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OCCURRENCE OF *CUCUMBER MOSAIC CUCUMOVIRUS* ON ORNAMENTAL PLANTS IN LITHUANIA

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Abstract

Cucumber mosaic cucumovirus (CMV) is pathogenic to an extremely wide variety of plant species including ornamentals. Using the methods of test-plants and electron microscopy CMV was identified in ornamental plant species of genera: *Asclepias* L., *Canna* L., *Crocus* L., *Dahlia* Cav., *Delphinium* L., *Gaillardia* Foug., *Gladiolus* L., *Iris* L., *Lilium* L., *Lupinus* L., *Crocosmia* Planch., *Muscari* Mill., *Narcissus* L., *Phlox* L., *Pelargonium* L'Her., *Primula* L., *Rudbeckia* L., *Tulipa* L., *Viola* L., representing 13 families. Virus identification was confirmed by modern methods of molecular biology.

Key words: *Cucumber mosaic cucumovirus,* ornamental plants, identification.

Introduction

During the last years great attention has been paid to the development of field floriculture in Lithuania. For small land property farmers field floriculture is perspective as family business. The farmers grow seedlings of perennial ornamental plants not only for Lithuanian domestic market, but also for neighbouring countries. Like other segments of agriculture this sector is threatened by plant diseases. The quality and quantity of ornamental plants is affected by viral diseases. Since viral infections are systemic in diseased plants, propagation by division may contribute to geographical spread of pathogens. The scientific workers of Plant Virus Laboratory of the Institute of Botany carry out regular survey controlling the phytosanitary state of ornamental plants grown at the Botanical Gardens of Vilnius, Kaunas Vytautas Magnus, Klaipėda Universities, Experimental Station of Field Floriculture and according to opportunities and requests in other floriculture farms and cities' parterres in order to help the growers to control plant diseases and also to collect plant samples for investigation. As a result of this investigation a wide distribution of *Cucumber mosaic cucumovirus* (CMV) has been revealed.

CMV, the type member of the genus *Cucumovirus*, in the family *Bromoviridae* is one of the most widespread plant viruses in the world. It is transmitted by at least 75 species of aphids in non-persistent manner and through seeds. CMV has an extremely broad host range, infecting more than 1000 plant species in over 85 families, causing important diseases and economic losses in cereals, forages, woody and herbaceous ornamentals, vegetables, fruits and other important crops /Francki et al., 1979; Brunt et al., 1996; Dijkstra, Khan, 2006; Aramburu et al., 2007/. The most common symptom

incited by CMV is mosaic; however, severity of disease may range from no obvious symptoms in some crops to death of the host species. Some of the intermediate symptoms include ringspot, fruit woodness and necrosis of bulbs. The virus also often causes plant dwarfing and flower breaking /Kaper, Waterworth, 1981/. CMV particles are isometric. The genome consists of three plus sense single-stranded RNAs, packaged in separate particles. In addition, CMV can be a helper virus for single-stranded satellite RNA that can modulate the symptoms induced by CMV, either attenuating or exacerbating them, depending on CMV strain. Numerous strains of CMV have been described which, based on phylogenetic and diversity studies, have been classified into two subgroups designed I and II /Quemada et al., 1989; Aramburu et al., 2007/. Previously CMV was identified in leguminous, vegetable and ornamental crops in Lithuania /Staniulis, 1994; Navalinskienė, 1994; Staniulis et al., 2000/.

This study is designed to identify plant species that serve as hosts for CMV in major ornamental species in Lithuania. Here we report the occurrence of CMV in perennial, bulb and corm ornamental plants and procedures of virus identification, including modern methods of molecular biology.

Materials and Methods

The plant material was collected in Botanical Gardens of Vilnius, Kaunas Vytautas Magnus, Klaipėda Universities, Experimental Station of Field Floriculture, and in private gardens.

