

## **Detection and characterization of *Cucumber mosaic virus* isolated from sweet peppers**

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*Cucumber mosaic virus* (CMV) causing viral diseases in forage, fruit, ornamental and vegetable crops worldwide has been isolated in Lithuania from sweet pepper (*Capsicum annuum* L.) plants exhibiting mottle-mosaic and distortion of leaves and fruits, and plant stunt symptoms. The plant material was collected in the private gardens of Vilnius, Kaišiadorys, Kėdainiai regions. The identification of CMV has been performed on the basis of determination of host range, symptom expression on the test plant species and morphological properties of the virus particles by the methods of test plants and transmission electron microscopy, and by using of specific oligonucleotide primers in reverse transcription-polymerase chain reaction (RT-PCR). In this work the primers designed on the basis of published sequences were applied for amplification of CMV RNA fragments in RT-PCRs using experimentally CMV infected host plants. The detection of CMV in inoculated test plants was confirmed by RT-PCR technique. Analysis of PCR products in acrylamide gel electrophoresis revealed amplification of about 540 bp (base pair) fragments which were in agreement with size of the fragment expected from the sequence data.

**Key words:** *Cucumber mosaic virus*, isolation, RT-PCR, sweet pepper.

**Introduction.** Sweet pepper (*Capsicum annuum* L.) is now grown worldwide under various environmental and climatic conditions and is the second most important crop among solanaceous fruits. Observation showed that the production of this crop has been banned with viral infection. Viral diseases are the major limiting factors for successful pepper cultivation in the world (Francki, 1979; Fujisawa et al., 1986; Florini, Zitter, 1987). Viruses that may occur on peppers include *Tobacco mosaic*, *Cucumber mosaic*, *Potato Y*, *Tomato spotted wilt*, *Alfalfa mosaic*, *Pepper mottle*, *Pepper veinal mottle*, *Pepper ringspot* and others (Šutic et al., 1999; Hiskias et al., 1999; Buzkan et al., 2006; Ryu et al., 2009). In order of importance are *Cucumber mosaic virus* (CMV). It as a type species of *Cucumoviruses* is reported to infect 1287 plants species in 518 genera belonging to 100 families (Edwardson, Christie, 1997). It is geographically wide spread and has been reported in Europe, Australia, North America. It is one of the most important virus disease agent of pepper worldwide. It is transmitted by numerous species of aphid in a non-persistent manner (Francki et al., 1979; Kaper, Wa-

terworth, 1981). CMV is not transmitted through pepper seeds. It also has an extremely wide host range and causes fern leaf, stunting of pepper and malformation of fruits.

Morphologically CMV has rather characteristic about 30 nm polyhedral particles with hollow center (Palukaitis et al., 1992). CMV particles contain about 18 % RNA. 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 (Foster, Taylor, 1998). Great number of different CMV strains and serogroups has been described (Kaper, Waterworth, 1981; Perry et al., 1993). In Lithuania, this virus spread on leguminous (Staniulis, 1994), ornamental (Navalinskienė, Samuitienė, 2006), cucumber and tomato (Zitikaitė, 2002; Zitikaitė et al., 2006) plants.

The purpose of this work was to study properties of virus isolates extracted from sweet pepper samples and to identify the causal agent.

**Object, methods and conditions.** Twelve leaf samples of cultivated 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, kept at 4 °C, when triturated in 0.1 M sodium phosphate buffer at pH 7.0–7.1 and rubbed on 600 mesh carborundum dusted leaves of test plants for virus propagation. For investigation of virus host range and induced symptoms about twenty herbaceous plant species from families of *Solanaceae* Juss., *Cucurbitaceae* Juss., *Aizoaceae* Rudolphi, *Chenopodiaceae* Vent. were tested as indicator plants (Table) (Matthews, 1993). Copper grids were floated on drops of 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 transmission electron microscope (EM) (Dijkstra, de Jager, 1998).

For detection of CMV isolated from sweet pepper plants by RT-PCR technique the frozen plant material was used. Nucleic acids of CMV were extracted using the small-scale procedure as proposed for extraction of nucleic acids from woody plants (Zhang et al., 1998) with slight modifications. Tissue samples of infected test-plants were ground in liquid nitrogen and transferred to microtubes. 600 µl 1 × STE buffer (0.1 M NaCl, 0.001 M Tris, 0.001 EDTA, pH 6.9), 80 µl of 10 % SDS and 800 µl of 2 × STE-saturated phenol was added to the powdered tissues. The mixture was centrifuged 5 min at 16 000 g. Aqueous phase was removed and transferred to a clean microfuge tube. Ethanol to a final concentration of 30 % was added, then ~ 10 mg cellulose (whatman CF-11). Cellulose CF-11 was washed by vortexing 3 times with 1 ml of 1 × STE/30 % ethanol, collecting cellulose by centrifugation between washes and discarding supernatants. RNA from cellulose CF-11 was eluted by adding 200 µl of 1 × STE buffer, and centrifugation for 5 min. Supernatant was transferred to a clean tube. For precipitation of the RNA 40 µl of 3 M sodium acetate and 1 ml of ethanol was added. The tube was incubated at -20 °C for 2 h., centrifuged for 10 min at 16 000 g, and the pellet was incubated with 80 % of ethanol at -20 °C, and air-dried.

