments were visualized using a UV transilluminator. The DNA fragment size standard was Φx174 DNA /BsuRI (HaeIII) digest (Fermentas) (from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp).

RESULTS AND DISCUSSION

Naturally infected tomato plants exhibiting the general interveinal-yellowing of leaves, bright mottling, reduced leaf size (Fig. 1 a) and stunted plant growth were observed in Vilnius district. Symptoms associated with ArMV were described as reduced growth, shoots with short internodes, and leaf chlorosis and distortion [6]. Overall, 10 leaf samples with these symptoms were collected from tomato plants. Two samples originating from tomato were inoculated onto the test plants. The symptomatology of test plants is shown in Table. This virus infected 22 out of 32 mechanically inoculated test plants. Several herbaceous indicator plants reacted with typical local and systemic symptoms reported for ArMV. Inoculated plants of the genus *Chenopodium* L. developed chlorotic local lesions on leaves. The systemic reaction was expressed by stunting and top distortion of these plants (Fig. 1 b). Symptoms characteristic of this virus were found on some infected plants of *Datura stramonium* L. their leaflets had local white lesions and systemic spotting followed by stunting of plants (Fig. 1 c). This virus causes local and systemic diffusive spots on inoculated leaves of *Phaseolus vulgaris* L. plants (Fig. 1 d). Similar results were obtained with test plants from the genus *Nicotiana* L. The inoculated leaves of *Nicotiana tabacum* L. cv. White Burley (Fig. 1 e) and *N. debneyi* plants showed severe local necrotic lesions. Virus isolates induced different systemic symptoms on leaves of *N. debneyi* plants: light and dark green mottle (Fig. 1 f) followed by vein-necrotic banding of older plant leaves. As the affected leaves turned brown and shrivelled, growth declined and the plant usually reduced in size until only a small central cluster of malformed leaves remained. Middle-aged inoculated leaves of tomato showed interveinal chlorosis, while more mature leaves showed more intense interveinal chlorosis and leaf size distortion in some cases. All tomato cultivars inoculated by the virus were susceptible to this virus. The symptoms induced by the virus on various test plant species were typical of ArMV. Based on the test plant reaction results, we can conclude that the study isolates belong to a typical strain of ArMV, because their biological properties correspond to literature data [6].

EM examination of crude sap preparations of tomato samples from a greenhouse revealed a complex infection: isometric particles of about 30 nm in diameter and flexuous filamentous particles 480–500 nm in lenght. A high concentration of isometric virus particles approximately 30 nm in diameter was found in the preparations made from inoculated and infected test plants (Fig. 2). The morphology of such particles was characteristic of ArMV.

The identification of ArMV isolates was confirmed by a positive reaction in the indirect double sandwich ELISA