Virus has been identified by the methods of test-plant /Francki et al., 1979, Brunt et al., 1996/, electron microscopy (EM) /Dijkstra, de Jager, 1998/, DAS-ELISA /Clark, Adams, 1977/ and RT-PCR /De Blas et al., 1994/.

The test-plants were inoculated in early stages of growth by mechanical sap transmission, applying carborundum as an abrasive. The inocula were prepared by homogenizing infected plant tissue in 0.1 M phosphate buffer pH 7.0, containing 0.2% 2–mercaptoethanol or 0.01 M sodium diethyldithiocarbamate as virus-stabilizing additives.

Virus particles were examined in leaf dip preparations negatively stained with 3% uranyl acetate electron microscopically using a JEM-100S electron microscope, at magnification 25000.

Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was carried out using commercial kit (DSMZ Plant Virus Collection, Germany), according to standard procedure. CMV IgG was used at a dilution of 1/1000 and alkaline phosphatase conjugate – at a dilution of 1/500. 50 mg of sample was extracted in 1 ml of sample buffer. 0.1% p-nitrophenylphosphate was used as substrate. The reactions were measured after 90 min incubation with substrate photometrically at 405 nm (Labsystems Multiskan RC).

Reverse transcription polymerase chain reaction (RT-PCR) was accomplished using the primers designed from non-coding intergenic region in the coat protein gene. An upstream primer: 5'-GTAGACATCTGTGACGCGA-3' homologous to nucleotides 114–132 and a downstream primer: 5'-GCGCGAAACAAGCTTCTTATC-3' complementary to position 633–653 were selected, resulting in a 540 bp amplification product /Quemada et al., 1989/.

Total RNA was extracted from symptomatic test-plant material stored frozen at −20° C using QuickPrep™ Total RNA Extraction Kit (Amersham Biosciences UK). Extraction procedure was carried out according to the manufacturer's instructions.

All PCR procedures were carried out in Eppendorf Master Cycler Personal. For RNA denaturation mixture of 10 µl RNA (for each sample) and 1 µl of downstream primer was incubated 5 min at 70° C and 5 min at 4° C.

Reverse transcription (RT, cDNA synthesis) reaction mixture (for one sample): 4 µl 5x PCR buffer; 1 µl RNasin; 2 µl 10 mM dNTPs; 1 µl MulRev Transcriptase and 11 µl of denatured RNA mix. Reaction was performed incubating at 37° C for 60 min, at 70° C for 10 min and at 4° C for 5 min.

PCR reaction mixture contained (for one sample): 11 µl c DNA; 34.75 µl PCR water, 4 µl 2 mM dNTPmix, 1 µl upstream primer, 1 µl downstream primer, 5 µl 10xPCR buffer without Mg, 3 µl $MgCl_2$, 0.25 µl Taq DNA polymerase. Reaction mixtures were incubated at 94° C for 4 min (for first step), 40 cycles of 94° C for 1 min, 52° C for 2 min, 72° C for 2 min, and at 72° C for 10 min (final step).

Resulting PCR products were analyzed by electrophoresis through 5% plyacrilamide gel, stained with ethidium bromide, and DNA bands visualized using a UV transilluminator. DNA fragment size standard was PhiX174 RFI DNA HaeIII digest, fragment sizes (from top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp.

Results and Discussion

CMV was detected in species of 19 ornamental plant genera representing 13 families. Virus was isolated and identified from following naturally infected ornamental plants expressing further described symptoms. These symptoms may be not specific only to CMV, because many ornamental plants were naturally infected by mixed virus infection.

Asclepiadaceae R. Br.

Asclepias L. Veins of infected *A. syriaca* plants are shortened and leaves are slight wrinkled and show dark spots.

Asteraceae Dumort.

Dahlia Cav. Symptoms vary depending on cultivar and mixed infection. CMV causes a light mosaic with an extreme narrowing of the leaf, producing the typical "fern leaf". Some cultivars show chlorotic lines across the middle of the leaf, like outline of an oakleaf. In some cases flowers may show deformations and colour breaking.

