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Cucumber mosaic virus identification in pumpkin plants

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Abstract

Cucumber mosaic virus (CMV) causing viral diseases of many important plants worldwide has been isolated from pumpkin (*Cucurbita pepo* L.) plant leaves collected in Ukraine. Diseased pumpkin plant samples were collected in Ternopol region (Zalischyky district) and Chernivtsi region (Khotyn district) in 2009. Diseased plants had light green mottled foliage. Leaves were smaller than normal, yellow mottled and crinkled. The diagnostic study of the pathogen was done in 2009–2010 at the Plant Virus Laboratory of the Institute of Botany, Nature Research Centre. The determination of causal agent has been based on host range, symptom expression in the test plant species and morphological properties of the virus particles using transmission electron microscopy (EM), and using specific oligonucleotide primers in reverse transcription-polymerase chain reaction (RT-PCR). An experimental host range and induced symptoms for causal agent were determined by mechanically inoculating the group of herbaceous hosts representing seven botanical families (*Fabaceae* Lindl., *Solanaceae* Juss., *Cucurbitaceae* Juss., *Aizoaceae* Rudolphi, *Chenopodiaceae* Vent., *Amaranthaceae* Juss., *Asteraceae* Dumort.). The virus infected a wide experimental host range and developed specific symptoms for CMV. EM investigation revealed presence of isometric virions with hollow centre about 28–30 nm in diameter characteristic of CMV. Total nucleic acids from virus infected plants were isolated and purified by TRIzol Reagent method. Primers to CMV1 and CMV2 complementary to conserved region of CMV RNA sequences were designed to produce amplicons of different lengths of CMV isolates belonging to subgroups I or II (Singh et al., 1995). The RT-PCR was directly performed in crude sap extracts of CMV infected pumpkin and test plants. All PCR procedures were carried out in "T-Gradient Thermocycler" ("Biometra", Germany). Cycling parameters for DNA amplification were used as developed by Parrella and Sorrentino (2009). The RT-PCR assay of infected pumpkin plants and test plants yielded a band of 499 to 502 base pairs specific to CMV subgroup II and confirmed that virus isolates from pumpkin samples collected in Ternopil and Chernivtsi regions of Ukraine belong to subgroup II of CMV.

Key words: RT-PCR, test-plants, *Cucumber mosaic virus*, *Cucurbita pepo*.

Introduction

Cucumber mosaic virus (CMV) 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 (Edwardson, Christie, 1987). It is geographically widespread and has been reported in Europe, Asia, Australia, and North America. In Lithuania, this virus is spread on black currant (Samuitienė, Savičienė, 1987), leguminous (Staniulis, 1994), ornamental (Samuitienė, Navalinskienė, 2008) and vegetable (Zitikaitė, 2009) plants, however not detected on pumpkin. The most common symptom induced by CMV is mosaic; however, severity of disease may range from no obvious symptoms in some crops to death of the host species. The virus causes fern leaf, stunting of vegetable crops and malformation of their fruits. It is transmitted by numerous species of aphid, through the sap, the seeds and dodder (Francki et al., 1979; Kaper, Waterworth, 1981; Dijkstra, Khan, 2006).

Morphologically CMV has rather characteristic about 30 nm polyhedral particles with hollow centre (Palukaitis et al., 1992). The genome consists of three plus sense single-stranded RNAs, packaged in separate particles. CMV particles contain about 18% RNA. The RNA consists of 4 RNAs. Only largest RNA3 are required for infectivity (Roossinck et al., 1999). 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 −20ºC (Roossinck, White, 1998). Great number of different CMV strains, serogroups, subgroups and biological variations has been described (Finetti-Sialer et al., 1999; Hord et al., 2001; Yordanova, Hristova, 2002; Lin et al., 2003; Aramburu et al., 2007).

The aim of our work was to identify and characterize the causal agent detected in mottled pumpkin plants from Ukraine, collected during survey of virus diseases of sugar beet and ornamental plants for implementation of joint research project.

