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Detection of natural infection by *Cucumber mosaic virus* in vegetable crops

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Cucumber mosaic virus (CMV) causing viral diseases in forage, fruit, ornamental and other important plants worldwide has been isolated in Lithuania from vegetable (cucumber, tomato and sweet pepper) species exhibiting mottle-mosaic and distortion of leaves and fruits, and plant stunt symptoms. Diseased vegetable samples were found in Vilnius, Kaišiadorys, Kėdainiai and Širvintos regions. The detection of CMV was performed on the basis of determining host range, symptom expression on the test plant species, and the morphological properties of the virus particles by the methods of test plants and transmission electron microscopy and by using specific oligonucleotides in the reverse transcription-polymerase chain reaction (RT-PCR). Primers complementary to conserved sequences of CMV RNA were designed, and RT-PCR was directly performed in crude sap extracts of CMV-infected cucumber, tomato and sweet pepper plants. Analysis of PCR products in acrylamide gel electrophoresis revealed amplification of about 540 base pair fragments which were in agreement with the size of the fragment expected from the sequence data. CMV detection in vegetables was confirmed by the RT-PCR technique.

Key words: vegetables, RT-PCR, identification, *Cucumber mosaic virus*

INTRODUCTION

Cucumber mosaic virus (CMV) is a sap and aphid transmissible virus affecting vegetables. It has one of the broadest host ranges. CMV as a type species of the genus *Cucumovirus* in the family *Bromoviridae* is reported to infect 1287 plant species in 518 genera belonging to 100 families [1–3]. It is geographically widespread and has been reported in Europe, Asia, Australia, North America. The CMV causes fern leaf, stunting of vegetable crops and malformation of their fruits. It is one of the most important virus disease agents of vegetables worldwide. The disease affects a number of important vegetables: melons, squash, spinach, celery and beets. It is transmitted by numerous aphid species in a non-persistent manner [4–6].

Morphologically, the CMV has rather characteristic about 30 nm polyhedral particles with a hollow center [7]. CMV particles contain about 18% of RNA. RNA consists of 4 RNAs. Only the largest RNA3 is required for infectivity [8]. The virions are not stable to freezing. Long-term storage of CMV is most reliable in the form of viral RNA which is highly infectious and very stable at –20 °C [9]. A great number of different CMV strains, serogroups, subgroups and biological variations has been described [5, 10–14]. The numerous serological isolates, instability of CMV particles and a comparatively poor immunological response comprise difficulties in the serological identification of CMV.

In Lithuania, this virus spreads on leguminous (*Lupinus* L. and *Trifolium* L. species [15], ornamental (*Dahlia* Cav., *Delphinium* L., *Gladiolus* L., *Petunia* Juss., *Phlox* L. and other [16]) plants. CMV was identified from *Ribes nigrum* L. leaves [17].

The aim of the present study was to investigate the properties of virus isolates extracted from vegetable samples and to identify the causal agent on the basis of its symptomatology, virion morphology and RNA sequence analysis.

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MATERIALS AND METHODS

Virus sourse, maintenanse and isolation. Diseased vegetables were found in Vilnius, Kaišiadorys, Kėdainiai, Širvintos regions. Twenty leaf and fruit samples of cultivated cucumber, tomato and sweet pepper were collected by visual screening of greenhouses and grown fields under plastic on presence of symptoms of viral etiology. Leaves showing disease symptoms were collected in plastic bags and kept at 4 ºC. For virus presence, eight vegetable samples were investigated.

Host range and symptom determination. An experimental host range and induced symptoms for CMV were determined by mechanically inoculating a group of 28 species of herbaceous hosts representing eight families (*Fabaceae* Lindl., *Solanaceae* Juss., *Cucurbitaceae* Juss., *Aizoaceae* Rudolphi, *Chenopodiaceae* Vent., *Amaranthaceae* Juss., *Asteraceae* Dumort., *Labiatae* Juss.). All experimental hosts (Table) were grown from seed and maintained in greenhouse under natural lighting at a daytime temperature of 28–30 °C. All plants were mechanically inoculated with virions at the two-to-three-true-leaf stage. Crude sap extracted from infected vegetable leaf or fruit samples was inoculated onto herbaceous indicator test plants. Leaves of test plants were triturated in 0.1 M sodium phosphate buffer, pH 7.0–7.1, containing 0.02% 2-mercaptoethanol or 0.01 M sodium diethyldithiocarbamate (NaDIECA) and rubbed on 600 mesh carborundum for virus propagation [18]. Plants were evaluated weekly for symptoms.

