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Two Hungarian isolates of cucumber mosaic virus from sweet pepper (Capsicum annuum) and melon (Cucumis melo): identification and antiserum preparation

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Abstract

Two Hungarian virus isolates from sweet pepper (K8) and melon (S4) were identified as cucumber mosaic virus (CMV) on the basis of host plant reactions and serology. The isolates were purified and antisera prepared. Homologous antiserum titers in double-diffusion tests were 256 (K8) and 512 (S4). They were serologically closely related to each other and to other CMV isolates. On the basis of symptoms they belong to different symptomatological groups of CMV; this was supported by serological properties. Sedimentation coefficients were c. 93 S, at 2 mg ml^{-1}. Purified preparations, stained with 2% uranyl acetate, showed spherical particles. In ELISA purified preparations reacted with each other's antisera.

Additional keywords: serology, ELISA, sedimentation coefficient.

Introduction

Cucumber mosaic virus (CMV) has a very wide host range and a world-wide distribution. It is known to infect 775 species, representing 85 families (Douine et al., 1979) and is transmissible in the non-persistent manner by more than 60 species of aphids (Francki et al., 1979).

In Hungary, CMV is one of the most common viruses occurring in vegetable crops outdoors. Infection in pepper *(Capsicum annuum)* and melon *(Cucumis melo)* may reach 80-100%, depending on environmental conditions. The losses caused by CMV in pepper may be 40-70% (Tóbiás et al., 1978) and in melon 80-100% (Tóbiás, unpublished).

One isolate from sweet pepper (K8) and one from melon (S4) were chosen for further identification studies and for preparation of antisera to enable quick diagnosis of CMV infections in these crops.

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Materials and methods

Virus origin. K8 originates from a commercial pepper field, located near Kondoros, Hungary, and S4 from an experimental melon field at Budatétény, Hungary. Prior to use in the experiments described below, single-lesion isolation was applied using *Chenopodium quinoa* as a local lesion host.

Test plants, symptomatology, and virus propagation. Plants were grown in an insect-proof, temperature-controlled greenhouse at 20-24 °C. During the experiments (from October till January) additional light (20 000 lx at plant level for 8 h/day) was supplied by Philips SON/T lamps.

Mechanical inoculations were made with freshly prepared sap (pestle and mortar) diluted approximately 1 : 1 with 50 mM potassium phosphate buffer (pH 7.2), using carborundum 500 mesh as an abrasive. Immediately after the inoculations were completed, plants were rinsed with tap water.

The test plants used are listed in Table 1. *Nicotiana clevelandii* and *N. rustica* were used for virus propagation.

Virus purification. Infected leaves of *N. clevelandii* and *N. rustica* were harvested 12-15 days after inoculation and chilled. Initial purification was largely according to the modified procedure of Lot et al. (1972) as described by Francki et al. (1979). However, only one cycle of differential centrifugation was applied (1 h at 250 700 *g;* 10 min at 8000 *g).* Further purification was by two successive sucrose-gradient centrifugations. Gradients (10-40%) were spun for 2 h at 103 800 *g* in a Beckman rotor SW27. Virus fractions collected after each sucrose-gradient centrifugation were diluted 1 : 1 with buffer before concentrating by high-speed centrifugation (1 h at 250 700 *g).* EDTA-containing borate buffer (pH 9) (Francki et al., 1979) was used to dissolve sucrose and to resuspend virus sediments or to dilute virus fractions. To the final suspensions 0.05% sodium azide was added. They were stored at 4 °C. All *g* values given are at R_{max}.

Virus concentrations in purified preparations were determined in a Beckman model 25 spectrophotometer, using an extinction coefficient of $E_{260}^{1\text{ cm},\ 0.1\%} = 5$ (Francki et al., 1979).

Antiserum preparation. Stored purified sodium azide-containing virus preparations were subjected to a third sucrose-gradient centrifugation and concentration procedure just before injection into rabbits, using one rabbit per virus isolate. Intravenous injections were with c. 7.5 mg of virus. For intramuscular or subcutaneous injections the same amount was emulsified with Freund's incomplete adjuvant. Several sets consisting of one intravenous and one intramuscular or subcutaneous injection were administered at varying intervals. Intervals for the S4 rabbit were 2, 2, and 7 weeks and for the K8 rabbit 2, 3, and 5 weeks. Blood samples were taken as listed in Table 2.