Materials and methods

Diseased pumpkin (*Cucurbita pepo* L.) plant samples were collected in Ternopil region (Zalischyky distr.) and Chernivtsi region (Khotyn distr.) in 2009. Leaf samples of these plants were collected by visual screening of grown fields for the presence of symptoms of viral etiology. The diagnostic study of the pathogen was done in 2009–2010 at Plant Virus Laboratory and greenhouse of the Institute of Botany of Nature Research Centre. An experimental host range and induced symptoms for causal agent were determined by mechanically inoculating the group of herbaceous hosts representing seven families (*Fabaceae* Lindl., *Solanaceae* Juss., *Cucurbitaceae* Juss., *Aizoaceae* Rudolphi, *Chenopodiaceae* Vent., *Amaranthaceae* Juss., *Asteraceae* Dumort.) (Table). Crude sap extracted from infected pumpkin leaf samples was inoculated onto herbaceous indicator plants. Leaves of test plants were triturated in 0.1 M sodium phosphate buffer at pH 7.0–7.1, containing 0.02% 2-mercaptoethanol and rubbed on experimental plant leaves dusted with 400 mesh carborundum for virus propagation (Jones, 1993). Plants were evaluated weekly for symptom expression.

Leaf samples collected from naturally infected pumpkin plants and test plants were prepared for transmission electron microscopy (TEM). Carbon coated palladium 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% solution of uranyl acetate. Grids were examined under a JEOL JEM-100S TEM at the instrumental magnification of 25 000 (Dijkstra, de Jager, 1998).

Reverse transcriptase-polymerase chain reactions (RT-PCR) for detection of causal agent isolated from pumpkin crop were accomplished using the primers designed from sequence of the protein gene of RNA of CMV (Henson, French, 1993). For this investigation we used the stored frozen at −20ºC systemically or locally infected plant material collected 10–12 days after inoculation.

Isolation method of total nucleic acids of virus infected plants with TRIzolReagent developed by Chomczynski and Sacchi (1987) was used. All PCR procedures were carried out in "T-Gradient Thermocycler" ("Biometra", Germany). For cDNA synthesis and PCR amplification of the CMV coat protein (CP) gene a downstream primer CMV1 (5'-GCC GTA AGC TGG ATG GAC AA-3') was designed complementary to position 2019 to 2038 in the CP gene and upstream primer CMV2 (5'-TAT GAT AAG AAG CTT GTT TCG CG-3') was designed homologous to nucleotides 1551 to 1573 of CMV RNA according to (Singh et al., 1995). Primers CMV1 and CMV2 were designed to produce amplicons of different lengths of CMV isolates belonging to subgroups I or II.

Total RNA resuspended from pellets in the solution containing RNAse inhibitor, 1 µl 20 pM downstream primer and deionized water and incubated at 70°C for 10 min were used for DNA first-strand synthesis. After denaturation, 11 µl of RNA solution was added to the mixture containing 4 µl of 5 x reaction buffer, 1 µl of 40 U/µl RNAse inhibitor, 2 µl 10 mM deoxynucleoside triphosphate (dNTP) mixture and 1 µl 200 U/µl RevertAid™ M-MuLV Reverse Transcriptase (for one sample) ("Fermentas", Lithuania). The synthesis of DNA first-strand was carried out at 42°C for 60 min and at 70°C for 10 min.

For DNA amplification PCR reaction mixture contained (for one sample): 34.75 µl PCR water, 1 µl of 20 pM each primers CMV1 and CMV2, 4 µl of 10 mM dNTP mixture at a concentration of 200 µM, 5 µl of 10 x PCR buffer without detergents, 3 µl of 25 mM $MgCl^{2+}$, 0.25 µl 5U/µl recombinant Taq DNA polymerase ("Fermentas", Lithuania) and 9 µl of cDNA were prepared. Cycling parameters were follows: pre-denaturation 92°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing: at 60°C for 1 min; elongation at 72°C for 1.5 min and a final extension of amplification products for 7 min at 72°C (Parrella, Sorrentino, 2009).