Electron microscopy (EM). Samples from vegetable leaves and fruits were collected from naturally infected cucumber, tomato and sweet pepper plants and experimentally inoculated test plants and prepared for transmission EM. Copper grids were floated on drops of a crude extract of virus-infected plants for 1 to 2 minutes, rinsed with bidistilled water and subsequently stained with 3% uranyl acetate. Grids were examined under a JEOL-100S EM at the instrumental magnification of 25 000 [19].

Reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR for detection of CMV isolated from some vegetable crops was accomplished using the primers designed from sequence of the 16 k protein gene of CMV RNA. For this investigation, infected plant material stored frozen at –20 °C was used. Systemically or locally infected test plant leaves were collected 10–12 days after inoculation. Nucleic acids of CMV-infected plants were extracted using a small-scale procedure as proposed for extraction of nucleic acids from woody plants [20] with slight modifications.

All PCR procedures were carried out in Eppendorf Mastercycler Personal [21]. For cDNA synthesis and PCR amplification of the CMV coat protein (CP) gene, a 21-mer downstream primer D, (5'-GCG CGA AAC AAG CTT CTT ATC-3') was designed complementary to position 633–653

Table. **Experimental host range and symptoms caused by *Cucumber mosaic virus***

No	Test plants	Local	Systemic
1	*Amaranthus caudatus* L.	N LL	0
2	*Atriplex hortensis* L.	Chl LL	0
3	*Capsicum annuum* L. cv. Gloria	0	M, Dis
	cv. Podarok Moldovy	0	Mo, Dis, St
	cv. Kristal	0	VC, Mo, M
4	*Celosia argentea* f. *Cristata* (L.) Kuntze	0	0
5	*Chenopodium amaranticolor* Coste et Reyn	Chl LL	0
6	*Chenopodium ambrosioides* L.	N LL	0
7	*Chenopodium foetidum* Schrad.	0	0
8	*Chenopodium murale* L.	Chl LL	0
9	*Chenopodium quinoa* Willd.	Chl LL	0
10	*Cucumis sativus* L. cv. Alfa	0	M, Dis
	cv. Movir	0	VC, YM
	cv. Libelle	0	Dif Chl Sp
	cv. Trakų pagerinti	0	M, Mo
	cv. Rodničok	0	Mo, St
11	*Cucurbita pepo* L.	0	YM, Dis
12	*Datura stramonium* L.	0	DifMo, LeDis
13	*Gomphrena globosa* L.	N LL	0
14	*Lycopersicon esculentum* Mill. cv. Nevskij	0	M, TDis
	cv. Ryčiai	0	Mo, St
15	*Lupinus albus* L.	0	Mo, Dis, TW
16	*Nicandra physalodes* (L.) Gaertn.	N LL	0
17	*Nicotiana benthamiana* Domin	0	Mo, LeRu
18	*Nicotiana debneyi* Domin	0	YMo, LeCr
19	*Nicotiana glutinosa* L.	0	VC, Mo, TDis
20	*Nicotiana rustica* L.	0	Dif Mo
21	*Nicotiana tabacum* L. cv. Samsun	0	Mo, Dis, St
	cv. White Burley	0	VC, M, LeDis
	cv. Xanthi	0	VN, Mo
22	*Ocimum basilicum* L.	0	0
23	*Phaseolus vulgaris* L. cv. Red Kidney	0	VN, dif Chl Sp
	cv. Bataaf	0	ChlMo, LeRu
24	*Pisum sativum* L. cv. Žalsviai	0	DifMo, M
25	*Tetragonia expansa* Murr.	Chl LL	0
26	*Vicia faba* L. cv. Aušra	0	M, Mo
27	*Vigna sinensis* Endl.	0	ChlMo
28	*Zinnia elegans* Jacq.	NSp	0

Abbreviations: L – local reaction, S – systemic reaction, NLL – necrotic local lesions, ChlLL – chlorotic local lesions, M – mosaic, Mo – mottling, Ma – malformation, VC – vein clearing, VN – vein necrosis, Dis – distortion, dif – diffuse, Cr – crincling, Ru – rugosity, TW – top wilting, y – yellow, Sp – spotting, St – stunting, 0 = no symptoms.

in the CP gene. A 19-mer upstream primer U was designed homologous to nucleotides 114–132 of CMV RNA (5'-GTA GAC ATC TGT GAC GCG A-3'). Primers D and U define a target sequence of 540 bp [22].