Immuno-diffusion tests. Double-diffusion tests were performed in 0.8% agarose in buffer (40 mM sodium barbital and 0.5 mM EDTA, adjusted to pH 8.6 with hydrochloric acid). Wells had a diameter of 4 mm and were 6 mm apart. Results were read after 24 and 48 h.

Enzyme-linked immunosorbent assay (ELISA). To isolate γ-globulins from antisera, a modification of the procedure described by Steinbuch and Audran (1969) using caprylic (octanoic) acid was followed by ammonium sulfate precipitation as follows. Five ml of antiserum was diluted with 10 ml of 60 mM sodium acetate (pH 4.8.), and dialyzed against this buffer (250 ml) for approximately 24 h at 4 °C, the buffer being refreshed after about 8 h. Under vigorous stirring, 0.41 ml of octanoic acid was added dropwise to the diluted and dialyzed antiserum and stirring was continued for 30 min at room temperature. After centrifugation for 10 min at 8000 *g* the liquid fraction was dialyzed against 250 ml of phosphate-buffered saline (PBS; 50 mM phosphate buffer (pH 7.4), containing 0.8% sodium chloride and 0.05% sodium azide) for 4 h at 4 °C, the buffer being refreshed after 2 h. The material was then diluted with PBS to make 20 ml, and 20 ml of a saturated ammonium sulfate solution was added at room temperature under stirring, and stirring was continued for 30 min. The precipitate was then collected by centrifugation for 10 min at 8000 *g* and resuspended in 10 ml of distilled water. Half of this solution was dialyzed against PBS (250 ml) at 4 °C for 24 h, the PBS being refreshed twice. The concentration was adjusted to 1 mg ml^{-1} ($E^{1\,cm}_{280}$ = 1.4) to be used as a standard solution for coating of ELISA plates. The other half was dialyzed against 50 mM potassium phosphate buffer (pH 7.2) (without sodium azide!) and the concentration adjusted to 3 mg ml^{-1} to be used for conjugation with alkaline phosphatase (Boehringer, Mannheim, West Germany, Nr 567744 or 567752; 10 mg enzyme per ml of a solution (pH 7.6) containing 3 M sodium chloride, 1 mM magnesium chloride, 0.1 mM zinc chloride and 30 mM triethanolamine).

The conjugation procedure (a modification of a procedure obtained from Boehringer, Mannheim) was as follows. Alkaline phosphatase (0.35 ml) was mixed with 1.20 ml of potassium phosphate buffer (pH 7.2) and 10 μl of a 25% solution of glutardialdehyde in water. The mixture was incubated in a water bath at 25 °C for 50 min. Then 1.5 mg of γ-globulin (in potassium phosphate buffer) was added and incubation was continued for 75 min. After cooling in an ice bath, the conjugate was dialyzed against 100 ml of Tris buffer (50 mM tris(hydroxymethyl)aminomethane, supplemented with 0.1 M sodium chloride, 1 mM magnesium chloride and 0.1% sodium azide, and adjusted to pH 8 with hydrochloric acid) at 4 °C for c. 24 h, the buffer being refreshed three times. Finally the volume was brought up to 3 ml with Tris buffer, and 15 mg of bovine serum albumin was added.

Preparations containing sodium azide were stored at 4 °C. Preparations without sodium azide were divided into small portions and stored at −20 °C.

Tests were performed largely as described by Clark and Adams (1977). Microtiter plates (M 129A, Dynatech) were coated with γ-globulin (1 μg ml^{-1}), during 18 h at 4 °C. Incubation with antigen preparations was for 3.5 h at 30 °C. Incubation with conjugate, diluted 4000×, was during 18 h at 4 °C. P-nitrophenyl phosphate was used at a concentration of 0.75 mg ml^{-1}, 200 μl per well. Results were read with a Titertek Multiskan photometer at 405 nm, after 1 h substrate incubation.

Sedimentation coefficient. The sedimentation coefficient was determined by the graphical method of Markham (1960), using a Spinco Model E analytical ultracentrifuge with schlieren optics.