Resulting PCR products were analysed by electrophoresis through 5% polyacrylamide gel which run in 1 x TBE buffer, pH 8.2. Gels were run at 125 V for about 100 min, stained with ethidium bromide (1 µg of buffer per ml) and DNA bands were visualized using the gel documenting system Bio-Rad Gel DocXR. DNA fragment size standard was GeneRuler™ 50 bp DNA Ladder ("Fermentas", Lithuania), fragment sizes (from top to bottom): 1000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100, 50 bp.

Results and discussion

Symptoms of naturally affected pumpkin (*Cucurbita pepo* L.) plants vary widely. One of the most common symptom expressions was severe stunting. The plants have light green foliages, smaller than normal, yellow mottled and crinkled (Fig. 1). Older plants of pumpkin show foliar mottling followed by diffuse chlorosis. Two isolates (No. 0907 and 0908) were selected from the pumpkin samples. The experimental host range of the virus isolates from pumpkin is listed in Table 1. In our experiment, the causal agent of both isolates infected practically all of 18 mechanically inoculated test plants.

The pathogen caused vein brightening, mottling and various deformations of growing leaves of *Nicotiana glutinosa* L. (Fig. 2 a). Systemic reaction in the form of growth disorder, mosaic or mottling and deformation of young leaves of infected *N. tabacum* L. cv. 'Samsun' was also noticed (Fig. 2 b). Virus infection developed the most conspicuous symptoms on the leaves of *Datura stramonium* L. − diffusive changeable light and dark green areas.

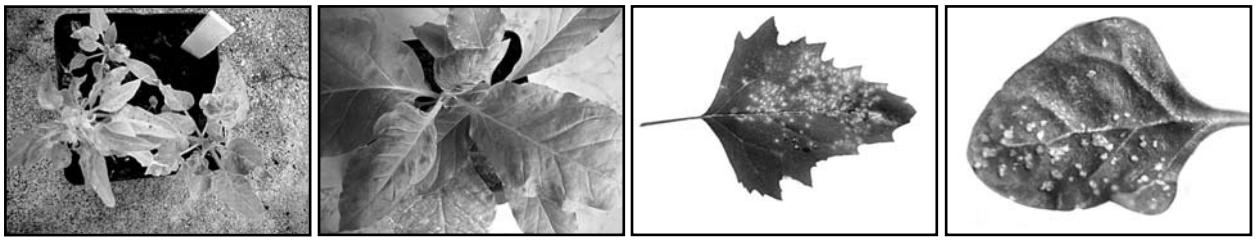
In the leaves of test plants of *Chenopodium* L. genus a local reaction in the form of red, white or chlorotic lesions was revealed (Fig. 2 c). Only local lesions appeared on the leaves of *Tetragonia expansa* Murr. (Fig. 2 d). Virus developed diffusive chlorotic spots, which later formed up a clear mosaic picture on the upper leaves of *Cucumis sativus* L. cv. 'Trakų pagerinti' and 'Krukiai'. Bright systemic symptoms were obtained on *Phaseolus vulgaris* L. 'Bataaf'. 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 Ukrainian pumpkin virus isolates on 18 diagnostic plant species were similar to those reported by other researchers and could be attributed to CMV. These test-plants are presented in plant virus descriptions as diagnostic species for this virus.

Figure 1. Symptoms of naturally infected pumpkin leaves with CMV

Table. The reaction of test-plants, inoculated with *Cucumber mosaic virus* isolated from pumpkin plants

