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Purification and serology of GE36 virus from apple and pear

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Abstract

A virus isolated from apple and pear, and coded GE36, was purified from sap of *Chenopodium quinoa* by bentonite clarification followed by differential centrifugation and rate-zonal centrifugation on a sucrose gradient in a zonal rotor. Infectious fractions contained spherical virus-like particles. An antiserum with a titer of 64 was prepared. No serological relation was found with 22 known spherical viruses and alfalfa mosaic virus. GE36 virus differs from any other sap-transmissible virus from apple and pear previously described.

Introduction

Van der Meer (1968) isolated a virus from apple and pear trees which he tentatively called GE36 virus because it superficially resembled E36 virus (Sequeira and Lister, 1969). Data on symptoms in herbaceous test plants, thermal inactivation point, and longevity in vitro of GE36 virus were given by van der Meer. Purification experiments and serological tests to further identify this virus are described in this paper.

Materials and methods

Virus isolate. The GE36 isolate (A.t.14) used for purification and antiserum preparation was obtained from the apple cultivar 'Schone van Boskoop' by mechanical transmission to *Amaranthus tricolor* (van der Meer, 1968).

Virus propagation and testing. Chenopodium quinoa was the propagation host in most experiments. The plants were grown in the greenhouse or in climate chambers at temperatures around 20 °C. Greenhouses were shaded with whitewash and cheesecloth during summer; during winter additional light was given using fluorescent tubes. Infectivity of virus preparations was tested on 6-8 primary leaves of *Phaseolus vulgaris* 'Bataaf', mostly grown in climate chambers.

The inoculum for virus propagation was generally prepared by homogenizing leaves in double their weight of a 0.02% bentonite suspension in distilled water and squeezing the homogenate through cheesecloth.

Virus stabilization and clarification. Unless otherwise stated, crude sap was prepared by homogenizing infected leaves in a Waring blendor together with twice their weight of distilled water, and squeezing the homogenate through cheesecloth.

Table 1. Virus-stabilizing effect of several buffers (0.02 M), as indicated by the average number of lesions per leaf of *Phaseolus vulgaris*, inoculated with sap diluted 1/10 with different buffers and stored for different periods at 4 °C.

Sap diluted with	Buffer pH	Average number of lesions per leaf after storage of mixture during			
		1 day	2 days	4 days	8 days
Distilled water		9	6	1	0
Phosphate-citric acid buffer	5	3	5	1	0
	6	9	3	2	0
	7	5	6	4	2
Na-K-phosphate buffer	6	8	5	1	0
	7	13	19	4	0
	8	25	20	6	0
Na-citrate	4	0	0	0	
	5	0	1	0	
	6	0	0	0	
0.02 M NaDIECA		1	1	0	
0.02 M Na-thiogl.		5	9	2	0
Undiluted sap		69	35	4	3

Tabel 1. Virus-stabiliserend effect van verschillende buffers (0,02 M), gemeten aan het gemiddeld aantal lokale lesies per blad van Phaseolus vulgaris, geïnoculeerd met sap dat 1 op 10 is verdund met verschillende buffers en vervolgens bewaard gedurende verschillende tijden bij 4 °C.

To test buffers for their stabilizing effect two procedures were followed. In one experiment crude sap was diluted 1/10 with the buffers mentioned in Table 1, and infectivity was measured after 1, 2, 4 and 8 days of storage at 4 °C. In the other experiment infected leaves were directly homogenized in three times their weight of the buffers mentioned in Table 2. Sap samples were tested for infectivity after 2, 5, 9 and 16 days' storage at 4 °C.

Table 2. Stabilizing effect of 0.02 M phosphate-citric acid buffers of different pH with and without 0.02 M Na-thioglycolate or 0.02 M Na_2SO_3, as indicated by the average number of lesions per leaf of *Phaseolus vulgaris* when inoculated with sap prepared with these buffers and subsequently stored for varying numbers of days at 4 °C.

