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Identification of ryegrass mosaic rymovirus in *Poaceae* plants

Laima Urbanavičienė

Plant Virus Laboratory, Institute of Botany, Žaliųjų Ežerų 49, LT-08406 Vilnius, Lithuania
E-mail: laima.urbanaviciene@botanika.lt

For the first time annual ryegrass (*Lolium multiflorum* Lam.), perennial ryegrass (*L. perenne* L.), meadow fescue (*Festuca pratensis* Huds.) and *Festulolium loliaceum* (Huds.) P. Fourn. (*Festuca pratensis* L. × *Lolium perenne* L.) showing symptoms of mosaic spotting, chlorotic and necrotic streaks on leaves and stems were collected at the Plant Breeding Centre of Lithuanian Institute of Agriculture and also at the Vilnius State Plant Varieties Testing Station in 2002. Virus infection was observed also in the season next year in this place and in other locations of Vilnius and Kaunas districts. Virus isolates were investigated by the methods of test-plants, electron microscopy (EM), serology, double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). The identification of the virus was based on the results of symptomology on host-plants, transmission of viral infection by mechanical inoculation to test plants, the morphology of virus particles filaments (about 700 nm long), positive reaction in DAS-ELISA. Ryegrass mosaic rymovirus (RGMV) identification was confirmed also by the RT-PCR technique.

Key words: the family *Poaceae*, identification, DAS-ELISA, RT-PCR, ryegrass mosaic virus

INTRODUCTION

Ryegrasses are annual and perennial graminaceous plants belonging to the family *Poaceae*, widespread in meadows and pastures. They are grown also as cultivated crops. During the vegetation period, the yield of perennial forage grass is influenced not only by ecological factors, but also by diseases caused by pathogens. Ryegrasses are the most important hosts, but also other grasses such as cocksfoot (*Dactylis glomerata* L.), fescue (*Festuca* L.) and annual meadow grass (*Poa annua* L.) may be infected [1]. Considering that forage feeds are important in Lithuania, much attention was paid to the prevalence of virus diseases in these crops.

Investigation of graminaceous plants with respect to viral diseases revealed a prevalence of ryegrass mosaic rymovirus (RGMV) on these plants in Lithuania.

RGMV belongs to the genus *Rymovirus* of the family *Potyvirideae*. It has been described as a common ryegrass pathogen in Canada, numerous European countries, Australia, New Zealand and South Africa [1–3]. RGMV is considered, after strains of barley yellow dwarf virus, the most serious and widespread virus affecting fodder grasses [4]. Studies indicate that infections with virulent RGMV strains may decrease the yield of pastures of Italian ryegrass by up to 27% and by 5 to 50% in perennial ryegrass [2, 5].

In addition to Italian and perennial ryegrasses, other important natural hosts of RGMV are cocksfoot (*Dactylis glomerata*), fescue (*Festuca pratensis*), and oat (*Avena sativa*) [6].

The symptoms caused by RGMV range from faint to severe mosaic with chlorotic and necrotic streaking, especially in perenial ryegrass [7]. RGMV is transmitted by the eriophyid mite *Abacarus hystrix* (Nalepa) [8] and by mechanical inoculation [9], and is also thought to be spread mechanically by livestock treading and haycutting machinery [5].

The virus particles are flexuous filaments 675–705 nm in length with a diameter of 15–18 nm. The thermal inactivation point is 60 °C, longevity *in vitro* is about 1 day at room temperature, and dilution endpoint is 10^{-3}. The virus contain one molecule of positive sense single-stranded RNA of ~9.5 kb encoding a single large open reading frame and one major structural protein, the capsid protein (CP) of Mr 45×10^3 kDa. [4].

The main goal of this study was to detect and identify the virus in symptomatic *Poaceae* family plants in Lithuania.

MATERIALS AND METHODS

Naturally infected symptomatic annual ryegrass (*Lolium multiflorum* Lam.), perenial ryegrass (*Lolium perenne* L.), meadow fescue (*Festuca pratensis* Huds.) and *Festulolium loliaceum* (Huds.) P. Fourn. showing viral symptoms (light green to yellow mosaic, chlorotic flecking, yellow and necrotic streaks on leaves and stems) were collected at the Vilnius State Plant Varieties Testing Station and at the Plant Breeding Centre of Lithuanian Institute of Agriculture and in fields of other regions of Lithuania. While investigating the RGM disease of gramineous forage plants, 60 virus samples were collected from 5 annual ryegrass, 35 perenial ryegrass, 6 fescue and 14 festulolium plants in experimental and other fields. The experimental work was carried out in the greenhouse and Plant Virus Laboratory of the Institute of Botany.