Primer pair for CMV detection by RT-PCR was used: D, 5' – GCG CGA AAC AAG CTT CTT ATC – 3' (nt 633 to 653) and U, 5' – GTA GAC ATC TGT GAC GCG A – 3' (nt 114 to 132) (de Blass et al., 1994). Pellets of total RNA were resuspended in the solution containing 1 % RNase inhibitor, 0.4 µM primer Reverse and PCR water

and incubated at 70 °C for 10 min. For the first strand copy DNA synthesis the RNA pellet solutions to the mixture containing 5 × Reaction buffer, RNase inhibitor, dNTP mixture and RevertAid™M-MuLV Reverse Transcriptase (MBI Fermentas, Lithuania) were added. The first strand cDNA synthesis was carried out at 37 °C for 60 min and 70 °C for 10 min. DNA amplifications were performed in reaction mixtures containing PCR water, 10 mM dNTP mixture, both primers, 10 × PCR buffer with MgCl<sub>2</sub> and recombinant *Taq* polymerase (MBI Fermentas) using Eppendorf Mastercycler Personal. PCRs were carried out for 40 cycles using the following parameters: 1 min at 94 °C (4 min for the first cycle), 2 min at 55 °C and primers extension for 2 min (10 min in the final cycle) at 72 °C (Saiki et al., 1988). DNA fragment size standard was × 174DNA/BsuRI(HaeIII) digest (MBI Fermentas) (from top to bottom: 1 353, 1 078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp). Resulting PCR products were analysed by electrophoresis through 5 % polyacrylamide gel, stained with etidium bromide (EB), and DNA bands were visualized under UV light.

**Results.** Symptoms of naturally affected sweet pepper (*C. annuum* L.) plants vary widely. One of the most common expressions was a severely stunted. Plants have light green foliages. In some cases the leaves become narrow and no longer expand, while in other cases small necrotic spots with oak leaf patterns develop. Leaves were smaller than normal and mild mottled (Fig. 1). Older plants of sweet pepper show foliar mottling followed by diffuse chlorosis or no symptoms on foliage or fruit. Fruits of some sweet pepper were deformed and reduced in size and form.



**Fig. 1.** Sweet pepper leaves affected with CMV  
**1 pav.** CMV pažeisti saldžiujų paprikų lapai

CMV infected practically all of 20 mechanically inoculated test plants in four isolates (Table). The pathogen caused vein brightening, mottling and various deformations of growing leaves of *Nicotiana glutinosa* L., *N. rustica* L. (Fig. 2). Systemic reaction in the form of growth disorder, mosaic or mottling and deformation of young leaves of infected *N. tabacum* L. 'Samsun' was also noticed. CMV infection developed the most

conspicuous symptoms on the leaves of *Datura stramonium* L. – diffusive changeable light and dark green areas (Fig. 3). In the leaves of test plant of *Chenopodium* L. genus a local reaction in the form white or chlorotic lesions was revealed (Fig. 4). Under the influence of CMV only local necrotic lesions appeared on the leaves of *Nicandra physalodes* (L.) Gaertn (Fig. 5). Virus developed diffusive chlorotic spots, which later formed up a clear mosaic picture on the upper leaves of *Cucumis sativus* L.

Numerous isometric particles with hollow center were commonly observed in leaf dip EM preparations of leaf samples of infected plants. They were about 28–30 nm in diameter (Fig. 6).



Fig. 2 / 2 pav. *Nicotiana rustica* (S)



Fig. 3 / 3 pav. *Datura stramonium* (S)



Fig. 4 / 4 pav. *Chenopodium amaranticolor* (chLL)



Fig. 5 / 5 pav. *Nicandra physalodes* (nLL)

**Table.** Test plant reaction to CMV isolated from sweet peppers  
**Lentelė.** Augalų indikatorių reakcija į CMV, izoliuotą iš saldžiųjų paprikų

