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NEW CUCUMOVIRUS ON BEANS IN BULGARIA – AN ATTEMPT FOR CHARACTERIZATION

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Summary

A virus was isolated from naturally infected bean plants, grown in different areas in Bulgaria. The investigated isolates were characterized using electron microscopy, serology, biological tests, electrophoretic analysis and reverse transcription-polymerase chain reaction (RT-PCR). Isometric particles about 30 nm in diameter were observed in crude sap preparations. The tested isolates were serologically distantly related to cucumber mosaic virus (CMV) but in ELISA experiments and immunoelectrophoresis they demonstrated a close serological relationship with a cucumovirus from China named Ch 39. The Bulgarian isolates were identified as a cucumovirus Ch 39-BG. The electrophoretic analysis of this virus showed the presence of two protein bands. CMV primers amplified an unspecific DNA fragment of 500 bp. All the performed investigations suggested the presence of a cucumovirus closer to CMV though different from the known CMV strains.

Key words: *cucumovirus, bean, electron microscopy, ELISA, immunoelectrophoresis, SDS-PAGE, RT-PCR.*

Introduction

The genus *Cucumovirus*, belonging to the *Bromoviridae* family, comprises three viruses: cucumber mosaic virus (CMV) - the type species, tomato aspermy virus (TAV) and peanut stunt virus (PSV) [13, 24]. Cucumoviruses are distributed worldwide and include numerous strains. Virions are isometric (29-30 nm in diameter) and contain one structural protein.

A great similarity in characteristics between CMV, PSV from one side [5, 21] and TAV from the other [11, 27] supports the union of these three viruses in one group. A comparative serological study of a large number of isolates of CMV, PSV and TAV has also led to the establishment of relationships among these viruses and a possibility of breeding "new" strains [6, 7]. Some researchers succeeded to construct infectious viruses through recombination of genomic segments of CMV with complementary parts of either the PSV [18] or TAV genome [11], which confirmed the close relationships between these viruses.

As a result of the investigation on bean viruses in Bulgaria, a new virus was isolated from bean plants with severe mosaic symptoms. The aim of the present study is to characterize this virus using different methods: electron microscopy, serology, biological tests, electrophoretic analysis and reverse transcription-polymerase chain reaction (RT-PCR).

Materials and Methods

Investigated virus isolates. Three virus isolates from naturally infected bean plants (No 75, 77 and 113), collected from 3 production regions (Markovo, Hrabrino and Plovdiv), were investigated. They were propagated and maintained in *Phaseolus vulgaris* in a growing chamber at temperatures 24 °C/16 °C (day/night). The isolates No 75, 77 and 113 were deposited in National Bank for Industrial Microorganisms and Cell Cultures, Sofia (NBIMCC 3690, 3691, 3692). Strain B1, isolated by Dr. V. Lisa from bean in Bulgaria and related to CMV [15, 28] (NBIMCC 3330), was also tested in some comparative investigations. The virus isolates were preserved by freeze-drying according to Donev et al. [9].

Virus purification. The tested isolates were purified by the method of Lot et al. [17], with some modifications. Virus preparations, obtained after one cycle of differential centrifugation, were used in electrophoretic and immunoelectrophoretic analyses.

Host plants. Biological characterization of the studied isolates was carried out on 16 plant species: *Chenopodium quinoa, C. amaranticolor, P. vulgaris* cv. Dubbele Witte, *Nicotiana bentamiana, N. clevelandii, N. glutinosa, N. tabacum* cv. White Burley, *Gomphrena globosa, Datura stramonium, Petunia hybrida, Lycopersicon esculentum, Cucumis sativus, Pisum sativum,* and *Vicia faba*. Inoculation was done using systemically infected bean leaves ground in ice-cold 1 % K_2HPO_4 solution, containing 0.1 % Na_2SO_3 (ratio 1:1 w/v). Carborundum 600 mesh was used as an abrasive. Plants were scored at weekly intervals.

Electron microscopy. Immunosorbent electron microscopy (ISEM) methods of attraction and decoration were used [20]. The following polyclonal antibodies (PAbs) from Istituto di Virologia Vegetale del CNR (IVV), Torino, were used: Antiserum (AS) 326 and AS 343 against CMV; AS 52 against broad bean mottle bromovirus (BBMV); AS 263 - brome mosaic bromovirus (BMV); AS 287 - melandrium yellow fleck bromovirus (MYFV); AS 229 - broad bean stain comovirus (BBSV); AS 157 - southern bean mosaic sobemovirus (SBMV).

