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WEEDS AND WILD PLANTS AS NATURAL HOSTS OF CUCUMBER MOSAIC VIRUS: A CASE STUDY FROM WESTERN SLOVAKIA

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ABSTRACT

Cucumber mosaic virus (Bromoviridae) is a worldwide widespread pathogen affecting more than 1200 plant species. Although more attention is paid to CMV research on agricultural crops, vegetables or ornamental plants, this work was focused on the analysis of several weeds and wild plants as potential natural hosts of CMV. For this purpose, samples from two different agroecological locations were tested by DAS-ELISA using CMV-specific antibodies, revealing nine CMV-positive plant species. CMV isolates from greater burdock (Arctium lappa L.), rough hawksbeard (Crepis biennis L.), wood avens (Geum urbanum L.), prickly lettuce (Lactuca serriola L.), black medick (Medicago lupulina L.), bitter dock (Rumex obtusifolius L.), field sowthistle (Sonchus arvensis L.), white clover (Trifolium repens) and white violet (Viola alba Besser.) were further characterized by sequencing the complete CP gene, disclosing their affiliation to the CMV Group II. We have shown that weeds and wild plant species can be effective hosts for CMV and can serve as virus reservoirs from which the virus can be spread to cultivated crops.

Keywords: cucumovirus, CMV, detection, molecular variability, weeds

INTRODUCTION

Cucumber mosaic virus (CMV, known as Cucumovirus CMV based on the binomial nomenclature), is the type species of the Cucumovirus genus, belonging to the Bromoviridae family (**Bujarski *et al*., 2019**). The CMV genome consists of three single-stranded messenger sense RNAs (RNA1, 2 and 3) encoding at least five open reading frames (ORFs). The capsid protein (CP), translated from subgenomic RNA 4, is required for intercellular and long-distance movement and aphid transmission (**Mochizuki & Ohki, 2012**). Based on the genomic distinctions, CMV isolates divide into three main molecular groups, namely IA, IB, and II (**Jacquemond, 2012**). During a recent study based on high-throughput sequencing (HTS), isolates of group IA and II were found in Solanaceae crops in Slovakia (**Mrkvová *et al*., 2022**).
Although the virus is transmitted mechanically and partly also by seeds (**Ali & Kobayashi, 2010**), under natural conditions it is mainly vectored by more than 70 aphid species in a stylet-borne non-persistent manner (**Gildow *et al*., 2008**). CMV is distributed worldwide and infects more than 1200 species of 100 plant families, including monocot and dicot plants (**Mochizuki & Ohki, 2012**).
Most epidemiologic studies related to CMV have in the past focused on economically important agricultural crops. Less attention has been paid to the occurrence and molecular diversity of the virus in weeds and wild plants. Such plants, however, are an important part of intensively cultivated crops, of the agroecological interface, or of wild plant communities (**Hasiów-Jaroszewska *et al*., 2021, Slavíková *et al*., 2023**). The virus can spread in both directions between weeds and crops and cause the emergence of new local epidemics. Knowledge about the host range, occurrence and diversity of CMV in wild plants and weeds can therefore significantly contribute to the understanding of virus epidemiology and help to the adoption of effective preventive and control measures.
In a previous work, in addition to Solanaceae hosts, CMV was also detected in weeds from the *Papaveraceae* family (**Mrkvová *et al*., 2022**). In order to expand the knowledge about the natural host range of CMV in Slovakia, several weeds and wild plants were tested in two localities, which are characterized by different ecological contexts. Moreover, the affiliation of CMV isolates to molecular groups was evaluated based on the complete sequence analysis of the CP gene.