Total RNA resuspended from pellets in the solution containing 1% RNAse inhibitor, 1 µl 20 pM downstream primer and deionized water and incubated at 70 °C for 5 min were used for first-strand DNA synthesis. After denaturation, 11 µl RNA solution was added to the mixture containing 4 µl of 5x reaction buffer, 1 µl of 40 U/µl RNAse inhibitor, 2 µl 10 mM dNTPs and 1 µl 200 U/µl RevertAid™ M-MuLV Reverse Transcriptase (for one sample) (MBI Fermentas, Vilnius, Lithuania). The synthesis of first-strand DNA was carried out at 37 °C for 60 min and at 70 °C for 10 min.

For DNA amplification reaction, mixtures containing dNTPs mixture, both primers, 1o × PCR buffer with detergents and recombinant *Taq* DNA polymerase (MBI Fermentas) were prepared. The PCR reaction mixture contained (for one sample): 37.75 µl PCR water, 1 µl 20 pM primer D, 1 µl 20 pM primer U, 4 µl 10 mM dNTP mix, 5 µl of 10 × PCR buffer with $MgCl^{2+}$, 0.25 µl 5U/µl Taq DNA polymerase and 10 µl cDNA. The cycling parameters were as follows: pre-denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing: at 55 °C for 2 min; elongation at 72 °C for 2 min, and the final extension of amplification products for 10 min at 72 °C.

The resulting PCR products were analysed by electrophoresis through 5% polyacrylamide gel run in 1 × TBE buffer. Gels were run at 120 V, stained with ethidium bromide, and DNA bands were visualized using the 254 nm Type UVT-20S transilluminator. DNA Ladder was ΦX174 DNA/*BsuRI* (*Hae*III) digest (MBI Fermentas), fragment size (from top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp.

RESULTS AND DISCUSSION

Symptomatic samples of vegetable plants were observed under greenhouse and field conditions in different locations. Symptoms of naturally affected sweet pepper (*Capsicum annuum* L.), cucumber (*Cucumis sativus* L.) and tomato (*Lycopersicon esculentum* Mill.) plants were very diverse. One of the most common expressions was severe stunting. Plants had light green foliages. In some cases leaves became narrow and did not expand any longer, while in other cases small necrotic spots with oak leaf patterns developed. The symptoms caused by this virus in cucumbers varied (typical leaf yellow or light green mosaic and decreased leaves, shortened stem internodes, and slow growth). Fruits were mottled and small (Fig. 1a). CMV was identified in isolates from cucumber plants of cultivars 'Alfa', 'Mirabelle', 'Movir' and 'Rodnichok'. Tomatoes of cultivars 'Luseta' and 'Svara' infected with CMV develop a slight yellowing and mottling of leaves, which are smaller than normal and distorted (Fig. 1b). Sweet pepper plants have light green foliages. Leaves of cultivars 'Niezhnost' and 'Podarok Moldovy' were smaller and mildly mottled. Older plants of sweet pepper show foliar chlorotic spotting followed by diffuse chlorosis and distortion (Fig. 1c).

CMV infected practically all of 28 mechanically inoculated test plants in all isolates (Table). The inoculated cucumber, tomato and sweet pepper as test plants showed virus symptoms similar to those observed in the field (in nature). The pathogen caused vein brightening, mottling and various deformations of growing leaves of *Nicotiana glutinosa* L. (Fig. 2a), *N. debneyi* Domin. Systemic reaction in the form of growth disorder, mosaic or mottling and