The virus was identified by symptoms of test-plants inoculated by mechanical inoculation. The following test-plants were used: *Agrostis stolonifera* L. 'Guoda', *Avena sativa* L. 'Jaugila', *Bromopsis inermis* Leyss. Holub., *Chenopodium amaranticolor* Coste et Reyn, *C. quinoa* Willd., *Dactylis glomerata* L. 'Asta', *Festuca arundinacea* Schreb., *Festuca rubra* L., *Festuca pratensis* Huds. 'Dotnuva', *Festulolium* Asch. et Gaebn., *Hordeum distichon* L. 'Anni', *Lolium multiflorum* Lam., *Lolium perenne* L. – 'Sodrė', 'Žvilgė', *Phleum pratense* L. 'Vėlenis', *Poa annua* L., *P. pratensis* L. 'Lanka', *Secale cereale* L. 'Owid', *Triticum aestivum* L. (variety uknown), *Zea mays* L. 'Pionier' (about 20 plants of each species). The inoculum for mechanical sap inoculation was prepared by grinding infected leaves in 0.1 M sodium phosphate buffer, pH 6.8–7.0. 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. Presence of virus particles was observed in dip preparations using a JEM-100S electron microscope (EM) after negative staining with 3% uranyl acetate solution [10, 11]. Serological detection and confirmation of virus presence in the tested samples of ryegrass, fescues, festulolium and experimentally infected test-plants were conducted by the direct double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) technique [12]. DAS-ELISA was carried out at the Plant Virus Laboratory, using a commercial kit (DSMZ Plant Virus Collection, Germany) according to standard protocols.

Reverse transcription – polymerase chain reaction (RT–PCR) was accomplished using frozen tissues of host plants (*L. perenne* and *Festulolium*). Primarily total RNA was extracted using the QuickPrep total RNA extraction kit (Amersham Pharmacia Biotech) according to manufacturer's instructions.

Investigation by the RT–PCR method was performed using two primers pairs selected for two different strains: Australian strain of RGMV (forward primer (RGMV-1)-GAACATGACTTCCACGACATCACCGACA-3'(28 nt); (reverse primer (RGMV-2) - 5'-AGCAAGTAAGTGGGCAGACAG-3'(21 nt) [5] and for RgMV-Bulgaria and -Czech strains -RGMV-F1 5'- GCA CGC ACC ATA CCT AGC TG- 3' (20 nt) and RGMV-R2: 5'- CAG CAT ATT CGC ACC CGA GA- 3'(20 nt). A 1126-bp fragment from the coding region of the CP gene was sequenced from RgMV-Bulgaria and RgMV-Czech strains after PCR amplification with RgMV-F1 and RgMV-R2 primers [13]. The synthesis of cDNA was performed under the following conditions. For RNA denaturation, solution was incubated in a mixture containing RNAse inhibitor, primer RGMV-R2 and PCR water. Samples were incubated for 10 min at 70 °C. For synthesis of the first cDNA strand, denaturated RNA solutions were added to a mixture containing reaction buffer, RNAse inhibitor, dNTP mix and M-MLV reverse transcriptase (MBI; "Fermentas", Vilnius, Lithuania). The first strand cDNA synthesis was carried out for 60 min at 37 °C and terminated by incubation at 70 °C for 10 min.

DNA amplification was carried out in a reaction mixture containing dNTP mix, RGMV-1 and RGMV-2 primers (Australian strain) [5] and accordingly RgMV-F1 and RgMV-R2 (Bulgarian and Czech strain) primers [13]. PCR buffer with magnesium chloride and Taq DNA polymerase (MBI "Fermentas"). Samples were placed in an Eppendorf Mastercycler Personal programmed to give one cycle at 94 °C (5 min), followed by 30 cycles at 94 °C (45 s), 55 °C (30 s), and 72 °C (2 min) (Australian strain) [5] and 94 °C (3 min), followed by 30 cycles at 94 °C (25 s), 58 °C (30 s), and 72 °C (45 s), with a final cycle of 72 °C (5 min) (Bulgarian and Czech strains) [13]. PCRs products were analysed by electrophoresis through 1% agarose gel, stained with ethidium bromide, and DNA bands were visualized using a UV transilluminator. DNA fragment size standard was a #SMO 311 Gene Ruler 100 bp DNA Ladder Plus (MBI "Fermentas") from top to bottom 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp.