MATERIAL AND METHODS

Collection of samples and initial detection

To monitor the presence of CMV, samples of weeds and wild plants were taken during the growing season (June - September 2023) from two different locations in western Slovakia: i/ a private production garden in Pezinok (GPS coordinates: 48.30308, 17.27645) situated in the vicinity of other gardens and small agricultural plots and ii/ a small forest area inside the city of Bratislava (GPS: 48.17189, 17.06226) surrounded by the urban environment without connection with agricultural plantings. Leaf samples were collected from actively growing plant parts, preferably from plants displaying virus-like symptoms or disorders (Table 1, Table2). Where necessary the identity of plant species was confirmed using online tools (https://www.plant.id/, https://identify.plantnet.org).
For the initial detection of CMV, a double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) was performed according to the protocol described by **Clark & Adams (1977)**. Briefly, microtitre plate wells (no. 82.1581, Sarstedt, Numbrecht, Germany) were coated with polyclonal antibody (Bioreba, no. 071490) diluted in carbonate buffer (pH 9.6) according to the supplier's specifications and incubated for 4 h at 37°C. Plant leaf samples (ca. 0.1-0.2 g per sample) were then homogenized in PBS (1/10 w/v) containing 0.05% Tween-20 and 2% polyvinylpyrrolidone 40. Crude plant extracts were incubated overnight at 4°C. CMV-specific alkaline phosphatase-conjugated antibody diluted in conjugate buffer (Bioreba, no. 081490) was then added and incubated for 4 hours at 37°C. After each step, the plates were washed with phosphate-buffered saline (PBS, pH 7.4). The presence of antigen-antibody complexes was detected by adding 1 mg/ml para-nitrophenyl phosphate to the substrate buffer. The composition of the buffers is available at https://www.dsmz.de/fileadmin/_migrated/content_uploads/DAS-ELISA_01.pdf. Absorbance values were measured repeatedly (generally over 10-60 minutes) at 405 nm using an ELISA reader (Multiscan EX, ThermoFisher Scientific, Waltham, MA, USA). A sample was considered positive if its absorbance value was at least three times the standard deviation over the mean absorbance of the negative control samples (*Cucumis sativus* cv. Desana, *Pisum sativum* cv. Alderman and *Nicotiana benthamiana*). *C. sativus* plants cv. Desana, experimentally inoculated with isolates CP2 (group IA) and LAS (group II), were used as positive controls (**Mrkvová *et al*., 2022**).
For mechanical inoculation, inoculum prepared by grinding weed leaves in Norit buffer (dilution 1/10 w/v) was applied to *C. sativus* Desana plants at the cotyledon stage, as previously described (**Mrkvová *et al*., 2022**).

RT-PCR, partial genome sequencing and sequence analyses

Total RNAs were extracted from leaf tissues using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). First strand cDNA was synthesized by reverse transcription of total RNAs using random hexamer primers and Avian myeloblastosis virus (AMV) reverse transcriptase (both from Promega Corp., Madison, WI, USA). An approximately 875 bp long segment of the CMV

RNA3 genome encompassing the complete capsid protein gene was amplified in RT-PCR using the CMV-specific primer pair CMV-CP-F (5′-TYTCATGGATGCTTCTCCRC-3′, sense) / CMV-CP-R (5′-CTGGATGGACAACCCGTTC-3′, antisense). PCR was performed using Go Taq G2 Mastermix (Promega Corp., Madison, WI, USA) under the following cycling conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s and a final extension step at 72°C for 10 min. Amplified products were purified using the Wizard PCR Preps DNA purification system (Promega Corp., Madison, WI, USA) and directly Sanger sequenced by priming the sequencing reaction with the same oligonucleotides as used for PCR (Eurofins Genomics, Ebersberg, Germany).

The flanking untranslated parts were trimmed from the generated sequences to obtain the complete sequences encoding the CP gene (657 bp). CP gene sequences were compared with CMV sequences representing groups IA, IB and II obtained from the GenBank database (www.ncbi.nlm.nih.gov, accessed 22 April 2024). Phylogenetic analyses and comparisons were conducted using MEGA v.7 (Kumar et al., 2016). The presence of satellite CMV was tested by RT-PCR using the primer pair satCMV_skF (5′-GGTTATATCTACGTGAGGATC-3, sense′)/satCMV_skR (5′-ACCACCTAACAGAGTGTTTC-3′, antisense) as described previously (**Mrkvová *et al.*, 2022**).

RESULTS AND DISCUSSION

Wild annual and perennial plants are an essential part of the agro-ecological environment, which, like cultivated crops, can be hosts to various pathogens, including viruses. Pathogenic viruses cause macroscopic changes (symptoms) on plants, which often lead to a decrease in plant vitality and growth (**Jiang & Zhou, 2023, Tatineni & Hein, 2023**). Visual symptoms recorded in this work on wild plants consisted of e.g. mosaics, mottling, local necrotic spots, deformation of leaves and/or stunting of the whole plant (Fig. 1).