Electron microscopy. Purified preparations were applied to formvar-backed carbon films and stained with 20% aqueous uranyl acetate. They were examined in a Philips EM300 electron microscope at an accelerating voltage of 60 kV.

Results

Results of test plant studies. The reactions of test plants are summarized in Table 1. Symptoms observed in these plants corresponded to those described for CMV in general. However, differences between S4 and K8 were obvious. S4 in general caused more severe symptoms and growth reduction than K8. The reduction in growth caused by both isolates in *N. clevelandii,* 4 weeks after inoculation, is shown in Fig. 1. In *N. tabacum* 'Xanthi-nc', S4 induced chlorotic spots on inoculated leaves (Fig. 2) and a severe mosaic and leaf deformations in systemically infected leaves, whereas K8 caused etching in the inoculated leaves (Fig. 3) and sometimes also in systemically infected leaves. In *N. glutinosa* inoculation with S4 resulted in severe mosaic and typical leaf deformations (Fig. 4) which were absent when inoculated with K8 (Fig. 5).

Virus purification. Yields of both isolates purified from *N. clevelandii* as well as from *N. rustica* were high and reached 70 mg of virus per 100 g of leaf material.

Table 1. Reactions of test plants.

Test plant	Virus isolate	
	K8	S4
Chenopodium amaranticolor	L[1]	L
Chenopodium quinoa	L	L
Capsicum annuum 'Javitott cecei'	(L), S	S
Cucumis sativus 'Gele tros'	L, S	L, S
Cucumis melo 'Tétényi cseres'	S	S
Nicotiana benthamiana	S	S
Nicotiana clevelandii	L, S	L, S
Nicotiana megalosiphon	L, S	L, S
Nicotiana glutinosa	L, S	S
Nicotiana rustica	S	S
Nicotiana tabacum 'White Burley'	S	S
Nicotiana tabacum 'Xanthi-nc'	L, S	(L), S
Phaseolus vulgaris 'Bataaf'	(L)	(L)
Pisum sativum 'Koroza'	L	L
Vicia faba 'Kompakta'	L	L
Vigna unguiculata 'Black'	L	L

[1] L = local symptoms; S = systemic symptoms; ( ) = symptoms not always observed or not clear.

Tabel 1. Samenvatting van de reacties op toetsplanten.

Fig. 1. *Nicotiana clevelandii:* healthy (left), and 4 weeks after inoculation with K8 (middle) and S4 (right).

Fig. 1. Nicotiana clevelandii, niet geïnoculeerd (links) en 4 weken na inoculatie met K8 (midden) en S4 (rechts).

Antiserum preparation. Homologous and heterologous titers in double-diffusion tests, obtained with sera from four different bleedings are presented in Table 2. The highest homologous titers were 256 for the K8 antiserum and 512 for the S4 antiserum. Heterologous titers were 1 or 2 two-fold dilution steps lower than homologous titers.

Immuno-diffusion tests. In double-diffusion tests, K8 and S4 were compared with other CMV isolates and a CMV-related virus from chrysanthemum (CV-Noordam). The virus isolates and the antisera together with the results obtained are given in Table 3. In these tests, highly purified virus preparations were used. Of the antisera used, only the one indicated CMV-ToRS in separate tests reacted with a concentrated extract of healthy *N. clevelandii* (titer 16). The results show that K8 and S4 are closely related but not identical. Both isolates are also closely related to the other CMV isolates tested, but not to CV-Noordam.

To differentiate further between K8 and S4 and to check whether they belong to the ToRS or the DTL serological groups of CMV (Devergne and Cardin, 1973, 1975) they were compared with representatives of these groups, and with each other, using the spur test (Fig. 6). When K8 and To antigens were placed in adjacent wells, with 3 of the 4 antisera (K8, ToRS, and D) the precipitation lines coalesced completely. They crossed only with the S4 antiserum. If D and S4 antigens were placed in adjacent

Fig. 2. Leaf of *Nicotiana tabacum* 'Xanthi-nc', c. 10 days after inoculation with S4.