RESULTS AND DISCUSSION

Ryegrass mosaic virus

The RGMV was isolated from naturally infected *L. multiflorum*, *L. perenne*, *F. pratensis*, and *Festulolium* 'Punia' cultural plants showing symptoms of light green to yellow mosaic, chlorotic flecking, yellow and necrotic streaks (Fig. 1).

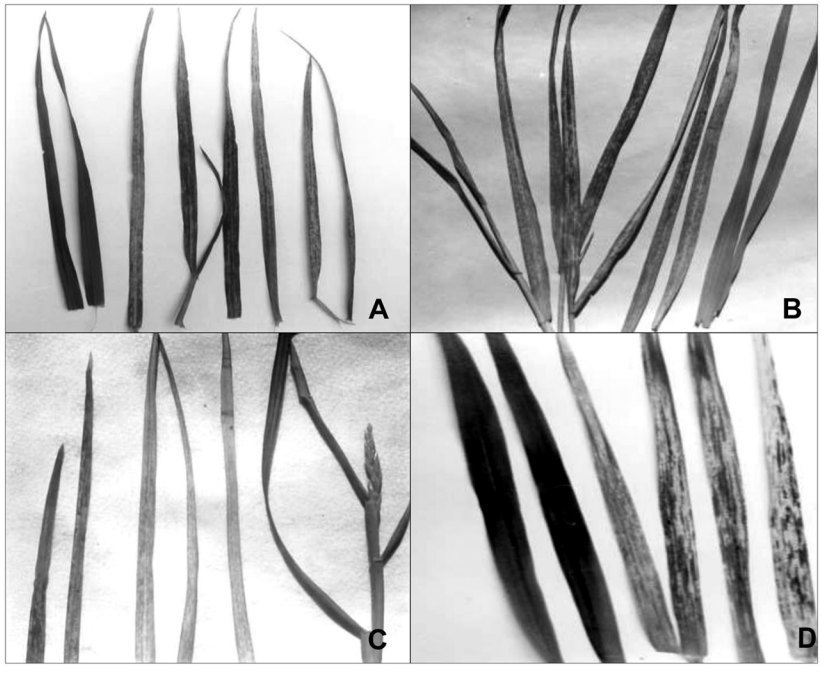

Fig. 1. Chlorotic streaks on naturally infected host plants: A – *Lolium perenne*, B – *Festuca pratensis*, C – *Festulolium* leaves, and D – systemic reaction: chlorotic and necrotic streaks induced by ryegrass mosaic rymovirus in test-plants *Avena sativa*; two healthy leaves on the left

Table. **Reaction of test-plants to inoculation with ryegrass mosaic virus isolated from annual ryegrass, perennial ryegrass, fescue and festulolium plants**

Test-plants	Diagnostical symptoms			
	Annual ryegrass	Perennial ryegrass	Fescue	Festulolium
Agrostis stolonifera L. cv. 'Guoda'	ChlFl	ChlStr	ChlFl	ChlStr
Avena sativa L. cv. 'Jaugila'	S : ChlStr	S : ChlStr	S : M,ChlNStr	S : ChlStr, NStr
Bromopsis inermis Leyss. Holub.	S : M	S : M	S : M	S : M
Chenopodium amaranticolor Coste et Reyn	0	0	0	0
C. quinoa Willd.	0	0	0	0
Dactylis glomerata L. cv. 'Asta'	S : M,ChlFl	S : M,ChlStr	S : M,ChlFl,Str	S : M,ChlFl
Festuca pratensis Huds. cv. 'Dotnuva'	S : M, ChlFl, Str	S : M, ChlStr	S : M, ChlStr	S : M, ChlStr
Festuca rubra L.	0	0	0	0
Festuca arundinacea Schreb.	S : M	S : M	S : M	S : M
Festulolium Asch. et Gaebn.	S : M,ChlStr	S : MChlStr	S : M,ChlStr	S : ChlStr,NStr
Hordeum distichon L. cv. : 'Anni'	0	0	0	0
Lolium multiflorum Lam	S : M, ChlStr	S : ChlStr	S : ChlStr	S : ChlStr, NStr
L. perenne L. cv. : 'Sodrė'	S : M, ChlStr	S : ChlStr	S : ChlFl, NStr	S : M, ChlStr
'Žvilgė'	S : M, ChlStr	S : ChlStr	S : M, ChlFl	S : M, ChlStr
Phleum pratense L. cv. 'Vėlenis'	0	0	0	–
Poa annua L.	S : M, ChlFl	S : M, ChlFl	S : M	S : M
P. pratensis L. cv. 'Lanka'	0	0	0	0
Secale cereale L. cv. 'Owid'	0	0	0	0
Triticum aestivum L.	0	0	0	0
Zea mays L. cv. 'Pionier'	0	0	0	0

Abbreviations: S – systemic reactions, Chl – chlorotic, Fl – flecking, M – mosaic, N – necrotic, Str – streaking, 0 – no infection, – not inoculated.