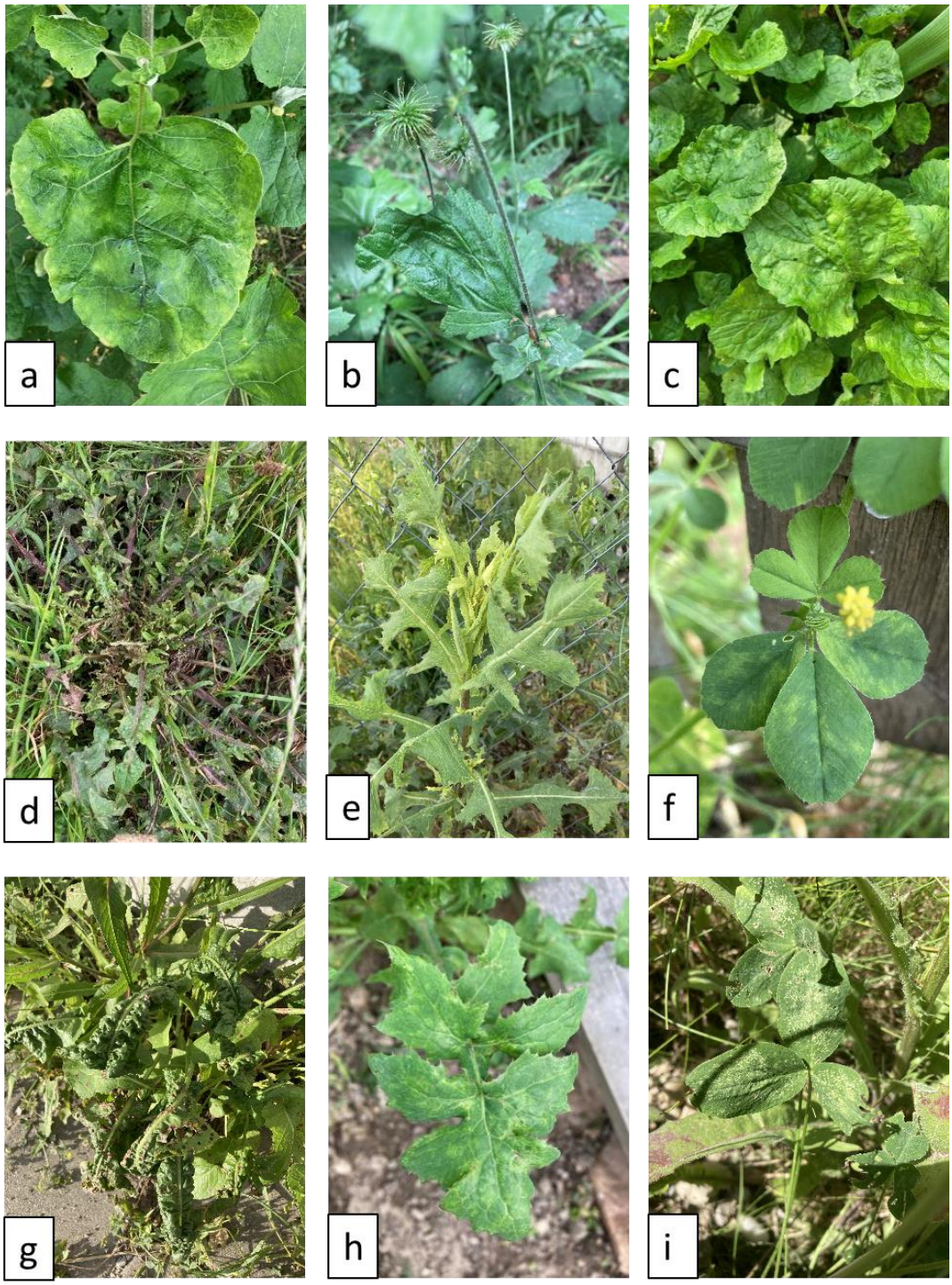

Figure 1 Symptoms observed on plants that tested positive for CMV; Arctium lappa (a), Geum urbanum (b), Viola alba (c), Crepis biennis (d), Lactuca serriola (e), Medicago lupulina (f), Rumex obtusifolius (g), Sonchus arvensis (h), Trifolium repens (i).

To study the occurrence of CMV in weeds and uncultivated wild plants, we focused on two different ecological locations. The first of them represented an intensively managed garden with the occurrence of CMV-sensitive crops (Pezinok). The second location consisted of the edge of a small forest area all around adjacent to the urban environment, without a direct connection to the agricultural landscape (Bratislava). Out of a total of 43 different plant species (Table 1 and 2), 9 were tested as CMV positive by DAS-ELISA using CMV-specific antibodies. CMV-positive samples included rough hawksbeard (Crepis biennis L.), prickly lettuce (Lactuca serriola L.), black medick (Medicago lupulina L.), bitter dock (Rumex obtusifolius L.), field sowthistle (Sonchus arvensis L.), white clover (Trifolium repens) from the locality Pezinok and greater burdock (Arctium lappa L.), wood avens (Geum urbanum L.) and white violet (Viola alba Besser.) from Bratislava. All plants tested positive for CMV showed symptoms similar to viral infection (Fig. 1), although symptoms of virus infection were sometimes difficult to distinguish as often masked by environmental stresses and pest damages.

