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Identification of *Brome mosaic virus* in cocksfoot (*Dactylis glomerata* L.) and meadow fescue (*Festuca pratensis* Huds.) in Lithuania

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Abstract

Brome mosaic virus (BMV) causing viral diseases in *graminaceous* plants worldwide has been isolated in Lithuania from common cocksfoot (*Dactylis glomerata* L.) and meadow fescue (*Festuca pratensis* Huds.) plants exhibiting mosaic, chlorotic mottling and streaks of leaf and stem symptoms. The plant material was collected in the fields and roadsides of Vilnius and Kaunas regions. Virus isolates were investigated by test-plants, serology and reverse transcription-polymerase chain reaction (RT-PCR) methods. The identification of the virus was based on the results of symptomology on host-plants, transmission of viral infection by mechanical inoculation on test-plants, positive double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) test and a specific amplification fragment size (450 bp) of virus RNA in RT-PCR. This is the first report of common cocksfoot and meadow fescue as a natural host for BMV in Lithuania.

Key words: identification, DAS-ELISA, RT-PCR, *Brome mosaic virus*, *Dactylis glomerata*, *Festuca pratensis*.

Introduction

Brome mosaic virus (BMV) is a small, positive-stranded, icosahedral RNA plant virus belonging to the family *Bromoviridae* (Lane, 1981; Kao, Sivakumaran, 2000).

BMV was first isolated from bromegrass (*Bromus inermis* Leyss) in the USA in 1942 (McKinney, 1944). BMV is widespread in Africa, North America and continental Europe (Bancroft, 1970; Lane, 1974; Gibson, Kenten, 1978; utic et al., 1999; Gadiou, Kundu, 2010). The virus infects species of about 50 genera of the family *Poaceae.* BMV can infect plants both systemically, e.g., monocotyledonous cereal crops or dicotyledonous *Nicotiana benthamiana*, and locally, e.g., *Chenopodium* species. Among dicotyledonous plants, its host range is restricted to a few genera in about six families (Lane, 1974). Although it is not generally considered to be an economically damaging plant pathogen, many cause forage crop and wheat yield reductions of 50% to 100%, as well as decreases in germination of seed from infected plants (Spaar, Schumann, 1977). The virus produces typical mosaic, mottling, or streaking symptoms in wheat, barley, oat, rye, sorghum, Johnson grass, lolium, and many other grasses (Brunt et al., 1996; Vacke, 1997).

The genome of BMV has three RNA components, RNA1, RNA2, and RNA3 (Ahlquist, 1992). RNA1 (3.2 kb) and RNA2 (2.9 kb) encode viral RNA replicase proteins 1a and 2a, respectively (Kroner et al., 1989; 1990). RNA3 encodes the non-structural cell-to-cell movement protein 3a (Schmitz, Rao, 1996; Takeda et al., 2004), and the coat protein. The coat protein is expressed from subgenomic RNA4, which co-encapsidates with RNA3 (Choi et al., 2002). BMV virions are non-enveloped icosahedrons about 26 nm in diameter and containing 180 coat protein subunits (Brunt et al., 1996). The capsid structure and coat protein sequence of this virus closely resembles that of *Cowpea chlorotic mottle virus* (CCMV) (Lane, 1981; Speir et al., 1995). Temperature for the virus inactivation is about 70–79ºC and limiting dilution is l:10000. The virus survives in air-dried leaf tissue for more than 1 year (Bancroft, 1970).

Several vectors have been reported to be involved in the spread of BMV. The virus often is spread from infected smooth brome grass to previously uninfected areas through animal and machinery traffic. Mechanical transmission by humans also can become significant in certain areas. Beetles (*Chaetocnema aridula* and *Oulema melanopus*) are reported to be the main natural vectors for transmission of BMV. In general, BMV is not transmitted through seed or by the aphids *Aceria tosichella*, *Myzus persicae*, *Schizaphis graminum*, and *Rhopalosiphon maydis.* Nematode species in the genus *Xiphenema* have transmitted BMV in laboratory settings (Ding et al., 2001; Rouf Mian et al., 2005).

