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OCCURRENCE AND DISTRIBUTION OF VIRUSES INFECTING THE BEAN IN SERBIA

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Abstract - This work describes the incidence and distribution of the most important bean viruses in Serbia: *Bean common mosaic virus* (BCMV), *Bean common mosaic necrosis virus* (BCMNV), *Bean yellow mosaic virus* (BYMV), *Cucumber mosaic virus* (CMV) and *Alfalfa mosaic virus* (AMV). The viral isolates were characterized serologically and biologically. BCMV was found in the largest number of plants (30.53%), followed by BCMNV (2.67%), CMV (5.34%), and AMV (3.41%), since BYMV was not determined. Mixed viral infections were found in several samples. The RT-PCR method was used to prove that the tested isolates belong to the BCMV, family Potyviridae and strains *Russian* and NL-3 D. Results obtained in this work will enable further studies of the genetic variability of bean virus isolates from Serbia.

Keyword: Phaseolus vulgaris, BCMV, BCMNV, BYMV, CMV, AMV, ELISA test, RT-PCR.

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INTRODUCTION

The bean (*Phaseolus vulgaris* L.) is one of the major grain legume crops in Serbia. Beans and their grain occupy an important place in human nutrition with a high nutritive value. They are used for animal feed and the industrial production of citric acid (Todorović et al., 2008). Reduced yields of this plant species may arise as a result of errors in agrotechnique, which are even greater when weather conditions are unfavorable for its growth and development (Vasić, 2003). Possible causes of low yield are the numerous pathogenic microorganisms. Like various kinds of fungi and bacteria, a virus is very widespread and can be regularly found in all grown plant species, as well as those from the spontaneous flora. Damaging consequences, caused by phytopathogenic virus attack, are manifested primarily in a reduced yield of infected plants, premature decline and extinction of infected plants, and poor quality products from those plants (Babovic, 2003). In Serbia, the most important causal agents of viral diseases in the bean are: *Bean common mosaic virus* (BCMV), *Bean common mosaic necrosis virus* (BCMNV), *Cucumber mosaic virus* (CMV) and *Alfalfa mosaic virus* (AMV) (Petrović, 2008). All these viruses can cause great damage to plant production, especially due to the presence of family *Aphididae* vectors on bean the plants, which represent one of the important factors for the spread of the virus in the field (Spence and Walkey, 1995). The harmfulness of a given viral disease will depend on bean cultivar susceptibility, the stage of plant development at which the infection occurs, and environmental factors and their effect on vector activity (Šutić, 1995).

There is a need to develop resistant bean varieties to overcome this disease threat. A sound knowledge of the pathogen and its variants or strains is a vital prerequisite for reliable breeding for resistance. In order to examine the incidence and distribution of the most important bean viruses in Serbia, during 2006 we collected and biologically characterized isolates of BCMV, BCMNV, BYMV, CMV and AMV. The RT-PCR method was used to confirm that the tested isolates belong to the BCMV, family Potyviridae and strains *Russian* and NL-3D.

MATERIALS AND METHODS

Areas surveyed and sample collection

To determine the presence, distribution and frequency of BCMV, BCMNV, BYMV, CMV and AMV, we collected a total of 262 bean leaves in 22 bean-growing areas of Serbia from June to July 2006. The collected leaves showed symptoms of leaf deformation, mosaic, wilting, leaf curling, vein banding, dwarfing and local necrotic lesions. The samples were labeled and brought to the laboratory by placing in an ice bucket kept at -20°C.

ELISA analysis (Enzyme Linked Immunosorbent Assay) of the collected samples

Clear polystyrene 96-well plates (Nunc-96) and a polyclonal antiserum kits for BCMV, BCMNV, BYMV, CMV and AMV (Loewe Biochemica GmbH, Germany) were used in the study. The antisera set included IgG antibodies, phosphatase-conjugated IgG antibodies, and positive and negative controls. The results of ELISA analysis were read with a Multiscan Ascent plate reader at 405 nm. The DAS ELISA test was conducted according to the standard protocol for this serological method (Clark and Adams, 1977), following the instructions supplied by the manufacturer of the antibodies.