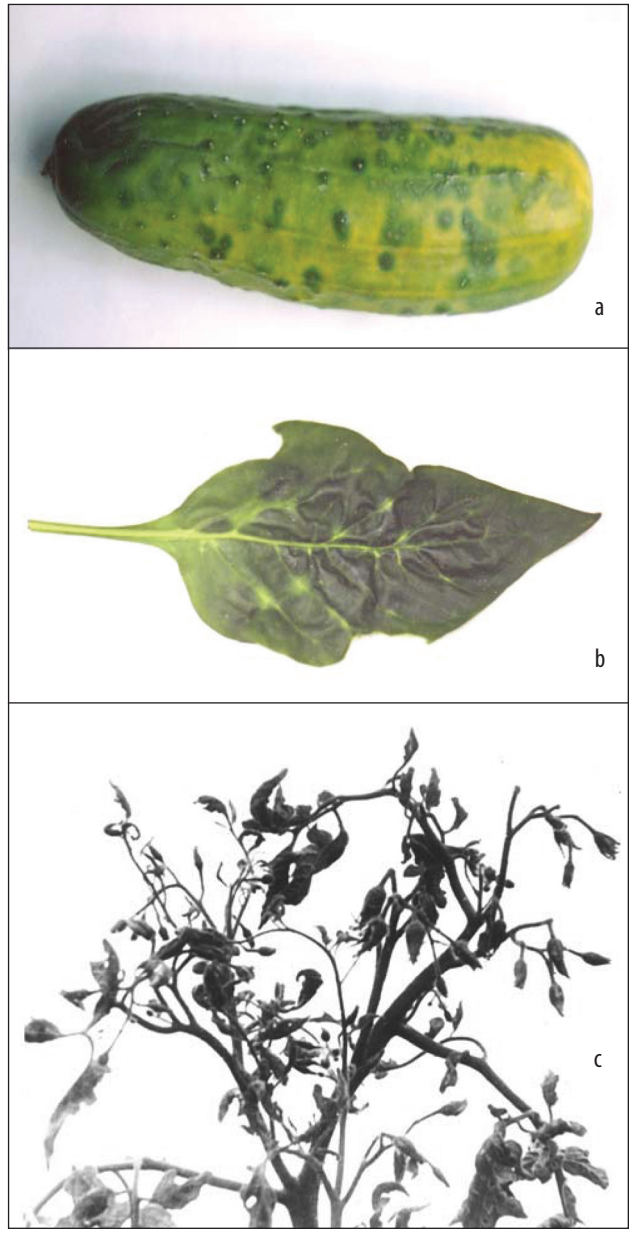

Fig. 1. Symptoms on vegetables naturally infected with CMV: a – cucumber, b – sweet pepper, c – tomato

deformation of young leaves of infected *N. tabacum* L.'White Burley' was also noticed. The virus developed diffusive chlorotic spots which later formed up a clear mosaic picture on the leaves of *Phaseolus vulgaris* L.'Bataaf' (Fig. 2b) and 'Red Kidney'. CMV infection developed the most conspicuous symptoms on the leaves of *Datura stramonium* L. – diffusive light and dark green areas and distortion. In leaves of test plants of *Amaranthus caudatus* L., *Atriplex hortensis* L., *Gomphrena globosa* L. (Fig. 2c), *Tetragonia expansa* Murr., *Nicandra physalodes* (L.) Gaertn., *Chenopodium* L., a local reaction in the form of white, chlorotic or necrotic lesions was revealed. Back-inoculation from the symptomless indicator plants onto healthy test plants showed no virus infection. The resulting reaction of test plants was typical of CMV described in the literature [3–5].

Numerous isometric particles with a hollow center were commonly observed in leaf dip EM preparations of leaf samples of naturally infected vegetable plants and experimentally infected test plants. According to EM data, they were about 28–30 nm in diameter (Fig. 3).

CMV identification by test plant reactions and EM test were verified in RT-PCR using as samples test plants inoculated with the virus isolated from diseased cucumber, tomato and sweet pepper. RT-PCR using a specific primer