The aim of this study was to isolate, identify and characterize the causal agent of gramineous plants' virus

disease in Lithuania expressing mosaic, chlorotic mottling and streaks symptoms.

Materials and methods

The plant material for investigation was collected from 8 locations (fields and roadsides) in Vilnius and Kaunas regions. The samples were collected from common cocksfoot (*Dactylis glomerata* L.) and meadow fescue (*Festuca pratensis* Huds.) plants showing visual virus symptoms on leaves and stems. While investigating the BMV disease of gramineous forage plants, 18 virus samples were collected from 10 common cocksfoot and 8 meadow fescue plants in fields and roadsides.

The diagnostic study of the pathogen was done in 2010–2011 at Plant Virus Laboratory and greenhouse of the Institute of Botany of Nature Research Centre. The virus was identified by the methods of test-plant (Bancroft, 1970; Brunt et al., 1996), DAS-ELISA (Clark, Adams, 1977) and RT-PCR (Rao et al., 1990).

Test-plants were grown in a greenhouse at 16–30ºC. The virus was identified by the symptoms of the mechanically inoculated test-plants: *Chenopodium amaranticolor* Coste et Reyn., *C. quinoa* Willd., *Avena sativa* L., *Dactylis glomerata* L., *Festuca pratensis* Huds., *Hordeum vulgare* L., *Lolium multiflorum* Lam., *Secale cereale* L., *Triticum aestivum* L., *Zea mays* L., *Datura stramonium* L., *Nicotiana rustica* L., *N. tabacum* L. 'Samsun' (about 20 plants of each species). The inoculum for mechanical sap inoculation was prepared by grinding infected leaves in 0.1 M phosphate bufer pH 6.8. Monocotyledonous test-plants were inoculated at the stage of three leaves and dicotyledonous test-plants at the four-leaf stage. Carborundum was added to the crude extract prior to mechanical inoculation.

Serological detection and confirmation of virus presence in the tested samples of fescue and cocksfoot and experimentally infected test-plants were conducted by the direct double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) technique. DAS-ELISA was carried out using a commercial kit (DSMZ Plant Virus Collection, Germany) according to the standard procedure. BMV IgG was used at a 1:1000 dilution and alkaline phosphatase conjugate at a l:1000 dilution. 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", Finland). Reverse transcription-polymerase chain reaction (RT-PCR) was accomplished using primers designed for BMV coat protein gene. The upstream primer d(5'-GAC ATG GTC TCT TTT AG-3') and the downstream primer d(5'-AGG ACC ACA CAA CGC TTG-3') were selected, resulting in 450 bp amplification product (Rao et al., 1990). Total RNA was extracted from symptomatic test-plant material stored frozen at −20ºC, according to TRIzol Reagent method developed by (Chomczynski, Sacchi, 1987). Total RNA was dissolved in PCR water – containing RNAse inhibitor and was stored at −20ºC. All PCR procedures were carried out in "T-Gradient Thermocycler" ("Biometra", Germany). Extracted total RNA with 1 µl 20 pM downstream primer and deionized water were incubated at 70°C for 10 min and were used for cDNA synthesis. After denaturation 11 µl of RNA solution was added to the mixture containing 4 µl of 5 × PCR buffer, 1 µl of 40 Uµl^{-1} RNAse inhibitor, 2 µl 10 mM deoxynucleoside triphosphate (dNTP) mixture and 1 µl 200 Uµl^{-1} "RevertAid™ M-MuLV Reverse Transcriptase" (for one sample) ("Fermentas", Lithuania). The synthesis of cDNA was carried out at 42°C for 60 min and 70°C for 10 min. PCR reaction mixture of 60 µl were used for DNA amplification: 34.75 µl PCR water, 1 µl upstream and downstream primers, 4µl of 2 mM dNTP mix, 5 µl of 10 × PCR buffer without detergents, 3 µl of 25 mM $MgCl^{2+}$, 0.25 µl 5Uµl^{-1} recombinant Taq DNA polymerase ("Fermentas", Lithuania) and 11 µl of 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 54°C for 2 min, elongation at 72°C for 2 min, and the final extension of amplification products for 5 min at 72°C.