Bioassays

We used the DAS-ELISA test for the biological characterization of selected samples of beans originating from different bean-growing localities in Serbia. Isolates were identified based on the reaction of test plants of the following species: *Glycine max, Lupinus albus, Datura stramonium*, *Zinnia elegans*, *Nicotiana glutinosa*, and *Nicotiana tabacum* var. *samsun.*

Inoculums from BCMV-, BCMNV-, AMV- and CMV-infected bean leaves were prepared in a phosphate buffer (0.01 M, pH 7.2, 1 ml per 1g of leaf material) and were applied to the test plants. The inoculated plants were grown in a greenhouse at 22°C-28°C. The occurrence and type of symptoms were observed on inoculated leaves and leaves formed after inoculation, three weeks after inoculation. Plants without symptoms were tested by DAS-ELISA.

BCMV detection by the RT-PCR method

Selected isolates were assayed by the Reverse Transcription and Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from symptomatic leaf tissue using an RNeasy Plant Mini Kit (Qiagen, USA). The extraction was done following the procedure described by the manufacturer. The reverse transcription and polymerase chain reaction amplification was performed using a OneStep RT-PCR Kit (Qiagen, USA). Primers pairs Dbcmv / Ubcmv specific for BCMV (Xu and Hampton, 1996), X / Y universal for the family *Potyviridae* (Chen et al. 2001), and specific primers for the strains *Russian* and NL-3 D were used (Tab.1).

Reverse transcription was performed in a 40µl reaction mixture containing 25µl H2O, a 10µl 5x QIAGEN One Step RT-PCR buffer (containing 12.5 mM $MgCl_2$), a 2µl 10mM dNTP mix (containing 10 mM of each dNTP), a 2 µl QIAGEN One Step RT-PCR enzyme mix, a 0.5µl 100mM primers and 10µl total RNK. For the specific primer Dbcmv/Ubcmv amplification was used for 35 cycles each of reverse transcription 30 min at 50°C; initial PCR activation for 15 min at 95°C, denaturation for 1 min at 94 C, annealing for 1 min at 37C and extension for 1 min at 72°C with final extension for 10 min at 72°C. For primer X/Y amplification was used for 30 cycles each of reverse transcription for 30 min at 50°C; 15 min at 95°C, 30 sec at 94°C, 1 min at 47°C and 2 min at 72°C and final extension 10 min at 72°C. For specific primer *Russian* and NL-3D amplification was used 30 cycles each of reverse transcription 30 min at 50°C; 15 min at 95°C, 30 sec at 94°C, 30 sec at 58°C and 30 sec at 72°C and final extension 7 min at 72°C.

PCR product analysis

The PCR products were analyzed by electrophoresis in a 5% polyacrylamide gel at 150 V for two and a half hours followed by silver nitrate staining

Table 1. Primer pairs used in RT-PCR identification of BCMV.

Target sequences	Primer name	Sequence 5′-3′	Fragment size	Source
Bean common mosaic virus	Dbcmv	ACCACGCTGCAGCTAAAGAGAACA	1456 bp	Xu and Hampton (1996)
	Ubcmv	AATCTAGATGATATCATACTCTCTA		
fam. Potyviridae	X	GTTTTCCCAGTCACGAC(T)15	1700 bp	Chen et al. (2001)
	Y	GGNAAYAAYAGYGGNCARCC		
Russiani	RU1f	CACCGTGCCACTTGTATG	710 bp	Larsen et al. (2005)
	RU1r	GCCGATGTATTCCTTCTG		
NL-3D	NL3Df	CCATTGCTGCTGAGATTC	714 bp	Larsen et al. (2005)
	NL3Dr	AGTTCACCGTGAGATGTC		

according to Schumacher et al. (1986). The samples were prepared with 3µl of the stain (DNA Gel Loading Buffer 10x, Eppendorf, Germany) and 10 µl of the PCR product. DNA markers (100 bp DNA ladder, GE Healthcare, USA) were used in each electrophoretic run