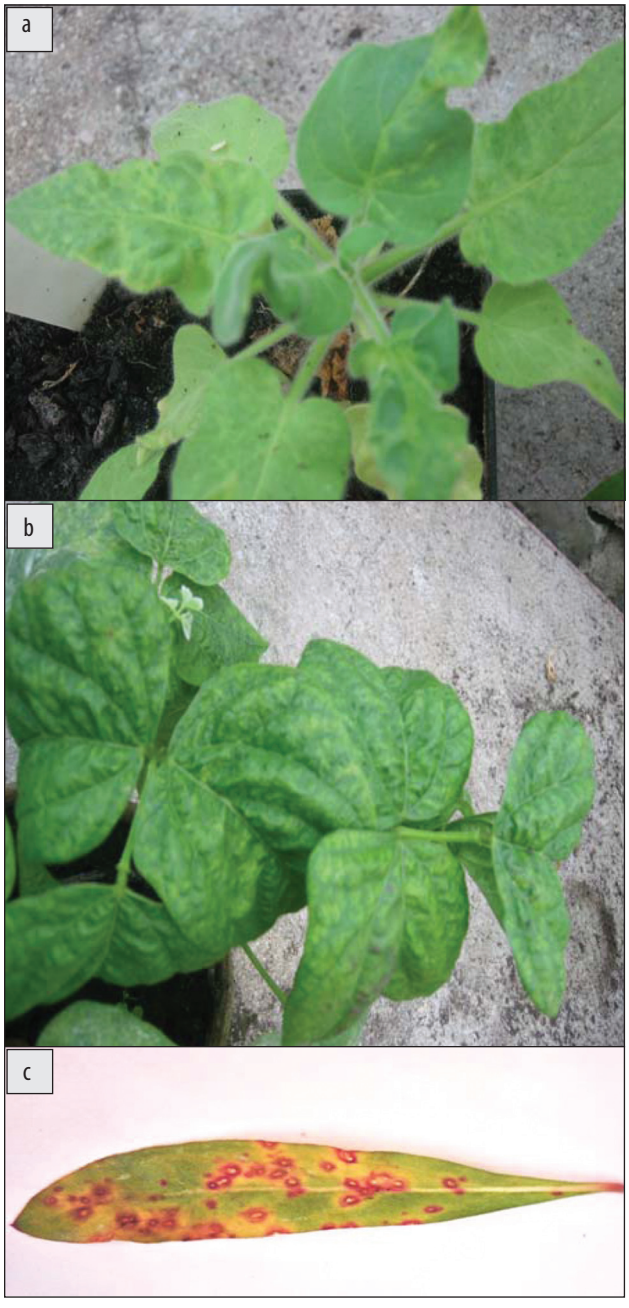

Fig. 2. Symptoms on test plants caused by CMV: a – *Nicotiana glutinosa*, b – *Phaseolus vulgaris*, c – *Gomphrena globosa*

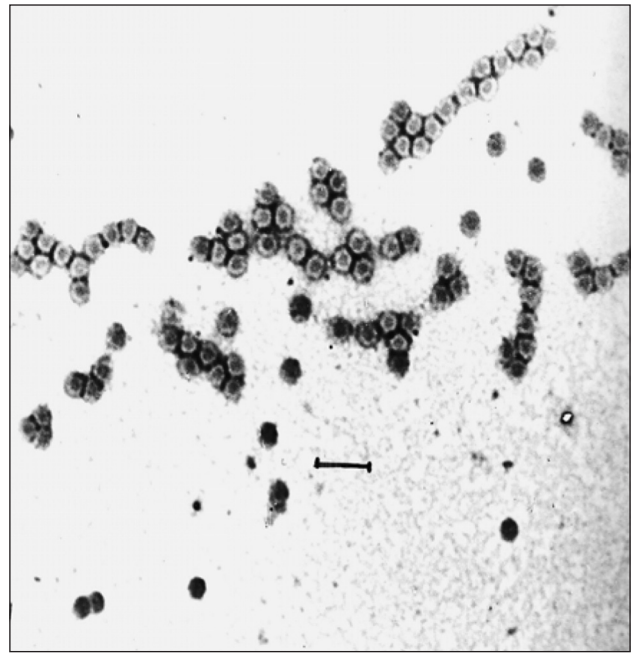

Fig. 3. Electronomicrograph of CMV particles. Bar represents 100 nm

pair for CMV detection primed amplification of about 540 bp DNA sequences from CMV samples of symptomatic plants. PCR products specific of CMV were obtained with systemically infected *D. stramonium, N. rustica, N. tabacum, Ph. vulgaris* and locally infected *Chenopodium amaranticolor* plant tissues (Fig. 4), confirming CMV identity. A sample of CMV-infected white clover plant as a positive control yielded a visible specific DNA band. Amplification was not observed in samples with negative controls: healthy plant tissue and PCR buffer + PCR water. Molecular investigation confirmed that the investigated cucumber, sweet pepper and tomato plants had been infected by the CMV.