Resulting PCR products were analysed by electrophoresis through 1.5% agarose gel, stained with ethidium bromide, and DNA bands were visualized using the documenting system "Bio-Rad Gel DocXR" (USA). 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.

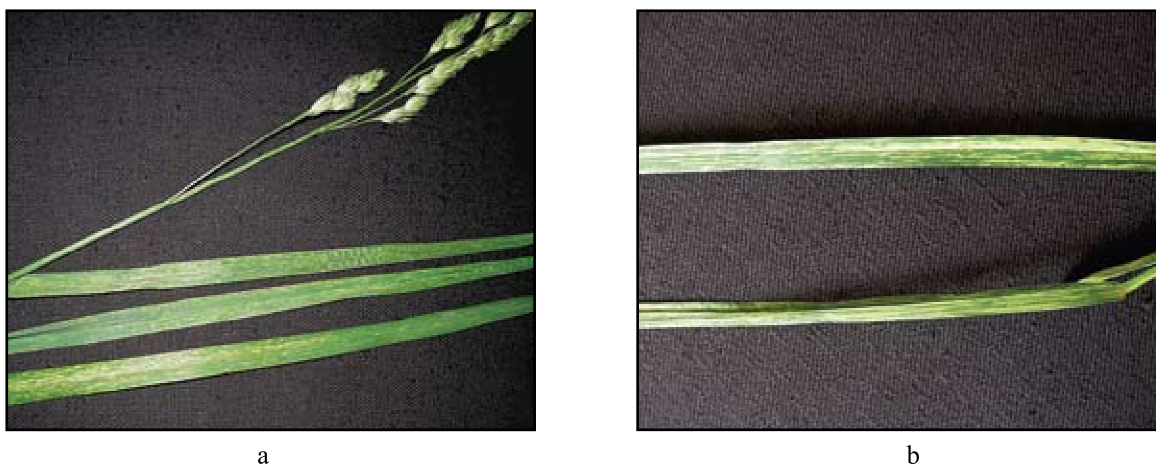
Results and discussion

BMV infection in host-plants (common cocksfoot and meadow fescue) samples expressing systemic mosaic, chlorotic mottling and light green streaks on leaves and stems (Fig. 1) was examined in DAS-ELISA using specific antiserum to BMV. Results confirmed BMV in three virus isolates (D1002, D1003, F1102) from 18 tested plants.

Isolate D1002 was separated from *Dactylis glomerata*. Leaves of infected plants showed light green streaks and chlorotic mottling. Isolate D1103 was separated from *Dactylis glomerata*. Leaves were covered by chlorotic mottling and systemic mosaic. Isolate F1102 was separated from *Festuca pratensis*. Leaves and stems showed light green streaks. These virus isolates were selected as material for investigations. Tissue extracts of BMV-infected plants were used for mechanical inoculation, BMV propagation and RT-PCR analysis.

Using mechanical inoculation, a group of test-plants consisting of 13 species representing three families was inoculated with virus isolates separated from naturally infected *Dactylis glomerata* and *Festuca pratensis* plants. The symptoms appeared in 2–5 weeks after inoculation and the severity of their expression depended on test-plant species and the temperature in the greenhouse.