Buffer pH	Addition	Average number of lesions per leaf after storage during			
		2 days	5 days	9 days	16 days
6		167	167	55	8
7		87	119	16	10
8		173	110	42	1
6	Na-thiogl.	216	201	24	17
7	Na-thiogl.	176	199	33	6
8	Na-thiogl.	200	184	30	6
6	Na_2SO_3	46	2		
7	Na_2SO_3	48	1		
8	Na_2SO_3	14	0		
Distilled water		147	148	14	18

Tabel 2. Stabiliserend effect van 0,02 M fosfaat-citroenzuur buffers van verschillende pH, met en zonder toevoeging van 0,02 M natriumthioglycolaat of 0,02 M Na_2SO_3, gemeten aan het gemiddeld aantal lokale lesies per blad van Phaseolus vulgaris, geïnoculeerd met sap dat met de verschillende buffers is bereid en vervolgens bewaard gedurende verschillende aantallen dagen bij 4 °C.

To test clarification procedures, crude sap (prepared with 0.02 M phosphate-citric acid buffer at pH 7) was divided into portions of 10 ml. These were used as such or were subjected to one of the following treatments: 1. centrifugation at low speed; 2. freezing and thawing followed by low-speed centrifugation; 3-8. shaking with: 1 ml of n-butanol, 2 ml of chloroform, 2 ml of carbon tetrachloride, a mixture of diethyl ether and carbon tetrachloride (2 ml), 2 ml of a 1% bentonite suspension, and 2 ml of hydrated calcium phosphate, respectively, and all followed by low-speed centrifugation. Moreover we tried to precipitate the virus from crude sap by adding twice the volume of cold ethanol, followed by low-speed centrifugation and resuspending the sediment in 0.02 M buffer (pH 7). Clarified suspensions were tested for infectivity undiluted and diluted 1/10.

To determine the maximum amount of bentonite that could be added without losing the virus, the procedure was as follows. Ten ml of a buffer and 2 ml of a 1% bentonite suspension prepared in the same buffer were added to 10 ml of crude sap. After centrifugation for 20 min at 4,000 g a small sample of the supernatant was tested for infectivity, while the rest was again mixed with 2 ml of bentonite suspension, and centrifuged. This procedure was repeated three times. We used 0.001 M Na-K-phosphate (pH 7.4) + 0.001 M $MgSO_4$ (Dunn and Hitchborn, 1965) and 0.02 M phosphate-citric acid (pH 7) as buffers.

Bentonite suspensions were prepared with a Waring blendor, homogenizing 1 g of dry bentonite (bentonite powder, technical, The British Drug Houses Ltd., England) in 100 ml of distilled water or buffer as required. Homogenizing was done for 2 min, interrupted for 5 min, and repeated for another 2 min. For every experiment freshly prepared bentonite suspensions were used.

Partial purification. Partially purified virus preparations for rate-zonal centrifugation were prepared as follows. To crude sap, obtained from 300 g of leaf material, 1/5 of its volume of 1% bentonite suspension in distilled water was added and the mixture centrifuged for 10 min at 7,500 g. If the supernatant was still greenish, again some bentonite was added (1/20-1/10 of the sap volume, depending on colour) and centrifugation repeated. The clear supernatants were then ultracentrifuged for 2 h at 80,000 g. The sediment was resuspended in 60 ml of 0.002 M phosphate-citric acid buffer (pH 7) and kept overnight in a refrigerator. After centrifuging for 10 min at 7,500 g the supernatant again was ultracentrifuged for 2 h at 80,000 g, and the sediment resuspended this time in 10 ml 0.002 M phosphate-citric acid buffer (pH 7). After 1 h it was centrifuged at low speed and then 2.5 ml of a 10% sucrose solution were added to the supernatant before introduction into the zonal rotor.

Rate-zonal centrifugation. A Beckman B14 titanium zonal rotor (Anderson and Cline, 1967) was used for rate-zonal centrifugations. Sucrose gradients were from 10-40% (w/v). They were prepared linearly with volume using a Beckman gradient pump. Samples of partially purified preparations of 12.5 ml containing 2% sucrose were layered onto the gradient with a syringe, followed by an overlay of 100 ml of distilled water. It was then centrifuged for 2 h at 45,000 rpm. Effluent streams were monitored for absorbance at 254 nm using an LKB-Uvicord absorption meter in conjunction with a Beckman 10″ linear/log laboratory recorder.

Ultraviolet absorbing fractions were collected separately, diluted 1/2 with distilled water and ultracentrifuged for 3 h at 80,000 g. Sediments were resuspended in 2-4 ml of 0.002 M phosphate-citric acid buffer (pH 7). For electron microscopy and serology they were stored in a deep-freezer after addition of glycerol (final concentration 10%).