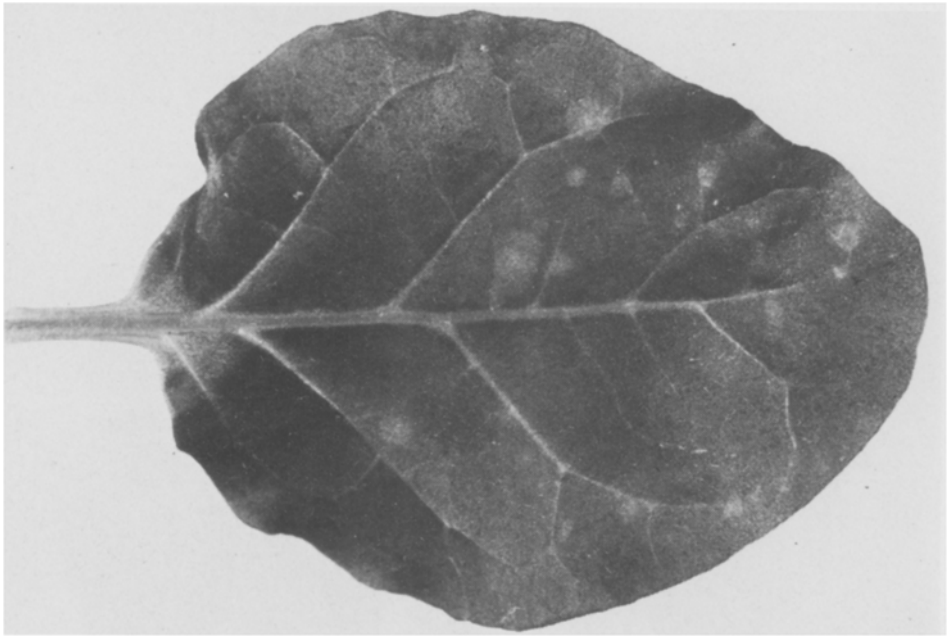

Fig. 2. Blad van Nicotiana tabacum 'Xanthi-nc', ca 10 dagen na incoculatie met S4.

Table 2. Homologous and heterologous antiserum titers of 4 different bleedings in double-diffusion tests.

Antiserum	Bleeding (weeks after first injection)	Antigen	
		K8	S4
K8	3	128	64
	5	256	128
	8	256	64
	10	64	32
S4	4	128	256
	6	256	512
	9	128	256
	11	64	256

Tabel 2. Homologe en heterologe antiserumtiters van 4 verschillende bloedafnamen in de dubbele-diffusietoets.

Fig. 3. Leaf of *Nicotiana tabacum* 'Xanthi-nc', 5 days after inoculation with K8.

Fig. 3. Blad van Nicotiana tabacum 'Xanthi-nc', 5 dagen na inoculatie met K8.

wells, with only 2 antisera (K8 and D) complete fusion of precipitation lines occurred. With all other combinations spurs occurred or lines crossed. The results indicate that K8 is more closely related to To than to D or S4, and that S4 is more closely related to D than to K8 and To. However, with this test all 4 isolates could be differentiated.

ELISA. Table 4 presents the extinction values (average of two experiments) after testing purified preparations of K8 and S4, diluted with PBS or EDTA-containing borate buffer (pH 9), with antisera to both isolates. To these buffers 0.05% Tween 20 and 2% polyvinyl pyrrolidone were added. The results show that reactions were much stronger when antigens were in PBS. When diluted with PBS both antigens were detected by both antisera. With both diluents homologous reactions were stronger than heterologous reactions. Testing crude extracts from several healthy test plants, diluted 10 × with PBS, yielded extinction values between 0.03 and 0.08.

Sedimentation coefficient. In the analytical ultracentrifuge, purified preparations of each of both isolates, containing c. 2 mg per ml of 5 mM sodium borate (pH 9), showed one peak. Sedimentation coefficients at 20 °C were 92.7 S and 93.3 S for K8 and S4, respectively. With mixed preparations only one peak was observed (Fig. 7).

Fig. 4. *Nicotiana glutinosa,* 3 weeks after inoculation with S4.