Test-plants and the results of their reaction to inoculation are presented in Table. The isolates produced similar symptoms in inoculated test-plant species.



a b

Figure 1. Chlorotic mottling and streaks on naturally infec-ted host plants: a) *Dactylis glomerata*, b) *Festuca pratensis*

Table. Reaction of test-plants inoculated with virus isolated from *Dactylis glomerata* and *Festuca pratensis*

Test-plants	Symptoms
Chenopodiaceae Vent.	
Chenopodium amaranticolor Coste et Reyn.	Chlorotic and necrotic local lesions
C. quinoa Willd.	Chlorotic and necrotic local lesions
Poaceae (R. Br.) Bernhart)	
Avena sativa L.	Systemic mosaic, chlorotic mottling
Dactylis glomerata L.	Systemic mosaic
Festuca pratensis Huds.	Systemic chlorotic mosaic
Hordeum vulgare L.	Systemic mild mosaic
Lolium multiflorum Lam.	Systemic chlorotic mosaic
Secale cereale L.	Systemic mild mosaic
Triticum aestivum L.	Systemic chlorotic mosaic
Zea mays L.	Chlorosis
Solanaceae Juss.	
Datura stramonium L.	Chlorotic spots
Nicotiana rustica L.	No symptoms
N. tabacum L. 'Samsun'	No symptoms

Virus isolates induced systemic reactions (mosaic, mild mosaic, chlorotic mosaic and streaking, chlorotic mottling, chlorosis) in test-plant representatives of the *Poaceae* family. *C. amaranticolor* and *C. quinoa* reacted with chlorotic and necrotic local lesions on leaves. *D. stramonium* reacted with chlorotic spotting on leaves (Fig. 2). According to plant virus descriptions such type of test-plant reactions is specific for BMV (Gibson, Kenten, 1978; Haber, 1989; Brunt et al., 1996; Mise, Pocsai, 2004).

Mechanically inoculated test-plants were tested for BMV infection by DAS-ELISA. The identification of BMV in test-plants was confirmed by positive reaction in DAS-ELISA using alkaline phosphatase linked to the BMV antibodies with glutaraldehyde and nitrophenyl phosphate as substrate. The strongly expressed positive results in this test for detection of BMV were obtained with inoculated test-plants: *A. sativa*, *D. glomerata*, *D. stramonium*, *F. pratensis*, *L. multiflorum*, *T. aestivum* and *C. quinoa*. The other infected test-plants (*Chenopodium amaranticolor*, *Hordeum vulgare*, *Secale cereale*, *Zea mays*) reacted weakly in DAS-ELISA.

For molecular confirmation of detection of BMV in common cocksfoot and meadow fescue RT-PCR, isolates (D1002, D1003, F1102) of BMV were used. The BMV-specific PCR products were obtained with investigated isolates, but not with negative controls (PCR water and healthy plant). Specific bands in agarose gel of analyzed products at a position corresponding to the expected size of amplification product of 450 bp were obtained, confirming BMV identity (Fig. 3).

The results of biological, serological and molecular confirmation of this virus disease agent confirm that it is BMV isolated from naturally infected common cocksfoot and meadow fescue plants. On the basis of the results of experiments and literature data (Brunt et al., 1996; utic et al., 1999; Mise, Pocsai, 2004) these virus isolates could be attributed to BMV from the genus *Bromovirus.*

BMV has by far the broadest distribution and may be cosmopolitan. The virus has been reported to infect hosts *Dactylis glomerata* and *Festuca pratensis* in Bulgaria, Yugoslavia, Finland (Lane, 1981).