Gaillardia Foug. CMV was isolated from two species *G. aristata* and *G. pulchella*. Plants generally are stunted and have conspicuous symptoms on flowers. Petals are distorted, display variously shaped and coloured spots and streaks.

Rudbeckia L. Infected *R. hirta* plants are stunted, internodes shortened. There are spots, necrosis on leaves. Flowers are smaller, distorted, petals uneven in length. In some cases severe colour breaking symptoms are observed.

Amaryllidaceae J.St.-Hil.

Narcissus L. Leaves of infected plants are hardened, variegated with light green streaks.

Cannaceae Juss.

Canna L. Infected *C. indica* plants are smaller than normal. Young leaves display chlorotic spots and streaks, which later turn to necrosis. Flowers are poorly developed and show severe colour breaking symptoms.

Fabaceae Lindl.

Lupinus L. Leaves of infected *L. polyphyllus* are severely distorted with light brown and necrotic spots. Stems display necrotic streaks. Severely affected plants do not blossom at all or have rarefacted inflorescences.

Geraniaceae Juss.

Pelargonium L'Her. CMV was isolated from plants expressing symptoms of leaf distortion and mosaic.

Hyacinthaceae Batsch ex Borkh.

Muscari Mill. Plants are smaller than normal, leaves distorted with chlorotic spots and streaks which later in season turn to necrosis.

Iridaceae Juss.

Crocosmia Planch. CMV was isolated from plants expressing symptoms of stem distortion, poorly developed flowers, light green streaks on leaves.

Crocus L. Infected plants are smaller than normal, leaves distorted having shape of zigzag. Petals are distorted, variegated with spots and streaks of various shape.

Gladiolus L. Severe colour breaking and deformation of flowers are the most common symptoms associated with CMV in gladioli. Flower stems have fewer and smaller flowers.

Iris L. Symptoms tend to develop 1–2 months after emergence. Leaf symptoms appeared as a light green mosaic pattern which may be more conspicuous on the flower bud sheaths. Symptoms on flowers are expressed by spots of various colour and shape.

Liliaceae Juss.

Lilium L. Leaves show chlorotic, yellow spots and stripes or vein-clearing. Later grey or brown necrotic spots and leaf curling may develop. A coarse breaking pattern in the flower is possible in some cultivars. In others, flower buds and flowers are degenerated, do not blossom out.

Tulipa L. Leaves of infected plants show light stripes and streaks. Plant growth is reduced, plants are distorted, flowers smaller than normal and variegated.

Ranunculaceae Juss.

Delphinium L. CMV was isolated from *D. cultorum* plants showing mosaic pattern consisting of yellow spots of various shape including ringspots.

Polemoniaceae Juss.

Phlox L. Naturally infected *P. paniculata* plants are stunted, show light green mosaic, mottling. Leaves, especially uppers, are severely narrowed, obtain filamentous shape, with uneven margins.

Primulaceae Vent.

Primula L. Infected plants are chlorotic, smaller than normal. Leaf lamina is crinkled with blisters. Later symptoms of vein chlorosis and necrotic spots develop. Petals, especially in margin area, are variegated with whitish spots. Severely diseased plants have uneven petals with indented margins.

Violaceae Batsch

CMV was isolated from *V. cornuta* and *V.* × *witrockiana* plants expressing symptoms of plant stunting, slight leaf and flower distortion, greenish mottling on leaves, colour breaking on flowers.

Using mechanical inoculation the group of 16 test-plants representing six families was inoculated with virus isolates separated from naturally infected ornamental plants. Test-plants and results of their reaction to inoculation are presented in Table.