The experimental host range and specific symptoms on all test plants indicated that the virus isolated from these vegetable crops most closely corresponded to the CMV [4]. Based on particle size and morphology, the virus was considered to be a member of the genus *Cucumovirus* [5]. RT-PCR data confirmed the identification obtained by investigating

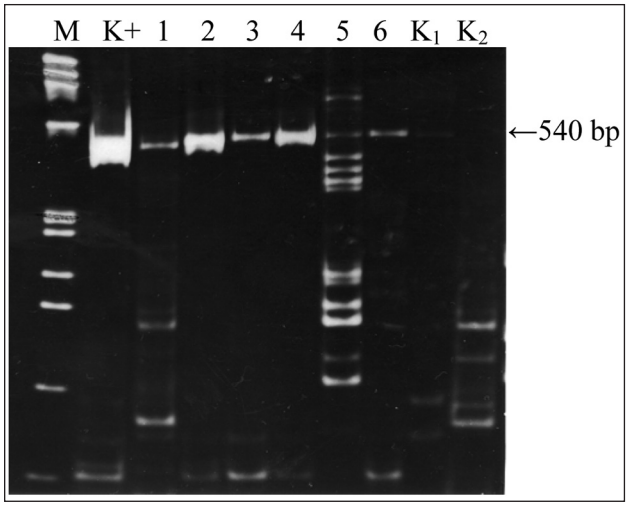

Fig. 4. RT-PCR DNA amplification of CMV

RT-PCR DNA amplification of CMV. M – DNA ladder; K+ – positive control, 1 and 2 – CMV from cucumber infected with *Datura stramonium* and *Nicotiana rustica*; 3 and 4 – CMV from tomato infected with *Gomphrena globosa* and *Phaseolus vulgaris*; 5 and 6 – CMV from sweet pepper infected with *N. tabacum* and *Chenopodium amaranticolor*; K_1 – healthy tomato plant; K_2 – water control

the host range, symptomatology and virus morphology. The RT-PCR product size of CMV isolates from infected vegetable samples showed identity with CMV isolates, identified in other plants in Lithuania.

The CMV is among the most economically damaging pathogens in many vegetable crops. It naturally affects parsley, celery, potato, pea, bean, lupine, clover, fruit and many ornamental plants [3, 23, 24]. The CMV overwinters in perennial weeds and may be transmitted to healthy plants by aphid vectors non-persistently in nature. Among more than 70 aphid species vectors, the most efficient are *Myzus persicae* Sulz., *Aphis gossypii* Glov., *A. craccivora* Koch. and *A. fabae* Scop. The large population of aphis vectors is one reason for the widespread nature of CMV. The CMV spreads through the sap of infected plants by leaf contact, through seeds of 19 plant species and by dodder [3, 4].

CONCLUSIONS

1. The virus disease agent detected in vegetable (cucumber, tomato and sweet pepper) samples found in Lithuania has been identified as a CMV by using classical virological methods and the modern method of molecular biology.

2. Methods of controlling vegetable or other plant virus diseases consist in growing healthy plants, visual inspection of plants for symptoms, elimination of affected plants and propagation of virus-free plant material.

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NATŪRALI AGURKŲ MOZAIKOS VIRUSO INFEKCIJA DARŽOVĖSE

Santrauka

Agurkų mozaikos virusas (*Cucumber mosaic virus* – CMV) buvo išskirtas iš žemaūgių agurkų mozaikiškai dėmėtais ir deformuotais lapais (*Cucumis sativus* L.), pomidorų (*Lycopersicon esculentum* L.) ir saldžiųjų ankštinių pipirų (*Capsicum annuum* L.). Daugumos jų vaisiai buvo dėmėti, deformuoti ir smulkūs. Tyrimams medžiaga surinkta Vilniaus, Kaišiadorių, Kėdainių, Širvintų r. privačiuose daržuose ir įmonėse. Virusinio pažeidimo sukėlėjas nustatytas pagal simptomus, pažeidžiamų augalų spektrą ir virionų morfologiją panaudojus augalų indikatorių, peršviečiamąją EM bei PGR technologiją. Nustatytas platus viruso pažeistų augalų spektras ir labai specifinė simptomatika tam tikruose augaluose indikatoriuose. Infekuotų augalų ekstraktuose EM aptikti izometriniai CMV būdingi virionai su centru. Pritaikius PGR technologiją ir specifinius oligonukleotidus elektroforezės akrilamidiniame gelyje gauti DNR kopijos PGR produktai (~540 bp dydžio) atitiko panaudotus pradmenis ir patvirtino CMV infekciją tirtose daržovėse.

Raktažodžiai: daržovės, *Cucumber mosaic virus*, EM ir PGR technologijos