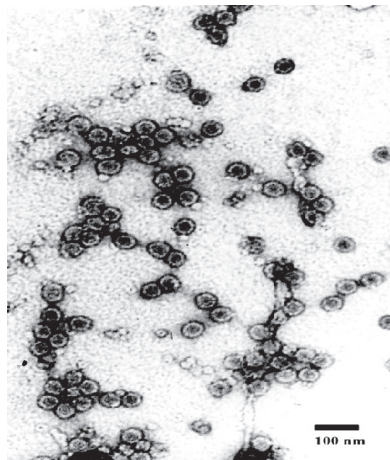
| Test plants<br>Augalai indikatoriai                    | Virus isolates and symptoms<br>Izoliatai ir simptomai |                        |                         |                         |
|--|---|------------------------|-------------------------|-------------------------|
|  | 9007  | 9707                   | 0211                    | 0413                    |
| 1  | 2   | 3                      | 4                       | 5                       |
| <i>Amaranthus caudatus</i>                             | L: nLL S:   | -                      | 0                       | L: nLL                  |
| <i>Capsicum annuum</i> ‘Kristal’,<br>‘Podarok Moldovy’ | Mo, Ma;<br>S: Mo, Dis                                 | S: VC, Cr;<br>S: M, Mo | S: VC, Mo<br>S: Mo, Dis | S: VC, Ru;<br>S: VN, Cr |

**Table continued**  
**Lentelės tęsinys**

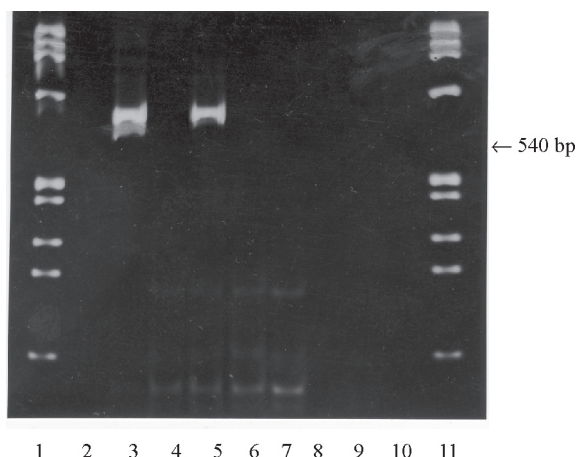
| 1  | 2                | 3              | 4          | 5            |
|--|------------------|----------------|------------|--------------|
| <i>Celosia argentea</i> f. <i>cristata</i> | -                | L: nLL         | L: nLL     | L: nLL       |
| <i>Chenopodium amaranticolor</i>           | L: chlLL         | L: chlLL       | L: chlLL   | L: chlLL     |
| <i>C. ambrosioides</i>                     | L: nLL           | 0              | L: nLL     | L: nLL       |
| <i>C. quinoa</i>                           | L: chlLL         | L: chlLL       | L: chlLL   | L: chlLL     |
| <i>Cucumis sativus</i> ‘Visconsin’         | S: M, Ma         | S: VC, Ru, Mo  | S: VC, Mo  | S: difMo     |
| <i>Datura stramonium</i>                   | S: M, chlMo      | S: VC, Cr      | S: yM      | S: M, Mo     |
| <i>Gomphrena globosa</i>                   | L: nLL           | L: nLL         | L: nLL     | L: nLL       |
| <i>Lupinus albus</i>                       | S: Mo, Dis       | S: Mo, Ru      | S: Mo, TW  | -            |
| <i>Nicandra physalodes</i>                 | L: nLL           | L: nLL         | 0          | L: nLL       |
| <i>Nicotiana debneyi</i>                   | S: Mo            | S: VC, Dis     | S: Ru, St  | S: VC, difMo |
| <i>N. glutinosa</i>                        | S: VC, M, Ru     | S: VC, Mo, Dis | S: M, Ru   | S: M, Mo     |
| <i>N. rustica</i>                          | S: VC, Cr        | 0              | S: Mo, Ru  | S: difMo     |
| <i>N. tabacum</i> ‘Xanthi’                 | S: VC, Mo        | S: M, Mo       | S: Cr, Dis | S: VN, Mo    |
| <i>Phaseolus vulgaris</i> ‘Bataaf’         | L: difSp; S: Epi | S: chlSp       | S: difSp   | S: VC, Mo    |
| <i>Pisum sativum</i> ‘Žalsviai’            | S: chlMo         | S: difMo       | S: M, Mo   | S: chlMo     |
| <i>Tetragonia expansa</i>                  | L: chlLL         | L: chlLL       | L: chlLL   | L: chlLL     |
| <i>Vicia faba</i> ‘Aušra’                  | S: Mo            | S: Mo          | S: VC      | S: Mo        |

**Abbreviations:** L – local reaction, S – systemic reaction, nLL – necrotic local lesions, chlLL – chlorotic local lesions, whLL – white local lesions, M – mosaic, Mo – mottling, Ma – malformation, VC – vein clearing, VN – vein necrosis, Dis – distortion, dif – diffuse, Cr – crincling, Ru – rugosity, Epi – epinasty, TW – top wilting, y – yellow, Sp – spotting, St – stunt, 0 – no symptoms, – - not tested.