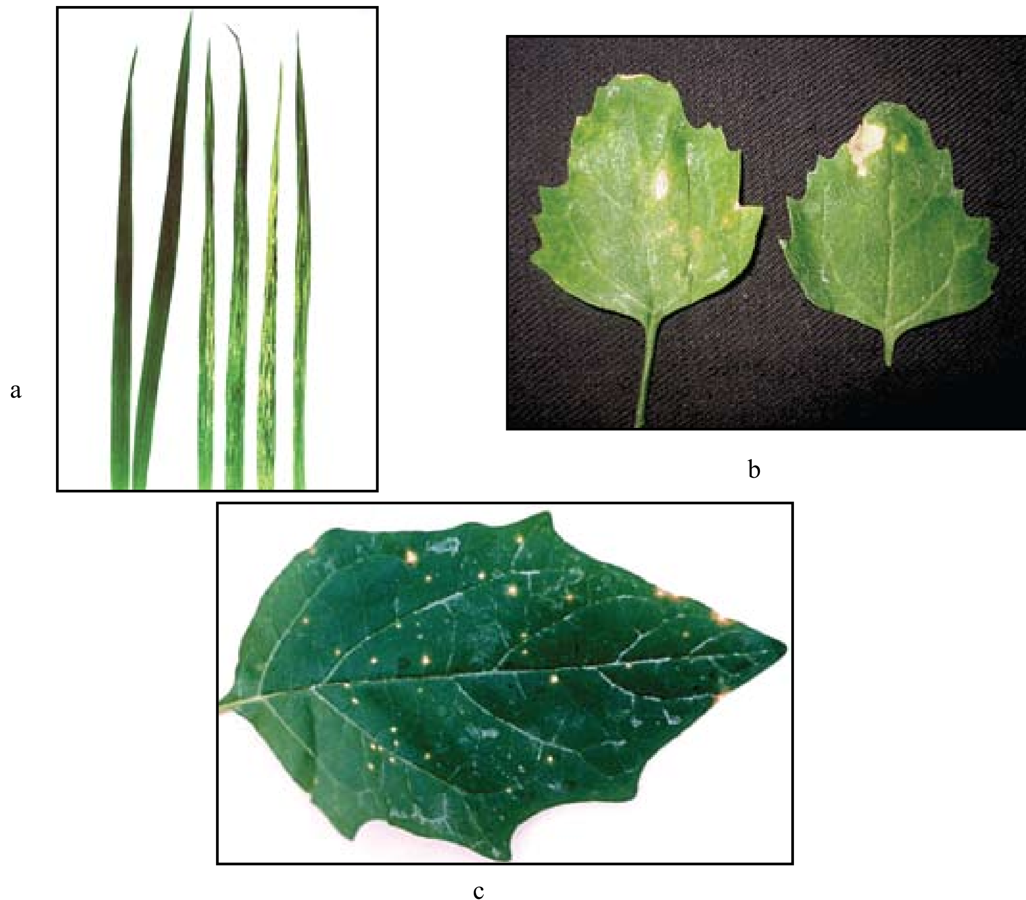
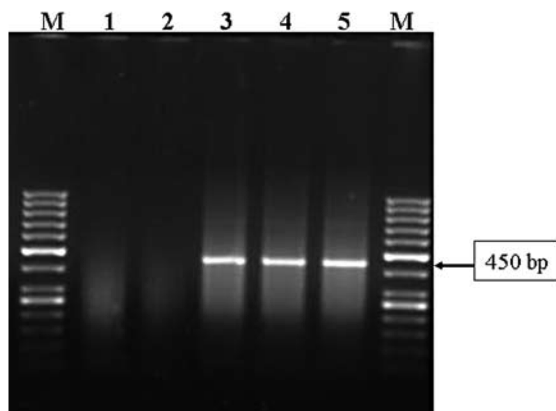


Figure 2. BMV induced symptoms on: a) *Avena sativa*, b) *Chenopodium quinoa*, c) *Datura stramonium*



Notes. Lanes: M – DNA size standard "GeneRuler™ 50 bp DNA Ladder", fragment sizes (from top to bottom): 1000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100, 50 bp; 1 – Kw (PCR water), 2 – K (negative control from healthy plant), 3 – *Dactylis glomerata* (No. D1002), 4 – *Dactylis glomerata* (No. D1103), 5 – *Festuca pratensis* (No. F 1102). Size of product – 450 bp.

Figure 3. RT-PCR products of amplified DNA fragments from BMV isolated from cocksfoot and fescue (EF 1. 5% agarose gel)

In Europe, BMV has minor effects on crops. Tošić (1971) observed in experimental infections that BMV reduced tillering, plant growth, and yields. The pathological effect of BMV on individual plants is serious, but the extent of overall crop damage depends on the number of infected plants in the field. BMV remains infective and is both preserved and transmitted from year to year in perennial hosts (*Bromus inermis* and others) (utic et al., 1999).