Electron microscopy and serology. For electron microscopy and antiserum preparation, samples were fixed with formaldehyde. Purified preparations were dialysed against a 2% formaldehyde solution for at least 6 h and then against diluted buffer to remove the formaldehyde. For antiserum preparation fixation with 5% formaldehyde was also tried. As in this case the virus precipitated it was resuspended by a short ultrasonic treatment.

Electron micrographs of purified virus preparations were made with a Philips EM 300 electron microscope after negative staining with 1% phosphotungstate at pH 6.8.

Rabbits were injected intravenously as well as intramuscularly. In the latter case an emulsion with Freund's incomplete adjuvant was used. Blood samples were taken from the ear vein, for large quantities (up to 75 ml of blood) the rabbits being anaesthetized with Nembutal (Nembutal, veterinary; Abbott Laboratories Ltd., Queenborough, Kent, England).

Serological tests were in accordance with the Ouchterlony double diffusion test with 1% agar gel in saline. Wells in agar were 3 mm wide, the centres of antiserum and antibody wells being 1/2√2 cm apart.

For serological testing purified preparations (concentrated after rate-zonal centrifugation) or partially purified preparations were used. For partial purification the procedure was similar to that used for rate-zonal centrifugation, except that only one cycle of differential centrifugation was given. In general, sap from 50 g of leaf material was concentrated to 1-2 ml. The procedure was the same for GE36, E36, and apple chlorotic leaf spot virus (CLSV) isolates.

Antisera against *Pelargonium* leaf curl virus and tomato bushy stunt virus were kindly supplied by Dr M. Hollings, England, and against tobacco streak virus by Dr R. W. Fulton, U.S.A. Dr A. F. Murant, Scotland, kindly provided us with antiserum against raspberry bushy dwarf virus, Dr J. Dunez, France, with antiserum against Myrobalan latent ringspot virus, and Dr G. Morvan, France, with antiserum against sowbane mosaic virus.

Results

In preliminary experiments *Chenopodium quinoa* proved to be a better propagation host than *Nicotiana rustica, N. glutinosa,* or *N. tabacum* 'White Burley'. *C. quinoa* gave the best and most consistent results as far as infectivity of expressed sap on *Phaseolus vulgaris* 'Bataaf' was concerned, and was therefore chosen as host for virus propagation.

Plant sap was diluted with various buffer solutions to test their virus-stabilizing effect. The buffers concerned and the results with their 0.02 M solutions, with 0.02 M sodium diethyldithiocarbamate (NaDIECA), with 0.02 M sodium thioglycolate (Na-thiogl.), and with distilled water are given in Table 1. With 0.2 M concen-

Table 3. Infectivity of sap after treatment with increasing quantities of 1 % bentonite suspension, as measured by the average number of local lesions per leaf of *Phaseolus vulgaris*.

Bentonite (ml) added to 10 ml of sap	Average number of lesions/leaf using:	
	phosphate-citric acid buffer pH 7	Na-K-phosphate buffer pH 7.4 + $MgSO_4$
2	444	435
4	396	>400
6	41	325
8	8	103
10	5	191

Tabel 3. Infectiositeit van het sap na behandeling met toenemende hoeveelheden 1% bentonietsuspensie, gemeten aan het gemiddeld aantal lokale lesies per blad van Phaseolus vulgaris.

trations of the buffers mentioned in Table 1, 0.2 M NaCl, and 0.2 M NaCl + 0.02 M NaDIECA, infectivity of sap was completely lost within 1 day. The results of a second experiment, when directly homogenizing leaves in a number of buffer solutions, are given in Table 2.

In the two clarification experiments no infectivity could be detected after precipitation with ethanol, after freezing and thawing followed by low-speed centrifugation, or even after just low-speed centrifugation. The clarified extracts obtained following the other treatments were infectious. The best clarification judged by the naked eye was obtained with bentonite. After bentonite treatments undiluted suspensions were far more infectious than after other treatments. Comparing suspensions diluted 1/10, difference in infectivity was very small.

The results of experiments to determine the maximum amount of bentonite that could be added to crude sap without losing the virus are recorded in Table 3. Every supernatant was tested on 6-8 bean leaves. After addition of 2 ml of bentonite supernatants were still greenish, but after 4 ml of bentonite they were clear

Fig. 1. Absorbance at 254 nm of effluent stream from zonal rotor after centrifugation of partially purified preparations of healthy and GE36-infected *Chenopodium quinoa* plants during 2 h at 45,000 rpm.