No	Test plants	Local	Systemic
1.	*Amaranthus caudatus* L.	NLL	0
2.	*Celosia argentea* f. *cristata* (L.) Kuntze	0	0
3.	*Chenopodium amaranticolor* Coste et Reyn	ChlLL	0
4.	*Chenopodium ambrosioides* L.	NLL	0
5.	*Chenopodium foetidum* Schrad	NLL	0
6.	*Cucumis sativus* L. cv. 'Trakų pagerinti', 'Krukiai'	0	VC, M, LeDis
7.	*Cucurbita pepo* L. cv. 'Black Beauty'	0	VC, YM, TDis
8.	*Datura stramonium* L.	0	DifMo, LeDis
9.	*Gomphrena globosa* L.	NLL	0
10.	*Lycopersicon esculentum* Mill. cv. 'Ryčiai'	0	M, LeDis, TDis
11.	*Nicandra physalodes* (L.) Gaertn.	NLL	VC, YM
12.	*Nicotiana debneyi* Domin	0	YMo, LeCr
13.	*Nicotiana glutinosa* L.	0	VC, Mo, Ma, Stunt
14.	*Nicotiana rustica* L.	0	DifMo, TDis
15.	*Nicotiana tabacum* L. cv. 'Samsun'	0	Mo, LeDis, Stunt
16.	*Phaseolus vulgaris* L. cv. 'Bataaf'	0	VN, DifChlSp
17.	*Tetragonia expansa* Murr.	ChlLL	0
18.	*Zinnia elegans* Jacq.	NSp	0

Note. NLL – necrotic local lesions, ChlLL – chlorotic local lesions, N – necrosis, necrotic, Sp – spotting, VC – vein clearing, M – mosaic, Le – leaf, Dis – distortion, Y – yellow, T – top, Dif – diffusive, Mo – mottling, Cr – crinkling, Ma – malformation, Stunt – stunting, VN – vein necrosis, 0 = no symptoms.



a b c d

Figure 2. CMV induced symptoms on: a) *Nicotiana glutinosa*, b) *N. tabacum*, c) *Chenopodium amaranticolor*, d) *Tetragonia expansa*

By EM investigation numerous isometric partic-les with hollow centre were commonly observed in leaf dip EM preparations of leaf samples of naturally infected pumpkin plants and test plants. They were about 28–30 nm in diameter and characteristic of CMV (Fig. 3).

The presence of CMV in tissues of pumpkin leaves and indicator plants was proved by RT-PCR using specific primer pair (Singh et al., 1995). PCR reaction of three tested samples resulted in specific amplification of about 500 bp DNA fragment of coat protein region from refrigerated symptomatic *C. pepo* (infection source), *N. glutinosa* and *Nicandra physalodes* plant tissues (Fig. 4). A samples of pumpkin found in Chernivtsi region did not yield visible specific DNA band. Amplification was not observed in sample with negative control (PCR buffer and PCR water). The molecular investigation confirmed that pumpkin plants found in Ukraine have been infected by the CMV belonging to subgroup II (Singh et al., 1995).

The experimental host range and specific symptoms on all test plants indicated that the virus isolated from pumpkin 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 pumpkin showed identity with CMV isolates, identified in other important plants in Lithuania (Staniulis, 1994; Samuitienė, Navalinskienė, 2008; Zitikaitė, 2009). No literary data confirming definite identification of CMV on pumpkin in Ukraine have been traced yet.

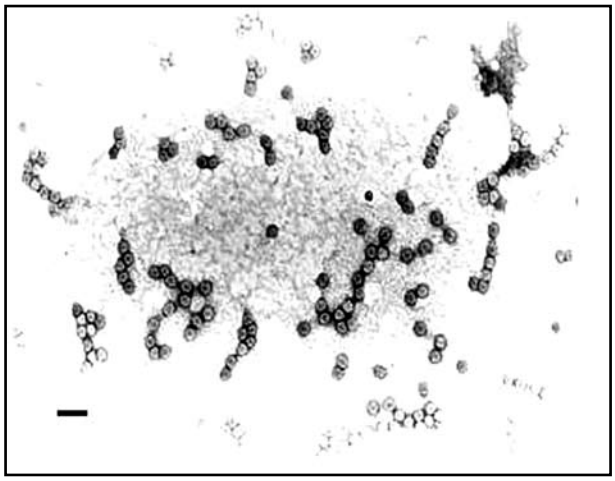


Figure 3. CMV particles in pumpkin extract. Bar represents 100 nm

CMV is among the most economically damaging pathogens in cucurbits and other vegetable crops. Besides cucurbit plants, CMV naturally affects tomato, pepper, parsley, celery, spinach, potato, pea, bean, lupine, clover, beets, fruit, and ornamental plants. 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 aphid vectors is one of the reasons for the widespread nature of CMV. The 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; Dijkstra, Khan, 2006). The CMV overwinters in perennial weeds and may be transmitted to healthy plants.