Control of BMV is difficult to ensure because possibly not all modes of its transmission and spread are sufficiently known. Vector control in the cases in which virus vectors are known is also recommended. All plant residues, as well as native plants in which the virus survives from the time of harvest to the next sowing, should be destroyed. Adequate crop rotation contributes to the decrease of the presence of nematode virus vectors in the soil (utic et al., 1999).

This is a first report of identification and characterization of BMV in *Dactylis glomerata* and *Festuca pratensis* in Lithuania.

Conclusions

1. The causal agent of the disease found in Lithuania in common cocksfoot (*Dactylis glomerata* L.) and meadow fescue (*Festuca pratensis* Huds.) was identified as *Brome mosaic virus* from *Bromovirus* genus.

2. The identify of the detected virus disease agent with BMV was confirmed by the methods based on test-plant reaction, double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) data.

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References

Ahlquist P. *Bromovirus* RNA replication and transcription // Current Opinion in Genetics and Development. – 1992, vol. 2, p. 71–76

Bancroft J. B. *Brome mosaic virus* // CMI/AAB Descriptions of plant viruses. – 1970, No. 3, p. 1–4

Brunt A. A., Crabtree K., Dallwitz M. J., Gibbs A. J., Watson L. *Brome mosaic bromovirus* // Viruses of plants. Descriptions and lists from VIDE database. – Cambridge, UK, 1996, p. 267–270

Choi Y. G., Dreher T. W., Rao A. L. tRNA elements mediate the assembly of an icosahedral RNA virus: proceedings of the National Academy of Sciences of the United States of America. – 2002, vol. 99, p. 655–660

Chomczynski P., Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanater-phenol-chloroform extraction // Analytical Biochemistry. – 1987, vol. 162, No. 1, p. 156–159

Clark M. F., Adams A. N. Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses // Journal of General Virology. – 1977, vol. 34, p. 475–483

Ding X. S., Boydston C. M., Nelson R. S. Presence of *Brome mosaic virus* in barley guttation fluid and its association with localized cell death response // Phytopathology. – 2001, vol. 91, No. 5, p. 440–448

Gadiou S., Kundu K. Complete genome sequence of a *Brome mosaic virus* isolate from the Czech Republic // *Czech Journal of* Genetics *and* Plant Breeding. – 2010, vol. 46, No. 4, p. 178–182

Gibson R. W., Kenten R. H. The occurrence of *Brome mosaic virus* in Britain // Journal of Phytopathology. – 1978, vol. 27, p. 66–67

Haber S. *Brome mosaic virus* isolated in Manitoba, Canada // Plant Disease. – 1989, vol. 73, No. 3, p. 195–199

Kao C. C., Sivakumaran K. *Brome mosaic virus*, good for an RNA virologist's basic needs // Molecular Plant Pathology. – 2000, vol. 1, p. 91–97

Kroner P., Richards D., Traynor P., Ahlquist P. Defined mutations in a small region of the *Brome mosaic virus* 2a gene cause diverse temperature-sensitive RNA replication phenotypes // Journal of Virology. – 1989, vol. 63, p. 5302–5309

Kroner P., Young B. M., Ahlquist P. Analysis of the role of *Brome mosaic virus* 1a protein domains in RNA replication, using linker insertion mutagenesis // Journal of Virology. – 1990, vol. 64, p. 6110–6120

Lane L. C. *Brome mosaic virus* // Advances in Virus Research. – 1974, vol. 19, p. 151–220

Lane L. C. Bromoviruses // Handbook of plant virus infection and comparative diagnosis / E. Kurstak (ed.). – Amsterdam, Netherlands, 1981, p. 333–376

McKinney H. H. Studies on the virus of brome-grass mosaic (abstract) // Phytopathology. – 1944, vol. 34, p. 993

Mise K., Pocsai M. *Brome mosaic virus* // Viruses and virus diseases of *Poaceae* (*Gramineae*) / Lapierre H., Signoret P. A. (eds). – Paris, France, 2004, p. 735–739