Enzyme-linked immunosorbent assay (ELISA). Different ELISA methods were used: plate-trapped antigen (PTA)-ELISA, double antibody sandwich (DAS)-ELISA and triple antibody sandwich (TAS)-ELISA [4, 10].

PTA-ELISA. Samples were ground in carbonate coating buffer at dilution 1:100. An universal PAb against CMV (AS 326) diluted 1:1000 was used in the assay. Antispecies antibody AP-conjugate was used as specified by manufacturer (Sigma).

DAS-ELISA. Plates were coated with CMV PAbs - ALS and CMV S4 from DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands. An antiserum, provisionally named Ch 39, was also used. It was raised against a cucumovirus, isolated from *P. vulgaris* in northern China by Dr. V. Lisa in 1990 (P. Roggero, V. Lisa and G. Dellavalle, personal communication). The PAbs were used at dilution 1:1000. The virus samples were applied at dilution 1:100 and the specific AP-conjugate - 1:400.

TAS-ELISA. The CMV PAbs were used at a concentration of 1 mg/ml in coating buffer. At the next step plates were loaded with samples diluted 1:100. CMV monoclonal antibodies (MAbs) were used at dilution 1:1000. Rabbit anti-mouse IgG-AP-conjugate was used at a dilution 1:10000. The CMV MAbs 2+185, M85 and M172 were from IVV, Torino, Italy and CMV MAb Agdia from Horticulture Research International (HRI), Wellesbourne, U.K.

In ELISA the colour reaction was developed by adding 0.4 mg/ml p-nitrophenylphosphate in diethanolamine buffer, pH 9.8. Absorbance values were read at 405 nm and considered positive when exceeded three times those of the mean healthy controls.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of the capsid protein (CP) was estimated by SDS-PAGE according to Laemmli [16]. The electrophoresis was done in 15 % resolving gel at 22 mA for 1.5 h. The gels were stained with Coomassie Brilliant Blue R-250. LMW electrophoresis calibration kit (Pharmacia) was used as a standard. Purified preparations from the collection of the NBIMCC were used as control strains: CMV Fny - I subgroup [26], CMV GR - II subgroup; tobacco mosaic virus (TMV) type strain U1.

Counter immunoelectrophoresis. Counter immunoelectrophoresis was carried out in 0.9 % agarose gel in 0.075 M veronal buffer, pH 8.6, containing 5 mM EDTA, at 150 V, 10 °C for 2.5 h. AS Uo, raised against CMV [29] was applied in this assay as well as AS Ch39. CMV Fny was used as a control strain. Plates were stained with Coomassie Bril-liant Blue R-250 [29].

RT-PCR and RFLP (restriction fragment length polymorphism) analysis. The amplifi-

cation of the CP gene cDNA was done according to the protocols described by Rizos et al. [25]. The primers used were: 5'CP (5' CTCGAA TTCGGATCCGCTTCTCCGCGAG 3'), corresponding to the nucleotide region from 1149 to 1161 and 3'CP (5' GGCGAATTCGAGCTCGCC GTAAGCTGGATGGAC 3') - to the nucleotide region from 1998 to 2015 of CMV-Q RNA-3. These primers amplify a product of about 870 bp with CMV isolates [25]. The amplified PCR products were separated in a 1.5 % agarose gel in TBE buffer and stained with ethidium bromide. As a size marker ϕX174 DNA/Hae III was used (Boehringer). Restriction digest of the PCR products was performed using the restriction endonuclease MspI (Gibco, BRL) [25].

Results

Electron Microscopy

Isometric particles about 30 nm in diameter were observed in crude sap preparations from naturally infected plants (isolates No 75 and 77). A small number of similar particles was found in the same preparations trapped on grids coated with 4 antisera raised against several isometric viruses (Table 1). The largest number of trapped virions was established with AS against BMV (about 100), whereas with CMV AS it was 15-20. Grids, treated with AS against BBSV and SBMV did not attract particles. No decoration was observed after incubation on grids coated with all the 6 antisera (Table 1).

Table 1. ISEM of virus isolates No 75 and 77 with different antisera.

AS	Isolate No 75		Isolate No 77	
	Attraction	Decoration	Attraction	Decoration
AS 52 - BBMV	30 particles per field	few particles, no decoration	25 particles per field	few particles, no decoration
AS 263 - BMV	100 particles per field	20 particles per field, no decoration	92 particles per field	20 particles per field, no decoration
AS 287 - MYFV	10 particles per field	virus is seen, no decoration	10 particles per field	virus is seen, no decoration
AS 326 - CMV	20 particles per field	virus is seen, no decoration	15 particles per field	virus is seen, no decoration
AS 229 - BBSV	-	-	-	-
AS 157 - SBMV	-	-	-	-
Control with buffer	400 particles per field	-	320 particles per field	-

Note: AS dilution 1:1000; colour reaction - uranil acetate; (-), no virus particles.