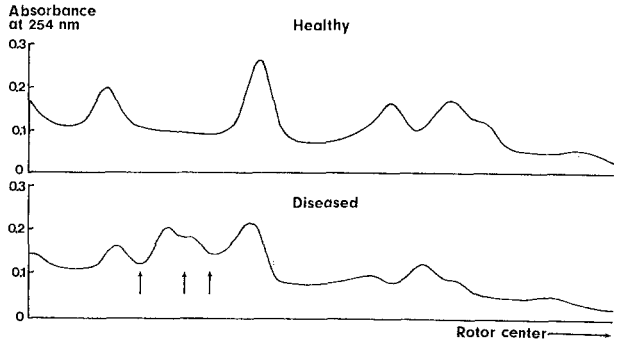

Fig. 1. Extinctie bij 254 nm van het effluent uit de zonerotor na centrifugering van gedeeltelijk gezuiverde preparaten van gezonde en zieke planten van Chenopodium quinoa gedurende 2 uur bij 45.000 omwentelingen per minuut.

Fig. 2. Electron micrograph of GE36 virus, negatively stained with PTA. Bar represents 100 nm (Photograph T.F.D.L., Wageningen).

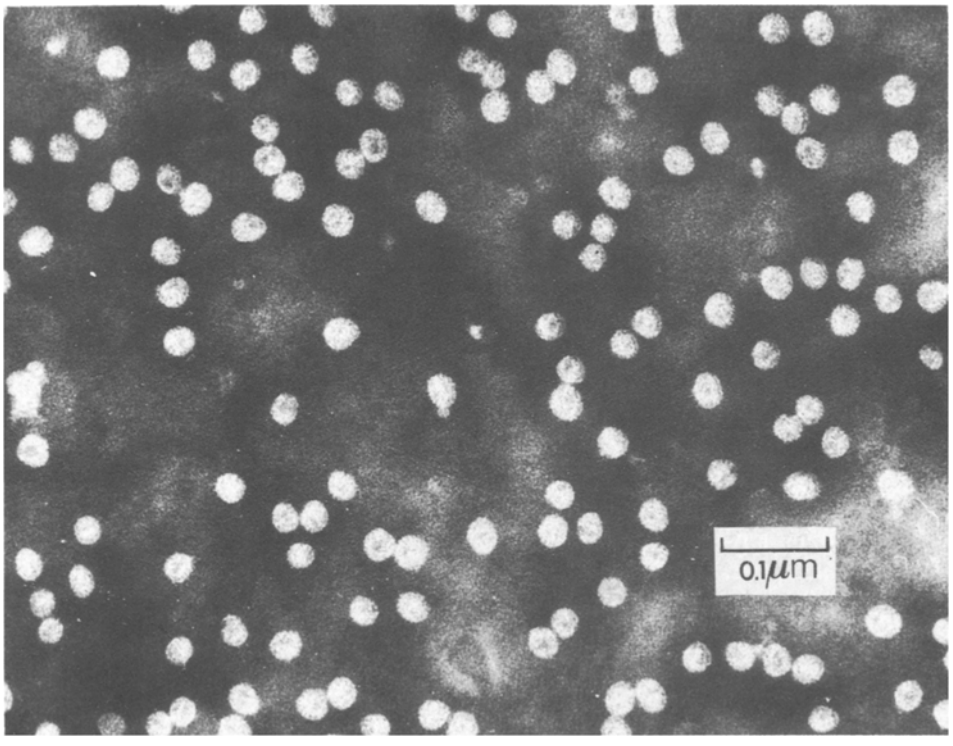

Fig. 2. Elektronenmicroscopische foto van GE36 virus, negatief gekleurd met fosforwolfraamzuur. Ingetekende schaal: 100 nm (Foto T.F.D.L., Wageningen).

with both buffers used. After 6 ml there was a sharp decrease in the number of lesions using phosphate-citric acid buffer (pH 7).

In another experiment with bentonite crude sap was subjected to the following treatments: 1. storing crude sap at 4 °C; 2. adding 1% bentonite suspension to crude sap (1:5) and storing at 4 °C; 3. as 2 but followed by low-speed centrifugation and storing the supernatant; 4. as 1 but adding 1% bentonite (1 ml bentonite to 5 ml sap) just before inoculation; 5. as 4 but followed by low-speed centrifugation. The results in terms of average number of local lesions per leaf of *P. vulgaris* are given in Table 4. They show that addition of bentonite results in a much greater number of lesions.