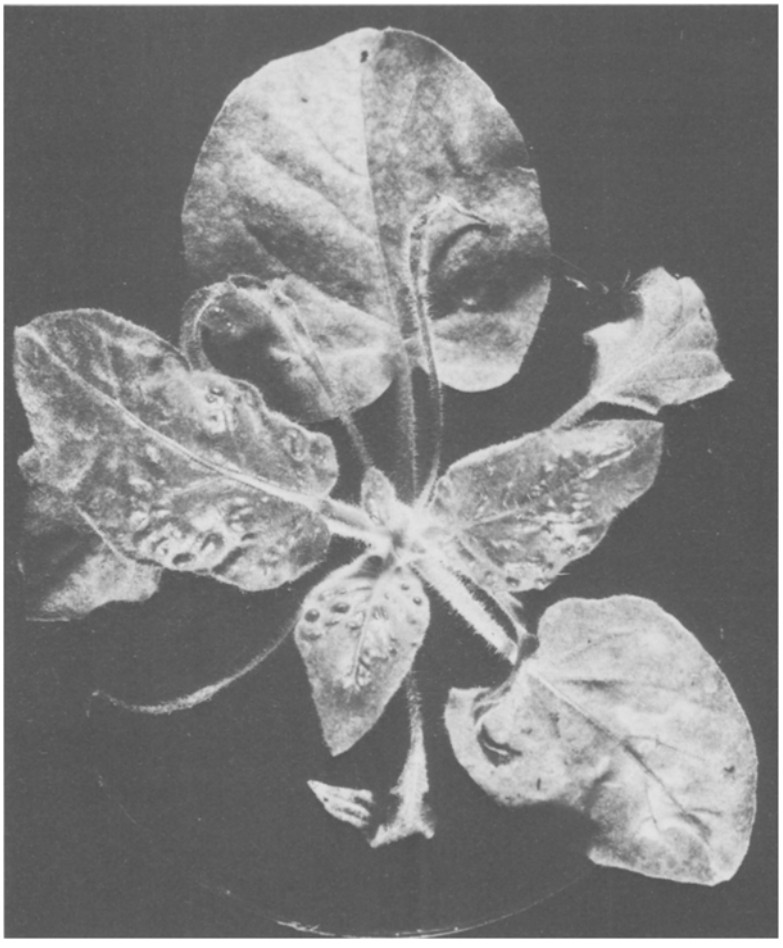

Fig. 4. Nicotiana glutinosa, 3 weken na inoculatie met S4.

Electron microscopy. Fig. 8 shows the spherical particles of a purified preparation of K8, stained with 2% uranyl acetate. Similar particles were found in preparations of S4. No particles were found after staining with potassium phosphotungstate.

Discussion and conclusions

On the basis of host plants reactions and the results of serological experiments, K8 and S4 can be considered as isolates of CMV.

Symptomatologically (etching in *N. tabacum* 'Xanthi-nc') K8 would belong to the symptomatological group B of CMV as defined by Marrou et al. (1975), together with CMV-To. Serologically K8 is more closely related to CMV-To and together with the

Fig. 5. *Nicotiana glutinosa,* c. 2 weeks after inoculation with K8.

Fig. 5. Nicotiana glutinosa, ca 2 weken na inoculatie met K8.

Fig. 6. Double-diffusion tests (spur test), comparing K8 and S4 with CMV-To and CMV-D. Dotted lines: after 48 h.

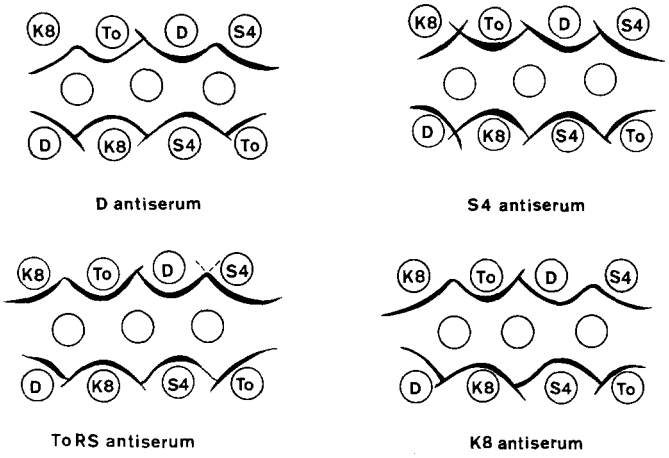

Fig. 6. Dubbele-diffusietoets ter nadere vergelijking van K8 en S4 met CMV-To en CMV-D. Stippellijntjes: na 48 uur.