Host range

The virus isolates were easily transmitted to the inoculated plant species (Table 2). Usual systemic symptoms were mosaic, yellow or necrotic spots, followed in some cases by systemic necrosis or stunting in the growth. It is worth to emphasize the systemic spread of the three isolates in *C. quinoa* and *C. amaranticolor* (Table 2).

Serology

In PTA-ELISA virus isolates were positive for CMV, whereas in DAS- and TAS-ELISA the samples were negative for the same virus (Table 3).

Surprisingly, all virus isolates reacted positively in PTA- and DAS-ELISA with AS Ch 39. The cucumovirus Ch 39 was distantly related to subgroups I and II of CMV and more closely related to TAV and PSV (P. Roggero, V. Lisa and G. Dellavalle, personal communication). The presented results suggested a cucumovirus, serologically related to that isolated in China (Table 4). It was designated as cucumovirus Ch 39-BG.

Partially purified preparations of cucumovirus Ch 39-BG (No 77, 113), isolate B1 and the

Table 2. Biological test with virus isolates No 75, 77 and 113.

Isolates	*C. quinoa*	*C. amaranticolor*	*P .vulgaris* cv .D. Witte	*N. benthamiana*	*N. clevelandii*	*N. glutinosa*	*N. tabacum* cv. W. Burley	*G. globosa*	*D. stramonium*	*P. hybrida*	*L. esculentum*	*C. annuum*	*C. sativus*	*P. sativum*	*V. faba*
75	*/YS	*/YS	DCS E/ St M	DCS/ M	DCS/ M	SNS/ M St	SNS/ M St	SNS/ M St	n.t.	NS/ M St Y	ns/YS	DCS LA/ Mo	ns/ns	ns/ns	SBN
77	*/YS	ns/YS	DCS E/ St M	ns/M LD E	n.t.	ns/M LD	CRS/ M Bls	RNS/ RNS LD	n.t.	n.t.	ns/ns	ns/ns	ns/ns	Chl/LC LD St W	SBN
113	*/YSp	*/YSp	DCS E/ St M	ns/M St	ns/M	SCRS/ LD FF	CRS/ns	n.t.	n.t.	n.t.	ns/ns	ns/ns	ns/ns	Chl/LC LD W	SBN
CMV control	CLL/ns	CLL/ns	DCS E/ M	ns/M	ns/M	ns/M	n.t.	ns/ns	DCS/ SCS	n.t.	ns/ns	ns/ns	ns/M	ns/ns	n.t.

Legend: primary/systemic symptoms (/), no symptoms (ns), chlorotic local lesions (CLL), single chlorotic spots (SCS), diffuse chlorotic spots (DCS), chlorotic spots (CS), single chlorotic spots (SCS), chlorosis (Ch), epynasty (E), necrotic spots (NS), small necrotic spots (SNS), redish necrotic spots (RNS), leaf abscission (LA), mottling (Mo), clear mottling (CMo), mosaic (M), severe mosaic (SM), yellow spots (YS), yellow local lesions (YLL), yellowing (Y), leaf curling (LC), blisters (Bls), leaf deformation (LD), filiform leaves (FF), stunting (St), wilting (W), systemic black necrosis (SBN), symptom variation between CLL, CS, DCS and YLL (*),not tested (n.t.).

Table 3. PTA-, DAS- and TAS-ELISA of three bean isolates (No 75, 77 and 113), using PAbs and MAbs against CMV.

Isolates	PTA-ELISA (PAb)	DAS-ELISA (PAbs)		TAS-ELISA* (MAbs)		
	AS 326	AS ALS	AS S4	M85	M172	Agdia
75	+	-	-	-	-	-
77	+	-	-	-	-	-
113	+	-	-	-	-	-
Positive CMV control	+++	++	+++	++	++	+++

Legend: <0.25 (+), 0.25–0.6 (++), 0.65 – 1.0 (+++), >1.0 OD (++++).
* In TAS-ELISA plates were coated with AS 326.

Table 4. ELISA of isolates No 75 and 77, using AS Ch 39.