Results of rate-zonal centrifugations comparing partially purified preparations from healthy and GE36-infected *C. quinoa* plants are shown in Fig. 1. The ultraviolet-absorbing fractions were collected separately and after concentration were tested for infectivity and for the presence of virus particles. Infectivity was concentrated in fractions between arrows in Fig. 1, whereas the two adjacent fractions

Table 4. Infectivity of sap after addition of bentonite at different times before inoculation, as measured by the average number of local lesions per leaf of *Phaseolus vulgaris*.

Treatment[1]	Average number of lesions/leaf after storage of original sab during				
	1 h	3 h	6 h	3 days	6 days
1	7	52	44	7	13
2	>400	350	350	117	2
3	>400	>400	>400	>400	196
4	>400	100	350	271	208
5	>400	>400	>400	278	279

1[1] = storage of crude sap at 4 °C;
2 = addition of 1 % bentonite suspension to crude sap and storage at 4 °C;
3 = as 2, but followed by low-speed centrifugation and storage of the supernatant;
4 = as 1, but addition of 1 % bentonite just before inoculation;
5 = as 4, but followed by low-speed centrifugation.

Tabel 4. Infectiositeit van sap na toevoeging van bentoniet op verschillende tijdstippen voor inoculatie (1 = geen bentoniet), gemeten aan het gemiddeld aantal lokale lesies per blad van Phaseolus vulgaris.

were only slightly infective. The infective fractions also contained virus-like particles (Fig. 2).

Antisera prepared against the fractions between the arrows in Fig. 1 reached titers of 64 in the agar-gel diffusion test, when tested against purified, partially purified, and, occasionally, crude preparations from GE36-infected *C. quinoa* plants. The antisera did not react with crude sap or partially purified preparations from healthy *C. quinoa*. Partially purified preparations of 4 isolates giving typical GE36 symptoms on *C. quinoa*, all reacted with the GE36 virus antiserum up to the same antiserum dilution (1/64). None of the 4 E36 isolates nor the apple chlorotic leaf spot isolate (CLSV) tested, reacted with this antiserum. The E36 and CLSV isolates were partially purified in the same way as the GE36 isolates.

Partially purified preparations of two GE36, two E36, and one CLSV isolate, all prepared according to the procedure described for GE36, were tested with antisera to the following viruses: GE36 virus, *Arabis* mosaic virus, raspberry ringspot virus, tomato black ring virus, cherry leaf roll virus, strawberry latent ringspot virus, tobacco ringspot virus, tobacco necrosis virus, red clover mottle virus, two isolates of cowpea mosaic virus, turnip yellow mosaic virus, cucumber mosaic virus, tomato aspermy virus, apple mosaic virus, *Prunus* necrotic ringspot virus (cherry isolate), carnation mottle virus, alfalfa mosaic virus, *Pelargonium* leaf curl virus, tomato bushy stunt virus, tobacco streak virus, bean pod mottle virus, raspberry bushy dwarf virus, sowbane mosaic virus, and Myrobalan latent ringspot virus. The only positive reactions obtained were between GE36 virus antiserum and the two GE36 virus isolates. The partially purified preparations of the three viruses proved to be highly infectious when tested on *C. quinoa*.

In electron micrographs of formalin-fixed purified virus preparations spherical virus-like particles were observed. An example is given in Fig. 2. In unfixed preparations it was very difficult to detect these particles. In preparations from healthy plants such particles were absent.

Discussion

GE36 virus was only isolated from apple and pear in 1965 and 1966 (van der Meer, 1968; Ragozzino et al., 1970).

The virus was very sensitive to high salt concentrations. As none of the 0.02 M solutions tested gave much better results than distilled water (Tables 1 and 2), for convenience the latter was chosen for homogenization.

Clarification experiments using varying quantities of bentonite suspension showed that 6 ml of bentonite added to 10 ml of sap, strongly decreased the infectivity when the suspensions were in phosphate-citric acid buffer (pH 7). In phosphate buffer (pH 7.4) + $MgSO_4$ there was not such a sharp decrease in infectivity (Table 3). Distilled water was not included in these experiments. A better clarification, without losing infectivity, may therefore be possible when using the phosphate buffer + $MgSO_4$ instead of the distilled water, used in further experiments.