Table 3. Antiserum titers in double-diffusion tests, performed to compare K8 and S4 with other CMV isolates and a CMV-related virus from chrysanthemum.

Antigen	Antiserum						
	CMV-ToRS[1]	K8	CMV-N11	CMV-Y	CMV-D[1]	S4	CV-Noordam
CMV-To[1]	4096	256	1024	32	1024	64	<4
K8	4096	256	512	64	512	128	<4
CMV-N11	4096	256	1024	64	512	128	<4
CMV-Y	2048	128	128	64	256	64	<4
CMV-D[1]	4096	128	256	64	1024	128	<4
S4	4096	64	128	64	1024	256	<4
CV-Noordam	4	<4	32	<4	<4	<4	512

[1] Antisera and antigens from Dr J.C. Devergne, Antibes, France (see Devergne and Cardin, 1973, 1975); the other antisera and antigens were prepared at Wageningen. CMV-N11: cucumber mosaic virus isolated from alstroemeria; CMV-Y: cucumber mosaic virus, Y-strain (Bos and Maat, 1974); CV-Noordam: CMV-related chrysanthemum virus (Bos and Maat, 1974).

Tabel 3. Antiserumtiters in de dubbele-diffusietoets. Vergelijking van K8 en S4 met andere komkommermozaïekisolaten en met een aan het komkommermozaïekvirus verwant chrysantevirus.

Table 4. Extinction values, obtained in ELISA, with purified preparations of K8 and S4 in two different buffers.

Antigens	Concentration ($\mu g\ ml^{-1}$)	Buffer	Antisera	
			K8	S4
K8	5	PBS	>2.00[1]	1.05
		Borate (pH 9)	1.22	0.04
	1	PBS	1.54	0.24
		Borate (pH 9)	0.22	0.04
S4	5	PBS	0.52	>2.00
		Borate (pH 9)	0.13	0.25
	1	PBS	0.32	1.27
		Borate (pH 9)	0.00	0.05

[1] Values represent the average of two experiments.

Tabel 4. Extinctiewaarden in ELISA, verkregen met gezuiverde preparaten van K8 en S4 in twee verschillende buffers.

Fig. 7. Analytical ultracentrifuge pattern of a mixture of K8 and S4. Sedimentation is from left to right. Photograph taken c. 8 min after rotor reached speed of 32 000 rpm.

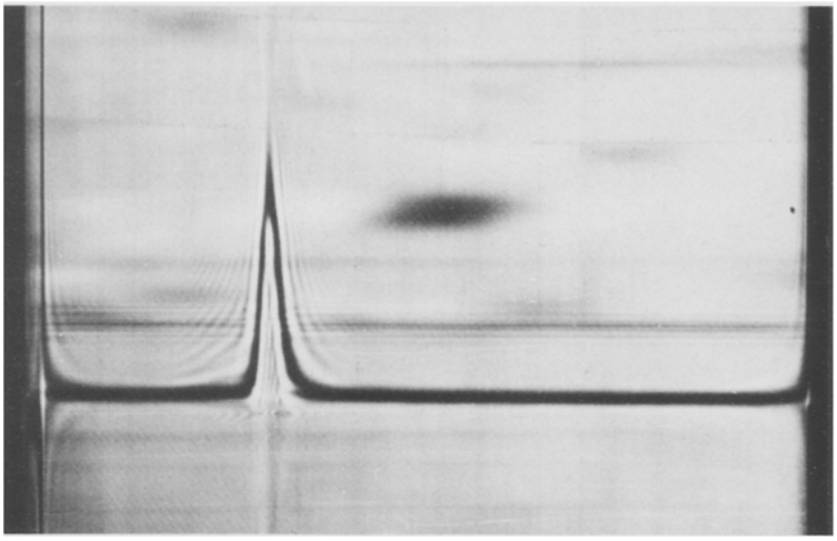

Fig. 7. Beeld in de analytische ultracentrifuge van een mengsel van K8 en S4. Sedimentatie van links naar rechts. Opname ca 8 min nadat de rotor op de ingestelde snelheid (32 000 tpm) was gekomen.

latter may belong to the serological group ToRS of CMV (Devergne and Cardin, 1973; 1975).