Isolates	PTA-ELISA		DAS-ELISA	TAS-ELISA*
	AS Ch 39	CMV AS 326	AS Ch 39	CMV MAb 2+185
75	+++	++	++	-
77	+++	+	++	-
Positive CMV control	+++	+++	-	++++

Legend: <0.25 (+), 0.25–0.6 (++), 0.65 – 1.0 (+++), >1.0 OD (++++).
* In TAS-ELISA plates were coated with AS Ch39.

control CMV strain Fny were analyzed by immunoelectrophoresis. When Ch 39-BG isolates diffused against CMV AS (Fig. 1a), no precipitin lines were observed. In contrast, Ch 39-BG isolates and B1 as well as CMV Fny gave precipitin bands formation against AS Ch 39 (Fig. 1b).

The results regarding the serological relationships between isolates 77, 113, B1 and CMV Fny are presented in Fig. 1c. Isolate B1 produced an apparent spur over the precipitin line of CMV Fny when diffused against AS Ch 39 (Fig. 1c). No spurs were observed between bands from adjacent antigens of B1 and 77 as well as between 77 and 113.

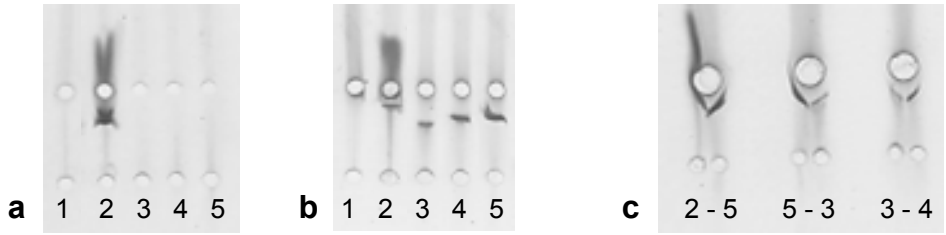

Fig. 1. Immunoelectrophoretic analysis of cucumovirus isolates.
Antisera in upper holes: CMV (**a**), Ch39 (**b** and **c**).
Antigens: healthy plant sap (1), CMV Fny (2), isolate 77 (3), isolate 113 (4) and isolate B1 (5).

SDS-PAGE

The CP of the isolates No 77, 113 and B1 were subjected to SDS-PAGE (Fig. 2). The three viruses formed two bands with very similar electrophoretic mobility. The molecular weight of these peptids was estimated of about 22 and 28 kDa. Similar bands were observed by V. Mavrodieva with isolate B1 (personal communication).

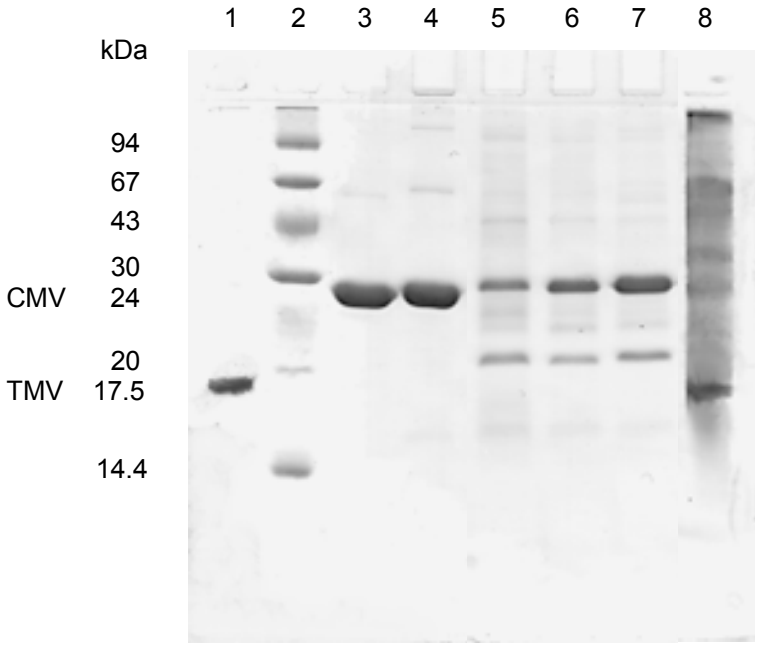

Fig. 2. SDS-PAGE of capsid proteins of cucumovirus isolates. Lines: TMV U1 (1), protein markers (2), CMV GR - II subgroup (3), CMV Fny - I subgroup (4), isolate 77 (5), isolate 113 (6), isolate B1 (7), healthy plant (8).

RT-PCR

The investigated cucumovirus isolates amplified a DNA fragment of approximately 500 bp, completely different from that of CMV - 872 bp (results are not shown in details). No restriction of the fragment was observed in RFLP analysis using the restriction enzyme MspI, whereas the control CMV strains produced a MspI restricttion pattern typical for subgroup I (fragment length 535 and 335 bp) and subgroup II (250, 200, 160 and 130 bp), respectively.