RESULTS

Serological analysis of the virus using ELISA assay

122 samples (46.54%) out of 262 were found to be infected with at least one of the four examined viruses. Of the samples tested, the percentages of single infections were 30.53% with BCMV, 2.67% with BCMNV, 5.34% with CMV, 3.41% with AMV (Fig 1).

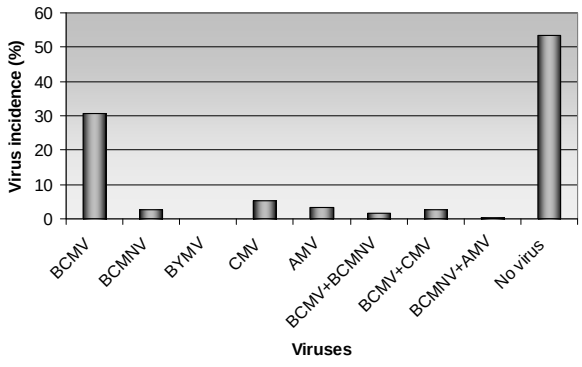

Fig.1. Frequency of the viruses isolated from bean in Serbia.

The results showed that 90.1% of the diseased plants were single-infected, whereas 9.9% were double-infected. The most common double infections were BCMV+CMV (2.67%), followed by BCMV+ BCMNV (1.53%) and BCMNV+AMV (0.39%) (Fig 1).

53.46% (140) samples showed viral disease-like symptoms although none of the above-mentioned viruses were detected. These symptoms were probably caused by pesticide damage, physiological disorders, or unidentified virus (Choi et al., 2005).

Results of testing bean plants from different localities in Serbia indicate that BCMV was the most common virus. It was represented in 16 locations, with the largest percentage of plants infected with these virus were found in Titel (75%). As for the other localities, the number of samples infected with this virus was somewhat lower. CMV was recorded in seven localities in a small percentage, while BCMNV and AMV were recorded in 3 locations (Fig.2).

Fig. 2. presents an overview of the representation of the viruses BCMV, BCMNV, BYMV, CMV and AMV in the investigated localities in Serbia during 2006.

Table 2. Symptoms produced on indicator plants of BCMV, BCMNV, CMV and AMV

Test plant	BCMV	BCMNV	CMV	AMV
Glycine max	MM	SM	MC	DM
Lupinus albus	SMV	MMN	-	-
Zinnia elegans	-	-	-	CM
Datura stramonium	-	-	CM	-
N. tabacum var. samsun	-	-	MM	SM
N. glutinosa	-	-	DLM	PL

- resistant plant, no symptoms; MM= mild mosaic in the form of scattered yellow-green areas; SM= stronger mosaic in the form of scattered yellow-green areas, MC = mosaic and light curling of leaves; DM = distortion of leaves and mosaic, SMV = strong mosaic in the form of dark green areas around the veins, MMN = mild mosaic in the form of less discernible areas along the veins; CM = mosaic in the form of chlorotic areas; DLM = dashed line mosaic, PL = puckering of the leaf blade.