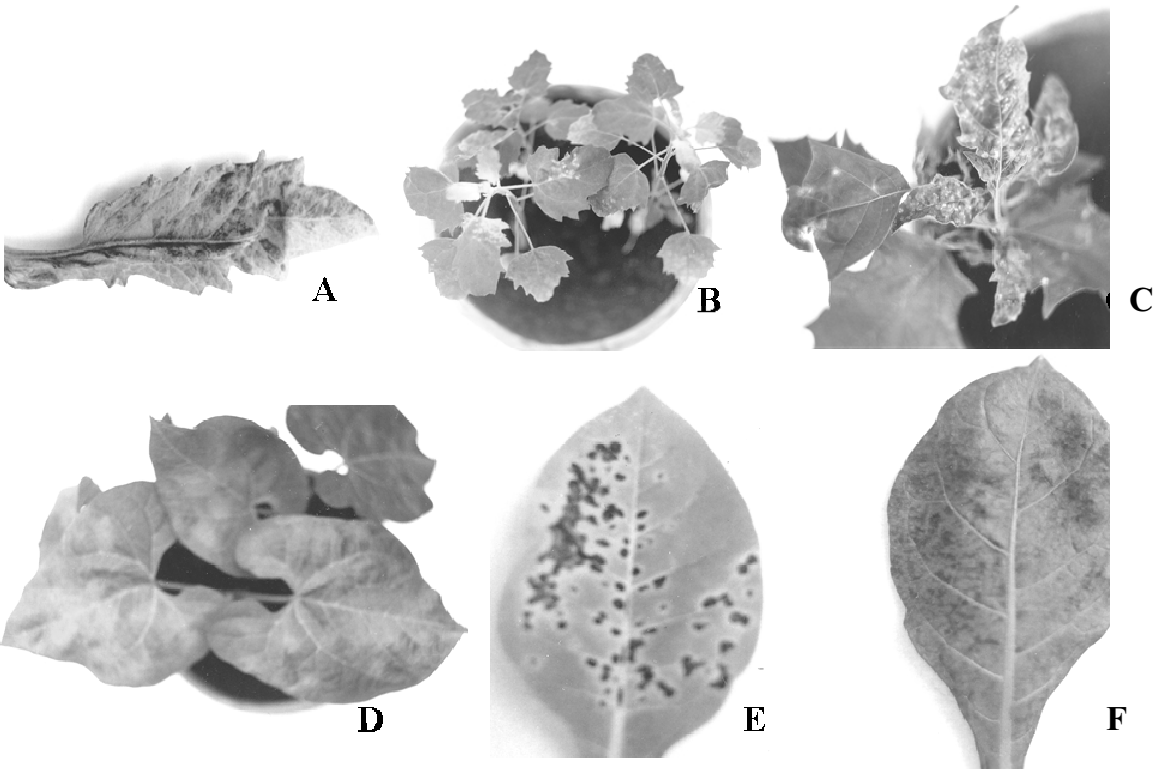

Fig. 1. Local and systemic reactions of test plants infected with ArMV: A – *Lycopersicon esculentum*, B – *Chenopodium quinoa*, C – *Datura stramonium*, D – *Phaseolus vulgaris* ‚Bataaf', E – *Nicotiana tabacum* 'White Burley', F – *Nicotiana debneyi.*

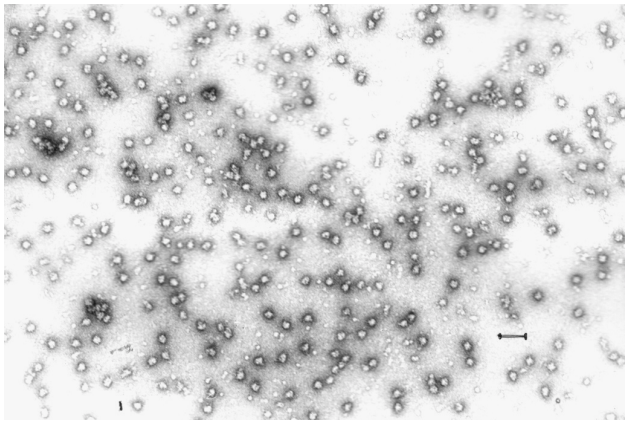

Fig. 2. ArMV particles. Bar marker represents 100 nm

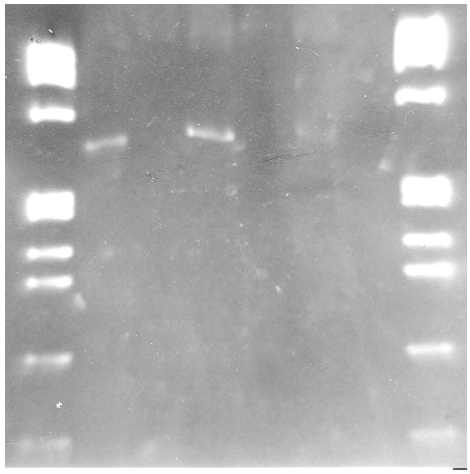

Fig. 3. 5% polyacrylamide gel electrophoresis of RT-PCR products of amplified ArMV samples using primer pair specific for ArMV. Lane 1 and Lane 8 – DNA fragment size standard; Lane 2 – *N. glutinosa*; Lane 3 – *G. globosa*, Lane 4 – *N. debneyi* (iz. 0408); Lane 5 – healthy *N. debneyi* plant; Lane 6 – *N. debneyi* (iz. 0422); Lane 7 – negative control

technique using alkaline phosphatase linked to the ArMV antibodies with glutaraldehyde, and nitrophenyl phosphate as a substrate. Samples of infected *N. debneyi* plants were positive for the presence of ArMV in DAS-ELISA tests. Leaf extracts that had been positive for ArMV by ELISA were mechanically inoculated onto indicator plants to confirm the authenticity of the virus.

The samples positive for ArMV in biological and immunological tests were tested by the RT-PCR technique using previously described specific primers. The PCR reaction resulted in a specifical amplification of the 420 bp genome fragment of ArMV RNA (Fig. 3). No product was amplified from the negative (healthy *N debneyi* tissue) and water controls. The primers designed on the basis of published sequences successfully amplified ArMV cDNA templates in RT-PCR of ArMV isolates from tomato plants. Molecular studies revealed and confirmed that ArMV as a new nepovirus infected tomato plants in Lithuania. It was found and isolated from mixed infection with *Potato X potexvirus.*

Our study was conducted to provide information on the response of a greenhouse-grown tomato crop to ArMV infection. ArMV has a wide host range including a number of important crop plants. 93 species from 28 dicotyledonous families can be experimentally infected by mechanical inoculation. ArMV are widely distributed in Europe (Belgium, Bulgaria, Czech Republik, Denmark, Finland, Sweden, UK and Poland). The virus has been reported from Asia (Japan, Kazakhstan), North America (Canada) and Oceania. Vegetatively propagated plant material is the most effective means of ArMV spread. Seed transmission was found in at least 15 species of 12 plant families. Some evidence exists for pollen transmission of ArMV [6].