Table. Test-plant reactions to inoculation with virus isolated from ornamental plants

Test-plant	Symptoms
Chenopodiaceae Vent.	
Atriplex hortensis L.	L: GNLL
Chenopodium amaranticolor Coste et Reyn	L: ClLL
C. urbicum L.	L: ClSp;
C. quinoa Willd.	L: ClLL
Cucurbitaceae Juss.	
Cucumis sativus L.	L: DifSp; S: Mo, YSp
Amaranthaceae Juss.	
Amaranthus caudatus L.	L: NLL
Gomphrena globosa L.	L: NLL
Solanaceae Juss.	
Nicandra physalodes (L.) Gaertn.	L:NLLSp; S: M, LeDis, NDot, LeTN
Nicotiana alata Link et Otto	L: NSp; S: ClGrRiSp, NRi
Nicotiana debneyi Domin.	L: DifSp; S: LeMo, VC, Dis
N. glutinosa L.	S: MiMo, LeDis
N. rustica L.	L: NRiSp, Sp; S: ClGrMo
N. tabacum L.	L: NSp; S: NSp, Str
Petunia hybrida Vilm.	L: ClSp; S: M, Mo
Aizoaceae F. Rudolphi	
Tetragonia expansa Murr.	L: NSp, LeR
Asteraceae Dumort	
Zinnia elegans Jacq.	S: Mo

Abbreviations: L – local reaction; S – systemic reaction; Cl – chlorosis, chlorotic; Dif – difussional; Dis – distortion; Dot – dots; G – grey; Gr – green; LL – local lesions; Le – leaf; M – mosaic; Mi – mild; Mo – mottling; N – necrosis, necrotic; R – rolling; Ri – rings; RiSp – ringspot; Sp – spots; Str – streaks; T – top; VC – vein clearing; Y – yellow.

Virus isolates induced local reaction (chlorotic, necrotic local lesions, spots) in test-plant representatives of *Amaranthaceae*, *Aizoaceae*, *Asteraceae*, *Chenopodiaceae* (Figure 1); local chlorotic lesions, necrotic spots, ringspots (Figure 2) and various systemic symptoms in test-plants of *Asteraceae*, *Cucurbitaceae* and *Solanaceae* families (Figures 3, 4). According to plant virus descriptions such type of test-plant reactions is specific for CMV /Francki et al., 1979; Kaper, Waterworth, 1981; Brunt et al., 1996/.

Figure 1. Necrotic local lesions caused by CMV in leaves of *Atriplex hortensis*

Figure 2. Local necrotic ringspots caused by CMV in leaf of *Nicotiana rustica*

Figure 3. Mottling incited by CMV in systemically infected leaf of *Cucumis sativus*

Figure 4. Systemic necrotic rings induced by CMV in leaves of *Nicotiana alata*

Electron microscopic (EM) examination of naturally infected host plants and inoculated test-plant tissue revealed the presence of isometric particles 30 nm in diameter (Figure 5). Such morphology of particles is characteristic for CMV /Francki et al., 1979; Palukaitis et al., 1992; Brunt et al., 1996/.

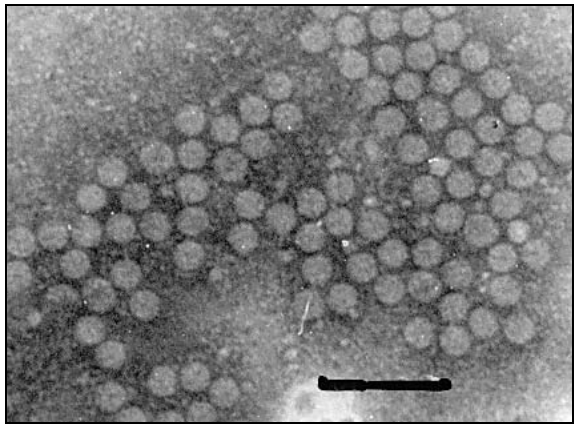

Figure 5. Particles of CMV. Bar represents 100 nm.

Symptomatic host plants and inoculated test-plants were tested in DAS-ELISA. Reaction was considered positive when absorbance at 405 nm was higher than twice the mean of healthy (negative) controls. All tested plants gave clearly expressed positive reaction confirming CMV infection (data not shown).