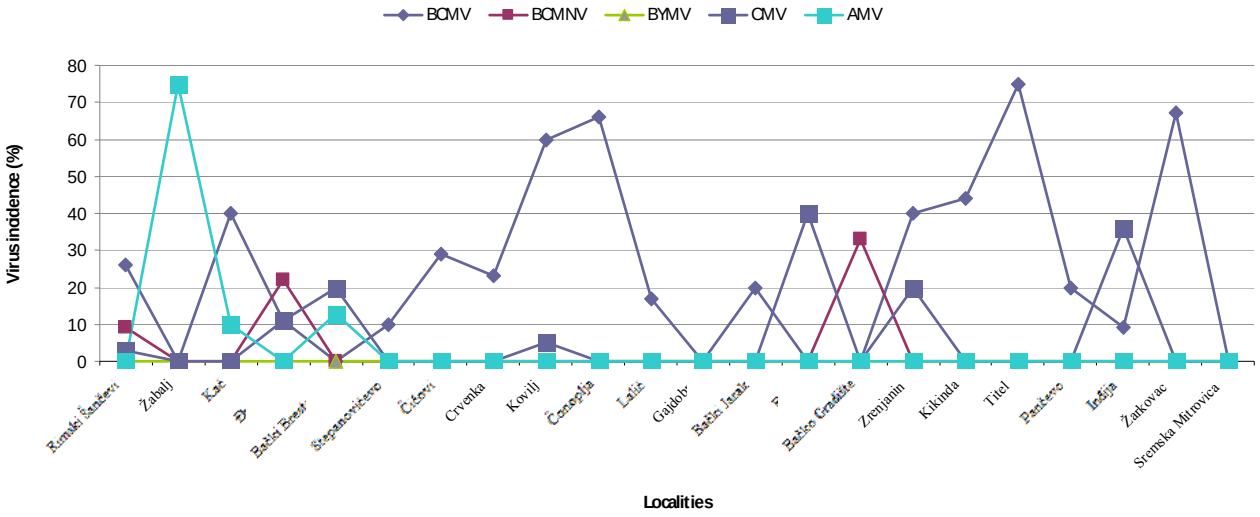

Fig. 2. Representation of BCMV, BCMNV, CMV and AMV by locality in Serbia during 2006.

Reactions of indicator plants

The biological characterization of the five selected isolates of each virus (BCMV BCMNV, AMV and CMV) was performed on the basis of expressions and types of symptoms on a number of species of plants belonging to the host range for the studied viruses and plant species that are not described in the literature as hosts of this virus. The reactions of the test plants (*Glycine max, Lupinus albus, Datura stramonium, Zinnia elegans, Nicotiana glutinosa, Nicotiana tabacum* var. *samsun*) are shown in Table 2.

Results of PCR reaction

The presence of BCMV in 3 of the tested samples was confirmed using specific RT-PCR. Comparison of the amplifiable fragments of the tested samples and used marker (M) determined the presence of the expected size of fragment of about 1456 bp (Fig. 3), which allowed for the amplification of primers Dbcmv/Ubcmv. Amplification did not occur in the negative control (PCR mixture with molecular water). The presence of a 1700 bp fragment (Fig. 4) was determined using primer X/Y. Amplification

fragments were not obtained for the tested samples when primers *Russian* and NL-3D were used.

DISCUSSION

DM = distortion of leaves and mosaic, SMV = strong mosaic in the form of dark green areas around the veins, MMN = mild mosaic in the form of less discernible areas along the veins; CM = mosaic in the form of chlorotic areas; DLM = dashed line mosaic, PL = puckering of the leaf blade.

The *Bean common mosaic virus* and the closely related *Bean common mosaic necrosis virus* are two bean-infecting potyviruses that are seed-transmitted and widely distributed in bean crops throughout the world (Galvez and Morales, 1989). There are also a number of other viruses that infect beans in California, including the *Bean yellow mosaic virus, Cucumber mosaic virus,* and *Alfalfa mosaic virus.* All these viruses pose significant potential problems for the California bean seed industry and they can be difficult, if not impossible, to identify based upon symptoms alone (Gilbertson et al. 2002).