Nineteen test-plant species of the *Poaceae* and *Chenopodiaceae* families were experimentally inoculated with RGMV (Table). Fifteen-eighteen days after inoculation, the majority of *Poaceae* plants showed disease symptoms on leaves. Virus isolates from the naturally infected host plants *L. multiflorum*, *L perenne*, *F. pratensis*, *Festulolium* 'Punia' were transsmitted to test-plants: *L. multiflorum, L. perenne, F. pratensis, F. arundinacea, Festulolium*, *D. glomerata, A. stolonifera, B. inermis*, *Poa annua* and *A. sativa* 'Jaugila' but did not infect *C. amaranticolor, C. quinoa, F. rubra, H. distichon, S. cereale, P. pratensis, T. aestivum* and *Z. mays*. The most susceptible test-plants for virus infection (*L. multiflorum, L. perenne, F. pratensis, Festulolium* and *A. sativa*) developed systemic chlorotic flecking, yellow and necrotic streaking in inoculated leaves (Fig. 1). Virus infection was transmitted to *A. stolonifera, F. arundinacea* which developed systemic mosaic, chlorotic fleking and chlorotic streaking on leaves.

Electron microscopy analysis revealed the presence of viral particles in the specimen grid preparations prepared from naturally infected ryegrass, fescues and festulolium plant samples or from inoculated monocotyledonous plants. Virus particles are flexuous filaments about 700 nm in length with a diameter of 15–18 nm. Such a morphology of particles is specific of *Ryegrass mosaic rymovirus* (Fig. 2) [4, 6, 7].

RGMV infection in host-plants (ryegrass, fescues, festulolium) and inoculated test-plants expressing systemic mosaic, yellow and necrotic streaks was comfirmed by DAS-ELISA. The identification of RGMV isolates was confirmed by a positive reaction in DAS-ELISA using alkaline phosphatase linked to the RGMV antibodies with glutaraldehyde and nitrophenyl phosphate as a substrat. The positive results in this test for detection of RGMV were obtained with inoculated test-plants *A. sativa* 'Jaugila', *D. glomerata* 'Asta', *F. pratensis* 'Dotnuva', *L. multiflorum*, *L. perenne* 'Sodrė', *Festulolium.*

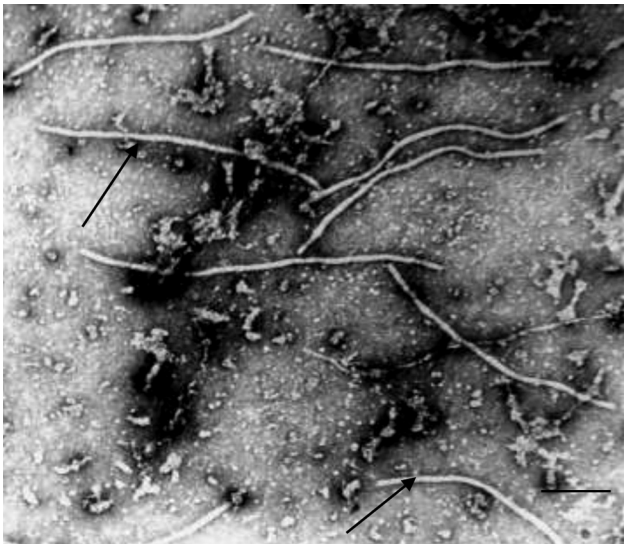

Fig. 2. Particles of ryegrass mosaic rymovirus. Bar represents 100 nm

RT–PCR (with primers selected for Bulgarian and Czech strain) was used for detection of RGMV isolates in ryegrass and festulolium infected leaf materials. A major PCR product of the expected size (1126 nucleotides) was obtained from five ryegrass and two festulolium samples, but it was not observed in the negative controls with healthy tissue or water (Fig. 3). Results of the RT-PCR test show that the ryegrass and festulolium plants were infected with a rymovirus – RGMV. Using the RT-PCR method with Australian strain primers as desribed in the literature [5], no RGMV was identified in our experiment. The specific PCR product was not obtained in either of the isolates with Australian strain primers. It is possible to suppose that the RGMV strain detected in Lithuania is similar to the strain described in the literature [13].