S4 has more features in common with the symptomatological group C of CMV, together with CMV-D. Serologically it is more closely related to CMV-D, and so may belong to the serological group DTL of CMV.

The symptomatological classification of K8 and S4 corresponded well with their serological classification. However, Beczner et al. (1978) reported that the grouping of several other Hungarian cucumovirus isolates on the basis of symptoms did not correlate with their serological classification.

S4 and K8 are closely related serologically, both mutually and to the other CMV isolates tested. No serological relationship was found with the chrysanthemum virus. In ELISA, using purified antigens, K8 and S4 could be detected with both antisera. Devergne et al. (1981), using direct ELISA, were also able to detect closely related CMV isolates (a.o. CMV-To and CMV-D) with antisera to each of them. However, using the same antisera to detect more distantly related cucumovirus isolates, indirect ELISA proved more useful.

The usefulness of the antisera to K8 and S4 to detect CMV infections in crops remains to be investigated.

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Fig. 8. Electron micrograph of purified K8, stained with 2% aqueous uranyl acetate. Bar represents 100 nm.

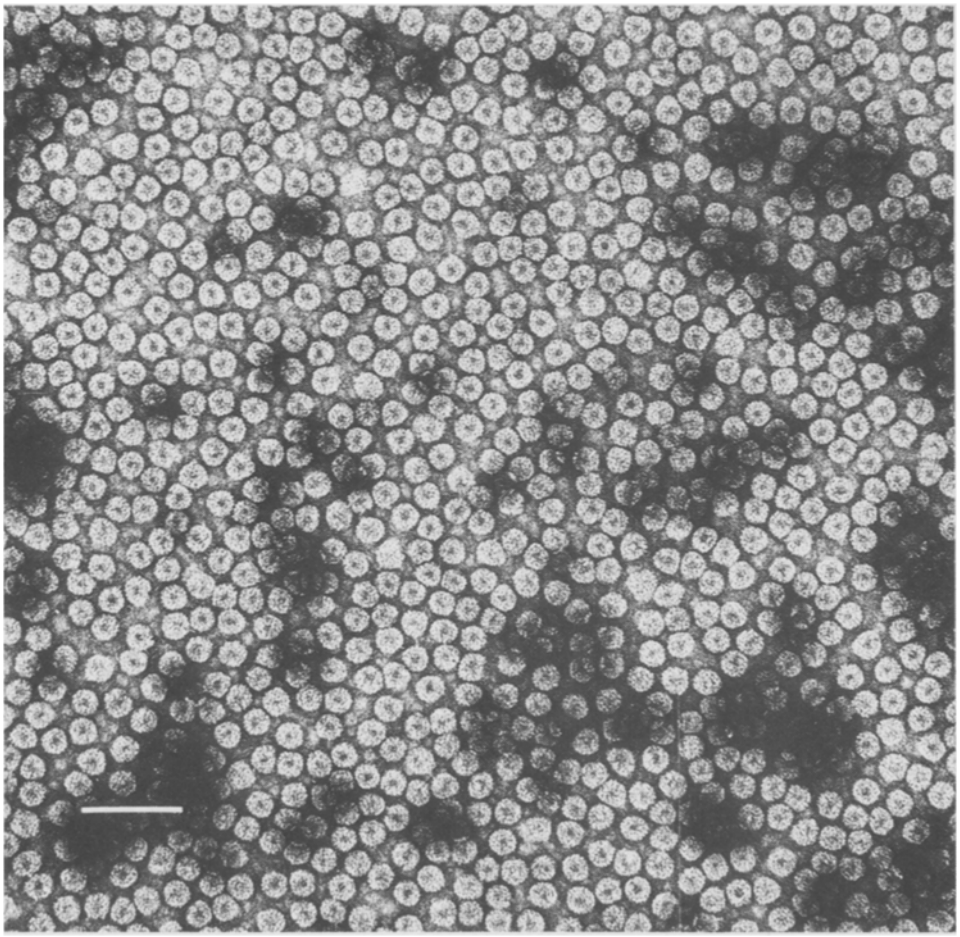

Fig. 8. Elektronenmicroscopische opname van gezuiverd K8, gekleurd met 2% uranylacetaat. Vergrotingsstaaf geeft 100 nm weer.

purified antigens of CMV-To and CMV-D, and antisera to CMV-D and to a member of the serological ToRS group of CMV.