Identification by test-plant reactions, EM and DAS-ELISA results were verified in RT-PCR using as samples virus isolates from *Crocus*, *Delphinium*, *Gladiolus*, *Iris*, *Lilium*, *Muscari*. Total RNA was extracted from frozen leaf tissue of infected test–plants. Leaf tissue from healthy *N. rustica* plant was used as negative control. Specific for CMV PCR products were obtained with all investigated isolates and positive control, but not with negative control. Specific bands in polyacrilamide gel of analyzed products

after electrophoresis at a position corresponding to the expected size of amplification product of 540 bp were obtained, confirming CMV identity (Figure 6).

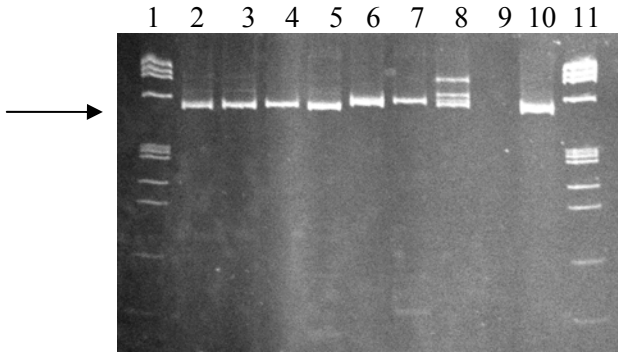

Figure 6. Electrophoresis of RT-PCR products of amplified DNA fragments from CMV isolates. Lanes: 1, 11 – DNA size standard PhiX174 RFI DNA *Hae*III digest, fragment sizes (from top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp., 2 – is. from *Delphinium*, 3 – is. from *Crocus*, 4 – is. from *Asclepias*; 5 – is. from *Iris*, 6 – is. from *Lilium*, 7, 8 – is. from *Gladiolus*, 9 – K–, 10 – K+. Size of product – 540 bp.

Based on the data of test-plant reactions, particle morphology, positive reaction with CMV specific antiserum in DAS-ELISA test, RT-PCR results it was ascertained, that agent causing virus diseases in investigated ornamental plants was CMV.

The presented results gave evidence that CMV is widespread on perennial, bulb and corm ornamental plants and causes harmful diseases, retards plant growth, damages any or all parts of a plant, distorts its standard properties, reduces aesthetic quality and marketability. Severity of disease symptoms may be exacerbated by common in ornamentals mixed virus infection. Virus-infected plants are more susceptible to fungal and bacterial pathogens, which lead to premature death.

Methods of controlling virus diseases have preventive character, consist of growing and propagation selected healthy planting material, inspection plants during vegetation for symptoms presence and eradication of affected plants in order to eliminate the source of infection. Control of aphids with insecticides and other means (using oil sprays, barriers to movement of aphids etc.) is an effective means in controlling the spread of aphid-transmitted viruses. Disease problems can be minimized by application of appropriate agrotechnical measures, weed control. Virus free stocks of valuable cultivars can be produced by heat treatment and meristem culture. The long-term effective control measure is creating and growing of virus resistant or tolerant varieties.

Conclusions

1. *Cucumber mosaic cucumovirus* (CMV) was identified as one of causal agents of viral diseases in ornamental plant species of genera: *Asclepias* L., *Canna* L., *Crocus* L., *Dahlia* Cav., *Delphinium* L., *Gaillardia* Foug., *Gladiolus* L., *Iris* L., *Lilium* L., *Lupinus* L., *Crocosmia* Planch., *Muscari* Mill., *Narcissus* L., *Phlox* L., *Pelargonium* L'Her., *Primula* L., *Rudbeckia* L., *Tulipa* L., *Viola* L., representing 13 families.

2. CMV is widespread on perennial, bulb and corm of ornamental plants and causes harmful diseases especially in the cases of mixed infections with other viruses.

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