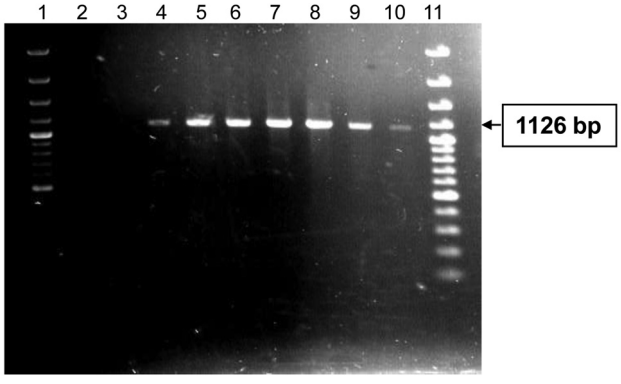

Fig. 3. 1% agarose gel electrophoresis analysis of RT–PCR products from RGMV-infected *Lolium perenne* (lanes 4, 5, 6, 9, 10) and *Festulolium* (lanes 7, 8) leaves using rymovirus primers. Lanes 1, 11, #SMO 311 Gene Ruler 100 bp DNA Ladder Plus (MBI "Fermentas"). Digest size markers; lane 2, water blank; lane 3, healthy *Lolium perenne*; lanes 4 – 10 RGMV. DNA fragment size (bp) standard was #SMO 311 Gene Ruler 100 bp DNA Ladder Plus (MBI "Fermentas") 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp

RGMV causing a disease in ryegrass was for the first time found in Lithuania in 2002 [14]. Symptoms of the disease caused by RGMV resemble those caused by other viruses affecting graminoid plants – light green to yellow mosaic, chlorotic and necrotic streaks. European RGMV isolates differ in virulence, whereas all isolates from the USA and Canada constitute a group of mild strains [15]. In crude preparations of the study host plants, the presence of filamentous particles about 700 nm long was revealed. The modal length of RGMV investigated in Czechoslovakia was estimated to be 665–700 nm [16], in Germany 650–750 nm [17], in Canada 705 nm [15]. In the description of the virus, a modal length of 700 nm is indicated [7].

Based on symptomology on the host plants and test plants, the length of the incubation period, the morphology of virus particles, positive reaction with RGMV-specific antiserum in the DAS-ELISA test, RT–PCR data, the viruses isolated from *L. perenne*, *L. multiflore, F. pratensis and Festulolium* plants are identical to the ryegrass mosaic virus from the genus *Rymovirus* described in the literature [4, 6, 7].

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Laima Urbanavičienė

SVIDRĖS MOZAIKOS VIRUSO (*RYEGRASS MOSAIC RYMOVIRUS*) NUSTATYMAS *POACEAE* ŠEIMOS AUGALUOSE

Santrauka

Vilniaus augalų veislių tyrimo stoties bei Lietuvos Žemdirbystės instituto Selekcijos centro įvairių veislių varpinių žolių bandyminiuose laukeliuose, taip pat atskirų pasėlių laukuose bei pakelėse aptikti vienmetės svidrės (*L. multiflorun* Lam.), daugiametės svidrės (*Lolium perenne* L.), tikrojo eraičino (*Festuca pratensis* Huds.) ir paprastosios eraičinsvidrės (*Festulolium loliaceum* (Huds.), P. Fourn. (*Festuca pratensis* L. × *Lolium perenne* L.) augalai su virusinės ligos požymiais – mozaikiniu dėmėtumu, chlorotiniais ir nekrotiniais dryželiais lapuose ir stiebuose. Išskirti viruso izoliatai, kurie buvo tiriami augalų indikatorių, elektroninės mikroskopijos, imunofermentinės analizės (DAS-ELISA) ir atvirkštinės transkriptazės polimerazės ciklinės reakcijos (AT-PCR) metodais. Remiantis elektroninės mikroskopijos, AT-PCR ir DAS-ELISA tyrimais nustatyta, kad vienmetės ir daugiametės svidrių, tikrojo eraičino ir eraičinsvidrės ligos, pasireiškiančios lapus pažeidžiančiais ryškiais chlorotiniais dryželiais, sukėlėjas yra svidrės mozaikos virusas (*Ryegrass mosaic rymovirus*).