Samenvatting

Twee hongaarse isolaten van het komkommermozaïekvirus uit paprika (Capsicum annuum) en meloen (Cucumis melo): identificatie en antiserumbereiding

Twee hongaarse virusisolaten uit paprika (K8) en meloen (S4) werden geïdentificeerd als komkommermozaïekvirus (CMV) met behulp van toetsplanten en serologie. Beide isolaten werden gezuiverd en er werden antisera tegen bereid. De homologe titers van

de antisera in de agar-geldiffusietoets bedroegen 256 (K8) en 512 (S4). K8 en S4 waren serologisch nauw verwant aan elkaar, evenals aan andere CMV-isolaten. Op grond van hun symptomen op toetsplanten behoren ze tot verschillende symptomatologische groepen van CMV. Dit laatste werd gesteund door de serologische eigenschappen. Beide isolaten hebben een sedimentatiecoëfficiënt van ca 93 S, bij een concentratie van 2 mg ml^{-1}. Gezuiverde preparaten, gekleurd met 2% uranylacetaat, bleken bolvormige deeltjes te bevatten. In ELISA reageerden gezuiverde preparaten van K8 en S4 met elkaars antisera.

References

Beczner, L., Devergne, J.C. & Vassányi, R., 1978. Symptomatological and serological characterization of some Hungarian cucumovirus isolates. Abstr. 3rd Int. Congr. Pl. Path., München, p. 45.

Bos, L. & Maat, D.Z., 1974. A strain of cucumber mosaic virus, seed-transmitted in beans. Neth. J. Pl. Path. 80: 113-123.

Clark, M.F. & Adams, A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. gen. Virol. 34: 475-483.

Devergne, J.C. & Cardin, L., 1973. Contribution à l'étude du virus de la mosaïque de concombre (CMV). IV. Essay de classification de plusieurs isolats sur la base de leur structure antigénique. Ann. Phytopathol. 5: 409-430.

Devergne, J.C. & Cardin, L., 1975. Relations sérologiques entre cucumovirus (CMV, TAV, PSV). Ann. Phytopathol. 7: 255-276.

Devergne, J.C., Cardin, L., Burckard, J. & Regenmortel, M.H.V. van, 1981. Comparison of direct and indirect ELISA for detecting antigenically related cucumoviruses. J. virol. Methods 3: 193-200.

Douine, L., Quiot, J.B., Marchoux, G. & Archange, P., 1979. Recensement des espèces végétales sensibles au virus de la mosaïque du concombre (CMV). Étude bibliographique. Ann. Phytopathol. 11: 439-475.

Francki, R.I.B., Mossop, D.W. & Hatta, T., 1979. Cucumber mosaic virus. CMI/AAB Descriptions of plant viruses No. 213, 6 pp.

Lot, H., Marrou, J., Quiot, J.B. & Esvan, C., 1972. Contribution à l'étude du virus de la mosaïque du concombre (CMV). II. Méthode de purification rapide du virus. Ann. Phytopathol. 4: 25-38.

Markham, R., 1960. A graphical method for the rapid determination of sedimentation coefficients. Biochem. J. 77: 516-519.

Marrou, J., Quiot, J.B., Marchoux, G. & Duteil, M., 1975. Caractérisation par la symptomatologie de quatorze souches du virus de la mosaïque du concombre et de deux autres cucumovirus: tentative de classification. Meded. Fac. Landb. wet. Rijksuniv. Gent 40: 107-121.

Steinbuch, M. & Audran, R., 1969. The isolation of IgG from mammalian sera with the aid of caprylic acid. Archs Biochem. Biophys. 134: 279-284.

Tóbiás, I., Molnár, B., Salamon, P. & Beczner, L., 1978. A paprikapatogén vírusok fertözésének hatása néhány étkezési paprikafajtára. Kertgazdaság 10: 51-60.

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