Analysis of our DAS ELISA results showed that BCMV was the main viral disease of beans in Serbia in 2006. BCMV was found in 30.53% of the 122 leaf samples that tested positive for the presence of a virus. Our findings are in agreement with those of some other authors. Sáiz et al. (1995) studied the incidence of viruses in bean plants originating from 11 different regions of Spain during the period 1989-1993 and determined that BCMV predominated over BCMNV, as the former virus was found in 56% of the samples tested, while the latter was observed in only 5% of the samples. In 1998, Ruiz et al. investigated the presence of BCMV

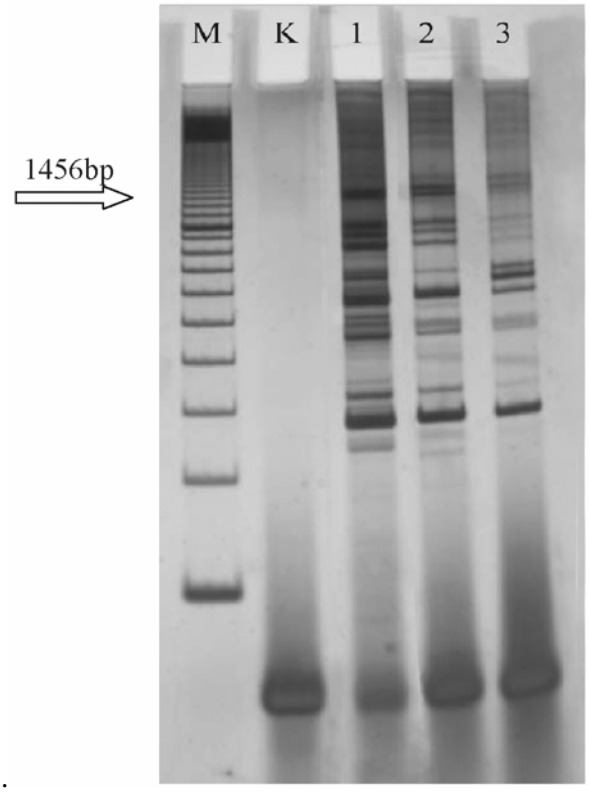

Fig. 3. Tests of RT-PCR amplification of BCMV isolates with primer pairs Dbcmv/Ubcmv M = marker, K = negative control, 1-3 tested isolates

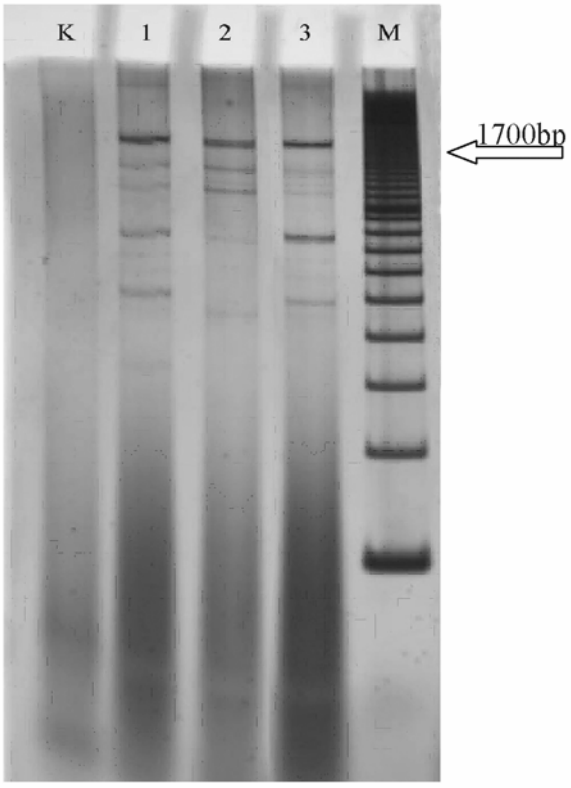

Fig. 4. Tests of RT-PCR amplification of BCMV isolates with primer pairs X/Y , M = marker, K = negative control, 1-3 tested isolates

and BCMNV in three areas of the Basque region in Spain using the ELISA test. Only one of the locations studied was found to be virus-free, while all the others had a high incidence of viral diseases, which is illustrative of the high prevalence of the viruses studied in the present paper. Chiumia and Msuku (2001) also used the ELISA test to study BCMV and BCMNV.