Bentonite may be helpful in the mechanical transmission of viruses (among others Yarwood, 1966). In our experiments addition of bentonite to crude sap from *C. quinoa* stimulated lesion formation on *P. vulgaris* (clarification experiments and Table 4). Even when sap was stored for several days before bentonite was added, a large number of lesions developed. So the effect of bentonite seems to be rather that of binding an inhibitor of infection, than of protecting the virus by adsorbing virus degrading substances. Such an inhibitor has been described earlier by Saksena and Mink (1969). They found that sap from *C. quinoa* reduced the number of lesions of CLSV on *C. quinoa* as well as on *P. vulgaris*. As a routine we therefore used bentonite when preparing inoculum to infect large numbers of *C. quinoa* plants for virus propagation.

The purification method finally adopted resembles the one described for two sap-transmissible viruses from apple by Lister et al. (1965). An important difference, however, is the use of a zonal rotor for density-gradient centrifugation. Results of the rate-zonal centrifugations varied considerably between experiments, concerning the heights of the peaks as well as (to a lesser extent) the number of ultraviolet-absorbing fractions and their position. However, the difference between healthy and diseased material was fairly consistent in the arrow-indicated region of Fig. 1. The results suggest that the virus has at least two components with a slight difference in sedimentation. The function of these two components was not investigated.

The antisera prepared reacted only with extracts from diseased plants. The purified virus therefore must have been antigenically fairly pure, indicating that a zonal rotor is a very valuable tool in virus purification. In our laboratory the technique was also successfully used to remove normal host constituents from partially purified preparations of *Arabis* mosaic virus, raspberry ringspot virus, tobacco ringspot virus, strawberry latent ringspot virus, cherry leaf roll virus, tomato black ring virus, apple mosaic virus, and *Prunus* necrotic ringspot virus (cherry isolate). Of the antisera prepared for these viruses only that against tomato black ring virus reacted slightly with normal plant material.

We found no evidence for a close relationship of the GE36 virus to any other virus. Serological tests performed excluded a close serological relationship with 22 spherical viruses and alfalfa mosaic virus, while on the basis of symptomatology

and host range (van der Meer, 1968) a close relation with these and other viruses is also unlikely. GE36 virus differs clearly from E36 virus and from CLSV on basis of particle morphology, the latter two having threadlike particles (Lister et al., 1965; de Sequeira and Lister, 1969). At present the cryptogram of the virus is (*/* : */* : S/S : S/*).

Samenvatting

Zuivering en serologie van GE36 virus uit appel en peer

Proeven werden uitgevoerd om te komen tot een nadere identificatie van het eerder door van der Meer (1968) geïsoleerde GE36 virus. Deze proeven hadden voornamelijk betrekking op de zuivering en de serologie. Voor vermeerdering van het virus werd *Chenopodium quinoa* gebruikt. In ruw sap van deze planten, 1 op 10 verdund met 0,2 M buffers, verloor het virus zijn infectievermogen reeds binnen 24 uur. Werd het sap 1 op 10 verdund met 0,02 M buffers of met gedestilleerd water, dan bleef het virus aanmerkelijk langer infectieus (Tabel 1 en 2).

Een goede klaring van het sap met behoud van infectievermogen kon worden verkregen door toevoeging van een bepaalde hoeveelheid van een 1% bentoniet-suspensie gevolgd door centrifugering bij laag toerental van het mengsel (Tabel 3). Bovendien bleek het aantal vlekjes bij inoculatie op *Phaseolus vulgaris* door toevoeging van bentoniet sterk toe te nemen (Tabel 4).

Het virus kon worden geconcentreerd door ultracentrifugeren. Werden voorgezuiverde en geconcentreerde preparaten van gezonde en zieke planten van *C. quinoa* vergeleken door middel van centrifugeren op een suikergradiënt in een zonerotor, dan bevatte het zieke materiaal twee componenten extra (Fig. 1). Deze waren infectieus en bevatten bolvormige virusachtige deeltjes (Fig. 2).

Antiserum (titer 64), bereid tegen gezuiverde viruspreparaten, reageerde alleen met preparaten van zieke planten van *C. quinoa* en niet met preparaten van gezonde planten. Antisera tegen 22 bekende bolvormige virussen en het luzernemozaïekvirus reageerden niet met GE36 virus. Het virus bleek niet identiek te zijn met enig ander reeds beschreven virus. Het cryptogram van GE36 is (*/* : */* : S/S : S/*).

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