The results of biological, immunological and partial molecular characterization of ArMV imply that this virus is a nepovirus sufficiently distinct from ToRSV isolated previously from tomato plants in Lithuania [5]. This is the first report of ArMV detected in a tomato crop in Lithuania.

AKNOWLEDGEMENTS

This research work was supported by a grant of the Lithuanian State Science and Studies Foundation No T-85/05.

The authors would like thank to virologist E. Jackevičienė from Lithuanian State Plant Protection Service for the immunological test.

Received 14 February 2006
Accepted 15 May 2006

References

1. Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ, Watson L. Viruses of Plants. Descriptions and Lists from the VIDE Database. Cambridge, 1996.
2. Edwardson JR, Christie RG. Viruses infecting peppers and other solanaceous crops. University of Florida, Gainesville, 1997.
3. Šutic DD, Ford RE, Tošic MT. Handbook of Plant Virus Diseases. CRS Press, New York, 1999.
4. Murant AF. Nepoviruses. In Handbook of Plant Virus Infections and Comparative Diagnosis (ed. E. Kurstak). Amsterdam, New York; Elsevier, North-Holland Biomedical Press, 1981: 198–238.
5. Zitikaitė I, Staniulis J, Jackevičienė E. Agronomijas vestis. Latvian J Agronomy 2004; 7: 55–59.
6. Murant AF. CMI/AAB Descriptions of Plant Viruses. Association of Applied Biologists, Wellesbourne, UK. 1970; 16.
7. Trudgill DL, Brown DJF, McNamara DG. Rev de Nematologie 1983; 6: 133–141.
8. Kulshrestha S, Hallan V, Raikhy G, Adekunle OK, Verma N, Haq QMR, Zaidi AA. Current Sci 2005: 89(10): 1759–1762.
9. Navalinskienė M, Samuitienė M. Lietuvos biologinė įvairovė: būklė, struktūra, apsauga. MSR serija. 2005: 1: 54–59.
10. Hayat MA. Principles and techniques of electron microscopy. Biological applications. 1970.
11. Dijkstra J, de Jager CP. Practical Plant Virology. Protocols and Exercises. Berlin–Heidelberg. 1998.
12. Clark MF, Adams AN. J Gen Virology 1977; 34: 475-483.
13. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA Science 1988; 239: 487–491.
14. Pantaleo V, Saponari M, Gallitelli D J Plant Pathol 2001; 83(2): 143–6.

I. Zitikaitė, E. Survilienė, Z. Jančys

IŠ POMIDORŲ IŠSKIRTO NAUJO NEPOVIRUSO NUSTATYMAS IR APIBŪDINIMAS

Santrauka

Straipsnyje apibūdinamas pomidorus (*Lycopersicon esculentum* Mill.) Lietuvoje pažeidęs nepovirusas. Pomidorų pavyzdžiai su virusinei infekcijai būdingais simptomais buvo aptikti privačiuose šiltnamiuose Vilniaus rajone. Pomidorų lapų virusinis pažeidimas pasireiškė šviesiai žalia mozaika ar margumu, geltonu ar tamsiai žaliu difuziniu dėmėtumu, senesnių lapų gyslų nekroze ir lapalakščių deformacija. Augalų augimas ir vaisių formavimasis buvo sutrikdytas. Iš pomidorų lapų ir vaisių ekstraktų išskirtas virusas buvo nustatomas naudojant augalus indikatorius, peršviečiantįjį elektroninį mikroskopą, imunofermentinį DAS-ELISA testą ir atvirkštinės transkripcijos polimerazės grandininę reakciją. Pagal nustatytus specifinius simptomus augale-maitintojuje ir eksperimentiniuose augaluose, platų šiam patogenui jautrių augalų spektrą, virionų morfologiją (izometriniai, apie 30 nm skersmens) ir viruso specifinio cDNR fragmento (420 bp) amplifikaciją iš pomidorų buvo išskirtas ir identifikuotas vaistučio mozaikos virusas (*Arabis mosaic virus,* ArMV) iš *Nepovirus* genties. Tai yra pirmas pranešimas apie aptiktą ArMV pomidoruose Lietuvoje.