Table 1 List of plant samples tested positive for CMV, host characteristics and availability of partial CMV genome sequences in public database.

sample (isolate)	species	family	life cycle	locality	Genbank accession number
B3	*Sonchus arvensis*	*Asteraceae*	perennial	PK	PP982859
B4	*Viola alba*	*Violaceae*	perennial	BA	PP982855
B9	*Arctium lappa*	*Asteraceae*	biennial	BA	PP982856
B10	*Lactuca serriola*	*Asteraceae*	annual / biennial	PK	PP982851
B12	*Geum urbanum*	*Rosaceae*	perennial	BA	PP982854
B18	*Medicago lupulina*	*Fabaceae*	annual/ perennial	PK	PP982852
B22	*Rumex obtusifolius*	*Polygonaceae*	annual/ perennial	PK	PP982853
B31	*Crepis biennis*	*Asteraceae*	biennial	PK	PP982857
B36	*Trifolium repens*	*Fabaceae*	perennial	PK	PP982858

Legend: PK – Pezinok (cultural environment), BA – Bratislava (wild environment)

Table 2 List of plant species (including families) whose samples tested negative in DAS-ELISA

Pezinok (cultural environment)	Bratislava (wild environment)
Achillea millefolium (Asteraceae)	
Borago officinalis (Boraginaceae)	*Aegopodium podagraria (Apiaceae)*
Calystegia sepium (Convolvulaceae)	*Capsella bursa-pastoris (Brassicaceae)*
Chenopodium album (Amaranthaceae)	*Cirsium arvense (Asteraceae)*
Conium maculatum (Apiaceae)	*Erigeron annuus (Asteraceae)*
Convolvulus arvensis (Convolvulaceae)	*Fragaria vesca (Rosaceae)*
Elymus repens (Poaceae)	*Geranium molle (Geraniaceae)*
Euphorbia peplus (Euphorbiaceae)	*Geranium robertianum (Geraniaceae)*
Glechoma hederacea (Lamiaceae)	*Hedera helix (Araliaceae)*
Mentha piperita (Lamiaceae)	*Impatiens parviflora (Balsaminaceae)*
Origanum vulgare (Lamiaceae)	*Lamium purpureum (Lamiaceae)*
Oxalis dillenii (Oxalidaceae)	*Oxalis acetosella (Oxalidaceae)*
Plantago lanceolate (Plantaginaceae)	*Parietaria officinalis (Urticaceae)*
Plantago major (Plantaginaceae)	*Picris hieracioides (Asteraceae)*
Senecio vulgaris (Asteraceae)	*Sisymbrium officinale (Brassicaceae)*
Stellaria media (Caryophyllaceae)	*Taraxacum officinale (Asteraceae)*
Taraxacum officinale (Asteraceae)	*Urtica dioica (Urticaceae)*
Verbascum densiflorum (Scrophulariaceae)	

However, it is clear from previous works that the virome of a plant host can be complex and the plant is often attacked simultaneously by several viruses (**Minicka *et al*., 2019, Susi *et al*., 2019, Jo *et al*., 2022**). It should be stressed that no other viral co-infections besides CMV were tested in our work. Therefore, it is not possible to unequivocally state that the observed morphological changes in CMV-infected plants are due only to the presence of this virus. In addition, infection with multiple viruses may provide atypical symptomatology due to the synergism or antagonism of two or more viruses (**Moreno & Lopez-Moya, 2020, Alcaide *et al*. 2020**).

To confirm the infectivity of the detected CMV isolates, we mechanically transferred the virus from three weeds (Arctium lappa, Sonchus arvensis and Viola alba) to healthy cucumber plants cv. Desana, which we subsequently verified by DAS-ELISA (Fig. 2).

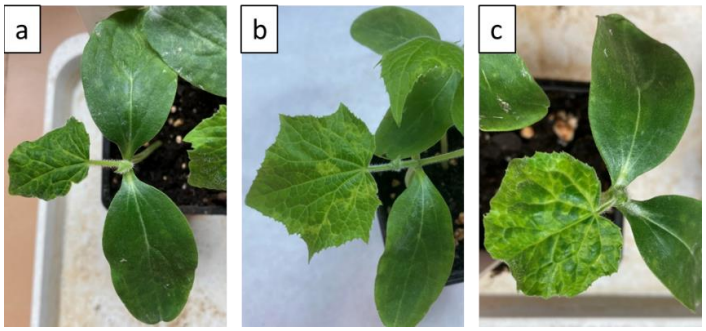

Figure 2 Symptoms induced in *C. sativum* cv. Desana plants 8 days post mechanical inoculation with sap from CMV-positive plants *Arctium lappa* (a), *Sonchus arvensis* (b) and *Viola alba* (c).