The second most common virus in the present study was CMV (5.34%). Fletcher (2001) studied the symptoms of bean viruses in New Zealand using ELISA and, along with many other viruses, found CMV in a fairly large number of the samples. During the period 1991-2000, Gilbertson et al. (2002) tested bean plants in California for the presence of a number of viruses, including CMV. The virus was detected in only some of the years and its incidence was low, much lower than that of BCMV, which agrees with our present findings.

The third most common virus in our experiment was AMV (3.41%). This low incidence of AMV agrees with the results of Fletcher (2001) in New Zealand. The aforementioned study by Gilbertson et al. (2002) included testing for the presence of AMV, which was observed in some years but not others and at a low percentage at that. The incidence of other viruses (BCMV, CMV, PVY, SDV and TSWV) was higher.

In the present study, the least common virus was BCMNV (2.67%). This is the first report of BCMNV in Serbia. These results support those of Sáiz et al. (1995), Ruiz et al. (1998), and Chiumia and Msuku (2001), all of whom studied infection of bean plants by BCMV and BCMNV. Their findings and ours are quite similar. This is especially true in the case of Sáiz et al. (1995), who found BCMNV in 5% of the plants studied in their paper, which is almost the exact same percentage we observed in our study. The other two studies reported that BCMNV was much less common in the bean plants than BCMV. Gilbertson et al. (2002) did record BCMNV in their study in California, but only in some years and at a very low percentageUsing the RT-PCR method, in 1996 Xu and Hampton investigated BCMV and BCMNV. Five of the isolates were identified as BCMNV and 17 as BCMV. In the present study, we used the Dbcmv and Ubcmv primers and also detected BCMV in all three samples. Larsen et al. (2005) identified BCMV and BCMNV using the RT-PCR method as well as the strains *Russsian* (RU 1) and NL-3 D. In our study, we used the same two sets of primers and found that BCMV from our isolates did not belong to those strains. Chen et al. (2001) studied whether particular viruses were part of the *Potyviridae* group using a primer universal to that family. The results showed that many species of the genera *Potyvirus*, *Bymovirus*, *Rymovirus* and *Tritimovirus* belonged to the family *Potyviridae*. In the present study, the same conclusion was reached, the use of the same primers showed that BCMV belonged to the family *Potyviridae.*

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ПРИСУСТВО И РАСПРОСТРАЊЕНОСТ ВИРУСА ПАСУЉА У СРБИЈИ

ДРАГАНА ПЕТРОВИЋ[1], МАЈА ИГЊАТОВ [1], ЗОРИЦА НИКОЛИЋ [1], МИЛКА ВУЈАКОВИЋ [1], МИРЈАНА ВАСИЋ[1], МИРЈАНА МИЛОШЕВИЋ[2], и КСЕНИЈА ТАШКИ-АЈДУКОВИЋ[1]

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Циљ истраживања је био утврђивање присуства и распрострањености економски најштетнијих вируса пасуља на подручју Војводине: *Bean common mosaic virus* (BCMV), *Bean common mosaic necrosis virus* (BCMNV), *Bean yellow mosaic virus* (BYMV), *Alfalfa mosaic virus*-(AMV) и *Cucumber mosaic virus* (CMV). Изолати вируса окарактерисани су серолошки и биолошки. У највећем броју узорака констатован је BCMV (30,53%), у знатно мањем броју констатовани су BCMNV (2,67%), CMV (5,35%), и AMV (3,41%), док ниједан узорак није показао позитивну реакцију на BYMV. У неколико узорака је утврђена мешана инфекције вируса. RT-PCR метода је коришћена да би се утврдило да ли испитивани изолати припадају BCMV, фам. Potyviridae и сојевима *Russian* и NL-3D. Резултати добијени у овом раду ће омогућити даља истраживања генетичке варијабилности изолата вируса пасуља из Србија.