**Santrumpos:** L – vietinė reakcija, S – sisteminė reakcija, nLL – nekrotinės vietinės žaizdos, chlLL – chlorotinės vietinės žaizdos, whLL – balkšvos vietinės žaizdos, M – mozaika, Mo – margumas, Ma – neišsivystęs, VC – gyslų išryškėjimas, VN – gyslų nekrozė, Dis – išsikraipymas, dif – išskydęs, Cr – garbanė, Ru – raukšlėtumas, Epi – išaugos, TW – viršūnės vytimas, y – geltonas, Sp – dėmėtumas, St – žemaūgė, 0 – be simptomų, – – netestuota.



**Fig. 6.** CMV particles  
**6 pav.** CMV dalelės



**Fig. 7.** DNA products amplified in PCRs from pepper infected by CMV:  
 1 and 11 – DNA Ladder; 2 – healthy plant; 3 and 5 – CMV infected test plants;  
 4 – control

**7 pav.** PGR amplifikuoti DNR produktai iš CMV pažeistų paprikų: 1 ir 11 – DNR markeris;  
 2 – sveikas augalas; 3 ir 5 – CMV užkrėsti augalai; 4 – kontrolė

RT-PCR using specific primer pair for CMV detection primed amplification of about 540 bp DNA sequences from two CMV samples from refrigerated symptomatic *D. stramonium* and *N. glutinosa* plant tissues (Fig. 7). A sample of non-infected *D. stramonium* plant did not yield visible specific DNA band. Amplification was not observed in sample with negative control (PCR buffer and PCR water), too. The molecular investigation confirmed that sweet pepper plants have been infected by the CMV.

**Discussion.** The experimental host range and specific symptoms on all test plants indicated that the virus isolated from sweet pepper crop most closely correspond with the CMV (Francki et al., 1979). Based on particles size and morphology, the virus was considered to be a member of the Cucumovirus genus (Kaper, Waterworth, 1981). RT-PCR data confirmed identification obtained by investigation of the host range, symptomatology and virus morphology. RT-PCR product size of CMV isolates from sweet pepper showed identity with CMV isolates, identified in other plants in Lithuania.

CMV is among the most economically damaging pathogens in cucurbits and other vegetable crops. Besides cucumber and pepper plants, CMV naturally affects tomato, parsley, celery, potato, pea, bean, lupine, clover, fruit and ornamental plants. Several aphids readily transmit CMV non-persistently in nature. Among more than 60 aphid species vectors, the most efficient are *Myzus persicae* Sulz., *Aphis gossypii* Glov., *A. craccivora* Koch. and *A. fabae* Scop. The large population of aphid vectors is one reason for the widespread nature of CMV. CMV spreads through the sap of infected plants by leaf contact, through the seeds of 19 plant species and dodder (Francki et al., 1979; Brunt et al., 1996).

**Conclusions.** The virus disease agent detected in sweet peppers in Lithuania has been identified as a CMV by using of different virological methods. Sweet pepper is a new natural host of CMV in Lithuania.

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### **Iš saldžiųjų paprikų izoliuoto agurkų mozaikos viruso nustatymas ir charakterizavimas**

**I. Zitikaitė, M. Samuitienė**

*Santrauka*

Pasaulyje pašarinius, vaisinius-uoginius, dekoratyvinius ir daržovinius augalus pažeidžiantis agurkų mozaikos virusas (*Cucumber mosaic virus*, CMV) buvo izoliuotas iš saldžiųjų paprikų (*Capsicum annuum* L.) Lietuvoje. Augalai buvo žemaūgiai, margai mozaikiškais ir deformuotais lapais bei vaisiais. Tyrimams medžiaga surinkta privačiuose daržuose Vilniaus, Kaišiadorių, Kėdainių rajonuose. Virusas identifikuotas pagal simptomų pasireiškimą augaluose indikatoriuose, pažeidžiamų augalų spektrą ir virionų morfologiją, panaudojant augalų indikatorius, peršviečiamosios elektroninės mikroskopijos bei atvirkštinės transkriptazės-polimerazės grandininės reakcijos (AT-PGR) testus. Nustatytas platus viruso pažeidžiamų augalų spektras. Labai specifinė simptomatika ir virionų morfologija yra būdingi CMV. Šiame darbe pritaikius publikuotą nukleotidinių sekų pagrindu parinktą specifinių oligonukleotidų porą, elektroforezės akrilamidiniame gelyje išryškėję kopijinės DNR PGR produktų fragmentai (~ 540 bp dydžio) atitiko panaudotus pradmenis ir patvirtino CMV saldžiosiose paprikose infekciją.

**Reikšminiai žodžiai:** agurkų mozaikos virusas, AT-PGR, izoliavimas, saldžioji paprika.