To assess the molecular variability of CMV in 9 DAS-ELISA positive plants, an RT-PCR was performed targeting CMV RNA3. The specific primers encompass part of the intergenic region, the complete CP gene and a part of 3´untranslated region (nucleotide positions 1155-2023 based on the reference genome NC_001440). Besides the high polyvalence of primers, this arrangement enabled us to obtain complete CP gene for subsequent phylogenetic analysis. The complete nucleotide CP sequences reported herein has been deposited in the GenBank database under accession no. PP982851-PP982859. All CP sequences were collinear and molecularly closely related, consisting of 657 nucleotides including the stop codon. BLAST search and phylogenetic analysis based on the complete CP gene clearly showed that the CMV isolates identified in this work belong to the Group II CMV isolates. In addition, the analyzes showed very low nucleotide divergence among the 9 isolates, regardless of their location (mean genetic distance reached 1%).

Interestingly, contrary to previous work (**Mrkvová *et al*., 2022**) no satellite CMV RNA have been detected to be associated to CMV infection of tested weeds. The biological or epidemiological significance of sat RNA in CMV epidemics involving weed plant communities remains thus to be investigated further (**Betancourt *et al*., 2011**).

The importance of biennial or perennial weeds and wild plants lies in their role as overwintering hosts from which a new cycle of viral disease can be initiated in the spring through effective transmission by aphids or transmission of the virus by seeds (**Tomlinson *et al*., 1970, Hobbs *et al*., 2000**). Therefore, the elimination of weeds susceptible to CMV is one of the key parameters of control measures aimed to slow viral epidemics, although it is very difficult to follow in practice.

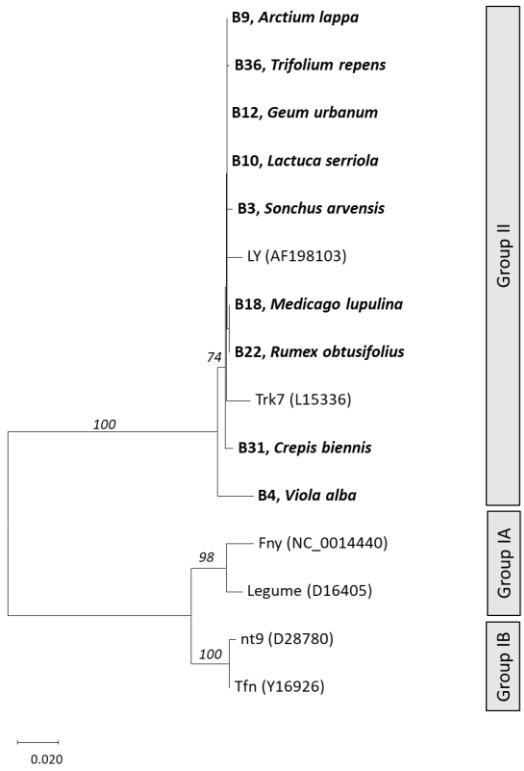

Figure 3 Neighbor-joining tree of Slovak CMV isolates generated from the complete CP gene obtained in this study. For each Group (**Jacquemond et al., 2012**), reference database isolates are added, identified by their Genbank accession number. The characteristics of Slovak isolates are described in Table 1. Scale bar indicates a genetic distance of 0.02. Bootstrap values >70 (500 bootstrap resamplings) are indicated as percentages.

CONCLUSION

CMV is a globally distributed viral pathogen with an extremely large host range that causes significant yield losses and poor crop quality in susceptible hosts. We have shown that 9 weeds and wild plant species can be effective hosts for CMV and can serve as virus reservoirs from which the virus can be spread to cultivated crops. It should be emphasized that selection pressure and evolutionary processes operating in new or changing ecological areas, changes in agro-ecological conditions due to global warming, emergence of new invasive or non-native plant hosts and co-infection(s) with other viruses may cause the emergence of new virus variants, which potentially increase the epidemiological threat associated with CMV. Although some of the weed species mentioned here have already been reported as a natural host of CMV (e.g. Škorić et al., 2000, Dunich et al., 2022), there is still a great need for continued research efforts to study the variability of the virus and its host range in a specific geographic region.

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