Rao A. L. N., Sullivan B. P., Hall T. C. Use of *Chenopodium hybridum* facilitates isolation of *Brome mosaic virus* RNA recombinants // Journal of General Virology. – 1990, vol. 71, p. 1403–1407

Rouf Mian M. A., Zwonitzer J. C., Hopkins A. A., Ding X. S., Nelson R. S. Response of tall fescue genotypes to a new strain of *Brome mosaic virus* // Plant Disease. – 2005, vol. 89, No. 3, p. 224–227

Schmitz I., Rao A. L. N. Molecular studies on *bromovirus* capsid protein. I. Characterization of cellto-cell movement-defective RNA3 variants of *brome mosaic virus* // Virology. – 1996, vol. 226, p. 281–293

Spaar D., Schumann K. Getreidearten und Gräser // Pflanzliche Virologie / Klinkowski M. et al. (eds). – Berlin, Germany, 1977, Bd. 3, S. 1–62

Speir J. A., Munshi S., Wang G., Baker T. S., Johnson J.E. Structures of the native and swollen forms of *cowpea chlorotic mottle virus* determined by X-ray crystallography and cryo-electron microscopy // Structure. – 1995, vol. 3, p. 63–78

utic D. D., Ford R. E., To ic M. T. Virus diseases of forage feed plants // Handbook of Plant Virus Diseases. – New York, USA, 1999, p. 85–96

Takeda A., Kaido M., Okuno T., Mise K. The C terminus of the movement protein of *Brome mosaic virus* controls the requirement for coat protein in cell-to-cell movement and plays a role in long-distance movement // *Journal of General* Virology. – 2004, vol. 85, p. 1751–1761

To ić M. Virus diseases of wheat in Serbia. II. Some changes in wheat plants infected with *Wheat streak mosaic virus* (WSMV) and *Brome mosaic virus* (BMV) // Phytopathology. – 1971, vol. 71, No. 4, p. 327–340

Vacke J. To the occurrence and harmfulness of *Brome mosaic virus* in cereal crops: proceedings of the International Conference Protection of Cereal Crops against Harmful Organisms / Tvaružek L. (ed.). – Prague, Czech Republic, 1997, p. 85–87

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Dirsės mozaikos viruso (*Brome mosaic virus*) identifikacija paprastosios šunažolės (*Dactylis glomerata* L.) ir tikrojo eraičino (*Festuca pratensis* Huds.) augaluose Lietuvoje

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Santrauka

Pasaulyje miglinius augalus pažeidžiantis dirsės mozaikos virusas (*Brome mosaic virus*, BMV) Lietuvoje buvo išskirtas iš paprastosios šunažolės (*Dactylis glomerata* L.) ir tikrojo eraičino (*Festuca pratensis* Huds.) augalų. Sergančių augalų lapų ir stiebų virusinis pažeidimas pasireiškė mozaika, chlorotiniu margumu ir dryžuotumu. Medžiaga tyrimams surinkta Vilniaus ir Kauno rajonuose laukuose bei pakelėse. Išskirti viruso izoliatai tirti augalų indikatorių, imunofermentinės analizės (DAS-ELISA) ir atvirkštinės transkripcijos polimerazės ciklinės reakcijos (AT-PCR) metodais. Pagal nustatytus specifinius simptomus augaluose šeimininkuose ir tirtuose augaluose, teigiamus DAS-ELISA testo duomenis ir viruso specifinio cDNR fragmento (450 bp) amplifikaciją, iš paprastosios šunažolės ir tikrojo eraičino augalų buvo išskirtas bei identifikuotas dirsės mozaikos virusas iš *Bromovirus* genties. Tai pirmasis Lietuvoje paprastojoje šunažolėje ir tikrajame eraičine aptikto BMV tyrimas.

Reikšminiai žodžiai: identifikacija, DAS-ELISA, AT-PCR, *Brome mosaic virus*, *Dactylis glomerata*, *Festuca pratensis*.