Discussion

A cucumovirus was isolated from naturally infected bean plants, grown in different areas in Bulgaria. The virus was identified as a cucumovirus Ch 39-BG based on a close serological relationship with a cucumovirus from China named Ch 39. The tested Bulgarian isolates demonstrated a strong positive reaction with AS Ch 39 in DAS-ELISA and a negative reaction for CMV in DAS and TAS-ELISA. The positive reaction of the same isolates for CMV in PTA-ELISA could be explained by the ability of the indirect ELISA to detect cucumoviruses that differ by a SDI up to 7 [8]. DAS ELISA is regarded as possessing a narrow specificity allowing discrimination between closely related CMV strains in both subgroups I and II, which generally differ by a SDI of only 1-2 [24]. Immunoelectrophoresis data confirmed the serological identity of the investigated isolates. They seemed closely related to the Chinese isolate Ch 39 and did not react with CMV AS.

The biological assay of the cucumovirus isolates did not help much for virus identification but gave some characteristics of the new virus. Local and systemic symptoms observed in *C. quinoa* and *C. amaranticolor* were of some similarities with PSV [1, 3]. It is noteworthy that there are CMV isolates causing systemic reaction in *C. quinoa* [1] where some PSV isolates are localized [30].

ISEM results proved the presence of isometric but different from CMV particles. The lack of decoration with antisera to BMV, BBMV, and MYFV led to the conclusion that the investigated virus was also different from these viruses and needed further investigation.

SDS-PAGE data were disputable. It is well known that CP of all cucumoviruses has a size of about 24,500 kDa [11]. It is unlikely to consider the existence of a virus from the cucumovirus group having two protein subunits. Probably, the larger polypeptide is the CP, which differs considerably in amino acid composition from the cucumovirus CP and therefore manifests lower electrophoretic mobility. The smaller polypeptide could represent a degradation pro-

duct of CP due to proteolytic activity in the plant sap during virus extraction [12, 14, 22, 23]. Okuda et al. found two protein bands of a clover yellow vein virus isolate from Japan despite the fact that potyviruses possess only one CP [23]. These are questions to be answered.

RT-PCR results indicated the presence of a virus different from CMV. CMV specific primers amplified a DNA fragment of 500 bp instead of 872 bp. In similar experiments with PSV and TAV isolates, amplification of any DNA fragment did not find [2]. The fact that some product was observed with the CMV primers and our cucumovirus isolates indicates that this virus is closer to CMV rather than to PSV and TAV. A similar fragment of 500 bp was observed also by V. Mavrodieva in RT-PCR of isolate B1 [20]. The same author revealed no homology between the nucleotide sequences of the CP gene of isolate B1 and the CP gene of CMV (personal communication). In SDS-PAGE, isolate B1 formed two bands of 22 and 28 kDa, typical for isolates 77 and 113. In counter immunoelectrophoresis B1 reacted as serologically identical to the tested isolates and expressed stronger relationship to Ch 39 than to CMV. These results are the reason to join B1 isolate to the investigated isolates No 75, 77 and 113.

In conclusion, all the performed investigations suggested the existence of a cucumovirus different from the known CMV strains. The fact that the three isolates studied were identical to the isolate B1, found on beans more than 15 years ago, indicate that this virus was spread on beans in Bulgaria long time ago.

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НОВ КУКУМОВИРУС ПО ФАСУЛА В БЪЛГАРИЯ – ОПИТ ЗА ХАРАКТЕРИЗИРАНЕ

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Резюме

Получени са вирусни изолати от естествено заразени фасулеви растения, отглеждани в различни райони на България. Те са характеризирани с електронна микроскопия, серологични и биологични тестове, електрофоретичен анализ и полимеразна верижна реакция с обратна транскрипция (RT-PCR). В растителен сок се наблюдават изометрични частици с диаметър около 30 нм. Проучваните изолати са серологично далечно родствени на краставично-мозаичния вирус (CMV), но при ELISA и имуноелектрофореза показват тясно родство с кукумовирус от Китай, означен като Ch 39. Българските изолати са определени като кукумовирус Ch 39-BG. Електрофоретичният анализ на този вирус показва присъствие на две белтъчни линии. При използване на праймери за CMV се амплифицира неспецифичен ДНК фрагмент с дължина 500 bp. Всички проведени изследвания предполагат наличие на кукумовирус, по-близък до CMV, макар и различен от известните щамове на вируса.