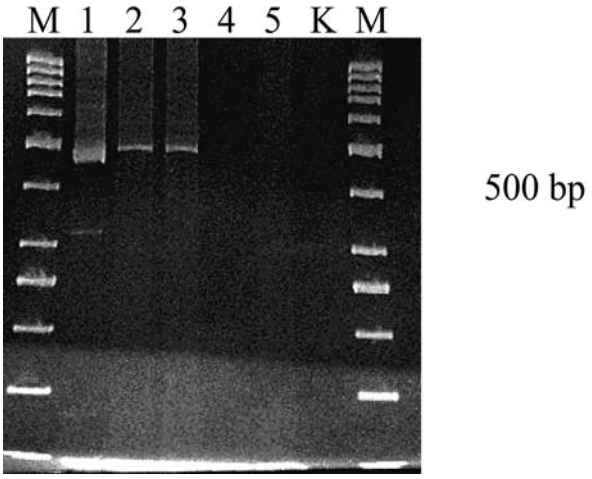


Figure 4. CMV specific 500 bp fragment: M – DNA Ladder, 1 – pumpkin sample from Ternopil region, 2 – *N. glutinosa*, 3 – *N. physalodes*, 4 and 5 – pumpkin samples from Chernivtsi region, K – control

Conclusion

The virus disease agent detected in pumpkin plants from Ukraine has been identified as a *Cucumber mosaic virus* (CMV) of subgroup II, applying the classical and modern molecular biology methods. To our knowledge, CMV so far has not been definitely identified as infecting pumpkin in natural conditions of the Ukraine.

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Agurkų mozaikos viruso (*Cucumber mosaic virus*) identifikacija *Cucurbita pepo*

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Santrauka

Agurkų mozaikos virusas (*Cucumber mosaic virus*, CMV*)*, visame pasaulyje pažeidžiantis daugelio rūšių augalus, buvo išskirtas iš paprastojo aguročio (*Cucurbita pepo* L.), aptikto Ukrainoje. Sergantys aguročiai 2009 m. buvo aptikti Ternopolio (Zališčikai) ir Černivcų (Chotinas) srityse. Sergančių augalų lapai buvo mažesni už normalius, geltonai numargę ir raukšlėti. Siekiant identifikuoti ligos sukėlėją, tyrimai atlikti 2009–2010 m. Gamtos tyrimų centro Botanikos instituto Fitovirusų laboratorijoje. Ligos sukėlėjas nustatytas pagal natūraliai pažeistų ir inokuliuotų augalų indikatorių požymius, eksperimentiškai pažeistų augalų spektrą, virionų morfologijos tyrimus elektroniniu mikroskopu (EM) ir atvirkštinės transkriptazės polimerazinės grandininės reakcijos (AT-PGR) duomenis. Sukėlėjo eksperimentinis augalų šeimininkų spektras ir išryškėję ligos požymiai nustatyti mechaniškai inokuliuojant septynių botaninių šeimų (*Fabaceae* Lindl., *Solanaceae* Juss., *Cucurbitaceae* Juss., *Aizoaceae* Rudolphi, *Chenopodiaceae* Vent., *Amaranthaceae* Juss., *Asteraceae* Dumort.) augalus. Eksperimentiškai užkrėstuose augaluose indikatoriuose išryškėjo būdingi CMV požymiai. EM preparatuose rasta izometrinių su išreikštu centru 28–30 nm skersmens virionų, būdingų CMV. Natūraliai CMV infekuotų aguročių, aptiktų Ukrainos Ternopolio bei Černivcų srityse, ir bandyminių augalų mėginių tyrimai parodė, kad AT-PGR gauti DNR amplifikacijos 499–502 bp. dydžio fragmentai atitiko panaudotų specifinių pradmenų dydį ir patvirtino ligos sukėlėjo priklausymą CMV II pogrupiui.

Reikšminiai žodžiai: AT-PGR, augalai indikatoriai, *Cucumber mosaic virus*, *Cucurbita pepo*.